AMINO ACID INTERACTIONS OF SPIROPYRAN

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AUTHOR'S DECLARATION

I hereby certify that this material, which I now submit for assessment on the programme of study leading to the award of Master of Science, is entirely my own work and has not been taken from the work of others save and to the extent that such work has been cited and acknowledged within the text of my work.

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ABSTRACT

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A solution phase study was carried out to explore the interactions between the spiropyran molecule in its merocyanine zwitterionic merocyanine 'open' form and amino acids. The principal amino acids chosen for investigation were β-Alanine, L-Phenylalanine and 8-Aminocaprylic acid. Both single phase and two-phase switching experiments were undertaken to document the photochromic interconversion between spiropyran and merocyanine in the presence of these amino acids, compared to a water blank. A kinetics study was then carried out to examine how the rate of decay of the coloured merocyanine back to the closed spiropyran was affected by the presence of amino acids. Results show that there are significant differences in the decay rate depending on which amino acid was present. There are several factors which may be involved in how the different amino acids affect the decay rate of the merocyanine, such as the relative polarity of the solution. A significant stabilisation effect was also observed in the presence of water (i.e. no amino acid present), suggesting that hydrogen bonding may play an important role in stabilising the merocyanine isomer at the molecular level.

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1.Introduction

1.1. Spiropyran background

Spiropyrans are a group of organic molecules that exhibit photochromism. Photochromism is a chemical process by which a compound undergoes a reversible change between two states which have different visible absorption spectra, i.e. different colours. As a general rule, the change in one direction is induced by electromagnetic radiation, usually UV light, and in the other direction by altering or removing the light source, or alternatively by thermal means[1].

The photochromism of spiropyrans was first observed in 1952 by Fischer and Hirshberg[2]. On exposure to UV light, the colourless "closed" spiro form (referred to as SP) is transformed to the highly coloured, "open" merocyanine form (referred to as MC). The UV light induces a heterocyclic ring cleavage, changing the conformation of the molecule to produce a planar conjugated molecule with absorbance in the visible region. This merocyanine structure is zwitterionic, a property which may be exploited to facilitate both physical and chemical sensing. This charged form can isomerise to a quinoidal molecule, but due to the loss of aromaticity in this neutral structure, the zwitterion is the major contributor to the open form[3]. In subsequent experiments it is assumed that the zwitterionic form dominates the switching. Irradiation with visible light reverses this isomerisation, as does thermal energy. The interconversion is shown in Figure 1.1. An example of the UV-Vis spectra of the open and closed forms can be seen in the experimental section in Figure 3.4.

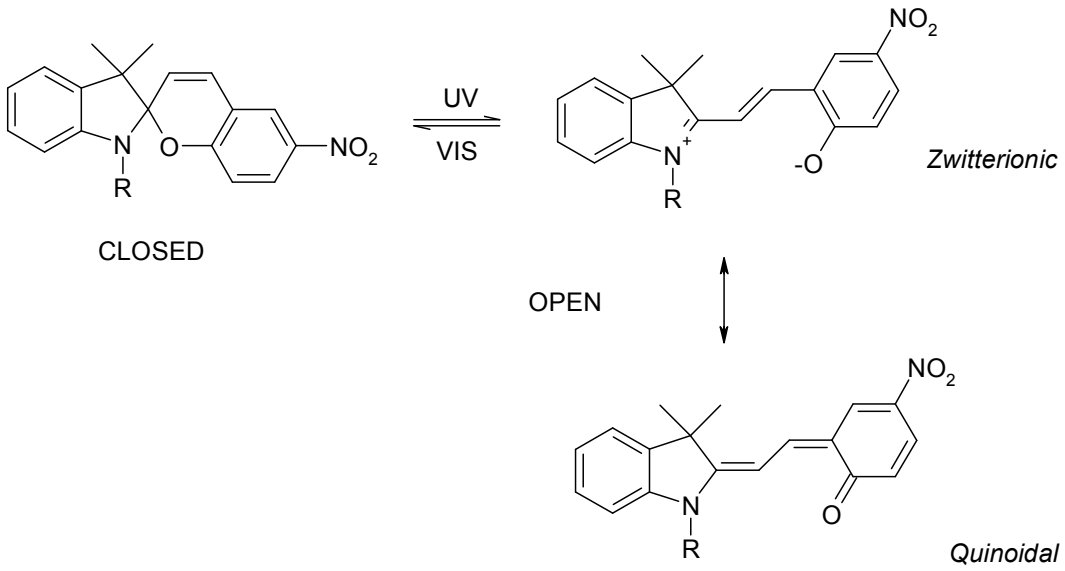


Figure 1.1 The photochromic conversion of the spiropyran from a colourless, closed form to a coloured, zwitterionic open form when exposed to UV irradiation. The open form also exists as a neutral quinoidal structure, but the zwitterion is stabilised due to the loss of aromaticity in the quinoidal form[3]. The reaction is reversed by visible irradiation, or thermally.

1.1.1. Dynamic Equilibrium

When the spiropyran merocyanine interconversion is in a state of dynamic equilibrium, there is no net change in reactant or product concentrations over time as the rate of the forward reaction is equal to the rate of the reverse reaction. The system is a typical intramolecular Lewis acid-base equilibrium[4]. The position of the equilibrium depends mainly on the degree of solvation of SP and MC, which in turn is linked to the polarity of the solvent used[5].

However, the delicate equilibrium can also be upset by other factors, such as exposure to light, changes in ambient temperature, or addition of more solvent to the spiropyran solution. In all these cases the equilibrium is affected, and the rates of forward and

reverse reactions change in order to re-establish equilibrium, according to Le Chatêlier's principle, which states:

"A system at equilibrium, when subjected to a disturbance, responds in a way that tends to minimise the effect of the disturbance"[6]

In practical terms, experimental procedures exploiting the photochromism of spiropyran must be performed with a certain level of exposure to ambient light, which of course affects this equilibrium. To reduce the effect that this might have on the reproducibility of the results, it is vital to be aware of the delicate nature of the dynamic equilibrium, to minimise interferences and try to keep unavoidable exposure to interferences constant between samples and experiments. Therefore the spiropyran solution must be left in the dark for a sustained period of time to re-establish the initial starting conditions.

1.1.2. Solvent Effects

The switching characteristics of spiropyran are also sensitive to the polarity of the local microenvironment. Solvatochromic studies have been carried out on various spiropyran derivatives[3,5]. Solvatochromism is the change in position and sometimes intensity of the absorption bands, and therefore colour, of solutes when measured in different solvents[7].

A range of common solvents are compared in Table 1.1 using various solvent polarity scales. The terms ε_r, n, and E_T^N represent the dielectric constant, refractive index and normalised Reichardt's polarity factor respectively, and the terms π^*, α, and β refer to

the Kamlet-Taft scales[8,9,10] for solvent dipolarity-polarisability, hydrogen bond donor acidity and hydrogen bond acceptor basicity. These parameters are used as a measure of the polarity of a solvent and may be used to predict the behaviour of the spiropyran in solvents of different polarity.

Table 1.1 Solvent polarity parameters[11].

Solvent	**ε_r**	**n**	**E_T^N**	**π^***	**α**	**β**
Toluene	2.38	1.497	0.099	0.54	0	0.11
Tetrahydrofuran	7.58	1.407	0.207	0.58	0	0.55
Acetone	20.56	1.359	0.355	0.71	0.08	0.48
1-Butanol	17.51	1.399	0.602	0.41	0.68	1.01
2-Propanol	19.92	1.377	0.546	0.48	0.76	0.95
Ethanol	24.55	1.361	0.654	0.54	0.83	0.77
Methanol	32.66	1.328	0.762	0.60	0.93	0.62
Water	78.30	1.333	1.000	1.09	1.17	0.18

Intermolecular interactions between the solute and solvent modify the energy gap between the ground and excited states of the absorbing species, thus altering its absorption wavelength[12], as shown in Figure 1.2.

Figure 1.2 Solvatochromic effect. A refers to the ground state MC in a non-polar solvent such as toluene, A* refers to the photoexcited MC in that solvent. B and B* indicate the ground state and photoexcited MC respectively in a more polar solvent, such acetonitrile. C and C* denote the ground state and photoexcited MC respectively in a very polar solvent such as methanol. The ground state is stabilised in more polar solvents, leading to an increase in the energy gap between ground and excited states, and hence a decrease in the λ_{max} of the absorbance waveband.

In a comprehensive study carried out by Garcia *et al*[3] it was reported that the solvent stabilisation of the merocyanine isomer depends on a whole variety of solvent-solute interactions, for example hydrogen bonding and dipole-dipole interactions[13,14]. Reichardt's polarity scale is regarded as a comprehensive measure of solvent polarity, as it takes into account both the dipolarity of the solvent, and its hydrogen bond donating ability.

Garcia and colleagues found that the polarity of the microenvironment of spiropyrans dramatically affects their photochromism[3]. They found the open chain merocyanine forms of spiropyrans to be negatively solvatochromic, meaning that their absorption and fluorescence emission bands undergo a hypsochromic (blue) shift in solvents of increasing polarity, with solvent polarity classified using the Reichardt E_T^N parameter.

This parameter is a measure of the solvation power of a solvent, taking into account all intermolecular interactions between solute and solvent molecules, excluding those which result in chemical alteration of the solute[15]. The most important intermolecular interactions in the case of spiropyrans are thought to be hydrogen bonding between the solute and solvent molecules[3]. The E_T^N parameter is calculated from the maximum wavenumber of the longest wavelength electronic absorption band of Reichardt's dye (Figure 1.3) in any given solvent[16].

2,6-diphenyl-4-(2,4,6-tripheynylpyidinio)phenolate

Figure 1.3 Reichardt's dye. Reichardt's polarity scale is based on the maximum wavenumber of the longest wavelength electronic absorption band of this molecule in a given solvent.

Garcia and co-workers reported evidence of the effect of hydrogen bonding on the stabilisation of merocyanine. They emphasised the strong stabilisation effect present in solvents with hydrogen bonding capability, finding that the hydrogen bond donor acidity played a significant role in solvent stabilisation.

Song *et al*[5] reported an in-depth study of the correlation between solvatochromism, Lewis acid-base equilibrium and the photochromism of spiropyran in pure and mixed organic solvents. They used the transition energy for the open MC as an empirical way to measure solvent polarity, and to investigate the position of the Lewis acid-base equilibrium of the SP-MC system, and the rate of decoloration of the MC back to SP. The results showed that as the equilibrium position depended on the relative solvation of the MC and SP, the value of the equilibrium constant was solvent dependant. The

results suggested that the MC was solvated to a greater extent in polar solvents, whereas the SP was solvated more strongly in non-polar solvents, which is to be expected, given the zwitterionic nature of the MC. They also reiterated the commonly reported solvatochromic effect, with a hypsochromic (blue) shift observed in the UV-Vis spectra on increasing solvent polarity. The rate of decay from the MC to SP form was also found to be dependant on solvent polarity. It was reported that the decoloration rate constants decrease as the solvent polarity increases, which again can be explained intuitively due to the increased stabilisation of the MC zwitterions in more polar solvents. The suggested mechanism for decoloration is shown in Fig 1.4.

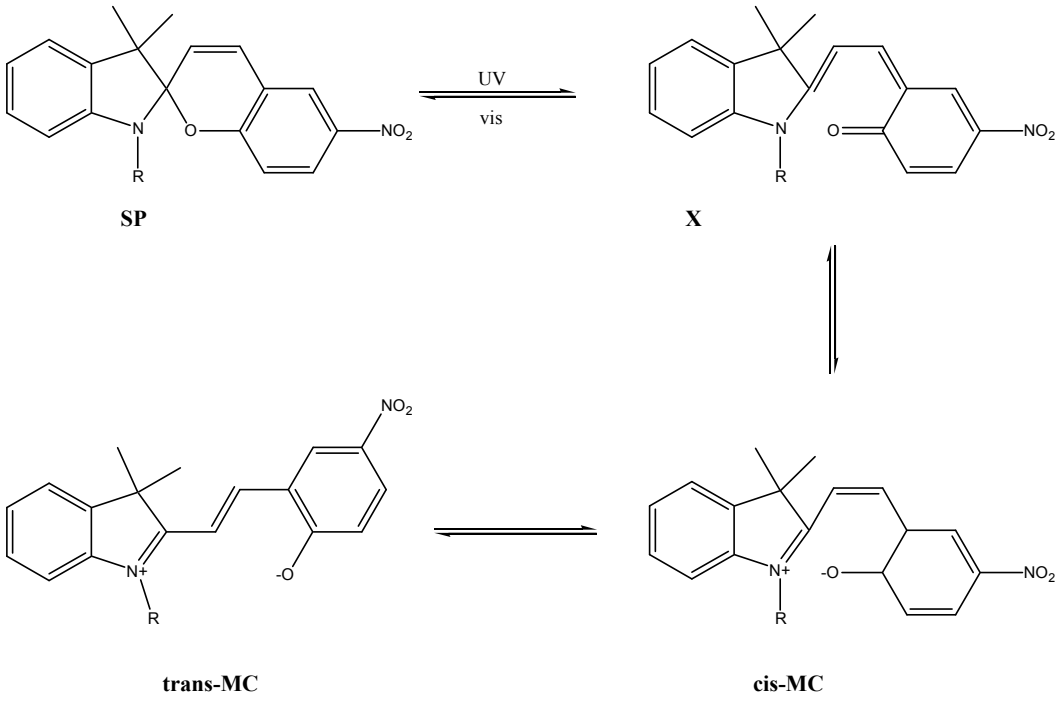


Figure 1.4 The proposed mechanism for interconversion between SP and MC via intermediate X.

This mechanism was first suggested by Flannery in 1968[17]. The interconversion between SP and MC takes place via an intermediate X[18,19]. During the ring opening process, X is formed as the spiro carbon - pyran oxygen atom bond is broken. The X form then interconverts to the trans- and cis- forms of MC. The solvent effect on the rate of ring opening is very weak, since the conversion of SP to X is an electrocyclic reaction, and this is the rate determining step. However, for the reverse reaction the rate determining step is the trans-cis isomerisation and internal rotation around the olefinic double bond. These are sensitive to solvent polarity, hence a large difference in decoloration rates can be observed on varying the polarity of the solvent used.

Görner[20,21,22] has also carried out detailed studies on a range of spiropyrans to investigate the effects of structure, solvent and temperature on photochromism. The

results he obtained were broadly similar to Song *et al*, with one interesting variation. His results show a higher absolute quantum yield of MC in non-polar solvents than in polar, when the SP is irradiated with UV light. This contrasts with Song's claim that the effect of solvent polarity on ring opening is negligible. Görner attributes the decrease in quantum yield with increase of solvent polarity to a suggested mechanism of ring opening – i.e. via the n,π triplet state and an increasing energy gap with respect to the $^3(\pi,\pi)^*$ state.

1.1.3. Temperature effects

The effect of temperature on the SP-MC equilibrium needs to be taken into account in any study of the molecule's photochromism. Spiropyrans have long been known to be thermochromic – i.e. their colour depends on temperature. Hirschberg and Fischer reported that the absorption spectra of several quinoidal merocyanines changed with temperature[23]. The general trend was a decrease in the intensity of the absorption band at longer wavelengths and an increase in that at shorter wavelengths, as the temperature decreased.

In 1951 Knott carried out a comprehensive study of the temperature dependence of the absorption spectra of various merocyanines, and classified them into groups accordingly[24]. These reversible temperature effects can be attributed to the change in position of the equilibrium between the different species. In the various current applications of spiropyran, the exploitation of the photochromic properties of SP-MC usually involves a thermal relaxation from the coloured to colourless forms[25]. The intensity of colour observed, and the length of time taken to revert to the closed form

are both temperature dependant. Görner[22] reported that there was a large variation in the equilibrium constant and rate of MC decay when the temperature is changed.

1.1.4. pH effects

The phenolate anion present in the MC form is a reactive functional group. For example, in the presence of acid the MC may become protonated, with the yellow-green MCH^+ being formed[26], as shown in Fig. 1.5. This means that the pH of the solution must be carefully controlled. The pK_a of the protonation of the merocyanine is of the order of 4-5[27]. The formation of the protonated MCH^+ may be exploited for various applications such as the use of the SP-MC-MCH^+ interconversion for a molecular switch, as discussed below. An example of the UV-Vis spectrum of a protonated merocyanine is shown in Fig. 1.6.

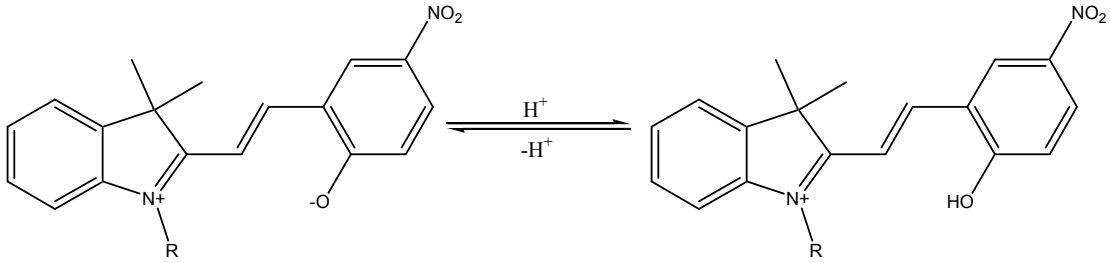
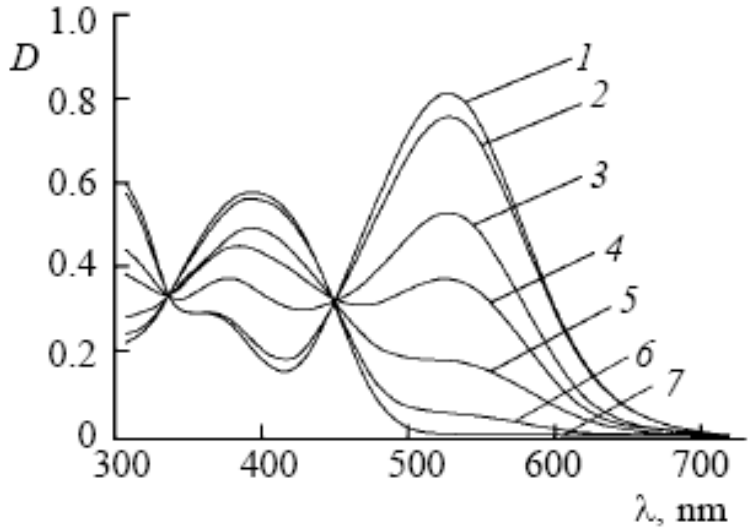


Figure 1.5 The protonation of the merocyanine in the presence of acid. The protonated form, denoted by MCH, is yellow-green in colour.



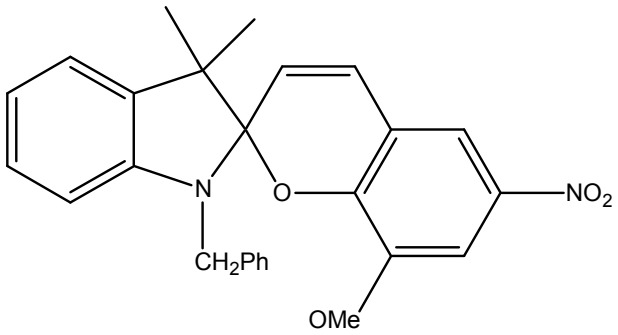


Figure 1.6 Part A shows the absorption spectra of the spiropyran shown in part B at various pHs of the aqueous-acetone (1 : 1) solutions[27]. pH: (*1*) 7.0, (*2*) 6.4, (*3*) 5.3, (*4*) 4.9, (*5*) 4.4, (*6*) 3.7, and (*7*) 2.0 (concentration 2.5×10^{-5} M). The pKa for this spiropyran is 5. It can be seen that a peak around 400 nm increases with increasing acidity, with a corresponding decrease in absorbance at about 530 nm[28]. Although the structure of this molecule differs slightly from the spiropyran studied here, it displays similar behaviour upon protonation.

