# An investigation into the synthesis of potentially chemiluminescent novel molecules.

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# An Investigation into the Synthesis of Potentially Chemiluminescent Novel Molecules

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A thesis submitted in fulfilment of the requirements for the degree of Doctor of Philosophy in the University of Wales



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To my wife

Ruth

Thank you

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## Acknowledgements

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Finally I would like to thank my wife Ruth whose self-giving love and support throughout the duration of my project has helped me to keep my focus.

## Abbreviations

| Abbreviation | Description |
|---|---|
| $\lambda$ | Wavelength of electromagnetic energy |
| °C | Degrees of centigrade |
| $\Phi_{CL}$ | The chemiluminescence efficiency |
| $\Delta H_r$ | Differences in the heats of reactions |
| $^1$HNMR | Proton nuclear magnetic resonance |
| Acc. Mass | Accurate mass spectrometry |
| AMP | Adenosine monophosphate |
| AMPPD | 3-(4-methoxyspiro{1,2-dioxetane-3,2'-tricyclo-[3.3.1.13,7 ]decan} -4-yl) phenyl phosphate |
| ATP | Adenosine triphosphate |
| CGD | Chronic granomatous disease |
| CI | Chemical ionisation |
| CIEEL | Chemically initiated electron exchange luminescence |
| CL | Chemiluminescence |
| CL ET | Chemiluminescence energy transfer |
| CoA | Co-enzyme |
| COSY | Correlated spectroscopy |
| d | Doublet |
| $d_6$-DMSO | Deutorated dimethylsulfoxide |
| DCCI | N,N'-dicyclohexylcarbodiimide |
| dd | Double doublet |
| ddd | Double doublet doublet |
| DDQ | 2,3-dichloro-5,6-dicyanobenzoquinone |
| DEPT | Distortionless enhancement by polarisation transfer |
| DMSO | Dimethylsulfoxide |
| dt | Double triplet |
| EI | Electron impact |
| ELISA | Enzyme linked immunosorbant assay |
| EPSRC | The engineering and physical sciences research council |
| ESI | Electrospray ionisation |
| ET | Energy Transfer |
| EtOAc | Ethyl acetate |
| EtOH | Ethanol |
| FAB | Fast atom bombardment |
| FMN | Flavin mononucleotide |
| $FMNH_2$ | Reduced flavin mononucleotide |
| Hz | Hertz |
| IR | Infrared spectrometry |
| LDLs | Low density lipoproteins |
| M | Mass value (in context of reporting mass data) |
| M | Moles / Litre (in context of reporting solution concentrations) |
| M+Na | Mass + Sodium (in context of reporting mass data) |
| MeI | Methyl iodide |
| MHz | Megahertz |

| | |
|---|---|
| mmol | Millimoles |
| MP | Melting point |
| mRNA | Messenger ribonucleic acid |
| MS | Mass spectrometry |
| NAD(P)H | Nicotinamide adenine dinucleotide phosphate |
| NADPH | Nicotinamide adenine dinucleotide phosphate |
| NMA | *N*-methylacridone |
| NMR | Nuclear magnetic resonance |
| PMNs | Polymorphonuclear leukocytes |
| ppm | Parts-per-million |
| psi | Pounds-per-square inch |
| Pyr | Pyridine |
| Rf | Retardation factor |
| RNA | Ribonucleic acid |
| $S_NAr$ | Aromatic nucleophillic substitution |
| $S_NI$ | Internal nucleophilic substitution |
| t | Triplet |
| td | Triple doublet |
| THF | Tetrahydrofuran |
| tt | Triple triplet |
| UV | Ultraviolet |
| $\Delta G$ | Gibbs free energy |

## Summary

Chemiluminescent compounds are essential tools as probes for the analysis of biological molecules. One such family, the Acridinium Esters, are safe to use and more sensitive than radio labels. The chemistry of the light-emitting reaction of the acridinium esters is a simple hydroperoxidation in basic conditions However, like all chemiluminescent systems, the light-emitting reaction and chemical stability of these molecules can be vastly improved. Chapter Two explores the chemistry around synthesising a sulfonate ester derivative of the standard ester (i.e. a carboxylate). From molecular modelling studies, it was predicted that sulfonate esters would react more energetically during hydroperoxidation. However, synthesis of 9-substituted acridinium sulfonate esters was impossible to achieve. Methods for esterification resulted in substitution of the sulfonate for a nucleophile, such as $HO^-$ or $Cl^-$. Moreover, the sulfonate anion was proven to be a very poor nucleophile, thus direct attack of the sulfonate ligand onto a nucleophile such as methyl iodide, was found to be unfavorable. Chapter Three explores the synthesis of a novel group potentially chemiluminescent molecules based on the original acridinium ester design but substituting the acridine ring for a pyridine ring. It was hoped through inter or intramolecular energy transfer to a bound fluor, that light of a desired wavelength could be selected based on choice of fluor. Finally, chapter four describes the synthesis of a family of molecules based on the parent molecule acridine-9-carboxylic acid pyridin-2-ylamide. Although classically, acridinium amides have proven to be too unreactive on hydroperoxidation, it was hoped that as the pyridine ring is electron withdrawing that it would improve said reactivity. However, when methylating these esters to potentially form an *N*-methyl acridinium, methylation preferentially occurred on the pyridine ring. Following this, work was then conducted to see if a doubly-methylated derivative could be made, however even under forcing conditions, only the monomethylated species was produced.

## Contents

Chapter 3 Synthesis and potential chemiluminescent properties of novel energy transfer molecules



Chapter 4 Synthesis of a range of molecules based on the parent molecule; acridine-9-carboxylic acid pyridin-2-ylamide

# Chapter 1

# Introduction

# 1 What is chemiluminescence?

Chemiluminescence occurs when a chemical reaction yields a certain electronically excited product and when this product relaxes to its ground state, light is evolved. Chemiluminescence comes under the umbrella of the much larger phenomenon of luminescence. Irrespective of how an excited state is generated, luminescence describes the light emitted from an excited molecule as it relaxes to its ground state. There are many different ways to generate the excited state apart from a chemical reaction (see table 1 for some examples), the most common way being by irradiation with light, when the process is called photoluminescence. When a compound photoluminesces, light of an appropriate wavelength promotes the formation of an excited state and when this relaxes, light of a longer wavelength is evolved.

**Table 1 Some types of luminescence**

| **Type of luminescence** | **Origin of luminescence** |
|---|---|
| Pyroluminescence | From heat |
| Photoluminescence | From light of a smaller λ |
| Chemiluminescence | From a chemical reaction |
| Triboluminescence | From friction |

Chemiluminescence is known as cold light, as heat is not evolved when the excited molecule relaxes from the excited state. However, light can be evolved from a solid or a liquid when it is heated to in excess of 525 °C. This is known as incandescence and an example of this is the light arising from the glowing embers of a fire. Incandescence arises from the loss of kinetic energy between atoms and molecules when they are heated to such temperatures.[1]

## 1.1 How is light generated by a chemical reaction?

The modern theory of light is summarised in the expression below:

| | |
|---|---|
| $E=h\upsilon=hc/\lambda$ | (1) |

Where:

**E** = Energy

**h** = Plank's constant

$\upsilon$ = frequency of the light waves

**c** = speed of light

$\lambda$ = length of the light waves

It is theorised that light acts both as a wave and a series of pockets of energy called photons. The above equation marries those two theories. It is also known that all particles have discrete energy levels, which can be subdivided as follows: [2]

1) Kinetic - translational, vibrational, rotational.
2) Potential - electronic, nuclear.

Since particles contain such energy levels, [1] they have the facility to absorb and/or emit electromagnetic radiation. The electromagnetic spectrum is shown in figure 1.

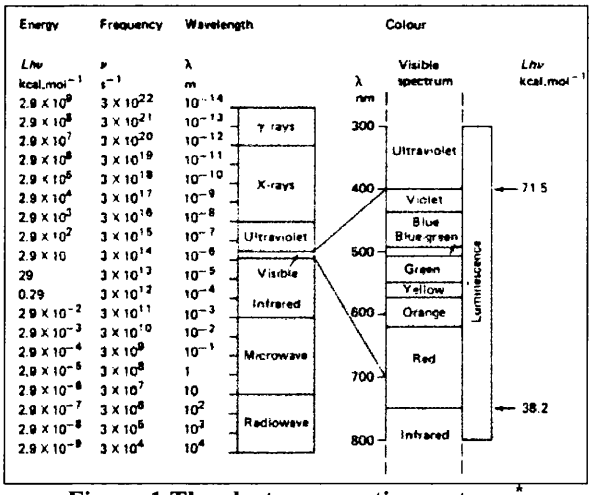


**Figure 1 The electromagnetic spectrum***

*Diagram reproduced from ref 1

Visible light occurs between 400 - 700nm, in between ultraviolet and infrared radiation, respectively. When a particle emits visible light, it will emit at these wavelengths. As the wavelength is inversely proportional to the amount of energy absorbed or emitted (equation1), the colour of the light indicates the energy of the transition which has formed the light. The energy of such energy transitions is between 159.8 and 362.7 kJ / mol for absorption and emission of visible light. The type of event which can absorb or yield such energies is an electronic excitation. Electronic excitations are the processes whereby ground state electrons are exposed to energy, at a magnitude of greater than 159.8 kJ / mol, and thereby they absorb energy. As a consequence, the electrons become excited and are promoted to higher electronic energy levels. However, the electrons do not remain excited, but fall to ground state levels and in doing so, release energy. This energy is sometimes released as electromagnetic radiation and for luminescent molecules, this is visible light. Not all molecules can absorb or emit visible light. In contrast, for example, some molecules only absorb and emit in the microwave or infrared regions. In doing so, the velocity of their molecular rotations or inter and intra molecular vibrations change, respectively. [1]

Electronic energy levels are made up of the following components see below:

| | |
|---|---|
| $E_{electronic} = E_{orbital} + E_{vibrational} + E_{rotational}$ | (2) |

Each electronic energy level contains a set of vibrational energies and each of these energies contains a set of rotational energies. It can be seen that there is a large number of energy levels to which an electron can be promoted. If these energy levels are contiguous, when the electron falls back to the ground state, no light will be emitted. This is because the energy is absorbed by each of the energy levels as the electron travels back to its ground state. Therefore to see an emission of visible light, the molecules responsible for such an emission do not have any overlap between their orbital levels. Moreover, the potential energy between these gaps must be between 159.8 and 362.7 kJ / mol.

Molecules having an even number of electrons generally exist in one of two spin states, these being triplet and singlet states. In the triplet state, the paired electrons are found in different orbitals but spin in the same direction. In the singlet state, the two electrons can be in the same orbital but have antiparallel spins or can be in different orbitals but still have antiparallel spins. For many molecules, the ground state is in the singlet configuration. Nevertheless, when a molecule gets excited, it can adopt either singlet or triplet electronic configurations. If an electron is promoted to an excited singlet state, when it falls back to the ground state, providing there is a sufficient energy gap between the two orbitals, it can emit light in the form of fluorescence. Electrons in the excited singlet state can also move to an excited triplet state, as long as the vibrational and rotational energies of the two states overlap one another. This transition is known as intersystem crossing. If an electron in the excited triplet state falls to the ground state, it can emit light in the form of phosphorescence (again provided there is a large enough energy gap between the two orbitals). The transition from triplet to singlet is known as a forbidden transition, which occurs a lot slower than an excited singlet to ground state singlet conversion ($100\text{-}10^6$ times slower). Phosphorescence is always found at a longer wavelength than fluorescence; the reason for this being that the triplet energy is lower than the excited singlet energy. Also, the longer it takes for the electron to relax, the greater the chance that energy will be emitted by a radiationless process. Such radiationless processes are: chemical quenching by ground state triplet molecules such as oxygen; internal conversion (where the electrons will move to lower energy levels of the same state in a radiationless manner) and collisional deactivation.[3] Figure 2 shows such processes.[4]

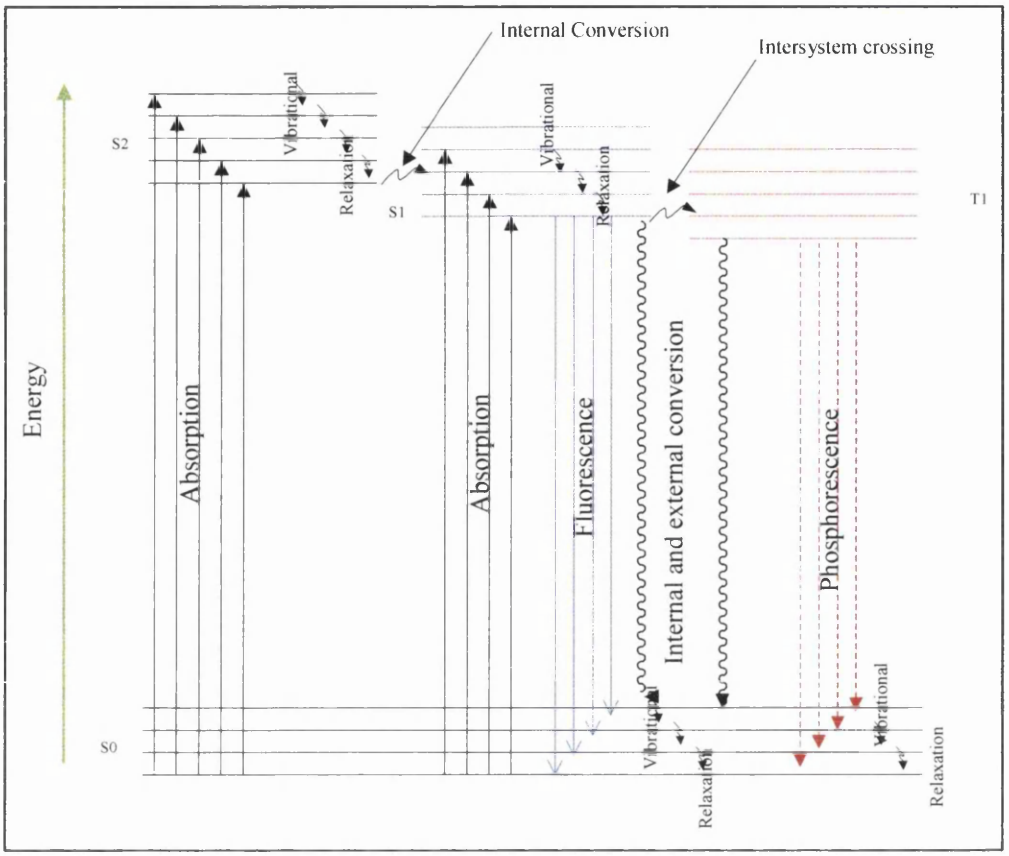


**Figure 2 Electronic transitions within energy levels**

Another way to represent electronic transitions is through potential energy diagrams, as shown in figure 3. Figures 2 and 3 both consider the transition of one electron between one interatomic distance within a diatomic species. However, the greater the number of atoms, the larger the number of interatomic distances or dimensions that must be considered. Thus for a molecule with more than two atoms, a multidimensional hypersurface is used to describe the electronic events which can occur. The number of dimensions depends upon the number of vibrational degrees of freedom within that molecule. If a molecule has $n$ number of atoms then it has a hypersurface of $3n$ - 6 dimensions.[5] However, this expression only predicts a hypersurface for molecules with 3 or more atoms because for two atoms the expression shows that a diatomic species would have zero dimensions when clearly it would have one.

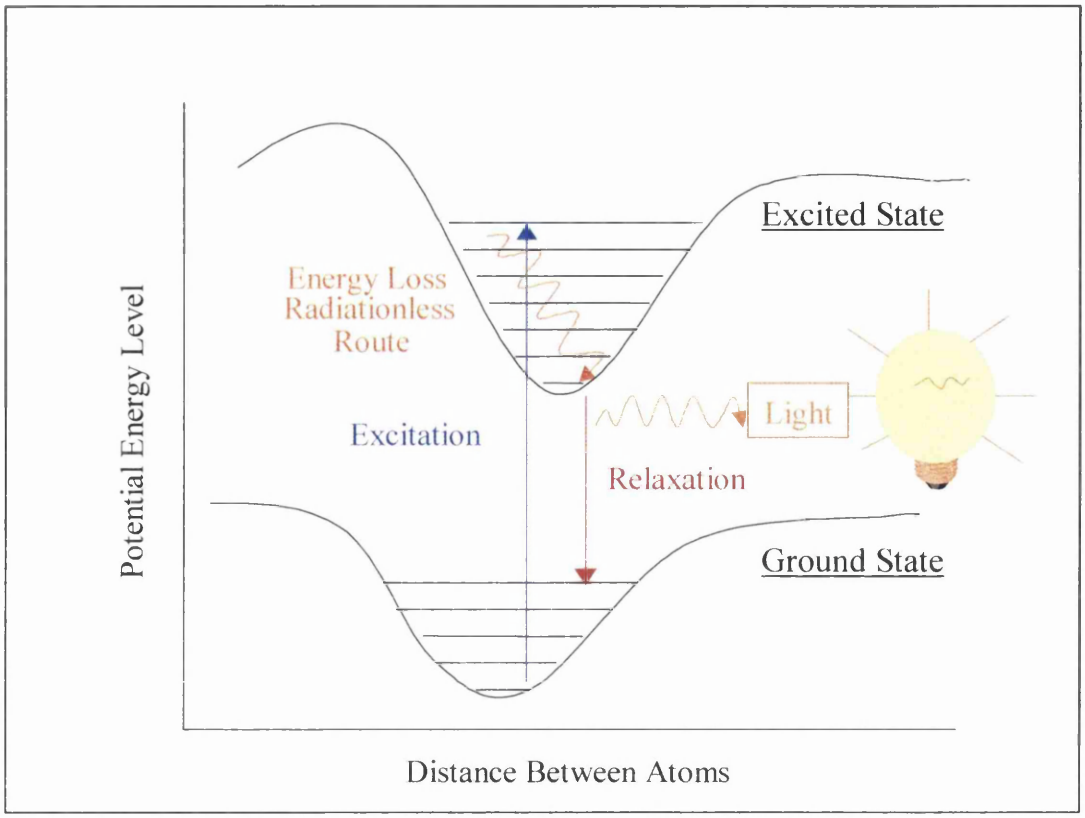


**Figure 3 A potential energy diagram showing how light is emitted from an excited molecule**

Figures 2 and 3 consider only transitions for a molecule that does not alter its chemical state on formation of the excited state. An example of this is photoluminescence. The energy which fuels an electronic transition in a chemiluminescent reaction is not radiation but Gibbs free energy which is released by certain chemical reactions (i.e. if $\Delta G$ is negative). Since the product of a chemiluminescent reaction is different in structure to its reactant, a different potential energy diagram is used to consider the above (shown in figure 4). Figure 4 shows that for any given chemiluminescent reaction, reactant to product formation is more favourable when the product is converted to an excited state prior to relaxation to its ground state, rather than converting directly to the product's ground state. This is because the activation energy required for reactant to product conversion is smaller if the path of the reaction is taken *via* an excited product formation. It is conceivable that many reactions go through the same path if the activation energies are lower for excited product formation. However, not all these reactions will yield light. As mentioned above, there must be between 159.8 and 362.7 kJ / mol energy difference between the lowest excited energy level and the highest ground state energy level to yield light.

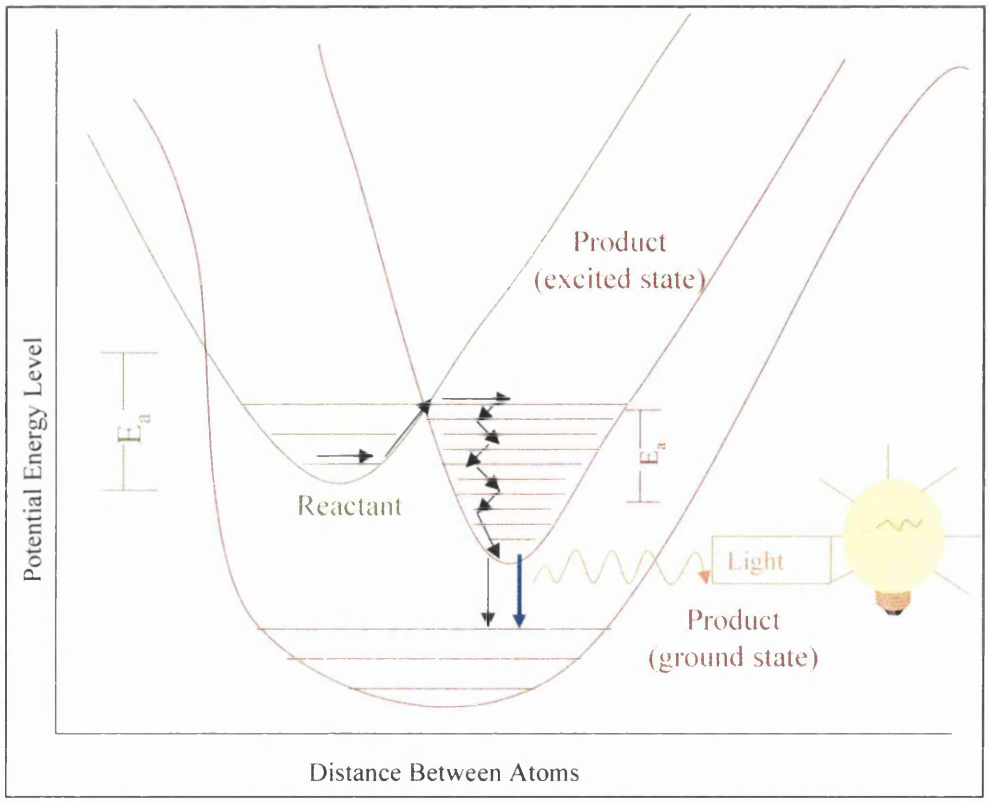


**Figure 4 A potential energy diagram showing how light is evolved from a chemiluminescent reaction**

The types of transitions which can lead to electronic excitations are between π-bonding and π*-antibonding and between n-bonding and π*–antibonding orbitals. π-π* transitions are common in aromatic systems and n-π* transitions are common in groups such as carbonyls. Thus the types of molecules which are luminescent must contain one or both of these groups. Even if a molecule has such groups in its structure, there are many other processes which can prevent it from emitting light. These are summarised in figure 5. [2]

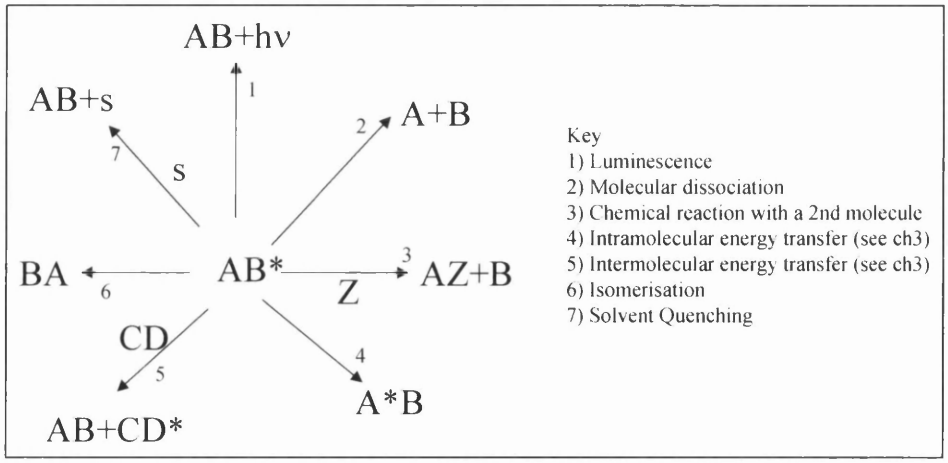


**Figure 5 Seven ways for an excited state to lose energy**

Since there is such competition for how the energy in an excited state can be channelled, there are relatively few reactions, with the capability to produce chemiluminescence. Moreover, those reactions which do chemiluminesce, are quite often inefficient. The chemiluminescence efficiency $\Phi_{CL}$ is composed of three parameters: the chemical yield of the reaction $\Phi_C$; the fraction of product molecules which enter an excited state $\Phi_E$ and the fluorescence quantum yield $\Phi_F$. For example if the $\Phi_C$ of a molecule was 90 % and the $\Phi_E$ was 50 % and furthermore the $\Phi_F$ was 10% then the overall $\Phi_{CL}$ would be 4.5 %.[5]

| $\Phi_{CL} = \Phi_C\Phi_E\Phi_F$ | (3) |
|---|---|

Although there are many factors which do not favour chemiluminescent pathways, chemiluminescent reactions are found in all areas of chemistry and can be classified as follows;

- Biological chemiluminescence
- Organic chemiluminescence
- Ultraweak chemiluminescence
- Inorganic chemiluminescence

## 1.2 Biological chemiluminescence

Bioluminescence - biological chemiluminescence, is found in many organisms, such as bacteria, fungi, dinoflagellates, jellyfish, worms, shrimps, insects, squid and fish. Bioluminescence is used for inter and intraspecies communication, such as mating and warding off attackers. There are up to six components which allow organisms to produce light and these are as follows. [6]

- Oxygen
- A luciferin (a chemiluminescent substrate)
- A luciferase (a protein catalyst)

- Cofactors
- Cations
- A fluor

Oxygen is an essential component in all bioluminescent reactions; it is used to oxidise a luciferin which as a consequence generates an electronically excited species. This excited species emits light or passes the excitation through energy transfer to a fluor, which is the light emitter. The compositions of luciferins differ between species but they are all low molecular weight molecules which contain carbonyls and / or conjugated $\pi$ systems such as arene rings. There are five major chemical classes of luciferins, as listed in table 2.

**Table 2 Major classes of luciferin**

| Type of molecule | Examples |
|---|---|
| Aldehydes coupled to a chromophore | Earthworms, Bacteria |
| Imidazolopyrazines | Decapods, Squids |
| Benzothiazoles | Beetles |
| Linear tetrapyrroles | Dinoflagellates |
| Flavins | Bacteria, Fungi |

Luciferases are mono-oxygenases, peroxidases or oxidases (this is dependent on the species) and act to catalyse oxidation of a luciferin. Some luciferases require cofactors, such as ATP for firefly luciferase and NADPH with the presence of long chain aldehydes for bacterial luciferase. Other luciferases require the presence of a cation such as $Mg^{2+}$, $Ca^{2+}$, $Fe^{2+}$ or $Fe^{3+}$. For example the chemiluminescent reaction of the earthworm requires $Ca^{2+}$ in part of the oxidation step. [7]

Both the firefly and bacterial chemiluminescent systems are used extensively in biology and medicine; their chemiluminescent reactions are illustrated as follows:

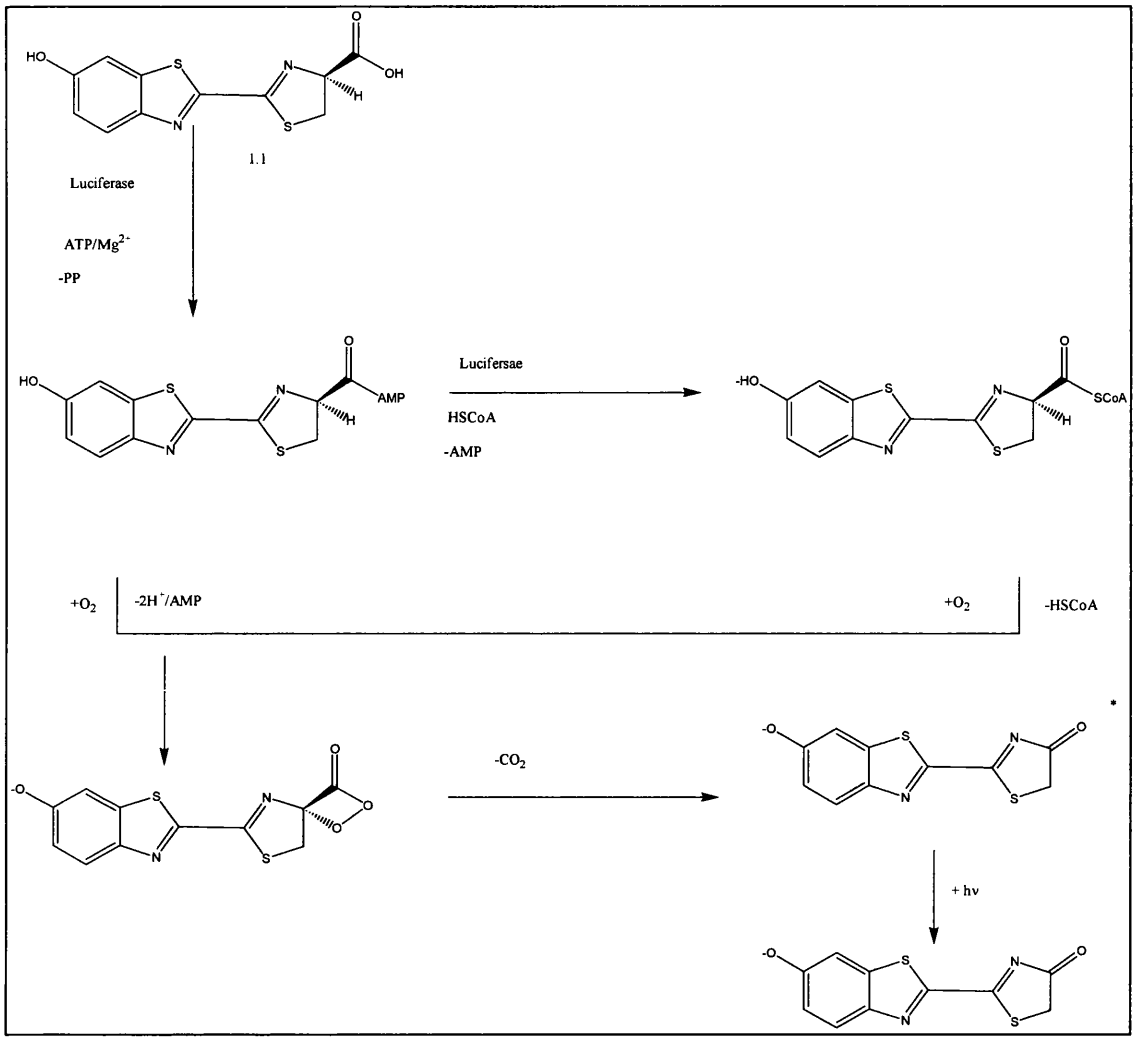


**Figure 6 The firefly (*photimus pyralus*) chemiluminescent reaction scheme**

## 1.2.1 The firefly (*Photimus pyralus*) chemiluminescent reaction

The luciferin of the firefly (a benzothiazole (1.1)) is adenylylated with ATP (in the presence of $Mg^{2+}$) on the carboxy terminus of the molecule. The reaction is catalysed by luciferase, which then catalyses a replacement of the adenylate moiety with coenzyme A. Either the adenylated or the coenzyme A derivative is then oxidised in the presence of $O_2$. As a result of oxidation, the AMP or CoA is lost from the molecule and a high energy dioxetane ring is formed. Upon formation of the dioxetane ring, an intramolecular charge-transfer process occurs, losing $CO_2$ as a result. The charge transfer results in the formation of an excited product which relaxes and releases yellow/green light (see figure 6 for the reaction scheme). This chemiluminescent reaction is the most efficient known, with a $\Phi_{CL}$ of 0.88 Einstein /

Mol. On account of this efficiency, the firefly system is ideal for use in biological and medical analysis, as it can be used to measure minute concentrations of ATP (up to one atomol).[8,9]

### 1.2.2 The chemiluminescent reaction mechanism of chemiluminescent marine bacteria

As previously mentioned, luminescent bacteria require the presence of NADH and a long chain aldehyde (1.2). It is known that an oxidoreductase reduces a FMN to $FMNH_2$ (1.3) using NADH. [3,8] The reduced flavin nucleotide (1.3), which is in fact the luciferin, associates with the luciferase and is subsequently oxidised into a 4a flavin hydroperoxide. The newly formed hydroperoxide forms a flavin peroxyhemiacetal with a long chain aldehyde (1.4). The aldehyde cleaves, oxidising to the carboxylic acid (1.5) and in doing so the flavin moiety forms an excited 4a hydroxide (1.6). As the excited flavin relaxes, it radiates light (490 nm). Finally the hydroxyl moiety on the flavin is hydrolysed and in doing so the flavin is released, which can then be recycled (see Figure 7 for a reaction scheme). An alternative theory suggested for the formation of the excited state, is that an intra or intermolecular energy transfer process occrurs at the active site on the luciferase which excites the flavin moiety. [10, 11]

Bacterial chemiluminescence is not so efficient as the firefly system but does give quantum yields of up to 0.3 Einsteins per Mol.

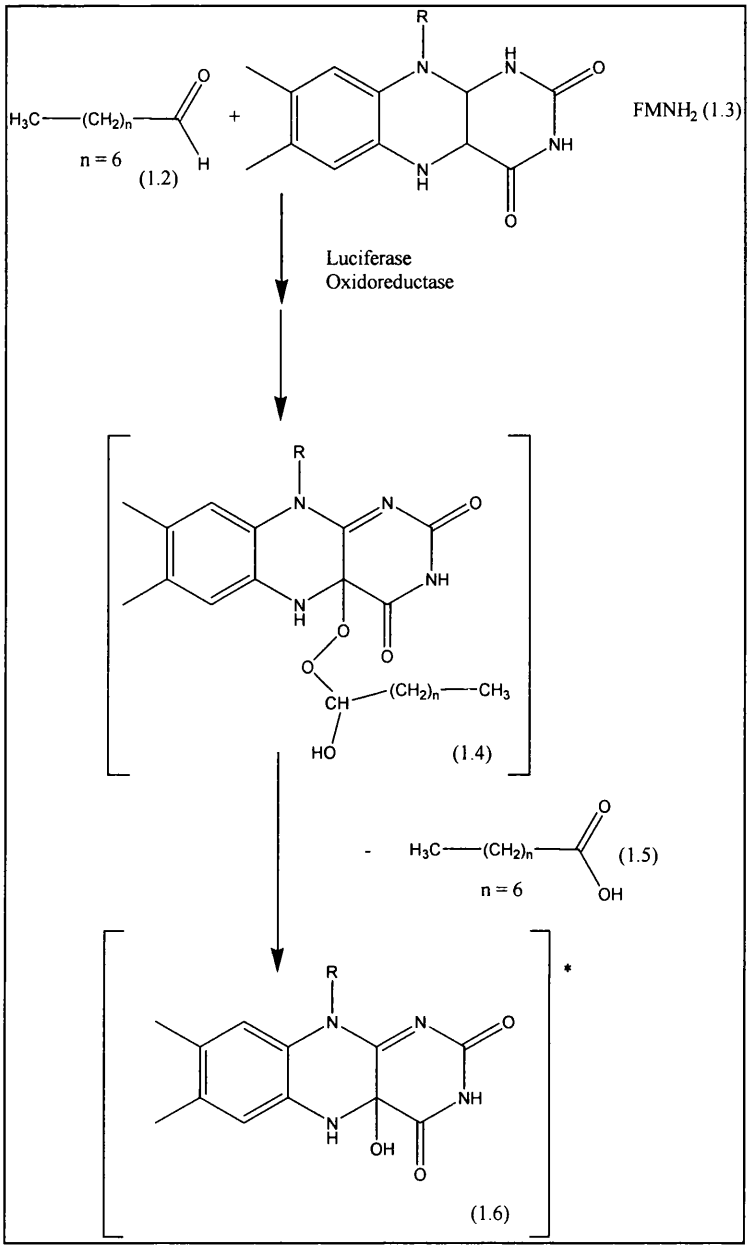


**Figure 7 The chemiluminescent reaction mechanism of chemiluminescent marine bacteria**

## 1.3 Organic chemiluminescence

The main classes of organic molecules which are known to undergo chemiluminescent reactions are as follows.

- Cyclic arylhydrazides e.g. luminol (1.7)
- Dioxetans, e.g. adamantylideneadamantane-1,2-dioxetan (1.8)

- Oxalic acid derivatives e.g. bis-(2,4,6-trichlorophenyl) oxalate (1.9)
- Acridinium derivatives, e.g. lucigenin (1.10)

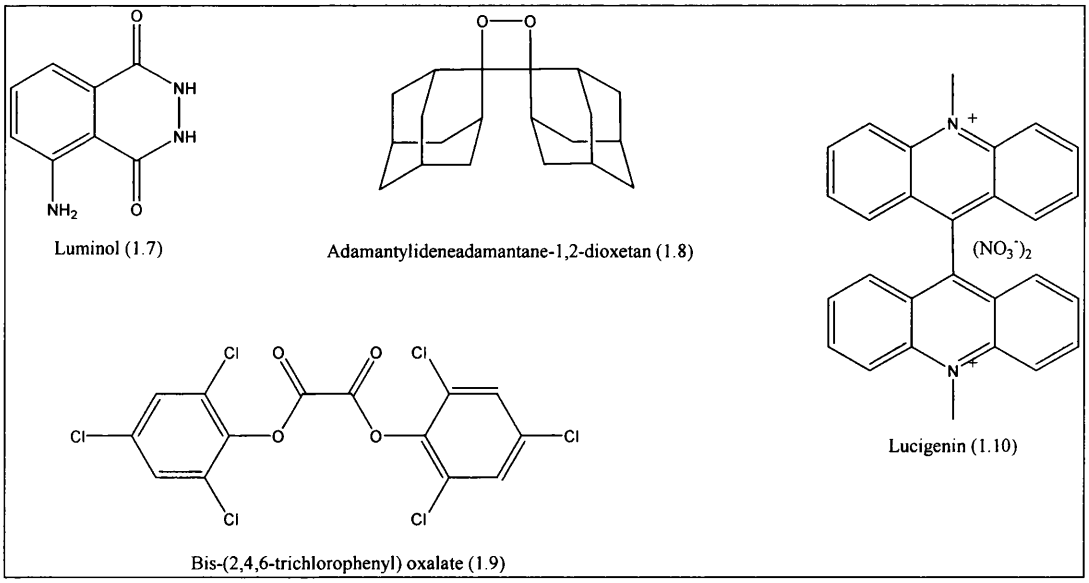


**Figure 8 Examples of chemiluminecent molecules**

Apart from dioxetans, the above classes of compounds undergo an oxidation to generate the chemiluminescent excited state. During this oxidation, peroxy derivatives, dioxetans or dioxetanones form as a result. Dioxetans, however, undergo a thermal decomposition rather than an oxidation to yield the chemiluminescent product. The chemiluminescent mechanisms for the said compounds are discussed below.

### 1.3.1 The chemiluminescent mechanism of the cyclic arylhydrazides

Luminol (5-amino-2,3-dihydro-1,4-phthalazinedione (1.7)) in aprotic organic solvents, can be oxidised by oxygen in the presence of a strong base, leading to chemiluminescence (see Figure 9). However, in aqueous conditions, superoxide or hydrogen peroxide can be used to oxidise the molecule. To increase the rate of the reaction, catalysts such as transition metals (e.g. cobalt (II)), biological molecules (e.g. hemin) and enzymes (e.g. horse radish peroxidase) are used. It is thought that the peroxidation, by either the superoxide or the hydrogen peroxide, leads to the formation of an α-hydroxyhydroperoxide intermediate which decomposes to form the light emitter (the monoanion or the dianion of aminophthalic acid (1.11)).[8,9]

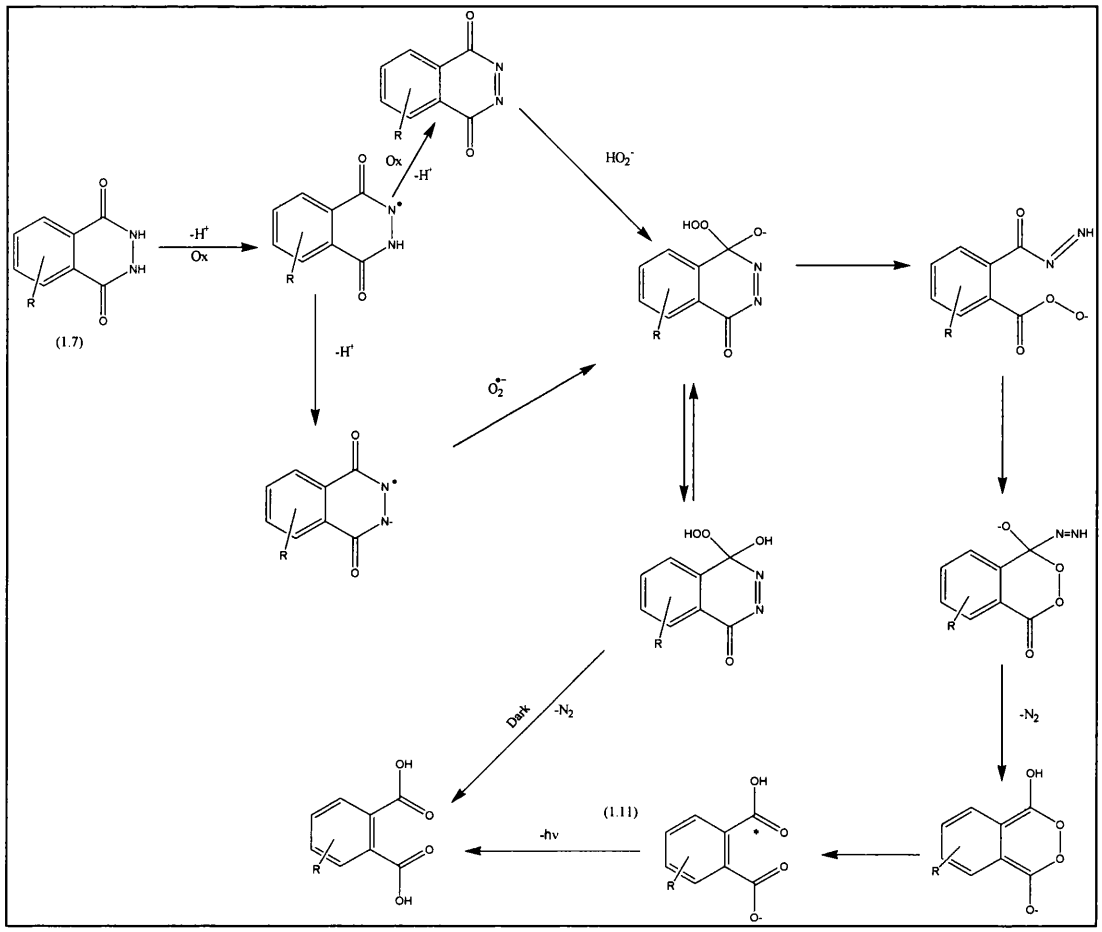


**Figure 9 The chemiluminescent reaction mechanism of the cyclic arylhydrazides**

## 1.3.2 The chemiluminescent mechanisms of the 1,2-dioxetans

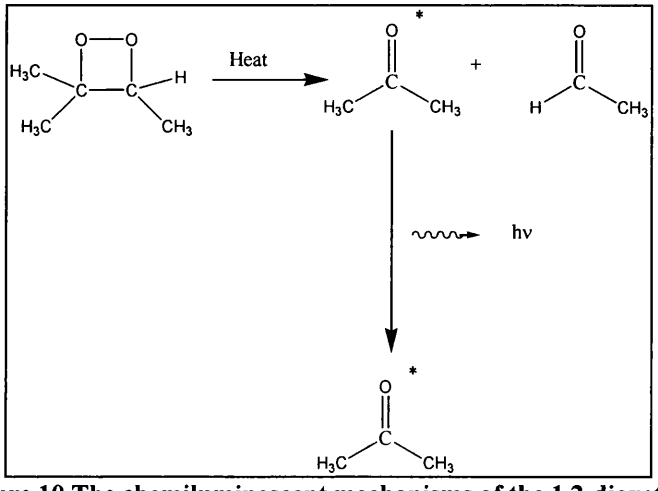


**Figure 10 The chemiluminescent mechanisms of the 1,2-dioxetans**

Dioxetans are a family of compounds which are characterised by containing a 1,2-dioxetane ring (see Figure 10). Flanking the ring are usually aryl or adamantane (1.8) groups.[12] The 1,2-dioxetane ring is four membered and thus is a strained system. The presence of aryl and or adamantane groups help to stabilise the ring through steric interactions preventing cleavage.

Unlike other chemiluminescent molecules, 1,2-dioxetans do not require the interactions of other molecules such as $H_2O_2$ or ATP to release light. Light is released by a thermally induced decomposition. During decomposition, the O-O bond stretches and the $C^1$-$C^2$ acts like a hinge which brings the groups at C1 and C2 together. If the groups have a low oxidation potential such as aryl-$O^-$, then the mechanism of decomposition is through a heterolytic cleavage of the O-O bond. This is initiated by the transfer of an electron from the oxidisable functionality and is called the concerted mechanism. If however the groups are of a higher oxidation potential, such as an adamantyloxy group, then the dioxetane group will break homolytically and this mechanism is called the diradical mechanism. Both the concerted and the diradical mechanisms yield two carbonyl products, one of which is found in an excited state and the other in a ground state.[13]

### 1.3.3 Chemiluminescent mechanisms of oxalic acid derivatives

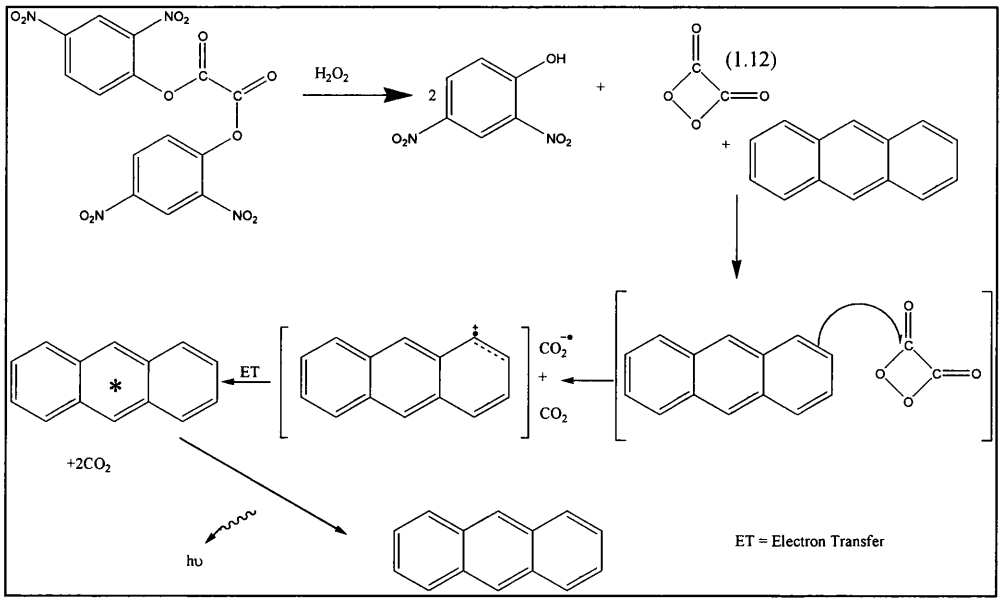


**Figure 11 Chemiluminescent mechanisms of oxalic acid derivatives**

During oxalate ester reactions (see Figure 11), peroxidation of the two carbonyl groups causes cleavage of the flanking groups forming dioxetanedione (1.12). This four-membered ring forms a charge transfer complex with an acceptor molecule which facilitates an electron transfer from the acceptor molecule to the dioxetandione, forming a radical cation and a radical anion The transfer facilitates the breakdown of the four membered ring resulting in the formation of two $CO_2$ molecules where one is a radical anion. The radical anion then transfers an electron back to the radical cation acceptor molecule which becomes excited; on relaxation, light is evolved. This is known as chemically initiated electron exchange luminescence or CIEEL. The CIEEL mechanism can occur both intermolecularly and intramolecularly.[14]

### 1.3.4 The chemiluminescent mechanism of the acridinium esters

Acridinium esters are the most studied chemiluminescent molecules. They are stable, can be used in aqueous media, and require only the addition of hydrogen peroxide to initiate the chemiluminescent reaction. Unlike the cyclic arylhydrazides they do not require a catalyst; unlike the dioxetans, they do not require heat to initiate the luminescence; and unlike the oxalic acid compounds, they do not require the presence of an external fluor.

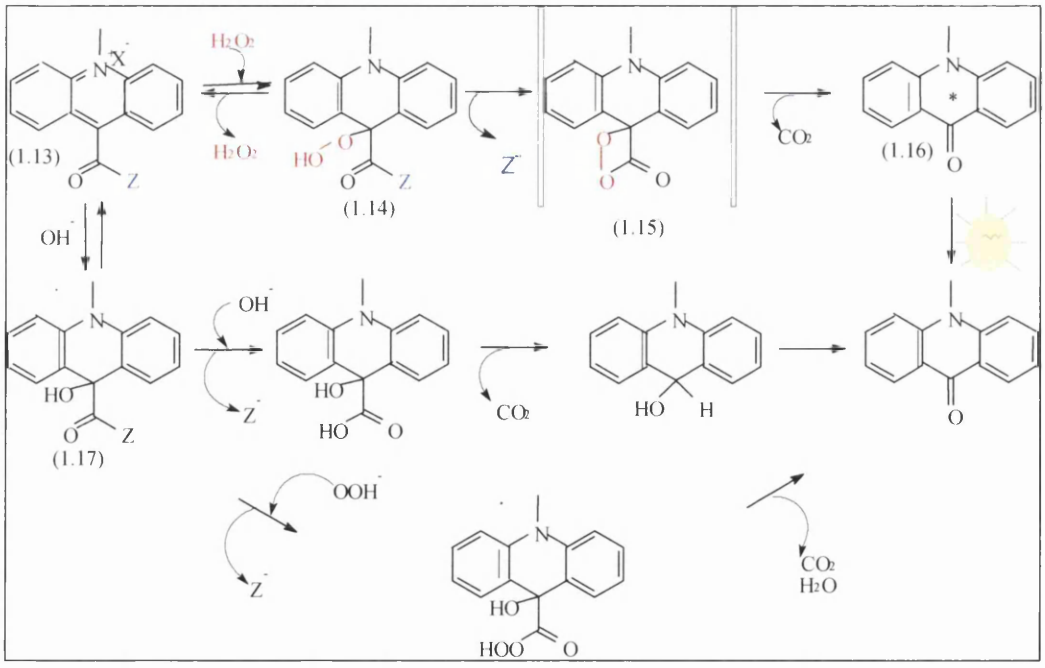


**Figure 12 The chemiluminescent mechanism of the acridinium esters**

Acridinium ester derivatives (1.13) can be oxidised to form *N*-methylacridone (1.16). There are two processes which can lead to the formation of this compound, the first one being the light reaction (see Figure 12). The light reaction proceeds when hydrogen peroxide attacks at the 9-position of the molecule and the hydroperoxide (1.14) formed then cyclises to form the dioxetanone (1.15). Upon cyclisation to the dioxetanone, the group bound to the oxygen, which is in turn bound to the 9-position, is eliminated. The dioxetanone then decomposes to form an excited *N*-methylacridone (1.16) and carbon dioxide. The excited *N*-methylacridone then relaxes to its ground state, releasing light. The second process is called the dark process, whereby acridone is formed in a non-radiative manner. It has been shown that acridinium esters can form pseudo bases with hydroxide ion; when this occurs, the hydroxide groups binds to the 9-position of the acridine ring and an equilibrium occurs between the *N*-methylacridinium-9-carboxylate ester (1.13) and its pseudo base (1.17). When the acridinium is hydroxylated, then *N*-methylacridone (1.16) is formed through this dark process (see section 1.7.1). [8,13]

## 1.4 Ultraweak chemiluminescence

Ultraweak chemiluminescence is one of the areas of chemiluminescence which is becoming increasingly important in biological and biomedical analysis. The definition of ultraweak chemiluminescence is: 'light emitted from chemical reactions in which the intensity is so low that it cannot be detected from the dark-adapted human eye' (below 3 x $10^4$ photons / s / $cm^2$). Surprisingly, ultraweak chemiluminecence is not just observed in classical chemiluminescent processes but is found in many chemical reactions. Moreover, many of these chemical reactions can be seen in biological processes and can be observed using equipment such as single photon counters.

The types of biological processes which can lead to the release of low level light are mitochondrial respiration, photosynthesis, lipid production, egg fertilisation, cell division, eicosenoid production, biocidal reactions in phagocytes and detoxification processes in the liver (using the cytochrome P450 and P448 system). As each of these

processes releases light, all have the potential to be monitored. The chemiluminescent systems which emit low level light have one of three light yielding systems, as discussed below.

1. Production of excited carbonyls, where oxidation of organic compounds forms dioxetane rings which, in turn, cleave forming excited carbonyls. An example of this is found in lipid peroxidation reactions, which occur in concerted reactions found in mitochondria and peroxisomes. Reactions such as this can release blue light (380-460nm).

2. Formation of singlet oxygen: many reactions can form singlet oxygen; examples are peroxide decomposition, aromatic endoperoxide decomposition, electron transfer reactions and energy transfer. An example of the formation of singlet oxygen is in the reactions of triplet state oxygen with excited triplet state chlorophyll in photosynthesis. This can lead to the formation of red light.

3. Energy transfer reactions: whereby the formation of an excited product can transfer its energy to a fluor such as a flavin or tetrapyrrole. An example is the transfer of energy from an active oxygen species to haemoglobin.

Ultraweak chemiluminescence is a non-invasive technique for the detection of metabolites and on account of this, biological processes can be observed in the *in vivo* state. By using such a non-intrusive technique, the chance of artefact formation is reduced, thus offering the investigator a true insight into specific biological processes. However, on account of the fact that the intensity of light is so low in ultraweak chemiluminescence, the concentration of analyte which can be observed is limited by the sensitivity of the detection system. To increase the sensitivity of the system, enhancer molecules can be used and these can enhance the chemiluminescence by 10,000 fold. There are three main enhancer molecules utilised in biological and biomedical analysis. These are luminol (1.7), lucigenin (1.10) and pholasin. Luminol (1.7) is oxidised by $O_2^{\cdot}$, $H_2O_2$, and $OCl^-$ yielding light but is oxidised by $^1O_2$ through a dark reaction. However, lucigenin (1.10) on the other hand can form light reactions with the above molecules but is more specific for singlet oxygen. Pholasin is a

protein-bound luciferin (from *Pholas dactylus*) which reacts with oxygen metabolites to release green light. It is used specifically to detect $O_2^-$ species from neutrophyls.[15]

## 1.5 Inorganic chemiluminescent reactions

Many inorganic elements, when in the right environment, chemiluminesce. For example, elements such as the alkali metals, lead, mercury, oxygen, sulfur, nitrogen, phosphorous and the halogens can all produce light. Inorganic chemiluminescence has not to date been widely used as a tool for biological and biomedical analysis and thus will not be further considered (chapter 1 of reference 1 gives a brief overview).

## 1.6 Applications for chemiluminescence in biology and medicine

There are five major applications in which chemiluminescence is being exploited for biomedical and biological analysis. The applications are shown as follows:

- measurement of metabolites, cofactors and ions using bioluminescence;
- detection of systems releasing ultraweak chemiluminescence;
- chemiluminescence immunoassay as an alternative to radioimmunoassay;
- production of gene probes;
- using luciferin genes in gene fusion experiments to investigate expression of desired proteins.

### 1.6.1 Measurements of metabolites, cofactors and ions

As discussed in section 2, some biological chemiluminescent reactions require the presence of cofactors such as ATP, NAD(P)H and long chain aldehydes. Other bioluminescent reactions require the presence of ions such as calcium. Since these chemiluminescent reactions require these factors to operate, chemiluminescence can therefore be used as a tool to measure these in both biological and medical systems.

Some biological/biomedical applications into the use of metabolites will now be discussed.

### *1.6.1.1 Measurement of ATP using the firefly system.*

It is well known that viable organisms contain much greater concentrations of the nucleotide ATP than dead organisms. On account of this, the detection of ATP can be used as a tool to test for the presence of viable biomass. On account of the fact that the firefly chemiluminescent system requires ATP to chemiluminesce, this system can be utilised to screen for viable biomass. The industries which benefit from this detection system are; clinical microbiology, hygiene, nutrition, brewing, water and milk purification.[16, 17, 18]

Two examples in which the firefly system has been used to detect ATP are urogenital infection and the effects of antibiotics.

### *1.6.1.2 Detection of urogenital infection*

Upon infection of the urogenital tract (this being the kidneys, urethra and bladder), the relative concentration of ATP increases in the urine. This increase of the nucleotide is due to the presence of an infectant, such as the salmonella bacteria or *candida* fungus, inhabiting the area and releasing ATP into the urine. A chemiluminescent assay has been developed to measure the concentration of ATP and thus help to diagnose the biochemical lesion. The system is so sensitive that it can measure down to 100 bacteria per ml. [18,]

### *1.6.1.3 The effect of antibiotics on organisms*

In a study conducted by L. Y. Brovoko *et al.,* [17] the firefly chemiluminescent reaction was used to monitor the concentration of ATP in various bacterial cultures. These cultures were grown for three hours in clinical samples incubated with or without antibiotics (erythromycin, gentamycin and penicillin). In all cases, the

concentrations of ATP in the control samples were significantly higher than the samples grown in the presence of antibiotics. Thus, on account of this novel screening system, patients suffering from bacterial infection can have their pathogens screened for the effectiveness of various antibiotics. Previous screening methods required several days, thus this system has provided a quick and easy screening method. Furthermore, this means that patients can be given the correct antibiotics within three hours of diagnosis.

#### *1.6.1.4 Analytical applications using a bacterial chemiluminescent system as an assay for the detection of NAD(P)H and long chain aldehydes*

The chemiluminescent reaction of marine bacteria (discussed in section 1.2.2) requires the presence of NADP(H) and a long chain aldehyde (1.2); both of these components can therefore be measured in biochemical reactions.

NAD(P)(H) is a very important biological molecule; it acts both to oxidise and reduce the intermediates in the majority of biochemical pathways. Biochemical pathways which yield or consume the nucleotide can be monitored by the bacterial chemiluminescence system. For example, glucose concentrations can be monitored using the reaction which is shown in Figure 13. However, it is the field of fatty acid metabolism which has benefited most from the use of a chemilumiescent detection system.

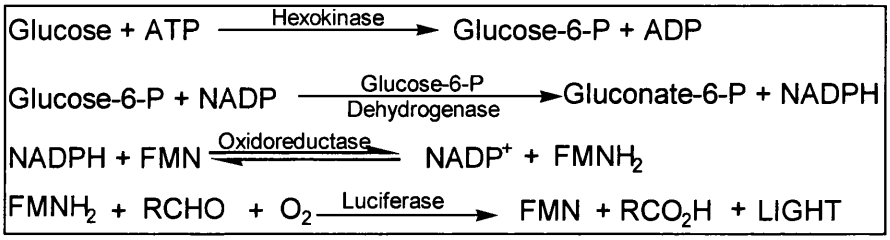


**Figure 13 Glucose concentration measurement using a bacterial chemiluminescent system**

### *1.6.1.5 The use of bacterial chemiluminescence in the study of lipid metabolism and heart disease*

Heart disease is the biggest killer in the United Kingdom. The causes of heart disease range from smoking and stress to genetic disorders such as congenital hypercholesterolemia and poor diet (fat and cholesterol rich foods). In recent years, there has been much investigation into the causes of heart disease and ways to prevent the onset of the illness. There has been much research into the metabolism of fats and it is hoped that a clearer understanding of this subject will lead to more effective treatments and protocols for prevention. The bacterial chemiluminescent system has been employed to help elucidate the metabolism of fats by monitoring pathways which yield NADH molecules quantitatively. Concentrations of glycerol, free fatty acids, lactate and β-hydoxybutyrate have been monitored from specimens of human adipose tissue using this system.[19]

It has been known that congenital disorders and cholesterol rich diets can lead to the blocking of arteries with cholesterol esters, which can lead to heart disease. There are a group of protein molecules which transport fats and cholesterol around the body which are collectively known as lipoproteins. Moreover, it has been discovered that a class of lipoproteins called LDLs (low density lipoproteins) carry the bulk of cholesterol around the body. The LDLs, which are synthesised in the liver, are mostly absorbed by peripheral cells and metabolised. Nevertheless, monocyte-macrophages (components of the immune system) can also absorb the LDLs. However, macrophages then act to block the arteries by depositing cholesterol esters into the subendothelial space.