1.1.5. Aggregation

Non-covalent interactions such as Van der Waals forces can induce the self-assembly of molecules into aggregates. Flannery[17] first proposed that the aggregation of molecules of spiropyran was taking place, after examining the absorption bands of the merocyanine form in neutral solvents. A review of the photoinduced aggregation of photochromic spiro compounds was published by Barachevskii and Karpov in early

2007[29], discussing recent studies of the aggregation of SP and potential applications. A spectral study of 6-nitrosubstituted spiropyran showed the appearance of a long wavelength absorption band in the region of 640-670 nm upon increasing the concentration of MC, indicating the presence of aggregates[30,31]. It was concluded that spiropyrans of this type tend to form J-aggregates, where the molecules are arranged in a head-to-tail pattern at the expense of electrostatic interactions, in the ratios AB and A_nB, where A is the initial SP and B is the ring-opened MC. This is demonstrated in Fig. 1.7 below.

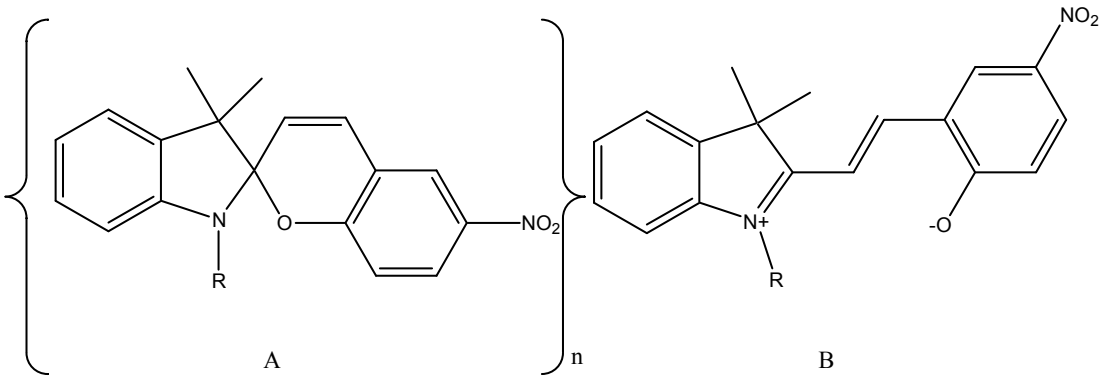


Figure 1.7 The head to tail molecular arrangement of SP and MC in J aggregates in the ratio A_nB, where A is the SP and B the MC.

In polar solvents, aggregates are only formed at high concentrations of MC. It was found that the activation energy for thermal decoloration and the free energy value for SP in solution were higher in toluene than in ethanol, meaning that the aggregation of molecules takes place more efficiently in non-polar solvents. The hydrogen bonding in polar protic solvents apparently leads to a decrease in the intermolecular interactions which lead to the formation of aggregates.

1.2. Current Applications

What makes spiropyran such an interesting potential chemical sensing agent is how its conformation, chemical and physical properties can be light modulated. The photoswitchability of spiropyrans has been exploited as a means of reversible light modulation of properties for a variety of chemical assemblies, including optical data storage, photoreceptors, molecular switches, among others[1].

Information transfer in contemporary telecommunication networks relies on the interplay of optical and electrical signals. The electronic processing of the optical signals slows down information transfer. Giordani *et al*[32] have described a spiropyran based three state optical molecular switch for multichannel digital transmission in an optical network of communicating molecules, demonstrating that molecular switches can be used to gate optical signals in response to optical signals. The simple optical network developed consisted of three light sources, one cell containing a solution of three fluorescent molecules, one cell containing the spiropyran based three-state molecular switch, and a detector. The three states in the molecular switch are spiropyran (SP), merocyanine (MC) and protonated merocyanine (MCH) (Fig. 1.8).

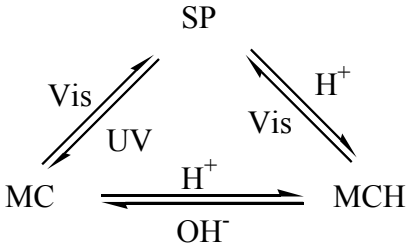


Figure 1.8 The three state molecular switch described by Giordani *et al*. The three states are spiropyran (SP), merocyanine (MC) and protonated merocyanine (MCH). The inputs are UV light, visible light, acid and base. The outputs are the purple absorption band of MC and the yellow green absorption of MCH.

Ultraviolet light, visible light, acid and base are the inputs that lead to a change from one state to the other. The SP form is colourless, MC is purple, and MCH is yellow-green. This change in absorbance between the three states can be exploited for optical signal switching. This is an important step towards the ultimate goal of eliminating the electronic element of existing communication networks. Giordani *et al*[33] also describe the exploitation of this same three-state molecular switch to execute a logic function equivalent to that of a combinational logic circuit integrating two AND, two NOT, and one OR gate. The logic gate produced two outputs that are spectroscopically observable; the absorption bands of the purple MC and the yellow-green MCH. This logic gate may be the starting point for the potential development of a more complex digital circuit based on molecular changes in the chemical system.

Electronically reconfigurable spiropyran-based molecular solid-state switching devices have also been constructed from monolayers sandwiched between two electrodes. Collier *et al*[34] reported that a well-characterised optical switching response was chemically designed into the molecular component. Previous work in molecular electronics showed that voltammetry experiments on molecular components in

solution often do not map onto solid-state device properties, but Collier demonstrated that even after harsh device fabrication conditions, the optical switching properties of spiropyrans were retained in the solid-state device, and in fact dominated the device characteristics, unlike similar devices with alternative molecular components. The two forms of the spiropyran, open and closed, were found to have completely different electrochemical behaviour. The fact that the photoswitching of the SP can still occur in the solid state makes the photocontrol of electrochemical properties possible. The authors also carried out control experiments with other amphiphilic molecules, but found that no optical switching was observed. This work is significant as it demonstrates that the basic molecular signatures of isolated molecules in solution may be retained in a solid-state device, and that it is possible to design a specific, unique molecular property into a solid-state tunnel junction.

Another electrochemical application of spiropyran is reported by Willner *et al*[35]. Photoisomerisable monolayers of SP were assembled on Au electrodes and used to control the electrooxidation of dopamine and (3,4-dihydroxyphenyl)acetic acid by means of electrostatic interactions that could be photomodulated. This functionalised electrode could be used for the transduction of optical, thermal or pH signals recorded by the monolayer, again exploiting the photochromic variation in the physical and chemical properties of SP and applying them to develop an effective sensing system.

The chelating ability of merocyanine was observed as far back as 1965 by Phillips[36]. More recently, Chibisov and Görner[37] have reported the use of spiropyran derivatives to complex metal ions, giving rise to the potential application of SP as a metal complexation agent. They carried out detailed kinetic studies to examine the

formation of the merocyanine-metal complex, and found that the complexation of metal ions by SP in solution is the result of two coupled reactions – the formation of the merocyanine-type ligand and substitution of the solvent (co-ordinated at the metal ion) by the ligand. Diamond *et al*[38] have also reported metal ion complexation by spiropyran, with the effect manifested with a number of ions such as Co^{2+}, Cr^{3+}, etc. Clearly the potential of spiropyran as an optical sensor has not yet been fully explored or exploited.

Another application which employs the difference in charge between the open and closed forms is the photocontrol of ionic conduction. Kobayashi[39] and co-workers presented a novel technique for the photocontrol of ionic conduction using spiropyrans. In the polymer electrolyte used, a photochromic chelation of spiropyran and a divalent cation occurred under UV irradiation, resulting in a decrease in ionic conductivity of the electrolyte. The photochromic chelation was suppressed by visible irradiation, leading to an increase in ionic conductivity.

Another application of the chelating capability of the photochromic spiropyran was reported by Filley *et al*[40]. A bis-spiropyran was prepared, and the magnesium and calcium chelating ability of this was investigated and compared to mono-spiropyrans. It was found that the binding was 8 times higher using the bis-spiropyran than the mono spiropyran. The colour of the merocyanine was shown to be strongly influenced by the metal cation, with a blue shift in the maximum absorbance of 43 nm for magnesium and 22 nm for calcium. Strong fluorescence was also observed, providing another technique to detect the chelation of the metal ions.

An additional interesting use of spiropyrans is for the recording of erasable holograms. This has been reported as far back as 1970 by Lesscinski *et al*[41], and illustrated more recently by Xue *et al*[42]. In this application it was the change in absorption and refractive index observed when irradiated with either UV or visible light that formed the basis for the method. The photochromic interconversion modulated the absorption and refractive index of spiropyran doped polymer films, allowing holograms to be recorded. These holograms could be erased by irradiating the polymer with visible light. Xue and co-workers found that this write-and-erase cycle could be carried out repeatedly, effectively producing erasable holograms. The wavelength of light used was 350 nm for the writing cycle, and 647 nm to erase the merocyanine hologram.

Min *et al*[43] describe a photoreceptor consisting of spiropyran-TCNQ films for image extraction. A spiropyran with a long alkyl chain was deposited by spin coating on a quartz substrate. TCNQ Langmuir-Blodgett films were deposited on the other side of the quartz substrate. The SP layer was irradiated with different wavelengths of light, producing different photocurrents in the TCNQ film. This enabled image extraction by the TCNQ photodetector without the need for computational circuits. The extraction efficiency was found to be 10%, with a 15 minute extraction time.

In a subsequent publication, Min *et al*[44] presented a photoreceptor consisting of spiropyran-bacteriorhodopsin films for photosignal enhancement. The experimental setup was similar to the TNCQ system described above, but in this case bacteriorhodopsin was chosen as a photoreceptor in an attempt to improve the signal to noise ratio. Bacteriorhodopsin is a protein which generates a photocurrent

proportional to light intensity. The extraction efficiency for this method was found to be 25% - an improvement from the TCNQ photoreceptor, however the extraction time was considerably longer, at 40 minutes.

The exploitation of the photochromism of spiropyran to produce a mechanical output was reported by Athanassiou *et al*[45]. They put forward a microsystem that underwent mechanical actuation induced exclusively by photons. The system consisted of a polymer substrate doped with photochromic spiropyran molecules. The photochromic interconversion led to the contraction and expansion of the polymer substrate in a controllable manner. The number and intensity of incident laser pulses controlled the optomechanical actuation. The advantages of using laser beams for the optical manipulation of microsystems were the high spatial control offered and the fact that the operation could be non-contact.

There are numerous other less common applications of spiropyran molecules to be found in the literature. Weston *et al*[46] have developed photo-modulation of horseradish peroxidase activity via covalent attachment of carboxylated spiropyran dyes. They reported a reduction in enzyme activity of greater than 90% under visible compared to UV irradiation, matching the greatest degree of photo-modulation previously reported in the literature. Other interesting applications include the use of spiropyran in dry colour printing[47], photo-controlled gating[48], potentiometric protein sensing[49], etc, but one of the most exciting areas of current research involves the exploitation of the zwitterionic nature of the merocyanine to develop an optical sensor for other zwitterions.

The merocyanine molecule is zwitterionic, as shown in Fig 1.1 above. This gives rise to the possibility of electrostatic interactions between the merocyanine and other charged species. If the target molecule is also a zwitterion, with similar spacing between charges, then the electrostatic interaction may be strong enough to form a host-guest complex. If this is the case, the light modulation of the merocyanine makes this a very attractive sensing agent, as the binding reaction, in principle, can be photonically switched on and off.

One potential group of zwitterionic target molecules is amino acids. Sunamoto *et al*[50] reported interactions between the zwitterionic merocyanine and zwitterionic amino acids. They reported that in a two-phase system an amino acid crossed from the aqueous phase to the organic phase after the SP in the organic phase had been irradiated with UV light and opened to become MC. They achieved the transport of amino acids across lipid membrane using spiropyran embedded in liposomal bilayers of egg phosphatidylcholine, as shown in Fig. 1.9.

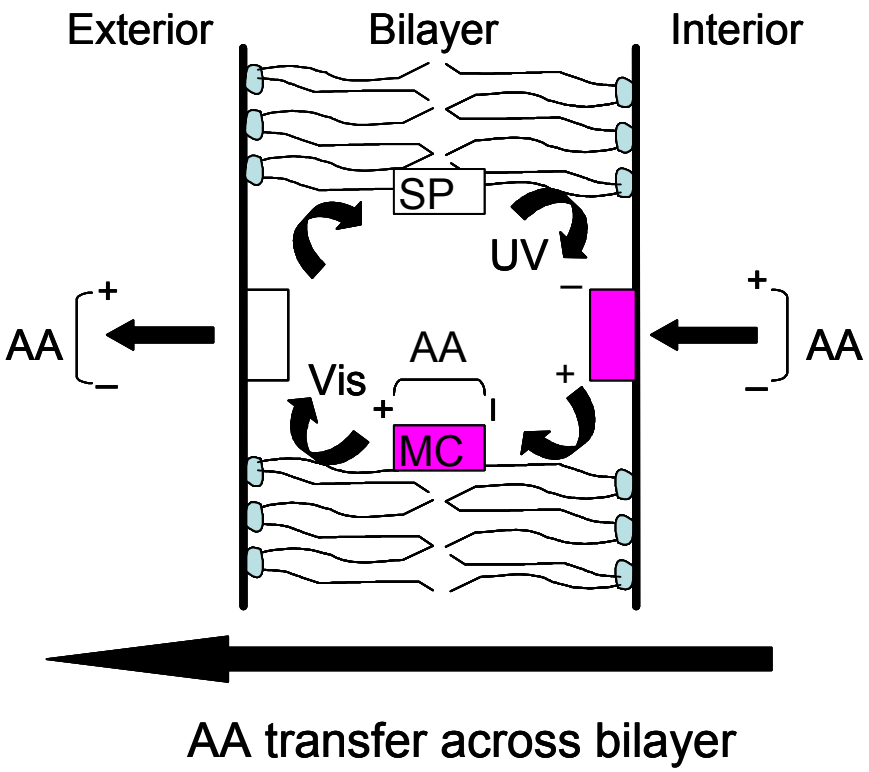
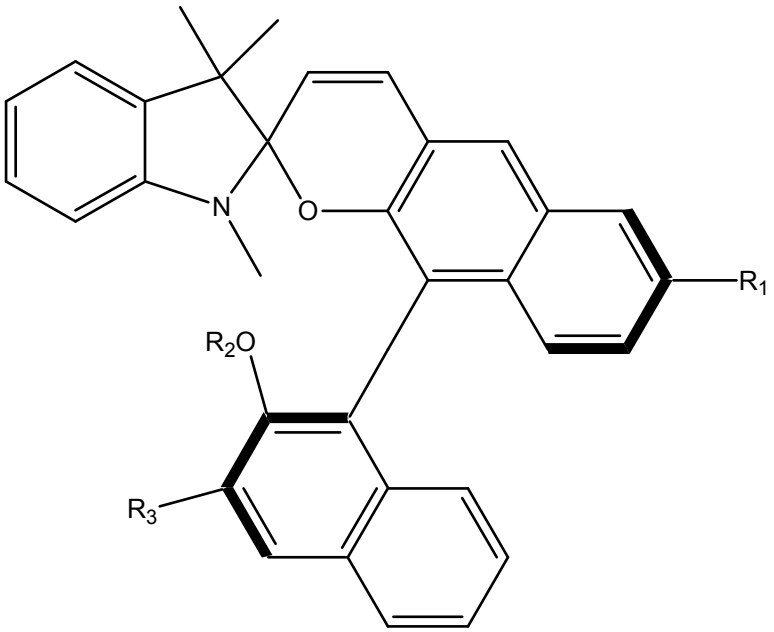


Figure 1.9 Representation of amino acid transfer across liposomal bilayers through the photochromic interconversion between SP and MC as reported by Sunamoto *et al*[50]. The SP present in the bilayer is irradiated with UV light, leading to the formation of the brightly coloured MC. This zwitterion interacts with the zwitterionic amino acid (AA) in the interior and transports it into the bilayer. When the MC-AA complex is irradiated with visible light, the MC returns to the SP form, releasing the AA into the exterior.

Seno and co-workers have also reported the photocontrolled extraction and transport of amino acids using functional reversed micelles containing a spiropyran moiety[51]. The spiropyran was incorporated into the reverse micelles of tetraethyleneglycol dodecylether (TEGDE) in n-decane. A three phase water/n-decane/water system was used, with the AA in the first aqueous layer, the SP-TEGDE in the organic layer. Transport of tryptophan was observed from the first aqueous layer to the second, with the first aqueous/organic interface irradiated with UV light, and the second irradiated with visible light.

Chiral spiropyrans have been used for the enantiomeric recognition of amino acids. Tsubaki *et al*[52] described the synthesis of an optically active spiropyran with a binaphthol moiety as a chiral source. The chiral spiropyrans used (1 and 2) are shown in Fig. 1.10.



1: R_1=R_3=H, R_2=Me
2: R_1=NO_2, R_2=CH_2CH_2OMe, R_3=Br

Figure 1.10 The chiral spiropyrans 1 and 2 used by Tsubaki *et al* in their system to differentiate between D- and L-amino acids.

It was found that the open merocyanine form obtained under UV irradiation was retained longer in D amino acids than in L amino acids. The half life of the coloured MC was measured in the presence and absence of different amino acids. The amino acids tested were alanine (Ala), valine (Val), tryptophan (Trp), histidine (His) and phenylalanine (Phe). The solvent system used was a 4:1 ratio of acetonitrile to water. The concentration of SP was 8.0×10^{-4} M. Using SP1 (see Fig. 1.10 above) it was found that the half lives of the MC in the presence of the amino acids were longer

than for the molecule alone. They found that the half lives decreased with increasing bulk of both the ammonium and the carboxylate moieties, suggesting that a two-point electrostatic interaction between the zwitterionic MC and amino acid was taking place. The results showed a difference in half-life that was directly related to the bulkiness of the side chain of the amino acid, facilitating differentiation between the D- and L-enantiomers. This is a novel method to differentiate between amino acid enantiomers. The synthesis of host 2 (see Fig. 1.10. above) led to epimerisation, resulting in a racemic mixture of spiropyran. No retention of amino acid was observed, and further studies were to be undertaken on this molecule.

More recently, Shao *et al*[53] have reported a spiropyran based system for the recognition and quantification of cysteine (Cys) and homocysteine (Hcy) at physiological levels. The spiropyran used is shown in Fig. 1.11. The system involves using the merocyanine of this spiropyran and mercury or copper ions as a sensing system for Cys and Hcy. It was found that when a range of amino acids were added to a merocyanine solution containing either Hg^{2+} or Cu^{2+}, a marked difference was observed in the absorbance spectra when Cys and Hcy were present, compared to the other amino acids. This difference in colour could be exploited to selectively detect Cys and Hcy. The divalent metal ion formed a complex with two MC molecules, which in turn acted as a host to the amino acid guest.