Enhanced uptake of the LDLs into macrophages is catalysed by an *in vitro* peroxidation of the fatty acids forming aldehydes such as hexanal (which has been found to be the most abundant). Until the development of a chemiluminescent assay, there was no sensitive assay available to measure the degree of lipid peroxidation. Nevertheless, an assay derived from the bacterial chemiluminescent system has been described and it utilises the fact that bacterial chemiluminescence requires the presence of a long chain aldehyde. As shown in Figure 14, oxidation of the

unsaturated fat from LDL molecules, provided a source of aldehyde for the chemiluminescent reaction and the intensity of light indicated the aldehyde concentration (see Figure 14). [20]

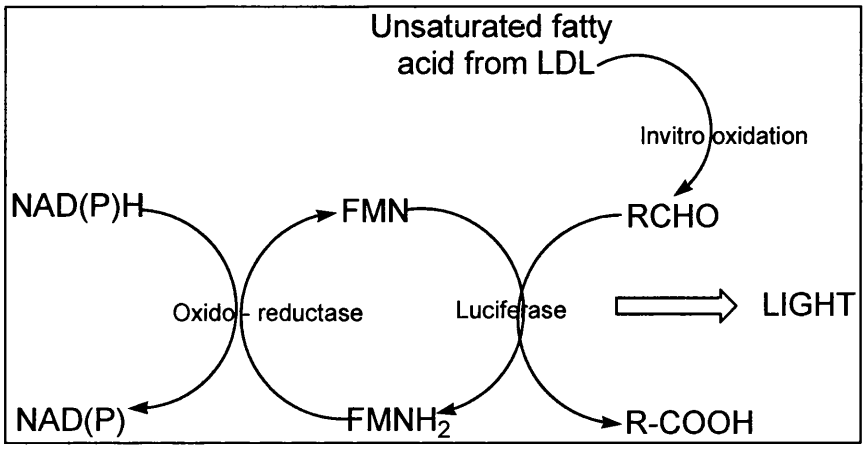


**Figure 14 Measurement of aldehydes using the bacterial chemiluminescent system**

### 1.6.2 Use of ultraweak chemiluminescence in biology and medicine

Much work has been carried out to develop techniques to measure the activity of polymorphonuclear leukocytes (PMNs), by monitoring their intrinsic production of ultraweak chemiluminescence.[21,22,23,24] PMNs, which include phagocytes and monocytes, are potent cells found in the mammalian immune system. They work in concert with other immune cells to destroy pathogenic organisms. The mechanism of detection and destruction of pathogens is complex and still not fully understood. However, it is known that on detection of a pathogen, the phagocyte engulfs the invading organism and destroys it from within.

One of the ways in which phagocytes destroy pathogens is to release active oxygen species, such as superoxide, singlet oxygen, hydroxyl radicals or hydrogen peroxide. These oxygen species are generated by activation of a membrane-bound NADPH-oxidase; the oxidase is activated by a cascade of intracellular messengers, which in turn is activated by recognition of the pathogen on the surface of the phagocyte. Hydrogen peroxide is a substrate for an enzyme called myeloperoxidase, which releases dioxygenate products. The metabolites from both NADPH-oxidase and myeloperoxidase induce death of the microbes by causing extensive oxidative damage. [21,22]

Each of the oxygen species releases low level light and this light can be enhanced by the enhancer molecules, luminol (1.7) and lucigenin (1.10). It has been found that lucigenin is more specific for detecting the activity of NADPH-oxidase by measuring for the presence of superoxide while luminol is more specific for detecting the oxygen species from myeloperoxidase.[22] Due to this, the activities of each of these enzymes can be monitored separately. On account of the fact that PMNs release light, their metabolism can be monitored.

Work has been conducted using the ultraweak chemiluminescence emitted from PMNs to gain a broad understanding of what can trigger the phagocyte function.[23] Recently, experiments using ultraweak chemiluminescence have elaborated more fully the role of fragment crystalline receptors (receptors which bind antibodies) and complement receptors (receptors which bind antibody-like molecules called complement) in the detection of pathogens.[25]

There has been increasing interest in using the chemiluminescence released from PMNs to help diagnose various diseases. The reason why PMNs can be used in this way is that the disease state either reduces the number of PMNs, or promotes the synthesis of dysfunctional PMNs. A reduction of the number of PMNs or their effectiveness will reduce the production of active oxygen species and thus reduce the light output. Diseases which show a dysfunction in PMN metabolism include: chronic granomatous disease (CGD), myeloperoxidase deficiency, mannosidosis, Downs syndrome, psoriasis, uremia, heamopathies (e.g. leukaemia), chediak-higashi and cystic fibrosis.[22,23]

A potent production of ultraweak chemiluminescence in mammals is during fatty acid metabolism. There are two groups of lipid metabolites called the eicosanoids and the leukotrienes. These metabolites are synthesised through enzymes called cycloxygenase and lipoxygenase, respectively. The substrate for these enzymes is the fatty acid called arachidonic acid and the synthesis of this metabolite requires the formation of peroxide intermediates via free radical reactions. The reactions go on to form products with excited carbonyls and singlet oxygen. Both of these products emit ultraweak chemiluminescence. The chemiluminescence from these reactions can be

exploited to measure the degree of oxidative damage caused by such free radical reactions. Vitamin E and glutathione are two of the most potent antioxidants found in mammals and work with perfused rat hearts has found that a reduction of serum vitamin E and glutathione enhances the cellular chemiluminescence resulting from oxidative stress. This indictes that vitamin E and glutathione have a positive role in preventing oxidative damage.[26]

There is some evidence that cancer cells emit significantly more ultraweak chemiluminescence than non cancerous tissues. Ultraweak chemiluminescence has been monitored for a variety of different cancers and non cancerous tissues. A report has indicated that non-cancerous tissues released an average value of 22 ± 6 photons/cm$^2$/min whereas cancerous tissues released on average the considerably higher light output of 300 ± 90 photons/cm$^2$/min.[27] The report does not elaborate as to why cancer cells should release a higher chemiluminescence in comparison with other tissues. However, it is a possibility that because cancer cells divide rapidly, their metabolic rates run at a higher level than non cancerous cells. Due to this, it is a possibility that the normal ultraweak chemiluminescence released is enhanced proportionally to the increase in metabolic rate. From the study it is hoped that in the future, cancers will be partially diagnosed by measuring their intrinsic ultraweak chemiluminescence.

### 1.6.3 Chemiluminescence immunoassay as an alternative to radioimmunoassay

Immunoassay is one of the most powerful techniques used in the measurement of metabolites in biology and medicine. Almost any biological substance can be measured using this technique and picomolar concentrations can be measured quantitatively. In the 1950s, radioimmunoassay was developed for the detection of insulin and since, a myriad of variations have been developed for the detection of countless compounds. The compounds range from molecular messengers to enzyme subunits to pathogens. Table 3 shows a selection of clinically important analytes. [28]

**Table 3 Analytes detected using chemiluminescent immunoassay and concentrations achieved**

| **Concentration** | | **Concentration** | | **Concentration** | |
|---|---|---|---|---|---|
| **µmol/l** | | **nmol/l** | | **pmol/l** | |
| Albumin | 600 | Estriol | 600 | Aldosterone | 180 |
| IgG | 90 | TBG | 500 | Insulin | 120 |
| IgM | 1 | Cortisol | 400 | PTH | 100 |
| | | hPL | 300 | HGH | 50 |
| | | T4 | 125 | LH | 10 |
| | | Corticosterone | 20 | fT3 | 10 |

### *1.6.3.1 The types of immunoassay*

#### 1.6.3.1.1 Immunoassay

The principal of immunoassay is to take the metabolite of interest and inject it into an animal which has the potential to raise specific antibodies against it. The antibodies are isolated and used to test for the presence of the metabolite via a competition assay where a known amount of antibody and a known amount of labelled antigen are added to an unknown concentration of the metabolite. The labelled and unlabeled antigens compete for the antibodies. The antibody / antigen complexes are separated from the free components and assayed for the detection of the label. The count is inversely proportional to the concentration of metabolite.

The rate equation for the competition assay is as follows:

$$Ab + Ag^* + Ag \underset{}{\overset{K}{\rightleftharpoons}} AbAg^* + AbAg \qquad (4)$$

Where:

**Ab** = Antibody

**Ag*** = Labelled antigen

**Ag** = Unlabelled antigen

**AbAg*** = Antibody / labelled antigen complex

**AbAg** = Antibody / unlabelled antigen complex

1.6.3.1.2 Immunometric assays

Immunometric assays are like immunoassays, where antibodies are used specifically to detect and quantitatively measure the existence of the analyte of interest. However, unlike immunoassays, immunometric assays use labelled antibodies rather than labelled antigens. The assay is non-competitive, thus the count is directly proportional to the amount of antigen antibody complex isolated, which in turn is directly proportional to the amount of the analyte of interest.

The rate equation of the immunometric assay is as follows:

| | |
|---|---|
| $*Ab + Ag \underset{}{\overset{K}{\rightleftharpoons}} *AbAg$ | (5) |

*1.6.3.2 Radio labels, dangerous markers*

Radioisotopes were first used as the labels of choice for immunoassays and still are widely used today in many assays. They are popular markers because they are non-intrusive and are very sensitive (down to attomolar concentrations). Popular isotopes used are $^{125}I$ , $^{32}P$ and $^{14}C$. However, there are many problems involved with using radioisotopes in immunoassays, including the following.

- The higher the specific activity, the shorter the half life, thus the shorter the shelf life of the label.
- The lower the specific activity, the less sensitive the label is.
- Dangers to health in handling radio isotopes, due to their mutagenic properties.
- Dangers and problems in disposing of the labels in a safe and environmentally 'friendly' way.
- Radiolytic degradation of the conjugated antigen or antibody.

*1.6.3.3 Alternatives to radio labels*

Stemming from the problems outlined above, alternative markers have been developed, which are both safe to handle and show comparable sensitivities. Some of

these alternatives involve the use of colorimetric dyes, fluorescent labels, enzymes and chemiluminescent labels.

There has been much success in the development of chemiluminescent labels as alternatives to radio labels for both immunoassays and immunometric assays. Many of the molecules described in sections 1.2 and 1.3 can be used as chemiluminescent probe molecules.

Figure 15 shows a selection of some of the chemiluminescent labels used in immunoassays. The acridinium esters (1.7) and the isoluminol (1.18) derivatives are used extensively in chemiluminescence immunoassays and chemiluminescence immunometric assays. It can be seen from Figure 15 that both labels have groups which can link onto proteins, these being the R groups for the isoluminol derivatives (1.18) and the Z groups for the acridinium esters. [29]

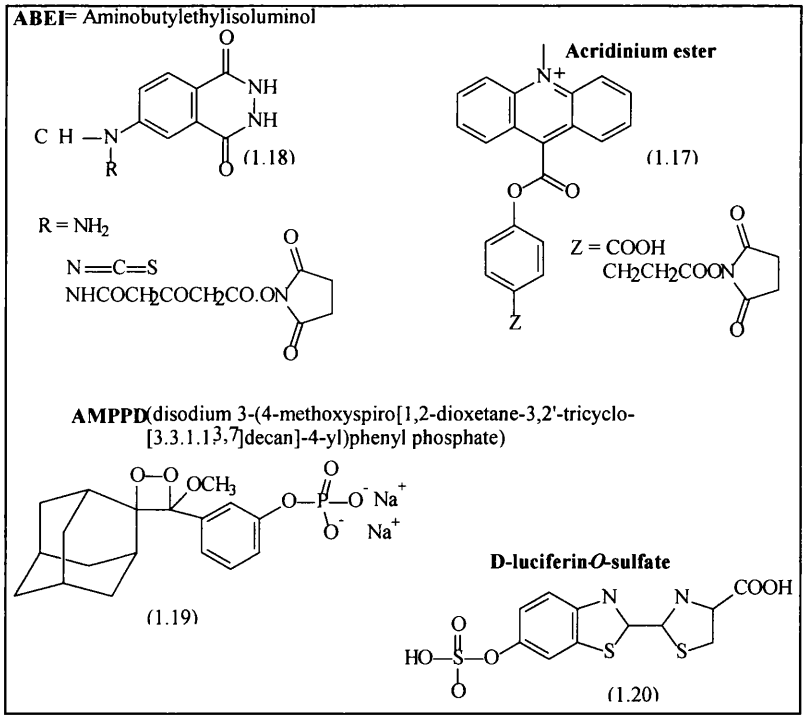


**Figure 15 A selection of labels used in immunoassays**

### *1.6.3.4 Enzyme linked immunosorbant assays*

Dioxetan (1.19), luminol (1.18) and luciferin (1.20) derivatives can be used as chemiluminescent substrates in enzyme linked immunoassays.[30,31,32,33] The enzyme assay which is extensively used is called enzyme linked immunosorbant assay (ELISA).[34] To perform an ELISA, an antibody ($Ab_1$) specific for the antigen of interest, is mounted on a solid support and is preincubated with the antigen (Ag). The $Ab_1Ag$ conjugate is washed to remove any free $Ab_1$ or Ag. Following this, a second antibody ($Ab_2$), which is linked to the enzyme, is incubated with the $Ab_1Ag$ conjugate to form a $Ab_1AgAb_2$-enzyme conjugate. The final conjugate is washed and exposed to the chemiluminescent substrates, which have the potential to release light when catalysed by the bound enzyme. The intensity and speed of the chemiluminescent reaction are parameters used to determine the amount of antigen present. The amount of antigen present is directly proportional to the concentration of enzyme present in the reaction medium. The higher the concentration of enzyme present, the faster the reaction and the higher the intensity of light.

For luminol and its derivatives (1.18), peroxidase enzymes are the sole enzymes which can be used in enzyme labelled assays (see page 9) Moreover, on account of the fact that luminol reacts with $H_2O_2$ without the presence of a peroxidase (albeit very slowly), there is always a very high blank associated with luminol / peroxidase reactions. On account of this, the sensitivity of luminol / peroxidase reactions is only in the femtomolar range.[35] To improve the sensitivity of the reactions and the range of enzymes which can be detected, work has been conducted to derivatise other chemiluminescent molecules such that they either chemiluminesce, or have the potential to chemiluminesce when in contact with other enzymes. Molecules which are being exploited to this end are derivatised dioxetans (1.19) and luciferins (1.20) .[30,31,32,33] These derivatives include phosphates, *O*-sulfates, and galactosides and their corresponding enzymes are phosphatases, sulfatases and galactosidases.

#### 1.6.3.4.1 Dioxetan derivatives

Dioxetan derivatives such as AMPPD (shown in Figure 16, (1.19)) work on the principal that whilst in the derivatised state, the dioxetan is very stable and will not thermolytically decompose (see page 9).[30,31,32] However, exposure to an enzyme such as phosphatase will cleave the cleavable moiety releasing a phenoxy anion. This anion, which is thermolytically unstable, will decompose and in doing so release light.

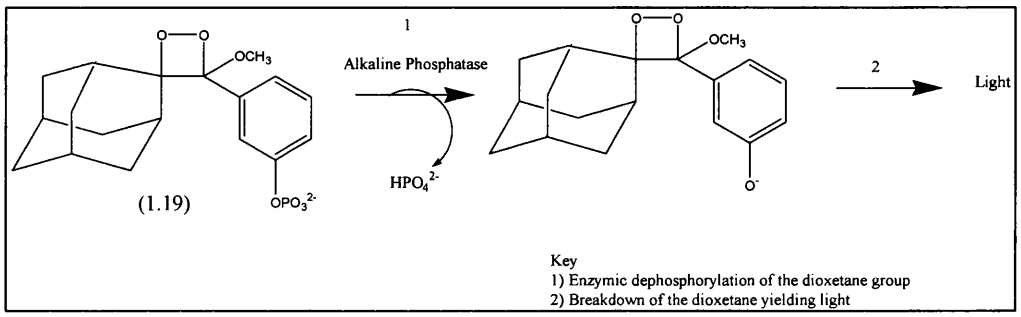


**Figure 16 Decomposition reaction of a 1,2 dioxetan DNA probe**

1.6.3.4.2 Luciferin derivatives

Derivatised luciferins work on the principal that they will not react with luciferase whist in the derivatised state and in doing so will not chemiluminesce. An example is D luciferin-*O*-sulfate (1.20), shown in Figure 15.[33] On exposure to an enzyme such as sulfatase, the *O*-sulfate is cleaved and in doing so releases free D luciferin which can then chemiluminesce when exposed to $H_2O_2$ and luciferase.

## 1.6.4 Chemiluminescent DNA probe molecules

DNA probes are oligonucleotides conjugated to a marker, such as a radioactive isotope or an enzyme. They are used in DNA hybridisation assays, where pieces of unknown DNA are exposed to the DNA probes (which have specific base sequences). If the unknown DNA strands contain base-pair sequences which are complementary to the probe, then the probe will anneal to the unknown DNA strand. However, if the unknown DNA strand does not contain a complementary base-pair sequence, then the probe will not anneal and will be washed off. On account of the fact that these DNA probes can anneal to complementary sequences on strands of DNA, these probes can be used to look for specific base sequences such as genes. To see which pieces of

DNA have probes annealed and which do not, reactions are conducted to visualise the marker adjoined to the probe.

Chemiluminescent labels, like those used in immunoassays, have been used as DNA probe labels. Indeed, both the acridinium esters (1.17) and the dioxetans have been used. In experiments using acridinium esters, the molecules were bound directly to the DNA probes and the release of chemiluminescence was accomplished by addition of $H_2O_2$. Moreover, excellent sensitivities of detection were attained, with a detection limit of up to $5 \times 10^{-19}$ mol/l.

In contrast to the acridinium esters, dioxetan derivatives have been used as substrates for enzymes, which are themselves conjugated to the DNA probes. An example of such enzymes are phosphatases; these can react with dioxetan derivatives, such as AMPPD (1.19), and in doing so chemiluminesce.[36] Good detection sensitivities have been found for reactions using the dioxetan system ($10^{-15}$ mol/l). Unfortunately though these sensitivities have been found to be lower than for the acridinium ester labels. The reason for this is because the background light levels were found to be high due to non-enzymic dephosphorylation of the dioxetan derivative.

On account of the fact that genes can be probed, the diagnosis of diseases can be accomplished by probing for infectant DNA and for malfunctionning genes caused by the disease state. Chemiluminescence, when used in conjunction with oligonucleotide probes will, in the future, be a specific, easy and safe method to visualise lesions in metabolism.

### 1.6.5 Detection of gene expression using bioluminescence

A definition of gene expression is: 'the biosynthesis of messenger RNA of a specific gene, which then has the potential to be translated into a protein'. More often than not, gene expression and protein synthesis can be regarded as the same phenomenon. The biosynthesis of proteins in cells is an amazingly regulated process, so much so that the correct protein is synthesised at the right time and in exactly the right amount. One way to detect for gene expression is to probe for the mRNA (see page 21). A

second method to detect for gene expression is to fuse a reporter gene to the gene of interest. By creating this fusion, when the cell expresses the gene of interest, it will synthesise a fusion protein. If the fusion protein contains luciferase as the reporter gene, then this will catalyse a light reaction which can quantitatively measure the gene expression.

On account of the fact that genes can be fused in this fashion, the expression of almost any gene can be monitored. For example, using the luciferase genes of the firefly, expression of oestrogen can be monitored. It is a well known fact that increases in oestrogen concentrations can enhance the synthesis of some cancers, such as breast cancer. Various drugs such as tamoxifen have been developed to halt the synthesis of oestrogen which in turn halts tumour synthesis. Until recently, the screening tests to characterise the oestrogenicity or antioestrogenicity of new drugs generally required several days. Recently, an assay has been developed to test the effectiveness of such drugs using the firefly luciferase genes linked to oestrogen genes. This assay can give significant results within 48 hours.[37] There are many other examples of the detection of gene expression using chemiluminescence and indeed the use of chemiluminescence to detect for gene expression will be more and more commonplace as the technology develops.

### 1.6.6 Conclusions

The applications of chemiluminescence in biology and medicine are vast. These applications range from the detection of metabolites through to the monitoring of gene expression. Chemiluminescence is a safe tool and thus has the potential to replace radioactive isotopes in practically all assays, where once radioactivity was the only option. Chemiluminescence can also be non-intrusive and also the monitoring of specific biological processes can be facilitated by exploiting the intrinsic ultraweak chemiluminescence released by some metabolites. Chemiluminescence is incredibly sensitive; it is hoped that soon some immunoassays will be so sensitive that they will detect down to the zeptomolar level (100 molecules/l). Due to the amazing sensitivity of chemiluminescence, novel metabolites will be detected in pathways which were

previously thought to be fully elucidated. Moreover, this sensitivity will aid in the elucidation of pathways which are not yet fully rationalised. Thus it should be recognised that chemiluminescence has the potential to 'light the future' for a better understanding of life.

## 1.7 The development of acridinium ester compounds for use as chemiluminescent probe molecules

### 1.7.1 History

The first record of light emission from an acridinium compound was in 1935 where lucigenin (1.10) was found to release light on the application of alkaline peroxide.[38] The reaction yielded two molecules of *N*-methylacridone (1.16) one of which was excited and upon relaxation to its ground state yielded light at 500 nm. Although this was of intellectual interest, this did not show any commercial benefit thus no further work was done with acridinium compounds until the mid 1960s. During the 1960's, several acridines were synthesised which included 9-carboxy-10-methylacridinium chloride (1.21), 9-cyano-10-methylacridinium nitrate (1.22) and 9-carboxy-10-methylacridine hydrochloride (1.23) (see Figure 17).[39,40]

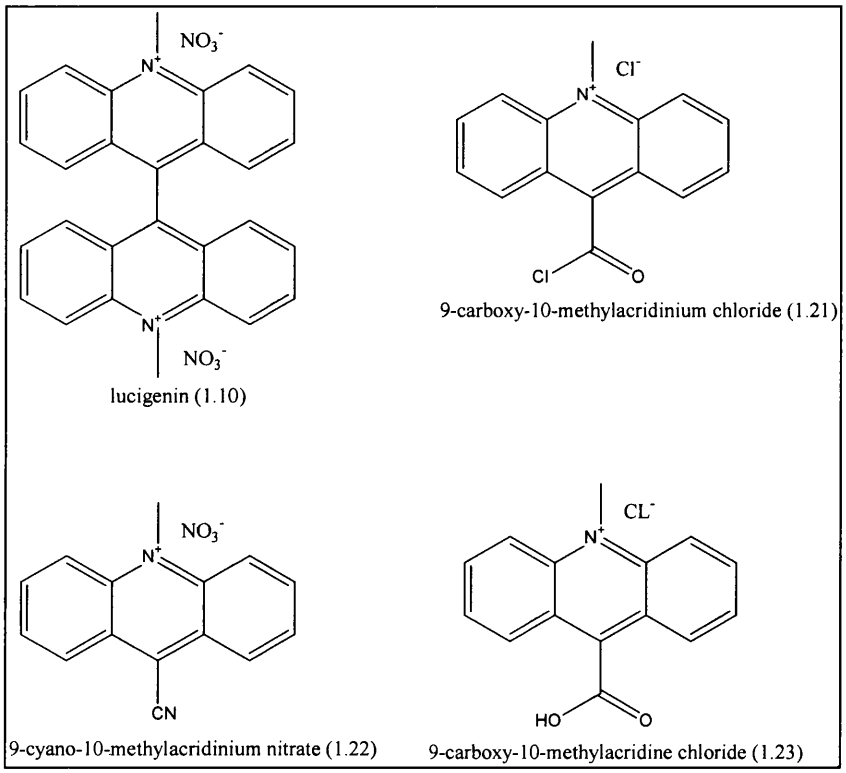


**Figure 17 Early acridnium molecules**

The first acridinium molecule synthesised for use as a probe molecule for chemiluminescence immunoassay was synthesised in the early 1980s by Ian Weeks, Stuart Woodhead and co workers.[41] The molecule; 4-(2-succinimidyloxycarbonylethyl)phenyl-10-methylacridinium-9-carboxylate fluorosulfonate (1.24), known as the Weeks-Woodhead label, is considered as the benchmark acridinium label (see Figure 18).

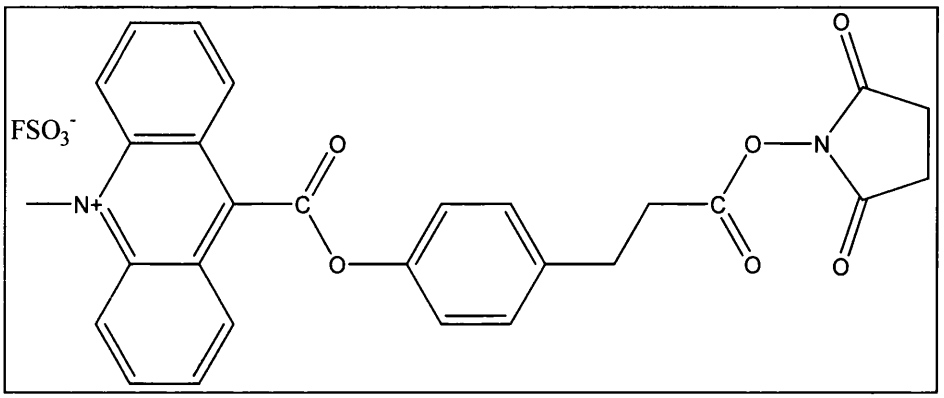


**Figure 18 The Weeks-Woodhead label (1.24)**

The Weeks-Woodhead label is designed for two functions; the first (which occurs at the acridinium head) is to emit light through a reaction using hydroperoxy attack in basic conditions (the mechanism of this is discussed in section 1.3.4). The second function (succinimidyl group) is to bind to free $NH_2$ moiety groups on protein molecules such as immunoglobulins (see Figure 19).

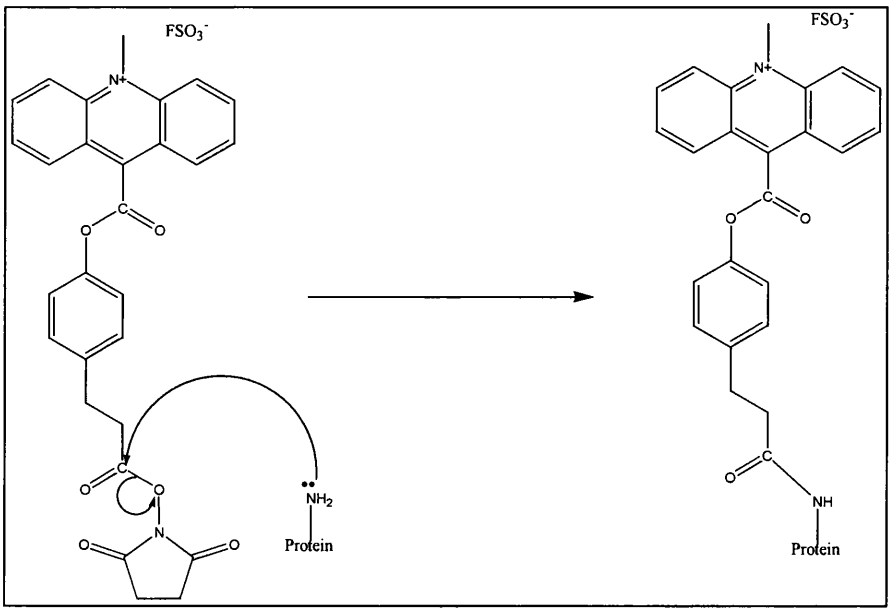


**Figure 19 Attachment of an acridinium probe molecule onto a protein**

The molecule is designed such that the two functional moieties are located at either end of the molecule. They are located in this fashion to minimise any intramolecular interaction. Furthermore, the molecule is designed so that when bound to a protein of interest and then exposed to hydrogen peroxide in basic conditions, the actual light emitter (*N*-methylacridone (1.16)) leaves the bound label. This has a distinct advantage over other chemiluminescent probes used for binding to proteins, on account of the fact that light quenching from the protein is minimised as the emitter is not bound. The spacer group (usually a dicarboxylate) links the acridinium head to the protein attachment site and has two functions. The first is to prevent the above mentioned interaction and the second to act as a good leaving group on hydroperoxy attack. However, the downside of this is that the ester group between the acridine group and the benzene ring does tend to be prone to hydrolysis. On account of this, acridinium ester molecules undergo dark reactions under basic conditions (see Figure 20).

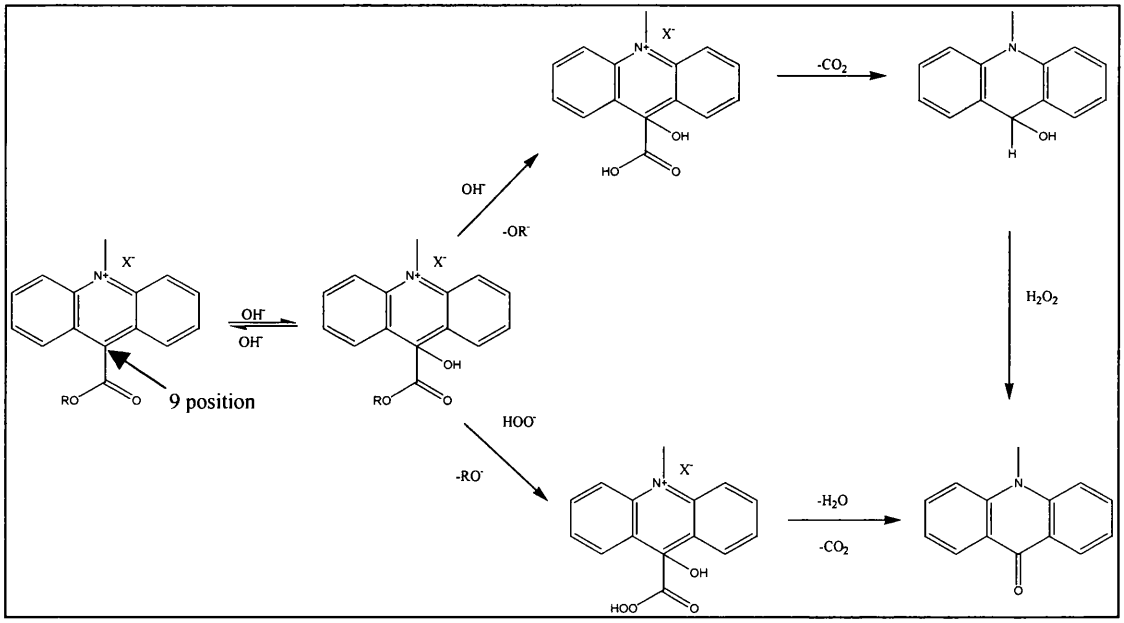


**Figure 20 Dark reactions of acridinum esters.**

For every chemiluminescent reaction of the acridinium series, there is always competition between nucleophiles for the 9-position. The nucleophiles, which are in abundance, are $HO^-$ and $HOO^-$. However, thankfully peroxide is 10,000 times more nucleophilic than hydroxide, thus peroxidation of the 9-position does predominate over hydroxide attack. However, hydrogen peroxide has a $pK_a$ of ~11 and so pH

concentrations below this protonate the peroxide thus significantly reducing its nucleophilic character.[42] On account of this, pHs below 11 do not favour either hydroperoxy or hydroxy attack. However, pHs above pH 11 favour hydroperoxy attack but also favour hydroxy attack and thus promote dark reactions. To attempt to minimise hydroxy attack, an excess of hydrogen peroxide could be used, however the problem is that hydrogen peroxide requires an excess of $OH^-$ to be deprotonated. Thus the order of addition of the components of the chemiluminescent reaction are the key to a successful chemiluminescent reaction and promote the light reaction over the dark pathways. The acridinium ester is pre-incubated at pH 5-6 in the presence of hydrogen peroxide. To initiate light an injection of base is used to raise the pH to above 11; this then elicits a short (~ 1 second blue [430 nm]) flash.[43]

A prerequisite of immunoassays is to be able to conduct these under aqueous conditions, thus a chemiluminescent probe molecule has to be stable for sometimes several days in aqueous environments. As mentioned in section 1.3.3, the chemiluminescence of oxalate ester molecules is significantly more efficient ($\Phi_{CL}$ 0.34) than their acridinium ester cousins ($\Phi_{CL}$ 0.05) but unfortunately due to the fact that they instantly hydrolyse in aqueous environments then the only useful application of these molecules is as light sticks.[44] Chemiluminescent immunoassay probe molecules have been developed using luciferins (1.20), luminol derivatives (1.18) and dioxetans (1.19) (see Figure 15). However these systems do have significant drawbacks, they rely on the presence of enzymes and or cofactors and light enhancers. This increases the likelihood of high background levels of light. Although the quantum yield of acridinium esters are only at best 5 %, due to the simplicity of the light reaction, a 10 to 100 fold decrease in background light is observed when compared with molecules which do require enzymes and / or co-factors.[45] Thus, the overall advantage of acridinium ester molecules is that their reaction is less complicated, and hence more robust and sensitive assays can be developed as a result.

Although the Weeks-Woodhead label demonstrated that acridinium ester molecules were suitable for chemilumescence immunoassay, there are many improvements that could be and have been subsequently made to modify the stability to hydrolysis,

colour choice, solubility in aqueous environments, kinetics of light output and overall $\Phi_{CL}$.

## 1.7.2 Improvements to the Weeks-Woodhead label

### *1.7.2.1 Modification of the head group*

Work has been done to modify the head group for several reasons, the first was to change the wavelength of light and thus allow for colour choice. For example the acridine head group has been modified with methoxy groups (1.25) which has resulted in light of longer wavelengths to be emitted. [46] Furthermore a tetracyclic homologue (1.26) of the acridininium head group has been synthesised by Law *et al* again this molecule shows a bathochromic shift when compared to the standard acridine group.[47] To increase steric hindrance and increase stability to hydrolysis, groups have been added to the flanking benzene rings on the positions closest to the carboxylate group (1.27). [48] Furthermore, acridinium head groups have been modified with the protein linker groups attached for example on the heterocyclic nitrogen (1.28) (see Figure 21).[49]

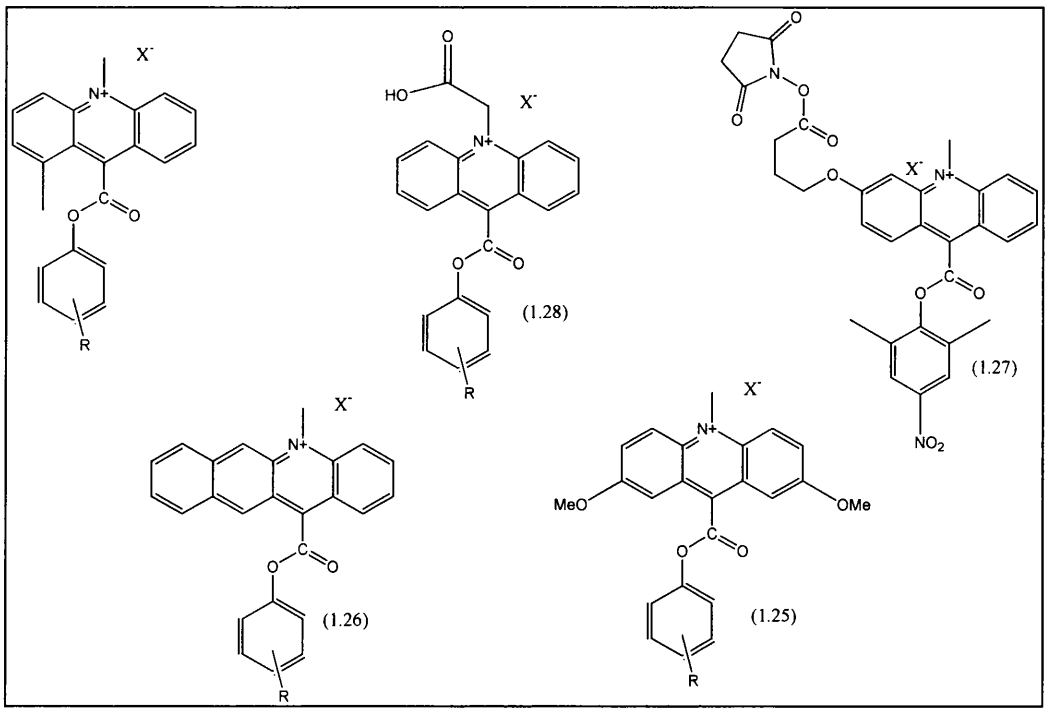


**Figure 21 Modification of the head group**

### *1.7.2.2 Modification of the protein linker*

The linking group can be essentially any group, which will link onto an amino, or carboxylate group on a protein molecule. For example glutamic acid or aspartate residues are ideal residues on proteins for attack by an amine linker terminus. Primary amino groups at the N-terminus such as those found on lysine residues are ideal sites for linker groups with carboxylate moieties. The most popular linker types are; *N*-succinimidyl esters (such as (1.24) and (1.27)) and, imidates (1.29) (see Figure 22), amines, carboxylic acids and 2-nitro-4-benzenesulfonic acid.[46, 50] Out of these the *N*-succinimidyl esters are by far the most popular.

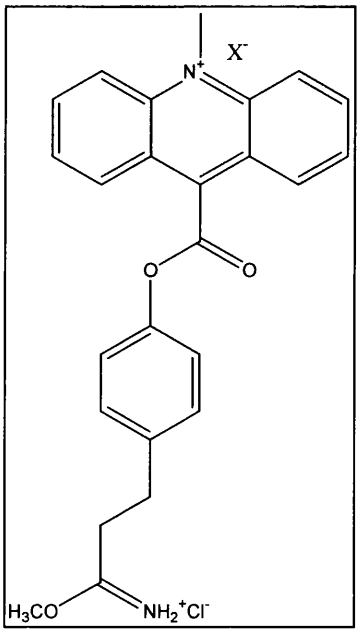


**Figure 22 Imidate linker group (1.29)**

### *1.7.2.3 Modification of the spacer / leaving group*

The spacer has been altered to investigate whether groups on the ortho positions on the benzene ring will give an increased stability to hydrolysis, for example a dimethyl derivative (1.30) has been syntheised and has shown greater stability in aqueous environments.[51] In our laboratory, Z. Li synthesised a mono and dimethoxy derivative too (1.31) and found that both were much more stable to hydrolysis than the Weeks Woodhead Label.[52] Furthermore, an isomer of the Weeks-Woodhead label has been synthesised where the linker chain has been moved from the para position of

the benzene ring to the ortho position to shield the leaving group to hydrolysis; this has proven to be a success especially when bound to the protein (see compound (1.32) of Figure 23).[53]

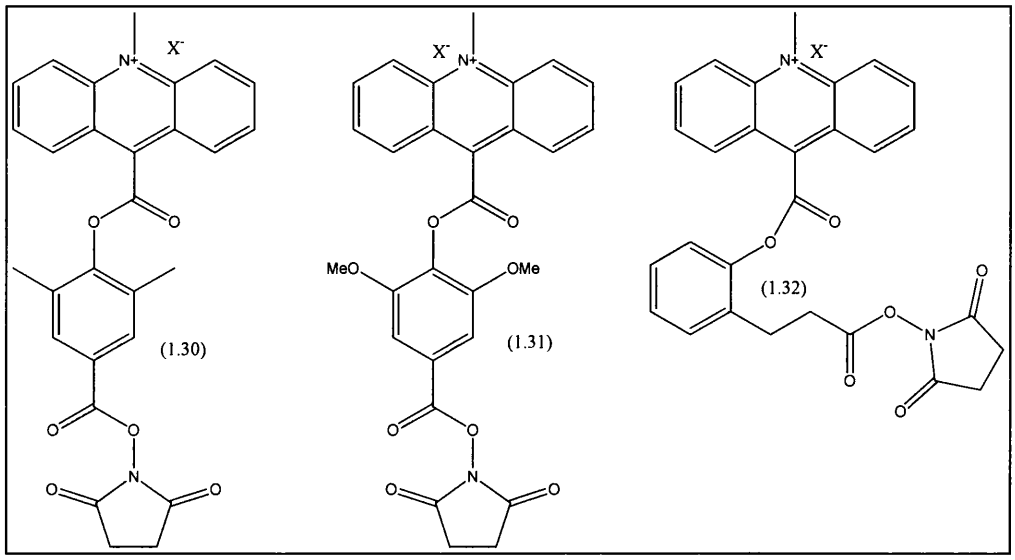


**Figure 23 Modification on the leaving group / spacer**

The main drive for modifying the leaving group is to provide stability under aqueous environments by adding groups, which sterically protect the 9-position but do not reduce the over all $\Phi_{CL}$. However, alteration in this manner invariably changes the kinetics of light output, and this is also dependent on the pKa of the conjugated acid of leaving group. The lower the pKa of the leaving group, the faster the light output. The reason is that the loss of the leaving group in the chemiluminescent reaction is the rate-determining step. To help improve the leaving group in terms of stability to hydrolysis and $\Phi_{CL}$, the carboxylate group has been, in some labels, changed to a variety of leaving groups such as thiols, sulfonamides, hydroxamic acids, sulphohydroxamic acids, thiolamines, oximes and chloroximes. In fact thiols and sulfonamides have been found to be five times more luminogenic than the Weeks-Woodhead label (see Figure 24).[42]

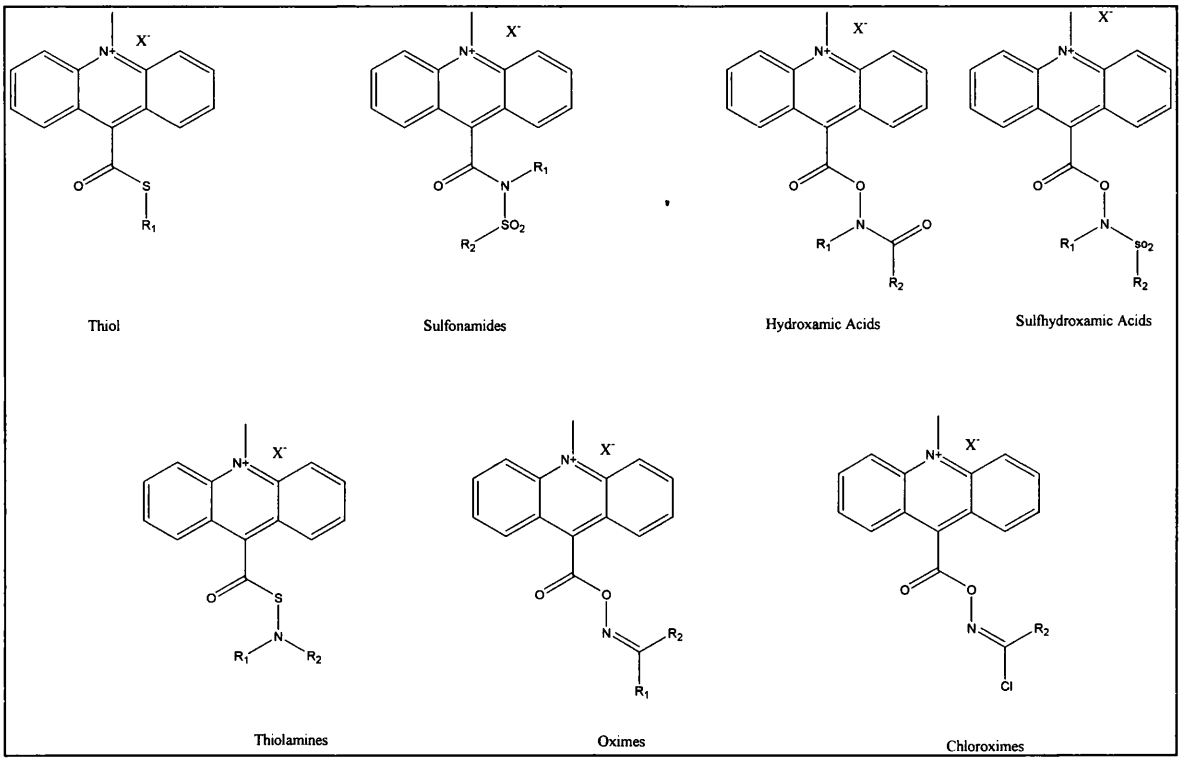


**Figure 24 Leaving groups available for acridinium probes**

Sulfonamides have proven to be very popular labels; they have been shown to combine high $\Phi_{CL}$ with stability in aqueous conditions. In fact, some sulfonamides have been shown to retain all their $\Phi_{CL}$ even after a year's incubation at room temperature. This is unique to sulfonamides; normally the lower the pKa of the conjugated acid of the leaving group, the more intense the flash but the more unstable the molecule is to hydrolysis.[42] Furthermore on account of the fact that the amine can form three covalent bonds then there is a spare electrophilic site where groups can be bound to either protect the 9-postion to hydrolysis or to increase the solubility of the compound in aqueous conditions. Recently, work with neopentyl 3-trifluoroxypropanesulfonate has been used to produce a range of sulfonamides with sulfonate groups in an attempt to increase the hydrophilicity of the molecules (see compounds (1.33) and (1.34) of figure 25).[54]

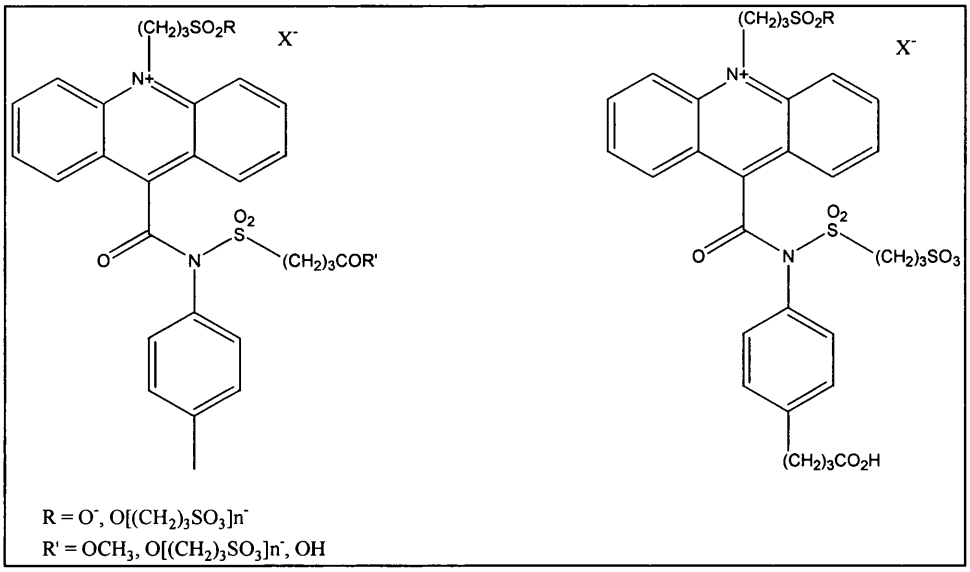


**Figure 25 Noval acridinium sulfonamides with hydrophilic groups**

## 1.8 Proposal of work for chapter 2

It has been demonstrated that choice of group directly attached to the 9-position affects the $\Phi_{CL}$. All the above groups share fact that directly bound to the 9-position on the acridine ring are always carbonyl groups. However, could a sulfonate ester derivative of the label create a more energetic reaction on breakdown of the dioxetan ring? It was already known that dioxetanones (e.g. (compound (1.24)) are more energetic and thus produce more light than dioxetans (e.g. compound (1.8)). Furthermore, dioxetanediones found in oxalate esters (e.g. compound (1.9) are more energetic than dioxetanones. On account of this, it seems that the presence of and number of carbonyl groups increases the reactivity and reduces the stability of the four membered ring. On account of the fact that attached to the sulfur are two sulfonyl oxygens, it is not inconceivable that this group could produce a four membered ring which is similar in its reactivity to dioxetanediones. On account of this, work detailed in chapter 2 investigates the feasibility of the synthesis of such a molecule.

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CH-T. An investigation into the synthesis of potentially chemiluminescent novel molecules.

James B. Rudge.

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# Chapter 2

# Attempted synthesis of a sulfonate ester analogue of the Weeks-Woodhead label

# 2 Attempted synthesis of a sulfonate ester analogue of the Weeks-Woodhead label

## 2.1 Introduction

The aim of this chapter was to synthesise the sulfonate ester analogue of the Weeks Woodhead label (see figure 1) to see if a sulfonate ester could give a more energetic chemiluminescent reaction than the standard carboxylate ester. It was proposed that the sulfonate ester would lead to a more energetic dioxetanone similar to the observed dioxetanedione of the oxalate system.

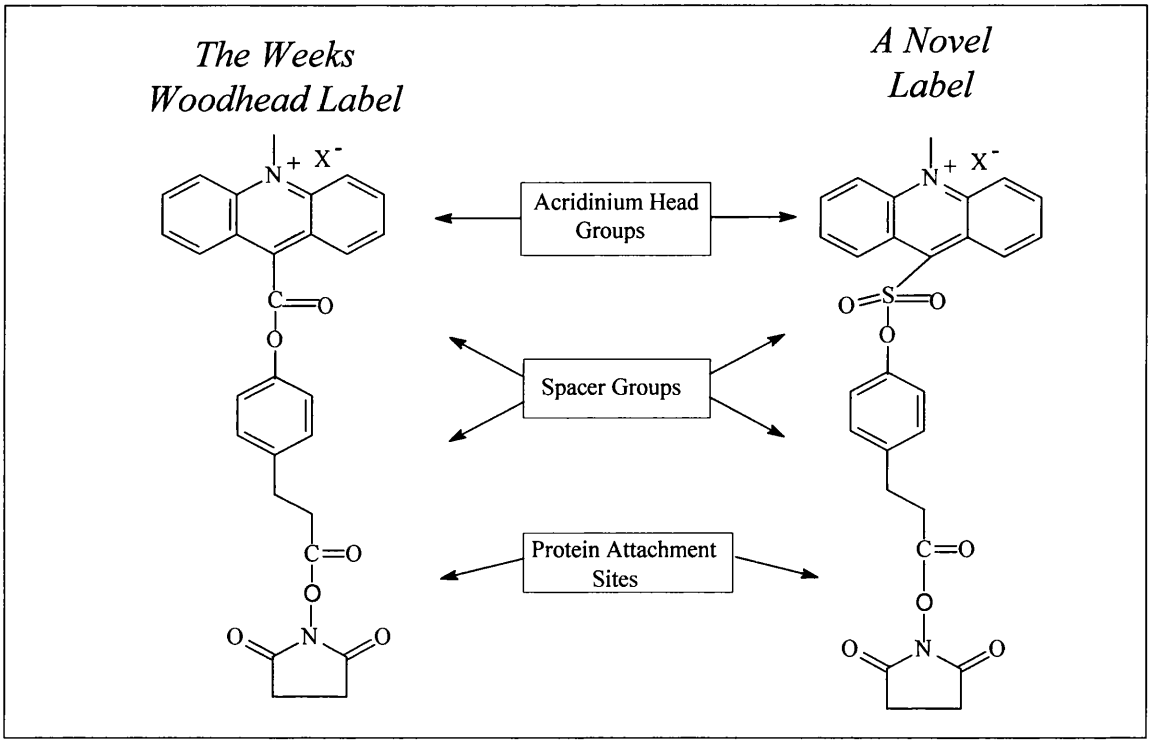



**Figure 1 A comparison between the Weeks Woodhead label and the proposed sulfonate ester**

Computational chemistry was initially conducted (by C. Nugyen in our laboratory) on a range of esters including the standard carboxylate. The computational chemistry was used to ascertain the likelihood of whether the amount of energy released from peroxidation of these proposed compounds would be sufficient to promote the expected product (*N*-methylacridone (1.16)) to its excited state (the standard carboxylate was used as a benchmark). Assuming the current mechanism of the light reaction of the acridinium ester synthesis was correct, values of differences in the heats of reactions ($\Delta H_r$) used to form *N*-methylacridone were calculated from the

decomposition of the dioxetanones listed in figure 2. However first, the geometries of the starting materials, transition states and products (assumed to be in the excited state) were optimised and then finally the $\Delta H_r$ calculations (using AM1 and PM3 packages) were conducted.

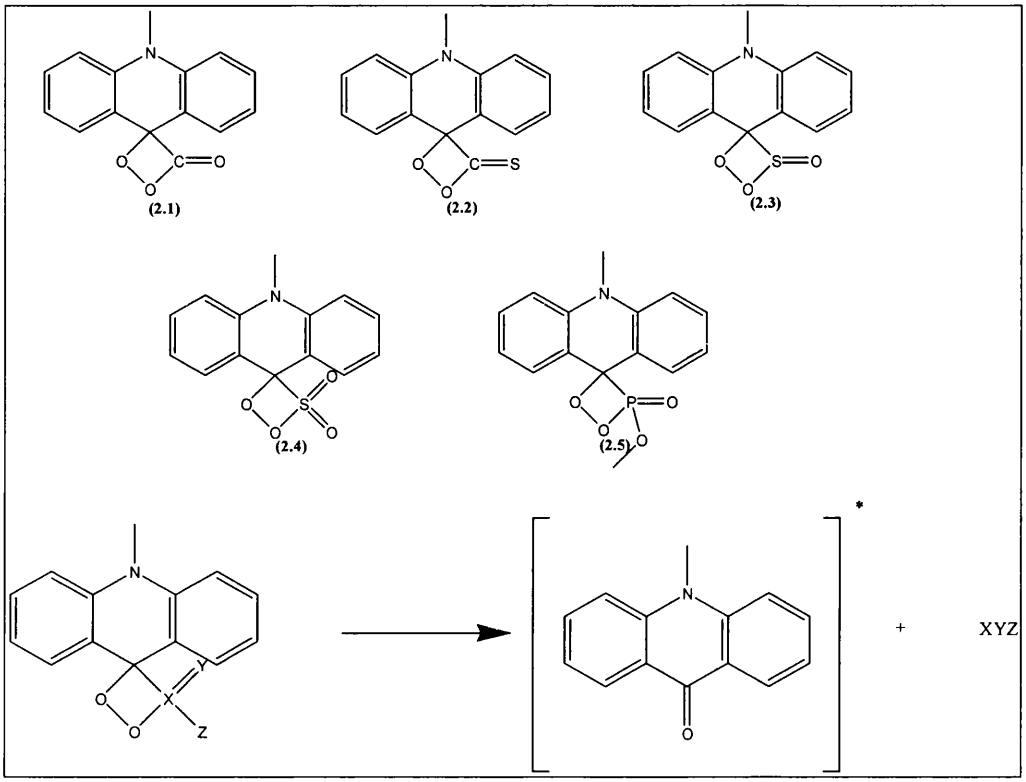



**Figure 2 Four membered intermediates of candidates for development of novel chemiluminescent probes**

## 2.1.1 Results of computational calculations

**Table 1 Calculated energy release ($\Delta H_r$) on cleavage of precursor molecules shown in figure 2**

| | | Calculated $\Delta H_r$ (kcal $mol^{-1}$) | |
|---|---|---|---|
| Precursor molecule | By-product (xyz) | AM1 | PM3 |
| (2.1) | $CO_2$ | -32.2 | -16.4 |
| (2.2) | COS | -43.9 | -32.7 |
| (2.3) | $SO_2$ | -15.1 | -16.5 |
| (2.4) | $SO_3$ | -52.4 | -44.8 |
| (2.5) | $MeOPO_2$ | -45.6 | -43.8 |

The computational modelling revealed out of the dioxetanones tested that precursors (2.2), (2.4) and (2.5) gave the most exothermic predicted heats of conversion. On account of this it was decided that the thionate (precursor (2.2)) and sulfonate (precursor (2.4)) would be synthesised for their chemiluminescence. Work in this chapter details the attempted synthesis of the sulfonate analogue.

## 2.2 Results and discussion (synthesis of phenyl *N*-methylacridinium-9-sulfonate trifluoromethanesulfonate (2.9))

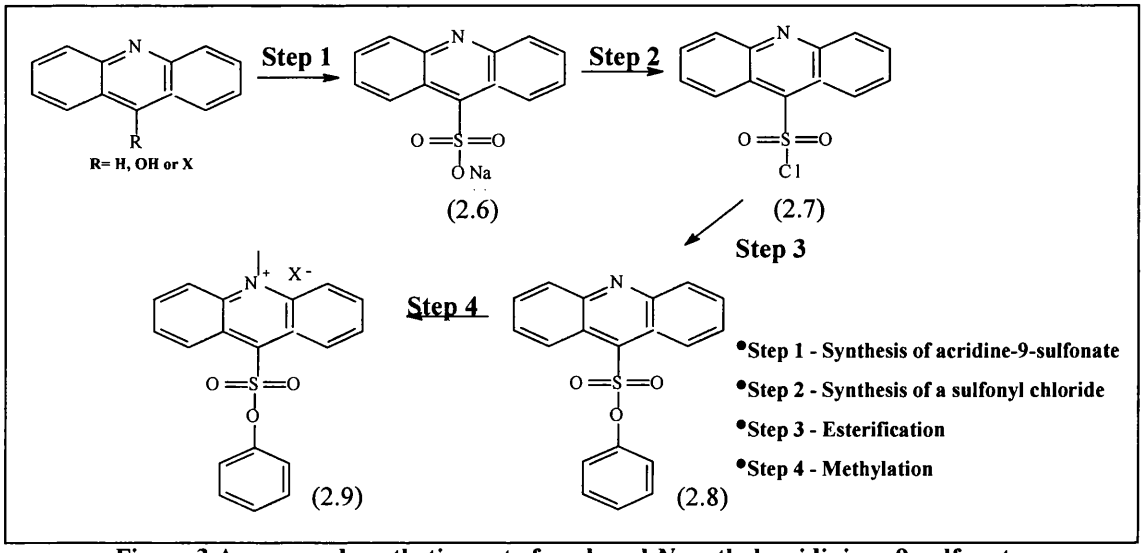



**Figure 3 A proposed synthetic route for phenyl *N*-methylacridinium-9-sulfonate trifluoromethanesulfonate (2.9)**

It was decided to first attempt the synthesis of the phenyl sulfonate ester analogue (figure 3) of the Weeks Woodhead Label (figure 1) rather than the full label with the spacer and linker groups attached. At this stage, the priority of this work was to see if sulfonate esters of acridine could be synthesised and whether in reality these were chemiluminescent. If this initial investigation was a success, then work would be conducted to synthesise the complete label (with the protein linker moiety attached).