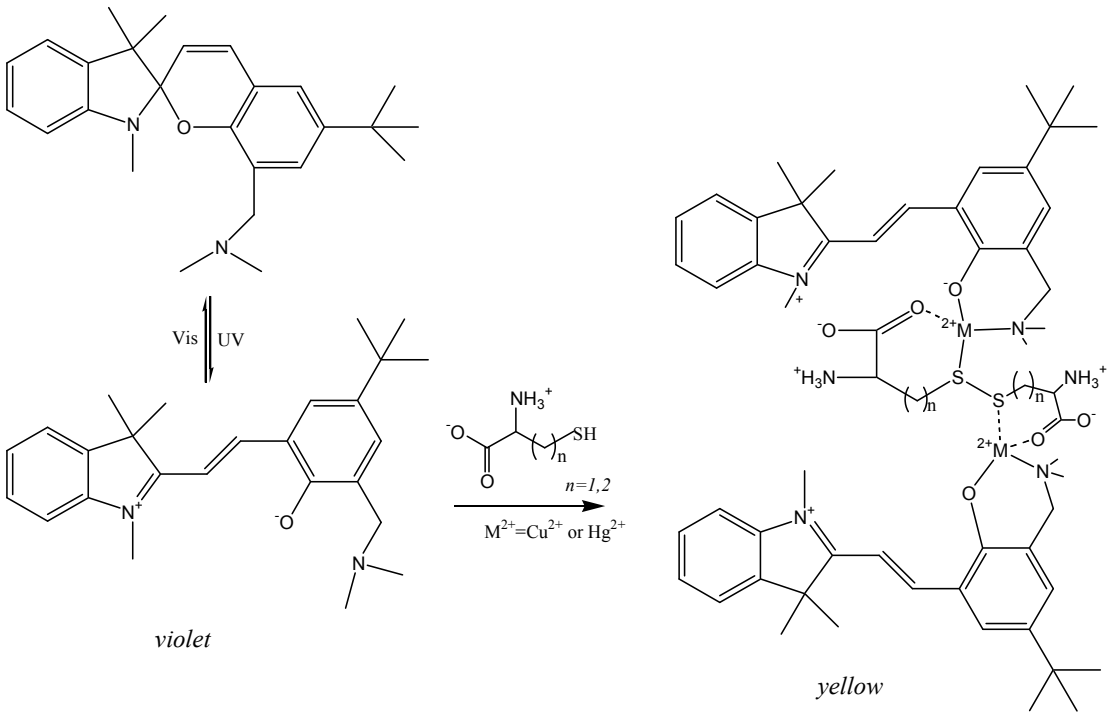


Figure 1.11 The spiropyran used by Shao *et al* in their system for the selective detection of Cysteine and Homocysteine in the presence of copper or mercury ions. Each metal ion forms a complex with a merocyanine, then two of these complexes act as a host for two amino acids, linked with a disulphide bridge. The system is dimeric, with two MCs, two metal ions and two amino acids being linked.

1.3. Proposed Research

In this study we investigate the possibility of light controlled interactions between the zwitterionic merocyanine and other charged molecules. Amino acids have been proposed as targets for this study, given that they may exist in a zwitterionic form at a specific pH (Fig 1.12).

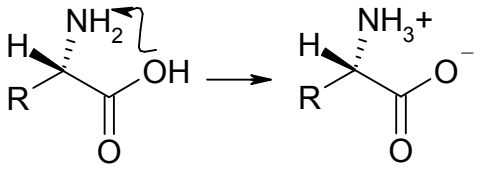


Figure 1.12 General structure of an amino acid in both its uncharged and zwitterionic forms. The R group refers to the side chain, which varies in different amino acids. The charge displayed by the molecule depends on the pH of the solution.

If docking takes place then the open form of the spiropyran is stabilised, meaning it stays coloured rather than reverting to the colourless closed form. This means that in principle spiropyran could be used to detect the presence of amino acid (Fig. 1.13).

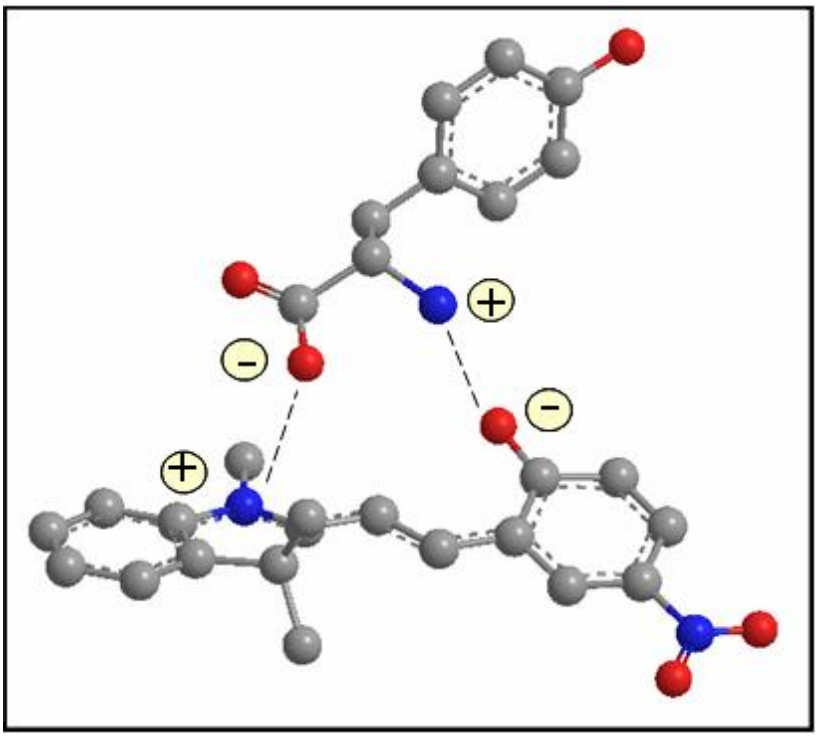


Figure 1.13 Schematic representation of possible "docking" interactions between merocyanine and zwitterionic amino acid.

One factor which may affect the MC-AA interactions is pH. Amino acids are ampholytes. An ampholyte is a molecule that contains both acidic and basic groups. The charge on these molecules depends on the pH, and they can exist in zwitterionic form at a specific pH known as the isoelectric point, or pI. Below this pH the molecule is protonated and the molecule has a net positive charge, whilst above this pH the molecule has a net negative charge. This is demonstrated graphically in Fig 1.14, where an example of a pH titration curve for an amino acid is shown[54].

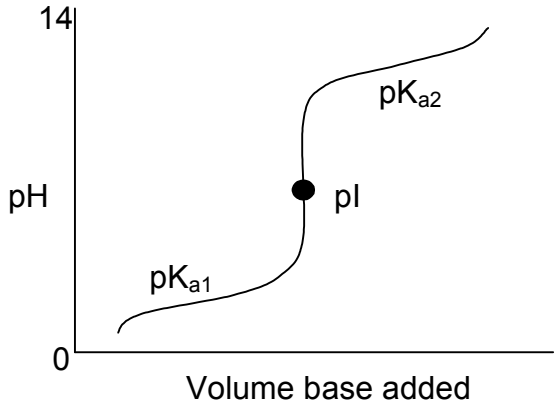
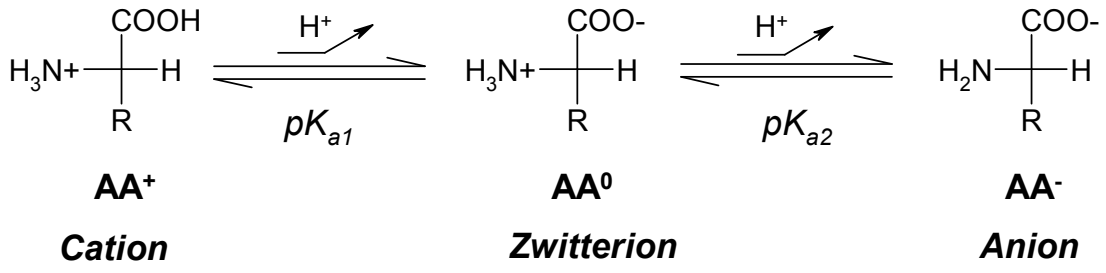


Figure 1.14 A sample pH titration curve for an amino acid.

The first deprotonation takes place at pK_{a1}, and the second at pK_{a2}. Below pK_{a1} the amino acid molecule exists as a positively charged ion. Then, as the pH increases, the carboxylic acid group on the amino acid loses a H^+ leading to the formation of the zwitterion. The deprotonation of the basic amine group takes place at a much higher pH, giving rise to a negatively charged ion. Between these two reactions the amino acid exists in its zwitterionic form.



The reactions taking place are as follows:

$$AA^+ + H_2O \overset{K_{a1}}{\rightleftharpoons} AA^0 + H_3O^+$$

$$AA^0 + H_2O \overset{K_{a2}}{\rightleftharpoons} AA^- + H_3O^+$$

From these reactions the rate constants K_{a1} and K_{a2} can be determined as shown:

$$K_{a1} = \frac{[AA^0][H_3O^+]}{[AA^+]} \qquad \textbf{Equation 1}$$

$$K_{a2} = \frac{[AA^-][H_3O^+]}{[AA^0]} \qquad \textbf{Equation 2}$$

The pI value is calculated from the pK_as of the molecule. For an amino acid with only one amine and one carboxyl group, the pI is calculated from the two pK_as as follows:

$$pI = \frac{pK_{a1} + pK_{a2}}{2} \qquad \textbf{Equation 3}$$

From the values of pI in Table 1.2 shown it can be seen that there is a general increase in pI upon increasing the spacer length between the positive and negative charges of the zwitterion. The higher the pK_a of an acid, the lower is its K_a and therefore the weaker is its proton donating power to water.

In order to optimise the potential for "docking" to take place, it is necessary to ensure that the amino acid is in the zwitterionic form, therefore the pH of each solution should be adjusted to the isoelectric point of the amino acid in question. A summary of some common amino acids and their isoelectric points is given in Table 1.2.

Table 1.2 Isoelectric points of some common amino acids[55].

Amino Acid	**Isoelectric point (pI)**
Phenylalanine	5.48
Tyrosine	5.66
Valine	5.96
Glycine	5.97
Leucine	5.98
Alanine	6.00
β-Alanine	6.90
γ-Aminobutyric acid	7.30
δ-Amino-n-valeric acid	7.52
ε-Amino-n-caproic acid	7.60
Lysine	9.59

The simple amino acid is the building block of peptides, and is often the basis for more complex drug molecules, for example dopamine (Fig. 1.15.). Therefore, if the detection of amino acids using spiropyran is optimised, there are potential applications for the monitoring and detection of both legal and illegal drugs. The spiropyran-drug molecular interactions could ultimately be monitored using a simple,

low-cost LED-based sensor system to control both the photo-switching of the SP-MC system and measurement of the resulting colour to determine whether binding with the guest species has occurred, as this often leads to changed in the visible absorbance spectrum of MC.

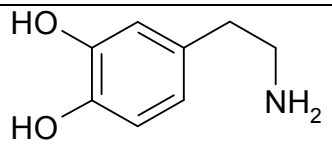
Dopamine	

Figure 1.15 The structure of the drug "dopamine", used in the treatment of Parkinson's disease.

2. Solvatochromic Study

2.1. Introduction

The solvatochromic nature of spiropyran has been investigated and reported in the literature. A solvatochromic study was undertaken to explore the effect of solvent polarity on the switching characteristics of spiropyran and the relative stabilities of the open and closed form, in order to better understand the various factors which can affect this sensitive equilibrium.

2.2. Experimental

2.2.1. Materials

The materials used in this section were as follows:

- Spiropyran: 6-Nitro-1', 3',3'-trimethylspiro[2H-1-benzopyran-2,2'-indolin]1',3'-Dihydro-1',3',3'-trimethl-6-nitrospiro, 98%, Sigma-Aldrich
- Methanol, Aldrich
- Ethanol, Aldrich
- Butan-1-ol, Riedel-de Haën
- Acetonitrile, Lab Scan
- Acetone, Aldrich
- Tetrahydrofuran, Aldrich
- Toluene, Aldrich

2.2.2. Instrumentation

The instrumentation used in this section was as follows:

- Electrolite Corporation Bond Wand UV lamp
- Ocean Optics S2000 Mini Fiber Optic Spectrometer

2.2.3. Method

A solvent study was carried out to investigate the switching characteristics of 6-Nitro-1', 3',3'-trimethylspiro[2H-1-benzopyran-2,2'-indolin]1',3'-Dihydro-1',3',3'-trimethl-6-nitrospiro (subsequently referred to as spiropyran) in solvents of different polarity. Spiropyran solutions were prepared in the concentration range of 10 μM – 1 mM in the solvents listed in Table 2.1.

Table 2.1 Solvents used and the concentration of spiropyran dissolved

Solvent	**SP Concentration**
Methanol	100 μM
Ethanol	100 μM
Butan-1-ol	1 mM
Acetonitrile	1 mM
Acetone	100 μM
Tetrahydrofuran	1 mM
Toluene	1 mM

Figure 2.1 Switching setup consisting of Bond Wand UV light source (1) and Ocean Optics mini spectrometer. The cuvette containing the spiropyran solution was placed in the optical cell (2) and irradiated with UV light facilitating real-time spectral analysis of sample. Light is conveyed from the light source to the optical cell via fibre optic cable (3), and after passing through the sample the transmitted light is sent via fibre optic cable (4) to the mini spectrometer.

The apparatus shown in Figure 2.1 was chosen as the photo-switching and spectrometric measurement set-up. The convenient size and portability of the Bond Wand meant that it could be used in conjunction with an Ocean Optics mini spectrometer for real time analysis. The experimental set-up consisted of the Bond Wand UV light source placed directly above the Ocean Optics mini spectrometer. The cuvette containing the spiropyran solution was placed in the mini spectrometer, the apparatus covered, and the UV light source switched on. The mini spectrometer was connected to a nearby PC using a fibre optic cable, facilitating real time spectral analysis of the sample as the molecule opened to the merocyanine form and the solution went from colourless to coloured. The UV-Vis spectra were recorded for the MC in the different solvents.

2.3. Results and Discussion

In the course of the switching experiments it was found that in some solvents the photochromic interconversion of the spiropyran to the merocyanine was not easily observable at concentrations below 100 μM. The optimum spiropyran concentration in each solvent for these preliminary switching experiments was determined by making up solutions in the concentration range of 100 μM - 1 mM. These are summarised in Table 2.2.

Table 2.2 The solvents studied are classed below in order of decreasing polarity (Reichardt), along with the concentrations of spiropyran used. It was necessary to increase the concentration in some solvents in order to optimise switching.

Solvent	**Concentration SP**	**Polarity (Reichardt E_T^{N} [3,56])**
Methanol	100 μM	0.762
Ethanol	100 μM	0.654
Butan-1-ol	1 mM	0.602
Acetonitrile	1 mM	0.47
Acetone	100 μM	0.355
Tetrahydrofuran	1 mM	0.207
Toluene	1 mM	0.099

There was a marked difference in the relative stability of the open/closed molecule in the solvents of different polarity. In toluene, for example, the open form was so unstable that the colour would decay within seconds of removing the UV light source.

For all the other solvents tested, irradiation with a visible light source was necessary to switch the solutions from coloured to colourless.

If we use the Beer-Lambert Law:

$$A = \varepsilon c l \qquad \textbf{Equation 4}$$

Where A is absorbance, ε is the molar extinction co-efficient, c is the molar concentration and l is the path length in cm, we can calculate the percentage of the SP in the open MC form. We estimate ε to be 40,000[27] from the literature values for similar molecules. It was estimated that in toluene, only 0.5% of the SP is in the MC form, compared to values of around 20% in methanol and ethanol. This demonstrates the effect that solvent polarity has on the relative stabilities of the open and closed forms. The non-polar solvents such as toluene stabilise the closed, uncharged spiropyran, whereas more polar solvents stabilise the charged merocyanine.

Table 2.3 Comparison of literature and experimental values of λmax, showing a solvatochromic shift in solvents of decreasing polarity according to Reichardt's E_T^N value of solvation power.

Solvent	**λ_{max} Literature[3]**	**λ_{max} Experimental**	**E_T^N**
Methanol	525	528	0.762
Ethanol	534	543	0.654
Butan-1-ol	548	545	0.602
Acetonitrile	Not tested	560	0.47
Acetone	562	567	0.355
Tetrahydrofuran	574	584	0.207
Toluene	596	590	0.099

This solvatochromic effect was observed in the solvents used, with Reichardt's polarity scale being used to measure the polarity of the solvents. A comparison of the literature values of absorbance wavelength with those experimentally obtained, along with the Reichardt E_T^N value, is summarised in Table 2.3. The experimental values are comparable with the literature values, as shown in Fig. 2.2, and there is an increase in absorbance wavelength as the Reichardt E_T^N value decreases.

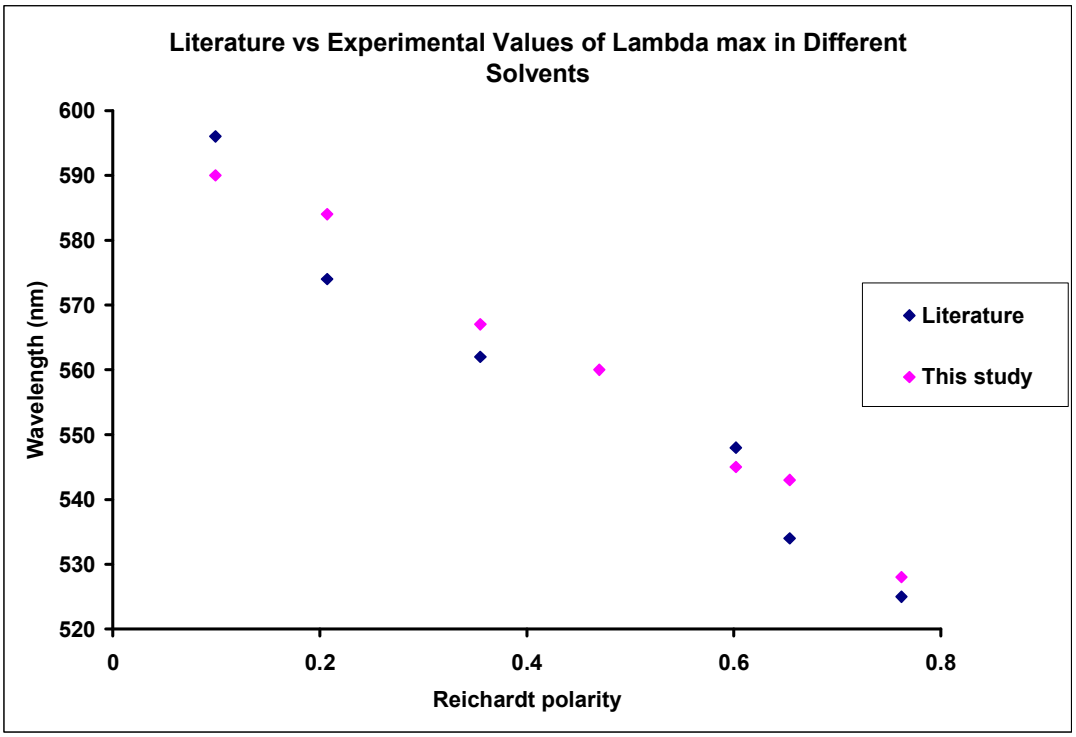


Figure 2.2 Comparison of the literature λ_{max} with the experimental values obtained in the course of this study. Both literature and experimental values show a decrease in λ_{max} as polarity increases. The experimental value found for toluene is ringed above, as it was less precise due to the relative instability of the MC in this highly non-polar solvent.

Figure 2.3 shows the solvatochromic shift in λ_{max} from 528 nm to 590 nm in solvents of decreasing polarity from methanol to toluene. Of course, water is the most polar

solvent, and would absorb at a lower wavelength than methanol, but water was not investigated in this study. The peak for toluene (ringed in Fig. 2.2) is highly irregular, due to the instability of the open form in this non-polar solvent. The coloured solution was highly sensitive even to light from the spectrometer, which switched the solution almost completely back to colourless thus resulting in low absorbance and irregular peak.