To synthesise phenyl *N*-methylacridinium-9-sulfonate trifluoromethanesulfonate (2.9), the pathway proposed was taken from the synthesis of the carboxylate ester. The first step in the pathway was to synthesise sodium acridine-9-sulfonate (2.6) followed by conversion to the acid chloride (2.7), followed by esterification (2.8) under basic conditions and then finally methylation (2.9).

### 2.2.1 Synthesis of sodium acridine-9-sulfonate (2.6))

The most popular method of sulfonation is electrophilic attack of an organic compound with sulfur trioxide or chlorosulfuric acid.[1] However, although acridine sulfonates have been synthesised through this method, the 9-position has never been sulfonated.[2] The reason for this is that the 9-position of the acridine ring is the most electron poor thus the electrophile would preferentially attack the more electron rich sites. On account of this, other methods of sulfonating the 9-position of acridine were sought. The literature showed that both sodium acridan-9-sulfonate (2.10) and pyridine-4-sulfonic acid had both been synthesised.[3,4] It was decided to initially look at the synthesis of (2.10) and attempt to convert this through either oxidation or dehydrogenation to form (2.6).

#### *2.2.1.1 Synthesis of sodium acridan-9-sulfonate (2.10) and attempts to oxidise it to the acridine analogue (2.6)*

The literature indicated that (2.10) could be synthesised by reaction of acridine with a mixture of aqueous $Na_2SO_3$ and $NaHSO_3$ at 80 °C.[4] However, the literature mentioned that oxidation of (2.10) did not form the acridine but resulted in the loss of the sulfonate group forming acridine and a biacridyl oxidation product (see figure 4).

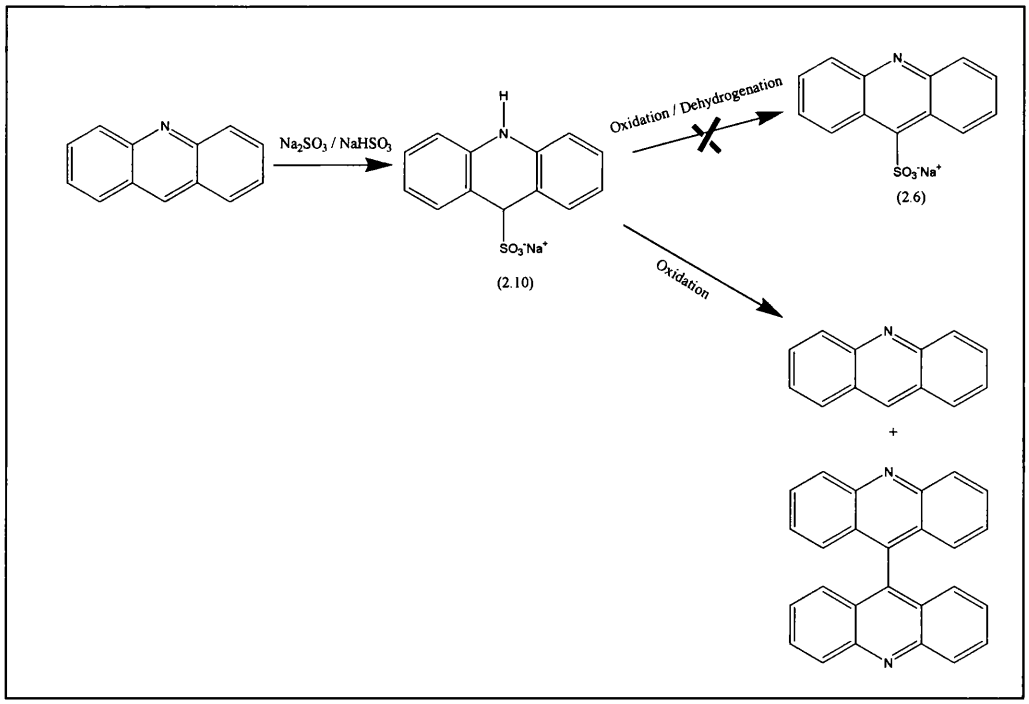



**Figure 4 Synthesis of sodium acridan-9-sulfonate (2.10) and then attempted oxidation / dehydrogenation**

Work was thus done to repeat what had been reported and the synthesis of (2.10) was found to be straightforward. As described in the literature, acridine was reacted with a mixture of sodium sulfite and bisulfite. The product crystals which formed as a result were then recrystallised in water, resulting in sodium acridan-9-sulfonate being purified in good yield (75 %). The crystallisation step provided an insoluble by-product which was sent for analysis along with the main product. The brown impurity was found to be acridine.

The proton NMR spectrum of the sulfonate showed 4 aromatic peaks integrating to two protons each, and the patterns of the peaks were double-doublet, double-triplet, double-triplet and double-doublet. It was assumed that the double-doublet and double-triplet found further downfield from the other two were those closest to the sulfonate group due the electronegative effect of this group. Furthermore there was a singlet integrating to one proton found up-field from the aromatic protons and this was identified as the 9-position proton. What was not evident was the proton on the heterocyclic nitrogen, however because $D_2O$ was used then exchange of this proton for deuterium would mask its presence. Negative ion mass spectrometry showed the molecular anion (m/z 260) of the sulfonate and positive ion mode showed the $M+H^+$ ion of acridine (m/z 180) which was thought to be a breakdown product. It was found that the sulfonate salt sublimed when a melting point test was conducted. Finally IR indicated characteristic absorbencies for the $SO_2O$- group in the finger print region (1176 and 1191 $cm^{-1}$), aromatic absorbencies (1484 and 1612 $cm^{-1}$) aromaitic C-H frequencies (2923 $cm^{-1}$) and finally an absorbance for the NH group (3446 and 3513 $cm^{-1}$).

As expected, attempts to oxidise the acridan resulted in loss of the sulfonate group yielding acridine. Interestingly the biacridyl product which was observed in the original paper was not observed in these attempts. Moreover, it was discovered that the sulfonate was not stable in aqueous conditions, observations showed that overnight left in water, (2.10) would decompose to form acridine. Work was then done to see if (2.10) was more stable in either acidic or basic conditions, however this seemed to accelerate the breakdown of the product to acridine and sodium bisulfite. It

was concluded from this that (2.10) would undergo acid and base catalysed elimination reactions readily (see figure 5).

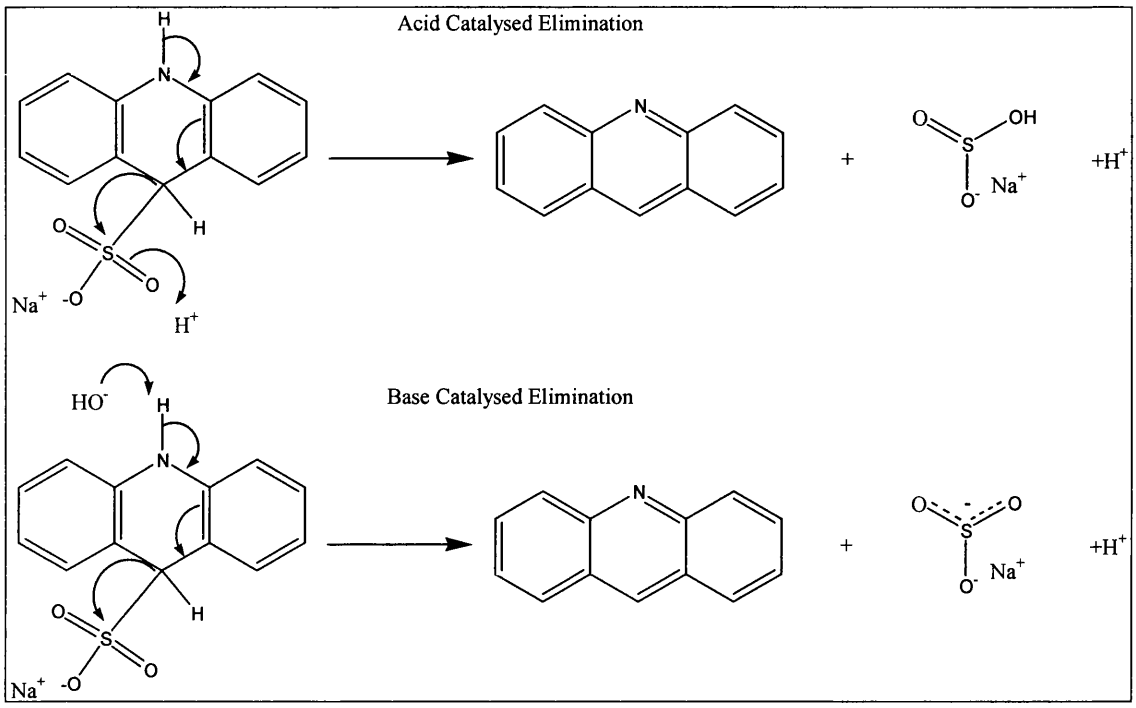



**Figure 5 acid and base catalysed elimination of (10)**

One last attempt was conducted to convert (2.10) into (2.6). It had been shown that the dehydrogenating reagent 2,3-dichloro-5,6-dicyanobenzoquinone (DDQ) had successfully dehydrogenated acridan to acridine in the presence of dry dioxane.[5] Unfortunately (2.10) did not dissolve in dioxane, which had been successfully used as the solvent for dehydrogenation of acridan. Attempts to dehydrogenate (2.10) with DDQ in methanol, and water resulted also in elimination of the sulfonate group. On account of this, the second proposed route was attempted.

### *2.2.1.2 Synthesis of acridine-9-thione (2.12) then attempted oxidation to the free acid of (2.6)*

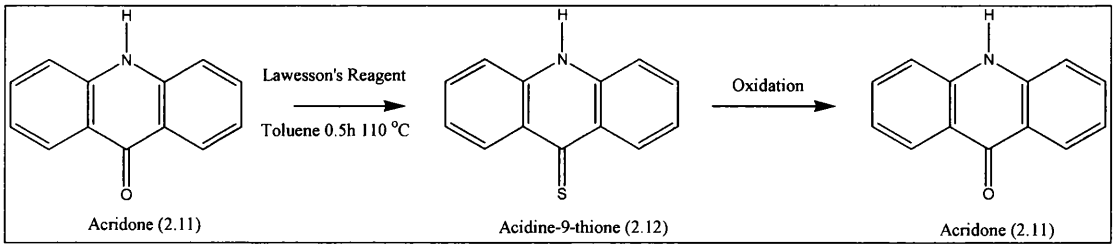



**Figure 6 Synthesis of acridine-9-thione (2.12) from acridone (2.11) and oxidation back to acridone**

Historically pyridine-4-sulfonic acid had been synthesised in a quantitative yield through oxidation of 4-thiopyridone using three molar equivalents of hydrogen peroxide in basic conditions.[6] This method was repeated, replacing 4-thiopyridone with (2.12), to attempt to synthesise (2.6) in its free acid form. Compound (2.12) was successfully synthesised from reaction of (2.11) with Lawesson's reagent (2,4-bis[4-methoxyphenyl]-1,2,3,4-dithiodiphosphetane-2,4-disulfide) in a 65 % yield.[7] However, when oxidation of this was attempted, using the same conditions as for 4-thiopyridone, unfortunately oxidation resulted in formation of (2.11) (see figure 6). It appeared that a nucleophilic substitution reaction occurred on the 9-position of the acridine ring rather than the hydrogen peroxide attacking the SH group and oxidizing it to the sulfonate. It was postulated that the sulfonate was being formed as an intermediate and it was then attacked by either hydrogen peroxide or hydroxide to form acridone. However on workup only (2.11) and (2.12) were found. Thus if the above idea was correct then it would have been unlikely that starting material was present when the reaction was worked up. On account of this, direct oxidation was more likely.

### *2.2.1.3 Synthesis of sodium acridine-9-sulfonate (2.6) through a nucleophilic substitution mechanism*[6,7]

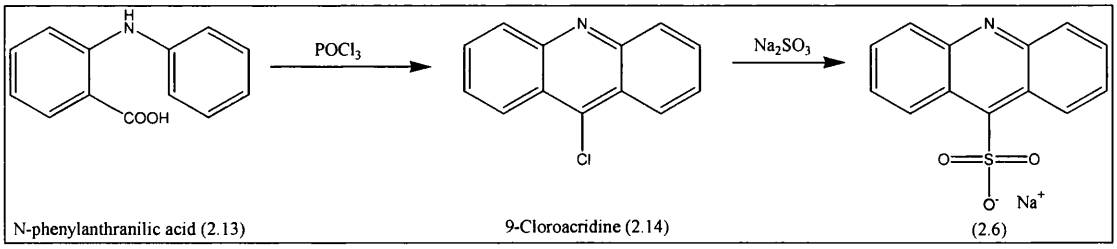



**Figure 7 Synthesis of (2.6) by a nucleophilic attack on 9-Chloroacrdine (2.14)**

The synthesis of (2.10) detailed in section 2.2.1.1 showed that the sulfite nucleophile attacked at the 9-position of the acridan ring and under these forcing conditions, a reductive nucleophilic addition reaction occurred. The fact that the sulfite attacked at the 9-position indicated that the 9-position is the most likely position to be attacked by a nucleophile. This is also true of other acridine and acridinium systems and it is well documented that the 9-position of the acridine is the carbon most likely to be attacked by nucleophiles.[2, 8] The reason why a nucleophilic substitution reaction did not occur when the sulfite molecule attacked the acridine molecule was because the potential leaving group, a hydride, is extremely poor at leaving.

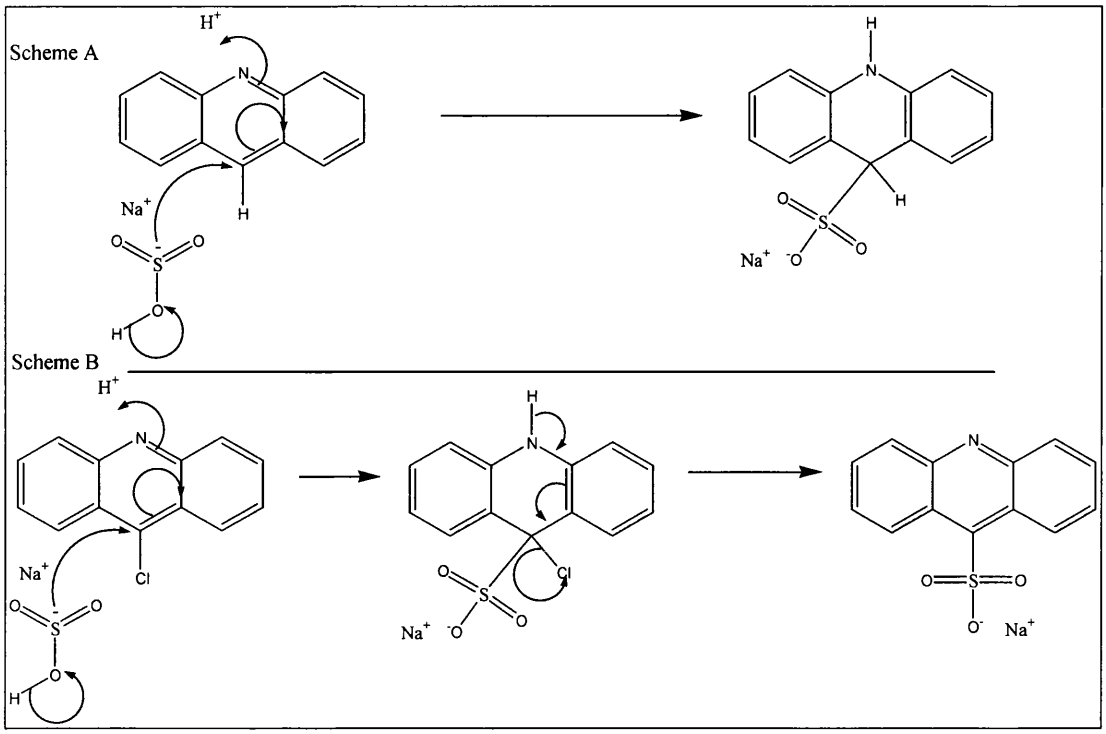



**Figure 8 A comparison between the nucleophilic addition of sulfite on acridine (scheme A) and a substitution reaction on (2.14) (Scheme B).**

However, groups such as halogens are excellent leaving groups; thus by attack of the sulfite nucleophile, the electronegative chlorine might leave as a chloride ion (see figure 8). Indeed when this theory was tested acridine-9-sulfonate was successfully synthesised through such a nucleophilic attack on 9-Chloroacridine (2.14).

2.2.1.3.1 Synthesis of 9-Chloroacridine (2.14)

The literature method for the synthesis of (2.14) was by reaction of *N*-phenylanthranilic acid (2.13) with a powerful chlorinating agent. The literature reference used phosphorus oxychloride forming (2.14) in quantitative yield.[17] The literature reaction was conducted and the desired product was indeed formed in excellent yield (100 %). The proton NMR showed that there were four aromatic signals characteristic of a 9-substituted acridine. The order of the signals, from higher field to lower field was; triplet, triplet, doublet, doublet. The doublets were found further down field due to the fact that they were closest to the heterocyclic nitrogen or chlorine group. The mass spectrometry data complemented the NMR with a molecular ion evident at m/z 213 and 215. The ion at m/z 215 was a third of the

intensity of the ion at 213 indicating the presence of the two isotopes of chlorine. The product was also found to have an identical melting point to that reported in the literature.[17]

2.2.1.3.2 Synthesis of sodium acridine-9-sulfonate (2.6)

The first attempt to synthesise compound (2.6) used the same method used to synthesise the acridan analogue (2.10) but substituting acridine with (2.4) (see section 2.2.1.1). The method produced a product, which was a mixture of (2.14) and a novel organic compound, which appeared to be, (when analysed by NMR), a 9-substituted acridine. Furthermore, electrospray MS indicated the presence of the molecular anion of (2.6) (m/z 258). It was estimated (from the NMR data) that the product was formed in only a 24 % yield. However at this point it was difficult to purify the mix and / or synthesise the desired product in its pure form. The problems faced are summarised below.

- Working with the impure product containing (2.6) showed that over-exposure to aqueous conditions facilitated the formation of (2.11), which was exacerbated with heating.
- The product was a salt, and being a sulfonate would be charged at all pHs; thus clean-up by normal phase silica gel chromatography would be ruled out. Thus purification of the sulfonate from the sulfite would be a concern.
- Unlike acridine, which eventually went into solution on exposure to the sulfite salt (see section 2.2.1.1), (2.14) did not fully go into solution in the aqueous conditions.
- The sulfite salt would not dissolve in organic solvents, not even methanol.

Some success with a 50:50 mix of methanol and water was achieved, improving the yield of the reaction to about 66 %. However, it was found that again the major problem was that the (2.14) did not fully dissolve in the reaction mixture. Eventually it was found that replacing methanol with ethanol solved the problem. Clean up was achieved by first evaporating the solvent off using a rotary evaporator attached to high vacuum pump at a moderate temperature (30 °C) leaving a yellow solid as a result. Analytical grade ethanol was then added to the product and the mixture stirred for 30 minutes. After filtering, a white powder was left on the filter paper and a yellow

solution in the filtrate. The ethanol was then evaporated off again leaving a yellow powder. Chloroform was then added to the powder and again the product was stirred for another 30 minutes, filtered and then washed a further two times in the chloroform. Analysis of the initial chloroform wash showed it to be (2.14) with a small impurity of the desired product. The washed product was analysed by NMR IR and electrospray mass spectrometry and was found to be free of (2.14) and was hoped that the ethanol wash removed the majority of the sodium sulfate, yielding (2.6) in a good yield (83 %).

*2.2.1.3.2.1 Analysis of (2.6)*

The proton NMR showed four major peaks all integrating for the same number of protons, which indicated that the product was an acridine substituted at the 9-position. The order of the proton peaks (higher to lower field), was; triplet, triplet, doublet, doublet if seen at low resolution. This indicated that the protons closest to the heterocyclic nitrogen and sulfonate group (the doublets) were being pulled further down field by the electron-withdrawing properties of these moieties. Mass spectrometry showed, in positive ion mode, ions at m/z 282 and 260. These were identified as: the sodium salt protonated on the heterocyclic nitrogen (m/z 282) and the free acid (m/z 260) again protonated on the heterocyclic nitrogen. However ions at m/z 214 and 216 (showing a characteristic isotopic ratio of 1 chlorine in the molecule), indicated that a small amount of the starting material (2.14) was still present. Finally acridone (m/z 196) in its hydroxy tautomer, protonated on the heterocyclic nitrogen was also observed. In negative ion mode the mass data showed the molecular anion of the salt, plus a deprotonated acridone anion (see figure 9).

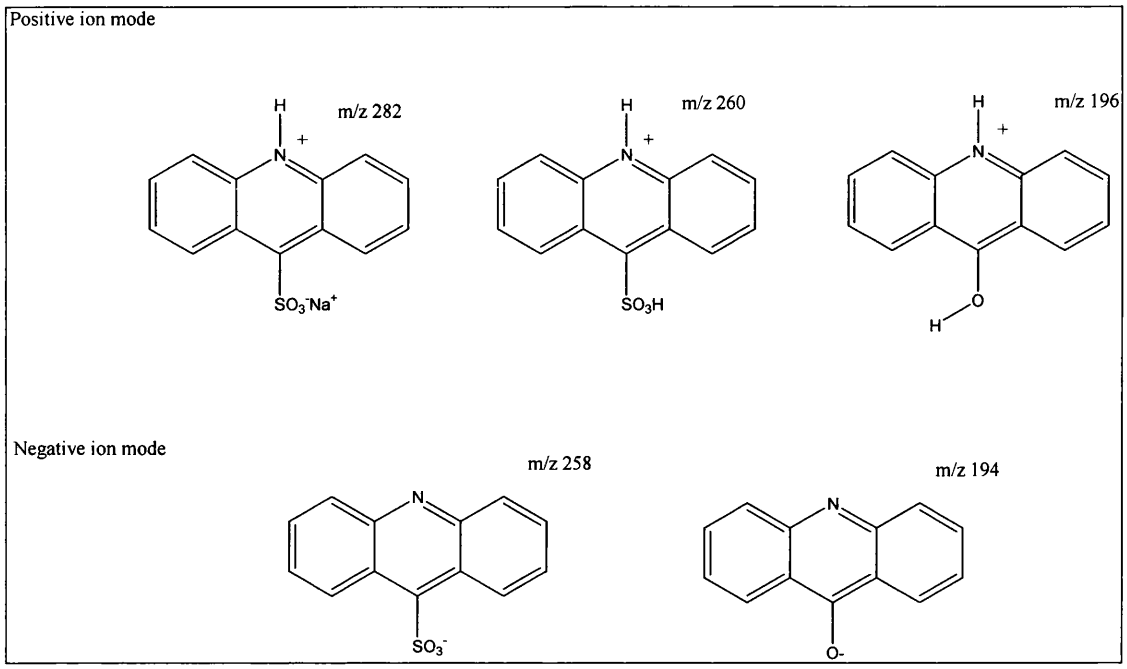
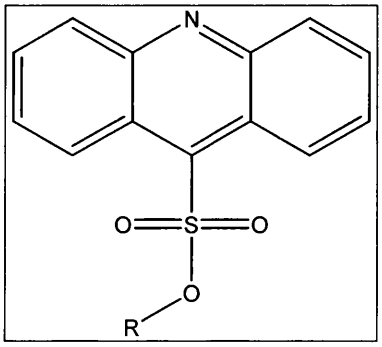



**Figure 9 Ions identified from mass spectrometry data**

Finally the mass data also indicated that in negative mode the sulfite ion (molecular weight 80) was totally absent from the spectrum indicating that washing the crude product in ethanol effectively removed the excess of the sodium sulfite. This complements the fact that excess sodium sulfite was recovered from the white powder. The IR data showed absorbancies characteristic of $SO_2O$- (1147 and 1394 $cm^{-1}$) and aromatic double bond stretching frequencies were also observed (1514.77 and 1604.77 $cm^{-1}$). Finally N bond absorbency was observed (3425 $cm^{-1}$).

The white powder was also analysed for presence of (2.6) and both NMR and mass spectrometry revealed that (2.6), was present in this too, but is was difficult to estimate the quantity of this.

### 2.2.2 Attempted esterifications of (2.6)



Following the successful synthesis of (2.6), work was then focused on synthesising the phenyl ester. The first route attempted was to synthesise the acid chloride using the same procedure used to synthesise the carboxylic acid chloride and then to react this with phenol in basic conditions.[9]

### *2.2.2.1 Attempted synthesis of phenyl acridine-9-sulfonate (2.8) through synthesis of acridine-9-sulfonyl chloride (2.7) followed by attack with phenol*

Compound (2.6) was refluxed in thionyl chloride, and the clear yellow solution that formed was concentrated to remove the excess chlorinating agent. The product was dried by rotary evaporation and then by high vacuum. The yellow powder that resulted, was added to a solution of pyridine and phenol and the solution was stirred overnight. Following this the product was taken up into chloroform and washed with both mild acid and base solutions. The chloroform layer was then dried over magnesium sulfate and concentrated to leave a yellow solid. This was then purified by column chromatography yielding one major product. This product was then analysed.

The NMR results appeared to suggest that the product had been successfully synthesised. The proton NMR results showed only seven peaks in the aromatic region of the spectrum and these integrating to 13 protons, six out of the seven peaks integrating for two protons each and a multiplet integrating for one proton. The multiplet corresponded to the para-position proton on a phenyl ring. It was difficult to assign most of the other peaks to specific protons due to the fact that both phenyl and acridine rings gave similar results. However, peaks at 7.45 and 7.78 ppm were thought to be the acridinium protons furthest away from the heterocyclic nitrogen and the 9-position. The reason for this is that in some spectra of 9-substituted acridines (such as this example) show a characteristic pattern, which is double double doublet in nature (from positions 2, 3, 6 and 7 on the acridine ring). Three J values result (6.6, 8.8 and 1.5 Hz) and this is due to these protons splitting each other (J = ~ 6.6), by the other proton ortho to them (J = ~ 8.8 Hz) and by protons meta to them (J = ~ 1.5 Hz). The carbon spectrum complemented the proton data. It showed the eleven expected signals, six double intensity CHs, one single intensity CH and four quaternary carbons. However, the mass spectrum showed only one molecular ion peak and this

was at m/z 272. The expected mass of 335 was absent from the spectrum.

There were two possible explanations for this data. The first was that in the mass spectrometer $SO_2$ might have been lost and thus rearranged to 9-Phenoxyacridine (2.15). The second and more plausible explanation is that the thionyl chloride attacked (2.6) causing a nucleophilic substitution and resulting in the loss of the sulfonate group in favour of a chloride, which was then displaced by phenoxide during the subsequent treatment with phenol.

To prove this, the reaction was repeated to the point of attack with thionyl chloride where at this point the product formed was isolated and tested for its identity. There were three possible products from this reaction. The first of these possibilities was the desired product, the sulfonyl chloride. The second was (2.14), and the third was the sulfonic acid, a possible hydrolysis product of the sulfonyl chloride.

The TLC and analytical data showed only one product had been formed. TLC indicated that the product had the same chromatographic properties as (2.14). The mass spectrum showed a $M+H^+$ ion for (2.14) (m/z 214 for the $^{35}Cl$ isotope), but the $^{37}Cl$ isotope peak for this compound was too high (m/z 216 at 80%). It was inferred that the compound may have ionised readily in the mass spectrum and in doing so overloaded the detector. This might have acted to give such inaccurate intensities.

It was thus concluded that (2.6) in the presence of thionyl chloride formed (2.14) which when exposed to phenol in basic conditions reacted to form (2.15) (see figure 10).

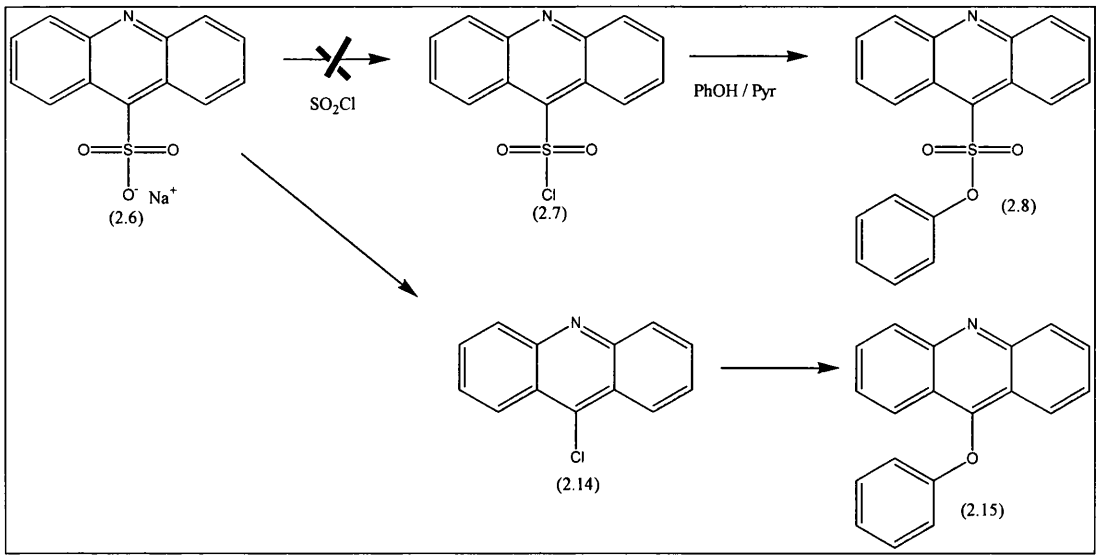



**Figure 10 (2.6) reacts with thionyl chloride to form (2.14)**

Although attempting to react the sulfonate with thionyl chloride formed an aryl halide it is interesting to consider why halide formation was favoured over conversion to the acid chloride.

It known that alcohols undergo an $S_Ni$ mechanism on reaction with thionyl chloride, as shown in see figure 11.[10]

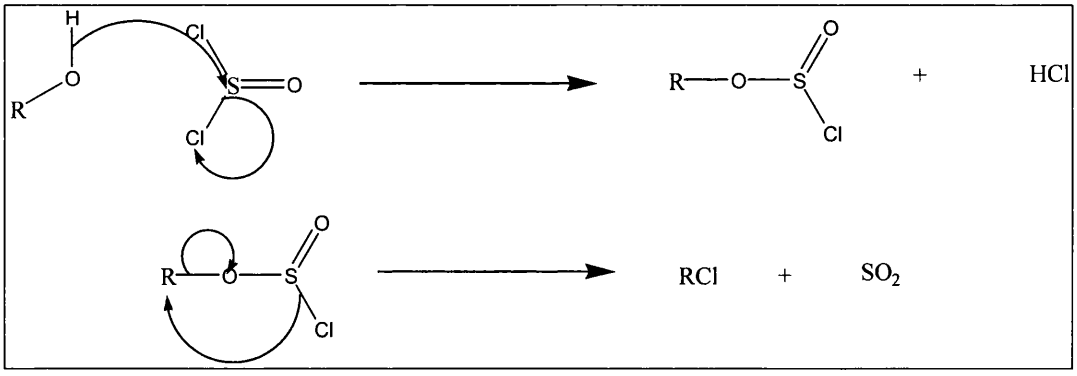



**Figure 11 The $S_{Ni}$ mechanism**

It is quite conceivable that the sulfonate could also have reacted by a similar mechanism to form the acid chloride. However, the first step of this mechanism is an initial nucleophilic attack of the hydroxy onto the sulfur of the thionyl chloride and thus assumes the oxygen anion on the sulfonate group acts as a good nucleophile. In the case of (2.6), this might not have been so. This is because the sulfonate was attached to a very electron poor carbon, which was pulling the electron density away

from the sulfonate. This would make the sulfonate oxygen a poor nucleophile. However, because sulfonate groups are electronegative in nature, then the carbon on the 9-position would be very prone to nucleophilic attack, in this case by a chloride ion.

It is also possible that the sulfonate oxygen did attack the thionyl chloride forming the initial $RSO_2.OSOCl$ group, but that there was an internal rearrangement where a chloride ion intermolecularly attacked the 9-position on the acridine ring, causing a sulfonicanhydride group to leave. This would result in decomposition to two molar equivalents of $SO_2$ and $Cl^-$. This is shown in figure 12.

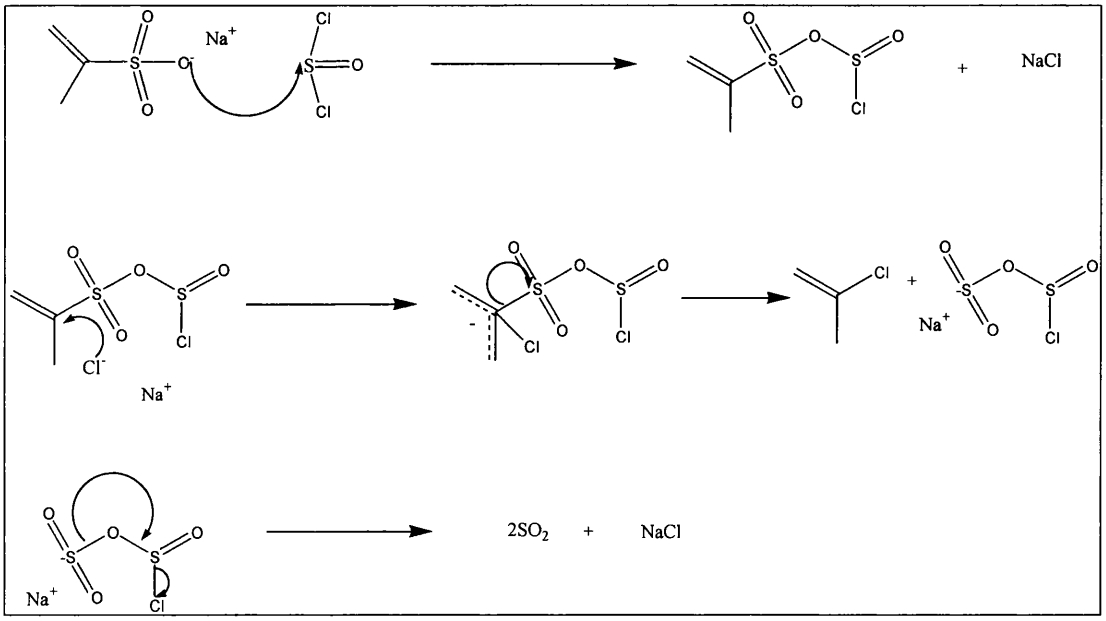
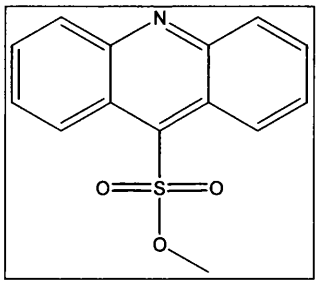



**Figure 12 Possible route to (2.14) by attack from thionyl chloride**

The conclusion which could be drawn so far from this was that in the presence of thionyl chloride, rather than forming the desired acid chloride, (2.6) lost the sulfonate group which was replaced by chloride. There are two possibilities why this is so, the first of which is shown in the mechanism in figure 12. The second possibility is that a free chloride in the solution (catalysed by any acid present) attacked via a $S_NAr$ mechanism. One way to prove this was to see how nucleophilic the sulfonate group was and thus attempt to form a methyl ester by reaction of the sulfonate with methyl iodide or methyl trifluoromethanesulfonate. If the methyl ester formed readily then this gave evidence for the above mechanism and more importantly showed that the sulfonate would act as a nucleophile. However, if the methyl ester did not form

readily then it indicated that forming the acid chloride might not be possible and thus another esterification method must be sought. Thus work was conducted to attempt to synthesise the methyl ester of (2.6) and this is described below.

### *2.2.2.2 Attempted synthesis of methyl acridine-9-sulfonate (2.16)*




**(2.16)**

Compound (2.6) was exposed to methyl iodide in each the following solvents: chloroform, acetonitrile and THF. The mixtures were heated to various temperatures and exposed to the methylating agent, but in no cases did the salt react with the methyl iodide. One of the problems found with this was the fact the salt was insoluble in the solvents tried. However, it was found that the salt would dissolve in DMSO and in this solvent a reaction did occur but unfortunately the product formed from the reaction was (2.11). Work was thus conducted to investigate why in the presence of methyl iodide and DMSO, (2.11) formed rather than the methyl ester. There was a possibility that the methyl ester formed but rapidly was hydrolysed by water in the DMSO. It was thus decided to conduct an NMR experiment to see when (2.6) was exposed to methyl iodide, whether the product directly converted to (2.11) or esterified first and then hydrolysed to (2.11).

### *2.2.2.3 An NMR investigation into the attempted synthesis of (2.16) in* d6-*DMSO using methyl iodide*

Two solutions of (2.6) in deuteriated DMSO were prepared, and to one of these a drop of methyl iodide was added. A third tube was prepared with deuteriated DMSO and 1 drop of methyl iodide, for comparison. These were then transferred to NMR tubes (see table 2) and analysed immediately by NMR (a1, b1 and c1). Following this, the tubes were then incubated for 0.5 h at 60 °C and reanalysed (a2, b2 and c2). Finally

the tubes were incubated for 2 h at 80 °C (a3, b3 and c3). The NMR results were then analysed.

**Table 2 Contents of NMR tubes**

| NMR tube label | Contents |
|---|---|
| a | Compound (2.6) + *d6-DMSO* |
| b | Compound (2.6) + *d6-DMSO* + MeI |
| c | $D_6$-DMSO + MeI |

The results of the experiment are summarised in table 3

**Table 3 Differences in the acridine species formed from exposure of (2.6) with methyl iodide – a comparison of their ppm values**

| Label | Time & temp. | Mean PPM Values | | | | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| a1 | 0h, 25 °C | 6.54 | 6.65 | 6.99 | | | 7.56 | | 7.79 | | 8.1 | | | 8.77 | 8.45 | 9.58 | |
| b1 | 0h, 25 °C | 6.54 | 6.62 | 6.98 | | | 7.56 | | 7.79 | | 8.1 | | | 8.77 | 8.45 | 9.58 | |
| | | | | | | | | | | | | | | | | | |
| a2 | 0.5h, 60 °C | | | | | | 7.56 | | 7.79 | | 8.1 | | | | | 9.58 | |
| b2 | 0.5h, 60 °C | | | | 7.26 | | 7.57 | 7.73 | 7.8 | | 8.11 | 8.23 | | | | 9.58 | 11.75 |
| | | | | | | | | | | | | | | | | | |
| a3 | 2.5h, 80 °C | | | | 7.26 | | 7.56 | 7.72 | 7.79 | | 8.11 | 8.23 | | | | 9.58 | 11.75 |
| b3 | 2.5h, 80 °C | | | | 7.25 | 7.33 | 7.56 | 7.73 | | 7.84 | | 8.22 | 8.33 | | | | 11.76 |
| | | | | | | | | | | | | | | | | | |
| (2.6) | | | | | | | 7.56 | | 7.79 | | 8.1 | | | | | 9.58 | |
| | | | | | | | | | | | | | | | | | |
| (2.11) | | | | | 7.25 | | 7.56 | 7.73 | | | | 8.22 | | | | | 11.76 |

***The shaded areas are the major acridine species which, were found to be in over 90% excess in each spectrum.**

It can be seen from the results that at the start of the reaction, (2.6) remained intact, as the peaks did not differ between tubes a and b. An interesting observation was that the minor peaks (ppm: 6.54m, 6.65m, 6.99m, 8.45s 8.77m,) detected at the start of the reaction, disappeared when the tubes were heated for 0.5h. The acridine species involved showed a singlet at 8.45 ppm and the other peaks were multiplets. This indicated that this impurity could have been acridine, which must have been a small

impurity in the salt. However its presence and why it disappeared still remains a mystery. When the salt was heated for 0.5h, it was noted that a small impurity of (2.11) had formed in tube b but not in a. A positive identification was possible because the values at 7.26, 7.73 8.22 and 11.75 were identical to those for (2.11).

After the tubes were heated to 80°C for 2h, there was a marked difference in the contents of the two tubes. The spectrum of the product a3 was essentially identical to that of b2 in that it consisted of mainly (2.6) but had a small impurity of (2.11). By contrast, tube b3 contained mainly (2.11) but had a tiny impurity of an unidentified acridine species. This evidence was based upon the presence of tiny peaks (δ 7.33, 7.84, and 8.33) around the acridine region, but the nature of this acridine species remains elusive.

The results showed that (2.6) did not form the desired ester in the reaction. They indicated that as in the previous reactions where an attempted methylation was conducted in DMSO, (2.11) was formed as the major product. The conclusion that could be drawn from this investigation was that the 9-position of (2.6) was too prone to attack by water. Furthermore, as described in section 2.2.2.1, the sulfonate moiety on the acridine ring was acting as a very poor nucleophile and so methylation was not favoured. A conclusion that also could be drawn was that the presence of MeI in the reaction mixture appeared to speed up the synthesis of (2.11). The evidence to prove this was that species b3 consisted of mainly (2.11) whereas the corresponding tube that did not contain MeI (a2), contained mainly the salt (with just a small contamination of (2.11)). Why the methyl iodide acted as a catalyst in the formation of (2.11) remains unclear. One suggestion, was that instead of the sulfonate being methylated, the nitrogen on the 10-position was temporarily methylated to provide the driving force for (2.11) synthesis through an intermediate. Figure 14 offers a hypothetical mechanism.

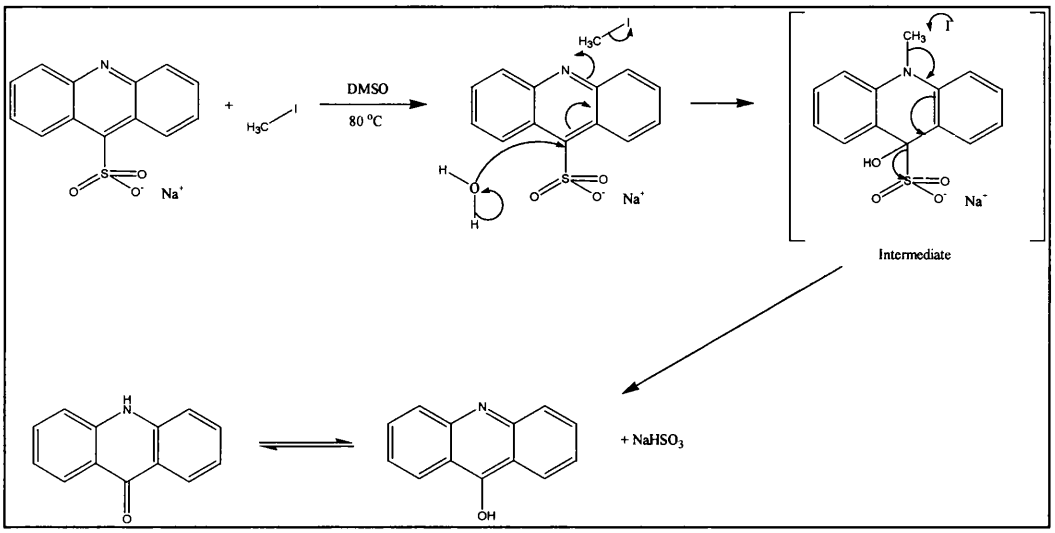
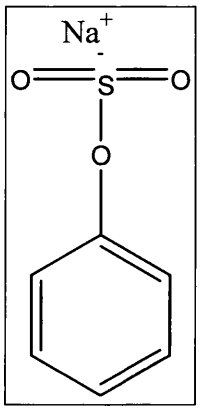



**Figure 14 Possible mechanism showing a methyl iodide catalysed formation of (2.11) from (2.6).**

The conclusion which can be drawn from this investigation is that (2.6) is a very poor nucleophile and any esterification mechanism which relies on nucleophilic attack of the acid, such as reaction with DCCI, is not favoured.[11] On account of this, the mechanism proposed in figure 12 would not be likely. It was suspected that during the synthesis of the phenyl ester, a trace of HCl in the thionyl chloride was the likely route to synthesis of the haloacridine. On account of this, it was decided to investigate an alternative route for synthesis of ester (2.8).

### 2.2.3 Attempted nucleophilic substitution of haloacridine by phenyl sodium sulfite

#### *2.2.3.1 Successful synthesis of phenyl sodium sulfite (2.17)*




**(2.17)**

On account of the fact that (2.6) could be successfully synthesised by reacting (2.14) with sodium sulfite through nucleophilic attack at the 9-position, it was thought that this reaction might also work for an analogue of this, (2.17). It was thought that one

possible route of synthesis would be to react sodium phenoxide (2.18) (see figure 15) with $SO_2$ gas. This assumption proved to be correct as it was found that in 1935 Voss and Lax made not only (2.17) but also the naphthalene analogue too.[12] It was hoped that formation of (2.8) could be formed by reacting (2.17) with (2.14) in a reaction similar to reaction of (2.14) with sodium sulfite. This is illustrated in figure 15.

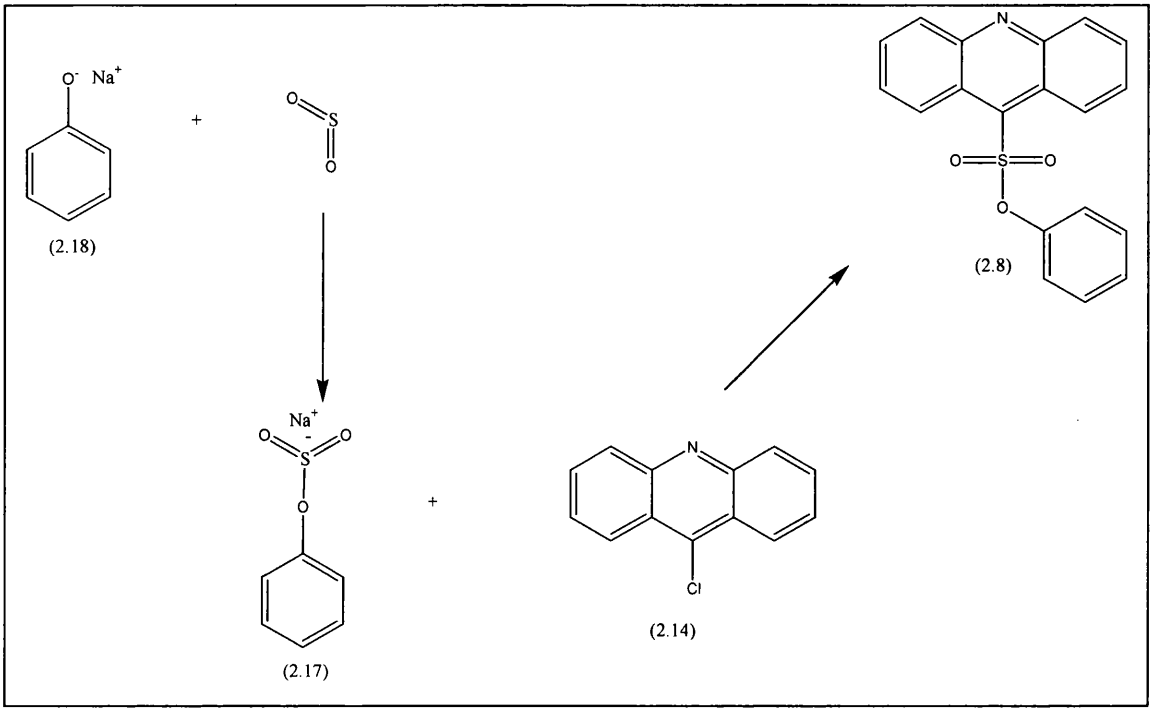



**Figure 15 A possible alternative route to synthesis of (2.8)**

2.2.3.1.1 Synthesis of $SO_2$ gas *in situ*

On account of the fact that a cylinder of $SO_2$ gas (500g) was relatively expensive (£100, Aldrich) it was decided to search for a more cost-effective method for producing the gas for the synthesis of (2.17). From the literature, it was found that $SO_2$ gas could be formed by several methods,[13,14,15] but the most straightforward method from these was the reduction of conc. $H_2SO_4$ by metallic copper and it was thus decided that this would be the method of choice. [13]

$$2H_2SO_4 + Cu \longrightarrow SO_2 + CuSO_4 + 2H_2O$$

A test reaction was first conducted to gain experience of the reaction and its behaviour (rate of gas evolution etc.). This was conducted by heating (90°C) copper turnings in conc. $H_2SO_4$. The solution went black and soon afterwards a gas evolved which was

dried over $H_2SO_4$. Confirmation that the gas evolved was $SO_2$ was by testing it with a wet pH indicator paper (full range), which turned red, indicating that sulfurous acid had formed from the $SO_2$ gas reacting with water on the indicator paper.

2.2.3.1.2 Synthesis of (2.17)

As discussed above, the synthesis of (2.17) had been previously achieved by reacting (2.18) with $SO_2$ gas.[12] In the literature, (2.18) was synthesised *in situ* by reacting sodium with phenol in a mix of xylenes and then reacting this resulting powder with $SO_2$ gas to obtain the product. The synthesis of (2.18) was deemed unnecessary as the trihydrate could be easily obtained commercially and was not very costly (~£22 for 100g from Aldrich). However, before the synthesis of (2.18) could be facilitated, the (2.18) and xylenes needed to be dried and this was conducted by a Dean and Stark azeotropic dehydration of the mixture. The resulting powder was then exposed to dry $SO_2$ gas under dry conditions. At the start of the reaction it was observed that the rate of gas flowing in the (2.18) suspension was the same as the gas flowing though the concentrated $H_2SO_4$ solution, which preceded it. However during the reaction, the rate of gas bubbling out of the vessel reduced to nothing, while the rate of gas flowing through the acid remained to the same. It was assumed that during the start of the reaction the $SO_2$ evolved was flushing out the dry nitrogen, which had preceded it. As the nitrogen was inert then it did not react with the (2.18) suspension but as the $SO_2$ replaced the nitrogen it did react with (2.18) and so the suspension was acting as a sink for the gas. As the reaction proceeded and the reaction was coming to completion, gas started to bubble though the solution again.

If all of the water had been azeotroped off the suspension of (2.18), then the weight of dry product would have been 4.835g. However, after exposure to $SO_2$ gas, the weight of the white powder which had formed was 7.193g. This weight increase indicated one of three possibilities; that the correct product had formed; that the (2.18) had not been properly azeotroped; or that upon work-up the very hygroscopic (2.18) gained water from the atmosphere. The latter two choices were not possibilities, as the $^1$NMR did not show any water peaks for the product. The carbon and proton NMR indicated that the aromatic peaks shifted towards low field, which was logical as addition of the $SO_2$ group on the phenoxide ion would act to shift the peaks in that direction.

The negative ion electrospray mass spectroscopic results did show the expected molecular anion, m/z 157, but it was not of substantial intensity (35%). The base peak was at m/z 95, while the relative molecular mass of the phenoxide ion is 93 so it was difficult to assign this anion without further information. The anion at m/z 171 was also difficult to assign. Three peaks at 979, 1065 and 1178 $cm^{-1}$ dominated the IR spectrum of the product and these were in the part of the spectrum where $-SO_2$-O- groups absorb.[16] Finally, the yield of product was correct (95 %) for the expected product. It was concluded that (2.17) had indeed formed in the reaction.

### *2.2.3.2 Attempted nucleophilic substitution of (2.14) using (2.17) to form phenyl acridine-9-sulfonate (2.8)*

Once (2.17) had been synthesised, it was then used to attempt synthesis of (2.8). Two molar equivalents of (2.17) were refluxed with one of (2.14) in acetonitrile for 6 h. The reason for reflux was that the (2.17) was sparingly soluble in organic solvents (even DMSO at room temperature). However (2.17) did dissolve in refluxing acetonitrile. The reaction was followed by TLC (toluene: EtOAc, 4: 1) and throughout the reaction there appeared to be little change to the TLC plate. There appeared to be two spots, one on the baseline (which did not move even when the TLC was conducted in EtOH), and the second with an Rf value of 0.67. On work-up, the solution was first cooled causing a product (product B) to precipitate out. The supernatant was then evaporated to dryness to give a residue (product A).

#### 2.2.3.2.1 Product A

The results of the $^1$HNMR analysis of product A indicated the presence of two, possibly three different compounds. These appeared to consist of two groups of peaks where the first group was 80% the intensity of the other.

##### *2.2.3.2.1.1 $^1$H Chemical shifts for product A (the major peaks)*

The $^1$HNMR spectrum showed 6 mutiplets at the following chemical shifts:
δ (ppm) 6.89, 7.21, **7.63, 7.79, 8.27** and **8.43**. The latter four shifts were almost identical to those of (2.14) (section 2.3.6).

The peaks at δ6.89 and 7.21 ppm corresponded, though not exactly, to those of compound (2.17) (δ 6.77 and 7.14 ppm). Although the chemical shifts for the six major peaks of product a were almost identical to the two starting materials it was conceded that these could also be peaks for either (2.15) or the desired product (2.8). This was because the integration values of the peaks matched nearly but not exactly to what would have been expected for the ester or the ether. However, the peaks 7.79 and 6.89 ppm appeared to be slightly larger than expected and thus it was thought that they might have contained peaks of another compound. Thus, as it stood, the integration values suggested the presence of the following protons:

δ (ppm) 6.89 (m, 3H), 7.21 (m, 2H), 7.63 (m, 2H), 7.79 (m 2H), 8.27 (d, 2H, 8.8 Hz) and 8.43 (dd, 2H, 8.7 and 0.5 Hz).

*2.2.3.2.1.2 $^{1}H$ Chemical shifts for product A (the minor peaks)*

The minor peaks present (20 % the intensity of the major peaks) are listed here:

δ (ppm) 7.07 (t, 1H, 7.4 Hz), 7.28 (dt, 2H, 1.1 and 7.1 Hz), 7.47 (m 2H), 8.11 (d, 2H 8.8 Hz) and 8.36 (d, 2H 8.9Hz).

As illustrated above, the major peaks at 7.79 and 6.89 ppm appeared to be too large in terms of their relative integrations. For example, the peak at 7.79 ppm appeared to integrate for 120 %, thus indicating that it contained a 20% impurity with the same shift. This was resolved by examining the fine structure of the peak at δ 7.79 on the Silicon Graphics computer. It revealed an ultra structure of another peak and it was thus concluded that the peak at 7.79 ppm contained also a peak for the minor component. Furthermore, for the same reasons as above, the peak at 6.89 ppm also contained a hidden minor peak.

The mass spectrometric results (EI) of product A showed molecular ions for compound (2.15) (m/z 271), compound (2.14), (m/z 213 / 215), and compound (2.11), (m/z 195). Disappointingly the molecular ion for compound (2.8) (m/z 335) was not

observed. Thus, it was becoming apparent that product A was a mixture of starting materials and (2.15).

#### 2.2.3.2.2 Product B

Product B was analysed first by mass spectroscopy; surprisingly the negative ion spectrum gave a molecular ion of m/z 258, which was identical to that expected for the acridine-9-sulfonate anion. When the proton NMR of product B was examined, it was found to be representative of at least two compounds where one appeared to be in a 2:1 excess. When comparing the major peaks against the peaks from a sample of pure (2.6), it was found that they had almost identical chemical shifts. It was however, very difficult to assign the peaks of the minor component other than to report that this was probably aromatic in nature and appeared to have an exchangeable proton, due to the presence of the wide singlet at 10.27 ppm. It was thus concluded that product B indeed contained (2.6) with a second impurity which was aromatic in nature and contained an exchangeable proton..

#### 2.2.3.2.3 Conclusions

The results; indicted that when (2.14) reacted with (2.17) in dry acetonitrile, two products formed. The products were (2.6) and (2.15). There are two possible explanations as to how these compounds had formed, the first requiring that (2.17) had broken down in the heat of the reaction to form (2.18) and sodium sulfite. The sulfite then reacted with (2.14) to form (2.6) and the phenoxide reacted with (2.14) to form (2.15). The problem with this explanation was that the reaction was conducted under totally anhydrous conditions, thus for (2.17) to decompose to (2.18), $SO_2$ would be released and this would need to react with base to form a sulfite anion or with water to form sulfurous acid. It is possible that (2.17) had some water associated with it when it was synthesised, however every attempt was made to keep it in totally anhydrous conditions and it was stored in these conditions until use. It is also a possibility that on workup, when product B was filtered, it reacted with moisture in the atmosphere and phenoxide was replaced with hydroxide. In the chemiluminescent reaction it is known that the phenoxy moiety has to be an excellent leaving group. Maybe for (2.8) the phenoxy group was too good a leaving group.

An alternative explanation of why (2.6) and (2.15) were found to be the major products in the reaction was that in the initial synthesis of (2.17), residual water (maybe of crystallisation) was still present in the (2.18) . After the azeotropic dehydration, when $SO_2$ gas was bubbled though the mixture, it not only reacted with (2.18) but also with this trace of water thus producing a sulfite. Figure 16 shows a possible mechanism for the products which formed in the attempted esterifiation.

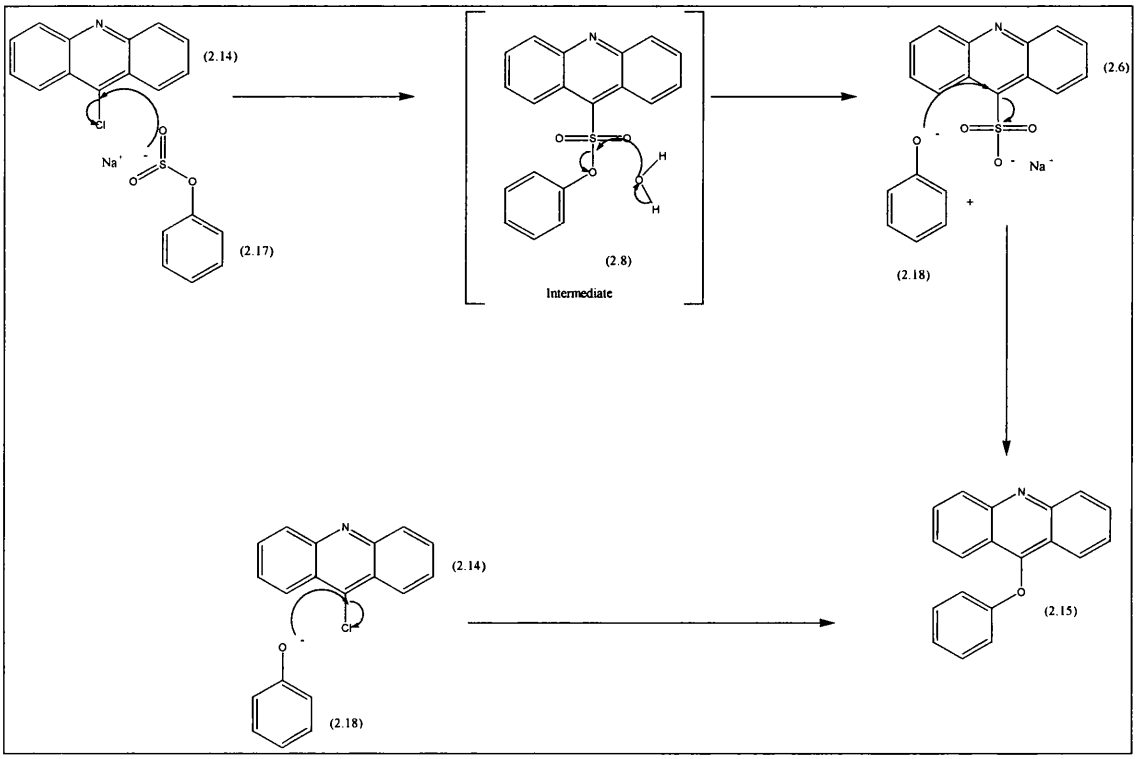



**Figure 16 A possible mechanism showing products formed when (2.17) attacks (2.14)**

Although the above partially explained why (2.6) was present, it did not fully explain why (2.15) was the only other product isolated. It is possible that in solution (2.17) was not stable but existed in an equilibrium with (2.18). Thus at any one moment the (2.17) nucleophile and (2.18) would exist with $SO_2$ in solution. Work was thus conducted to investigate whether this was a possibility.