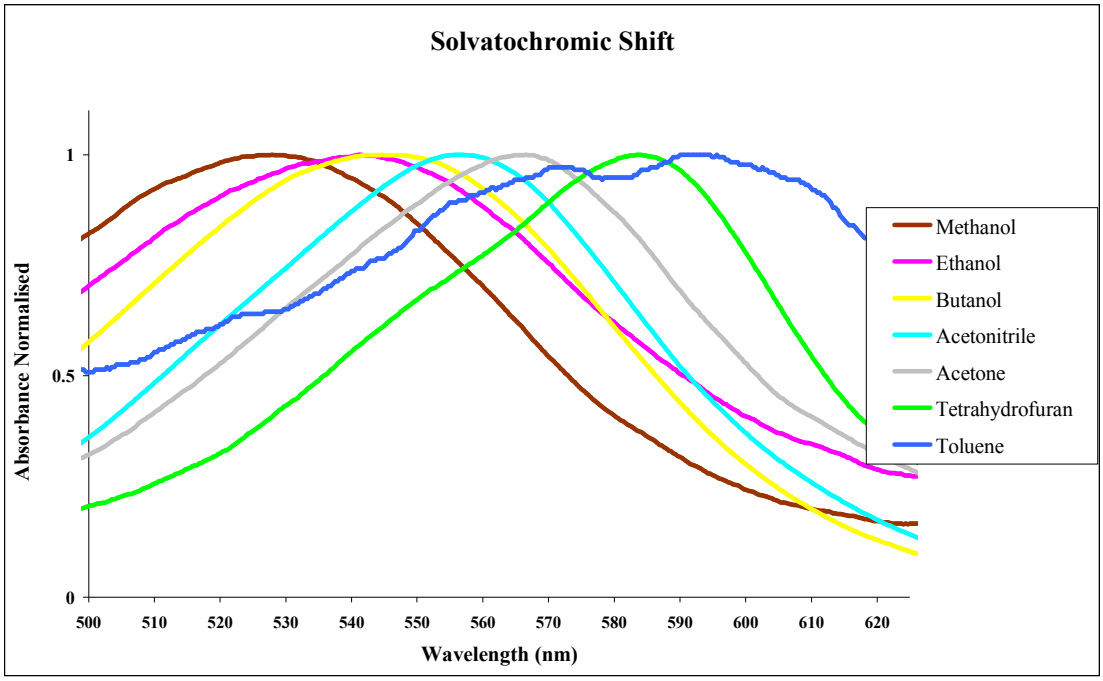


Figure 2.3 Hypsochromic solvatochromic shift of MC in solvents of decreasing polarity, classed according to Reichardt's polarity scale. The absorbance values are normalised, as different absorbance intensities were observed for different solvents. The peak for toluene is very irregular, as in this non-polar solvent, the open form was very unstable, and the solution was in fact switched back to colourless by the light from the spectrometer.

The solvatochromic effect can also be demonstrated photographically. Figure 2.4 shows spiropyran solutions in a range of solvents of varying polarity after UV irradiation. The solutions are highly coloured, with a blue solvatochromic shift observable as the colour varies from pink to purple to blue in solvents of decreasing

polarity. The same solutions after irradiation with visible light can be seen in Figure 2.5, and are colourless, demonstrating the reverse reaction to the closed spiropyran.

Figure 2.4 SP in solvents of decreasing polarity after UV irradiation. From left to right: methanol, ethanol, butan-1-ol, acetonitrile, acetone, tetrahydrofuran and toluene. The solvatochromic effect is observed, with a shift in colour from pink to purple to blue. A much weaker colour is observed for the toluene solution than the other solutions. This was due to the instability of the open form in toluene, the colour disappearing within seconds of removing the UV light source.

Figure 2.5 SP in solvents of decreasing polarity after visible irradiation. From left to right: methanol, ethanol, butan-1-ol, acetonitrile, acetone, tetrahydrofuran and toluene. Toluene switched back to colourless with ambient light, but all other solutions needed irradiation with desk lamp.

The variation in colour observed indicates the different wavelength of light absorbed by the chromophore. The colour seen by the naked eye can be equated to the colour absorbed, as described in Table 2.4. This indicates that the merocyanine is absorbing light in the wavelength range of 480-630 nm, depending on the polarity of its microenvironment.

Table 2.4 The relationship between colour observed, colour absorbed and wavelength (nm) of light absorbed – for example, a solution appearing violet absorbs in the yellow, and a red solution absorbs in the green, etc.

Colour observed	**Colour absorbed**	**Absorption wavelength**
Yellow	Violet	400-430 nm
Orange	Blue	430-480 nm
Red	Green	480-560 nm
Violet	Yellow	560-590 nm
Blue	Orange	590-630 nm
Green	Red	630-750 nm

There are many implications for the solvatochromism of spiropyran. One interesting potential application to exploit this phenomenon would be to use the merocyanine to probe polarity changes in local environments. In the absence of competing mechanisms (e.g. metal ion binding), a slight change in the absorbance wavelength of the merocyanine would indicate that an event had occurred to alter the polarity of the microenvironment.

2.4. Conclusions

In conclusion, this simple solvent study clearly demonstrated the solvatochromic behaviour of the spiropyran studied, with a blue solvatochromic shift observed in solvents of decreasing polarity. The experimental results obtained agree with those in the literature. In addition, the complex nature of the spiropyran-merocyanine equilibrium was highlighted, as it was found that polar solvents tended to stabilise the open MC, while non polar solvents favoured the closed SP. In any study of spiropyran as a chemical sensing agent, it is vital to have an understanding of all these issues, as small changes in the polarity of the microenvironment of the spiropyran can affect the equilibrium. In fact, this sensitivity to polarity could perhaps be exploited in using the spiropyran as a polarity probe. The remainder of this study however focuses on investigating the interactions between spiropyran and amino acid, building on the background understanding of the photochromic interconversion of spiropyran developed in this initial solvent investigation.

3. Spiropyran/Amino Acid Switching

3.1. Introduction

After investigating the switching characteristics of spiropyran in various solvents, the next phase was to add amino acid to the solution and spectrally characterise the switching in order to determine whether or not interactions between the zwitterionic merocyanine and amino acid are observed. For optimal stabilisation of the open merocyanine, it was important to ensure that the distance between the positive and negative charges on the amino acid was comparable to the distance between the charges on the merocyanine. The distance between the positive and negative charges on the merocyanine was estimated using molecular modelling[a] (4.67 Å). The energy minimised model is shown from different angles in Figures 3.1 and 3.2.

[a] The molecular modelling program used was Gaussian 03, used on a PC platform. Calculation Type: FOPT, Caluculation Method: RB3LYP, Basis Set: 6-311 G(d), E (HF): -1070.16908722 a.u., Dipole Moment: 11.8177 Debye.

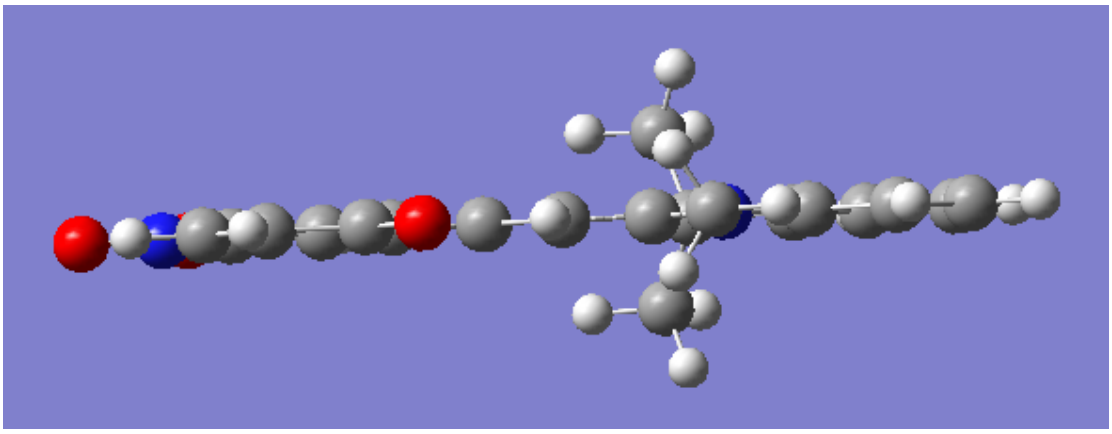


Figure 3.1 Side view of the merocyanine after Gaussian energy optimisation. The molecule can be seen to be planar.

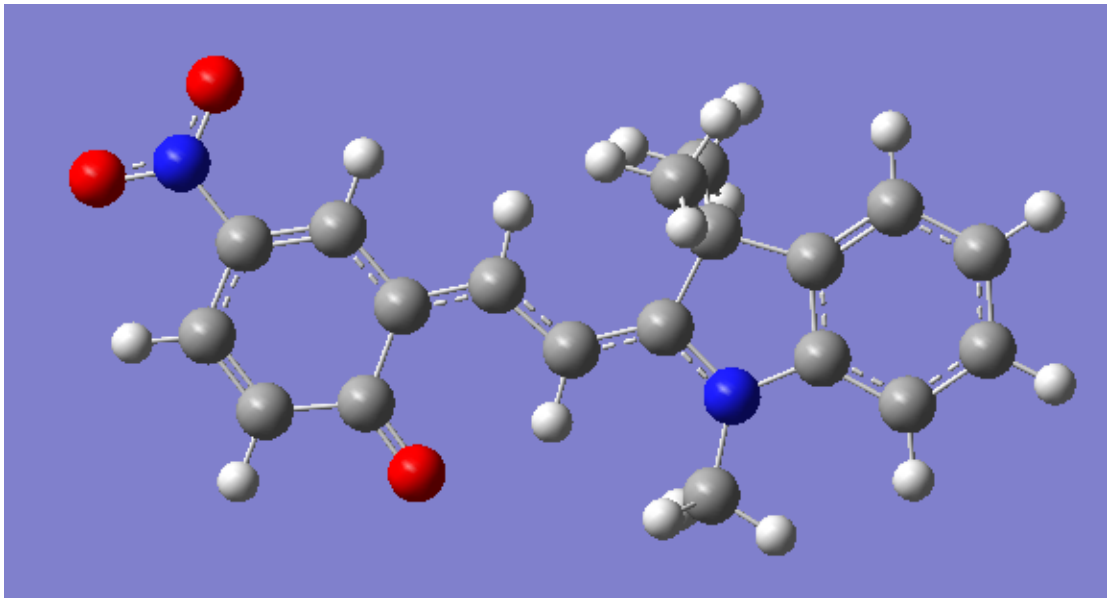


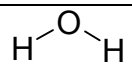
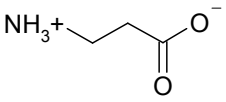
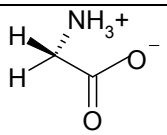
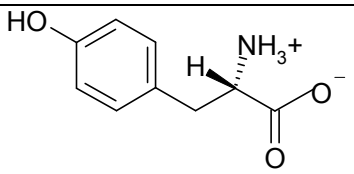
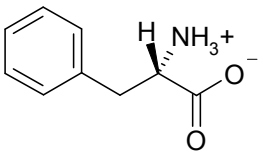
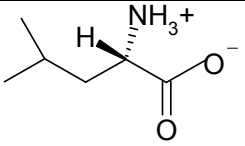
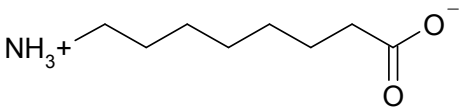
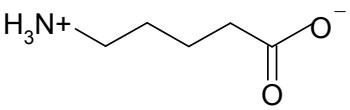
Figure 3.2 Aerial view of the merocyanine after Gaussian energy optimisation. The distance between the positively charged N and negatively charged O was found to be 4.67 Å.

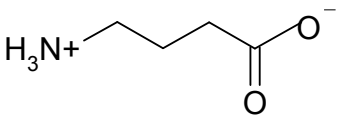
Depending on the spacer length of the amino acid, both intramolecular and intermolecular interactions are possible, though it would be expected that the intramolecular would be considerably stronger. This is demonstrated in Figure 3.3.

Figure 3.3 "Docking" of zwitterionic amino acids onto zwitterionic open form of spiropyran. In solution phase both intra- and intermolecular interactions can theoretically take place, depending on spacer length between the two charges on the amino acid. The relative strength of the intermolecular reactions compared to the intramolecular would be thought to be weaker, though this can only be determined by a comparative study. Surface immobilisation of the merocyanine could reduce the intermolecular interactions with amino acids observed in solution phase by controlling the distance between merocyanine units. This gives rise to greater selectivity for use of merocyanine in potential applications involving the detection of amino acids.

With this in mind, a range of amino acids were selected for study, and their spacer length measured using molecular modelling (MM2 energy minimisation in Chem 3D Ultra, version 10.0). These amino acids, their solution labels, abbreviated names and structures are summarised in Table 3.1. The 3D structures are shown in the Appendix.

Table 3.1 Amino acids used, with full name, abbreviated name, and structure of each amino acid tested, where d refers to the distance between the opposite charges in the zwitterions, calculated using Chem 3D Ultra version 10.0 MM2 energy minimisation. The 3D structures of these molecules are shown in the Appendix.

AA	AA abbreviation	Structure
Blank (Water)	-	
β-Alanine	β-Ala d=4.924 Å	
Glycine	Gly d=2.147 Å	
L-Tyrosine	L-Tyr d=2.147 Å	
L-Phenylalanine	L-Phe d=2.147 Å	
L-Leucine	L-Leu d=2.145 Å	
8-Aminocaprylic acid	8-ACA d=11.364 Å	
5-Aminovaleric acid	5-AVA d=7.511 Å	

γ -Aminobutyric acid	γ –ABA d=6.269 Å	

3.2.

3.3. Experimental

3.2.1. Materials

- Spiropyran: 6-Nitro-1', 3',3'-trimethylspiro[2H-1-benzopyran-2,2'-indolin]1',3'-Dihydro-1',3',3'-trimethl-6-nitrospiro, 98%, Sigma-Aldrich
- β-Alanine, >99%, Fluka
- Glycine, 99%, Sigma
- L-Tyrosine, >99%, Fluka
- L-Phenylalanine, >99%, Fluka
- L-Leucine, >99.5%, Fluka
- 8-Aminocaprylic acid, 99%, Aldrich
- 5-Aminovaleric acid, 97%, Aldrich
- γ -Aminobutyric acid, >99%, Sigma
- Acetonitrile, Aldrich
- Hexane, Aldrich

3.2.2. Instrumentation

The instrumentation used in this work was as follows:

- Electrolite Corporation Bond Wand UV lamp
- Perkin Elmer UV/VIS/NIR Spectrometer Lambda 900

3.2.3. Method

Single phase switching experiments

In an attempt to reproduce the stabilisation of the open form of the spiropyran by the zwitterionic amino acid described by Tsubaki *et al*[52], spiropyran solutions were prepared in acetonitrile, amino acid solutions were prepared in water and the two were mixed in a 4:1 ACN/H_2O v/v ratio, then exposed to UV light from the Bond Wand. The switching was monitored spectrally, with UV-Vis data being collected at various stages of the experiment. Eight solutions and their corresponding blanks were analysed, taking the UV-Vis spectrum of each solution before exposure to UV light, immediately after 2 minutes UV irradiation, and 1 hour after irradiation, after leaving the solutions exposed to ambient light. The eight solutions are described in Table 3.2.

Table 3.2 Composition of solutions tested where ACN is acetonitrile, amino acids are denoted by their three letter abbreviation, L-Tyr is L-Tyrosine, L-Ala is L-Alanine.

Solution	Volume Ratio	Spiropyran (SP)	Amino Acid
1	4:1	SP/ACN 10^{-4} M	L-Tyr/H_2O 10^{-4} M
2	10:1	SP/ACN 10^{-4} M	L-Tyr/H_2O 10^{-4} M
3	10:1	SP/ACN 10^{-4} M	L-Tyr/H_2O 10^{-3} M
4	4:1	SP/ACN 10^{-3} M	L-Tyr/H_2O 10^{-3} M
5	10:1	SP/ACN 10^{-3} M	L-Tyr/H_2O 10^{-3} M
6	4:1	SP/ACN 10^{-3} M	L-Ala/H_2O 10^{-3} M
7	4:1	SP/ACN 10^{-4} M	L-Tyr/H_2O 10^{-4} M
8	4:1	SP/ACN 10^{-4} M	L-Ala/H_2O 10^{-4} M

Two phase switching experiments

To counteract the stabilisation effect that the addition of water was having on the merocyanine, two-phase switching experiments were proposed. The aim of these experiments was to investigate whether the amino acid, normally insoluble in organic solvents, could be induced to transfer from the aqueous to the organic phase when the spiropyran was in its open, charged merocyanine form. The solutions were prepared by adding equal volumes of 10^{-4} M spiropyran (SP) in hexane and 10^{-4} M amino acid (AA) in water. For convenience the solutions were labelled numerically, as described in Table 3.3.

Table 3.3 Explanation of solution labels, with full name and abbreviated name of each amino acid tested, where d refers to the distance between the opposite charges in the zwitterions, calculated using Chem 3D Ultra Version 10.0 MM2 energy minimisation.

Solution No.	AA	AA abbreviation
1	Blank (Water)	-
2	β-Alanine d=4.924 Å	β-Ala
3	Glycine d=2.147 Å	Gly
4	L-Tyrosine d=2.147 Å	L-Tyr
5	L-Phenylalanine d=2.147 Å	L-Phe
6	L-Leucine d=2.145 Å	L-Leu
7	8-Aminocaprylic acid d=11.364 Å	8-ACA
8	5-Aminovaleric acid d=7.511 Å	5-AVA
9	γ -Aminobutyric acid d=6.269 Å	γ –ABA

The solutions were irradiated with UV light for 2 minutes, then photographed. The vials were shaken, first for 5 seconds and photographed, then for a further 30 seconds to maximize contact between organic and aqueous phases, and photographed again. Results were documented photographically and spectrophotometrically using the UV-Vis spectrometer. The vials were then exposed to ambient light for 10 minutes, and the colour monitored using UV-Vis spectroscopy and digital photography.

3.4. Results and Discussion

3.4.1. Single phase switching

When the UV-Vis spectra of the merocyanine in acetonitrile in the presence of aqueous solutions of Alanine and Tyrosine were compared to those of the water blank, no substantial difference could be observed. This can be seen in Figures 3.4 and 3.5. A similar colour intensity and decay were observed in the spiropyran solutions with or without amino acid present, and this was the case for all amino acids tested.

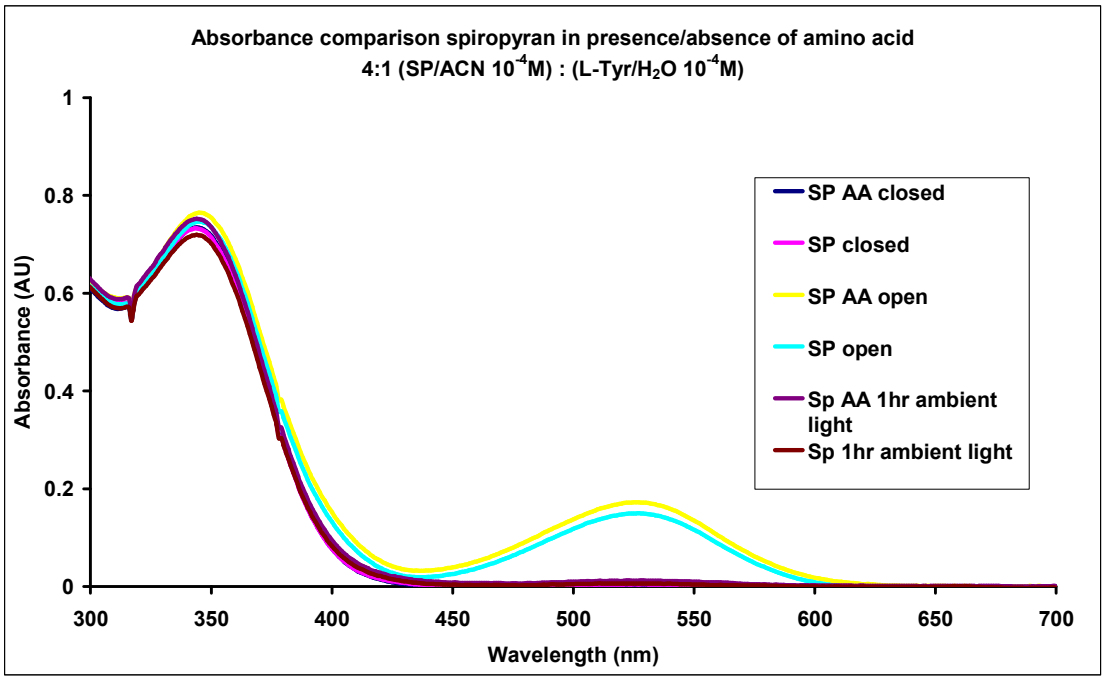


Figure 3.4 Absorbance vs. wavelength of 4:1 SP/ACN 10^{-4} M: L-Tyr/H_2O 10^{-4} M under conditions described in legend. The results show that there is no significant difference between curves 1 and 2, 3 and 4, 5 and 6. However, this experiment merely monitors the beginning and end of the colour change. This would indicate that amino acid docking is not having a significant effect on the steady state ring open and closed behaviour of the spiropyran.