### 2.2.4 An Investigation into the stability of (2.17) in $d_4$- methanol

To help confirm the stability of (2.17) in solution, an NMR experiment was conducted where (2.18) and (2.17) were mixed in different amounts. The thought here was that theoretically if (2.18) and (2.17) did not interact then there would be two species in

the NMR spectrum. However, if the $SO_2$ molecule were shared between the phenoxide moiety of (2.17) and (2.18) added to the mixture then there would be a timed average spectrum where the shift value would be proportional to the ratio of (2.17) and (2.18). If it were found that a timed average spectrum was a result of this test then a temperature experiment would be conducted to try to resolve both species at low temperatures.

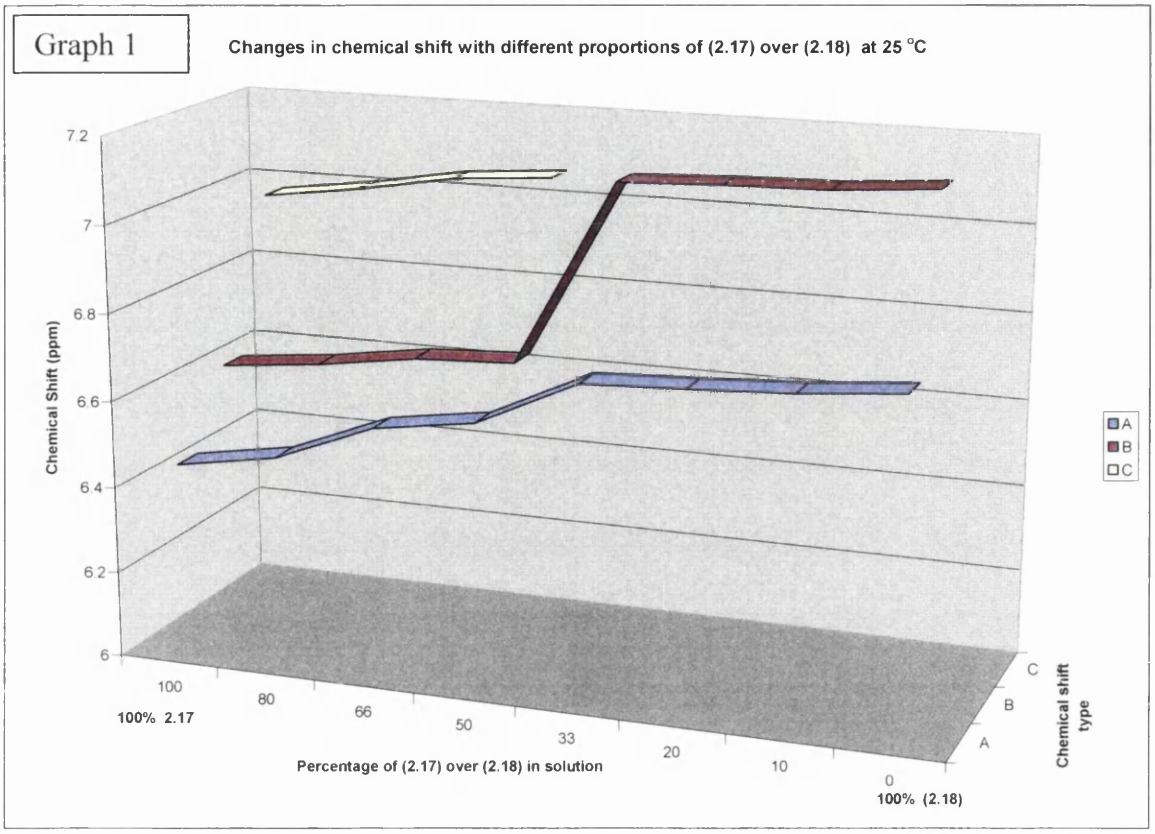
### *2.2.4.1 NMR experiment to investigate the behaviour of (2.17) in solution*

Eight NMR tubes were taken and eight (7 ml) samples were made up in these with (2.18), (2.17) or a mixture of both in $d_4$-methanol. Table 4 gives a list of the components in each tube.

**Table 4 A list of the components of the eight tubes tested in the 400MHz NMR spectrometer**

| Tube # | **Compound(s)** | **Amount ( mg)** |
|---|---|---|
| 1 | (2.17) | 5 |
| 2 | (2.18) | 5 |
| 3 | (2.17)/ (2.18) | 2/2 |
| 4 | (2.17)/ (2.18) | 1/3 |
| 5 | (2.17)/ (2.18) | 1/5 |
| 6 | (2.17)/ (2.18) | 1/10 |
| 7 | (2.17)/ (2.18) | 3/1 |
| 8 | (2.17)/ (2.18) | 5/1 |

Proton NMR measurements for tubes 1 to 3 were then taken on a NMR spectrometer (400 MHz) at the following temperatures; 25°C, -20°C, -40°C, -60°C. The proton NMR spectra of these tubes were then recorded at each of these temperatures. Following this, the proton NMR spectra was measured for tubes 4 to 8 at 25°C.



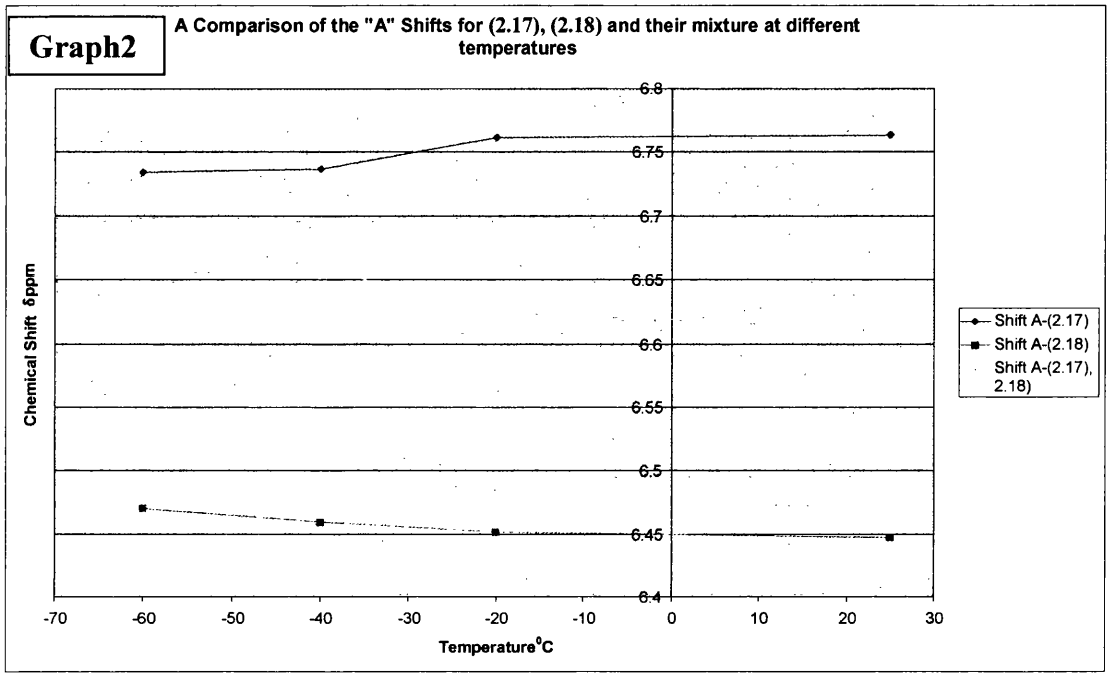

The ppm values were calculated as mean values for each of the multiplets which were representative of each spectrum. Each of the mean values was assigned letters A, B and C, which represented the characteristic multiplets with increasing chemical shift. The "A" shifts integrated always to two protons and the "C" shifts always integrated to one proton (the para proton on the phenyl) ring. However, not all the spectra showed the "C" proton as a separate signal. Some spectra showed only two multiplets, where the "B" multiplet contained also the proton of the "C" multiplet. Thus in these spectra, the "B" multiplets integrated to three protons. In the spectra displaying a "C" multiplet the "B" multiplet integrated to two protons.

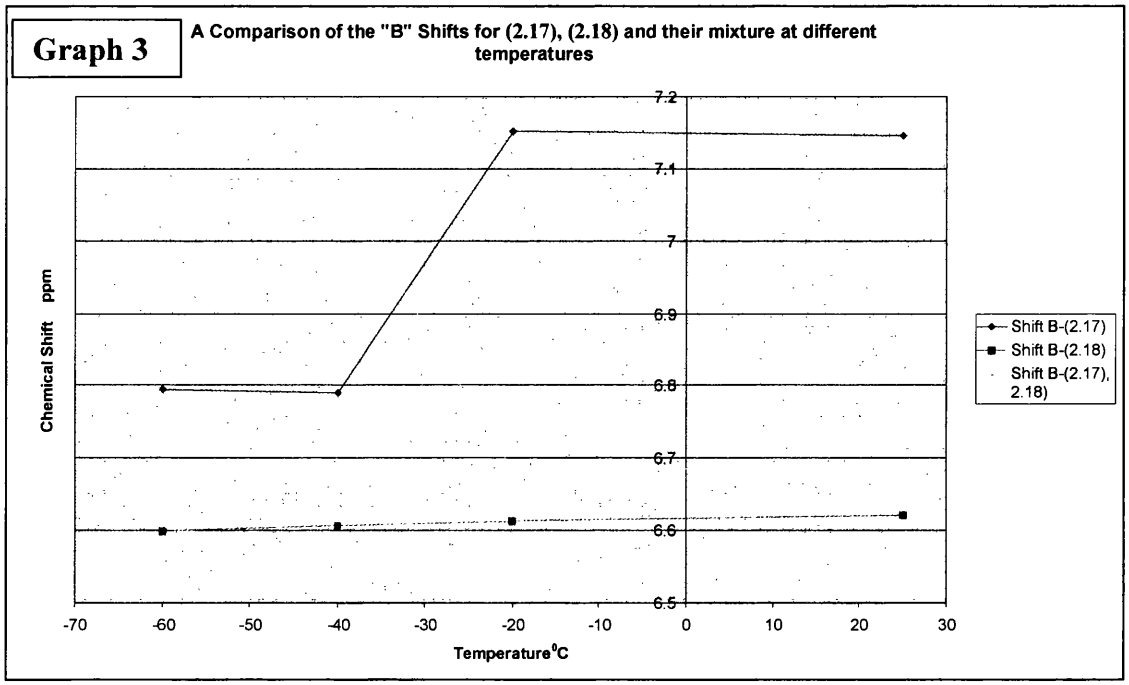
2.2.4.1.1 Effect of varying the proportions of (2.18) and (2.17)

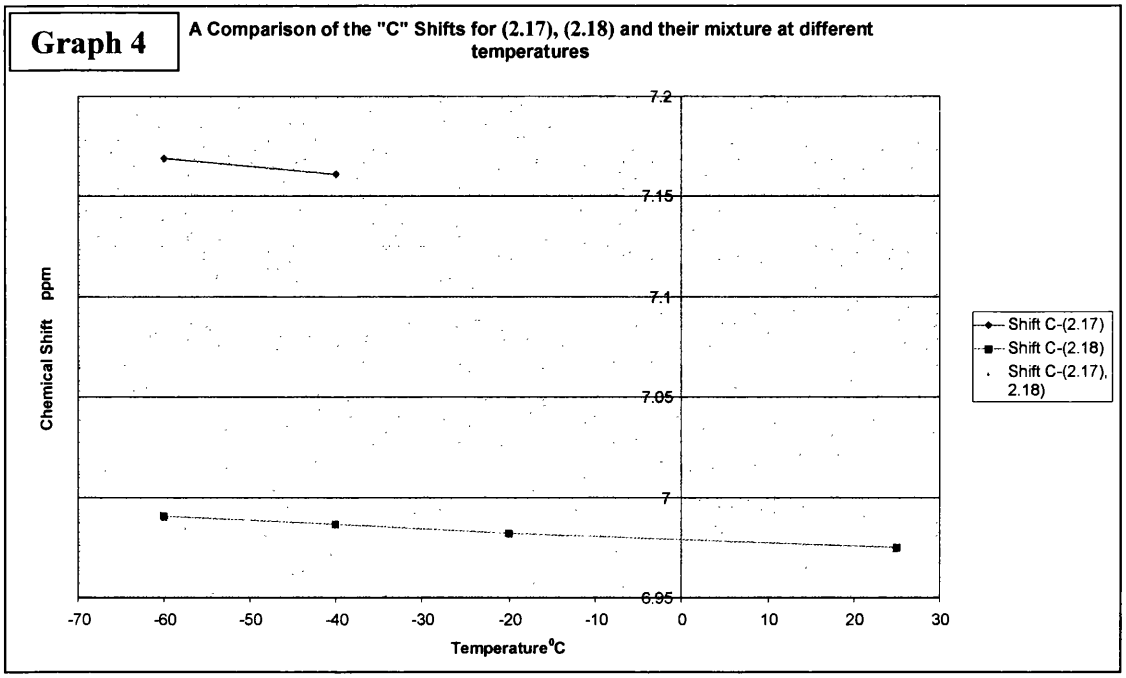
The results of the variable component experiment are represented in graph 1. The results show that mixtures of (2.17) and (2.18) do not give specific peaks for both substances but, as predicted, a timed average spectrum resulted. Moreover, as the mixtures changed from (2.17) to (2.18) the shifts moved towards those of (2.18). The change in the shifts was more apparent for the "B" shifts than the "A" shifts, which

was probably due to the fact that these were the protons ortho to the sulfite. If $SO_2$ were exchanging in the solution, then it would be predicted that the protons would be more affected than the meta protons.

The variable temperature experiment gave some very interesting results, which are represented in graphs 2 to 4.






2.2.4.1.2 The Effect of Temperature on the chemical shifts of (2.18)

On observing the results displayed in graphs 2 to 4, it can be seen that the temperature had little effect on the chemical shifts for this molecule. Shift "A" showed a slight increase with respect to decrease in temperature as did shift "C", but shift "B" showed a slight decrease.

2.2.4.1.3 The effect of temperature on the chemical shifts of (2.17)

In comparison to (2.18), (2.17) showed quite a large shift towards high field strength. This was most apparent for the "B" shift. In fact upon cooling the sample, the "B" shift split to give a "C" shift, thus, the NMR spectra had more of a (2.18) character than (2.17).

2.2.4.1.4 The effect of temperature on the chemical shifts of (2.17) / (2.18) Mix

The mix seemed to have an exactly opposite effect upon cooling than the pure (2.17). At room temperature, the "C" shift seemed to merge into the "B" shift when the solution was cooled down towards - 60 °C. Thus it can be seen that the mixture had

more (2.18) character when at room temperature but (2.17) character when cooled. A very simple explanation to these results is that as the mix cooled, (2.18) precipitated out of the solution leaving mainly (2.17) and a small amount of (2.18). This however did not explain why the pure (2.17) changed so dynamically when cooled.

#### 2.2.4.1.5 Conclusions

The results indicated that (2.17) was not stable in solution; this was shown to be the case in the variable concentration experiment where the mixture showed peaks for just one compound and not two. Thus this indicated that $SO_2$ exchange might have been occurring and thus (2.18) and (2.17) were in equilibrium. The temperature experiment showed that a change in temperature greatly affected the chemical shifts (especially the "B" and "C" shifts). However it must be noted that the changes in the temperature experiment were probably caused by solubility differences rather than the dynamics of the equilibrium.

Under ideal conditions, it should have been shown that a drop in the temperature actually should have caused the signals to broaden and then separate into the two components. The reason for this is that the drop in temperature would act to slow the exchange. As no such separation occurred, an alternative theory for explaining the results is that in solution, (2.17) and mixtures of it and (2.18) can actually form new molecules which consist of two or more phenyl rings sharing one $SO_4$ molecule. It is a possibility that this new molecule was more stable at colder temperatures and this would account for the changes in the NMR spectra upon cooling the tubes.

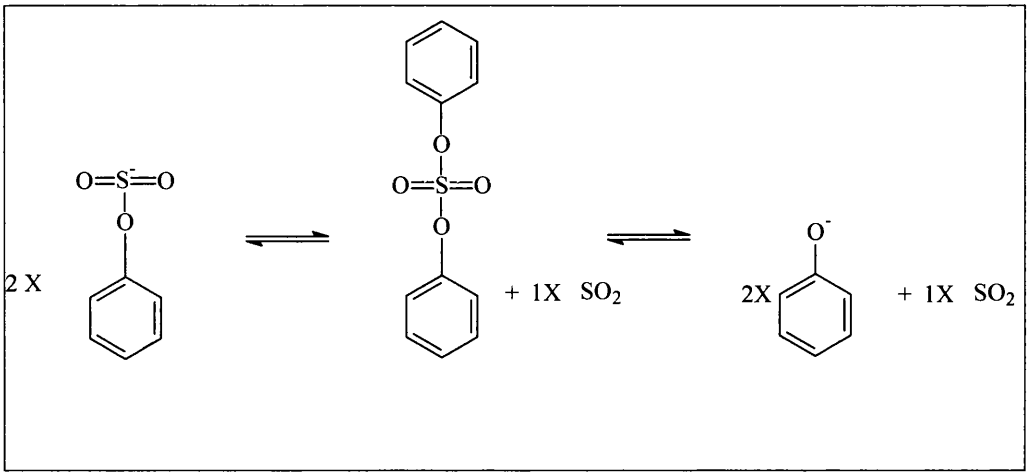



**Figure 17 Possible components of (2.17) found in equilibrium in solution**

As shown in figure 17, (2.17) and (2.18) might exchange through the bis-phenyl intermediate. The implication that (2.17) had the potential to be unstable in solution to form (2.18) and $SO_2$, meant that that (2.18) could compete for the same electrophile such as a haloacridine. This then provided further evidence as to why 9-phenoxy acridine was isolated from the reaction between (2.14) and (2.17).

## 2.3 Experimental

### 2.3.1 General Methods

#### *2.3.1.1 Melting points (m.p.)*

Recorded on a Griffin Melting Point Apparatus and were uncorrected.

#### *2.3.1.2 IR spectra*

Analysed using a Perkin Elmer FT-IR spectrometer 1725x. All IRs quoted were in KBr disk format.

#### *2.3.1.3 NMR*

$^1H$ NMR (400 MHz) and $^{13}C$ NMR (100 MHz) spectra were measured on a Bruker AC 400 spectrometer with TMS (tetramethylsilane) as an internal standard. Chemical ($\delta$) are expressed in ppm, coupling constants (J) are in Hz and abbreviations s = singlet, d = doublet, dd = double doublet, td = triple doubet, dt = double triplet, t = triplet, tt = triple triplet and m = multiplet.

*2.3.1.4 Mass Spectrometry*

Low resolution mass spectrometry with the following ionisation techniques: electron impact (EI), chemical ionisation (CI) and electrospray (ESI) was measured on a Quattro mass spectrometer (VG) and accurate mass fast ion bombardment (FAB) was analysed on a VG AutoSpec mass spectrometer. All mass spectrometric analyses were carried out by the EPSRC in the University of Wales Swansea.

*2.3.1.5 Microanalyses*

Analysed at Butterwoth Laboratories
www.butterworth-labs.co.uk

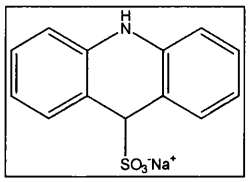
*2.3.1.6 Column and Thin-layer Chromatography*

Column chromatography was carried out with silica gel 60 (230-400 mesh, Merck). TLC were carried out on Watman aluminium backed silica gel plates and these were visualised by ultraviolet light (258 nm).

*2.3.1.7 Chemiluminescent measurements*

Chemiluminescent measurements were carried out using a Magic Lite Analyzer (Ciba-Corning Diagnostics, Medfield, MA 02052, USA). To a test tube containing the sample solution (10 μl, 1 mMol), 0.3 ml of 0.5 % w/v hydrogen peroxide solution in 0.1 M $HNO_3$ was delivered automatically, which was followed by 0.3 ml of 0.25 M NaOH solution containing a surfactant. The output of photons was counted for 2 seconds.

2.3.2 Synthesis of sodium acridan-9-sulfonate (2.10)




**(2.10)**

*2.3.2.1 Method*

Acridine.(1.79 g, 10 mmol), sodiumbisulfite (1.8 g, 22 mmol) and sodium sulfite (0.8 g, 6.3 mmol) was added to water (27 ml) in a round bottom flask (100 ml) fitted with

a reflux condenser. The mixture was then heated to 80 °C to dissolve the products. Once dissolved, the solution was left to cool to room temperature allowing cream crystals of the crude product to form. The crystals were filtered by vacuum filtration and allowed to dry under vacuum over phosphorus pentoxide to give a crude product (2.36 g, 8.4 mmol). The crude acridan-9-sulfonate was then placed in distilled water (5 ml) and heated until most of the solid had dissolved (leaving only a brown solid). When most of the solid had dissolved, the mother liquor was filtered whilst hot and on cooling white / cream crystals formed in the supernatant. After cooling, the crystals were filtered, washed with distilled water and dried under vacuum (2.13 g, 7.6 mmol). The brown insoluble product (122 mg) was then dried under vacuum and was analysed with the main product.

#### *2.3.2.2 Characterisation*

##### 2.3.2.2.1 The cream crystals

**$^1$HNMR (δ ppm, $D_2O$):** 4.44 (large water peak), 5.19 (s, 1H), 6.85 (dd, 2H, J = 8, 1 Hz), 6.93 (dt, 2H, J = 8, 1 Hz), 7.25 (dt, 2H, J = 8, 1 Hz), 7.32 (dd, 2H, J = 8, 1 Hz), **MS (ESI):** Negative Mode m/z, 61 (40 %), 75 (40 %), 80 (15 %), 95 (25 %), 97 (30 %), 260 (100 %, M) 261 (10 %), Positive Mode m/z, 180 (100 %), **MP:** 214 °C (sublimes). **IR $\upsilon_{max}$:** 609, 756, 1039, 1176, 1191, 1315, 1484, 1612, 2923, 3446, and 3513.

##### 2.3.2.2.2 The brown impurity

Same as acridine

##### 2.3.2.2.3 Acridine

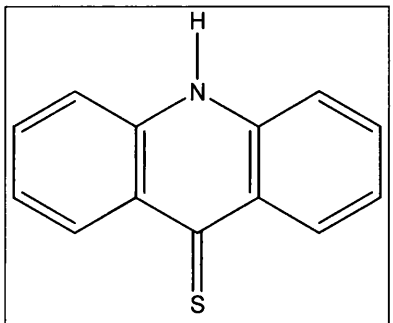
**$^1$HNMR (δ ppm, $CDCl_3$):** 7.55 (dt, 2H, J = 7, 1 Hz) 7.78 (dt, 2H, J = 7, 1 Hz), 7.99 (d, 2H, J = 9 Hz), 8.24 (dd, 2H, J = 9, 1 Hz), 8.81 (s, 1H), **MS (EI):** m/z 179.

### 2.3.3 Oxidation attempts of (2.10) – a general procedure.

Aliquots of 2.10 (50 mg) were taken and dissolved into the following solvent systems NaOH (pH9, 10 ml), HCl (pH4, 10 ml), water (10 ml), methanol / water (10 ml). To the basic and water solutions, a drop of hydrogen peroxide was added. To the water and methanol solution ferric chloride (10 mg) or DDQ (2,3-dichloro-5,6-dicyanobenzoquinone) (10 mg) was added. The solutions were allowed to mix (30

minutes at room temperature). The solvents were then evaporated off and samples of the products were analysed by proton NMR. The results for all attempts showed that (2.10) had decomposed to acridine (see section 2.3.2.2.3).

### 2.3.4 Synthesis of acridine-9-thione (2.12) using Lawesson's reagent




Acidine-9-thione (2.12)

**(2.12)**

#### *2.3.4.1 Method*

Lawesson's reagent (550 mg, 1.25 mmol) was added to a stirring solution of acridone (2.11) (490 mg, 2.5 mmol) in toluene (20 ml) and the mixture was stirred at 110 °C for 0.5h. The product was evaporated to dryness and recrystallised from methanol (342 mg, 1.63 mmol).

#### *2.3.4.2 Characterisation*

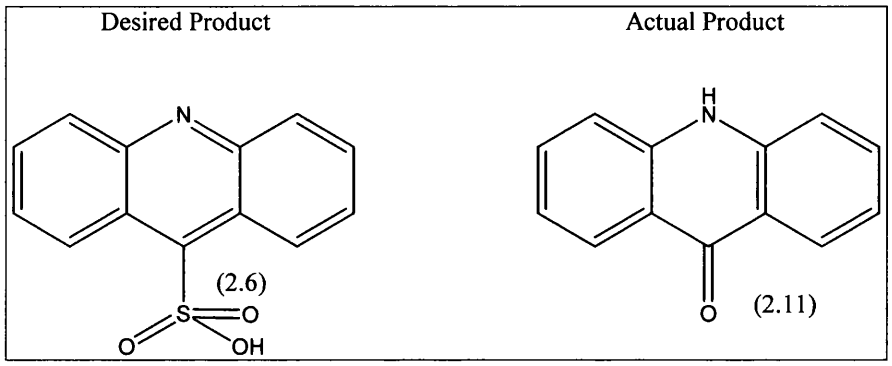
##### 2.3.4.2.1 Product

**$^1$HNMR (δ ppm, $CDCl_3$):** 6.85 (m, 2H); 7.19, (m, 2H); 7.22 (m, 2H); 8.61 (m, 2H). **MS (EI):** m/z: 167 (40 %), 211 (100 %). **(CI):** m/z 212 (100 %). **MP:** 256-8 °C lit 266.[17]

##### 2.3.4.2.2 Compound (2.11) to compare

**$^1$HNMR (δ ppm, $CDCl_3$):** 7.25 (dt, 2H, J = 8, 1 Hz); 7.54, (td, 2H, J= 8, 0.5 Hz); 7.73 (dt, 2H, CH, J = 8, 1, Hz); 8.23 (td, 2H, J = 8, 0.5 Hz), 11.75 (s, 1H, NH).

## 2.3.5 Attempted oxidation of (2.12) to form the free acid of (2.6)




### *2.3.5.1 Method*

Compound (2.12) (0.07 g, 0.33 mmol), was dissolved in NaOH solution (200 ml, 2M) whilst stirring. Following this, $H_2O_2$ (0.12 ml, 30 %) was slowly added and the solution was allowed to stir at room temperature for 1 h. An aliquot of the aqueous solution (0.5 ml) was removed and sent for analysis (mass spectrometry). The temperature of the reaction mixture was increased to 80 °C and the mixture was left to stir for 2 h. At the end of the reaction, HCl (41ml 35 %) was added to neutralise the base solution. From this, a red precipitate formed (0.023 g,) and was filtered, leaving a yellow filtrate. The yellow filtrate was then extracted with chloroform (3 x 200 ml) causing the yellow colour to move into the chloroform extract, which in turn was dried on $MgSO_4$ and evaporated down to dryness forming a yellow powder (0.062 g). Both the red precipitate and the material recovered from the yellow solution were sent for characterisation.

### *2.3.5.2 Characterisation*

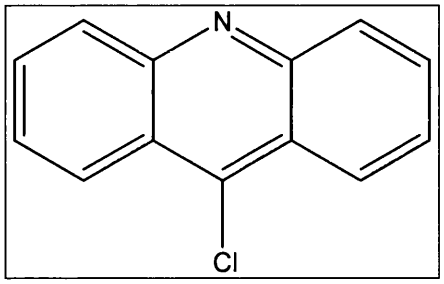
#### 2.3.5.2.1 Red precipitate

See compound (2.11)

#### 2.3.5.2.2 Yellow filtrate

The NMR spectrum showing peaks for (2.11) (see section 2.3.4.2.2) and compound (2.12), with (2.11) twice the intensity of (2.12).

2.3.6 Synthesis of 9-chloroacridine (2.14)




**(2.14)**

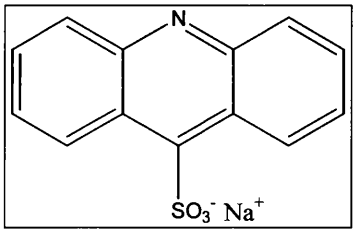
*2.3.6.1 Method*

In a round bottom flask (50 ml) fitted with a condenser and a drying tube, a mixture of (2.13) (1.052 g, 4.7 mmol) and phosphorus oxychloride (8 ml, 88 mmol) was slowly heated with stirring to 90 °C and maintained at this temperature for 30 minutes. Following this, the temperature was raised to 120 °C and the mixture was stirred for 2h. The $POCl_3$ was then distilled off under vacuum (15 mm Hg) at 120 °C. The brown / black solution was evaporated to form a black solid on the base of the flask. Cold DCM (50 ml) was then added to dissolve the solid. Three attempts were taken to acquire all the bulk of the product into solution. The dissolved product was added slowly to cracked ice (60g) and ammonia (40 ml). The chloroform layer was then separated, dried with $MgSO_4$ and evaporated on a rotary evaporator to yield a yellow solid (1.0 g, 4.7 mmol). The product was then analysed by $^1$HNMR and mass spectrometry (EI / CI).

*2.3.6.2 Characterisation*

**$^1$HNMR (δ ppm, $CDCl_3$):** 7.59 (dt, 2H, J = 9, 1 Hz), 7.78 (dt, 2H, J = 9, 1 Hz), 8.20 (dd, 2H, CH, J = 9, 1 Hz), 8.38 (d, 2H, CH, J = 8 Hz). Small impurities aromatic region **MS (EI):** m/z 177 (10%), 213 (100%), 215 (35%), **(CI):** m/z 213 (70%), 214 (100%), 215 (40%), 216 (40%). **MP:** 117-118 °C (lit 117-118 °C).[17]

2.3.7 Successful synthesis of sodium acridine-9-sulfonate(2.6)




**(2.6)**

### *2.3.7.1 Method*

Compound (2.14) (630 mg, 2.9 mmol) and $Na_2SO_3$ (750 mg, 5.5 mmol) were added to a solution of water and EtOH (200ml, at a 1:1 ratio) in a round bottom flask (250 ml) fitted with a condenser. The solution was heated at 60 °C and stirred for 5 h. Following this, the solvent was evaporated using a rotary evaporator attached to high vacuum pump and at a moderate temperature (30 °C) yielding yellow solid (1.285 g). The solid was added to ethanol (100 ml) and stirred for 0.5 h where it was filtered yielding a white powder (0.450 g, 0.0035 mol) and the liquid was evaporated to yield a yellow solid (0.742 mg). This solid was then added to chloroform (100 ml) and stirred for 30 minutes whereupon a yellow powder remained and was filtered off. This was repeated twice and the insoluble yellow powder was filtered (680 mg, 2.4 mmol). The resulting liquid was evaporated to produce a yellow solid (50 mg).

### *2.3.7.2 Characterisation*

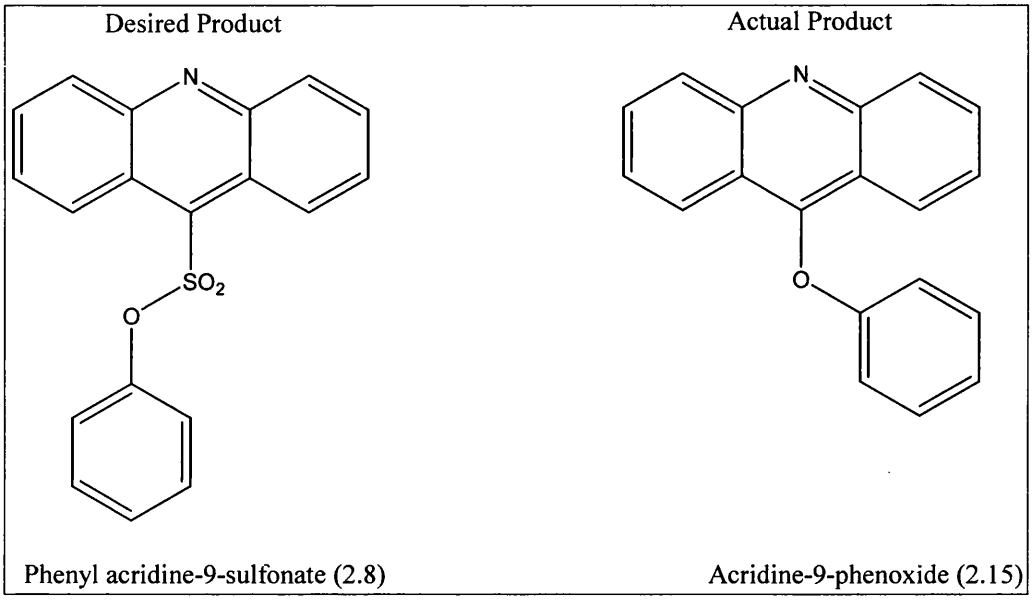
#### 2.3.7.2.1 The yellow solid

**$^1$HNMR (δ ppm, d6-DMSO):** 7.55 (ddd, 2H, J = 9, 7, 1 Hz), 7.76 (ddd 2H, J = 9, 7, 1 Hz), 8.09 (dd, 2H, J = 9, 1 Hz), 9.56 (d, 2H, J = 9 Hz), **MS (ESI):** Positive Mode m/z 55 (40%) 87 (50 %) 97 (100 %), 119 (50 %), 129 (50 %) 196 (50 %), 214 (40%) 216 (12%), 260 (60 %), 282 (60 %),. Negative Mode m/z 45 (30 %), 194 (60%), 258 (100 %),. **MP** 244-246°C. **IR $\upsilon_{max}$:** 564, 794, 944, 1147, 1259, 1315, 1394, 1434, 1514, 1604, 3425.

The chloroform extract

Same as (2.14) (see section 2.3.6.2)

### 2.3.8 Attempted Synthesis of (2.8) through synthesis of acridine-9-sulfonyl chloride and then attack with phenol




Phenyl acridine-9-sulfonate (2.8) Acridine-9-phenoxide (2.15)

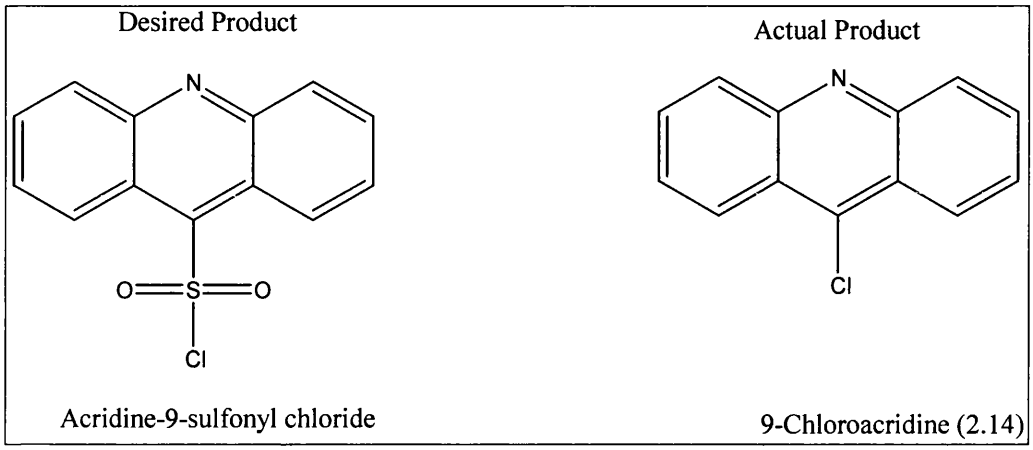
#### *2.3.8.1 Method*

A mixture of (2.6) (200 mg, 0.71 mmol) and thionyl chloride (20 ml), in an oven dried round bottom flask (50 ml) fitted with a dry reflux condenser and drying tube, was refluxed for 4 h, with the solution clarifying after 2 h. The solution was then allowed to cool and the thionyl chloride was removed buy rotary evaporation. The product was then put under high vacuum for 4 h to remove the remainder of the thionyl chloride. The product was then dissolved in dry DCM (20 ml). Following this, both phenol (135 mg, 1.4 mmol) and pyridine (1 ml) were added and the reaction mixture was allowed to stir at room temperature overnight. The crude product was first taken up into DCM (200 mg) and extracted successively with both dilute acid and base. The product was then dried over magnesium sulfate, filtered and evaporated to dryness. Following this it was separated by column chromatography. The solid phase used was silica 60 and the mobile phase was a gradient of the following solvents in increasing polarity (DCM, DCM 90 / EtOAc 10 and then DCM 80 / EtOAc 20). When monitored by TLC, the major product (Rf 0.55 on a silica gel plate, using DCM90 EtOAc10 as a mobile phase) was isolated in a yield of 94 %, (181 mg).

#### *2.3.8.2 Characterisation*

**$^1$HNMR (δ ppm, $CDCl_3$):** 6.85 (td, 2H, J = 9, 2 Hz), 7.04 (m, 1H), 7.26 (tt, 2H, J = 8, 2 Hz), 7.45 (ddd, 2H, J = 9, 7, 1 Hz), 7.78 (ddd, 2H, J = 9, 7, 1 Hz), 8.09 (d, 2H, J = 9 Hz), 8.27 (d, 2H, J = 9 Hz). **$^{13}$CNMR (δ ppm, $CDCl_3$):** 115.5 (2CH), 120.3 (C), 122.6 (CH), 122.7 (2CH), 125.8 (2CH), 129.6 (2CH), 129.9 (2CH), 130.6 (2CH), 150.5 (C), 155.2 (C), 159.5 (C). 171.1 (artifact). **MS (ESI):** Positive Mode m/z 272.

### 2.3.9 Reaction of (2.6) with thionyl chloride




#### *2.3.9.1 Method*

(2.6) (100 mg, 0.0035 mol) was added to thionyl chloride (10 ml) in a round bottom flask (25 ml) fitted with a reflux condenser and drying tube. The mixtue was refluxed at 85 °C for 3.5 h. During the reaction, the salt slowly dissolved in thionyl chloride. At the end of the reaction, the thionyl chloride was distilled off (85 °C, 20 mm Hg) and the resulting yellow solid placed under high vacuum (0.1 mm Hg) for 1 h to remove the rest of the thionyl chloride. Following this the product obtained was dissolved in dry chloroform (25 ml) and a white, water-soluble powder was filtered off (70 mg). The solution was tested for purity by TLC and was then sent for $^1$HNMR, IR and FAB mass spectroscopic analyses.

#### *2.3.9.2 Characterisation*

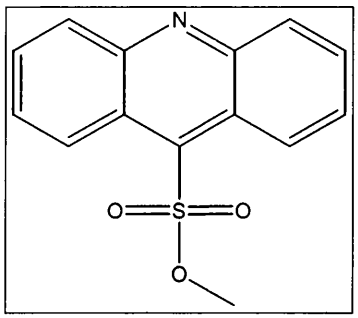
**TLC** (Silica gel solid phase, chloroform / ethyl acetate (75:25) mobile phase)

One single spot with an Rf of 0.83, which was identical to the Rf of (2.14) and furthermore when visualised at 266 nm the spot gave the colour fluorescence of (2.14) was observed.

2.3.9.2.1 Product from the reaction

**MS (FAB):** m/z 196 (25 %), 214 (100%), 215 (55%), 216 (80%), 217 (20%). **IR** $\upsilon_{max}$**:** 750, 838, 957, 1192, 1286, 1298, 1317, 1345, 1464, 1494, 1626, 1694, 1933, 2014, 2014, 2414

2.3.10 Attempted synthesis of methyl acridine-9-sulfonate using methyl iodide in THF or chloroform




*2.3.10.1 Method*

Compound (2.6) (100 mg, 0.35 mmol) was dispensed into two round bottom flasks (25 ml) so each contained equal quantities of the salt (50 mg). Into each flask was added a molar excess of MeI (~300 mg, 2.1 mmol) and an aliquot (10 ml) of either, chloroform or THF. The two round bottom flasks, attached to reflux condensers, were heated (70°C) and stirred for 4 h. As a control, (2.6) (10 mg) was added to THF (10 ml) and heated along side the two experimental flasks. The reaction was monitored by TLC. Following this, the round bottom flasks (experimental) containing chloroform and THF (called RBF 2 and 3 respectively) were removed from the heat. It was noted that the yellow solutions had turned into a red / brown colour, the two solvents (in RBF 2 and 3) were removed by rotary evaporation and the resulting solids were weighed (to give two products [58 mg] and [76 mg]). Aliquots of both products were taken added to chloroform (20 ml), stirred (0.5 h) and filtered.

*2.3.10.2 Characterisation*

Both samples were found to be starting material

### 2.3.11 An NMR investigation into the attempted synthesis of methyl acridine-9-sulfonate in $d_6$-DMSO using methyl iodide

#### *2.3.11.1 Method*

Aliquots of (2.6) (10 mg, 0.036 mmol) were added to each of two sample vials (10 ml) upon which $d_6$-DMSO (7 ml) was added to each tube. The tubes were capped and shaken to allow the salt to dissolve. To one of the tubes (b) a drop of MeI (13 mg, 0.092 mmol) was added and the tube was further shaken to allow mixing. A third sample vial (c) was taken and an aliquot of $d_6$-DMSO (7 ml) and to this MeI was added (13 mg, 0.0092 mmol was added). The contents of each tube were transferred to separate NMR tubes labelled a, b, and c. Please see table 5

**Table5 Contents of NMR tubes during an investigation of attempted methylation**

| NMR tube label | Contents |
|---|---|
| a | (2.6) + *d6-DMSO* |
| b | (2.6) + *d6-DMSO* + MeI |
| c | *d6-DMSO* + MeI |

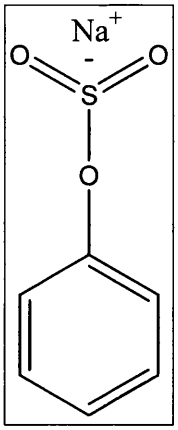
The tubes were sent for $^1$HNMR analysis (a, b and c), and following this, the tubes were heated in a water bath (60 °C) for 0.5 h and then sent for a second NMR analysis give (a2, b2, and c2). The tubes were then heated to 80 °C for 2h and reanalysed by NMR (a3, b3, and c3).

### 2.3.12 Synthesis of $SO_2$ gas

#### *2.3.12.1 Method*

Copper turnings (50 g) were added to a round bottomed flask attached to a gas tight seal and gas tap. A gas line was attached between this and a solution of concentrated sulphuric acid (200 ml). Moreover a second aliquot of sulphuric acid (200 ml) was added to the copper turnings and this solution was heated (90°C), resulting in gas formation. The gas was passed though the gas line and to dry the gas was bubbled though the extra vessel containing $H_2SO_4$.

2.3.13 Synthesis of (2.17)




**(2.17)**

*2.3.13.1 Method*

Sodium phenoxide trihydrate (2.18) (7.075 g, 41.4 mmol) was dispensed into a xylenes mixture (100 ml) in a round-bottomed flask (250 ml) fitted with a Dean Stark apparatus (25 ml) which in turn was fitted with a reflux condenser. The solution was refluxed for 5 h. The Dean Stark trap was removed and the round bottom flask containing the (2.18) (and xylenes) was placed in an ice bath and $SO_2$ gas (formed from the copper / $H_2SO_4$ reaction, see section 2.3.12) was bubbled though the mixture, with rapid stirring, for 2 h. Following this, the gas was removed and the round bottom flask containing the product was sealed and kept on ice stirring, for a further 4 h. After the reaction was complete, whilst stirring was maintained, the suspended product was decanted into test tubes and was centrifuged to form pellets. The supernatant was decanted off and evaporated to find the weight of any product in the supernatant. The pellets were then re-suspended in dry diethyl ether and then re-centrifuged. The supernatant was then decanted, evaporated and weighed. This washing procedure was repeated twice.

*2.3.13.2 Characterisation*

2.3.13.2.1 Sodium phenoxide trihydrate (for comparison)

**$^1$HNMR (δ ppm, $CD_3OD$):** 4.90 (s, 5H, water), 6.44 (tt, 1H, J = 7, 1 Hz); 6.62 (m, 2H), 6.95 (m, 2H). **IR $\upsilon_{max}$:** 526, 700, 768, 822, 880, 992, 1166, 1272, 1480, 1588, 1634, 2340, 3358, 3392

2.3.13.2.2 Product from the reaction (2.17)

**$^{1}$HNMR (δ ppm, d6-DMSO):** 6.77 (m, 3H), 7.13 (m, 2H). **$^{13}$CNMR (δ ppm):** 95.5 (C), 119.2 (CH), 128.9 (CH), 157.1 (C). **MS (ESI):** Negative mode (Cone Voltage 8V) m/z 95 (100 %), 109 (25%), 113 (30%), 121 (45%), 127 (40 %), 139 (%), 141 (50 %), 145 (40 %), 149 (50 %), 157 (35 %), 171 (30 %). **MP** 224-226 °C. **IR $\upsilon_{max}$:** 449, 516, 530, 651, 691, 754, 979, 1065, 1178, 1474, 1596, 2360, 3430

2.3.14 Attempted nucleophilic substitution of (2.14) using (2.17)

*2.3.14.1 Method*

Pure (2.14) (100 mg, 0.47 mmol) and (2.17) (160 mg, 0.88 mmol) were added to a dry (hot) two necked round bottom flask (25 ml), containing a magnetic flea, fitted with a dry (hot) condenser. Septa were added to the free neck of the round bottom flask and at the end of the condenser. Following this, dry nitrogen gas was passed through the septum of the free neck of the round bottom flask and out through the septum attached to the condenser. The glassware was allowed to cool and then dry acetonitrile (10 ml) was introduced into the round bottom flask *via* a dry syringe. The solution was stirred to reflux (90 °C) for 6 h. Following this, the solution was cooled then filtered giving a beige powder (product B, 34 mg) and the mother liquor was evaporated down to give a yellow product (product A, 108 mg). These were then sent for both NMR ($^{1}$H and $^{13}$C) and mass spectroscopic analyses.

*2.3.14.2 Characterisation of Product A (Crude NMR)*

See section 2.2.3.2

*2.3.14.3 Characterisation Product B (Crude NMR)*

Main peaks

**$^{1}$HNMR (δppm, d6-DMSO):** 7.56 (m, 2H), 7.78 (m, 2H), 8.10 (d, 2H), 9.57 (m, 2H).

Major impurities

**$^{1}$HNMR (δppm, d6-DMSO):** 8.02 (m), 8.83 (m), 10.27 (ws,).

**MS (ESI)** Negative Mode m/z 65 (70 %), 95 (70%), 99 (40 %), 127 (50 %), 194 (50 %), 258 (100 %).

2.3.14.3.1 Compound (2.6) (to compare)

**$^1$HNMR (δ ppm, d6-DMSO):** 7.55 (m), 7.78 (m), 8.09 (d), 9.57 (d).

## 2.4 Final conclusions

The work described in this chapter details the development of a successful synthesis of (2.6) through nucleophilic attack on (2.14). It was hoped that this could then be used as a starting material for the synthesis of a range of aromatic phenyl sulfonate esters. It was surmised that initially, the phenyl ester would be synthesised and then the chemistry could be developed such that a sulfonate analogue of the Weeks-Woodhead ester (1.24) could be produced. However, from the onset, synthesis of a phenyl ester was found to be extreme difficult to achieve. On attempts to synthesise acridine-9-sulfonyl chloride (2.7), the sulfonate was substituted for a chlorine forming (2.14). However, this relied on the fact that synthesis of acid chlorides required an initial nucleophilic attack of the sulfonate onto the chlorinating agent. However if the sulfonate was a poor nucleophile then this first step would be inhibited from occurring. Work was done to assess if the sulfonate was a good nucleophile and thus attempts to form the methyl ester were undertaken using methyl iodide as a methylating agent. It was soon to be discovered that under all conditions tried, methylation did not occur. If water was present then hydrolysis to (2.11) was observed. Interestingly, when an NMR experiment was conducted to investigate the unsuccessful attempts to form methyl esters it was found that in DMSO, rather than forming an ester, methyl iodide actually speeded the loss of the sulfonate group in favour of hydroxide. It was thought that this was due to the methylating agent attacking the heterocyclic nitrogen and thus significantly reducing the electron density around the 9-position carbon, thus promoting hydroxy attack.

On account of the instability of the sulfonate group to attack by nucleophiles and an apparent lack of functionality as a nucleophile, it was decided synthesis (2.17) and use this to attack (2.14). However, it was found that (2.17) was unstable in solution and when reacted with (2.14), compounds (2.15) and (2.6) were observed as a result (figure 16). Work in the future could be conducted to attack (2.14) or even (2.6) (as the sulfonate group has proven to be such an excellent leaving group) with phenyl sodium sulfonate with a continual stream of $SO_2$ gas passing though to try to keep the $SO_2$ group on the phenoxide.

The results of this investigation have in some respects complemented the initial computational data, which indicated that the sulfonate group would decompose significantly more energetically than the carboxylate analogue. It was concluded that the very feature which would make the sulfonate moiety such an energetic group meant that it was too prone to nucleophilic attack to be a useful probe in an aqueous system. Based on the above conclusion, it was decided to abandon synthesis of this elusive ester and focus on synthesis of energy transfer molecules which is described on the following chapter.

## 2.5 References

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# Chapter 3

# Synthesis and potential chemiluminescent properties of novel energy transfer molecules

# 3 Synthesis and potential chemiluminescent properties of novel energy transfer molecules

## 3.1 Introduction

Chapter 1 described chemiluminescence and its uses in both medicine and biotechnology. Chemiluminescent molecules although extremely useful have many drawbacks: some are extremely inefficient; however some, whilst more efficient, are not stable in aqueous conditions. Furthermore, some chemiluminescent molecules require catalysts and co-factors for light formation. For most chemiluminescent reactions, the wavelength of light is predetermined by the luminescent chemical reaction. Thus colour choice is severely restricted by the limitation of the choice of chemiluminescent system. Chemiluminescent energy transfer is a means to allow for a wider colour choice for a given chemiluminescent reaction and furthermore, in some incidences increases in the quantum yield of light output are observed.[1]

Chemiluminescent energy transfer (CL ET) is a combination of energy transfer and classical chemiluminescence. During classical chemiluminescent reactions such as those illustrated for acridinium esters and luminol, a chemical reaction facilitates the generation of excited molecules and when these excited molecules decay to their ground states, light is emitted (see chapter 1). CL ET differs from CL, in that it is a dual process whereby the chemically initiated generation of an excited state occurs on a donor molecule is followed by transfer of the energy to an acceptor molecule. The donor, may, or may not be luminescent. Following energy transfer the acceptor molecule in turn becomes excited and on relaxation, yields light. The transfer of the energy to an acceptor can be achieved though either intermolecular or intramolecular interactions. An example of CL ET is the well-understood chemiluminescent reaction found for the oxalate ester family. These react through a process known as CIEEL or chemically initiated electron exchange luminescence (see chapter 1 figure 10).

The CIEEL mechanism is one of several methods through which CL ET can occur. These mechanisms can be classified into two groups, trivial and non-trivial, where CIEEL is an example of the latter. Trivial ET involves the emission of light from

relaxation of an excited donor (D*) molecule. The emission of this light is then taken up by an acceptor molecule (A), which in turn becomes excited (A*) and then emits light of a longer wavelength on relaxation (see figure 1).

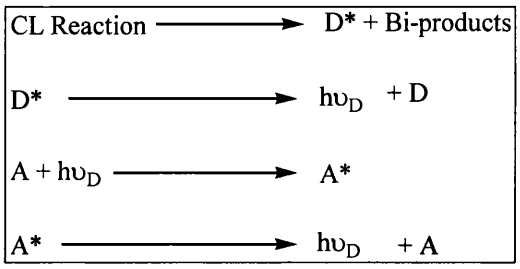



**Figure 1 Intermolecular energy transfer**

The quantum yield obtained from trivial ET is subject to the following limitations:

1. Quantum yield of emission form A* will always be less than that of D* alone, because A will never absorb all of the light emitted by D*
2. The wavelength of light from A* will always be longer than from D* because energy will only be transferred to a species with a lower energy excited state.
3. The degree of the spectral overlap between the excitation coefficient of D and absorbitivity coefficient of A.

Although trivial energy transfer certainly accounts for some of the energy transferred in many energy transfer reactions, it is radiationless energy transfer mechanisms that take precedence. As well as CIEEL, the other forms of energy transfer which are known to exist are: Resonance (or Dipole Dipole), Collisional, and Energy pooling. Campbell defines radiationless energy transfer as:

'the transfer of energy from one atom, or group of atoms, in an electronically excited state to another without the direct transfer of a photon' (ref.1, p477)

### 3.1.1 Differences between radiative energy transfer and radiationless energy transfer

There are 4 parameters which distinguish radiationless energy transfer from radiative energy transfer.

1) An increase in the concentration of both the donor and acceptor, keeping to the some molar ratio, will show an enhanced light output for radiationless ET when

compared to trivial energy transfer. This is quite often due to quenching of the excited donor molecules by solvent molecules before photon emission. Non radiative ET occurs much faster (pico to femto second range) than photon emission of the donor (nano second range) thus reducing the exposure to quenchers.[2]

2) Radiationless energy transfer can show an overall increase in the total quantum yield. This occurs when the acceptor molecule has a higher fluorescence quantum yield than the donor molecule. In other words, if the donor molecule has an excitation yield of 'x' amount, during trivial energy transfer, the acceptor molecule will only be excited by the photons emitted from the donor. However, during radiationless ET, the excited donor itself excites the fluorescer and if the fluorescer has a higher fluorescence yield than the donor, the system will show an increased quantum yield.
3) An increase in solution volume will show a greater increase in light output for trivial ET when compared to the same increase in volume in radiationless ET. This is due to the fact that a lager volume increases the path length of the light, which for trivial ET increases the probability of a photon acceptor encounter. By virtue of the fact that radiationless energy transfer does not use photon evolution and uptake as the mechanism for transfer then an increase in volume would not enhance light output.
4) Increased solvent viscosity will enhance some non-radiative ET mechanisms which require the formation of complexes such as the charge transfer complexes found for CIEEL type energy transfer. The more viscous the solution the more stable the complex.

### 3.1.2 Collisional vs dipolar resonance non-radiative energy transfer

The two major mechanisms for non-radiative energy transfer are: through collisional interactions and through dipolar resonance. The latter differs from collisional interactions in that the atoms/molecules do not have to actually touch but energy can be transferred through the same attractive dipolar forces that help to hold these molecules together. Furthermore, it has been calculated that such energy transfer can occur over distances of up to 120 Å.

### *3.1.2.1 Resonance energy transfer*

During the mid 1920s J. Perrin was the first person to mathematically demonstrate that resonance energy transfer was possible between molecules using the principals of classical physics.[3] He predicted that *r,* the critical distance between two molecules, was a function of the wavelength excitation maximum of the acceptor ($\lambda$) over $2\pi$. Several years later F. Perrin then improved this equation by adding a quantum mechanical aspect. Thus, the equation estimated *r* as:

$$r = \lambda/\pi\sqrt{t/\tau} \qquad (1)$$

where t is the mean interval between successive collisions and $\tau$ is the mean excitation time.[4] There were, however, two major flaws in J Perrin's equation; the first was that it did not take into account the imperfect overlap between the emission spectrum of the donor and the excitation spectrum of the acceptor. The second flaw was that the equation did not take into account that the donor and acceptor molecules would not be perfectly orientated. The ideal orientation of dipoles is between two molecules is 0° or 180°. At 90°, energy transfer is impossible, as the dipoles would not interact. T. Förster addressed these issues and derived the modern resonance energy transfer equation (2). As well as including a spectral overlap expression (*J*) and an orientation factor ($K^2$) he also argued that the efficiency of resonance energy transfer decreased in proportion to the sixth power of the distance. This is because the energy in dipole dipole interactions decreases with the third power of the molecular separation distance and the statistical chance of energy transfer through dipole dipole interactions is proportional to the square of the interaction energy.[2]

$$E = R_o^6/\left(R_o^6 + r^6\right) \qquad (2)$$

The expression $R_0$ is the critical transfer distance and r is the donor-acceptor intermolecular distance $R_0$ is calculated from equation 3

$$R_0 = \left[\left(9000(\ln 10)K^2\Phi_d\right)/\left(128\pi^5 n^4 N\right)\right]J \qquad (3)$$

$K$ is the relative special orientation factor and assumes a random orientation of 2/3 or $K^2$. $\phi_d$ is the donor fluorescence quantum yield, $n$ is the refractive index of the medium, $N$ is Avogadro's number and $J$ is the integral overlap. $J$ is calculated from equation (4)

$$J = \left[\int \varepsilon(\lambda) I_{fl}(\lambda) \lambda^4 d\lambda\right] / \left[\int I_{fl}(\lambda) d\lambda\right] \quad (4)$$

$\varepsilon(\lambda)$ is the acceptor molar absorbitivity and $I_{fl}(\lambda)$ is the donor fluorescence intensity at wavelength $\lambda$.

Förster's equation showed that for resonance energy transfer to be favoured, the distance between the donor and acceptor must be <100-120 Å. Furthermore, the donor and acceptor must be in the correct orientation at least some of the time and there should be overlap between the donor emission and acceptor absorption spectra. Morover, the larger the spectral overlap, the greater the amount of energy transfer that is possible.[2]

Two intermolecular energy transfer chemiluminescent systems which have been shown to transfer energy through resonance transfer are: 1) *N*-methyl acridone, from the oxidation of lucigenin, transferring energy to a second unreacted lucigenin, which then becomes the emitter; 2) the formation of the donor molecule, an aminopthalate derivative, from oxidation of luminol or isoluminol, which excites fluorescein though resonance energy transfer.[5] Moreover intramolecular energy transfer molecules have been synthesised from phthalhydrazide derivatives, which are poorly chemiluminescent in the underivatised form, but when bound to an energy acceptor such as *N*-methyl acridone show enhanced chemilumnescence.[6,7,8] See figure 2 for another example of intramolecular energy transfer.