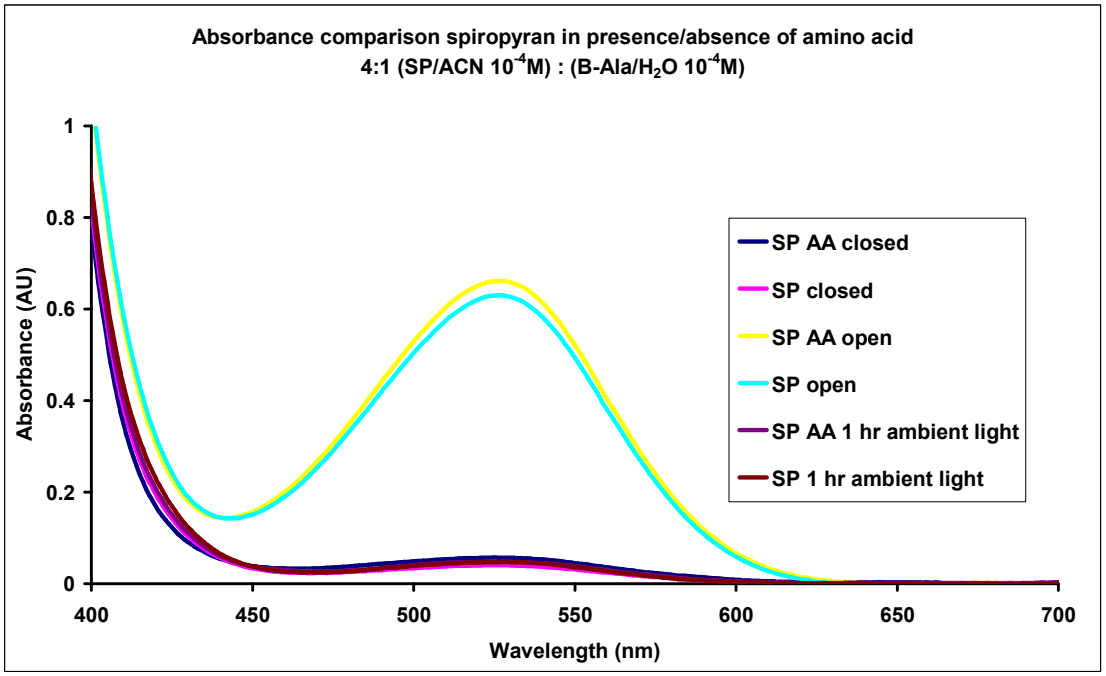


Figure 3.5 Absorbance vs. wavelength of 4:1 SP/ACN 10^{-4} M: ß-Ala/H_2O 10^{-4} M under conditions described in legend. The results show that there is no significant difference between curves 1 and 2, 3 and 4, 5 and 6. Again, this experiment merely monitors the beginning and end of the colour change. This would indicate that amino acid docking is not having a significant effect on the steady state ring open and closed behaviour of the spiropyran.

This behaviour may arise because of the presence of water in the solution, as this will stabilise the merocyanine. Ideally the docking experiments should be carried out in a much less polar environment, so that the amino acid interactions could be observed more closely. However, this is difficult to accomplish due to the limited solubility of amino acids in organic solvents. The amino acids used, namely L-Alanine and L-Tyrosine, were found to be insoluble in a range of organic solvents, including the polar organic solvent such as ethanol. Another contributing factor may be the concentration of amino acid used. In the experimental design used, the SP was almost always in excess. The problem with having such a low proportion of amino acid solution present is that there may not be enough amino acid molecules to drive the binding, as for the effect to be observed, perhaps it is necessary for the amino acid to

be in excess. In an attempt to counterbalance this problem, the concentration of the amino acid solution was increased by a factor of 10 to 10^{-3} M, while the spiropyran concentration was kept at 10^{-4} M. The dilution factor then means that the spiropyran and amino acid in solution are equimolar. The results are displayed in Figures 3.6 and 3.7. It was found that there was no significant difference between the merocyanine or spiropyran in the presence or absence of the amino acid. Perhaps the amino acid concentration was still too low to observe a difference in absorbance.

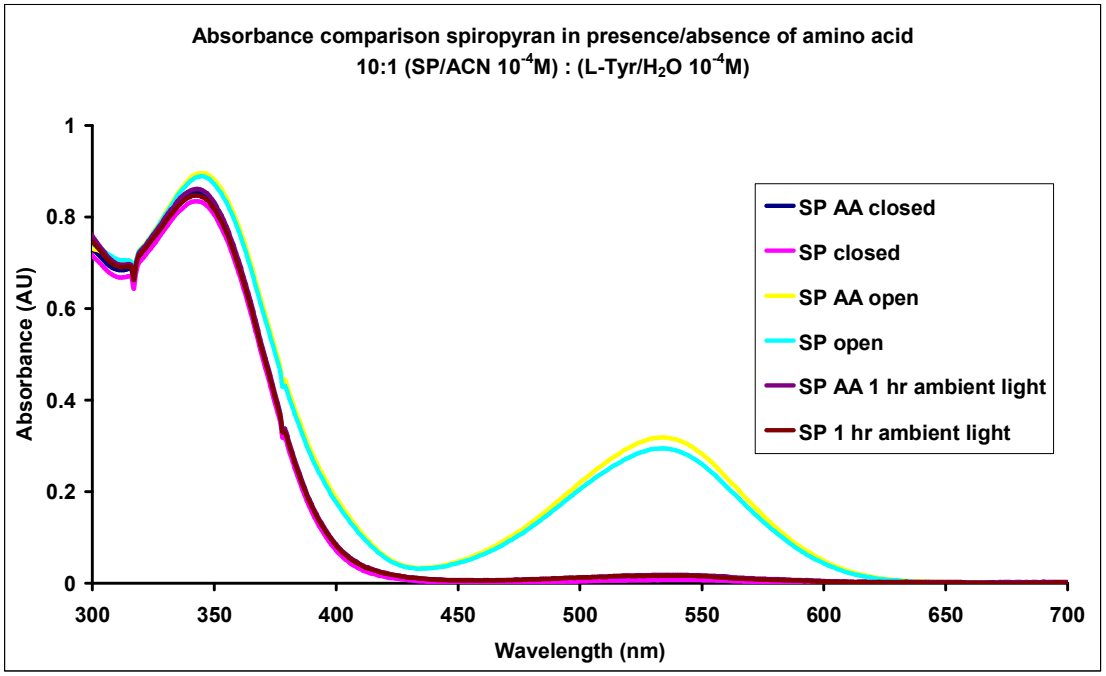


Figure 3.6 Absorbance vs. wavelength of 10:1 SP/ACN 10^{-4} M: L-Tyr/H_2O 10^{-4} M under conditions described in legend. The results show that there is no significant difference between the spiropyran in the presence or absence of AA before, immediately after irradiation with UV light, and after exposure to ambient light for 1 hour. Therefore, amino acid interactions with spiropyran cannot be confirmed using this method. The slight increase in absorbance at the λ_{max} for SP open in the presence of AA when compared to SP open without AA is minimal, and falls within the experimental error range.

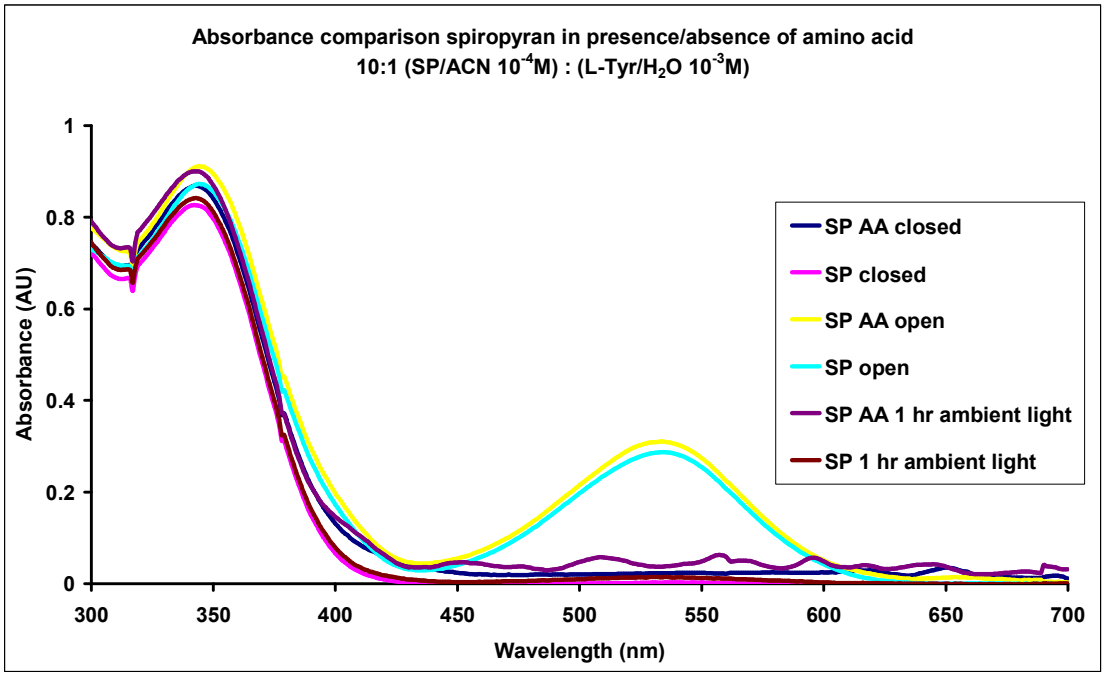


Figure 3.7 Absorbance vs. wavelength of 10:1 SP/ACN 10^{-4} M: L-Tyr/H_2O 10^{-3} M under conditions described in legend. The results show that there is no significant difference between spiropyran in the presence or absence of AA before, immediately after irradiation with UV light, and after exposure to ambient light for 1 hour. Therefore, amino acid interactions with spiropyran cannot be confirmed using this method, although, again, the slight increase in absorbance at the λ_{max} for SP open in the presence of AA when compared to SP open without AA suggests that a slight effect may be taking place.

3.4.2. Two phase switching

The concept behind these two-phase switching experiments was as follows. Under UV light spiropyran (10^{-4} M) ring opening takes place in the organic phase (hexane). Amino acid (10^{-4} M) present in an aqueous layer is in contact with the organic phase. When ring opening occurs the merocyanine may be able to act as a host and attract the charged amino acid across the phase barrier as an ion-pair, as suggested by Sunamoto *et al*[50]. Under normal conditions the amino acid would not be soluble in the organic phase, but in theory the presence of the highly charged merocyanine in the organic layer would favourise solubilisation of the amino acid by forming an electronically neutral host-guest pair through the electrostatic docking interaction. Figure 3.8 shows the nine two-phase solutions immediately before UV irradiation. Vial 1 is the water control, vials 2-9 are the amino acid solutions described in Table 3.3. All the solutions are colourless before exposure to UV light. After two minutes irradiation with the Bond Wand the organic (upper) phase is highly coloured in all vials (Fig. 3.9), indicating that ring opening of the spiropyran has taken place. After 5 seconds shaking some of the merocyanine has migrated to the aqueous phase in all vials, but some colour still remains in the organic phase (Fig. 3.10). However, after 30 seconds shaking, the merocyanine has been completely transferred to the aqueous phase of all the samples, as there is no longer any colour in the organic layer (Fig. 3.11). After 10 minutes ambient light, it is observed that in all the vials all colour has decayed (Fig. 3.12.), and some precipitate can be seen, suggesting that aggregation of spiropyran molecules may be taking place. Initially it was thought that by shaking the two phases the amino acid would come in contact with the charged merocyanine and transfer to the organic phase. However, the reverse was observed: the merocyanine rapidly

transferred to the polar aqueous solution. There appeared to be a slight difference in colour intensity between the spiropyran-water control and the spiropyran in the presence of the various amino acids. This could be due to different strengths of interaction between the different amino acids and the merocyanine.

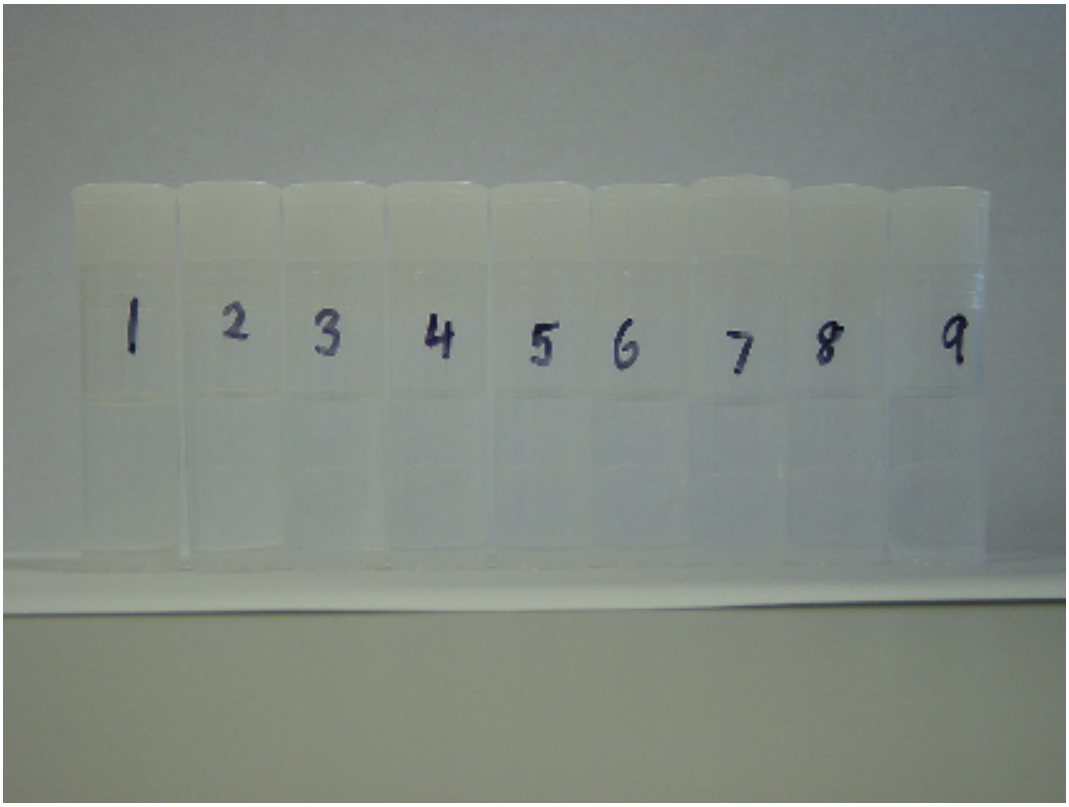


Figure 3.8 Solutions 1 to 9 before UV irradiation, all solutions colourless, organic phase (hexane) on top, aqueous phase below. SP 10^{-4} M in hexane, AA 10^{-4} M in water.

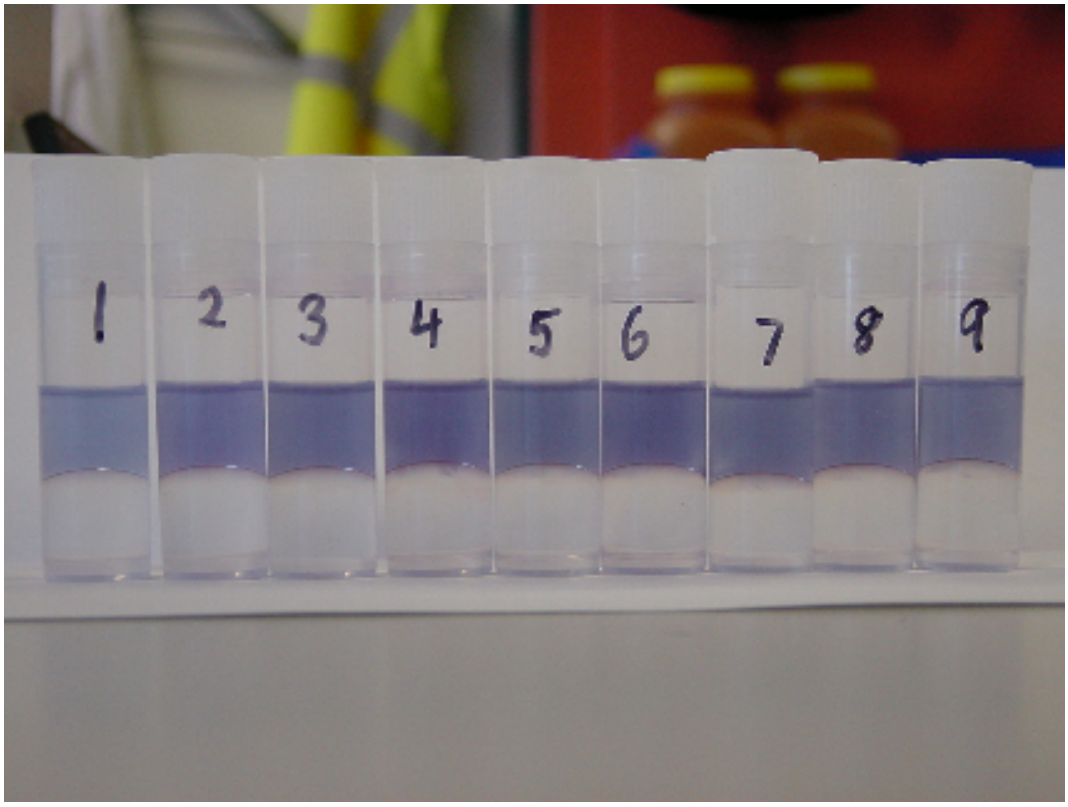


Figure 3.9 Solutions 1 to 9 immediately after 2 mins UV irradiation. Spiropyran is now in the MC open form in organic phase, some cross-phase migration observed at interface, and can be seen in picture inset. The pink colour observed as the spiropyran crosses the interface is consistent with the solvatochromic effect, as the absorbance shifts to a lower wavelength in the more polar solvent.

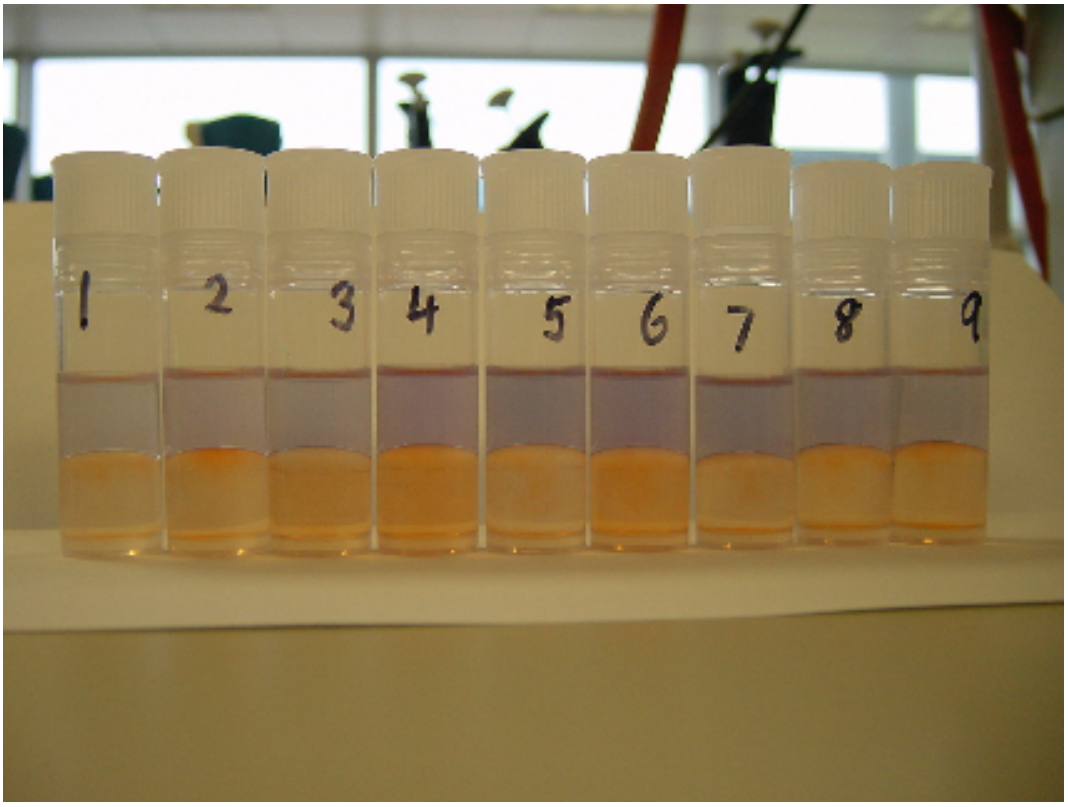


Figure 3.10 Solutions 1 to 9 after 2 mins UV irradiation and after 5s shaking. Merocyanine in organic phase, some MC has migrated to aqueous phase giving rise to the orange colour.

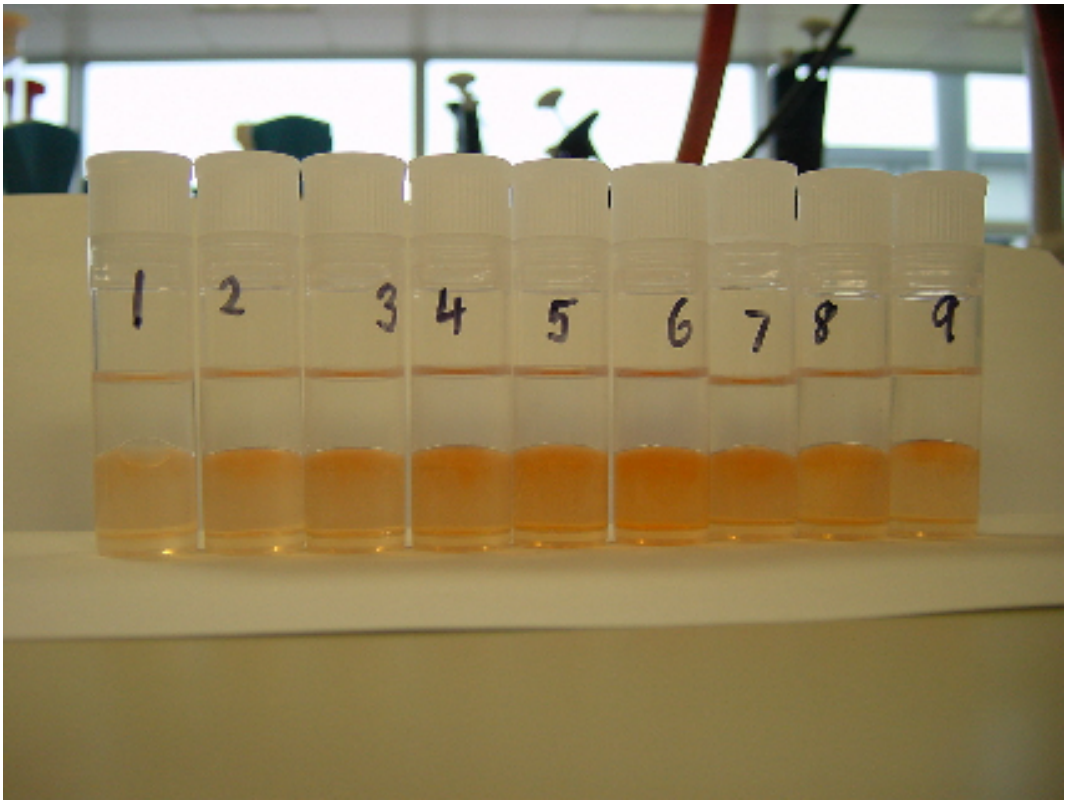


Figure 3.11 Solutions 1 to 9 after a further 30 seconds shaking. Orange colour in varying intensity in the presence of different amino acids. The amino acids were all present in the same concentration (10^{-4} M), which suggests that the variation in colour intensity may be due to different interactions between the MC and the different amino acids tested.

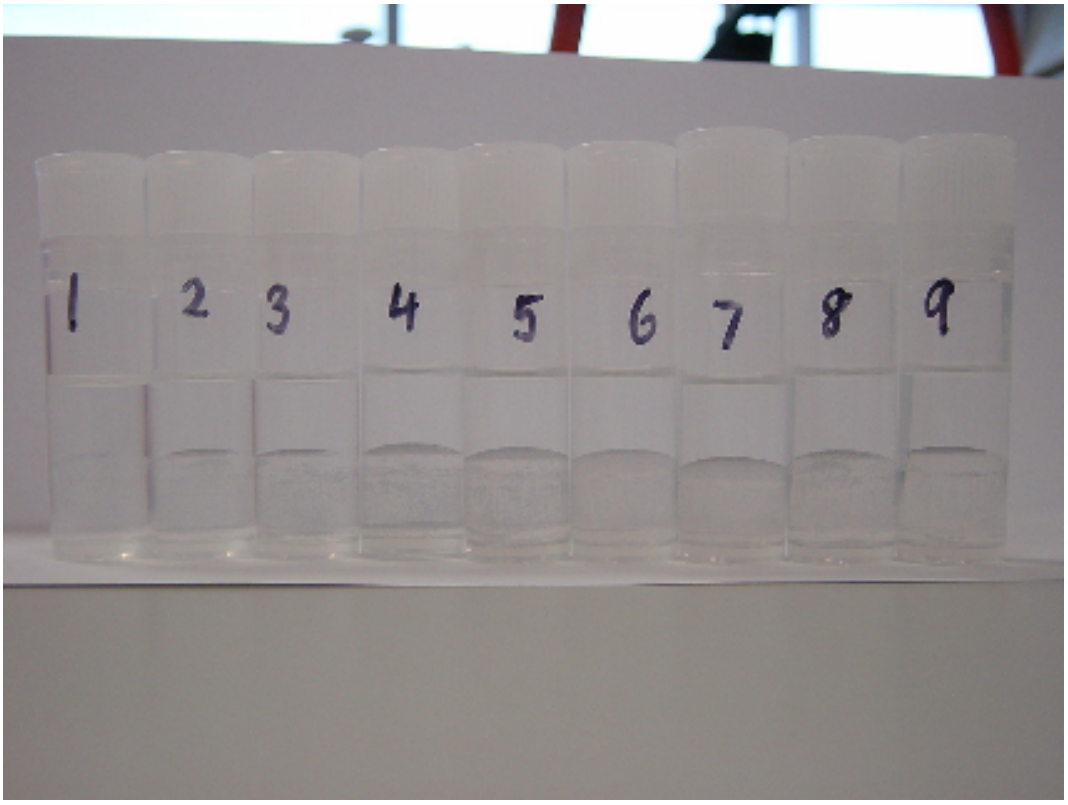
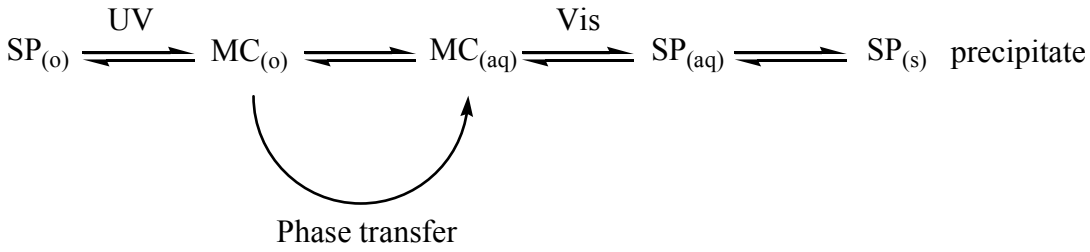


Figure 3.12 Solutions 1 to 9 after 10 minutes ambient light. Orange colour has disappeared. Some precipitate visible.

These results are very interesting as they demonstrate the possibility of photo-induced phase transfer. The processes happening are as follows:



The UV-Vis spectra of the aqueous phase after MC migration were obtained. The results are shown in Fig. 3.13. It was found that the absorbance intensity varied in decreasing order as follows: L-Phe, L-Tyr, L-Leu, γ-ABA, 5-AVA, 8-ACA, Gly, β-Ala, water. This suggests that the presence of the amino acids in some way stabilises

the merocyanine, increasing absorbance intensity compared to water. The variation in the colour intensity observed may be due to varying levels of interaction between the merocyanine and the different amino acids. These interactions may take the form of intimate association between the zwitterionic MC and AA, or alternatively the results observed could be due to a bulk effect, depending on the ionic strength or polarity of the solution, which again depends on the amino acid used. Also, the nature of the experimental set-up used meant that there were slight variations in UV exposure for spatial reasons, and the time taken to measure the UV-Vis spectrum of each sample. This means that the UV-Vis spectra obtained could have inherent errors and comparisons between samples cannot be fully relied upon.

There is no shift in the wavelength of maximum absorbance for all amino acids tested, indicating that there is no charge transfer complex forming between the merocyanine and amino acid, but ionic interactions may still have taken place.

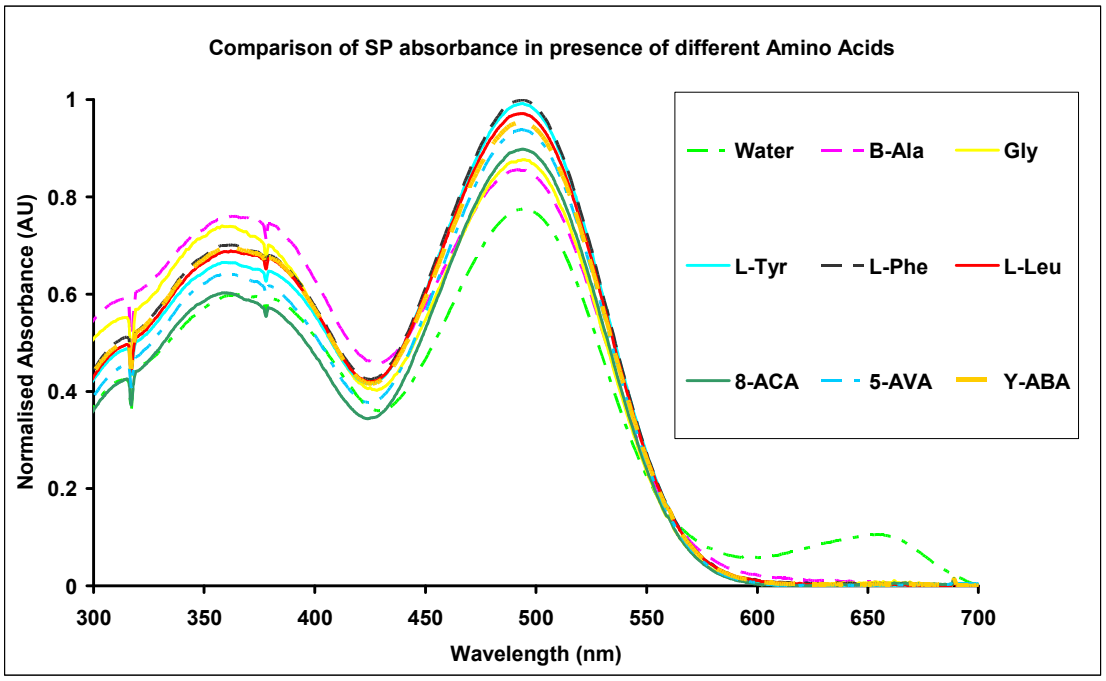


Figure 3.13 Normalised relative absorbance of merocyanine in the presence of different amino acids in aqueous phase. The maximum absorbance wavelength is 492 nm, indicating a blue shift from the wavelength observed in the solvents previously studied. This is in line with the solvatochromic shift to lower wavelengths in solvents of increasing polarity. The peak observed in water at 650 nm indicates that a contaminant may have been present.

Table 3.4 Table summarising and comparing the data obtained from the UV-Vis spectra of the MC in the presence of water and different amino acids. The amino acids are listed according to decreasing absorbance in two wavelength regions – approximately 360 nm and 500 nm. The pI values of the amino acids are also listed in order to determine whether a trend in absorbance emerges depending on the pI of the amino acid.

Absorbance Order		**Absorbance Order**	
λ_{max} ~360 nm	**pI**	**λ_{max} 492 nm**	**pI**
β-Ala	6.90	L-Phe	5.48
Gly	5.97	L-Tyr	5.66
L-Phe	5.48	L-Leu	5.98
L-Leu	5.98	γ-ABA	7.30
L-Tyr	5.66	5-AVA	7.52
γ-ABA	7.30	8-ACA	~8
5-AVA	7.52	Gly	5.97
Water	7	β-Ala	6.90
8-ACA	~8	Water	7

From examining the data in Table 3.4 we can see that at 492 nm there is a general increase in absorbance intensity as the pI decreases. It would appear that the amino acid with the lowest pI will shift the SP-MC equilibrium to the right to a greater extent than an amino acid with a higher pI. This is a very interesting observation. There are some exceptions to this trend, namely Gly and Ala, which have the lowest absorbances of the amino acids tested but have low pI values. Interestingly, these are the two smallest amino acids tested, and this may have an effect on any interactions taking place. These results show that the addition of amino acids with different

structures, polarities, and isoelectric points, definitely has an effect on the MC. The nature of this effect is indeterminate, but merits further study. An in depth examination of the MC-AA interactions in water could yield some very useful information, especially since the system would be reduced to a single phase.

Any interactions that may be taking place must be quite weak, or perhaps the interactions are such that they do not lead to a change in absorbance wavelength. Kinetics experiments to study the rate of decay or the merocyanine in the presence of amino acids would perhaps be more powerful at drawing out any interactions or structural effects. Therefore it was decided to concentrate on optimising an experiment to monitor and compare the kinetics of the reverse reaction from merocyanine to spiropyran in the presence of a carefully selected range of amino acids.

3.5. Conclusions

The single phase switching experiments carried out showed similar results in the presence of the two amino acids tested and the water blank. It appears that the polar water molecules stabilise the open MC to a great extent (which agrees with the results of our solvent study, above). It could not be confirmed that any spiropyran - amino acid interactions were taking place using this method, perhaps due to the low relative concentration of amino acid present. A similar experimental procedure was employed by Tsubaki *et al*[52] in their system for the enantiomeric recognition of amino acids using chiral spiropyran. However, the structure of the spiropyran used was quite different from the subject of this study. Tsubaki's spiropyran (Fig. 1.9) had a structure which could facilitate a three point interaction with the amino acid, potentially enhancing the host-guest binding affinity.

Two-phase switching experiments were then proposed to try to keep the spiropyran in a non-aqueous environment so that any zwitterionic interactions between the merocyanine and amino acids could be observed in the organic phase. However, in these experiments it was found that rather than the zwitterionic amino acid being attracted by the zwitterionic merocyanine, and brought into the organic phase, the merocyanine itself went into the aqueous phase. This demonstrated the strong affinity that the charged merocyanine has for water. Sunamoto *et al*[50] has reported the light-modulated transfer of amino acid from aqueous to organic phase through the photochromism of spiropyran, but there are several differences between his system and the one used in these experiments. Sunamoto's system employed a liposome membrane between phases (see Fig. 1.9), ensuring that the SP-MC was retained in the

organic phase, and not released into the aqueous on either side. This enabled recycling of the SP used, meaning that the SP was continuously being switched to the MC form and back again. Perhaps this is necessary to prevent the MC from crossing to the aqueous phase. In addition, Sunamoto's procedure did not involve sample shaking.

In this study, light-modulated phase transfer was shown to take place, and the spectral analysis of the merocyanine in the aqueous samples yielded some very interesting results. The variation in colour intensity between the amino acid samples suggests amino acid – spiropyran interactions, though a more in-depth study is needed to develop a better understanding of what is taking place at molecular level. The relative concentration of AA to SP may be very important for these interactions also, as previously mentioned in the single-phase study. Again, perhaps it is necessary for the amino acid to be present in a large excess in order to be able to see what interactions may be taking place. Otherwise, it is possible that the SP-MC interconversion could mask any other binding.

Therefore, the spectral characterisation of the spiropyran – merocyanine equilibrium suggested that some kind of interaction was taking place to varying degrees between the amino acids and merocyanine. Consequently, it was decided to move to a series of kinetics experiments in order to ascertain whether the rate of decoloration of the MC is affected by the presence of amino acids, which would help to shed light on the interactions taking place.

4. Kinetics study

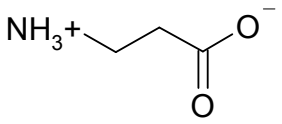
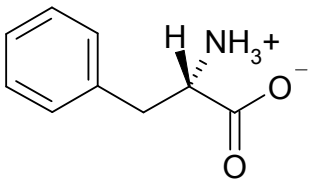
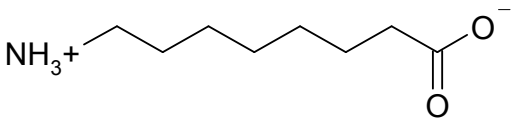
4.1. Introduction

Considering that it was difficult to detect merocyanine-amino acid interactions through comparing absorbance spectra, it was decided to carry out a kinetics study to investigate whether the presence of amino acid influenced the rate of ring closure after exposure to UV light. There are two types of kinetic study reported in the literature for the study of the thermal ring closure of the merocyanine – one over a long time scale, another over a very short time scale[57]. The first has been carried out at room temperature, measuring absorbance using a regular spectrophotometer over time periods ranging from a few seconds to several days. The second type of kinetic study is typically carried out at low temperatures measuring absorbance changes over time periods of less than one second.

The results from both types of studies indicate that spiropyran undergoes very complex and rapid molecular changes due to a series of photo or thermal reactions immediately after colouration, and a relatively simple first order colour decay over longer time periods. Therefore, the thermal decoloration rates over longer time periods are the most studied, and it is this type of experiment that was envisaged for this study. If strong interactions are taking place between the zwitterionic merocyanine and the zwitterionic amino acid, it is expected that the rate of decay of the coloured MC back to SP would be slowed down to an extent that would depend on the strength of the interaction.

The importance of matching the spacer length of the amino acid to that of the merocyanine has already been mentioned. To investigate this, the following amino acids were selected for an in-depth study; ß-Alanine (Ala), Phenylalanine (Phe) and 8-Aminocaprylic acid (ACA) (see Table 4.1). A comparison of the interactions between these zwitterionic molecules should clarify the effect of spacer length on interactions, and give an insight into the impact this has on intra and intermolecular interactions.