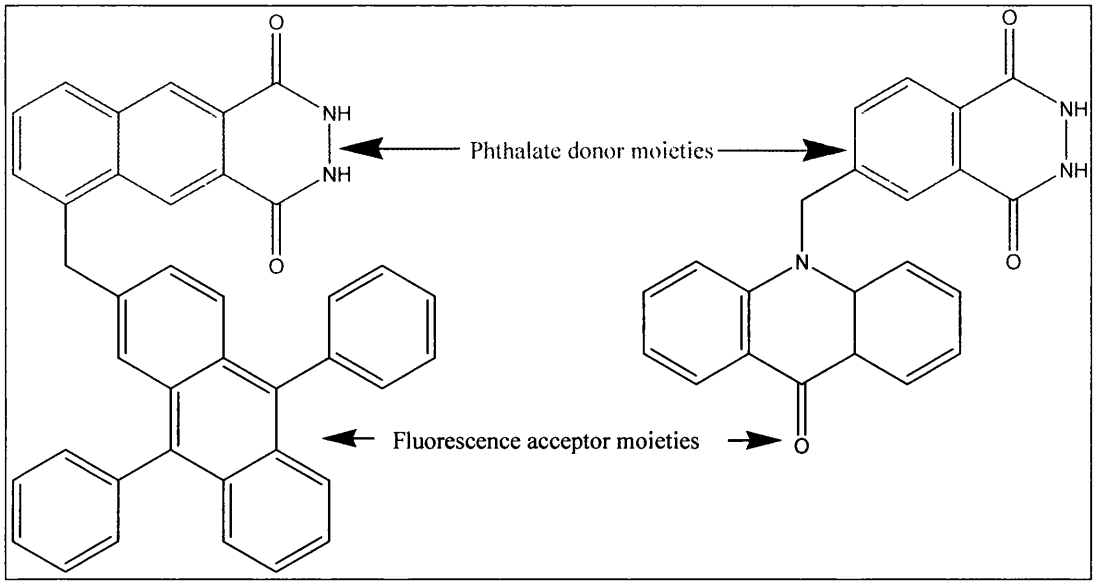



**Figure 2 Intramolecular chemiluminescent energy transfer molecule**

Although resonance energy transfer plays a major role in many energy transfer mechanisms, it is fair to say that most energy transfer is not exclusive by just one mechanism but is a combination of two or more. Recently Grayeski *et al.* examined the mechanisms of intermolecular energy transfer between acridinium esters and rhodamine 110, with or without the presence of micelles. As expected, more than one mechanism of energy transfer was found to exist (resonance, collisional and trivial). In cationic surfactant solutions, the major ET mechanisms were collisional and trivial. However resonance ET played a greater role in anionic solutions.[9]

### *3.1.2.2 Collisional energy transfer*

Collisional energy transfer can be subdivided into two major groups: electron exchange / transfer and energy pooling. The latter is less important in terms of accepted theories for the majority of CL ET mechanisms. Energy pooling occasionally occurs when two or more excited molecules form a complex and the sum of the energies of the excited states within the complex is sufficient to produce light. However, individually these excited molecules do not contain sufficient energy to emit outside of the complex. If the complexes are homogeneous in nature then they are known as excimers. However, if the complexes are heterogeneous then they are known as exciplexes. One example of an excimer is involved in the emission of excited oxygen. Outside of the excimer it emits at 1238 nm. However, when an excimer is formed, visible light is observed (705 – 634 nm).[10]

3.1.2.2.1 CIEEL

The most widely accepted collisional energy transfer mechanism is electron exchange and electron transfer. For CL ET, the CIEEL mechanism takes precedence. As mentioned before, the CIEEL mechanism is the primary mechanism for not only the chemiluminescence of oxalate esters but also of dioxetans too. During oxalate ester reaction (see figure a), peroxidation of the two carbonyl groups causes cleavage of the flanking groups forming dioxetandione. This four membered ring forms a charge transfer complex with an acceptor molecule which facilitates a radical transfer from the acceptor molecule, and this forming a radical cation, to the dioxetandione. The transfer facilitates the breakdown of the four membered ring resulting in the formation of two $CO_2$ molecules where one is a radical anion. The radical anion then transfers an electron to the radical cation acceptor molecules which becomes excited and on relaxation light is evolved. The CIEEL mechanism can occur both intermolecularly but also intramolecularly.[11]

In summary, collisional ET differs from resonance ET in the following ways.

1. Intermolecular distances are much shorter at about 1-5 Å, as opposed to up to 80-120 Å for resonance.
2. The donor and acceptor molecules require overlap of energy levels for ET to occur.
3. Collisional ET involves the exchange or transfer of electrons between the donor and acceptor molecules.
4. To allow for the electron transfer, charge transfer complexes are formed surrounded by a solvent cage.
5. Efficiency of the energy transfer does not follow the same restrictions as described for resonance; for example the amount of energy transferred is independent of the *J* integral.
6. The rate of the chemiluminescent reaction and efficiency of energy transfer can be enhanced by fluors, which are good electron donors.
7. Collisional energy transfer quite often involves single electron transfer. However, although the Förster equation does not take into consideration spin states, oxygen, which is naturally triplet in nature, would quench excited triplet formation certainly before intermolecular energy transfer could be facilitated.

### 3.1.3 Advantages of CL ET over classical CL

Chemiluminescence energy transfer has some distinct advantages over non-energy transfer chemiluminescent mechanisms such as those found for the acridinium ester, luminol or luciferin families. These advantages include selection of a fluor which allows not only the desired wavelength of light to be selected but also offers the chance of higher light output for acceptors which have large quantum yields of emission. This can be seen clearly when examining oxalate ester chemiluminescence. Not only are oxalate esters extremely efficient (quantum yields of up to 0.5) but there is a huge selection of highly efficient fluors available to allow colour choice to be made.[12] However, the major shortcoming of oxalate ester chemistry is that they are prone to hydrolysis and thus are limited in their uses. Thus, historically chemiluminescent assays requiring aqueous environments have been limited to systems such as the acridinium ester, luminol or luciferin families. Moreover as outlined in chapter 1, the acridinium ester system has shown some distinct advantages over rival systems such as the luminol or luciferin systems. These are outlined below.

1. The CL reaction requires no catalyst thus this helps to increase the sensitivity of the reaction.
2. The acridinium moiety detaches from the linker group during the CL reaction thus if the linker is bound to an antibody, the antibody will not quench the chemiluminescence.

### 3.1.4 The proposal

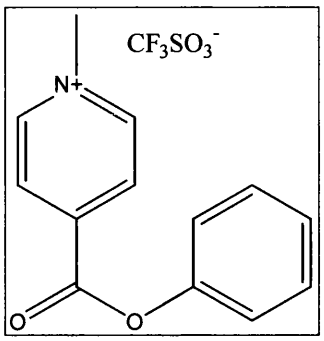
Acridinium esters are much less efficient than oxalate esters (typical quantum yields for acridinium esters are 0.05), and colour selection is limited to modification of the acridinium ring.[12] Nevertheless, acridinium ester molecules are stable in aqueous environments and hence can be viewed as a good compromise between stability and sensitivity. However, is the acridinium head group the best fluor available in terms of its fluorescence quantum yield? Could a molecule be synthesised which utilises the same mechanism for excited state generation but then transfers this energy to a second molecule that can more efficiently utilise this excited state generation for light emission? In other words could a system be designed such that it has the stability of

the acridinium ester system but efficiency of the oxalate ester system? If such a chemiluminescent system were produced it could be envisaged that not only colour selection but also a higher quantum yield with a good stability in aqueous environments would be a lot easier to achieve.

The oxalate and the acridinium ester systems share in some respects similar routes to light emission. They both are attacked by hydrogen peroxide and in doing so form dioxetane rings with the expense of leaving groups. In the case of the acridinium ester system, a dioxetanone is formed and for oxalates the dioxetane ring is a dioxetanedione. Moreover, both systems also share the fact that the breakdown of the dioxetane ring elicits the formation of an excited state in a fluor.[12] However, the mechanisms of how these facilitate this diverge. For the oxalate ester mechanism, CIEEL has been identified as the mechanism of conversion. However for the acridinium ester system excited N-methylacridone is formed as a result of the breakdown of the dioxetanone.

The work outlined below is an investigation into whether chemiluminescence can be achieved from an analogue of the acridinium ester system, the pyridinium ester system through intermolecular and or intramolecular energy transfer. The reason for choosing the pyridinium system is that by essentially removing the benzene rings from the acridinium molecule, the natural fluor will be removed. It is conceivable that removal of the fluor could alter the mechanism of light emission, thus on exposure to hydrogen peroxide, with a separate fluor present, the excited pyridinium molecule could transfer its energy to this fluor. Moreover, it is not inconceivable that this could be facilitated though resonance energy transfer or even though the CIEEL mechanism.

It was thus decided to first synthesise phenyl 1-methylpyridinium-4-carboxylate trifluoromethanesulfonate (3.1) and test this molecule for any chemiluminescence with and without the presence of fluors. Following this work, it was hoped to synthesise molecules based on the pyridinium ester but with fluors intramolecularly bound and then to test these for possible chemiluminescence energy transfer.




**Phenyl 1-methylpyridinium-4-carboxylate trifluoromethanesulfonate (3.1)**

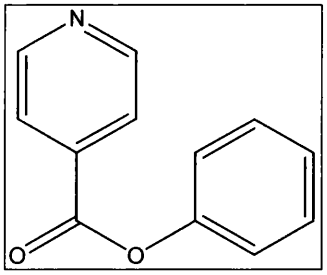
## 3.2 Results and discussion

### 3.2.1 Intermolecular energy transfer

#### *3.2.1.1 Phenyl 1-methylpyridinium-4-carboxylate trifluoromethanesulfonate (3.1).*

It was decided to synthesise (3.1) for preliminary studies and then if this looked promising the analogue with the protein linker chain would be synthesised. The method used for the synthesis of (3.1) was the in-house method used for the synthesis of acridinium esters.[13] Thus (3.1) was synthesised from the methylation of isonicotinic acid phenyl ester (3.2), which in turn was synthesised by reacting phenol with isonicotinic acid chloride.

##### 3.2.1.1.1 Successful synthesis of Isonicotinic acid phenyl ester (3.2)

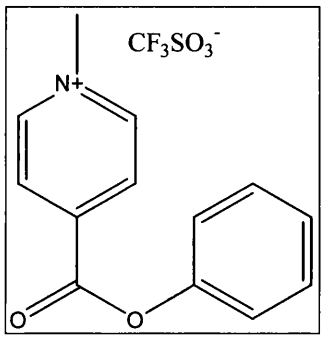



**(3.2)**

Synthesis of (3.2) was straightforward; isonicotinic acid was refluxed in thionyl chloride to form the acid chloride, which was then reacted with an excess of phenol in the presence of the base catalyst pyridine to from the ester. Compound (3.2) was then purified by column chromatography, which separated two fractions from the mix. The first fraction from the column was shown to be recovered phenol and the second, the desired product. This product was then analysed for confirmation of its structure. The proton NMR showed that the molecule had nine aromatic protons arranged in five multiplets. The three peaks found upfield were thought to be those of the phenyl

moiety, as these integrated to five protons and the all showed similar coupling constants. Furthermore the double doublets found downfield were assigned to the pyridine protons. As expected, the carbon NMR results showed 9 low field signals (however, one of these was thought to be an artefact [171 ppm] as it only appeared in the DEPT and was of very low intensity. Of the other 8 protons which were thought to be from the desired compound 3 of these which disappeared in the DEPT spectrum and thus were assigned as the quaternary carbons. Furthermore it was thought that the the carbonyl signal was that found furthest downfield (163.7 ppm). The 5 signals, that did not disappear in the DEPT spectrum were characterised as the CH carbons, and 4 of these were each twice the intensity of the 5th. This indicated that these signals were representing the 9 aromatic CH peaks. To confirm, electrospray mass spectroscopy gave an $M+H^+$ ion for the desired product. Moreover, infrared spectroscopy showed a large signal at 1739 $cm^{-1}$ indicating the presence of the carbonyl. The reaction gave a disappointing yield (47 %), and it was postulated that the reaction may not have gone to completion or perhaps that the ester hydrolysed on the silica gel during purification. Compound 1 was then used as the starting material for the methylation reaction which is discussed below.

3.2.1.1.2 Successful synthesis of (3.1)




**(3.1)**

Synthesis of (3.1) was successfully conducted by utilising the methylation procedure used for the methylation of acridine esters.[13] Compound (3.2) was methylated at room temperature in a stirring solution of dry DCM and $CF_3SO_3CH_3$. The white powder of compound (3.2) at first dissolved to form a clear solution but over time a white precipitate formed. At the end of the reaction, diethyl ether was added, which caused a further precipitation of the product. This solid was filtered and then

characterised. The results showed that not only had the desired product formed but also it was pure. The proton NMR showed that there were 9 aromatic protons, showing the presence of both the pyridine and phenyl rings. Moreover, there was a singlet integrating for 3 protons (4.48 ppm) in the aliphatic end of the spectrum, which indicated the presence of the methyl group. Two double doublets at 8.69 and 9.24 ppm were identified as the pyridine protons. Two signals found slightly upfield from the pyridine signals were assigned to the phenyl moiety as the combined areas integrated to five protons in total. The $^{13}C$ NMR showed a methyl carbon upfield from the aromatic carbons (48.6 ppm). There were three quaternary carbons (which disappeared on DEPT) corresponding to the gamma carbon of the pyridine ring, the carbonyl carbon (161.1 ppm) and the quaternary carbon of the phenyl ring. There were also 5 aromatic CH signals indicating that there were 9 CHs on the molecule because one of the aromatic CH signals was half the intensity of the other four. The $^{19}F$ NMR showed that fluorine was present in the salt, and hence indicating the presence of the trifluoromethanesulfonate counter ion. The electrospray mass spectrometry in positive mode showed the presence of the $M\text{-}CF_3SO_3^-$ (m/z 214) and in negative mode showed the counter ion (m/z 149, $CF_3SO_3^-$). The IR spectrum showed a strong signal for the carbonyl group (1752 $cm^1$).

### *3.2.1.2 An investigation into the chemiluminescent properties of (3.1)*

Following the synthesis of (3.1), work was then conducted to explore the possibilities of chemiluminescence when it was exposed to $H_2O_2$ in basic conditions. Furthermore, work was done to see if energy from the peroxidation of the pyridinium ester could be transferred to the acceptor molecule rhodamine 6G. Rhodamine has historically been found to be an excellent energy transfer acceptor molecule in intermolecular transfer reactions for energy transfer reactions of acridinium systems.[9] It was thus decided to use this molecule as a potential energy transfer acceptor for the pyridinium system.

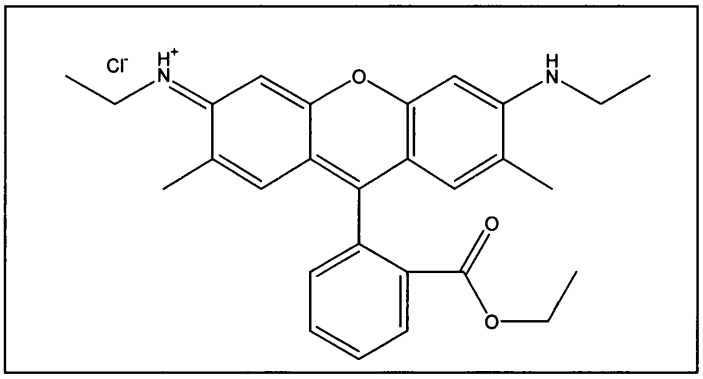



**Figure 3 Rhodamine 6G**

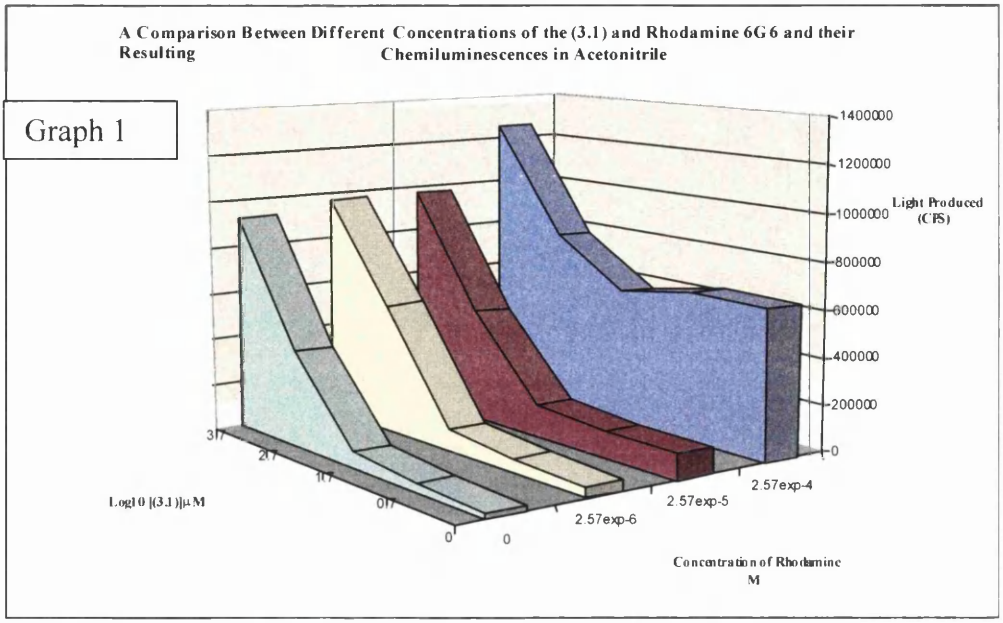
Serial dilutions of compound 2 in both acetonitrile and water were prepared. These solutions were then tested for potential chemiluminescence using the Magic Lite chemiluminometer. Following this, solutions of Rhodamine 6G were prepared in the same way as the pyridinium salt and were also tested for any background chemiluminesence. Finally mixes of the pyridinium salt (3.1) and Rhodamine were prepared as shown in table 1.

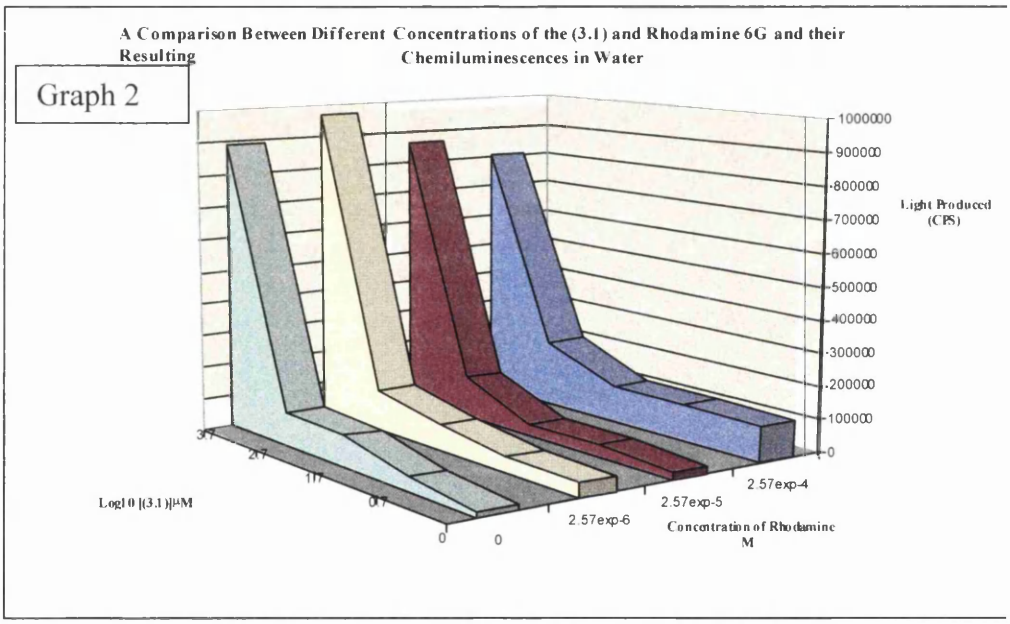
**Table 1 Preparation of solutions for energy transfer experiments*[1]**

| | | [Rhodamine 6G] M | | |
|---|---|---|---|---|
| | 2.33 exp -4 | 2.33 exp -5 | 2.33 exp -6 | 0 |
| [Salt] M | 0.5 ml of each | 0.5 ml of each | 0.5 ml of each | 0.5 ml of each |
| 4.65exp -3 | a1 | b1 | c1 | d1 |
| 4.65exp -4 | a2 | b2 | c2 | d2 |
| 4.65exp -5 | a3 | b3 | c3 | d3 |
| 4.65exp -6 | a4 | b4 | c4 | d4 |
| 0 | a5 | b5 | c5 | d5 |

Each of these mixes were then tested for chemiluminescence and the results of these are shown in graphs 1 and 2.

* A 0.5 ml aliquot of a solution of compound 2 ("salt") of the indicated concentration was mixed with a 0.5 ml aliquot of rhodamine solution of the indicated concentration. Separate solutions were prepared in acetonitrile and in water




### *3.2.1.3 Conclusions*

The results indicated that (3.1) did chemiluminesce but to obtain counts of around $10^6$ cps, mMol concentrations were required. Moreover, differences in solvent did not alter the light output. When compared to the known values for the acridinium analogue, the pyridine salt was a factor of $10^{-5}$ less sensitive.[13] Therefore, these results showed that without the presence of Rhodamine, (3.1) did not chemiluminesce

with the same intensity as the acridinium analogue. These results were expected as the pyridine ring is not a good fluor.

The presence of the rhodamine 6G did not in any way affect the chemiluminescence of the salt, it was first thought that there was a small enhancement at high salt and rhodamine concentrations; however, when this experiment was repeated there was no such enhancement. The increased rhodamine light output on graph 1 was probably due to a contaminant.

At first it was difficult to conceive as to why rhodamine did not have an effect on enhancing the chemiluminescence of the (3.1). However upon examining the literature, it was found that work had been conducted on measuring the fluorescence spectra of the product *N*-methylpyridone. The paper found that the product absorbed light at around 260-280 nm, but it did not fluoresce and in fact was found to phosphoresce between 340-360 nm at 77K.[14] Furthermore, the $\lambda_{abs}$ of rhodamine is 550 nm.[12] Thus on account of the fact that the triplet emission spectrum of the *N*-methylpyridone is far away from the absorption spectrum of the rhodamine, then the lack of spectral overlap would prevent any resonance energy transfer even if the triplet state would survive to allow for this. Nevertheless, a CIEEL could have been possible, but on account of the marked lack of chemiluminescence, then it was concluded that a CIEEL energy transfer reaction was not likely either.

The results for the intermolecular tests were disappointing as no energy transfer was detected, however it was decided to move forward and attach a fluor to the pyridine group due to the following reasons. The first reason was that by binding the fluor to the 'excited' group, it would significantly increase the likelihood of an energy transfer reaction by reducing the interstitial distance between the emitter and the acceptor. It could be argued though that as the emission from the pyridine moiety was through phosphorescence then if energy transfer occurred then the expected route would also be from triplet state to triplet state. On account of this, the aqueous environment would rapidly quench any excitation. Nevertheless, addition of the fluor onto the pyridine molecule would alter the physical properties of the molecule. On account of this, where excitation of (3.1) causes phosphorescence, the new molecule could have

its energy states altered such that an excited singlet state would be preferred on hydroperoxidation and thus increase the chance of resonance energy transfer. CIEEL mechanisms on the other hand are radical processes; thus it is difficult to predict the likelihood of such a mechanism. On account of this, without synthesising examples of such molecules it would be difficult to rule out energy transfer merely on the results of the intermolecular energy transfer experiment.

### 3.2.2 Intramolecular energy transfer

Work was then undertaken to synthesise an intramolecular energy transfer probe, based on the parent molecule – (3.1). The first decision to be taken was where the linker group was going to be located on the molecule. It was decided to place the linker molecule on the 2-position of the pyridine ring. This was on account of the fact that the 2-position was furthest away from the ester group and thus would minimise any interaction that the ester group may have with the fluor molecule when attempting to bind this to the pyridine group. A range of pyridine molecules substituted at the 2-position were then selected as potential candidates for synthesis, (figure 4).

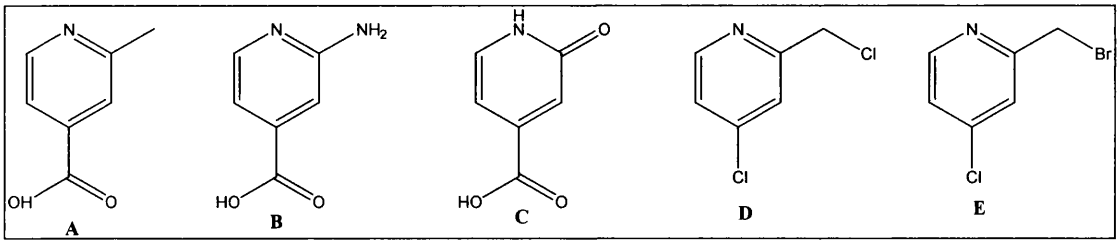



**Figure 4 A selection of potential candidates as starting materials for synthesis of energy transfer molecules**

Out of the five candidates selected it was decided that candidate B (2-amino-pyridine-4-carboxylic acid (3.3) would be initially used. The reason for this was that the amino group would act as an excellent nucleophile for attachment to a variety of electrophilic sites, such as carbonyls and isothiocyanates, on potential fluor moieties. Thus, work was then done to explore the synthesis of (3.3). From the literature, it was found that this molecule could be synthesised through two routes. The first route involved synthesising 2-acetylamino-4-methylpyridine from the commercially available 2-aminopicoline. The acetylation was necessary to protect the amino group

for the next step which was oxidation of the 4-methyl group to a carboxylic acid group. The final step was deprotection of the amino group by hydrolysis using a strong basic solution (see figure 5).[15]

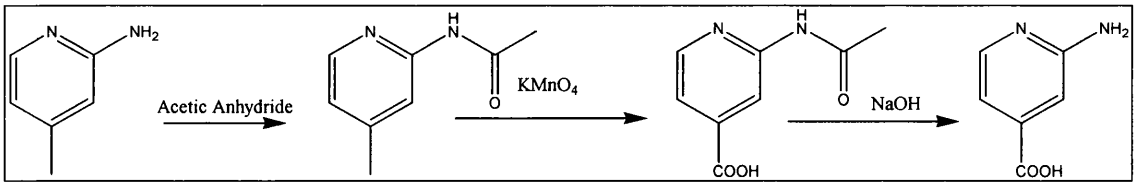



**Figure 5 A synthetic route to (3.3)**

The second method was to synthesise 2-chloroisonicotinic acid from 2,6-dichloropyridine-4-carboxylate or from pyridine-4-carboxylate.[16,17] The 2-chloroisonicotinic acid would then be autoclaved in the presence of aqueous ammonia to form the product.[18]

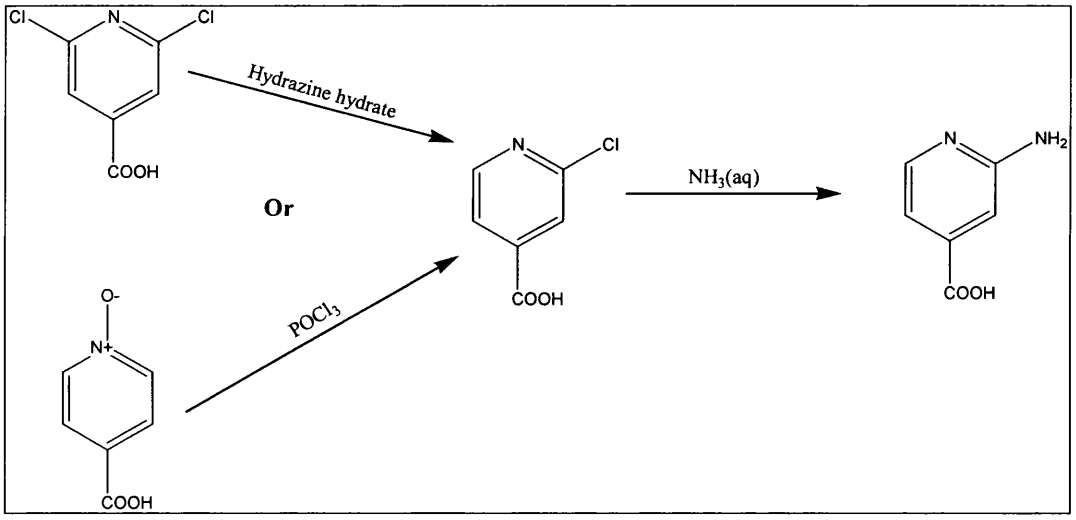



**Figure 6 Alternative route to synthesise (3.3)**

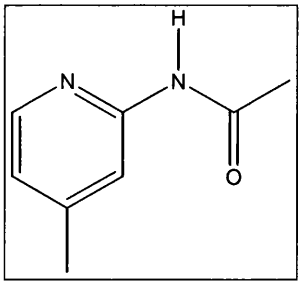
Although both routes were synthetically possible neither was ideal for the following reasons. The first reason was that the final yield from the first method was only 5%. The second reason was that for the second reaction, 2-chloropyridine needed to be autoclaved to allow for the amination. This restricted the quantity of product that could be prepared at one time. The final reason was that both required either a two or three step synthesis which would result in the loss of yield. On account of these reasons a more direct route was investigated. The literature indicated that pyridine could be very easily directly aminated through a Chichibabin reaction, which involved direct amination with sodium or potassium amide. However, a recent review

indicated that direct amination of pyridine carboxylates was almost impossible. It appears that electron withdrawing groups such as carboxylate reduce the basicity of the heterocyclic nitrogen preventing co-ordination of the metal cation. Co-ordination is necessary to pull electrons way from the α-position carbon allowing addition of the amide ion and then loss of hydride. Moreover it is thought that groups such as the carboxylates also co-ordinate with the metal preventing co-ordination with the heterocyclic nitrogen. What was evident from the review was that isonicotinic acid had never been aminated via a Chichibabin reaction and that nicotinic acid had only been aminated at high pressure (350 psi 7 % yield). On account of this it was decided not to continue down this avenue but instead to concentrate on the two methods outlined in figures 5 and 6.[19]

### *3.2.2.1 Synthesis of 2-aminopyridine-4-carboxylic acid (3.3)*

It was decided first to attempt the procedure shown in figure 5, which was essentially oxidaton of 2-aminopicoline into the carboxylic acid. The first step of this was the synthesis of 2-acetylamino-4-methylpyridine (3.4).

#### 3.2.2.1.1 Synthesis of 2-acetylamino-4-methylpyridine (3.4)




**(3.4)**

The synthesis of (3.4) was achieved by adding 2-amino-4-picoline to a mixture of acetic anhydride and triethylamine. The mixture, which became exothermic almost instantaneously, was capped and stirred overnight at room temperature. The solution changed colour from colourless to black. The solvent was removed by distillation and the remaining black oil was taken up in DCM and extracted with water to first convert the remaining acetic anhydride into the acid and then remove this acid from the organic layer. On removal of the DCM, a dirty white crystalline solid formed which

was first purified by normal phase column chromatography and then twice re-crystallised in warm diethyl ether, forming clear colourless crystals in a good yield (85 %). The characterisation showed that the product had formed and was pure. The proton NMR showed that two methyl groups were present due to 2 high field singlets integrating to 3 protons each. The results also showed 4 lower field peaks, each integrating to one proton. The furthest downfield was assigned as the NH proton on account of the fact that it was broader than the other peaks thus indicating an exchangeable proton. The three other low field peaks were assigned as the aromatic protons, two of these were doublets and were coupled to each other thus were assigned to positions 5 and 6. Furthermore, the remaining singlet was assigned as the 3-position proton. The carbon NMR results complemented the proton data; the two high field peaks, which did not disappear in the DEPT spectrum, were assigned as the two methyl carbons. The other three carbons, that also did not disappear in the DEPT spectrum were assigned as the aromatic CH carbons. Finally, the three carbons that did disappear on DEPT were assigned as the three quaternary carbons, with the lowest field carbon being the carbonyl. Mass spectrometry further complemented the NMR data, showing the $M+H^+$ for the product (m/z 151) and a key fragment ion at m/z 108, which was concluded to be a deacetylated fragment ion (figure 7).

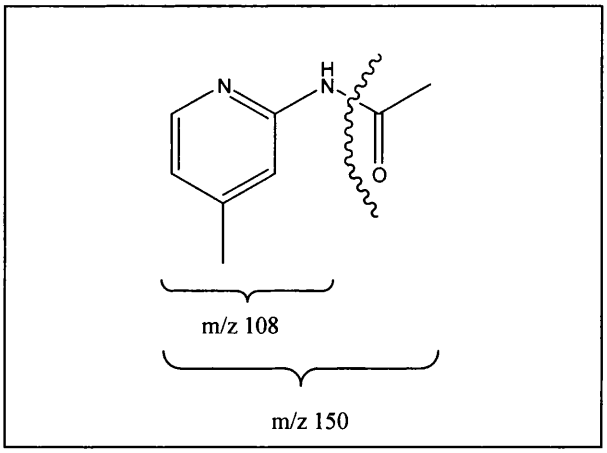
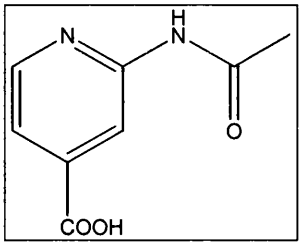



**Figure 7 ions in the mass spectrum of (3.4)**

3.2.2.1.2 Synthesis of 2-acetylaminopyridine-4-carboxylic acid (3.5)



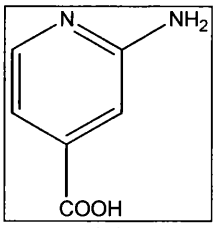

**(3.5)**

Synthesis of (3.5) was achieved by oxidation of (3.4) with a hot solution of aqueous $KMnO_4$. This reaction was conducted over 4h, where during the first 3h the $KMnO_4$ was slowly added to a stirring solution of the picoline (which had fully dissolved in the hot water). It was paramount that the order of addition was conducted in this sequence, as addition of the entire oxidising agent in one aliquot at the beginning of the reaction led to over oxidation of the starting material, forming a mass of oxidation products. During the reaction, the dark purple of the potassium permanganate solution formed the dark precipitate of its reduced form, manganese dioxide. At the end of the reaction, the solution was filtered without pre-cooling yielding a clear yellow solution. This solution was then allowed to cool and the potassium carboxylate salt of the product was then converted into its free acid by addition of HCl. This conversion caused the free acid to precipitate out of the aqueous environment forming a pure white crystalline powder. The pH of the solution was monitored carefully by frequent testing with litmus paper. The reason for this was that over acidification of the acid would favour the formation of the hydrochloride salt, which would be more soluble in the aqueous environment than the free acid. The powder was then filtered and weighed. On account of the fact that the product obtained was only a small amount of what was expected (25 % yield), the mother liquor was reduced to half its volume by rotary evaporation (15 mm Hg pressure). The solution was allowed to cool and found to be basic (when tested for its approximate pH with litmus paper). The reason for this was that during the evaporation, any free HCl in the solution would have evaporated leaving KOH and the product mainly in its $K^+$ salt form. Thus from this observation, after cooling, the solution was then neutralised with a further addition of HCl and as expected more of the product precipitated. This was finally repeated for a third time, thus yielding three crops from the solution. A fourth attempt was conducted but this resulted in no

precipitation. The combined weight of the three products gave a moderate yield (48 %).

The three products were then individually characterised and all found to be the desired product. The proton NMR showed only one methyl singlet indicating that one of the methyl groups had disappeared and assumed to have been oxidised. In the aromatic area of the spectrum, the peaks at positions 7.49 ppm, 8.45 ppm and 8.56 ppm were identified as protons at positions 6, 5 and 3 respectively. The first two were doublet in nature, exhibiting identical vicinal J coupling values. The latter two had very similar chemical shift values, which were further downfield from the first. This indicated that they were both ortho to an electron withdrawing group such as a carboxylic acid. The final two broad singlets found at low field were identified as the NH (10.71 ppm) and the COOH (13.6 ppm) protons. The carbon NMR data complemented the proton data. As expected, there was only one high field peak, which was identified as the methyl carbon. There were four quaternary carbon peaks, two of which were significantly shifted towards lower field than the remaining two. The low field ones were thus identified as the two carbonyl carbons. The remaining two quaternary carbons and three CH carbons were identified as the aromatic carbons. The mass spectrum further complemented the NMR results, showing a weak molecular ion for the desired product. The ionisation modes used for the MS analysis were EI and CI, on retrospect this was probably not optimal for obtaining a strong molecular ion or $M+H^+$. As well as the fact that these are both hard ionisation modes, due to the carboxylic acid moiety, negative ion ESI would have probably been more favourable. The IR showed a very strong peak at 1710 $cm^{-1}$ indicating an aromatic carboxylic acid group.

3.2.2.1.3 Synthesis of 2-aminopyridine-4-carboxylic acid (3.3)




**(3.3)**

The final step in the synthesis of (3.3) was the base hydrolysis of (3.5). This was achieved by first refluxing (3.5) in NaOH, cooling the solution and then neutralising with HCl (2M). Neutralisation caused the product to form a white milky precipitate, which was then collected and tested for purity and characterised. The proton NMR showed four signals all of which were found in the aromatic end of the spectrum. The first was a broad singlet, which was concluded to be the $NH_2$ signal. The two doublets were concluded to be due to the protons at positions 5 and 6, with the latter being pulled further downfield. Moreover, the peak at 7.01 ppm (thought to be position 5) was in fact a double doublet thus thought to be also split by the proton at position 3. However, the position-3 proton was singlet in nature, it was thus assumed that the NMR instrument could not resolve the fine coupling on this peak. The carbon NMR showed 6 signals, as expected, three of which were quaternary carbons as they disappeared in DEPT. The mass spectrum complemented the NMR data clearly showing a $M+H^+$ for the product (m/z 139) and a fragment ion showing decarboxylation and protonation (m/z 95). The infrared spectroscopy showed the presence of an $NH_2$ group with a N-H stretching frequency at 3278 $cm^{-1}$ and N-H bending at 1641 $cm^{-1}$. Moreover, the C=O stretching frequency of the carboxyl group was clearly visible at 1696 $cm^{-1}$.

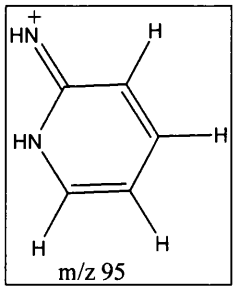
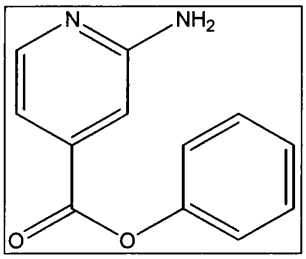



**Figure 8 fragment ion in the mass spectrum of (3.3)**

Following the synthesis of 3.3, work was then done to elaborate the two functional groups. The aim was to esterify the carboxyl group with a suitable leaving group (for the chemiluminescent reaction) such as a phenyl group and to use the amine group as a nucleophile for linking onto fluor molecules. There were two potential synthetic directions, the first was to esterify and then react the amine with a fluor of choice. The second choice was the opposite of the above.

It was decided to attempt the esterification of the carboxyl group and following this use this molecule to bind the desired fluors on to the amine group. There was a concern that if the amide reaction was attempted prior to esterification then the conditions required for esterification might run the risk of hydrolysis of the amide bond. The reason is that a classic method for esterifying carboxylates is formation of an acid chloride with a chlorinating agent such as thionyl chloride.[20] When reagents such as thionyl chloride are exposed to water they form HCl, which could catalyse hydrolysis of the amide. Thus it was decided to attempt the synthesis of phenyl 2-aminopyridine-4-carboxylate (3.6) and then to use this as a building block for addition of fluors.

### *3.2.2.2 Successful synthesis of phenyl 2-aminopyridine-4-carboxylate (3.6a)*




**(3.6a)**

Direct conversion of (3.3) into its acid chloride prior to esterification was the desired route for synthesis of (3.6a). However this did pose a potential problem. This was due to a potential self-coupling reaction which conceivably could occur. This is because the amine group could potentially intermolecularly react with an acid chloride forming 2- (2-aminopyridine-4-carbonylaminoisonicotinic acid (figure 9) or its acid chloride.

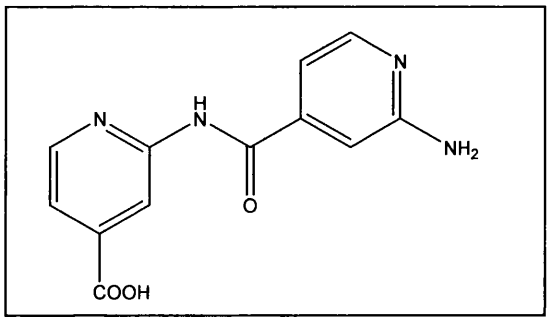



**Figure 9 2-(2-aminopyridine-4-carbonylamino) isonicotinic acid a possible side product of the synthesis of (3.6a)**

It was thus thought to protect the amino group first with BOC (tert-butyloxycarbonyl), esterify and then deprotect. However, the thionyl choride would react with any water in the reaction to form HCl that this would protonate the amine group forming $RNH_3^+$ and hence prevent nucleophilic attack by the amine group.

In actual fact, synthesis of (3.6a) proved remarkably straightforward. To form the acid chloride, (3.3) was refluxed in thionyl chloride. Over time, (3.3) went into solution and the colour of the solution changed from colourless to red. The thionyl chloride was then distilled off leaving a red powder (the acid chloride). Following this, anhydrous DCM was added *via* a dry syringe to the red powder, forming a partially dissolved red suspension. In a second flask, under anhydrous conditions, a solution of phenol in pyridine was prepared. Finally, the acid chloride suspension was slowly added to the basic phenolic solution. The reason why the acid chloride was added to the phenol and not the reverse was that statistically, the reaction would favour esterification and not self-coupling as at any point in the reaction there would be an excess of phenol. However, if the reverse was attempted, certainly initially, there would have been an excess of the amino acid chloride over the to phenol. Thus, in the process of adding a basic solution to the acid chloride to the protonated amino group would be deprotonated and hence increase the chance of self coupling.

Once the acid chloride had been added to the phenol, the solution was allowed to stir at room temperature and hence allow the reaction to come to completion. Following this, the product was then washed with a weak acid solution to remove any excess pyridine and then a weak basic solution to remove any excess phenol. The solution was then dried over magnesium sulfate and purified by gradient silica column chromatography. The chromatography yielded three significant products. All three products were then sent for analysis. The first product was found to be some excess phenol, which had not been extracted from the product during the liquid liquid extraction. The final two products appeared from the data to be two novel pure compounds. The later eluting of these (the major component of the mix (labelled as product (3.6a) was elucidated first and found to be the desired product. The second product (labelled as product (3.6b)) was found to be a chlorinated derivative of the desired product. A detailed analysis of the elucidation of these products is discussed below.

3.2.2.2.1 Analysis of (3.6a)

Proton NMR showed a broad singlet for the $NH_2$ peak, which integrated to two protons. There were four peaks in the aromatic region of the spectrum, which integrated in total to 8 protons, which tied in with the proposed product. Unfortunately, except for the final peak at the lowest field (8.25 ppm), the other three appeared to be multiplets. However the final peak at 8.25 ppm (integrated to 1 proton), was a double doublet and thus was assigned as the either the 5 or 6-position proton on the pyridine ring. The carbon spectrum was quite weak but showed clearly six CH protons. Two of these (121.4 and 129.6 ppm) were double the intensity of the others in the DEPT spectrum. This indicated that these relating to two identical carbons each, which corroborated with the identical ring carbons on the phenyl ring. From this it can be assumed then that there were in total 8 CH carbons, which matched the structure of the desired product. The DEPT spectrum also showed the absence of four quaternary carbons. The EI mass spectrum showed the molecular ion (m/z 214 = $M^+$) and also a high intensity ion at m/z 121 which corresponded to a fragment showing loss of the phenoxy group. The peak at m/z 93 was probably the aminopyridine head fragment ion. The various fragmentations are illustrated in figure 11. In CI mode, a protonated molecular ion was visible (m/z 215), but there were two other ions of interest. The first was the peak at m/z 153 (base peak in the spectrum), corresponding to the $M+H^+$ ion of methyl 2-aminopyridine-4-carboxylate.

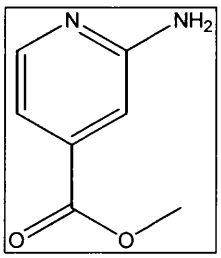



**Figure 10 Methyl 2-aminopyridine-4-carboxylate, suspected artifact from EI of (3.3).**

Upon consultation with the EPSRC centre Swansea, who carried out the analysis of (3.6), it was found that to produce the spectrum the sample was dissolved in methanol and the EI was conducted almost immediately. However the CI was conducted some 6-12 hours later and it is believed that during this time transesterification occurred.

The second ion of interest was at m/z 138 which was thought to be the free carboxylic acid. .

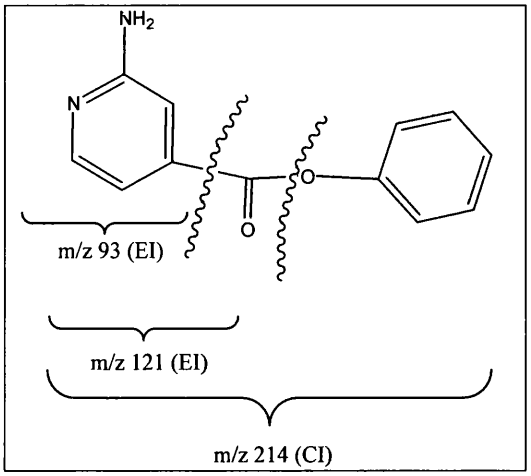



**Figure 11 Characteristic fragments elucidated from mass spectrometric analysis of (3.6a)**

3.2.2.2.2 Analysis of (3.6b)

The proton NMR of (3.6b) suggested that this compound was pure. However, the product displayed a NMR spectrum rather like the desired product except for the fact that an aromatic proton seemed to be missing. This was further corroborated with analysis of the carbon NMR with DEPT analysis, which seemed to suggest that one of the CH carbons had become a quaternary carbon. This indicated that a substitution had occurred on one of the aromatic carbons during the reaction. Thus, to summarise the NMR data; the proton NMR had a broad high field singlet integrating to 2 protons and thus indicating that $NH_2$ was present, like the desired compound. Further downfield were three multiplets integrating to 6 protons in total and a singlet integrating to one proton that was significantly further downfield from the aromatic cluster, indicating that one of the protons was ortho to an electronegative environment. The carbon NMR data showed that there were 5 quaternary carbons and 5 CH peaks two of which were twice the intensity of the rest, thus indicating that these were the 2 / 6 and 3 / 5 positions on the phenol ring. The NMR data strongly suggested that the product formed was an analogue of the desired compound, however substituted at one of the aromatic sites. Work was then done to elucidate the nature of novel compound. When a mass spectroscopic analysis was conducted it was soon realised that chlorination had occurred. This is because in both the CI and EI modes a number of the main peaks had peaks 2 mass units greater that were 33% the intensity

of these main peaks. One of these is was concluded was the molecular ion (m/z 248 / 250) as this was the predicted molecular weight for a chlorinated derivative of (3.6a). There was no real surprise that a substitution had occurred as the synthesis required a strong chlorinating agent. It was assumed that chlorination had occurred on the pyridine ring rather than the benzene ring on account of the fact that the double intensity ortho and meta CH peaks were still visible on the DEPT spectrum. Also, during the step involving treatment with thionyl chloride, during which chlorination probably occurred, the phenol was not present. Moreover, fragmentation analysis of the mass data showed fragments which indicated that the chlorine atom was indeed substituted on the pyridine ring. A summary of these fragment ions are shown in figure 12

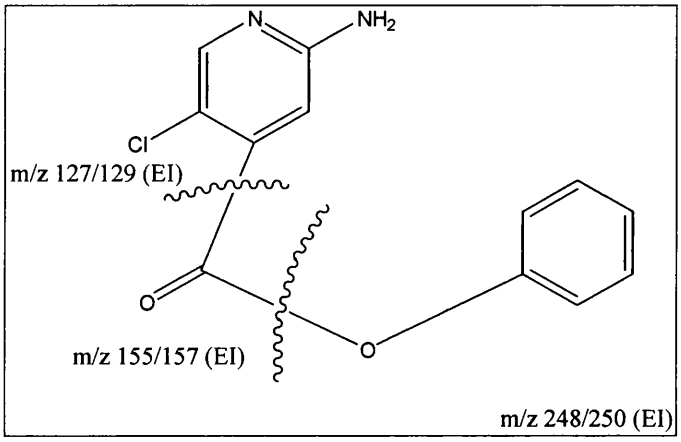
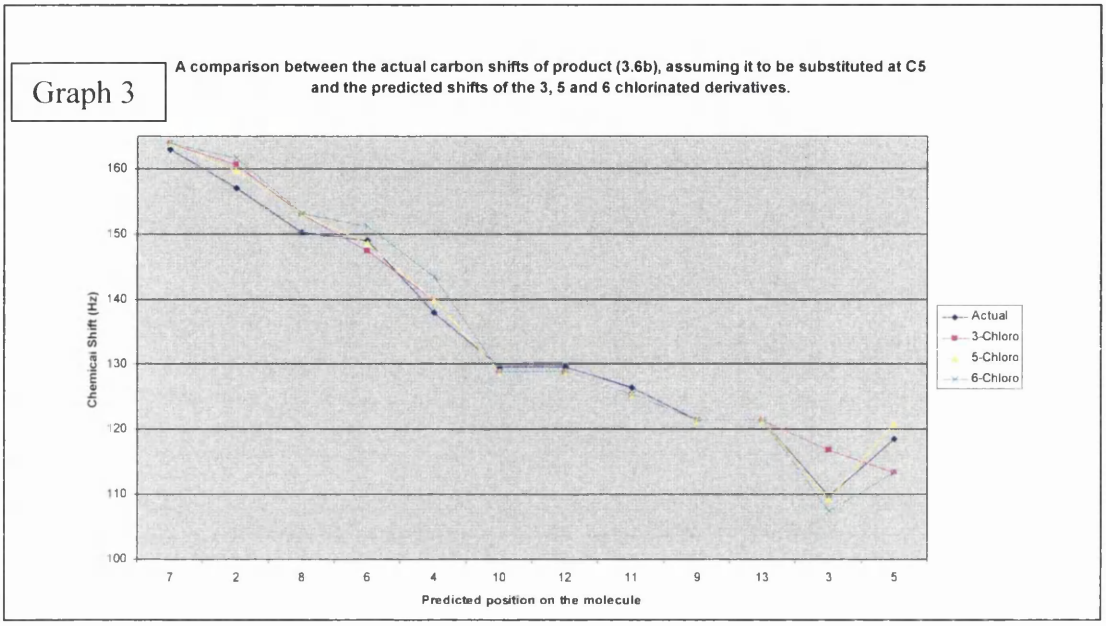



**Figure 12 Characteristic fragments elucidated from mass spectrometric analysis of (3.6b)**

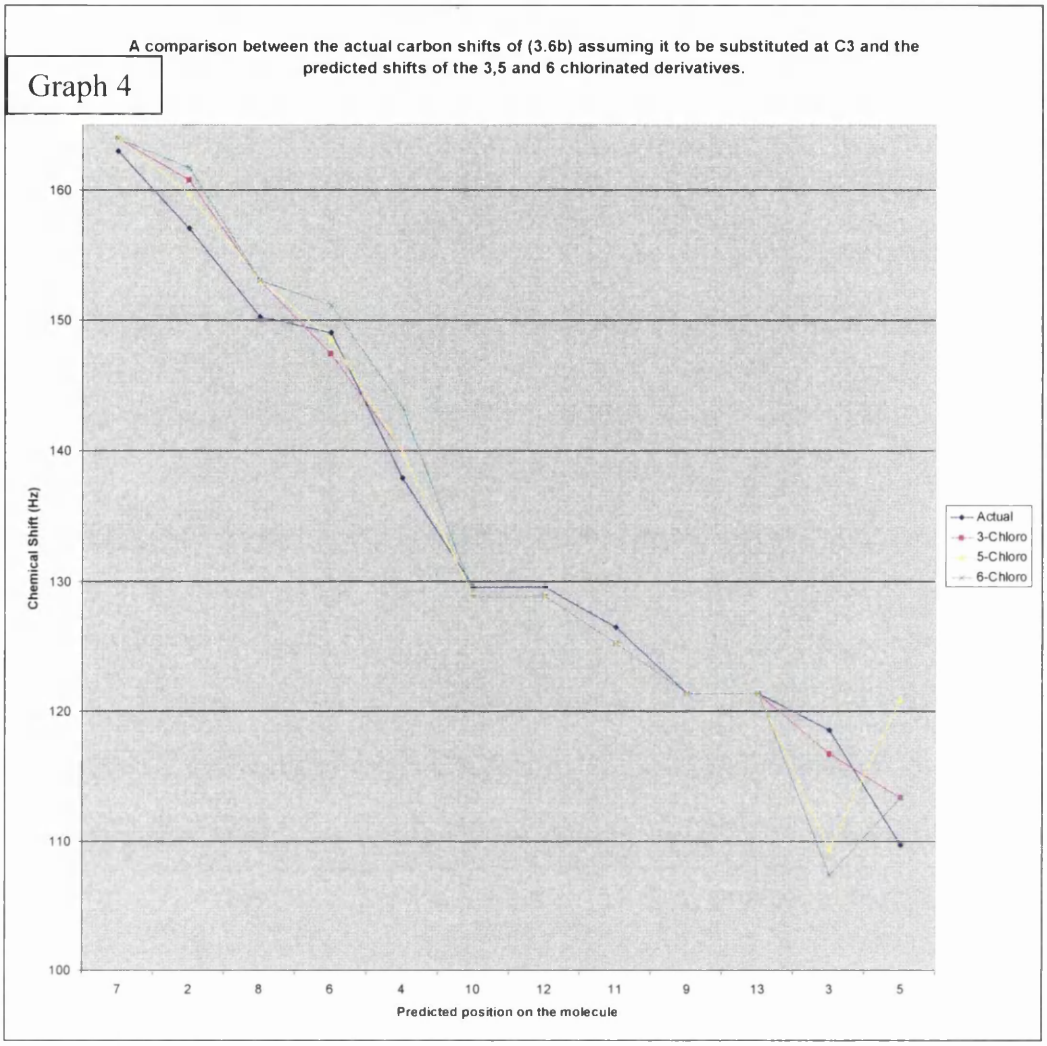
The remaining question to be solved was the actual site of chlorination. One clue towards solving this puzzle was from the proton NMR, which indicated that there were two singlets, one of which was significantly shifted downfield (8.21 ppm) from the other aromatic signals. This indicated that chlorination had occurred on the 5- or 6- position on the pyridine ring and not the 3-position. The reason for this was due to the fact that if chlorination had occurred on the 3-position then this would have resulted in the formation of 2 doublets coupled to each other, which was not evident from the spectrum. Out of the 5- and the 6- positions, chlorination at the 5- position seemed to be the most likely, this due to the fact that if chlorination had occurred on the 6- position then the protons at the 3- and 5- positions would more than likely to couple to each other resulting in coupling constants of 2-3 MHz. Also, the β(3/5) positions of pyridines are generally more reactive than the α (2/6) positions to

electrophilic substitution, and this tendency would be enhanced by the presence of an electron-donating amino group at position 6. To provide yet further evidence for this, $^{13}C$ NMR predictions on 3, 5, and 6 mono-chlorinated pyridines were conducted using ChemDraw Ultra and these were compared to the unknown compound. The comparisons are shown in graph 3 and 4.

Graph 3



Graph 4




Graphs 3 and 4 show that when compared to predicted shift values, the actual carbon shifts are very similar to those predicted for both the 3- and the 5- chloro derivatives for almost all the positions on the molecule except for the 3- and 5- positions, whereas the differences are a little bit greater for the 6-chloro derivative. Thus, the results suggest that the site of chlorination is either at the 3- or 5- positions. At the 3- and 5- positions, the effect of chlorination moves the chemical shift of the directly bonded carbon downfield. This is due to the electronegative inductive effect that the chlorine has on the carbon. At the 3- and 5- positions the actual data and the predicted data are extremely similar for if chlorination had occurred on the 5-position (please see graph 3). Although the trend is similar if chlorination had occurred on the 3-position the data does not match as well (please see graph 4).

In conclusion, it was suggested that nature of (3.6b) was thought to be the 5-chloro derivative. Substitution on this position would have been thought to have been more likely on account of the fact that the 3-position would be sterically more hindered with substitutions at both the 2- and 4- positions.

### *3.2.2.3 Attaching the fluorescent labels*

Following the successful synthesis of (3.6a), work was then conducted to couple this to possible fluor molecules. It was decided to attempt to synthesise 3 labels, an acridine derivative, a pyrene derivative and a fluorescein derivative. The reason why these labels were chosen was due to the following reasons. Acridine was chosen because not only was this molecule fluorescent but also acridine-9-carbonyl chloride was readily available in the laboratory and furthermore there was a lot of experience in handling this material in the laboratory. Pyrene was chosen because again it is fluorescent and the 4-(1-pyrenyl) butanoic acid was readily available and the procedure to convert this to the acid chloride was well documented.[21] Finally fluorescein isothiocyanate was chosen on account of the fact that it was a very popular fluorescent label and furthermore, work in the laboratory was being conducted by a colleague (Z Li) to bind fluorescein onto acridinium esters.

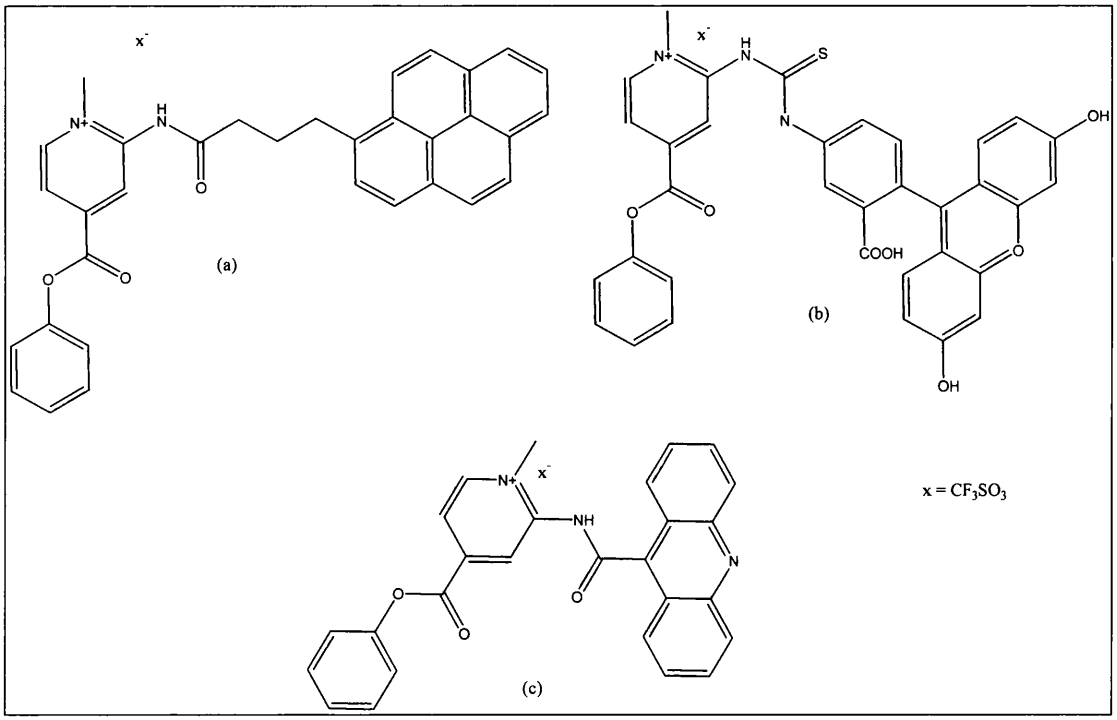
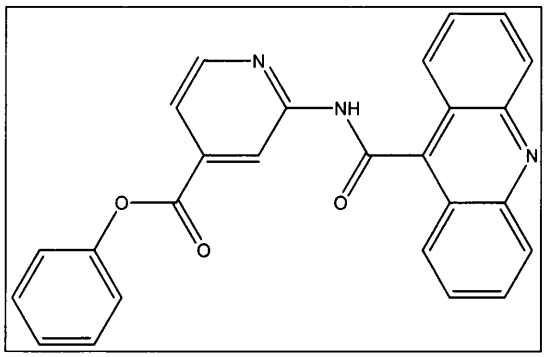



**Figure 13, Proposed structures for the three intramolecular energy transfer molecules to be synthesised; (a) a pyrene derivative; (b) a fluorescein derivative; (c) an acridine derivative**

3.2.2.3.1 Synthesis of 2-(acridine-9-carbonylamino)isonicotinic acid phenyl ester (3.7)




**(3.7)**

Chapter 4 discusses the synthesis of a range of acridine amides from 2- aminopyridine and derivatives of this molecule. It can be seen that the desired product is itself an analogue of such an amide; thus it was decided to use the methodology developed for the synthesis of these molecules. Equimolar concentrations of the acridine acid chloride and the amine were allowed to react under inert conditions at room temperature for 6 hours. The resulting reaction was followed by TLC (using a UV lamp at 254 nm) and showed three major spots. These had Rf values of 0.16 0.60 and 0.86, receptively. The staring materials were recognised as the spots at 0.16 (amine) and 0.86 (acid chloride). During the reaction these spots faded whereas the spot at 0.6 grew stronger in intensity. The solvent was then removed by rotary evaporation and the subsequent yellow powder was purified by silica gel column chromatography. The column seemed to yield only two the three major components. It appears that the acid chloride spot which had become faint on the TLC at the end of the reaction had hydrolysed on the silica surface yielding the acid which in turn had remained on the surface of the silica. However, the two other spots were collected and sent for characterisation.

Compound (3.6a) (the starting material) was confirmed as the spot with an Rf value of 0.16 on the TLC. However, it was evident that the second product isolated was something novel and in fact identified as the product of choice and was formed in high yield (90 %). The proton NMR showed that there were 17 protons in total and one of these was a broad singlet indicating that this was in fact the NH proton. The remaining signals were all in the aromatic end of the spectrum, of which was rather complicated, with two multiplets accounting for 6 of the protons. However, by close

examination of the data peaks were assigned as either originating from the pyridine, acridine or phenyl rings or as the NH proton (figure 14).

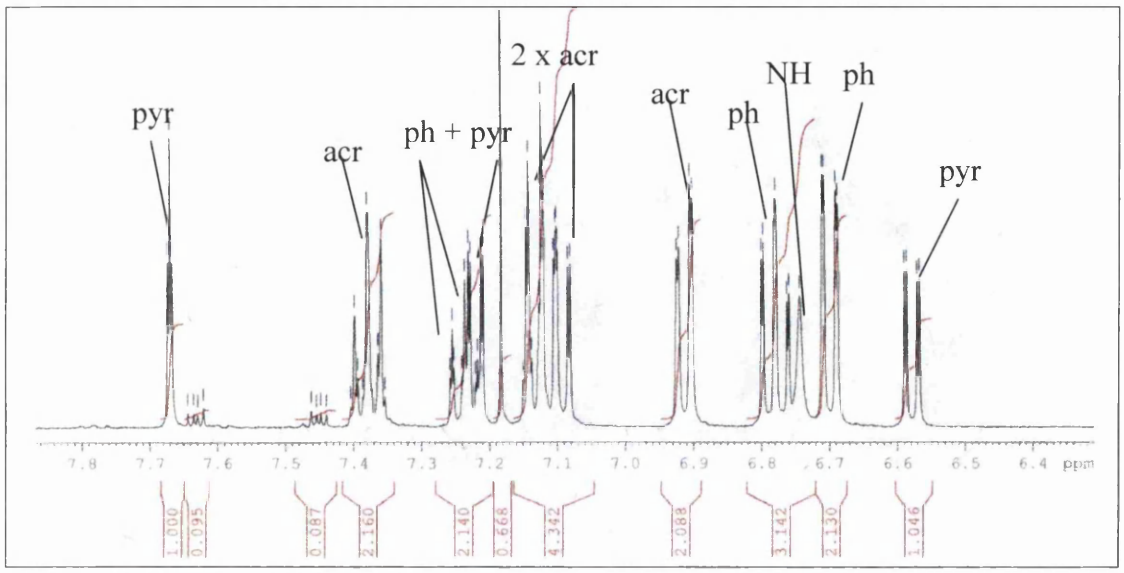


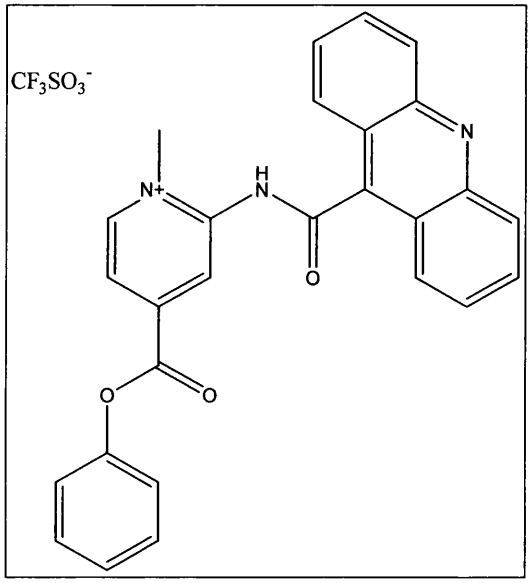

**Figure 14, Proton NMR spectrum of (3.7)**

The peak at 6.58 ppm was found to be for either position 5 or 6 of the pyridine ring as it was double doublet in nature and integrating to one proton. The fine coupling matched the coupling for the triplet at 7.67 ppm, which also integrated to one proton and hence was found to be the 3-position proton on the pyridine ring. The final pyridine proton proved at first to be a little elusive, but upon close examination of the data, the 2H multiplet at 7.23 ppm seemed to be the most likely candidate to contain the appropriate signal. This multiplet seemed to be a mixture of a triple triplet and a double doublet. The triple triplet was identified as the 4-position proton on the phenyl ring as it would have been split into a triple triplet by the surrounding protons. The double doublet was identified as the final pyridine proton (either position 5 or 6). The multiplet at 7.12 ppm was identified as two acridine peaks a triple triplet (7.12 ppm) and a double doublet (7.4 ppm), with had the following coupling constants: the triple triplet - 7.6 and 1.5 Hz, and the double doublet - 7.3 and 1.4 Hz. These coupled to peaks at 6.91 ppm and 7.38 ppm respectively. Finally, the double triplet and double doublet at positions 6.78 and 6.69 ppm were identified as positions 3/5 and 2/6 on the phenyl ring.

To complement the proton data the carbon data indicated that there were 16 aromatic CH protons, 6 of the peaks for which were double the intensity of the other 4 CH

peaks thus indicating that these represented the symmetrical CH carbons. Furthermore, there were 7 aromatic quaternary carbons peaks (identified by a DEPT analysis), which corresponded with the expected number of quaternary carbon peaks since there were 4 symmetrical quaternary carbons that would display only 2 peaks as a result of their identical shifts. Finally, mass spectroscopic analyses showed the $M+H^+$ ion (m/z 420).

3.2.2.3.2 Synthesis of 2-[acridine-9-carbonylamino]-1-methyl-4-phenoxycarbonylpyridinium trifluoromethanesulfonate (3.8)




**2-[acridine-9-carbonylamino]-1-methyl-4-phenoxycarbonylpyridinium trifluoromethanesulfonate (3.8)**

Methylation of (3.7) was taken from the known synthesis of acridinium esters.[20] Under anaerobic conditions, the newly formed amide was exposed to 1 molar equivalent of methyl trifluoromethanesulfonate and the reaction occurred almost instantaneously when monitored by TLC. Following this, the solvent was removed and crystals were obtained by cold crystallisation in acetone-diethyl ether. The crystals were then analysed and the analysis proved that methylation had occurred. The proton spectrum showed a peak in the aliphatic region that integrated to 3 protons thus indicating methylation had occurred, however the site of methylation was unclear. Moreover, the expected 16 aromatic protons were detected. The peaks at 8.34 and 8.27 ppm were the 1/8 and 4/5 position protons on the two flanking benzene

rings of the acridine system as these both were doublet in nature. From H,H COSY data (shown in figure 15), peaks 8.34 ppm and 8.27 ppm were coupled to peaks 8.21 ppm and 7.92 ppm respectively. The H,H COSY spectrum was recorded on account of the fact that the coupling constants for the two doublets were identical (8.7 Hz) but did not appear to be the same as those of the two triplets. However analysis by COSY showed clearly that these were coupled and presumably the apparent triplets were actually overlapping double doublets which gave the appearance of triplets with different coupling constants. The doublets at 8.07 ppm and 7.24 ppm were assigned to the 5 and 6 position protons on the pyridine ring. If methylation had occurred on the pyridine ring then the peak at 8.07 ppm would more than likely be the 6–position proton. Again the COSY spectrum indicated that these were coupled. The singlet at 7.67 ppm was assigned as the 3-position proton on the pyridine ring as it showed only a weak coupling to the multiplet at 7.33 ppm. Finally there was a broad singlet at 9.25 ppm which was assigned as the NH proton. The carbon spectrum complemented the proton spectrum. The peak at 28.9 ppm was assigned as the methyl carbon. The DEPT spectrum indicated that of the 16 aromatic CH carbons only 8 peaks were visible, thus indicating that there were multiple carbons to one peak. Two of these peaks appeared to be four times and two twice the intensity of the final four. The mass spectrometric data indicated only the presence of the anion of the expected salt. However there was concern over to which nitrogen the methyl group had actually added. Work in chapter four indicated that when methylating amides similar in structure to compound 1 of chapter 4, then methylation occurred exclusively on the pyridine nitrogen. However in this case it was a little unclear which nitrogen was methylated. Thus, a comparison of the proton shifts of the methylated and unmethylated protons was conducted, and this is displayed in table 2.

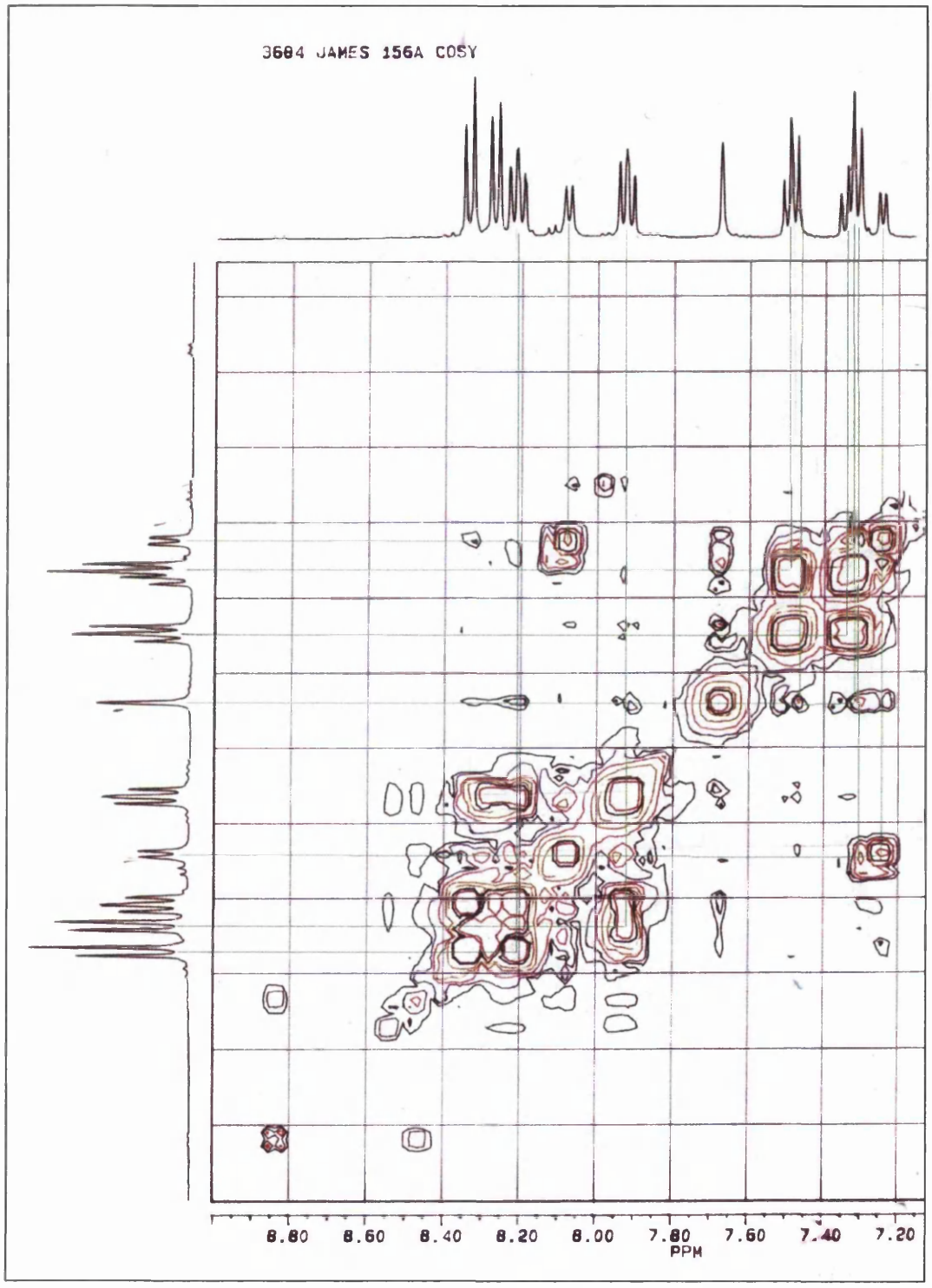



**Figure 15 H, H 2D $^{1}$HNMR COSY (400 MHz) of (3.8)**

**Table 2 A comparison of chemical shift values between the methylated (3.8) and unmethylated (3.7) amides.**

| Ring Type | Chemical Shift for product 3.7 | Chemical shift for product 3.8 | Difference in chemical shifts |
|---|---|---|---|
| **Pyridine (s, 3-position)** | **7.67** | **7.67** | **0** |
| **Pyridine d** | **7.23** | **8.07** | **0.85** |
| **Pyridine d** | **6.58** | **7.24** | **0.66** |
| **Acridine t** | **7.38** | **8.21** | **0.83** |
| **Acridine d** | **7.14** | **8.34** | **1.2** |
| **Acridine t** | **7.12** | **7.92** | **0.82** |
| **Acridine d** | **6.91** | **8.27** | **1.36** |
| **Phenyl t** | **7.23** | **7.33** | **0.09** |
| **Phenyl t** | **6.78** | **7.49** | **0.71** |
| **Phenyl d** | **6.69** | **7.33** | **0.63** |

The data in table 2 showed that the acridine protons seemed to be shifted by a greater degree than the pyridine peaks. Furthermore, the pyridine 3-position proton did not shift at all on methylation, thus indicated that methylation occurred on the acridine nitrogen. If methylation had occurred on the pyridine nitrogen then H3 of the pyridine ring would have shown a significant shift towards lower field. The fact that the 3-position pyridine did not change its chemical shift also ruled out any possibility of methylation of the pyridine nitrogen. Furthermore, for this same reason, it was also concluded that methylation had not occurred on the amide nitrogen either. Thus, the NMR evidence suggested that the methylation yielded the structure shown in figure 16.

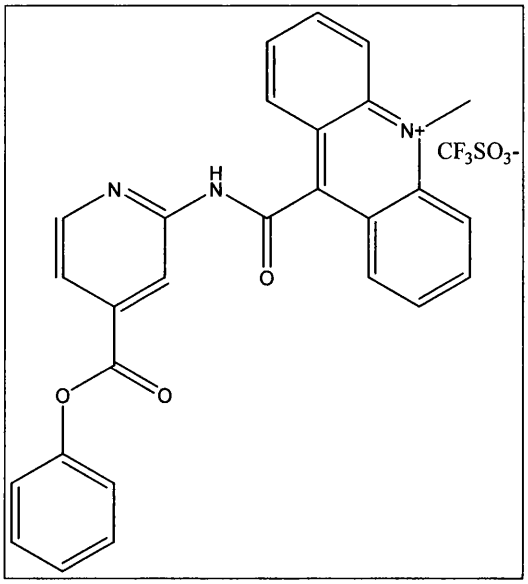
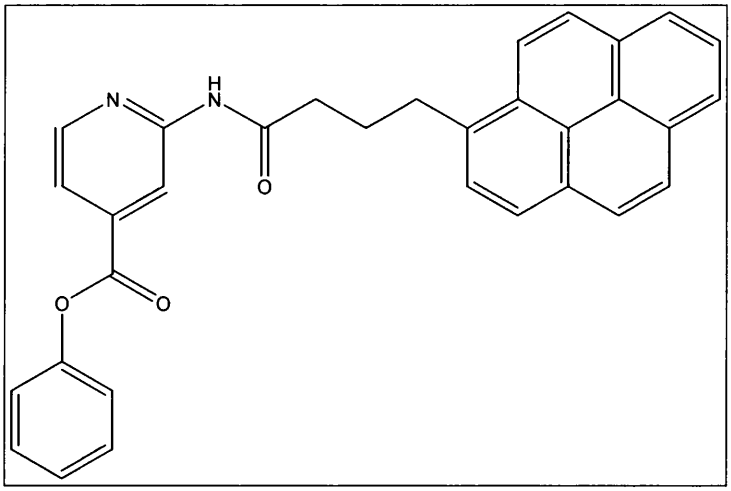



**Figure 16, 10-Methyl-9-(4-phenoxycarbonyl-pyridin-2-ylcarbamoyl)-acridinium trifluoromethanesulfonate**

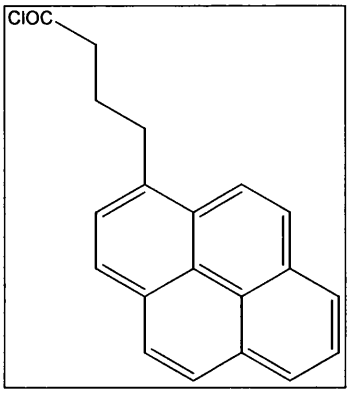
Work was then conducted to synthesise the pyrene derivative. This had the distinct advantage that it did not have any heterocyclic nitrogen atoms to compete with the pyridine nitrogen. Thus it was hoped that synthesis of this would be more straightforward.

3.2.2.3.3 2-[4-(Pyren-1-yl)butyrylamino]isonicotinic acid phenyl ester (3.9)




**(3.9)**

Synthesis of the amide was found to be straightforward. As in previous amide formations, the amine was reacted with an acid chloride. Thus, for the synthesis of (3.9), 4-(1-pyrenyl)butanoyl chloride would be required, which had historically been obtained by reacting 4-(1-pyrenyl) butanoic acid with thionyl chloride. [22]




**1-pyrene butyryl chloride**

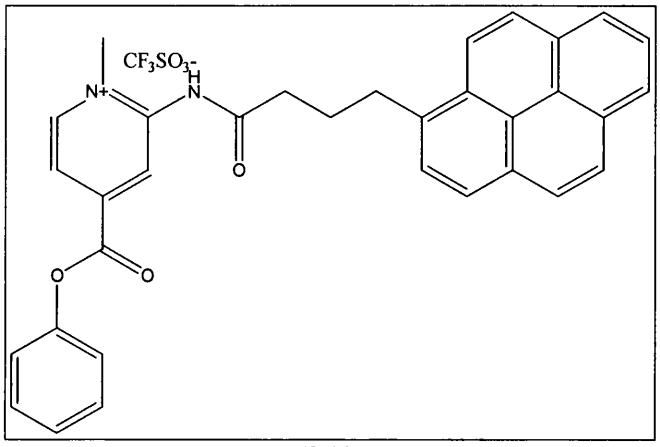
On account of the reactive nature of, it was decided to form the acid chloride and immediately convert this into the amide by conducting both reactions in the same flask. To ensure that hydrolysis did not occur, all reactions were conducted in flame-dried, glassware cooled under argon where possible using transfer needles. The acid chloride was thus formed by exposing the acid to a solution of thionyl chloride in anhydrous DCM. The mixture was stirred overnight, during which time the solid dissolved in the acid chloride solution forming a clear yellow solution. The solvents were then removed by attaching the round-bottom flask to a vacuum pump fitted with a solvent trap. A second round-bottom flask was taken and an anhydrous solution of (3.6a) and triethylamine in DCM was prepared. DCM was then added to the dry acid chloride powder in the first round bottom flask. Dropwise, the acid chloride was added to the amine solution, resulting in an exothermic reaction. The solution was then allowed to stir at room temperature for 2 hours. During this time, the yellow solution slowly turned to red and furthermore, a novel spot showed on a TLC plate. The solution was then evaporated to remove the DCM and base. Product was purified by silica gel column chromatography and crystallised *via* evaporative crystallisation to form yellow crystals in modest yield (60 %). The product was then characterised.

The proton NMR spectroscopic results indicated that the product had formed. The peaks at 2.22 ppm, 2.40 ppm and 3.34 ppm all integrated to 2 protons each and hence were indicative of the 3 $CH_2$ groups on the pyrene linker arm. Furthermore, the peaks in the aromatic end of the spectrum collectively integrated to 17 protons, which was the expected number. However, it was extremely difficult to assign the protons to the positions on the aromatic rings on account of their similarity. What was found to be interesting was that there appeared to be two NH protons, on account of the fact that

that there were 2 broad singlets at the low field end of the spectrum. Only one of these was expected to be present on account of the fact that this should represent the NH proton on the amide group. It was possible that this second proton could have been due to protonation of the pyridine nitrogen. On account of the fact that the reaction to form the amide was base catalysed in the presence of triethylamine, protonation of the product most probably had occurred during the purification process. One possible explanation is that the product could have formed a salt during the chromatography. The silica used in the column was standard 60 μm irregular sorbent, which is not pure in terms of metal contamination. Some of the metals result in silanol groups that are more acidic than usual (the average pKa of the surface silanols is around 4). Thus it would not be inconceivable that the silica protonated the base as it travelled down the column.

The carbon data complemented the proton data. There were three high field peaks, which were inverted when the DEPT analysis was conducted, demonstrating that they were attached to $CH_2$ groups. Only 13 CH peaks were observed out of a possible 17. However it was noted that 3 of the peaks were significantly higher in intensity than the rest (121.5, 124.8 and 129.6 ppm); thus, it was assumed that the remaining three protons were hidden under these peaks. However, all 12 quaternary carbon peaks were observed. CI mass spectrometry unfortunately did not show the desired molecular ion it seemed to show a product which contained a Cl as a number of peaks appeared to show a characteristic isotopic distribution. This was rather confusing as the NMR data seemed to suggest the desired product had been formed. It was decided to carry on with the synthetic route and see whether the product being a salt would provide a molecular ion and this would confirm that (3.9) was the desired product.

3.2.2.3.4 1-Methyl-4-phenoxycarbonyl-2-[(4-pyren-1-yl)-butanoylamino]pyridinium trifluoromethanesulfonate (3.10)




**(3.10)**

Synthesis of (3.10) was found to be straightforward. Like the synthesis of the acridinium analogue, the amide was reacted with methyl trifluoromethanesulfonate in DCM at room temperature, causing precipitation of the product. This was purified by crystallisation, giving a yield of 100%. The product was then characterised and the results showed that the desired product had been synthesised.

The proton NMR analysis showed that there were four aliphatic proton peaks, the first three (2.23, 2.82 and 3.49 ppm) of which were triplet in nature and all integrating for 2 protons. These were identified as the three $CH_2$ groups on the linking arm from the pyrene to the amide group. The final aliphatic signal (4.25 ppm) was singlet in nature and integrated for three protons and hence was found to be the pyridinium methyl. Although it was difficult to assign the aromatic protons, their combined integrations amounted to 17 protons which was the expected number of aromatic protons. Finally the broad singlet found significantly downfield (11.06 ppm) was identified as the NH proton. The carbon NMR spectrum complemented the proton spectrum. The DEPT analysis showed the presence of the three $CH_2$ carbons and also the $CH_3$ carbon too. On account of the complexity of the spectrum, it was difficult to account for all the aromatic peaks. The spectrum showed 14 CH peaks whereas there are 17 actual CH carbons. However, the 4 symmetrical phenyl carbons would have shown as only 2 peaks. These were more than likely the peaks at 129.9 and 121.6 ppm as these seemed to be over double the intensity of the other peaks. Also, the quaternary carbon

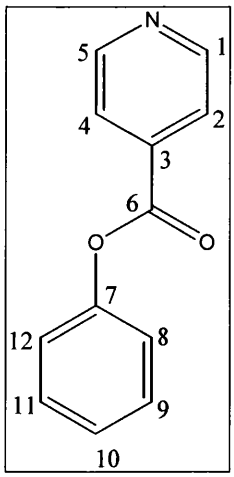
peaks appeared to be two carbons short; however, again the likely explanation for this was that one or two of the peaks were representative of two or more carbons. The mass spectrum showed the molecular ion of the cation (m/z 499) and also the sulfonate anion (m/z 149). This molecule was then tested for its chemiluminescence; however, unfortunately the molecule did not produce light on reaction with hydrogen peroxide.

#### 3.2.2.3.5 Attempted synthesis of the fluorescein derivative

Several attempts were made to bind fluorescein isothiocyanate to (3.6a). However, in each of the attempts either starting materials were recovered when gentle conditions were applied or when the starting materials were heated too strongly they decomposed, leaving complex mixtures of breakdown products. It was thought that steric hindrance prevented the two molecules from reacting. In future work a small chain linker arm could be added to either the fluorescein or the ester.

## 3.3 Experimental

### 3.3.1 Isonicotinic acid phenyl ester (3.2)




**(3.2)**

#### *3.3.1.1 Method*

Isonicotinic acid (2.019 g, 8.264 mmol) and thionyl chloride (20 ml, 32.62 g, 272mmol) were added to a round-bottom flask (50 ml) fitted with a condenser and a drying tube. The solution was refluxed for 3 h at 79 °C. Following this, the excess thionyl chloride was distilled off and the remainder was removed by a high vacuum

pump for 2h. The resulting solid, was dissolved in pyridine (40 ml) and phenol (1.49 g, 16 mmol) was added. The mixture was stirred at room temperature overnight. The pyridine was then removed by distillation and the resulting residue was taken up in DCM (120 mL), washed twice with cold NaOH solution (0.05 M, 2 x 25 ml) and then dried with $MgSO_4$. A brown oil was obtained (2.708 g), from which a portion (2.478 g) was then passed through a silica gel column using EtOAc / DCM solvent system (1:4 increasing to 2:2). The column was monitored by TLC (silica gel, EtOAc 1, DCM 4) and two fractions were isolated. Fraction 1 (924 mg) at Rf 0.85, fraction 2 (1368 mg) at Rf 0.5.

### *3.3.1.2 Characterisation*

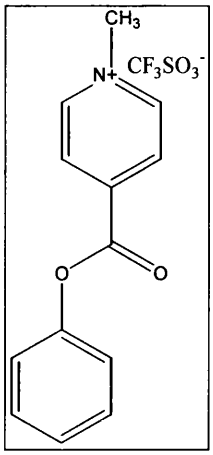
**Fraction 1**

**$^1$HNMR (δ ppm, $CDCl_3$):** 6.03 (ws, 1H), 6.89 (m, 2H), 6.96 (m, 1H), 7.27 (m, 2H). **$^{13}$CNMR (δ ppm, $CDCl_3$):** 115.4 (CH), 120.7 (CH), 129.7 (CH), 155.6 (C), 172.5 (very small intensity possibly an artefact). **MS (EI)** m/z 43, (35 %), 55 (20 %), 66 (45 %), 94 (100%) **MS (CI)** m/z 94 (100%), 98 (30 %), 100 (20%), 110 (20 %), 114 (40 %).

**Fraction 2**

**$^1$HNMR (δ ppm, $CDCl_3$):** 7.22, (d, 2H, J = 8 Hz), 7.29 (t, 1H, J = 7Hz), 7.43 (t, 2H, J = 8 Hz), 7.98 (dd, 2H, J = 4, 2 Hz), 8.84 (dd, 2H, J = 4, 2 Hz). **$^{13}$CNMR (δ ppm, $CDCl_3$)**: 121.4 (2CH), 123.2 (2CH), 126.3 (CH), 129.6 (2CH), 136.8 (C), 150.5 (C), 150.8 (2CH), 163.7 (C), 171.0 (very small peak possibly an artefact) **MS (ESI):** Positive Mode m/z 200 (100 %), Negative Mode m/z 35 (20 %), 66.9 (45 %), 92.9 (50 %), 127.9 (60 %), 148.8 (100 %), 156.9 (50 %). **IR $\upsilon_{max}$:** 508, 682, 692, 704, 747, 757, 816, 852, 916, 1066, 1099, 1160, 1205, 1219, 1284, 1328, 1408, 1456, 1484, 1561, 1591, 1739.

### 3.3.2 Phenyl 1-methyl-pyridineium-4-carboxylate trifluoromethanesulfonate (3.1)




**(3.1)**

#### *3.3.2.1 Method*

Phenyl pyridine-4-carboxylate (200 mg, 1.0 mMol) was added to a dry round-bottom flask (50 mL) which had been sealed with a septum and cooled under nitrogen. To this, dry DCM (8 ml) was added and the powder dissolved into a clear liquid. Using a dry syringe, methyl trifluoromethanesulfonate (0.5 mL, 3.75 mmol) was then added. The solution was then stirred for 3 h under an atmosphere of nitrogen. Following this, diethyl ether (2x 50 ml) was added and the solution was filtered to give a pure white crystalline solid (357 mg).

#### *3.3.2.2 Characterisation*

**$^{1}$HNMR (δ ppm, $CDCl_3$):** 4.48 (s, 3H), 7.39 (m, 3H), 7.53 (m, 2H), 8.69 (d, 2H, J = 7 Hz), 9.24 (d, 2H, J = 7 Hz). **$^{13}$CNMR (δ ppm)**: 48.6 ($CH_3$), 121.6 (CH), 126.8 (CH), 127.3 (CH), 129.8 (CH), 143.3 (C), 147.2 (CH), 150.2 (C), 161.1 (C). **$^{19}$FNMR (δ ppm):** 77.4 (m, $CF_3$). **MS (ESI):** Positive Mode m/z 93 (30 %), 152 (20 %) 214 (100 %), Negative Mode m/z 80 (20 %) 149 (100 %). **IR $\upsilon_{max}$:** 518, 636, 680, 698, 760,

827, 872, 1030, 1102, 1150, 1162, 1184, 1224, 1264, 1335, 1488, 1586, 1646.25, 1752, 3062.

### 3.3.3 Chemiluminescent experiments of compound (3.1) in isolation and in the presence of rhodamine 6G.

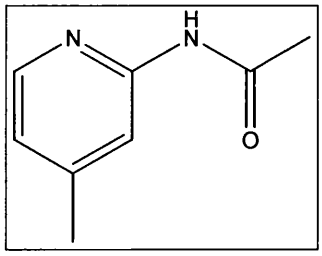
Preparation of the solutions

Compound 2 (10 mg, 4.64 $x10^{-5}$mol) was added to a volumetric flask (10 ml) and water or acetonitrile was added so that the final volume of the flask was 10 ml (4.65 $x10^{-3}$ M). A portion of this solution (1.00 ml) was added to a second volumetric flask (10 ml) and the solvent was added as before to make up the 10 ml, i.e. a 1 in 10 dilution. This serial dilution was twice repeated to yield four solutions of decreasing concentrations these are shown in table 1. Three solutions of rhodamine 6G were prepared in the same way as for (3.1), except that the starting solution contained only 1mg of the fluor (2.33 $x10^{-4}$ M). From this, aliquots of the stock solutions (0.5 ml each) and / or solvent (0.5 ml) were taken to make the solutions shown in table 1.

Assay procedure

Each of the solutions (labelled a1-d5 in table 1) were tested in triplicate using 2μ aliquots of each solution. Standard conditions were applied to the chemiluminometer and except the assay time, after each injection, was three seconds. The results were then graphically represented in graphs 5 and 6 as total light counts against both salt and rhodamine concentrations.

### 3.3.4 2-acetylamino-4-methylpyridine (3.4)




**(3.4)**

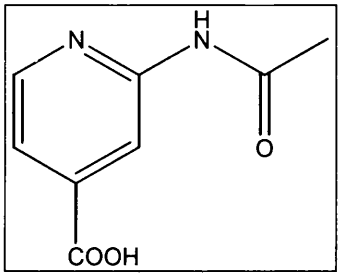
#### *3.3.4.1 Method*

2-Amino-4-picoline (5.0 g, 46 mmol) was added to acetic anhydride (12 ml) and triethylamine (20 ml) in a round-bottom flask (50 ml). Instantly the reaction became exothermic (~ 70 °C). The flask was capped with a rubber septum and the mixture stirred overnight at room temperature. During this time the solution changed colour from colourless to black. Most of the solvent was then removed by distillation (80 °C, 15 mm Hg). The remaining black oil was taken up into chloroform (100 ml) and an extraction was conducted with water (2 x 200ml) which destroyed any acetic anhydride and removed the resulting acetic acid. The chloroform layer was then dried with $MgSO_4$ and removed on a rotary evaporator to from a brown crystalline product (6.8 g). This product was tested by TLC (silica, EtOAc 100%) and showed one major spot (Rf 0.67, starting material = Rf 0.23). This product was purified on silica column (100 % EtOAc) yielding a cream crystalline solid (6.6g), which was twice recrystallised with hot diethyl ether to yield colourless crystals (5.9 g, 85 %).

#### *3.3.4.2 Characterisation*

**$^1$H (δ ppm, $CDCl_3$, main peaks)** 2.18 (s, 3H,), 2.37, (s, 3H) 6.87 (d, 1H, J = 5 Hz), 8.07 (s, 1H), 8.21 (d, 1H, J = 5 Hz), 9.42 (s, 1H, NH). **$^{13}$C (δ ppm)** 21.4 ($CH_3$), 24.6 ($CH_3$), 114.9 (CH), 120.9 (CH), 147.0 (CH), 150.1 (C), 151.9 (C), 169.0 (CO). **MS (CI)** m/z 44 (100 %), 45 (70 %), 58 (40 %), 60 (30 %), 94 (40 %), 108 (30 %), 109 (60 %), 151 ($M+H^+$). .

### 3.3.5 2-acetylaminopyridine-4-carboxylic acid (3.5)




**(3.5)**

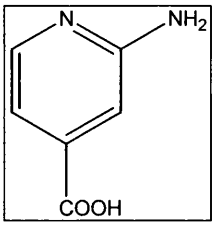
#### *3.3.5.1 Method*

In a two-necked round-bottom flask (250 ml) fitted with a reflux condenser, (3.4) (10.015 g, 0.080 mol) was dissolved in water by heating (85 °C). Over 3 h, $KMnO_4$ (23.0 g, 0.146 mol) was slowly added to the hot stirring solution. The black suspension was heated for a further 1h whereupon filtration of the $MnO_2$ yielded a clear yellow solution. HCl (2M) was slowly added until a white precipitate formed. This was filtered and the solid was collected (2.403 g). The solution was evaporated down and a further aliquot of HCl was added, which caused a second precipitation. The solution was filtered again yielding a white product which was isolated (1.038 mg). This was repeated a further time giving a third batch of the white product (1.651 mg). Thus, total product collected was 6.939g (38.55 mmol, 48 %).

#### *3.3.5.2 Characterisation*

**$^1$HNMR (δ ppm, DMSO, main peaks):** $^1$H (δppm) 2.11 (s, 3H,), 7.49 (dd, 1H, J = 5, 1 Hz), 8.45 (dd, 1H, J = 5, 1 Hz), 8.56 (s, 1H), 10.71 (s, 1H, NH), 13.6 (s, 1H, COOH). **$^{13}$CNMR (δ ppm)**: 23.8 ($CH_3$), 112.5 (CH), 118.1 (CH), 139.9 (C), 148.8 (CH), 152.9 (C), 166.1 (C), 169.5, (C). **MS (EI):** m/z 43 (45 %), 45 (85 %), 63 (100 %), 78 (70 %) 180 (M, 5 %). **MS (CI):** 79 (100 %), 96 (95 %) 181 ($M+H^+$, 2 %). **IR $\upsilon_{max}$:** 419, 560, 667, 771, 924, 1017, 1118, 1246, 1279, 1374, 1430, 1577, 1710, 3031. Proton NMR also shows tiny impurity peaks for the starting material too.

### 3.3.6 2-Aminopyridine-4-carboxylate (3.3)




**(3.3)**

#### *3.3.6.1 Method*

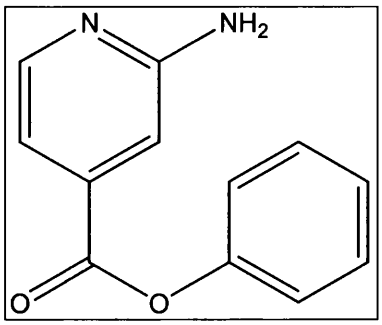
To a round-bottom flask (25 ml) fitted with a reflux condenser, (3.5) (2.00 g, 0.011 mol) was added with sodium hydroxide (10 ml, 10 %) and the solution was refluxed for 3 h. The solution was then cooled in ice and brought to neutrality with HCl (2M). This facilitated the precipitation of a fine white powder. In a previous attempt at synthesising (3.3), filtration with a grade 1 paper was found to trap the product in the fibres of the filter paper thus causing a lot of the product to be lost within the filter paper. Extraction of the product from the filter paper into solvent was found to be difficult due to the low solubility of the product in any solvent (even DMSO). Therefore, capture of the fine powder was achieved by centrifugation. The advantage of this technique was that all of the product could be isolated and the powder could be easily washed with water (3x) to remove the inorganic salts (NaCl) and acetone (3x) to remove any soluble organic contaminants. The acetone was removed by rotary evaporation and the sample dried on a high vacuum pump yielding the product (1.038 g). The product was then analysed.

#### *3.3.6.2 Characterisation*

**$^{1}$HNMR (δ ppm, NaOD, main peaks):** 4.82 (large water peak plus $NH_2$), 6.95 (s, 2H), 7.01 (dd, 1H, J = 5, 1 Hz), 7.98 (s, 1H), 7.98 (d, 1H, J = 5 Hz). **$^{13}$CNMR δ ppm**: 109.9 (CH), 114.2 (CH), 147.8, (C), 148.1 (CH), 159.9 (C), 174.6 (C). **MS (EI):** m/z 45 (55 %), 63 (100 %), 78 (90 %), 84 (%), 91 %), 138 (M, 10 %). **MS (CI):** m/z 80 (10 %), 95 (100 %), 109 (15 %), 139 ($M+H^+$, 45 %). **IR $\upsilon_{max}$:** 414, 669, 774, 809, 930, 994, 1374, 1478, 1596, 1641, 1696, 1955, 2798, 2944, 3278.

The proton NMR also showed a tiny impurity of the starting material.

### 3.3.7 Phenyl 2-aminopyridine-4-carboxylate (3.6a)




**(3.6a)**

#### *3.3.7.1 Method*

In a round-bottom flask (25 ml) containing freshly distilled thionyl chloride (15 ml) fitted with a reflux condenser and drying tube, (3.3) (100, mg, 0.73 mmol) was added. The mixture was stirred at reflux (80 °C) for 5 h with the solution clarifying after 2.5 h. The thionyl chloride was then distilled off and the red oil was dried under a high vacuum (0.5 mm Hg), leaving a red powder (109 mg, 0.73 mmol). Into a dry double-necked round-bottom flask (25 ml) pyridine (585 mg, 7.4 mmol) and phenol (511 mg, 5.4 mmol) were added. Dry DCM (8 ml) was then added to the acid chloride with a dry syringe. This was mixed and formed a red / brown suspension. The acid chloride suspension was then slowly transferred (using a dry syringe) to the phenol solution and the mixture was stirred overnight. The solution was then washed successively with dilute acid (2 x 50ml) and base (2 x 50 ml) solutions and dried over $MgSO_4$ for 10 min. The solution was then evaporated to yield a red tar (240 mg). The product was tested by TLC which showed multiple products (see table **3**)

**Table 3 Products of the red tar separated by TLC**

| Spot Code | Rf of Spot | Fluoresces at 280 nm |
|---|---|---|
| A | 0.93 | No |
| B | 0.66 | Yes |
| C | 0.56 | No |
| D | 0.46 | Yes |
| E | 0.36 | No |
| F | 0.30 | Yes |
| G | 0.13 | No |
| H | 0.1 | No |
| I | 0-0.06 | No |

TLC conducted on a normal phase silica gel plate eluted by chloroform EtOAc (1:1).

The red tar (240 mg) was then separated by silica gel column chromatography using a variable polarity elution system (chloroform, chloroform/EtOAc, EtOAc, EtOAc/EtOH).

The products shown in table 4 were isolated.

**Table 4 Products isolated from the column chromatography of the red tar**

| Spot Code on TLC | Amount obtained (mg) |
|---|---|
| A | 62 |
| B | 2 |
| D | 20 |
| F | 131 |
| G | 2 |
| H-I | 1 |
| **Total** | 158 |

Each product was then analysed and the results are shown as follows.

### *3.3.7.2 Characterisation*

#### 3.3.7.2.1 Product A

Phenol

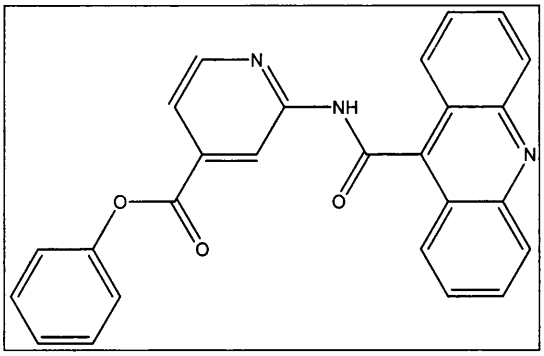
#### 3.3.7.2.2 (3.6a) (Fraction F)

**$^1$HNMR (δ ppm, $CDCl_3$):** 4.77 (s, 2H), 7.21 (m, 3H), 7.31 (m, 2H), 7.44 (m, 2H), 8.25 (dd, 1H, J = 5, 1 Hz). **$^{13}$CNMR (δ ppm)**: 108.8 (CH), 113.2 (CH), 121.4 (2CH), 126.2 (CH), 129.6 (2CH), 149.1 (CH), 150.3 (C), 159.1 (C), 164.1 (C), 171.2 (C), 198.82 (artifact). **MS (EI):** m/z 39 (70 %), 43 (70 %), 44 (40 %), 49 (100 %), 51 (50 %), 65 (60 %), 66 (85 %), 84 (70 %), 86 (40 %), 93 (55 %), 121 (60 %), 214 (10 %). **MS (CI):** m/z 79 (70 %), 94 (30 %), 95 (40 %), 96 (60 %), 108 (25 %), 130 (20 %), 138 (25 %), 153 (100 %), 215 ($M+H^+$, 15 %). **MP** 156°C. **Anal:.** Calcd for $C_{12}H_{10}N_2O_2$: C,67.28; H, 4.71; N, 13.08, found C, 66.86; H, 4.67; N, 13.04.

#### 3.3.7.2.3 (3.6b) (Fraction D)

**$^1$HNMR (δ ppm, $CDCl_3$):** 4.66 (s, 2H), 7.04 (d, 1H, J = 1 Hz), 7.24 (m, 2H), 7.31 (tt, 1H, J = 7, 2 Hz), 7.45 (tt, 2H, J = 7, 2 Hz), 8.21, (s, 1H). **$^{13}$CNMR (δ ppm)**: 109.7 (CH), 118.6 (C), 121.4, (2CH), 126.5 (CH), 129.6 (2CH), 138.0 (C) 149.1 (CH) 150.3 (C) 157.1 (C) 163.0 (C). **MS (EI):** 39 (60 %), 50 (30 %), 65 (70 %), 100 (80 %), 102 (25 %), 127 (40 %), 129 15 %), 155 (100 %), 157 (35 %), 248 (M [$^{35}$Cl], 20 %), 250 (M [$^{37}$Cl], 7 %). **MS (CI):** 95 (30 %), 129 (50 %), 131 (15 %), 153 (20 %), 187 (45 %), 189 (15 %), 215 (20 %), 249 ($M+H^+$ [$^{35}$Cl] 100 %), 251 ($M+H^+$ [$^{37}$Cl]35 %).

### 3.3.8 2-(Acridine-9-carbonylamino)isonicotinic acid phenyl ester (3.7)




**(3.7)**

#### *3.3.8.1 Method*

Into a flame-dried round-bottom flask (50ml), under an atmosphere of dry nitrogen, (3.6a) (200 mg, 0.93 mmol), anhydrous DCM (20 ml) and anhydrous triethylamine (2 ml) was added. A second round-bottom flask (50 ml) was prepared in the same was as the first except instead freshly prepared acridine-9-carbonyl chloride (218mg 0.93 mmol) and DCM (20 ml) were added. Finally, using a double-ended needle, the acid chloride solution was slowly added to the stirring basic solution at room temperature. The reaction was monitored by thin layer chromatography using a silica gel plate (DCM - ethyl acetate 50:50). After 6 h the plate showed three major spots with Rf values of 0.16, 0.60 and 0.86, respectively. The three components were purified by silica gel column chromatography. Only two of these eluted from the column (products with Rfs 0.16 [24 mg] and 0.60 [349 mg, 0.83 mmol, 90 %yield].

#### *3.3.8.2 Characterisation*

Product 1 (Rf 0.16)

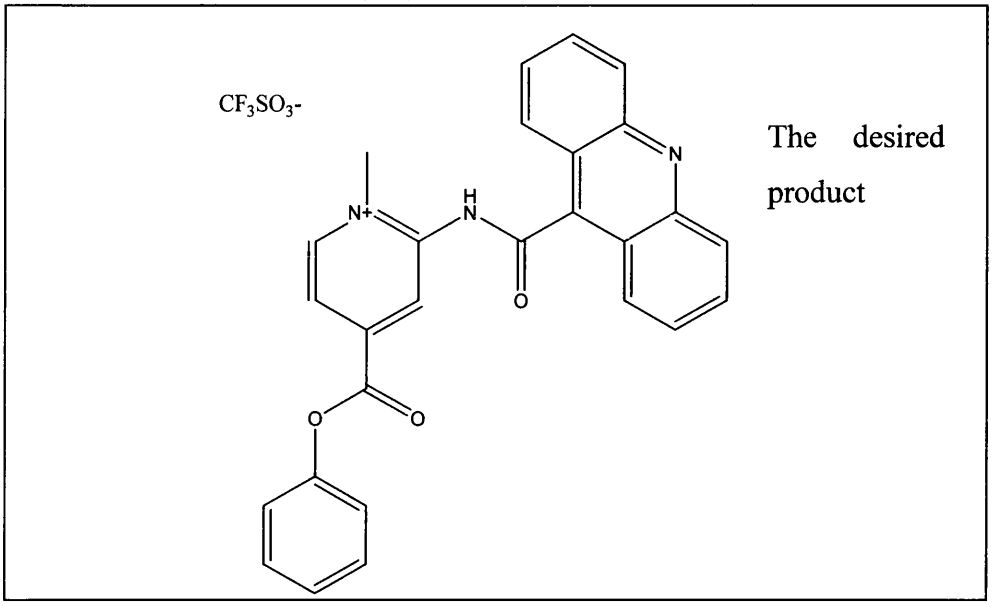
As (3.6a)

Product 2 (Rf 0.60)

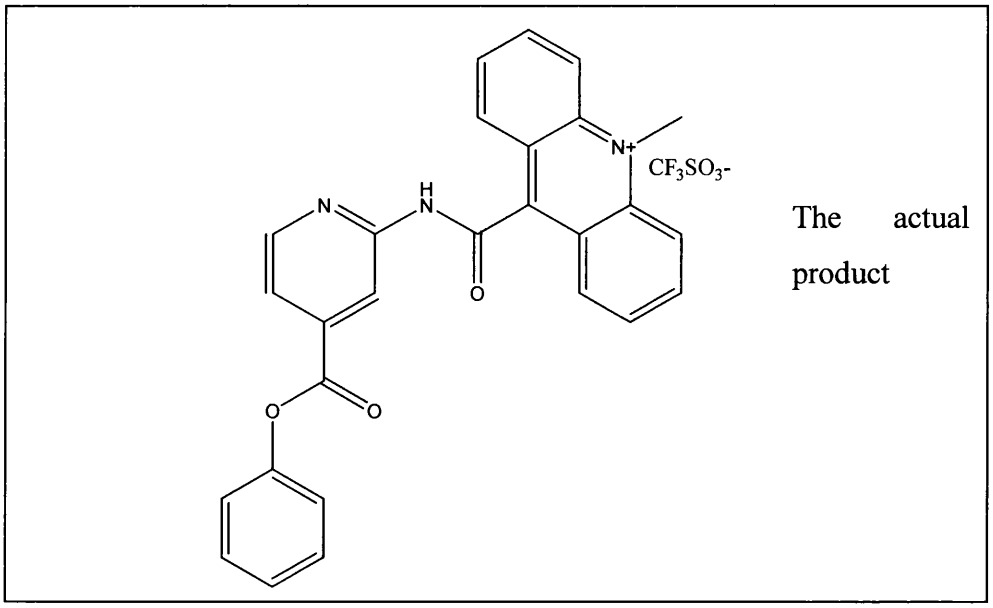
**$^1$HNMR (δ ppm, $CDCl_3$):** 6.58 (dd, 1H, J = 7, 2 Hz), 6.69 (dd, 2H, J = 8, 1 Hz), 6.74 (s, 1H, NH), 6.78 (dt, 2H, J = 8, 1 Hz), 6.91, (dd, 2H, J = 8, 1 Hz), 7.12 (m, 2H), 7.14 (m, 2H), 7.23 (m, 2H), 7.38 (tt, 2H, J = 8, 2 Hz), 7.67 (t, 1H, J = 1 Hz). **$^{13}$CNMR (δ ppm)**: 107.9 (CH), 115.2 (2CH), 115.5 (C), 120.8 (2CH), 121.2 (2CH), 124.4 (CH),

125.2 (CH), 125.6 (2CH), 126.5 (CH), 129.6 (2CH), 129.7 (2CH), 138.4 (2C), 138.6 (C). 150.3 (C), 156.2 (C), 162.3 (C), 180.9 (C). **MS (FAB):** Positive Mode m/z 107 (65 %), 123 (50 %), 149 (100 %), 167 (30 %), 392 (60 %), 413 (40 %), 414 (60 %), 420 (15 %).

### 3.3.9 2-[Acridine-9-carbonylamino]-1-methyl-4-phenoxycarbonylpyridinium trifluoromethanesulfonate (3.8)




The desired product




The actual product

**(3.8)**

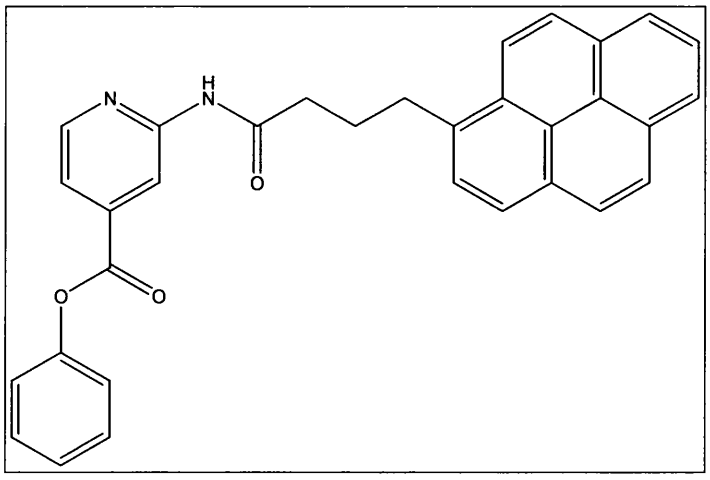
### *3.3.9.1 Method*

In a dry round-bottom flask (50 ml), (3.7) (50 mg, 0.12 mmol) was added to a stirring solution of anhydrous DCM (20 ml). Following this, $CF_3SO_3CH_3$ (0.2 ml, 1.7 mmol) was added to the solution and the mixture was then stirred for 6 h. The solvent was removed by rotary evaporation and the product was re-dissolved in acetone (3 ml). To obtain crystals of the product, diethyl ether (~ 10 ml) was added and the crystals were allowed to grow overnight in the refrigerator (66 mg, 0.11 mmol, 91% yield).

### *3.3.9.2 Characterisation*

**$^1$HNMR (δ ppm, d6-DMSO):** 3.02 (s, 3H), 7.24 (dd, 1H, J = 7, 1 Hz), 7.33 (m, 3H), 7.49 (t, 2H, J = 8 Hz), 7.67 (s, 1H), 7.92 (t, 2H, J = 8 Hz), 8.07 (d, 1H, J = 7 Hz), 8.21 (t, 2H, J = 8 Hz), 8.27 (d, 2H, J = 9 Hz), 8.34 (d, 2H, J = 9 Hz) 9.25 (s, NH). **$^{13}$CNMR (δ ppm):** 28.9 ($CH_3$), 109.8 (CH), 121.3 (2C), 121.6 (4CH), 123.5 (CH), 126.3 (4CH), 126.6 (2CH), 128.6 (CH), 129.7 (2CH), 135.4 (CH), 142.8 (C), 150.12 (C), 153.87 (C), 161.79 (C), 166.88 (C). **MS (ESI):** Positive Mode m/z 85 (100 %),107 (40 %),169 (40 %), 191 (40 %), 229 (90 %), 275 (50 %). Negative Mode m/z 80 (30 %), 149 ($CF_3SO_3$, 100 %). .

### 3.3.10 2-(4-Pyren-1-ylbutanoylamino)isonicotinic acid phenyl ester (3.9)




**(3.9)**

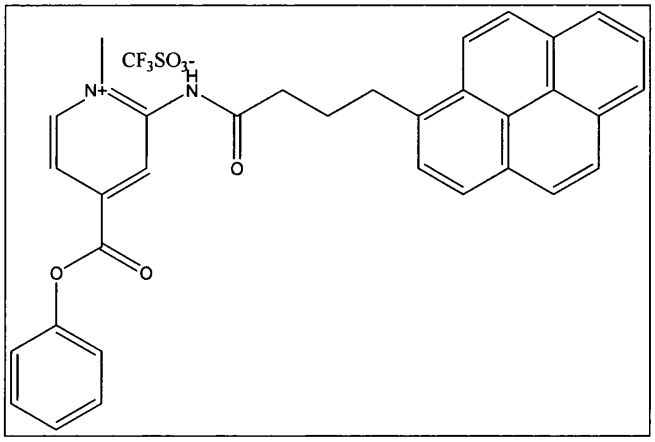
*3.3.10.1 Method*

Into a flame-dried round-bottomed flask (20 ml), capped with a rubber septum, dry DCM (10 ml) and anhydrous thionyl chloride (1 ml) were introduced through a transfer needle. To the stirring solution, 4-(-pyreneyl)butanoic acid (200 mg, 0.69 mmol) was added. The mixture was then stirred over argon at room temperature overnight and following this the solvent was removed using a vacuum line until a yellow powder formed. A second aliquot of DCM (5ml) was then added and the mixture was stirred to dissolve the yellow powder. To this solution, dry triethylamine (2 ml) was added. A second dry round-bottom flask (20 ml) was taken and phenyl 2-aminopyridine-4-carboxylic acid (150 mg, 0.7 mmol) was added. This was dissolved in dry DCM (10 ml) and allowed to stir for 5 min. Dropwise, using a transfer needle, the solution containing the pyrenylbutanoic acid chloride was dispensed under a dry argon atmosphere into the round-bottom flask containing the amino acid. The resulting solution was then stirred for 2h. The solvent was removed by rotary evaporation and the product (450 mg) then taken up into a small quantity of DCM (2ml) and separated using silica gel column chromatography using a gradient elution method from 100 % DCM to 100 % ethyl acetate. The major product was obtained then crystallised from ethyl acatate forming yellow crystals (200 mg, 0.41 mmol, 60%) and was sent for characterisation.

*3.3.10.2 Characterisation*

**$^{1}$HNMR (δ ppm, $CDCl_3$):** δppm: 2.22 (quintet., 2H, J = 7 Hz), 2.40 (t, 2H, J = 7 Hz), 3.34 (t, 2H, J = 7 Hz), 7.14 (m, 2H), 7.21 (t, 1H, J = 7 Hz), 7.30 (t, 2H, J = 8 Hz), 7.49 (dd, 1H, J = 5, 1 Hz), 7.75 (d, 1H, J = 8 Hz), 7.88 (m, 4H), 7.98 (d, 2H, J = 9 Hz), 8.05 (d, 2H, J = 7 Hz), 8.18 (m, 2H), 8.37 (s, 1H, NH), 8.77 (s, 1H, NH). **$^{13}$CNMR (δ ppm)**: 26.7 ($CH_2$), 32.5 ($CH_2$), 36.7 ($CH_2$), 113.9 (C), 119.3 (CH), 121.5 (CH), 123.2 (CH), 124.8 (CH), 124.8 (CH), 124.9 (CH), 125.0 (CH), 125.9 (CH), 126.3 (CH), 126.8 (CH), 127.4 (CH), 127.5 (CH), 128.8 (C), 129.6 (CH), 130.0 (C), 130.9 (C), 131.4 (C), 135.4 (C), 139.2 (C), 148.4 (C), 150.5 (C), 152.3 (C), 163.6 (C), 171.5 (C). **MS (CI):** Positive Mode m/z 138 (100 %), 142 (80 %), 153 (30 %), 305 (30 %), 423 (70 %), 425 (30 %).

### 3.3.11 1-Methyl-4-(phenoxycarbonyl)-2-[4-(pyren-1-yl)butanoylamino]pyridinium trifluoromethanesulfonate (3.10)




**(3.10)**

#### *3.3.11.1 Method*

To a flame-dried argon cooled round-bottom flask (10 ml), (3.9) (50mg, 0.10 mmol) and anhydrous DCM (5ml) were added and stirred until the solid dissolved. Methyl, trifluoromethanesulfonate (0.2 ml, 1.7 mmol) was added and the mixture was stirred for 1 h. The solvent was removed by evaporation and the resulting powder was dissolved in acetone (3 ml) to which was added a few drops of diethyl ether until crystallisation was observed. The crystals were allowed to develop overnight in the refrigerator. Following this, the crystals were filtered and weighed (55 mg, 0.1 mmol). The product (3.10) was then characterised.