Table 4.1 The amino acids selected for study with varying charge separation distance

β-Alanine (Ala) Charge separation 4.924 Å	
Phenylalanine (Phe) Charge separation 2.147 Å	
8-Aminocaprylic Acid (ACA) Charge separation 11.364 Å	

If indeed "docking" is taking place, it can be supposed that the merocyanine will be stabilised, thus altering the rate of the reverse reaction to the closed spiropyran molecule. However, in order to better understand what is happening at a molecular level when aqueous amino acid is added to spiropyran in an organic solvent, it is necessary to consider the various intermolecular interactions that can take place. In this case we regard electrostatic interactions and hydrogen bonding as the most

relevant. The electrostatic interaction is strictly defined as the Coulombic interaction between the charge distributions of the molecules, when they have not been distorted by the interactions[58]. It appears that the electrostatic interactions, when accurately calculated, often dominate the orientation dependence of the total intermolecular potential[59]. However, the analysis of Sokalski *et al* of the electrostatic potential around model blocked peptides showed that it can be considerably distorted around the functional groups which can form hydrogen bonds[60]. This is very interesting, as in this study so far it was found that the proposed strong electrostatic interactions were not in fact observed. This may be due to a number of factors, one of which could involve the hydrogen bond stabilisation of the merocyanine.

Although the hydrogen bond is weaker than covalent bonds, it is stronger than the Van der Waals forces between non-polar molecules. The Coulombic attraction between polar molecules contributes towards the force of the hydrogen bond, however the hydrogen bond is considered to be more than a simple electrostatic interaction. For a hydrogen bond to form, both proton donating and accepting groups are needed, and whether they are in separate molecules is irrelevant, so long as the proper spatial positioning can be attained. When a hydrogen atom which is covalently bonded to an electronegative atom A, such as oxygen, nitrogen or a halogen, is able to approach another electronegative atom B, a relatively strong interaction energy is observed between them[61]. As the proton donor, AH, approaches the acceptor, B, the hydrogen atom forms a bridge between them. The lone pair of B is drawn towards the bridging proton to form a weak bond, represented by $AH^{\cdots}B$[62]. Typical strengths of neutral hydrogen bonds are in the 2-16 kJ mol^{-1} range, whereas for ionic hydrogen bonds the range raises to 9-45 kJ mol^{-1} [63]. These values are compared with sample energies for

other bonding interactions in Table 4.2. These figures give a general indication of the value of bond strength across the various classes. Actual values vary widely depending on the specific interactions involved.

Table 4.2 Some sample energies for various bonds and interactions[64].

Interaction	**Sample Energy (kJ mol^{-1})**
Ionic bond	700
Covalent bond	400
Van der Waals interactions	8
Hydrogen bonding	20

Therefore, there are a number of factors which will affect the stabilisation of the merocyanine, which the proposed kinetic study should help to elucidate.

4.2. Experimental

4.2.1. Materials

- Spiropyran: 6-Nitro-1', 3',3'-trimethylspiro[2H-1-benzopyran-2,2'-indolin]1',3'-Dihydro-1',3',3'-trimethl-6-nitrospiro, 98%, Sigma-Aldrich
- β-Alanine, >99%, Fluka
- L-Phenylalanine, >99%, Fluka
- 8-Aminocaprylic acid, 99%, Aldrich
- Hydrochloric acid, Aldrich
- Sodium hydroxide, Aldrich
- Ethanol, Aldrich
- Toluene, Aldrich

4.2.2. Instrumentation

The instrumentation used in this work was as follows:

- Metrohm 713 pH Meter
- Electrolite Corporation Bond Wand UV lamp
- Bio-tek Instruments KC4 Universal Microplate Spectrophotometer

4.2.3. Method

A 10^{-3} M spiropyran in ethanol solution was prepared. The amino acids were made up in water at a concentration of 10^{-1} M. The pH of the amino acid solutions was adjusted using a calibrated Metrohm 713 pH meter and hydrochloric acid and sodium

hydroxide solutions so that 3 samples were obtained for each amino acid – one with the pH below the isoelectric point (pI), one at the pI, and another above the pI. The amino acids and their pHs are summarised in Table 4.3.

Table 4.3 Summary of amino acid solutions prepared at different pHs.

Amino Acid	**pH**
Alanine below pI	5.90
Alanine at pI	6.90
Alanine above pI	7.90
Phenylalanine below pI	4.48
Phenylalanine at pI	5.48
Phenylalanine above pI	6.48
Aminocaprylic acid below pI	7.00
Aminocaprylic acid at pI	8.00
Aminocaprylic acid above pI	9.00

15µL of the aqueous amino acid solution (10^{-1} M) was added to 200µL spiropyran ethanol solution (10^{-3} M) in a 96 well plate. Each sample was repeated 5 times. Water was used as a control, with 15µL being added to 200µL spiropyran. The delicate equilibrium between the SP and MC is affected both by the exposure to ambient light during sample preparation, and also the altered polarity of the solution due to the addition of the amino acid sample in water. Therefore, the samples were left to equilibrate overnight in the dark. Then the plate was illuminated with UV light from the Bond Wand for 3 minutes (the Bond Wand was allowed to heat up for 3 minutes before the experiment). Then the absorbance at 546 nm was noted every minute for 4

hours and 30 minutes. The procedure was repeated to investigate inter-day reproducibility, and Excel Solver was used to determine the rate constant for each sample.

The entire UV-Vis spectrum of each sample was then recorded. The plate was exposed to ambient light for 10 minutes, and the UV-Vis spectrum recorded again.

4.3. Results and Discussion

When the absorbance at 546 nm was monitored at 1 minute intervals over a 4 hour 30 minute period subsequent to 3 minutes irradiation with UV light, some interesting trends emerged. It was found that the absorbance was substantially lower in the solutions containing ACA (Fig. 4.1), and that the rate of decay of MC in these samples was much faster than the other amino acids tested. The sample containing water as a blank was found to have the highest absorbance after 4 hours 30 minutes, even though several of the amino acid containing solutions started with higher absorbance than water (Fig. 4.2.).

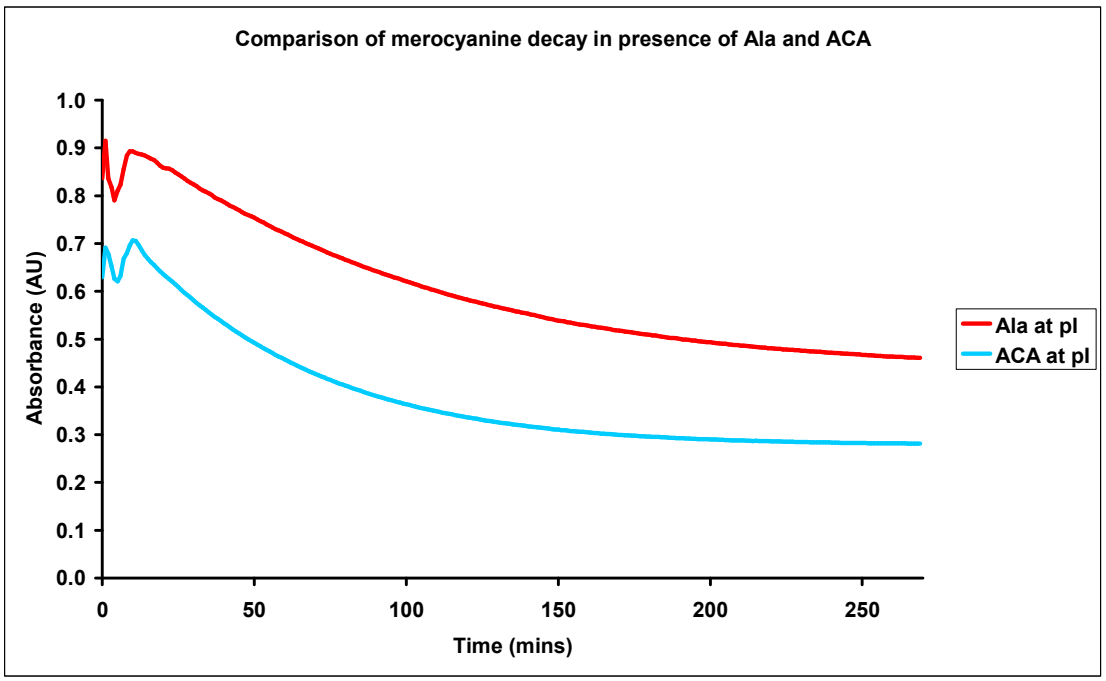


Figure 4.1 Comparison of absorbance and rate of decay of merocyanine in the presence of Ala and ACA. Though both were added at the same concentration, there is a large initial difference in absorbance between the two, which continues throughout the kinetics experiment. Ala can be seen to stabilise the merocyanine to a greater extent than ACA, and ACA has a faster rate of decay.

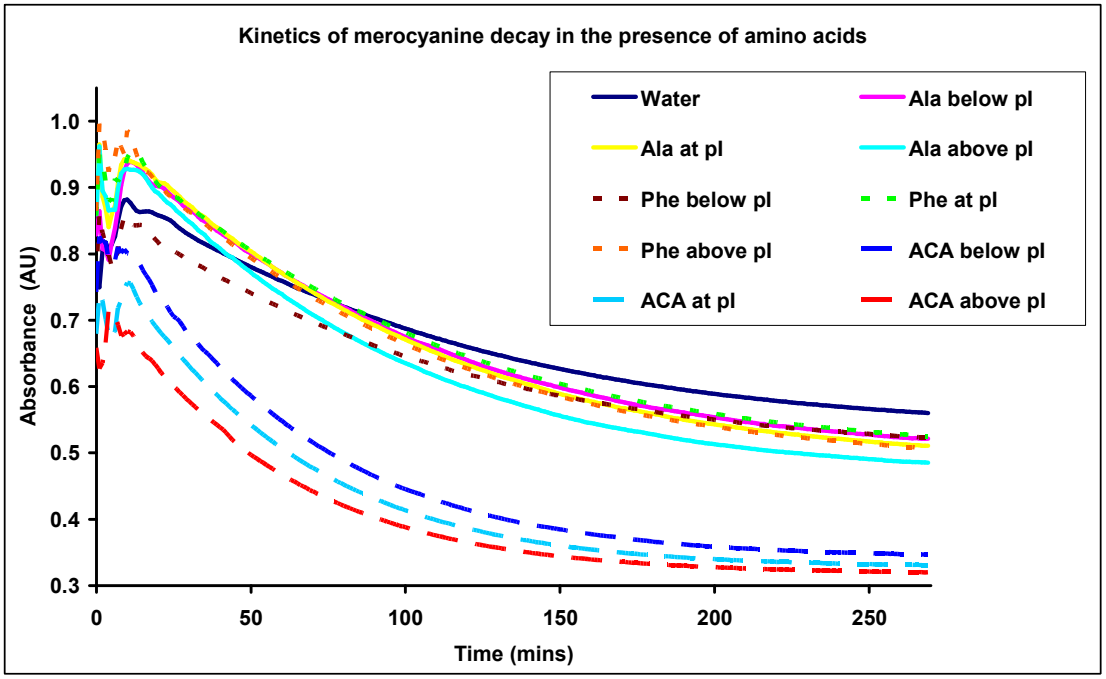


Figure 4.2 Decaying absorbance of merocyanine over time, in the presence of water and a selection of amino acids at different pHs. The irregularity in the spectra over the first 20 minutes is thought to be an artefact of the instrument used. Water has the highest absorbance after 4 and a half hours, followed closely by the Phe and Ala solutions, with a marked drop in absorbance for the ACA solutions. The ACA solutions are seen to have a faster rate of decay than the other amino acids and water.

As can be seen in Fig. 4.2, there is a large difference between the absorbance values for ACA and the other samples. This indicates that there are some interactions taking place between the amino acids and the merocyanine.

There are 2 effects observed when the results are compared. There is the variation in initial absorbance or colour intensity, and also the difference in the rate of decay of merocyanine between the amino acids tested. The difference in initial absorbance, showed clearly in Fig. 4.2 can be denoted by K_{eq}, where:

$$K_{eq} = \frac{[MC]}{[SP]} \qquad \textbf{Equation 5}$$

The difference in absorbance intensity is related to the relative amounts of merocyanine and spiropyran present in solution. This in turn depends on the stabilisation of the merocyanine by the solvent. The merocyanine tends to be more stable in a polar solvent, so perhaps the different amino acids affect the polarity of the local environment of the merocyanine to greater or lesser degree based on their own structure and charge.

More information about merocyanine-amino acid interactions may be extracted from the rate of the merocyanine decay back to spiropyran.

$$SP \underset{K_R}{\overset{K_F}{\rightleftharpoons}} MC$$

The rate of reverse reaction, indicated by K_R, varies between samples. In theory, this rate depends on the strength of interactions between the amino acid molecules and the merocyanine. The stronger the interactions the slower the rate of reversion to spiropyran.

The difference in colour intensity was visible to the naked eye, as demonstrated in Fig. 4.3.

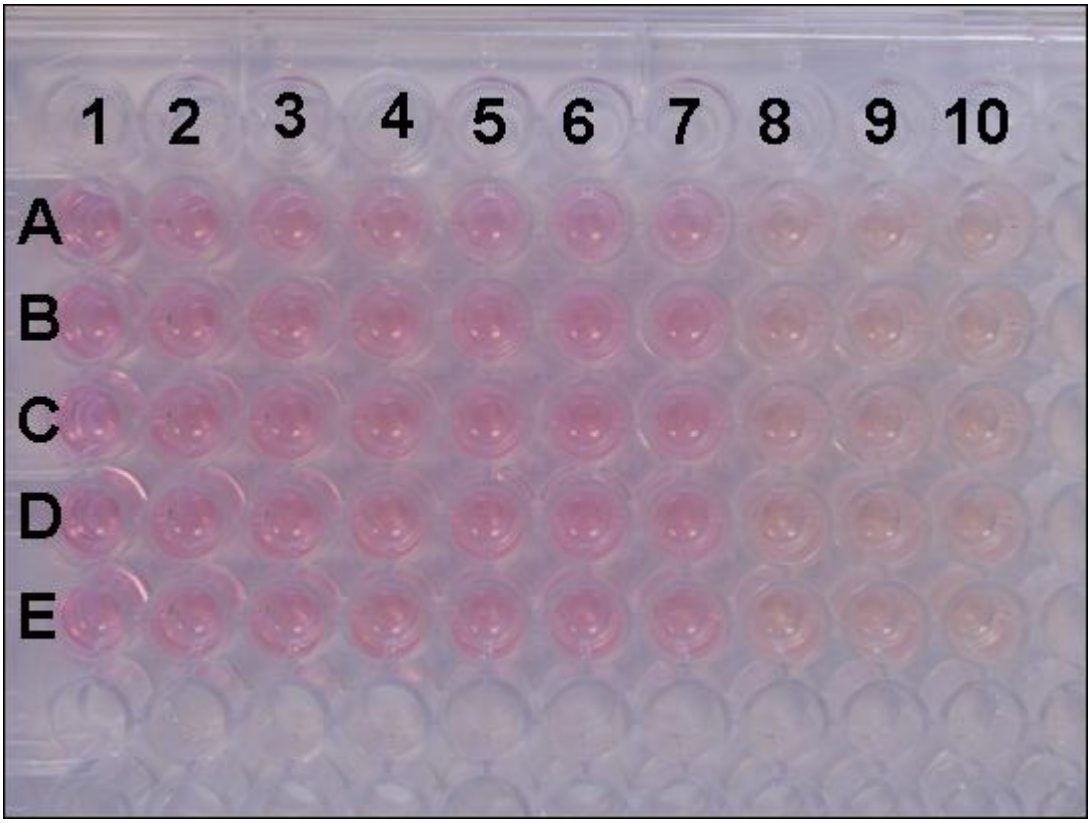
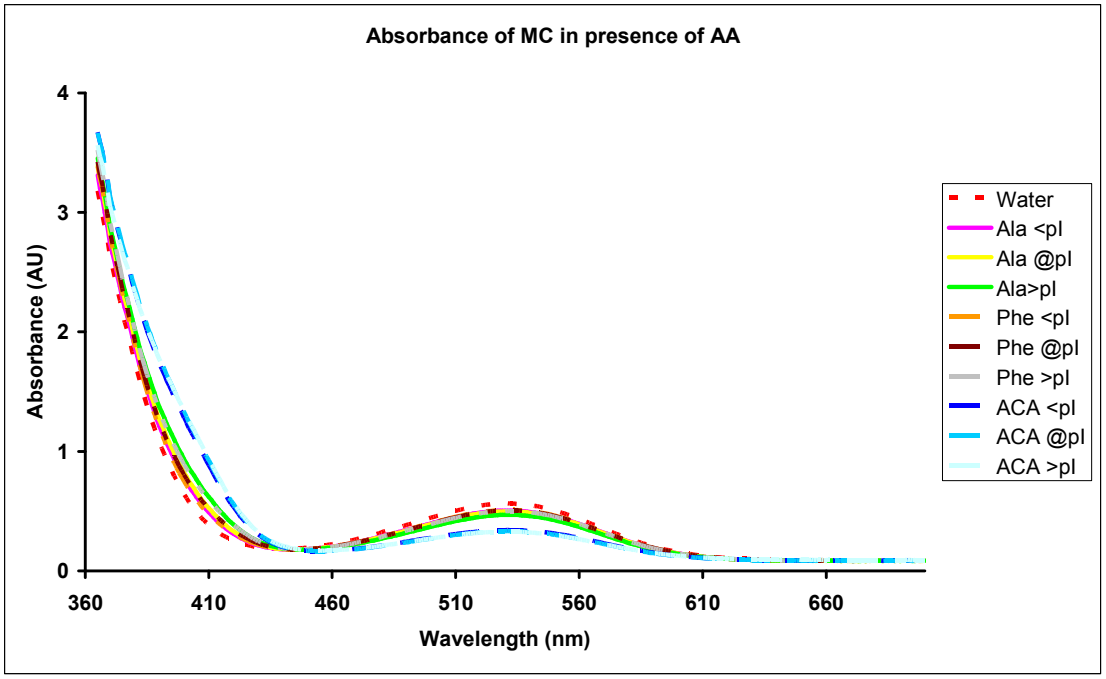


Figure 4.3 Photograph comparing colour of merocyanine in the presence of water and different amino acids. In each case 15 µL of 10^{-1} M AA was added to 200 µL of 10^{-3} SP. The different aqueous solutions added to the SP are as follows: Column 1: water, Column 2: Ala below pI, Column 3: Ala at pI, Column 4: Ala above pI, Column 5: Phe below pI, Column 6: Phe at pI, Column 7: Phe above pI, Column 8: ACA below pI, Column 9: ACA at pI, Column 10: ACA above pI. There is a noticeably lighter colour in the last 3 columns on the right hand side of the plate, when ACA is the amino acid present.

The wells A 8 to E10 contain ACA. There is a sharp contrast in the colour of the merocyanine in these wells compared to wells A1 to E7. In addition, when the UV-Vis spectrum of these samples was taken (Fig. 4.4) it was observed that in the ACA samples there was an increased absorption at around 400 nm, contributing to the more yellow-coloured solution. There is also a decrease in the intensity of the peak at 540 nm showing that there is a smaller proportion of spiropyran molecules in the open merocyanine form.