#### *3.3.11.2 Characterisation*

**$^{1}$HNMR (δ ppm, d6-DMSO):** δppm: 2.23 (t, 2H, J = 8 Hz), 2.82 (t, 2H, J = 7 Hz), 3.49 (t, 2H, J = 8 Hz), 4.25 (s, 3H, ), 7.41 (m, 3H), 7.55 (t, 2H, J = 8 Hz), 8.03 (d, 1H, J = 8 Hz), 8.09 (t, 1H, J = 8 Hz), 8.16 (dd, 2H, J = 11, 9 Hz), 8.28 (m, 5H), 8.47 (d, 1H, J = 9 Hz), 8.84 (d, 1H, J = 2 Hz), 8.99 (d, 1H, J = 7 Hz), 11.06 (s, 1H, NH). **$^{13}$CNMR (δ ppm)**: 26.4 ($CH_2$), 31.9 ($CH_2$), 35.9 ($CH_2$), 44.8 ($CH_3$), 121.4 (CH), 121.6 (2CH), 123.5 (CH), 124.1 (C), 124.3 (C), 124.9 (CH), 125.0 (CH), 125.1 (CH), 126.3 (CH), 126.7 (CH), 126.8 (CH), 127.4 (CH), 127.5 (CH), 127.7 (CH), 128.3 (C), 129.5 (C), 129.9 (2CH), 130.4 (C), 130.9 (C), 136.1 (C), 144.5 (C), 146.0 (CH), 150.1

(C), 161.2 (C). **MS (ESI):** Positive Mode m/z 299 (20 %), 271 )10 %) 499 (M-$CF_3SO_3^+$, 100 %), Negative Mode m/z 80 (40 %), 149 ($CF_3SO_3^-$ 100 %).

## 3.4 Final Conclusions

This chapter detailed an investigation into the synthesis and possible chemiluminescence energy transfer properties of compounds based on the parent molecule 1-methylpyridinium-4-carboxylate trifluoromethanesulfonate (3.1). Work was done initially to look at intermolecular chemiluminescent energy transfer. However, due to the fact that in its underivatised state *N*-methylpyridone, phosphoresces, any intermolecular energy transfer would be unlikely in aqueous conditions due to triplet state quenching. However, it was still plausible that a derivative of (3.1) with a fluor bound would have its electronic energy levels altered in such a way that fluorescence predominated over phosphorescence. Furthermore, even if resonance energy transfer was not possible, energy transfer though a CIEEL process could not be ruled out. Thus, phenyl 2-aminopyridine-4-carboxylic acid (3.6) was successfully synthesised and used to develop two potential energy transfer molecules, an acridine derivative (3.7) and a pyrene derivative (3.9). Unfortunately for the case of acridine derivative, the final methylation step formed (it is thought) an acridinium as opposed to a pyridinium salt. Although acridinium salt formation is a logical possibility, work in chapter 4 detailing development of acridine-9-carboxylic acid pyridin-2-ylamide and its derivatives showed that without fail, methylation formed pyridinium derivatives and not the acridinium salt observed when methylating (3.7). Interestingly, however, no chemiluminescence was detected for the methylated derivative of (3.7). Although methylation of (3.9) did yield a pyridinium again, very little chemiluminescence was detected. From these studies it can be concluded that the pyridinium system is not a viable chemiluminescent donor molecule although on paper it would be an ideal energy transfer molecule. It is also thus concluded that similarities between the CIEEL reactions of the oxalate system cannot be drawn from the tests done. However, future work could be conducted to expose (3.10) to conditions which yield light from oxalate ester oxidation.

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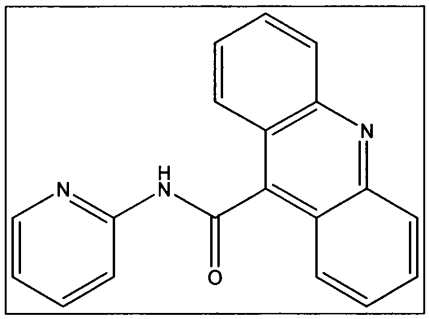
# Chapter 4

# Synthesis of a range of molecules based on the parent molecule; acridine-9-carboxylic acid pyridin-2-ylamide

# 4 Synthesis of a range of molecules based on the parent molecule; acridine-9-carboxylic acid pyridin-2-ylamide.




**Acridine-9-carboxylic acid pyridin-2-ylamide (4.1)**

## 4.1 Introduction

Work has shown that to date, acridinium amides show poor chemiluminescence in comparison to their acridinium ester cousins. This is due to the amide bond's increased stability to hydroperoxide attack over the ester bond. [1] To make an amide bond more prone to hydroperoxide attack, electron-withdrawing groups must be employed to remove electron density away from the carbonyl group. Work with acridinium sulfonamides has been previously investigated and has proven that such compounds can show comparable chemiluminescence to their ester cousins.[2] It was found that the presence of the sulfone group provided enough electron-withdrawing properties to allow for sufficient hydroperoxide attack.

As discussed in chapter one, a prerequisite for development of acridinium labels is that hydroperoxide attack is highly favoured over hydrolysis. Moreover, for effective hydroperoxidation, the leaving group has to have a pKa of $< 11$.[4] Acridinium esters have thus been developed to try to shield the carbonyl group from hydroxide attack (by both steric and electronic protection) without unduly affecting hydroperoxide attack.[3] Work with acridinium amides takes the opposite approach where groups are added to reduce the stability of the amide bond to hydroperoxide attack but keeping enough stability to prevent hydrolysis.[2,4]

Chapter three discussed work which had been conducted to develop a group of chemiluminescent energy transfer molecules which utilised a pyridinium ester energy generating 'engine' linked to a fluor though an amide bond. Following from this work, it was surmised that pyridine amides linked to acridinium groups could be an interesting group of novel chemiluminescent molecules. The reason for this was that heterocyclic nitrogen on the pyridine ring is electron withdrawing thus when attached *via* an amide bond to acridinium, it would act to reduce the stability of the amide bond enough to allow for hydroperoxide attack.

It was thus decided to attempt the synthesis of a range of the above-mentioned acridinium / pyridinium amides both mono and, if possible, bismethylated and then look at the chemiluminescence of these products. The proposed products to be synthesised are showed in figure 1. These included the unsubstituted pyridine, all four picolines, 5'-chloropyridine, 5'-nitropyiridine and 3'-hydroxypyridine derivatives.

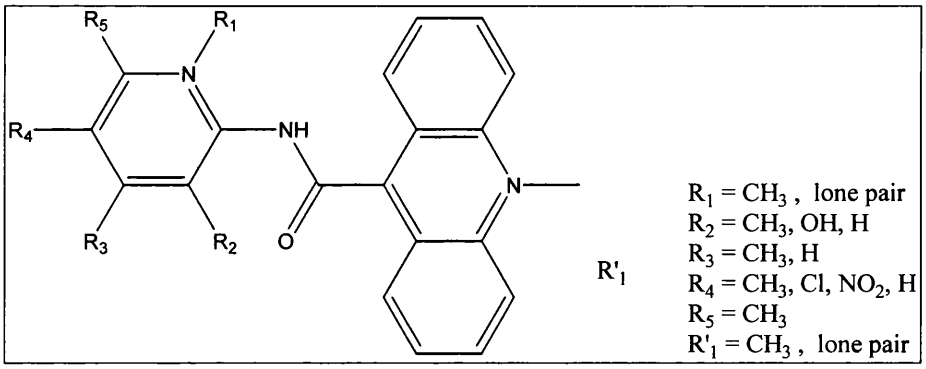



**Figure 1 A generic structure of the proposed compounds to be synthesised.**

The reasons why the 2-aminopyridines were selected as possible precursors for amide synthesis were as follows. The first reason was that it was hoped that the electron-withdrawing effect of the ortho-position pyridine nitrogen would minimise methylation of the 2-position nitrogen. The second reason was that there was a wide range of 2-aminopyridines, such as a complete range of the picolines, commercially available.

A wide range of compounds was selected for two reasons. The first was to test certain synthetic characteristics such as how the presence of electron-withdrawing/ donating

groups would promote methylation on which heterocyclic nitrogen. For example it was hoped that the 6-methyl-2-aminopyridine derivative would provide further steric shielding to thus reduce the chance of methyl attack on the pyridine nitrogen and thus promote attack on the acridine nitrogen. The second reason was to see whether the presence of such groups would affect the chemiluminescent characteristics. As well as for the above reasons, the hydroxyl derivative was chosen to see, given a choice, whether the hydroxyl react with the acid chloride or the amine would preferentially bind or whether a mixture of the two possible products would form. Furthermore, it was speculated that a free hydroxyl or conversely amine group could then be used for further derivatisation such as the addition of a linker group.

There were two possible synthetic routes for synthesis of the mono-methylated compounds summarised, the first synthetic route would be to adopt the procedure used for synthesis of the standard acridinium phenyl ester.[5] This route (shown in figure 2) would require the synthesis of acridine-9-carbonyl chloride (from the commercially available acid) and then, under anhydrous conditions, reaction of this acid chloride with the amine of choice in the presence of a dry base catalyst such as triethylamine. The amide would then be exposed to a methylating agent such as methyl trifluoromethanesulfonate to form a mono-methylated salt.

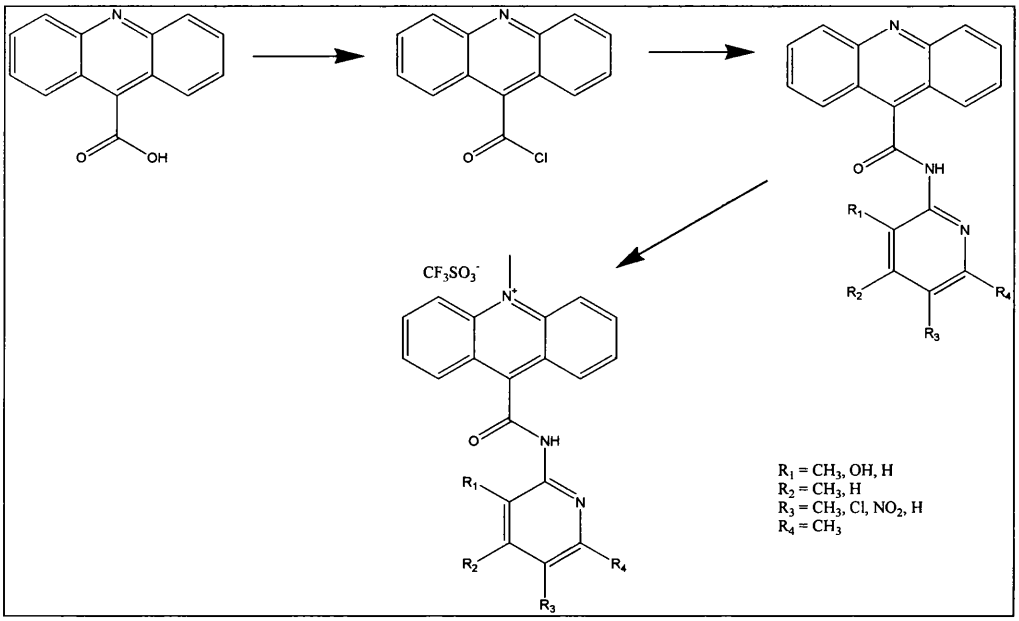



**Figure 2 Proposed synthesis of the desired acridinium amide series adopting the method used for acridinium ester synthesis**

The advantage of the above route is that it has been well documented; thus by adopting this procedure, a straightforward synthesis of the desired series might be achieved with greater success than using a novel synthetic pathway. The only disadvantage that could be seen from this synthetic route was possible difficulty in controlling methylation. It was thus decided that if this route failed to give the desired products then an alternative route would be saught. One such possible route that was postulated was to synthesise a 10-methylacridium-9-carbonyl chloride salt and to react this salt with the desired amine to form the amide of choice (see figure 3). Furthermore, this pathway would ensure that the methyl group would be on the acridine nitrogen.

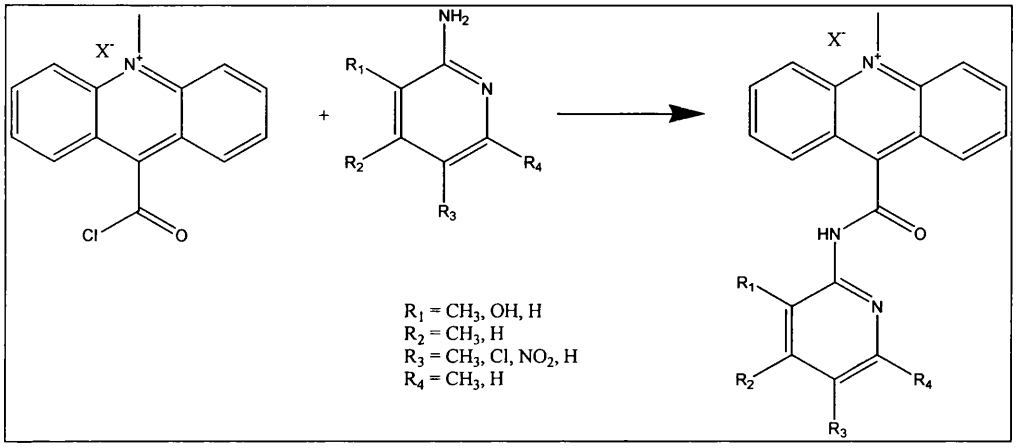



**Figure 3 Proposed acridinium amide synthesis using 10-methylacridium-9-carbonyl chloride and the desired amine**

10-Methylacridium-9-carbonyl chloride salt had been previously synthesised in the 1960's for investigation into its unique chemiluminescent properties.[6] This investigation showed that the molecule displayed very efficient and sharp chemiluminescence. Furthermore, intriguingly the molecule was also found to be extremely stable in acidic conditions. However, conversely 10-methylacridium-9-carbonyl chloride salts were found to be extremely prone to hydrolysis in basic conditions. 10-Methylacridium-9-carbonyl chloride was synthesised from refluxing 10-methylacridinium-9-carboxylic acid in thionyl chloride. On account of the fact that 10-methylacridinium-9-carboxylate was not commercially available, the molecule was synthesised from the base hydrolysis of a 10-methylacridinium-9-carboxylic acid methyl ester salt. The methyl ester was in turn synthesised by *N*-methylation of

methyl acridine-9-carboxylate. Finally, the acridine methyl ester was synthesised from acridine-9-carbonyl chloride (see figure 4).

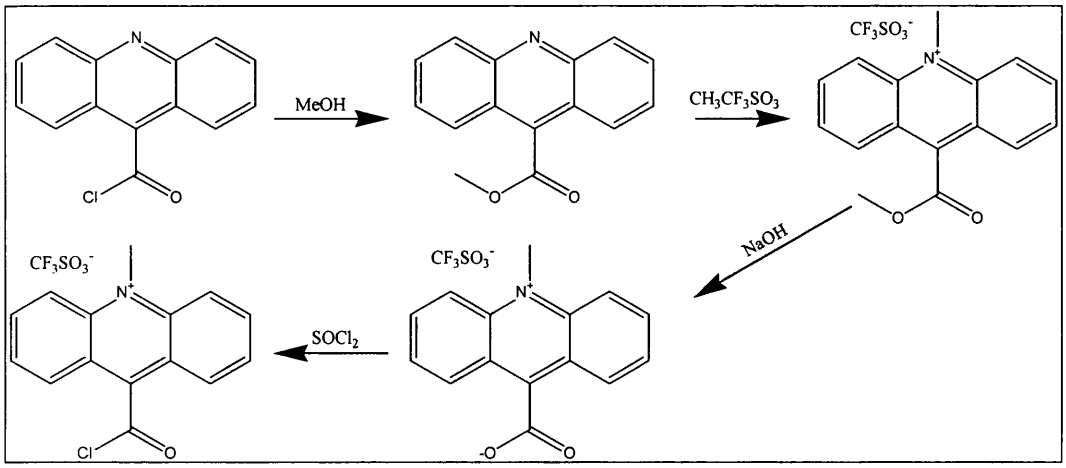



**Figure 4 Synthesis of 10-methylacridinium-9-carbonyl chloride trifluoromethanesulfonate**

The main disadvantages of using 10-methylacridinium-9-carbonyl chloride trifluoro-methanesulfonate were as follows.

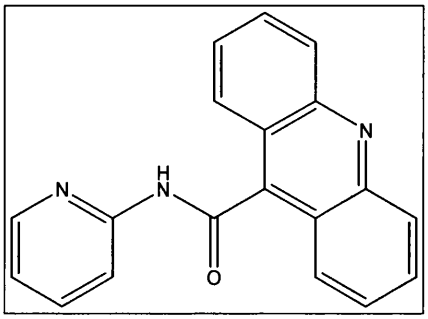
- 10-methylacridinium-9-carbonyl chloride trifluoromethanesulfonate is extremely prone to base hydrolysis and thus might lead to difficulties in conducting the reaction.[6]

- 9-Chlorocarbonyl-10-methylacridinium chloride is a salt and the product formed would also be a salt. Furthermore, any base catalyst used would convert into a hydrochloride salt during the reaction and hence this combined salt mixture might cause problems in purification of the desired product.

It was thus decided to initiate the experimentation with the first synthetic method discussed as this seemed to be the more straightforward of the two proposed. It was then decided that if this route failed then the second synthetic route discussed would be attempted.

## 4.2 Results and discussion

### 4.2.1 Acridine-9-carboxylic acid pyridin-2-ylamide (4.1): synthesis and *N*-methylation.




**Acridine-9-carboxylic acid pyridin-2-ylamide (4.1)**

#### *4.2.1.1 Synthesis of compound (4.1)*

Synthesis of this novel amide proved to be straightforward, the method used derived from the method used in-house for synthesis of the acridine esters, [5] however there were some changes to both the synthesis and the work-up. One of the major changes in the method was to use triethylamine in DCM instead of pyridine (acting as the base catalyst and the solvent) which had been previously used for the ester syntheses. The reason why this was chosen was purely a practical advantage; triethylamine has a lower boiling point (88 °C) than pyridine (114 °C) and thus is easier to remove. Moreover, pyridine tended to prevent visualisation of product spots on TLC plates using a UV lamp (254 nm) as the solvent itself absorbed strongly in this UV region.

The general method adopted for the synthesis was to react 2-aminopyridine with acridine-9-carbonyl chloride in equimolar quantities using the above-mentioned triethylamine as a base catalyst and anhydrous DCM as the solvent. The order of addition was important to prevent double addition of the acid chloride forming a tertiary amide. To help prevent tertiary amide formation, the acid chloride was dissolved in anhydrous DCM and added slowly (over 5 min through a dry syringe) into a stirring mixture of the amine, catalyst and solvent. It was hoped that this slow addition would keep the amine in greater excess throughout the course of the reaction and thus sequester the acid chloride before the resulting product could further react.

Upon addition of acridine-9-carbonyl chloride (a yellow milky solution) to the 2-aminopyridine (a clear solution), the resulting mixture darkened and turned black /

brown on completion. Over the course of the reaction, the mixture then precipitated a fine solid, which was yellow in pigmentation. It was assumed that this was either the desired product or triethylamonium hydrochloride salt precipitating out of solution, which would mirror such observations obtained from the synthesis of the acridine esters, where pyridinium hydrochloride precipitates out of solution. A second and less favourable possibility was that during the course of the reaction, water had contaminated the reaction mix. However this was thought to be unlikely as the reaction was carried out in anhydrous conditions.

The reaction work-up was straightforward; the product was filtered and the precipitate washed with diethyl ether, which successfully washed away the dark impurity from the fine yellow crystalline product (558 mg). The dark brown mother liquor was then evaporated to form a black solid (162 mg). Both products were then sent for proton NMR spectroscopic analyses. The results of the mother liquor showed it to be mainly triethylammonium hydrochloride salt with a small aromatic impurity, which appeared to consist of a mixture of products. No further purification was conducted on the mother liquor. The proton NMR of the precipitate indicated that the desired product had formed without a visible impurity; thus the product was then subjected to further analyses such as mass spectrometry and IR spectroscopy (see section 4.3.2.2 for results of the characterisation).

The proton NMR peaks, although not well resolved, integrated to 12 aromatic protons and one low field broad singlet, which integrated to one proton and thus was identified as the NH proton. The double doublet at 6.96 ppm was identified as one of pairs of the acridine protons either at positions 4/5 or 1/8 as it integrated to 2 protons. The carbon NMR also showed 12 peaks. The DEPT spectra, in comparison with the full carbon spectrum, showed an absence of four peaks (the peak at 138.7 ppm was twice the intensity of the other ipso carbons) in the aromatic region, which were identified as the five quaternary carbon peaks. It was surmised that as well as the peak at 138.7 there must have been another peak with overlapping signals for one of the 2 identical pairs of quaternary carbons in the acridine ring.. Since the DEPT spectrum clearly showed four peaks that were double the intensity to the others, it was surmised that these were the acridine ring CH carbons. The mass spectroscopic data also complemented the NMR results; the peak at m/z 300 was identified as the $M+H^+$ ion

from the results of CI. The peak at m/z 206 in the EI spectrum was identified as a loss of the pyridine ring with NH attached and the peak at m/z 178 was the acridine ring with a loss of the amide group (see figure 5).

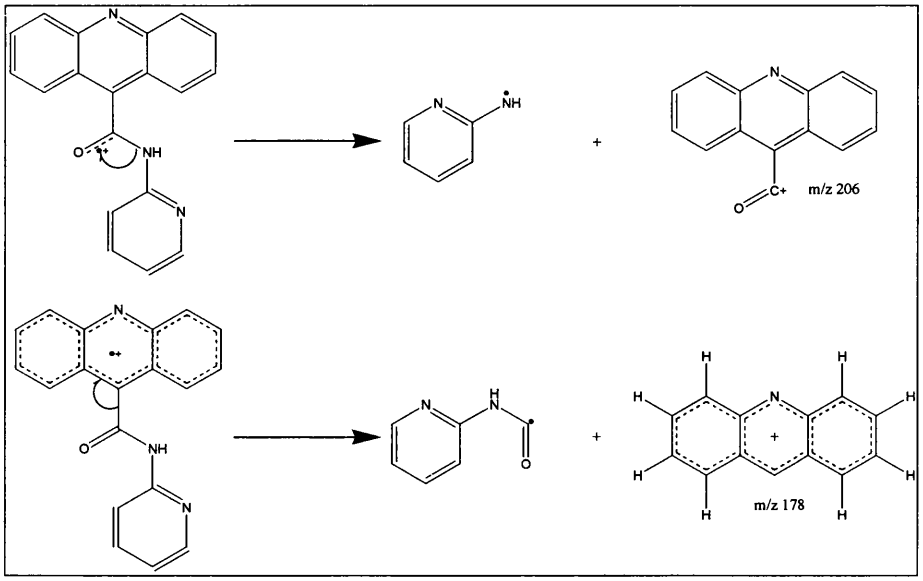



**Figure 5 Probable products formed from the electron impact ionisation mass spectroscopy of (4.1)**

On account of the fact that the product precipitated out of the reaction mixture as a pure compound, no further purification steps were deemed necessary and the product was thus used for the following methylation reaction. The yield of the reaction was 85 %.

### *4.2.1.2 Methylation of (4.1) to form compound (4.2)*

Methylation of (4.1) was conducted by adopting the methylating procedure used in-house.[5] As recorded in section 4.1, one limitation of this pathway was the fact that no control measures were employed for the addition of the methyl group. Therefore, it was possible for the methyl group to add to either heterocyclic nitrogen or both. However, it was decided to try the reaction to see what product would result from the standard methylating procedure.

Compound (4.1) was dissolved in a large volume of anhydrous DCM, which was required because of the fact that the amide was found to have low solubility in most solvents, 1 ml of DCM, was found to be just sufficient to almost fully solvate the amide (8 mg). Moreover, it was hoped that the methylating agent would act to further dissolve the mixture. This was based on the assumption that mixtures tend to have

better solubilising properties than single solvent systems. On account of the fact that the concentration of the amide solution was so low, there was some concern that this might adversely affect the reaction. For example, if there was a small contamination by water, then this would have had a more potent effect on solutions where the amine concentration was lower, as both the amine and the water would compete for the methylating agent. Therefore, to maximise yield and scavenge any water contamination, an excess of the methylating agent was added to (4.1). Upon addition of methane trifluoromethanesulfonate, the colour of the solution almost instantaneously changed from a milky yellow to a clear orange. Moreover, upon examining the solution by TLC, the starting material spot (Rf 0.6) was replaced by one at the baseline, indicating formation of the salt. At the end of the reaction the solvent was evaporated off to leave an orange solid. This was then crystallised from acetone / diethyl ether yielding red / orange crystals after cooling in the refrigerator. The product was then characterised (see section 4.3.3.2).

The results of the characterisation showed that indeed methylation had occurred, because a peak corresponding to the methyl peak was visible both in the proton (3.04 ppm) and the carbon (28.78 ppm) spectra. Furthermore in ESI mode, the mass spectrometric data showed an M – $CF_3SO_3^-$ ion (m/z 314) in positive ion mode and the negative ion mode showed the molecular ion for the anion (m/z 149). However the position of the methyl group still remained unclear at this stage.

As indicated in section 4.1, the most likely position for methylation was on the pyridine nitrogen and indeed this seemed to be the case according to the relative positions of the peaks in the NMR spectroscopy. The proton NMR data gave two pieces of evidence that indicated the positioning of the methyl group. The first of these was that the position of the methyl peak (3.04 ppm) was too far upfield for it to be an acridinium methyl, which usually shifts towards 5 ppm (see section 4.3.16.2 for an example of an acridinium methyl chemical shift value). The reason why acridinium *N*-methyls shift so much further downfield when compared to the corresponding pyridinium methyls is due to the fact that the flanking benzene rings of the acridine ring afford a significant electron-withdrawing effect on the heterocyclic nitrogen. The second piece of evidence, which complemented the above data, was the relative shifts of the aromatic peaks when compared with those of (4.1). The proton

shifts in the aromatic region in (4.1) included four multiplets at 6.78, 6.96 and 7.22 and 7.31 ppm respectively. The aromatic multiplets for (4.2) were better resolved than those of the starting material. On account of this, it was possible to assign which peaks were those of acridine and which were of pyridine. The peaks at 6.92, 7.14, 7.28 and 7.43 ppm were all acridine peaks as they all integrated for two protons each. When comparing the shifts of these to the shifts of the four low field aromatic multiplets for (4.1) (which contained both acridine and pyridine peaks), it can be seen that these shifted between 0.1 to 0.2 ppm towards lower field. However, the shifts for the pyridine ring protons (7.43, 7.74, 8.38, 8.43 ppm) were shifted to a much greater degree, indeed about 1 ppm when compared to the same 4 multiplets. This degree of chemical shift would have been expected for adjacent ring protons if a highly electron-withdrawing group were added or as in this example, where the heterocyclic nitrogen had become charged. Furthermore, like the pyridine peaks, the NH proton peak also shifted significantly further (a shift of about 0.7 ppm) than the acridine peaks. This indicated that the NH group was adjacent to a group affected by addition of an electron withdrawing adduct. Figure 6 shows the proton spectra for both unmethylated and methylated species.

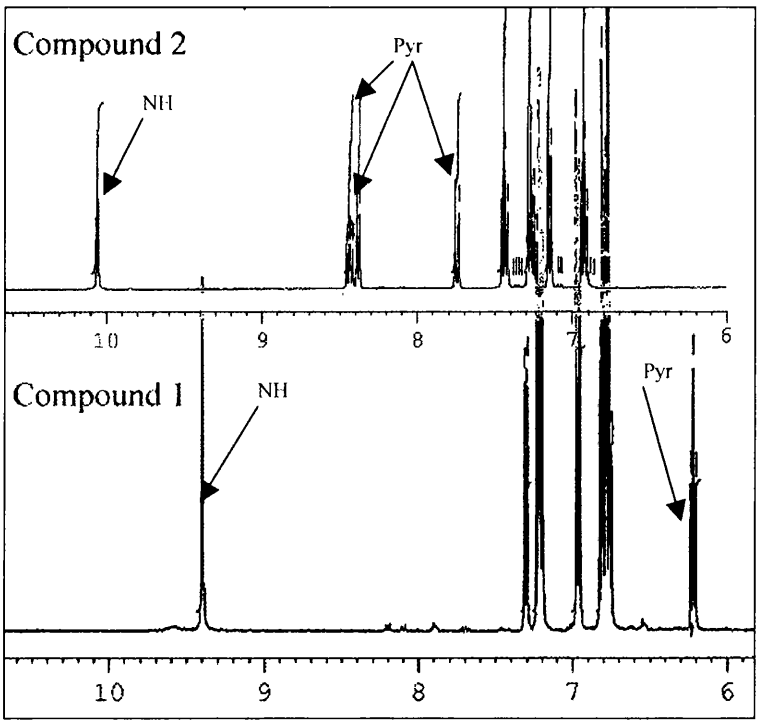
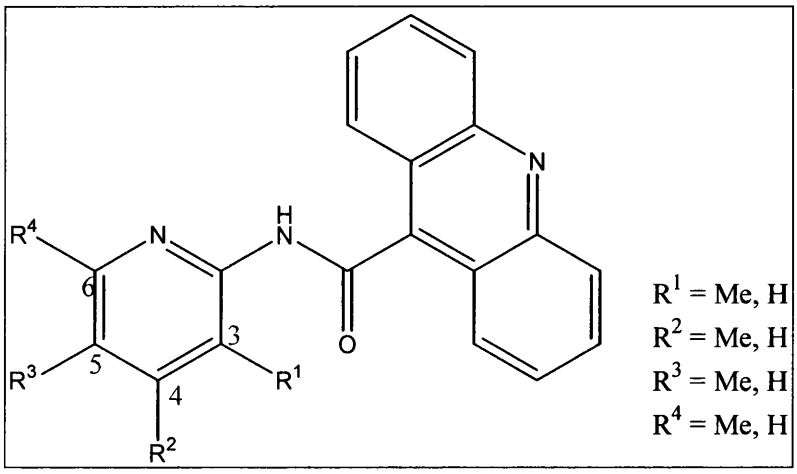



**Figure 6 Proton NMR (400MHz) data showing a comparison between the differences in chemical shifts of the unmethylated species (compound1) and the methylated species (4.2).**

The product yield from this reaction was 76 %.

The molecule was then tested for chemiluminescence however it was found not to emit light.

4.2.2 Synthesis of the series: 'acridine-9-carboxylic acid (#-methyl-pyridin-2-yl)-amide' where # represents positions 3, 4, 5 and 6 on the pyridine ring.




*4.2.2.1 Synthesis of the amides*

The synthesis of the four picolinyl-amides was successful; the procedure used for the synthesis of (4.1) was adopted for the synthesis of these amides. The only change to the method was the choice of amine. The yields for the four methyl pyridinylamides are shown in table 1.

**Table 1Yields obtained in the syntheses of acridine-9-carboxylic acid (#-methyl-pyridin-2-yl) amide**

| Compound N° | Position of $CH_3$ on pyridine ring (#) | Yield of amide isolated (%) | Reaction time (h) |
|---|---|---|---|
| (4.3) | 3 | 73 | 6 |
| (4.5) | 4 | 67 | 6 |
| (4.7) | 5 | 60 | 6 |
| (4.9) | 6 | 100 | 15 |

As it can be seen, good yields were obtained for all four methyl derivatives formed. The reason why the 6-position methyl product appeared to take longer to form was because unlike the other three products during the reaction nothing precipitated out. Furthermore, when monitoring the reaction by TLC (normal phase silica gel, eluted

using a DCM / EtOAc mix (50:50) a new product spot did not appear and moreover, the acid chloride spot (Rf 0.8) did not disappear during the first 6 h. It was first assumed that this was because this reaction for some unknown reason had slower reaction kinetics. Thus on account of this, the reaction was allowed to react overnight. However, when the reaction mixture was re-examined by TLC, nothing had changed; if nothing else, decomposition from carbonyl chloride to acid would have been a possible outcome. It was however soon realised that the desired product had formed but had a similar Rf value to the acid chloride and thus was co-eluting on the TLC plate. The product was precipitated out of solution by addition of diethyl ether. It was then filtered and twice washed with more diethyl ether. The fine yellow crystalline product was then analysed and found to be the desired product. The analyses of all four methyl pyridinylamides are discussed below (the results of the characterisation can be found in section 4.3.4 through to 4.3.10).

For each of the 4 products synthesised, the proton NMR result showed a high field singlet (2.11-2.4 ppm), integrating for three protons, assigned as the methyl groups on the picoline ring. As shown for (4.1), the aromatic proton peaks for compounds (4.3) and (4.7) were again not well resolved. Nevertheless, for both products, the aromatic signals did integrate for the 11 ring protons in each case. Furthermore for all four products, the broard singlets found between 9.31 and 11.55 ppm were identified as the NH peaks of the amide bond. However, the aromatic $^{1}$HNMR spectra for compounds (4.5) and (4.9) were well resolved, thus the possible peak assignments of one of these compounds, (4.9) will be now discussed.

The proton NMR showed three peaks integrating to 1 proton each thus these were confirmed as the pyridine protons. The pyridine peaks at 8.22, 7.85 and 7.13 ppm were assigned as H3, H4 and H5 respectively, on the pyridine ring. The 8.22 ppm peak was the furthest peak downfield due to the electron-withdrawing effect that the amide group was exerting ortho to this. The 7.85 ppm peak was a triplet in nature due to the almost identical coupling constants of its two neighbouring ring protons. The 7.13 ppm doublet was shifted upfield due to the electropositive inductive effect of the ortho methyl group. The remaining 4 aromatic peaks were from the acridine ring protons and they integrated to two protons each. The double, double, doublets were assigned as either the positions 2/7 or 3/6. The doublets were assigned to positions

1/8 or 4/5. A $^{1}$HNMR prediction (using 'Chemdraw Ultra') was conducted to try to further assign the positions of the peaks. Even though the actual numbers were not identical they did give an acceptable trend. Table 2 shows the predicted data for the acridine ring verses the actual data.

**Table 2 -Real vs. predicted $^{1}$H chemical shift data for the acridine ring on compound (4.9)**

| Position on acridine ring | Actual Chemical Shift (ppm) | Predicted Chemical Shift (ppm) |
|---|---|---|
| 1/8 | 8.22 | 8.44 |
| 2/7 | 7.85 | 7.54 |
| 3/6 | 7.97 | 7.68 |
| 4/5 | 8.00 | 8.08 |

All four products gave carbon and DEPT NMR spectra that substantiated the proton results. All four showed a high field peak at between 15.73 and 23.69 ppm and so were identified as the ring methyls. The amides with the methyl groups at positions 3 and 6 showed all thirteen possible peaks, seven of which were CH in nature. Four of these were double the intensity of the other three and were identified as the acridine CHs. The other six signals disappeared in DEPT and thus were identified as the quaternary carbons. Two of these signals represented the two pairs of identical carbons at positions 1a/8a and 4a/5a on the acridine ring. It appeared that two quaternary carbons shared the same shifts, however on account of the fact that quaternary carbons tend to be lower intensity than those with adjacent protons, it was difficult to assign the offending carbon.

All four amides gave molecular ions from the mass spectrometric analyses, which confirmed the NMR findings.

The methylpyridinylamides were then used for methylation.

### *4.2.2.2 Methylation of the methylpyridinylamides*

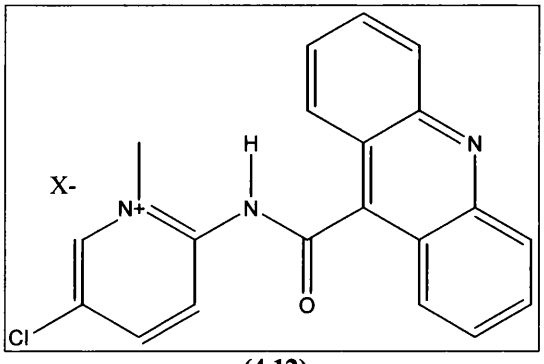
The methylation procedure mirrored that discussed in section 4.2.1.2, the only changes being choice of starting material and amounts used. Table 3 summarises the yields and colours of the products obtained.

**Table 3 The product characteristics and yields from the *N*-methylation of the methyl pyridinylamides**

| Compound N° | Position of ring methyl | Yield Of Crystals Obtained (%) | Product Characteristics |
|---|---|---|---|
| (4.4) | 3' | 71 | Orange Crystals |
| (4.6) | 4' | 65 | Red Crystals |
| (4.8) | 5' | 46 | Orange Crystals |
| (4.10) | 6' | 66 | Red Plates |

Table 3 showed that mixed yields were obtained from the methylation reactions, due to mixed success in obtaining crystals from the mother liquor. The most surprising result was that methylation in every reaction was found to be on the pyridine rather than the acridine nitrogen. The evidence to prove this was that the position in the proton NMR spectrum of the heterocyclic nitrogen methyl, in all four products synthesised, was found to be in the region of ~ 3 - 3.5 ppm. As indicated in section 4.2.1.2, due to the electron-withdrawing properties of the 2 benzene ring components of the acridine ring, the normal position of *N*-methyl is usually at ~ 5 ppm. Moreover, as found for (4.2), in all four cases, the pyridine ring protons shifted downfield by a factor of ~ 1 ppm in comparison to the pre-methylated starting materials. In contrast, the acridine ring protons showed a much reduced downfield shift of ~ 0.2 ppm. The carbon NMR and mass spectroscopic data further complemented the proton NMR results for identification of the four *N*-methylated products formed.

### 4.2.3 Synthesis of 2-[(acridine-9-carbonyl)amino]-5-chloro-1-methyl-pyridineium trifluoro-methanesulfonate (4.12)




**(4.12)**

#### *4.2.3.1 Synthesis of the amide (4.11)*

Compound (4.11) was successfully synthesised using the general method described for (4.1), but there were some changes to the method. The first was the choice of amine, 2-amino-5-chloropyridine instead of the unsubstituted aminopyridine. The second change was the reaction time, to compensate for the presence of the electronegative chlorine, the reaction was allowed to mix over 48 h under anhydrous conditions. Like (4.1), over the course of the reaction, the new product precipitated and thus was worked up in the same way as described in section 4.2.1.1, giving a yield of 71%. Proton NMR analysis identified the product to be pure. The NMR analysis showed 11 aromatic protons and a broad singlet, characteristic of the NH proton. The mass spectroscopic analysis gave $M+H^+$ ions (334 & 336) for the desired amide and furthermore the observed peak intensity ratio of 3:1 was indicative of a chlorine isotopic distribution.

#### *4.2.3.2 Methylation of compound (4.11) to form compound (4.12)*

The methylation procedure mirrored that discussed in section 4.2.2.1. However, two different crystals formed from the solution: red plates and small yellow crystals. It was assumed that these were either two different products, or the same product in two different crystalline forms. Analysis of the products showed that the yellow crystals were a mixture of two compounds the first was assumed to be acridinium-9-carboxylic acid and the second the desired product. The red plates were shown to be the pyridinyl methylated product (4.12) with a tiny impurity of acridinium-9-carboxylic acid.

##### 4.2.3.2.1 The Red Plates

The NMR result gave characteristic peaks for the mono-substituted acridinium and di-substituted pyridinium moieties. At a low cone voltage (8 V), the mass spectrometric peaks showed M - $CF_3SO_3$ peaks (m/z 348 and 350), which displayed characteristic ion ratios for a mono chlorinated product. When the cone voltage was increased (50 V) the peaks 28 mass units less than the M - $CF_3SO_3$ appeared (m/z 320 & 322), It was concluded to be a loss of carbon monoxide. The CO loss was due to a rearrangement in the high energy ionisation conditions. A plausible mechanism for this is shown in figure 7.

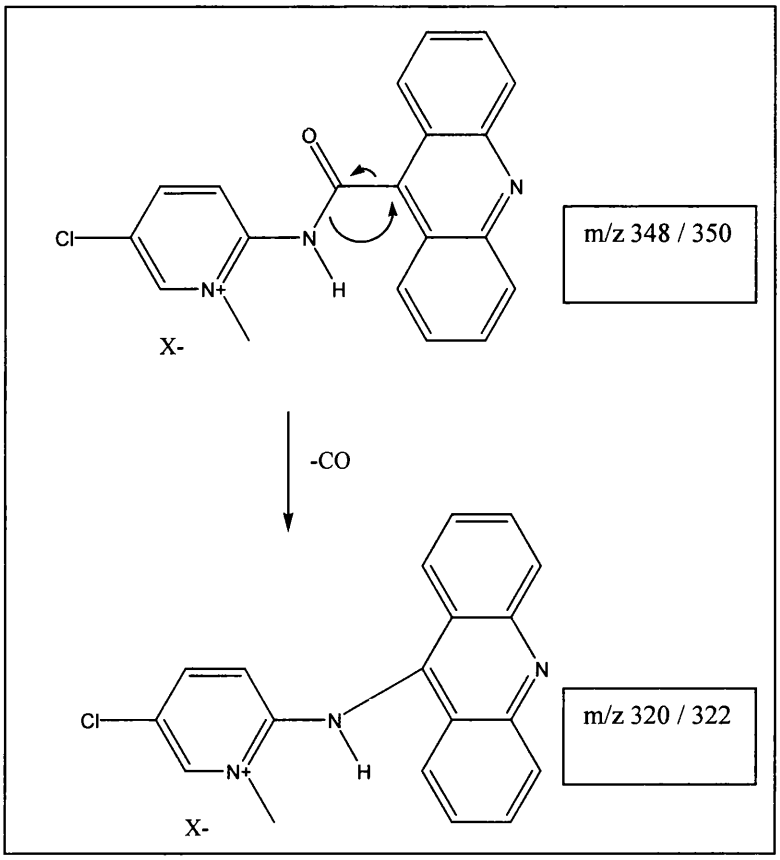



**Figure 7 Possible mechanism for the extrusion of CO from the electrospray ionisation.**

The results showed that again the pyridine nitrogen was preferentially methylated over the acridine nitrogen, even when an electron-withdrawing group was present on the pyridine. Furthermore, the results showed that the presence of the chloro group increased the overall electron-withdrawing property of the aminopyridinium group. This then weakened the NH-CO bond leading to an increased vulnerability to hydrolysis and hence explained why the yellow crystals (acridine-9-carboxylate) had co-crystallised with the red plates (4.12). On account of the fact that the methylation step was conducted in anhydrous conditions, it was assumed that acridine-9-carboxylate was formed during the crystallisation process by hydrolysis of the product (4.12) with water present in the crystallisation solvents. This was further supported by the fact that when a re-crystallisation attempt was conducted (using an acetone / ether crystallisation solvent mix), the product completely hydrolysed to form acridine-9-carboxylic acid.

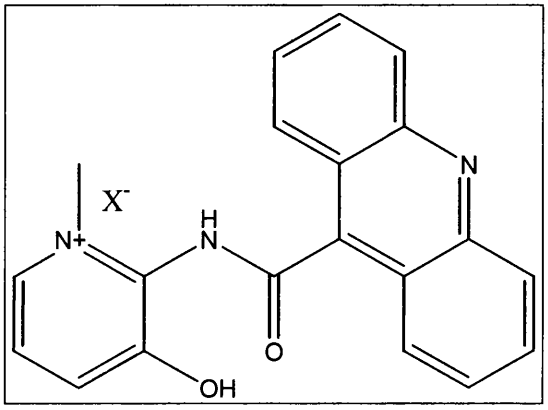
The observed vulnerability to hydrolysis of (4.12) raised two interesting points.

- The observed hydrolysis products confirmed that the methylation had indeed occurred on pyridine nitrogen and not the acridine nitrogen.

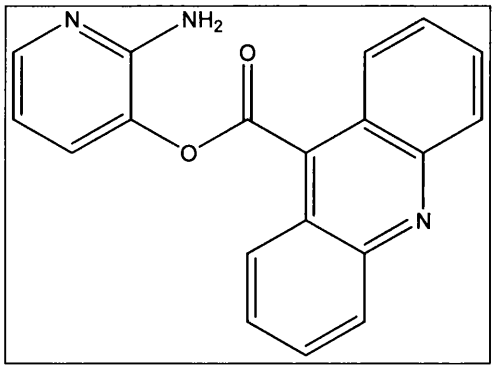
- ❖ The product should also be equally prone to hydroperoxidation thus was an interesting candidate for chemiluminescence testing.

The product was then tested for its chemiluminescence however again failed to produce light.

### 4.2.4 Attempted Synthesis of 2-[Acridine-9-carbonylamino]-3-hydroxy-1-methyl-pyridinium trifluoromethanesulfonate (4.13a)




**Desired product, 2-[acridine-9-carbonylamino]-3-hydroxy-1-methyl-pyridinium trifluoromethanesulfonate (4.13a)**




**Actual product, acridine-9-carboxylic acid 2-aminopyridin-3-yl ester (4.13b)**

#### *4.2.4.1 Attempted synthesis of the amide*

Synthesis of the above amide was found to be unsuccessful using standard method (see section 4.2.2.1), instead of yielding an amide (4.13a), this synthetic procedure formed an ester (4.13b) in a good yield (93%). Although the reaction was allowed the full time to react (4.5 h), in fact the product precipitated out as soon as the acid

chloride was added to the stirring solution of the 2-amino-3-pyridinol. The product was characterised as shown below.

As discussed in section 4.2.2.1, the $^1H$ NMR results showed the product peak with a small impurity thought to be the starting material (~10 %). The major peaks showed the presence of 11 aromatic protons. Unfortunately, it was difficult to assign these peaks because 2 out of the 4 aromatic peaks were multiplets. The peak at 6.10 ppm was assigned as the $NH_2$ peak as it was a broad singlet and integrated to two protons. Furthermore, the characteristic singlet at ~ 9-10 ppm, which had been previously characterised as the NH peak, was absent. The mass spectrometric analyses showed a weak $M+H^+$ ion (m/z 316).

It was at first unclear as to why the ester had formed in preference to the amide and why a mixture of both had not formed in the reaction. On account of the fact that hydroxy groups are harder nucleophiles than amino groups, the opposite was expected. However it was assumed that the hydroxy proton was hydrogen bonding to the amine, thus internally deactivating the amine group but in turn activating the oxygen for nucleophilic attack. Figure 8 shows a possible mechanism for this.

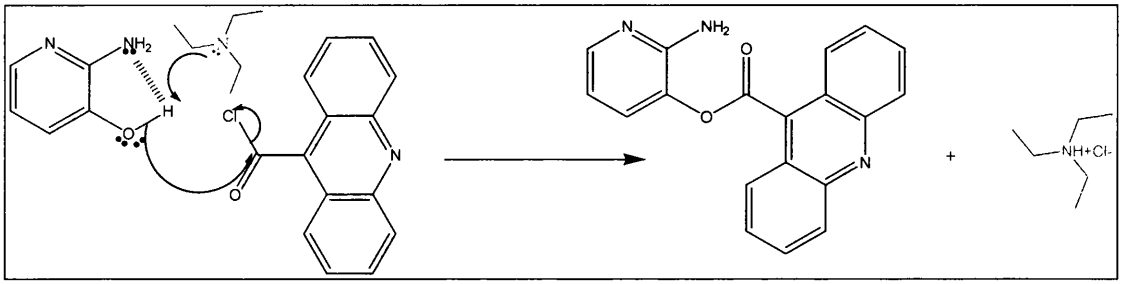


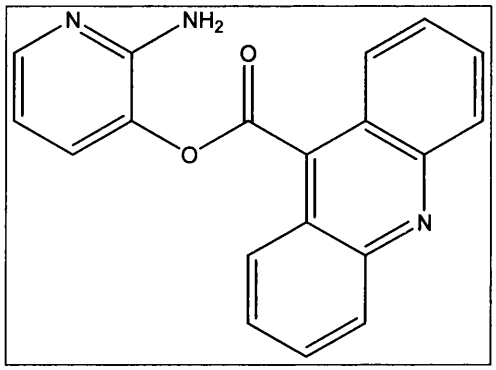

**Figure 8 A possible mechanism for ester synthesis by internal base supression of the amine group.**

Further attempts to synthesise the amide were made by conducting the reaction at 0 °C but this only resulted in slowing the inevitable esterification.

On account of the fact that the above ester was novel and that any acridinium ester synthesised was of interest as a candidate for use as a chemiluminescent probe molecule, it was decided to attempt to methylate this acridine ester to try to form the acridinium derivative. In the case of the above ester, presence of the amine group

could be used as site for building in desired functionalities such as linker and spacer groups.

### *4.2.4.2 Methylation of Acridine-9-carboxylic acid 2-amino-pyridin-3-yl ester (4.13b) forming (4.14)*




**Acridine-9-carboxylic acid 2-amino-pyridin-3-yl ester (4.13)**

Methylation of (4.13) was again a success, but the position of the methyl group was thought to be again on the pyridine ring. The evidence for this was the relative positions of the ring proton peaks in both the carbon and proton NMR spectra (see tables 4 and 5).

**Table 4 - $^1$H NMR (400 MHz) chemical shifts of the aromatic protons for the unmethylated species (4.13) and methylated species (4.14)**

| Compound (4.13) (ppm) | Compound (4.14) (ppm) |
|---|---|
| 6.76 (pyridine) | 7.17 (pyridine) |
| 7.79 (acridine) | 7.83 (acridine) |
| 7.97 (pyridine and acridine) | 8.03 (acridine) |
| 8.30 (pyridine and acridine) | 8.22 (pyridine) |
| | 8.35 (acridine) |
| | 8.66 (pyridine) |

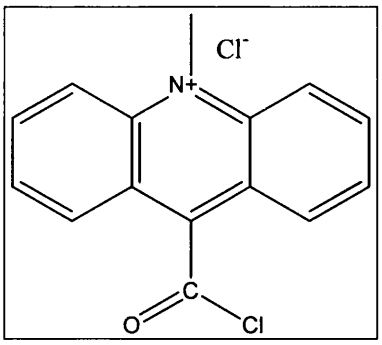
**Table 5 - $^{13}C$ NMR (100 MHz) chemical shifts of the acridine ring carbons of the unmethylated species (4.13) and methylated species (4.14)**

| Unmethylated species (4.13) (ppm) | Methylated species (4.14)(ppm) |
|---|---|
| 125.4 | 125.4 |
| 127.9 | 128.2 |
| 129.6 | 128.7 |
| 130.9 | 131.5 |

Table 4 shows that after methylation, the pyridine peaks shifted significantly further downfield than the acridine peaks. To corroborate this evidence, the CH carbon peaks of the acridine ring were compared (Table 5) and showed very little change on methylation. This product was then tested for its chemiluminescence however again no significant chemiluminescence.

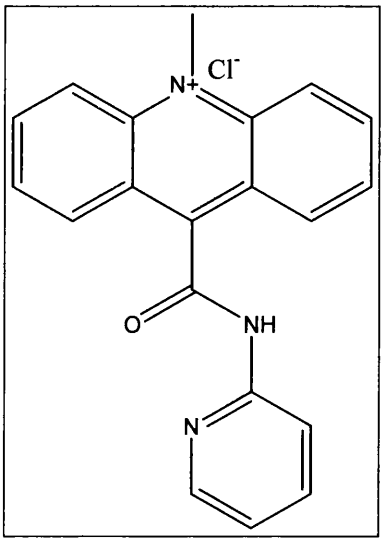
### 4.2.5 Attempted synthesis of a range of acridinium homologues of the pyridinium amides: (4.2), (4.4), (4.6), (4.8), (4.10), (4.12).

The experiments up to this point had concentrated on amide pre-synthesis and then methylation. For each amide synthesised, the methylating agent selectively methylated the pyridine nitrogen rather than the acridine heteroatom. Although these products were interesting candidates for chemiluminescence experimentation, it was also decided to synthesise a range of acridinium homologues of the fore-synthesised pyridinium amides and then compare their resulting chemiluminescences. However, on account of the aforementioned selectivity of methylation it was decided first to synthesise 9-chlorocarbonyl-10-methylacridinium chloride (4.17) and then to use this to hopefully react with the amine precursors, which were used to make the pyridinium range. The first of these to be attempted was unsubstituted analogue - 10-Methyl-9-(pyridin-2-ylaminocarbonyl)-acridinium chloride (4.18)



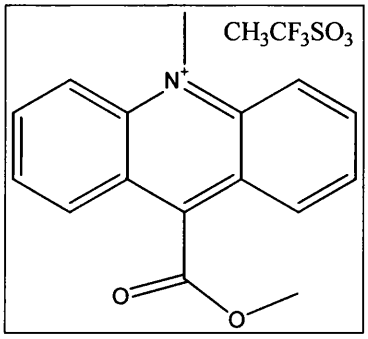

**9-Chlorocarbonyl-10-methylacridinium Chloride (4.17)**

### 4.2.6 Attempted synthesis of 10-methyl-9-(pyridin-2-ylaminocarbonyl)-acridinium chloride (4.18)




**10-Methyl-9-(pyridin-2-ylaminocarbonyl)-acridinium chloride (4.18)**

The first two steps of the synthetic process, methylation of acridine-9-carbonyl chloride to form the methyl ester and then *N*-methylation to form the acridinium salt were conducted as a one pot synthesis giving a 92 % yield of 9-methoxycarbonyl-10-methylacridinium trifluoromethanesulfonate (4.15).

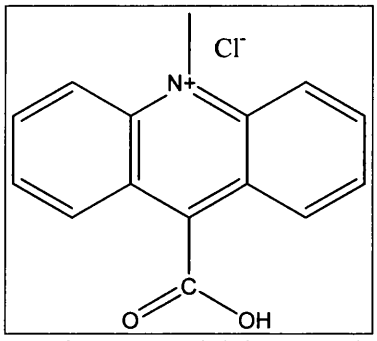



**9-Methoxycarbonyl-10-methylacridinium trifluoromethanesulfonate (4.15)**

The only major difference to the method used previously in the literature was that the methylating agent used in this synthesis was methyl trifluoromethanesulfonate rather than dimethyl sulfate.[6] The reason why methyl trifluoromethanesulfonate was used was that it was readily available in the lab and had been used extensively for many similar methylation reactions. The product was purified from crystallisation from acetone and diethyl ether yielding yellow crystals. This was characterised by both proton and carbon NMR spectroscopy.

The proton NMR spectroscopic results showed two peaks each integrating for three protons upfield in the spectrum. These indicated the presence of the two methyl groups, with one methyl at 4.32 ppm corresponding to the methyl ester, and a second at 4.91 ppm, the acridinium methyl. The remaining four peaks (found downfield) corresponded to the ring protons. The carbon NMR data complemented the proton results. The peaks which were observed upfield and which did not disappear after a DEPT analysis were assigned to the two methyl groups. The peak further upfield of the two was again assigned to the methyl ester. The remaining eight carbon peaks (found downfield) included four peaks that were double the intensity of the rest, which did not disappear in the DEPT spectrum, and hence were assigned as the acridine CH ring carbons. The remaining carbons did disappear in the DEPT spectrum, three of these were assigned as acridine ring carbons and the furthest downfield, the carbonyl (at 165.1 ppm).

The methyl ester (4.15) was then hydrolysed in the presence of hot (100 °C) sodium hydroxide solution to form the carboxylate, 9-carboxy-10-methylacridinium chloride (4.16).




**9-Carboxy-10-methylacridinium chloride (4.16)**

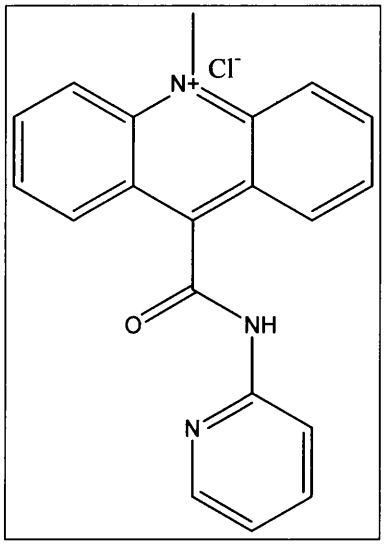
At the beginning of the hydrolysis, the yellow crystals very quickly discoloured, finally turning into a black tar-coated, hard solid. This solid was broken up with a spatula to speed the hydrolysis. Eventually over time, the solid dissolved forming a dark green solution. The work-up was straightforward; the solution was heated whilst hot and then was acidified with HCl. After cooling, the green carboxylic acid precipitated out. This product was the analysed by $^{1}$HNMR spectroscopy.

The proton NMR results showed that a methyl peak had disappeared, leaving only the acridinium heteroatom-bound methyl. The ring protons were all present at almost identical shifts to those of the starting material (see Table 6). Unfortunately, on account of the insolubility of the molecule in deuterated solvents, carbon NMR was not possible.

**Table 6 – A comparison of the aromatic proton chemical shifts of compounds (4.15 and 4.16)**

| Chemical Shifts For (4.15) | Chemical shifts for Compound (4.16) |
|---|---|
| 8.09t | 8.08t |
| 8.45d | 8.39d |
| 8.50t | 8.47t |
| 8.89d | 8.86d |

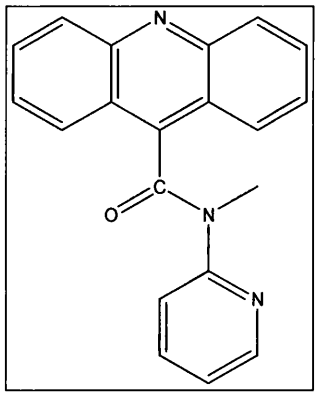
Work was then conducted to generate the acid chloride (4.17) and then react this *in situ* with 2-amino pyridine to form hopefully form the amide (4.18).




**10-Methyl-9-(pyridin-2-ylaminocarbonyl)-acridinium chloride (4.18)**

Compound (4.16) was refluxed in thionyl chloride for 6h to form the acid chloride (4.17). The solution was poured into hexane causing precipitation and the precipitate was collected and dried under vacuum.

An excess of 2-aminopyridine was then dissolved in anhydrous DCM and the acid chloride was also dissolved in DCM. Under an atmosphere of dry argon, the acid chloride solution was added to the amine solution, forming a brown solution. The solution was then allowed to stir overnight under argon, from which the solution formed a small precipitate. The precipitate was filtered from the solution and collected. The remaining solution was then evaporated down to an oil on a rotary evaporator. Then attempts to obtain crystals from the oil were carried out, including trituration with acetone and diethyl ether, and cooling the oil in the refrigerator overnight. However, all methods to encourage crystallisation failed. It was surmised that perhaps the reaction had not gone to completion and that starting materials were still present. It was thus decided to perform a liquid / liquid (DCM / $H_2O$) extraction to separate the salts from organic soluble material. The organic layer yielded a white solid, which was analysed by NMR. The results of the crude product showed the presence of a major product with a small impurity. When comparing the chemical shifts with those of 2-aminopyridine, the impurity was found to have exactly the same shifts indicating that the impurity was 2-aminopyridine. The product was thus dissolved in hot DCM and upon cooling, white crystals grew. The white crystals were sent for analysis and proved to yield rather surprising results. The characterisation seemed to indicate that acridine-9-carboxylic acid *N*-methylpyridin-2-ylamide (4.19) had been formed.




**Acridine-9-carboxylic acid *N*-methylpyridin-2-ylamide (4.19)**

The first reason was due to its physical nature; the product was highly soluble in organic solvents such as DCM, whereas it has been found that acridinium and pyridinium salts are much less soluble. Furthermore, the product was pure white and acridinium compounds tend to be bright yellow in nature. Moreover, the NMR showed that both a 9-substituted acridine ring and a 2-substituted pyridine were present. This is because on the proton spectrum there were eight aromatic peaks, four of which were double the intensity of the other four. These four of double intensity followed the pattern: triplet, doublet, doublet, triplet (ddd) and were assigned to the four protons of the acridine ring. The remaining four protons were again two lots of triplets and doublets and thus were assigned as the pyridine protons. The NMR also displayed a high field singlet integrating to three protons and hence was assigned as a methyl group. However, so far these results would have also been plausible for the desired product (4.18) but for one detail; the NH peak was missing from the spectrum. Furthermore, the mass spectroscopic results showed some very interesting confirmatory evidence. When measured using electron impact ionisation, a molecular ion of m/z 313 was shown. If the product had been an acridinium salt then the molecular ion of the cation would have been m/z 314. The electrospray ionisation (ESI) showed an m/z of 314, which proved to be an $M+H^+$ peak. However, equally this could have also been a peak for the molecular cation of the desired product. However, the ESI showed other ions that disproved this; peaks of m/z 336, 627 and 649, related to M+Na, $2M+H^+$, and 2M+Na, respectively, for the given structure (4.19). These results thus indicated that, during the attempted synthesis of the amide, a methyl transfer reaction had occurred. A plausible mechanism for this is shown in figure 10.

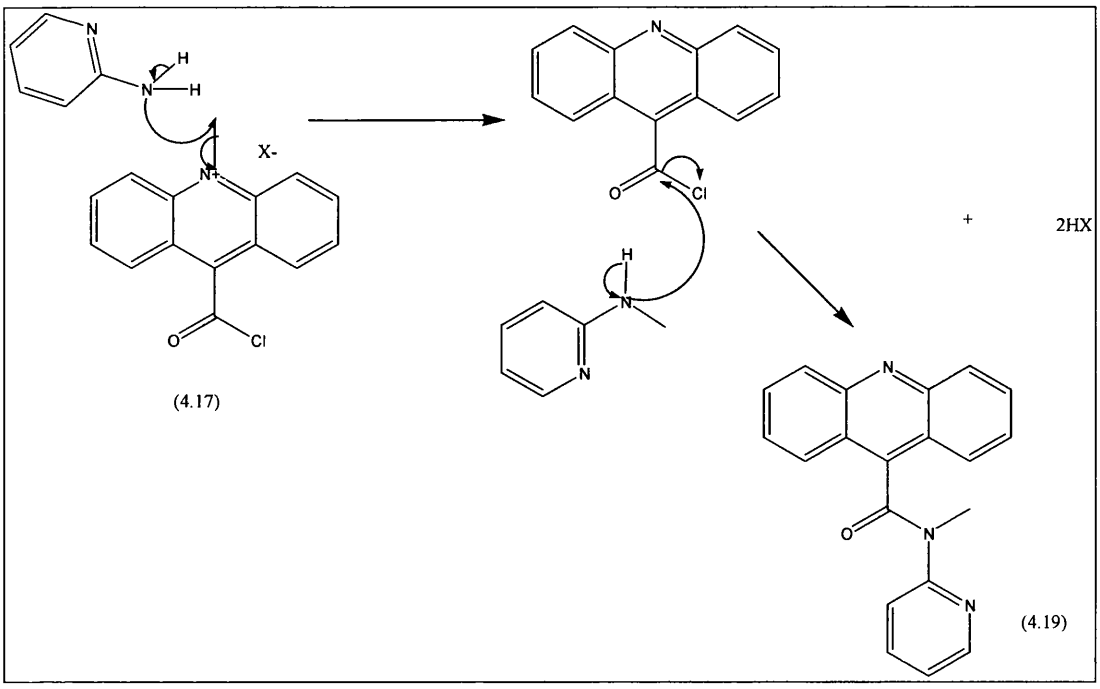



**Figure 10 A possible mechanism for the formation of compound (4.19)**

Figure 10 suggests that instead of attacking the carbonyl carbon, the 2-aminopyridine attacked the *N*-methyl on the acridinium ring, forming a secondary amine. It is plausible that this occurred because the methyl group was attached to an electron deficient nitrogen and this electron deficiency was further depleted by the acid chloride moiety. The enhanced positive charge on the nitrogen, through an inductive force, depleted the electron density on the methyl carbon, rendering it susceptible to nucleophilic attack. The reaction then yielded 9-chloroacridine and *N*-methylpyridin-2-ylamine. As a result of this nucleophilic attack, HCl would be formed and would react with a base to form the hydrochloride salt. The base used in this reaction was triethylamine and thus was expected to form triethylammonium hydrochloride salt. However, this product was not detected in either the organic or aqueous layers (see following paragraphs detailing this). It was thus conceivable that the 2-aminopyridine acted as both a catalyst and a reactant. If the above mechanism were correct then this would partly explain why a second molar equivalent of the 2-aminopyridine did not attack the acridine-9-carbonyl chloride, because, if 2-aminopyridine was both a base catalyst and reactant then this would have depleted the stock of this primary amine. Moreover, addition of the electron-donating methyl group would have activated the amino group increasing its nucleophilic character. Hence the reason why the

secondary amine would preferentially attack the acridine-9-carbonyl chloride over the primary amine. Finally the accurate mass helped to confirm the structure of (4.19).

The aqueous layer was evaporated down to yield another oil; it was decided to analyse this product to see what was present. As mentioned previously, the aqueous layer, like the organic layer, did not contain the expected triethylamonium hydrochloride salt but rather a mixture of two aromatic components. Even though the true nature of these two components is unknown, the NMR results seemed to indicate that two pyridine species substituted at the 2 position were present. Upon first examination of the spectrum, it was unclear as to whether the mystery species were 2-subsitituted pyridines or 9-substituted acridines or a mixture of both. However, the carbon spectrum strongly suggested that both species were substituted pyridines. The reason for this was that the carbon spectrum for both was very similar to that of 2-aminopyridine, where four CHs and one quaternary carbon were visualised for each species. Furthermore, in both cases the quaternary carbon was the furthest downfield. If the species were acridines substituted at the 9-position, then the carbon spectrum for both would have been expected to have been more complicated (see section 4.3.2.2 for an example of the carbon data for a 9-substituted acridine).

The mass spectrometric data proved to be rather more complicated. There appeared to be an ion at m/z 95, which appeared to be $M+H^+$ for 2-aminopyridine. However, the NMR results indicated that the expected amino group was absent. A second idea was that the amino group had hydrolysed to form 2-hydroxypyridine as the molecular weight for this compound would be 94. However, again the NMR results should have shown an OH peak. It is a small possibility that this OH was not visualised due to exchange and that the peak was too 'flat' to see. There appeared also to be peaks at m/z 110 and 127, where the former of these two weights was 16 mass units greater than 94, which thus suggested a second addition of oxygen. However, what is more likely is that methanol from the mass spectrometric solvent, under the energetic conditions of ionisation, replaced the group on the 2-position forming 2-methoxypyridine (m/z 110, $M+H^+$). If this ether was indeed an artefact of the mass spectroscopic results then this could explain the peak at m/z 127. This peak was 18 mass units greater than 109 and if an ammonium acetate buffer had been used in the

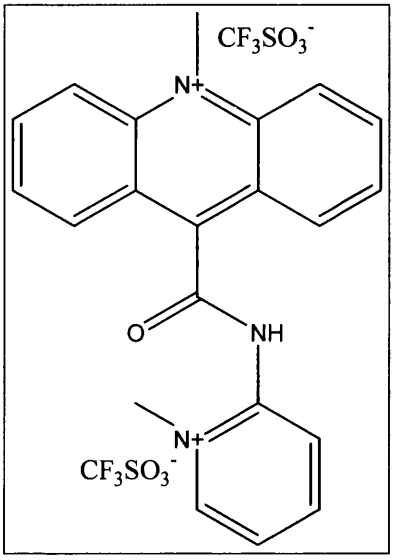
mass spectrometry solvent, then this would be 2-methoxypyridine plus an $NH_4^+$ adduct.

Unfortunately the true nature of the two compounds in the aqueous layer still remains unclear, but the results indicated that both were pyridine compounds substituted at the 2-position. Furthermore, it appears that the base used in the reaction (triethylamine) did not play a part in the reaction and so it can only be surmised that the 2-aminopyridine acted as a base. As this would have formed a water-soluble salt it is conceivable that the pyridine groups, substituted at the 2-positions, were hydrochloride salts from the reaction. It can be thus surmised that 2-aminopyridine self-catalysed this reaction and that the pyridine species in the aqueous layer were by-products of this.

The conclusion to be drawn from this experiment is that 9-chlorocarbonyl-10-methylacridinium chloride is too reactive at the *N*-methyl to allow for attack at the carbonyl carbon. It is conceivable also that if attack had not occurred on the methyl, the 9-position of the acridine ring would also have been a prime position for attack.

### 4.2.7 Attempted double methylation of acridine-9-carboxylic acid pyridin-2-ylamide (4.1).

Previous attempts to methylate (4.1) and its derivatives, discussed earlier in this chapter, led to methylation exclusively on the pyridine rather than the acridine heteroatom. In an effort to overcome this, attempts were made to synthesise 9-chlorocarbonyl-10-methylacridinium chloride and attack this with the desired amine such as 2-aminopyridine, as reported above. However, this resulted in a methyl transfer reaction forming (4.19). It was thus decided to attempt the synthesis of a dimethyl analogue of (4.1); *N*-(1-methylpyridinium-2-yl)-10-methylacridinium-9-carboxamide bis(trifluoromethanesulfonate (4.20).




***N*-(1-methylpyridinium-2-yl)-10-methylacridinium-9-carboxamide bis(trifluoromethanesulfonate (4.20)**

To attempt the synthesis of (4.20), it was decided to use (4.2) as a starting material and use the conditions for the synthesis of 2 (see section 4.3.2.1) to attempt the dimethylation. One of the first problems encountered, was the insolubility of (4.2) in most organic solvents. The first experiments were conducted to vary the amount of time that (4.2) was exposed to the methylating agent in DCM, under anhydrous conditions. This ranged from 4 hours to 5 days. However, in every attempt, only starting material was recovered. As mentioned above, on account of the fact that (4.2) was so insoluble in the methylation mixture, it was concluded that this insolubility was preventing the methylating agent from attacking the compound. After screening a range of solvents, it was discovered that acetonitrile could dissolve (4.2). Using acetonitrile rather than DCM, under anhydrous conditions, the method for the synthesis of (4.2) was again repeated. During this reaction (4.2) dissolved. After mixing at room temperature for 4 hours, the solvents were removed by evaporation. This yielded a brown oil, which was several times greater in mass than what was calculated for the desired product. When analysed by NMR, the proton spectrum showed a complicated spectrum where there were multiple peaks of various intensities in the high field area. The aromatic end of the spectrum looked equally as complicated too. It was surmised that the methylating agent (methyl trifluoromethanesulfonate) had methylated the solvent, forming a mixture of products. It was thus concluded to search for a solvent which would dissolve (4.2) without reacting with the methylating agent. It was found that anhydrous 1,2-dichloroethane

at 83 °C allowed the components to dissolve, however even after a six-hour reaction the mono methylated species exclusively precipitated on re-cooling.

It is thus concluded that the dimethylation reaction was difficult to achieve under the conditions tested. From the experiments conducted, the reaction appeared to be solvent sensitive In the halocarbon solvents tested no reaction was observed acetonitrile reacted with the methylating agent to form an oil. Thus future work could be conducted to continue to search for conditions, such as a suitable solvent system, which would favour dimethylation.

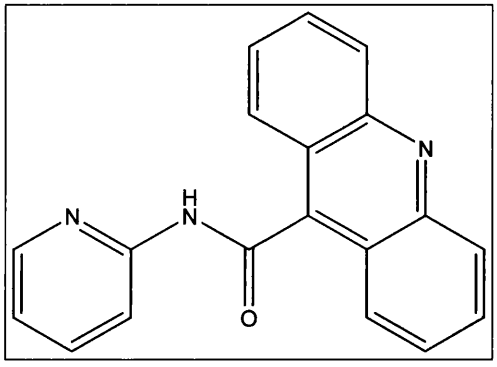
## 4.3 Experimental

### 4.3.1 Acridine-9-carbonyl chloride

#### *4.3.1.1 Method*[6]

Acridine-9-carboxylic acid (4.00 g, 17.9 mmol) was added to a round bottom flask containing freshly distilled thionyl chloride (60 ml). A dry reflux condenser attached to a drying tube was attached to the flask and the mixture was refluxed for 6 h. The solution that formed was then added to hexane slowly to precipitate the acid chloride, which was then filtered and dried under vacuum (3.876 g, 16.1 mmol, 90 %).

### 4.3.2 Acridine-9-carboxylic acid pyridin-2-ylamide (4.1)




**Acridine-9-carboxylic acid pyridin-2-ylamide (4.1)**

#### *4.3.2.1 Method*

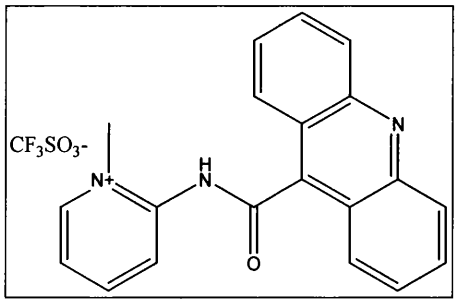
Into a flame-dried 25 ml round bottom flask fitted with a rubber septum (25 ml), 2-aminopyridine (247 mg, 2.63 mmol), anhydrous DCM (5 ml) and anhydrous triethylamine (1 ml) was added through a dry syringe. Into a second flame-dried

round bottom flask fitted with a rubber septum, acridine-9-carbonyl chloride (500 mg, 2.07 mmol) and DCM (10 ml). Both mixtures were allowed to stir until both were solutions. An oven-dried syringe was then cooled under an argon atmosphere; the acid chloride solution was then slowly transferred by syringe to the aminopyridine solution and the solution was allowed to stir. After 30 min, the dark brown solution became cloudy, resulting in the formation of a yellow precipitate. The solution was allowed to stir for a further 4.5 h, and the precipitate was then filtered off onto pre-weighed filter paper and weighed (558 mg). The precipitate, which showed one spot on TLC, was sent for analysis. The mother liquor was then evaporated down to form a black solid (162 mg).

#### *4.3.2.2 Characterisation*

**$^{1}$HNMR (δ ppm, d6-DMSO):** 6.23 (t, 1H, J = 7 Hz), 6.78 (m, 5H), 6.96 (dd, 2H, J = 8, 1 Hz), 7.22 (m, 3H), 7.31 (td, 1H, J = 7, 1 Hz), 9.40 (s, 1H, NH). **$^{13}$CNMR (δ ppm):** 109.5 (CH), 114.8 (2CH), 115.7 (C), 118.6 (CH), 119.7 (2CH), 124.8 (CH), 125.9 (2CH), 129.1, (2CH), 138.3 (CH), 138.7 (2C), 155.9 (C), 181.8 (C). **MS (EI/CI):** EI m/z 75 (50 %), 151 (65 %), 178 (100 %), 206 (50 %), 237 (90 %). CI m/z 95 (100 %), 180 (20 %), 240 (20 %), 300 (20 %). **MP:** 274-275 °C. **IR $\upsilon_{max}$:** 512, 550, 586, 618, 668, 690, 746, 846, 896, 944, 994, 104, 1104, 1142, 1196, 1268, 1332, 1384, 1430, 1456, 1486, 1524, 1579, 1620, 1644, 1748, 2940, 2996, 3024, 3096, 3184, 3210, 3246.

### 4.3.3 2-[Acridine-9-carbonyl-amino]-1-methylpyridinium trifluoro-methanesulfonate (4.2).




**2-[Acridine-9-carbonyl-amino]-1-methylpyridinium trifluoro-methanesulfonate (4.2)**

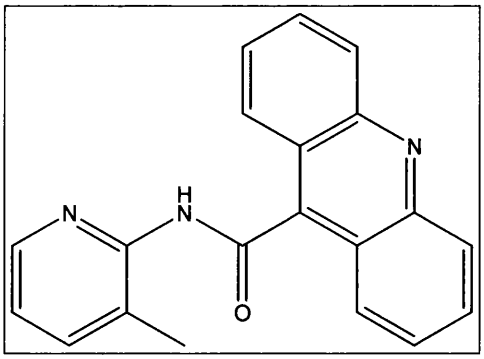
*4.3.3.1 Method*

Compound (4.1) (200 mg, 0.69 mmol) was added to a dry, 250 ml round bottom flask and DCM (anhydrous) was introduced until all of the solid had dissolved (200 ml). An excess of $CF_3SO_3CH_3$ (0.4 ml, 3.4 mmol) was then added and the solution was allowed to stir for 4 h. The solution was then evaporated, leaving an orange powder. This was dissolved in analytical grade acetone (5 ml) and diethyl ether was added (5 ml). The solution was then capped and left in the fridge overnight, whereupon red / orange crystals formed (243 mg, 76 % yield.).

*4.3.3.2 Characterisation*

**$^1$HNMR (δ ppm, d6-DMSO):** 3.04 (s, 3H, $CH_3$), 6.92 (t, 2H, J = 8 Hz), 7.14, (d, 2H, J = 8.0 Hz), 7.24 (d, 1H, J = 7 Hz), 7.28 (dd, 2H, J = 9, 1 Hz), 7.43 (t, 2H, J = 8 Hz), 7.74 (d, 1H, J = 9 Hz), 8.38 (d, 1H, J = 7 Hz), 8.43 (ddd, 1H, J = 9, 7, 1Hz), 10.05, (s, 1H, NH). **$^{13}$CNMR (δ ppm):** 28.8 ($CH_3$), 70.3 (unknown), 108.9 (CH), 110.6 (C), 115.4 (CH), 116.0 (2CH), 120.9 (2CH), 127.2 (2CH), 128.3 (CH), 131.5 (2CH), 139.1 (C), 150.4 (CH), 155.8 (C), 172.3 (C). **MS (ESI):** Positive Mode m/z 109 (100%), 141 (30 %), 238 (40 %), 314 (M – $CF_3SO_3^-$, 40 %), Negative mode m/z 149 ($CF_3SO_3^-$ ). **MP:** 212-213 °C. **IR $\upsilon_{max}$:** 636, 752, 1030, 1158, 1248, 1276, 1490, 1572, 1656, 1812, 3126, 3282. **Anal:.** Calcd for $C_{21}H_{16}F_3N_3O_4S$: C, 54.43; H, 3.48; N, 9.07, found C, 54.41; H, 3.66; N, 8.83.

4.3.4 Acridine-9-carboxylic acid (3-methylpyridin-2-yl)amide (4.3)




**Acridine-9-carboxylic acid (3-methylpyridin-2-yl)amide (4.3)**

*4.3.4.1 Method*

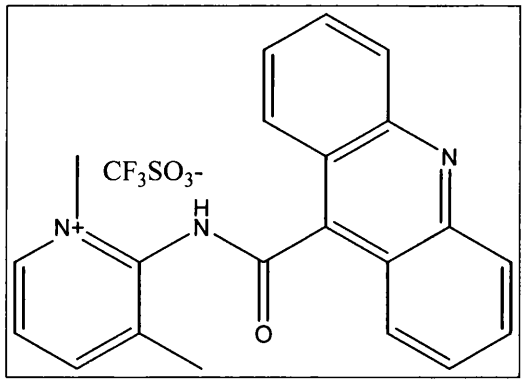
See section 4.3.2.1 for the general procedure. However, 2-amino-3-picoline (250 mg, 2.34 mmol) was used instead of 2-aminopyridine. Moreover, the reaction was

allowed to stir for 6 h, over which time a precipitate formed from the black solution. The precipitate was washed with diethyl ether and dried overnight (530 mg, 73 %).

*4.3.4.2 Characterisation*

**$^1$HNMR (δ ppm, d6-DMSO):** 2.11 (s, 3H, $CH_3$), 6.18 (m, 1H), 6.74 (m, 4H), 6.95 (d, 2H, J = 8 Hz), 7.03 (m, 1H), 7.19 (m, 3H), 9.37 (s, 1H, NH). **$^{13}$CNMR (δ ppm):** 15.7, ($CH_3$), 109.5 (CH), 114.8 (2CH), 116.0 (C), 119.9 (2CH), 122.1 (CH), 126.0, (2CH), 127.5 (C), 129.1 (2CH), 134.5, (CH), 138.7 (C), 156.6 (C), 182.4 (C). **MP:** 318-320 °C. **MS(EI):** 270 (30 %) 284 (100 %). **MS (CI):** 109 (100 %), 180 (40 %), 240 (30 %), 314 (M + $H^+$, 20 %). **IR $\upsilon_{max}$:** 740, 1154, 1326, 1344, 1410, 1490, 1530, 1554, 1630, 1752, 2942, 2996, 3082, 3172, 3246.

4.3.5 2-[Acridine-9-carbonylamino]-1,3-dimethylpyridinium trifluoromethanesulfonate (4.4).




**(4.4)**

*4.3.5.1 Method*

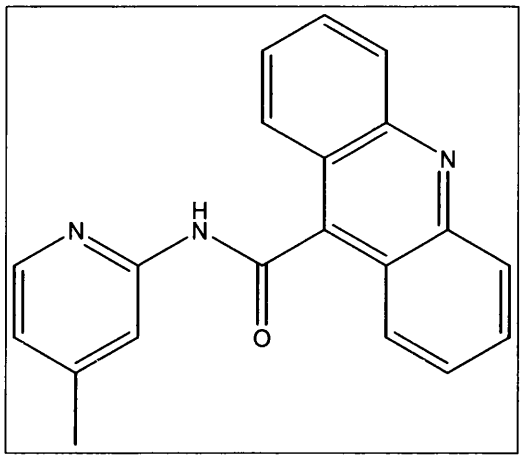
See section 4.3.3.1 for details of the procedure. However, (4.3) (200 mg, 0.64 mmol) was used as a starting material instead of (4.1). After crystallisation overnight in the fridge, orange crystals formed (217 mg, 0.455 mmol, 71 %).

*4.3.5.2 Characterisation*

**$^1$HNMR (δ ppm, d6-DMSO):** 2.71 (s, 3H, $CH_3$), 3.29 (s, 3H, $CH_3$), 6.93 (t, 2H, J = 8 Hz), 7.14 (m, 3H), 7.33 (d, 2H, J = 7 Hz), 7.43 (t, 2H, J = 8 Hz), 8.15 (d, 1H, J = 7 Hz), 8.23 (d, 1H, J = 7 Hz), 10.03 (s 1H, NH). **$^{13}$CNMR (δ ppm):** 18.7 ($CH_3$), 31.9 ($CH_3$), 109.1 (C), 115.4 (CH) 116.0 (2CH), 120.9 (2CH), 122.8 (C), 126.1 (CH), 127.2 (2CH), 131.5 (2CH), 139.2 (C) 150.2 (CH), 155.7 (C), 172.5 (C). **MS (ESI):** Positive mode m/z 123 (50 %), 300 (100 %), 328 (M - $CF_3SO_3$, 95 %), Negative

Mode: m/z 80 (40 %), 148.8 ($CF_3SO_3$, 100 %). **MP:** 235-236 °C. **IR $\upsilon_{max}$:** 638, 750, 1028, 1160, 1244, 1278, 1468, 1550, 1636, 1806, 3294. **Anal:.** Calcd for $C_{22}H_{18}F_3N_3O_4S$: C, 55.34; H, 3.80; N, 8.80, found C, 55.28; H, 3.72; N, 8.26.

### 4.3.6 Acridine-9-carboxylic acid (4-methylpyridin-2-yl)amide (4.5)



**(4.5)**

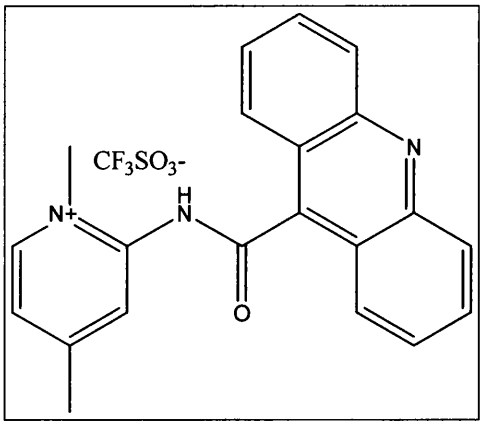
#### *4.3.6.1 Method*

See section 4.3.2.1 for the general procedure. However, 2-amino-4-picoline (233.5 mg, 2.15 mmol) was used instead of 2-aminopyridine. Moreover, the reaction was allowed to stir for 4 h, over which time a precipitate formed from the black solution. The precipitate was washed with diethyl ether and dried overnight (402 mg, 67.3 %).

#### *4.3.6.2 Characterisation*

**$^1$HNMR (δ ppm d6-DMSO):** 2.16 (d, 3H, $CH_3$, J = 1 Hz), 6.05 (dd, 1H, J = 7, 1 Hz) 6.52 (d, 1H, J = 1 Hz), 6.73 (t, 2H, 7 Hz), 6.80 (dd, 2H, 8, 1 Hz), 6.94 (d, 2H, J = 8 Hz), 7.18 (t, 3H, J = 8 Hz), 9.28 (s, 1H, NH). **$^{13}$C NMR (δ ppm):** 21.7 ($CH_3$), 112.5 (CH), 114.8 (2CH), 115.1 (CH), 116.0 (2C), 119.5 (2CH), 123.6 ($CH_2$), 125.8 (2CH), 128.9 (2CH), 138.7 (C), 149.0 (C), 156.0 (C), 181.2 (C). **MS (ESI):** Positive mode m/z 286 (90 %), 314 (30 %, M+ $H^+$). **Acc. Mass (ESI):** 314.1288 (calc. 314.1293). **IR $\upsilon_{max}$:** 752, 1268, 1312, 1338, 1484, 1580, 1652, 1752, 3022, 3078, 3174, 3249, 3328.

### 4.3.7 2-[Acridine-9-carbonyl)amino]-1,4-dimethylpyridinium trifluoro-methanesulfonate (4.6)




**(4.6)**

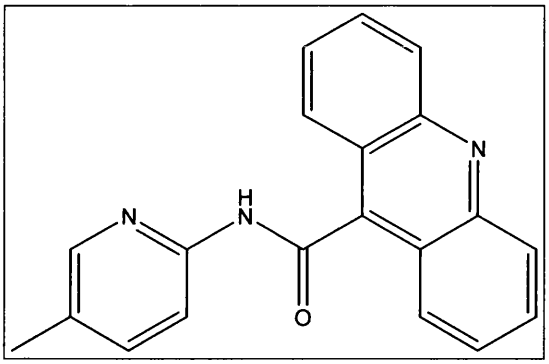
#### *4.3.7.1 Method*

See section 4.3.3.1 for details of the procedure. However, in this experiment only a reduced amount of the starting material was used ((4.5), 100mg, 0.30 mmol) was used and hence only half of the methylating agent was used (0.2 ml 1.7 mmol). After crystallisation overnight in the fridge, red crystals formed (100 mg, 0.195 mmol, 65 %).

#### *4.3.7.2 Characterisation*

**$^1$HNMR (δ ppm, $CDCl_3$):** 2.58 (s, 3H, $CH_3$), 2.99 (s, 3H, $CH_3$), 6.91 (t, 2H, J = 7 Hz), 7.14 (t, 3H, J = 6 Hz), 7.24 (d, 2H, J = 8 Hz), 7.43 (t, 2H, 8 Hz), 7.63 (s, 1H), 8.30 (d, 1H, J = 7 Hz), 10.03 (s, 1H, NH). **$^{13}$CNMR (δ ppm):** 23.0 ($CH_3$), 28.6 ($CH_3$), 108.5 (CH), 109.2 (2C), 115.9 (2CH), 117.7 (CH), 120.9 (2CH,), 127.1 (2CH), 127.3 (CH), 131.4 (2CH), 139.0 (3C), 155.2 (C), 164.6 (C), 171.9 (C). **MS (ESI):** – Positive mode m/z 123 (100 %), 328 (M - $CF_3SO_3^-$), 360 (M - $CF_3SO_3^-$ + MeOH), 395 (M - $CF_3SO_3^-$ + 2MeOH + $H^+$). Negative Mode m/z 149 ($CF_3SO_3$). **MP:** 221-222 °C. **IR $\upsilon_{max}$:** 636, 758, 1030, 1164, 1250 (ws), 1482, 1576, 1656, 1794, 3294. **Anal:.** Calcd for $C_{22}H_{18}F_3N_3O_4S$: C, 55.34; H, 3.80; N, 8.80, found C, 55.33; H, 3.72; N, 8.62.

### 4.3.8 Acridine-9-carboxylic acid (5-methylpyridin-2-yl)amide (4.7)



**(4.7)**

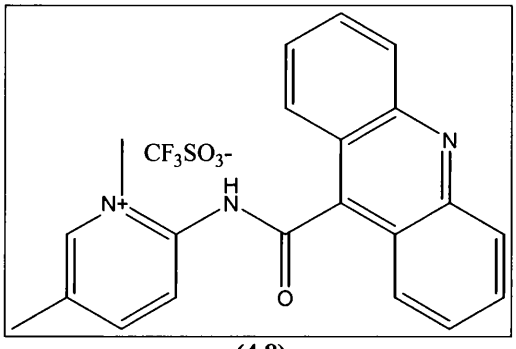
#### *4.3.8.1 Method*

See section 4.3.2.1 for the general procedure. However, 2-amino-5-picoline (250 mg, 2.34 mmol) was used instead of 2-aminopyridine. Moreover, the reaction was allowed to stir for 4 h, over which time a precipitate formed from the black solution. The precipitate was washed with diethyl ether and dried overnight (438 mg, 60 %).

#### *4.3.8.2 Characterisation*

**$^1$HNMR (δ ppm, d6-DMSO):** 2.11 (s, 3H, $CH_3$), 6.73 (m, 5H), 6.94 (d, 2H, 8 Hz), 7.10 (m, 2H), 7.18 (dd, 2H, J = 8, 7 Hz), 9.31 (s, 1H, NH). **$^{13}$C NMR (δ ppm):** 16.9 ($CH_3$), 114.8 (2CH), 15.8 (C), 118.1 (CH), 118.6 (C), 119.7 (2CH), 120.5 (CH), 125.9 (2CH), 129.1 (2CH), 138.7 (C), 141.8 (CH), 155.5 (C), 181.6 (C). **MS (CI):** m/z 314 ($M + H^+$, weak ion). **MP:** 258-261 °C. **IR $\upsilon_{max}$:** 742, 1138, 1256, 1340, 1490, 1582, 1658, 1742, 3090, 3180, 3210, 3244.

### 4.3.9 2-[Acridine-9-carbonylamino]-1,5-dimethylpyridinium trifluoro-methanesulfonate (4.8)



**(4.8)**

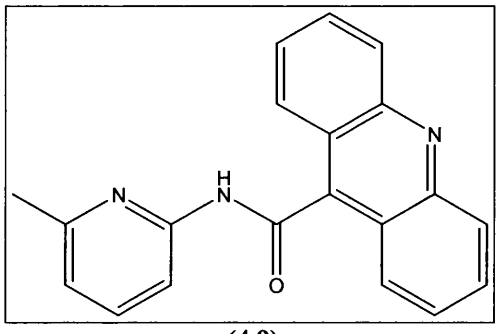
*4.3.9.1 Method*

See section 4.3.3.1 for details. The starting material used was (4.7) (100 mg, 0.3 mmol). The solution, milky yellow in character, went temporarily clear red when the methylation agent was added. Moreover, over 30 min the solution precipitated orange solid. On crystallisation, large red plates formed (70 mg, 0.318 mmol, 45 %).

*4.3.9.2 Characterisation*

**$^1$HNMR (δ ppm, d6-DMSO):** 2.39 (s, 3H), 3.02 (s, 3H), 6.91 (t, 2H, J = 8 Hz), 7.14 (dd, 2H, J = 8, 1 Hz), 7.24 (dd, 2H, J = 8, 1 Hz), 7.43 (dt, 2H, J = 8, 1 Hz), 7.72 (d, 1H, J = 9 Hz), 8.27 (s, 1H), 8.36 (dd, 1H, J = 10, 2 Hz), 10.08 (s, 1H). **$^{13}$CNMR (δ ppm):** 16.6 ($CH_3$), 28.3 ($CH_3$), 109.0 (C), 110.1 (CH), 116.0 (2CH), 120.9 (2CH), 124.8 (CH), 125.3 (C), 127.1 (2CH), 131.5 (2CH), 139.1 (C), 153.0 (CH), 155.04 (C), 172.40 (C). **MS (ESI):** Positive mode m/z 123 (40%) 328.1 (100 %) (M - $CF_3SO_3$). Negative Mode m/z 149 ($CF_3SO_3$, 100 %). **MP:** 190-194 °C. **IR $\upsilon_{max}$:** 638, 750, 1028, 1158, 1246, 1284, 1488, 1572, 1654, 1800, 3292.

4.3.10 Acridine-9-carboxylic acid (6-methylpyridin-2-yl)amide (4.9)




**(4.9)**

*4.3.10.1 Method*

See section 4.3.2.1 for details. However, 2-amino-6-picoline (250 mg, 2.34 mmol) was used as the starting material. On crystallisation, large red plates formed (730 mg, 2.34 mmol, 100 %).

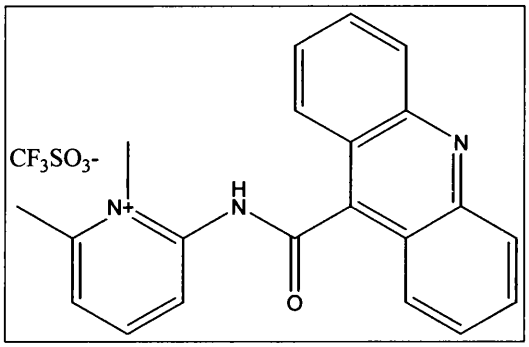
*4.3.10.2 Characterisation*

**$^1$HNMR (δ ppm, d6-DMSO):** 2.41 (s, 3H, $CH_3$), 7.13, (d, 1H, J = 8 Hz), 7.68 (ddd, 2H, J = 8, 7, 1 Hz), 7.85, (t, 1H, J = 8 Hz), 7.91 (ddd, 2H, J = 9, 7, 1 Hz), 8.00 (d, 2H,

J = 9 Hz), 8.22, (d, 2H, J = 9 Hz), 8.28, (d, 1H, J = 8 Hz), 11.55, (s, 1H, NH). **$^{13}$C NMR (δ ppm):** 23.7 ($CH_3$), 111.7 (CH), 119.8 (CH), 121.8 (C), 125.4 (2CH), 127.0 (2CH), 129.4 (2CH), 130.7 (2CH), 138.8 (CH), 148.2 (C), 150.9 (C), 157.0 (C), 165.8 (C). **MS (CI):** m/z 314 ($M + H^+$). **MP:** 204-210 °C. **IR $\upsilon_{max}$:** 758, 806, 1036, 1172, 1308, 1398, 1454, 1476, 1560, 1602, 1654, 2492, 2676, 2738, 2938, 2976.

There were also some impurity peaks in the aromatic end of the spectrum.

### 4.3.11 2-[Acridine-9-carbonyl)amino]-1,6-dimethylpyridinium trifluoromethanesulfonate (4.10)




**(4.10)**

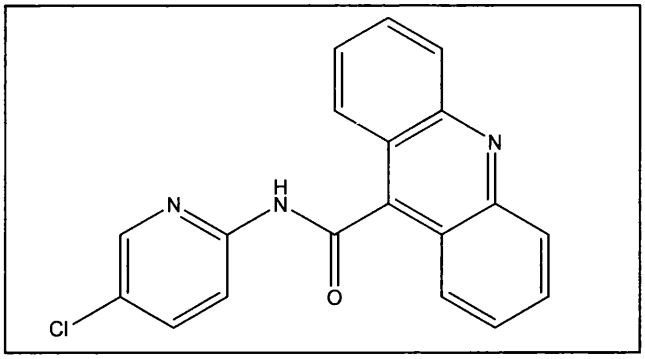
#### *4.3.11.1 Method*

See section 4.3.2.1 for details. The starting material used was (4.9) (100 mg, 0.3 mmol) in anhydrous DCM (50 ml). The solution, milky yellow in character, went temporarily clear red when the methylation agent was added. Moreover, over 30 min the solution precipitated an orange solid. On crystallisation, large red plates formed (100 mg, 0.196 mmol, 66 %).

#### *4.3.11.2 Characterisation*

**$^1$HNMR (δ ppm, d6-DMSO):** 1.92 (s, 3H, $CH_3$), 3.52 (s, 3H, $CH_3$), 6.89 (t, 2H, J = 8 Hz), 7.15 (m, 4H), 7.45 (m, 3H), 8.03 (d, 1H, J = 9 Hz), 8.55 (t, 1H, J = 8 Hz), 10.36 (s, 1H, NH). **$^{13}$CNMR (δ ppm):** 17.4 ($CH_3$), 27.7 ($CH_3$), 109.7 (C), 110.2 (CH), 115.6 (2CH), 121.3 (2CH), 122.8 (CH), 127.0 (2CH), 131.9 (2CH), 138.8 (C), 147.6 (CH), 149.9 (C), 152.1 (C), 171.4 (C). **MS (ESI):** Positive mode m/z 102 (70 %), 143 (20 %) 328.1 (M - $CF_3SO_3$); Negative mode m/z 80 (30 %), 148.8 ($CF_3SO_3$). **MP:** 188-190 °C. **IR $\upsilon_{max}$:** 638, 760, 792, 1028, 1168, 1250, 1280, 1492, 1508, 1586, 1632, 1752, 3282.

4.3.12 Acridine-9-carboxylic acid (5-chloropyridin-2-yl)amide (4.11)




**(4.11)**

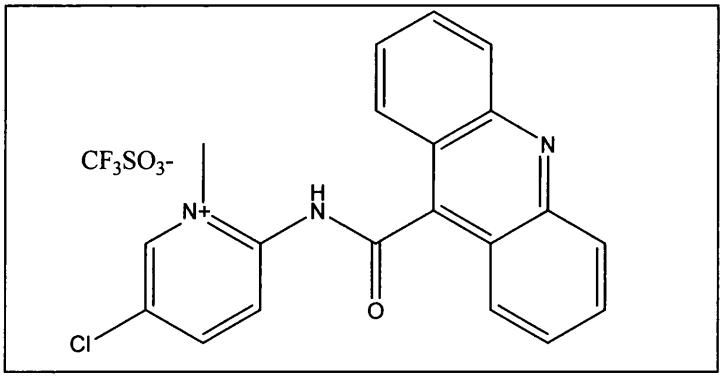
*4.3.12.1 Method*

See section 4.3.2.1 for the general procedure. However, 2-amino-5-pyridine (260 mg, 2.02 mmol) was used instead of 2-aminopyridine. Moreover, the reaction was allowed to stir for 48 h, over that time a precipitate formed from the black solution. The precipitate was washed with diethyl ether and dried overnight (475mg, 71 %).

*4.3.12.2 Characterisation*

**$^1$HNMR (δ ppm, d6-DMSO):** 6.76 (t, 2H, J = 8 Hz), 6.86 (m, 3H), 6.96 (dd, 2H, J = 8, 1 Hz), 7.22 (m, 3H), 7.57 (dd, 1H, J = 2, 1 Hz) 9.43 (s, 1H, NH). **$^{13}$C NMR (δ ppm):**72.6 (C), 14.8 (2CH), 115.2 (C), 116.1 (C), 119.6 (2CH), 120.0 (CH), 122.5 (CH), 126.2 (2CH), 129.2 (2CH), 138.5 (C), 139.1 (2CH), 153.8 (C), 180.6 (C). **MS (FAB):** m/z 334 [66.6 %] ($M+H^+$), 336 [33.3 %] ($M+H^+$). **MP:** 248°C. **IR $\upsilon_{max}$:** 668, 740, 1160, 1344, 1488, 1574, 1644, 1748, 3070, 3178, 3240.

4.3.13 2-[Acridine-9-carbonyl-amin0]-5-chloro-1-methylpyridinium trifluoromethanesulfonate (4.12)




**(4.12)**

*4.3.13.1 Method*

See section 4.3.3.1 for the general procedure. However, smaller quantities of the amide ((4.11), 100 mg, 0.29 mmol), anhydrous DCM (50 ml) and $CF_3SO_3CH_3$ (0.2 ml, 1.7 mmol) were used. After stirring at 25 °C for 0.5 h, the product was worked up as described in section 4.3.3.1. However in contrast to section 4.3.3.1, two products resulted from the crystallisation, small yellow crystals and large red plates. The red plates were separated from the yellow crystals by washing with diethyl ether (the red plates floated on the surface). Both products were then analysed.

*4.3.13.2 Characterisation of the two products*

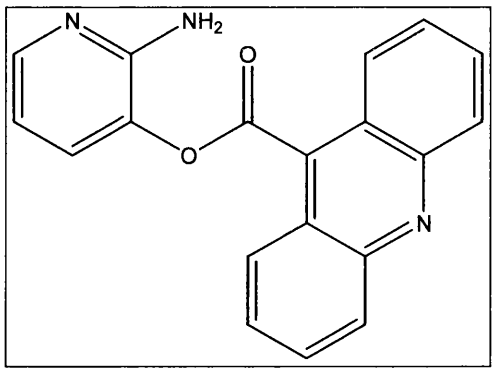
4.3.13.2.1 Product 1 - the yellow crystals

**$^1$HNMR (δ ppm, d6-DMSO)** Major peaks: 7.64 (t, 2H, J = 8 Hz), 7.86 (t, 2H, J = 9 Hz), 8.09 (d, 2H, J = 8 Hz), 8.17 (d, 2H, J = 9 Hz).
Minor peaks were also observed for the Red plates, the results of which are as listed in the following section.

4.3.13.2.2 Product 2 - the red plates

**$^1$HNMR (δ ppm, d6-DMSO):** 3.03 (s, 3H, $CH_3$), 6.92 (dt, 2H, J = 8, 1 Hz), 7.14 (dd, 2H, J = 8, 1 Hz), 7.35 (d, 2H, J = 8 Hz), 7.44 (dt, 2H, J = 8, 7 Hz), 7.83 (d, 1H, J = 10, 1 Hz), 8.53 (dd, 1H, J = 10, 2 Hz), 8.77 (dd, 1H, J = 2, 1 Hz), 10.09 (s, 1H, NH). **$^{13}$CNMR (δ ppm):** 28.9 ($CH_3$), 108.5 (C), 112.8 (2CH), 115.9 (2CH), 120.8 (2CH), 121.6 (C), 126.2 (CH), 127.3 (2CH), 131.5 (2CH), 138.9 (C), 149.9 (CH), 154.5 (C), 171.3 (C). **MS (ESI):** Positive mode m/z, 320 [100 %], 322 [33.3 %], 348 [100 %] (M - $CF_3SO_3$), 350 [33.3 %] (M - $CF_3SO_3$). Negative mode m/z 148.8 ($CF_3SO_3$). **MP:** 177-180 °C. **IR $\upsilon_{max}$:** 638, 744, 830, 1030, 1160, 1252, 1272, 1490, 1562, 1614, 1656, 1792, 1826, 3052, 3394.

4.3.14 Acridine-9-carboxylic acid 2-aminopyridin-3-yl ester (4.13b)




**(4.13b)**

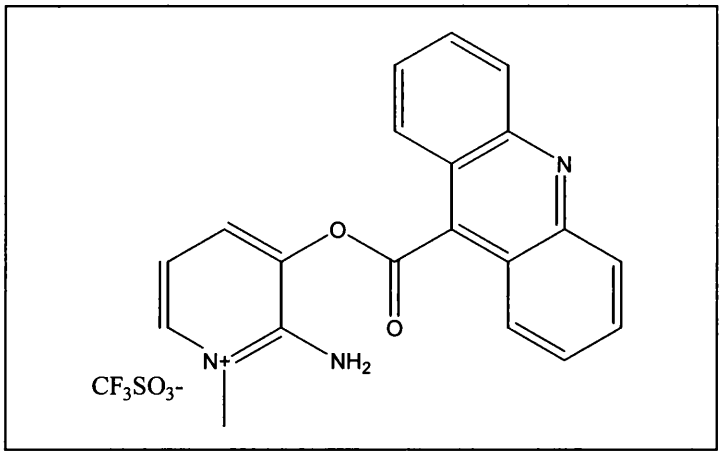
*4.3.14.1 Method*

See section 4.2.3.1 for the general procedure. However 2-amino-3-pyridinol (250 mg, 0.22 mmol) was used as the nucleophile. After the components had been added, a precipitate immediately formed. After stirring for 4.5 h the precipitate was filtered, washed with diethyl ether, weighed (646 mg, 0.20 mmol, 93%) and sent for analysis.

*4.3.14.2 Characterisation*

**$^1$HNMR (δ ppm, d6-DMSO) main peaks:** 6.10 (s, $NH_2$, 2H), 6.76 (dd, 1H, J = 8, 5 Hz), 7.79 (ddd, 2H, J = 9, 7, 1 Hz), 7.97 (m, 4H), 8.30 (m, 4H). **$^{13}$C NMR (δ ppm):** 112.4 (CH), 121.9 (C), 125.4 (2CH), 127.9 (2CH), 129.6 (2CH), 130.1 (CH), 130.9 (2CH), 132.2 (C), 135.1 (C), 145.9 (CH), 148.1 (C), 152.5 (C), 165.4 (C). **MS (ESI):** .Positive mode 102 (100 %) 143 (15 %), 316 (5 %) ($M+H^+$). **MP:** 235-237°C. **IR** $\upsilon_{max}$**:** 762, 1141, 1157, 1190, 1456, 1476, 1640, 3306.

4.3.15 3-(Acridine-9-carbonyloxy)-2-amino-1-methylpyridinium trifluoro-methanesulfonate (4.14)




**(4.14)**

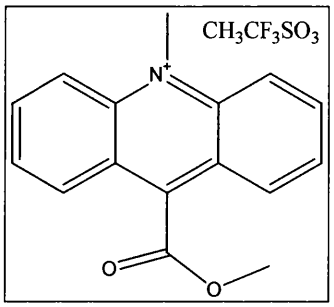
*4.3.15.1 Method*

See section 4.3.3.1 for details of the method. However, (4.13) (200 mg, 0.63 mmol) was used as the substrate for methylation. The methylation yielded a yellow solid, which formed yellow crystals (161 mg, 52 %, 0.33 mmol) after crystallisation.

*4.3.15.2 Characterisation*

**$^1$HNMR (δ ppm, d6-DMSO) main peaks:** 3.89 (s, 3H), 7.17 (dd, 1H, J = 8, 7 Hz), 7.83 (ddd, 2H, J = 8, 7, 1 Hz), 8.03 (ddd, 2H, J = 9, 7, 1 Hz), 8.22, (dd, 1H, J = 7, 1 Hz), 8.35 (m, 4H), 8.49 (s, 2H), 8.66 (d, 1H, J = 7 Hz). **$^{13}$CNMR (δ ppm):** 42.1 ($CH_3$), 111.2 (CH), 122.2 (C), 125.4 (2CH), 128.2 (2CH), 128.7 (2CH), 131.5 (2CH), 134.0 (CH), 134.3 (C), 139.0 (CH), 147.3 (C), 149.2 (C), 163.8 (C). **MS (ESI):** Positive mode m/z 100 (50 %),146 (25 %) 330 (100 %) (M - $CF_3SO_3$), Negative mode m/z 149 (100 %), ($CF_3SO_3$); **MP:** 282-285 °C. **IR $\upsilon_{max}$:** 516, 638, 1028, 1158, 1238, 1278, 1476, 1642, 1680.

4.3.16 9-Methoxycarbonyl-10-methylacridinium trifluoromethanesulfonate (4.15)




**4.15)**

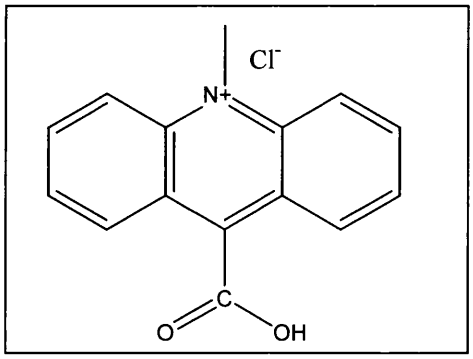
*4.3.16.1 Method*

Into a round bottom flask-containing methanol (20 ml), acridine-9-carbonyl chloride (400 mg, 1.65 mmol) was added and the solution was allowed to stir for 30 min at 25°C. During this time, the acid chloride dissolved, causing an exothermic reaction. Following this, saturated sodium hydrogen carbonate (100 ml) was added resulting in the formation of a yellow precipitate. The solution was twice extracted with DCM (2 x 100 ml), and the extract was dried with $MgSO_4$ (~ 50 g). The solvent was then removed, leaving a fluffy yellow product (364 mg, 96 %. A septum was added to the flask, the flask was then put under an argon atmosphere and dry DCM (10 ml) was injected into the flask. Once the ester had fully dissolved, $CF_3SO_3CH_3$ was added slowly *via* a syringe into the stirring mixture and was allowed to stir for 5 h. The precipitate that formed, was filtered and twice washed with diethyl ether, which caused a further precipitation. Thus the solution was again filtered, the solvent was then removed and the product was weighed (517 mg, 100 % yield). The majority (453 mg) of this product was then re-crystallised in acetone / diethyl ether producing two crops of yellow needles (339 mg and 80 mg, 92 % combined yield).

*4.3.16.2 Characterisation*

**$^1$HNMR (δ ppm d6-DMSO):** 4.32 (s, 3H), 4.91 (s, 3H), 8.09 (t 2H, J = 9 Hz), 8.45 (d, 2H, J = 9, Hz), 8.50 (t 2H, J = 8 Hz), 8.89 (d, 2H, J = 9 Hz). **$^{13}$CNMR (δ ppm):** 39.6 ($CH_3$), 54.9 ($CH_3$), 119.8 (2CH), 122.3 (C), 128.0 (2CH), 129.3 (2CH), 139.1 (2CH), 141.9 (C), 148.5 (C), 165.1 (C).

4.3.17 9-Carboxy-10-methylacridinium chloride (4.16)




**(4.16)**

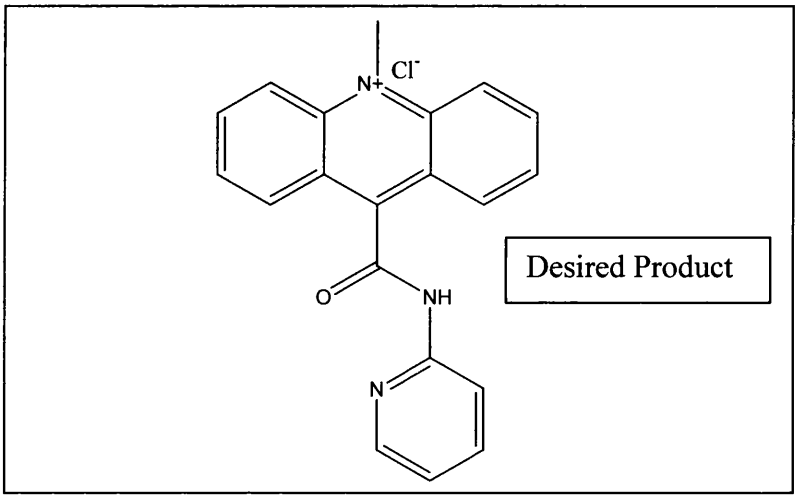
*4.3.17.1 Method*

Into a round bottom flask (10 ml) containing a solution of sodium hydroxide (6 ml, 5 %), compound (4.15) was added (310 mg 0.77 mmol) and the mixture was then heated to 100 °C for 2.15 h. Following this, the clear green solution was filtered whilst hot and then acidified with HCl (conc.). As the solution cooled the carboxylic acid precipitated out (158 mg, 0.58 mmol), giving a yield of 77 %.

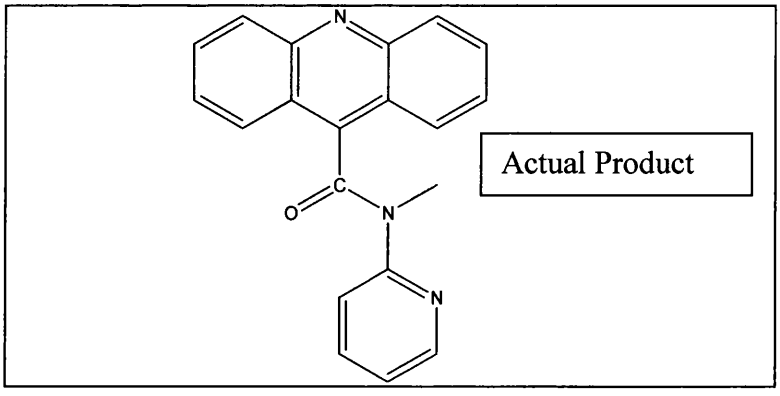
*4.3.17.2 Characterisation*

**$^1$HNMR (δ ppm, d6-DMSO):** 4.87 (s, 3H), 8.08 (t, 2H, J = 8 Hz), 8.39 (dd, 2H, J = 8,1 Hz), 8.47 (dt, 2H, J = 9, 1 Hz), 8.86 (d, 2H, J = 9 Hz).

4.3.18 First attempted synthesis of 10-methyl-9-(pyridin-2-ylcarbamoyl)-acridinium; chloride (4.18)




**(4.18)**




**Acridine-9-carboxylic acid *N*-methylpyridin-2-ylamide (4.19)**

*4.3.18.1 Method*

Into a dry round bottom flask, (4.16) (300 mg, 1.08 mmol) was added, followed by thionyl chloride (5 ml). The solution was refluxed for 6h, during which time, the solid dissolved. The hot solution was then poured into hexane (20 ml), which facilitated a precipitation. The solid was then filtered and the solvent was removed under high vacuum. Into a second dry round-bottom flask (25 ml) cooled under an argon atmosphere, an excess of pre-dried 2-aminopyridine (300 mg, 3.19 mmol) was added. Following this, a mixture of anhydrous DCM (5 ml) and anhydrous triethylamine (1 ml) was injected though a septum into the flask and the mixture was allowed to stir under the inert atmosphere. The flask containing the acid chloride (128 mg 3.35 mmol) was taken and placed under an argon atmosphere. Through a septum, anhydrous DCM (10 ml) was then added. A slurry was then formed and this was transferred into the second round bottom flask (containing the amine) using a double ended-needle. The mixture was then allowed to stir overnight, during which time a precipitate formed. The precipitate was filtered and washed with diethyl ether and the remaining solvent was removed by evaporation under a high vacuum giving product 1 (3 mg). The solution was then evaporated down, yielding an oil. The oil was then extracted twice with DCM (50 ml) and water (50 ml). The DCM layer was dried with $MgSO_4$ and evaporated down to yield a white solid (330mg, 1 mmol, 100% yield, identified as (4.19)). The aqueous layer was also evaporated down to yield an oil (200 mg).

*4.3.18.2 Characterisation*

4.3.18.2.1 The white precipitate

**$^1$HNMR (δ ppm, $CDCl_3$):** 3.57 (s, 3H, $CH_3$), 6.51, (dt, 1H, J = 1, 7 Hz), 6.96 (t, 2H, J = 8 Hz), 7.07, (dd, 2H, J = 1, 8 Hz), 7.15 (d, 2H, J = 8 Hz), 7.24 (d, 1H, J = 9 Hz), 7.34 (d, 1H, J = 6 Hz), 7.4 (ddd, 2H, J =1, 7, 9 Hz), 7.59 (ddd, 1H, J = 2, 7, 9 Hz). **$^{13}$C NMR (δ ppm):** 29.6(artefact) 33.4 ($CH_3$), 112.8 (CH), 113.6 (2CH), 115.7 (CH), 119.5 (C), 121.6 (2CH), 127.5 (2CH), 130.3 (2CH), 135.2 (CH), 141.2 (C), 142.0 (CH), 166.7 (C), 187.8 (C). **MS (EI):** 313 (M). **MS (ESI):** Positive Mode 179, 194, 314 ($M+H^+$), 336 ($M+Na^+$), 627 ($2M+H^+$) 649 ($2M+Na^+$). **MS (Acc. Mass):** m/z 314.1286 (calc. 314.1293). **MP:** 268 °C. **Anal:.** Calcd for $C_{20}H_{15}N_3O$: C, 76.66; H, 4.82; N, 13.41, found C, 73.99; H, 4.81; N, 12.73.

4.3.18.2.2 The aqueous layer

*4.3.18.2.2.1 2-Aminopyridine (for comparison with components 1 and 2)*

**$^1$HNMR (δ ppm, d6-DMSO):** 8.57 (s, 2H, $NH_2$), 6.44 (m, 2H), 7.33 (m, 1H), 7.88 (m, 1H). **$^{13}$C (δ ppm):** 108.0 (CH), 111.8 (CH), 136.9 (CH), 147.7 (CH), 159.8 (C).

*4.3.18.2.2.2 Component 1 (83 %)*

**$^1$HNMR (δ ppm, d6-DMSO):** 6.76 (d, 1H, J = 7 Hz), 6.90 (d, 1H, J = 9 Hz), 7.80 (dt, 1H, J = 7, 9 Hz), 7.93 (dd, 1H, J = 6, 1 Hz). **$^{13}$C NMR (δppm):** 111.9 (CH), 112.4 (CH), 137.8 (CH), 142.6 (CH), 155.0 (C).

*4.3.18.2.2.3 Component 2 (17 %)*

**$^1$HNMR (δ ppm, d6-DMSO):** 6.83 (t, 1H, J = 7 Hz), 7.17 (d, 1H, J = 9 Hz), 7.72 (dt, 1H, J = 9, 7 Hz), 8.0 (dd, 1H, J = 6, 1 Hz). **$^{13}$C NMR (δ ppm):**111.6 (CH), 114.54 (CH), 139.74 (CH), 143.25 (CH), 153.16 (C).

*4.3.18.2.2.4 Mass Spectroscopic data for both components 1 and 2*

CI (m/z) 95 ($M+H^+$), 110, 127, 112 ($M+NH_3$).

## 4.4 Final conclusions

An investigation into the synthesis of a range of acridinium amides based on the parent molecule, acridine-9-carboxylic acid pyridin-2-ylamide, was conducted for further investigation into their chemiluminescent properties. Under conventional routes, the amides selectively methylated on the pyridine rings. Thus to attempt to synthesise a range of acridinium analogues, *N*-methylacridinium-9-carbonyl chloride, was synthesised and allowed to react with 2-aminopyridine to test the viability of the route. This reaction resulted in a methyl transfer from acridine to the pyridine amine forming a secondary methylamine, which was then selectively reacted with acridine-9-carbonyl chloride to form a tertiary amide. It was concluded that the methyl group was thus a more likely site of nucleophilic attack than the acridinium carbonyl carbon. Finally some initial experimentation was conducted into the synthesis of doubly methylated species, but on account of issues with solvent compatibilities further

investigation will be required to facilitate a successful double methylation. The products synthesised were tested for possible chemiluminescence, however tests showed that no significant chemiluminescence was observed for the said compounds. Although disappointing this was expected as chemiluminescence required acridinium precursors to react with hydrogen peroxide. Unfortunately the findings in this chapter detail that acridinium derivatives of pyridine amides do not methylate on the acridine nitrogen.

## 4.5 References

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