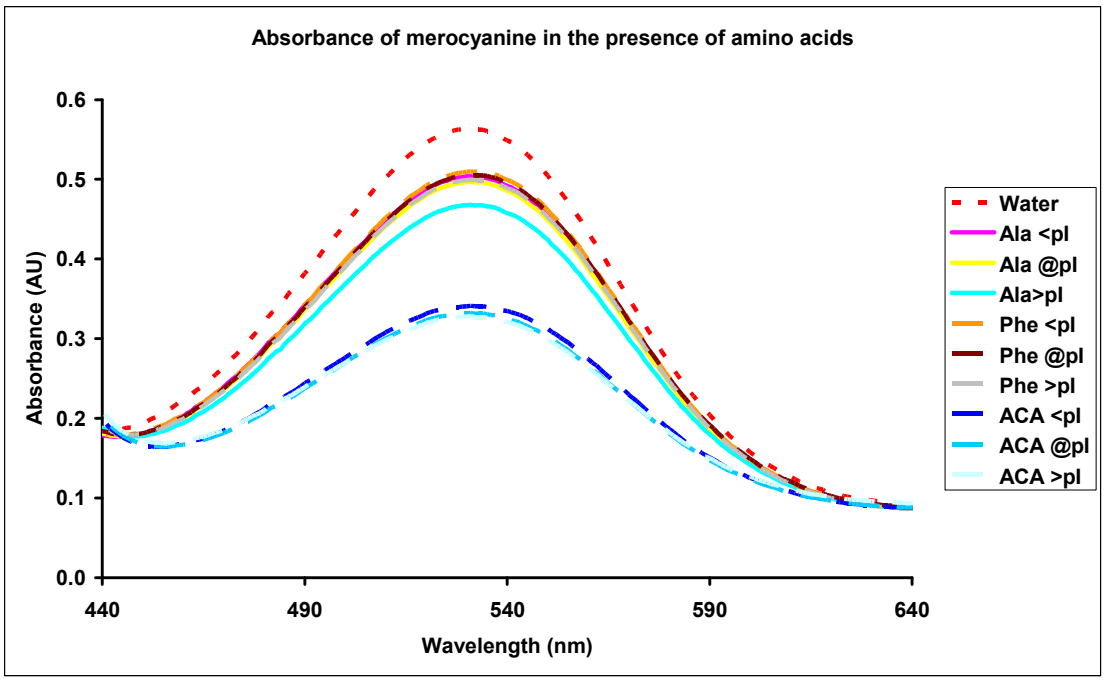


Figure 4.4 (A) UV-Vis spectra of merocyanine comparing absorbance in the presence of different amino acids at varying pHs, 4 and a half hours after UV irradiation. ACA has the lowest absorbance, and also shows an increase in absorbance in the region of 380 – 410 nm. **(B)** Detail from (A), shown at different scale. Water shows the highest absorbance, followed by Ala and Phe, with a sharp drop to the ACA absorbance.

After exposure to ambient light for 10 minutes, the pink colour decayed from all wells (Fig. 4.5). This suggests that any stabilisation effects that may be taking place due to the presence of the zwitterionic amino acids and highly polar water molecule are not strong enough to keep the merocyanine molecule from reverting to its closed SP form when exposed to light. When all the pink had decayed, a strong yellow colour was observed in the wells containing ACA. There is also a slight yellow tinge to the β-Ala and Phe solutions. This is confirmed by the UV-Vis spectra shown in Fig. 4.6, when an increase in the ~400 nm region is observed for all amino acids compared to the water blank, with the largest increase for ACA. This yellow colour will subsequently be discussed in further detail. The overall result however indicates that the amino acids have a different effect on the merocyanine to water, and this effect varies depending on the amino acid used.

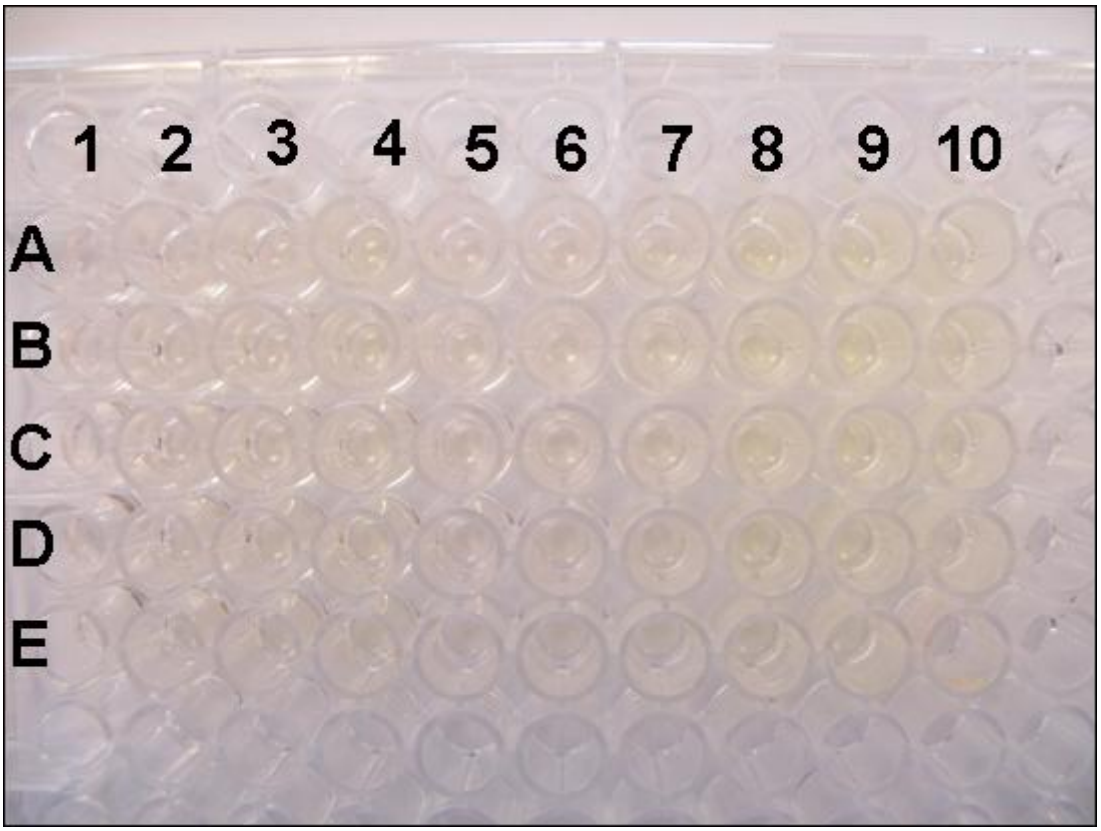


Figure 4.5 Photograph comparing colour of merocyanine in the presence of water and different amino acids after exposure to 10 minutes ambient light.. In each case 15 μL of 10^{-1} M AA was added to 200 μL of 10^{-3} SP. The different aqueous solutions added to the SP are as follows: Column 1: water, Column 2: Ala below pI, Column 3: Ala at pI, Column 4: Ala above pI, Column 5: Phe below pI, Column 6: Phe at pI, Column 7: Phe above pI, Column 8: ACA below pI, Column 9: ACA at pI, Column 10: ACA above pI. The pink colour has decayed, in leaving a yellow coloured solution for both ACA and Ala.

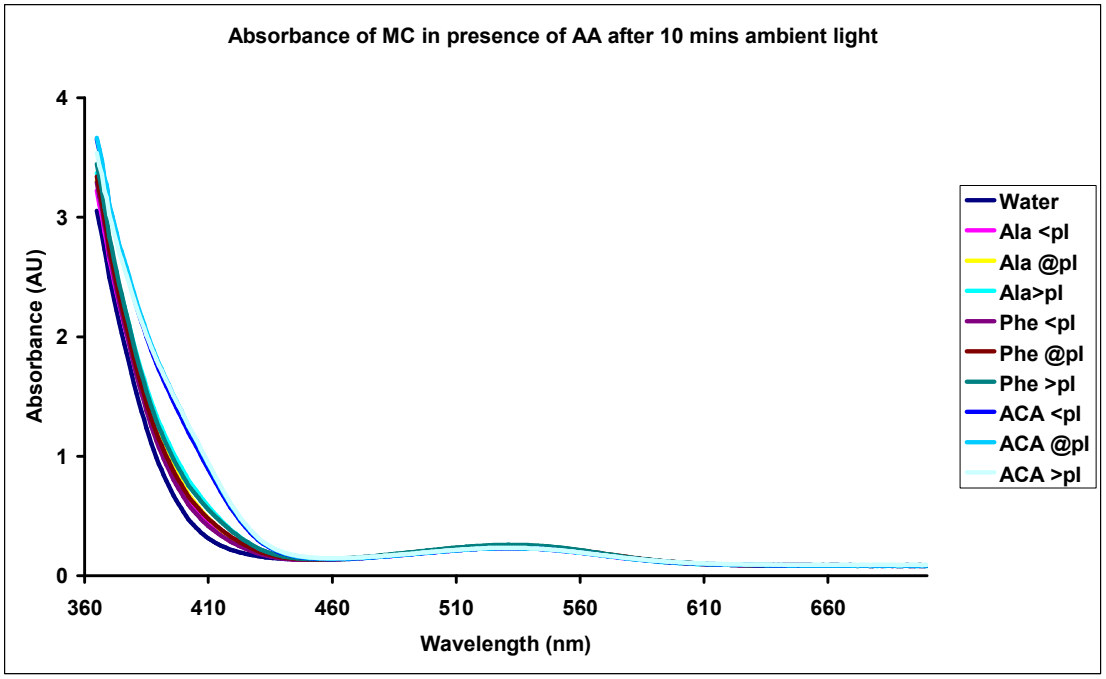


Figure 4.6 Absorbance spectra after 10 minutes exposure to ambient light. Pink colour has decayed, and absorbance shift for ACA samples can be observed from 360-460 nm.

In order to determine the rate constants for the decay of the merocyanine in the presence of water and amino acids, Microsoft Excel Solver was used. A model was created using the equation:

$$\text{Absorbance} = [A(1-e^{-Kt})]+B \qquad \textbf{Equation 6}$$

where K is the rate constant, t is time and A and B are constants. The experimental data was fitted to this model with an error of less than 1%. The data and models were plotted, and can be seen in Figures 4.7-9.

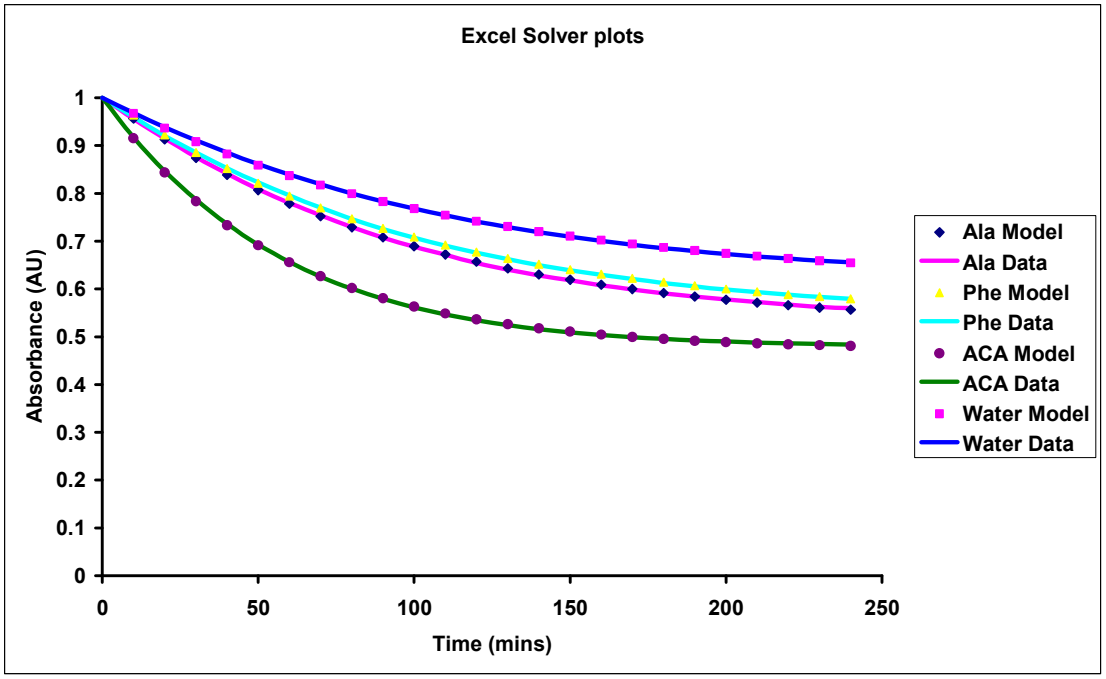


Figure 4.7 Microsoft Excel Solver plots, fitting the experimental data to a model in order to determine the rate constant. Ala, Phe and ACA are all plotted at their isoelectric points (pH measured in aqueous solution before adding to SP).

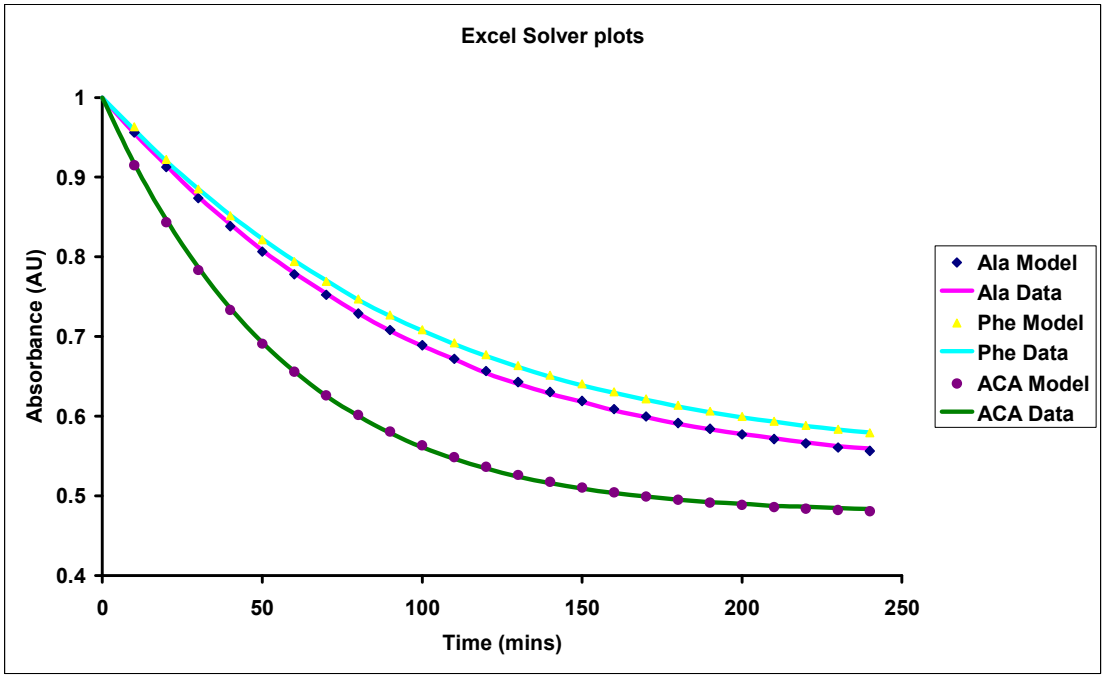


Figure 4.8 Microsoft Excel Solver plots comparing the rate of decay of the merocyanine in the presence of the three different amino acids. ACA can be seen to decay considerably faster than the other two.

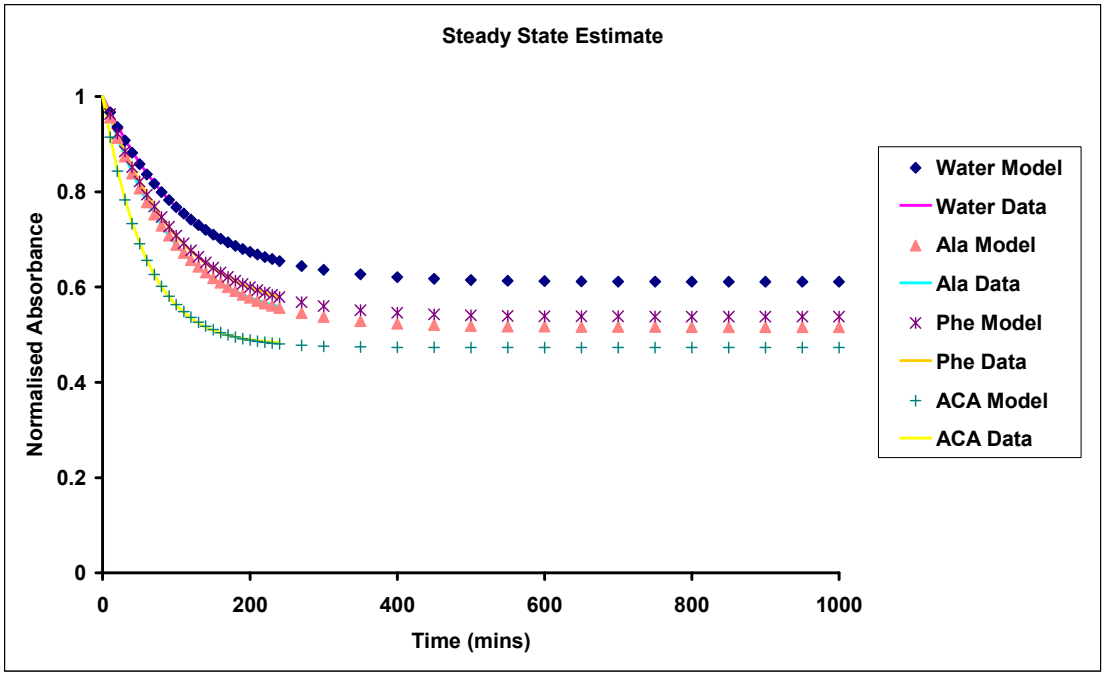


Figure 4.9 Microsoft Excel Solver plots, fitting the experimental data to a model, and then extending the model over time to estimate steady state absorbance, when the system had reached equilibrium. Ala, Phe and ACA are all plotted at their isoelectric points.

This data analysis was also applied to the repeat experiment. The rate constant values were extracted, and are compared in Table 4.4. The inter-day precision can be seen to be approximately 10%.

Table 4.4 Rate constants determined using MS Excel Solver on 2 different sets of experimental data.

	pH	rate constant k (s^{-1}) Run A	rate constant k (s^{-1}) Run B	Difference (B-A)	% Difference [(B-A)/B]x100
water	7	9.11E-03	9.30E-03	1.84E-04	2
Ala <pI	5	1.01E-02	1.07E-02	6.59E-04	6
Ala @pI	6	1.04E-02	1.14E-02	1.08E-03	9
Ala >pI	7	1.14E-02	1.25E-02	1.09E-03	9
Phe <pI	4.48	1.04E-02	1.10E-02	6.20E-04	6
Phe @pI	5.48	1.01E-02	1.12E-02	1.03E-03	9
Phe >pI	6.48	1.04E-02	1.19E-02	1.51E-03	13
ACA <pI	7	1.66E-02	1.83E-02	1.72E-03	9
ACA @pI	8	1.77E-02	1.91E-02	1.43E-03	7
ACA >pI	9	1.87E-02	1.98E-02	1.12E-03	6

The slowest rate constant in both cases was found to be that of water, indicating that the merocyanine is most stable in the presence of the blank water solution. Ala and Phe have the next slowest rate constant, demonstrating that they do have an effect on the stability and lifetime of the merocyanine. ACA has the largest rate constant, indicating that the MC is least stable in the presence of this amino acid. As can been seen in Table 4.5, the rate of MC decay in ACA is 69% faster than in Ala, and 73% faster in Phe, which is quite a large difference.

Table 4.5 Percentage increase in rate of ACA decay compared to other AAs

	Mean at pI	**% increase in rate of ACA relative to other AA**
Ala	1.09E-02	69%
Phe	1.065	73%
ACA	1.84	-

A significant difference can be seen between the results obtained on different days. This difference can be clearly observed when 2 sets of results for the absorbance decay over time of Ala and water are compared (Figures 4.10, 4.11). In both cases the absorbance was found to be higher the second time the experiment was carried out.

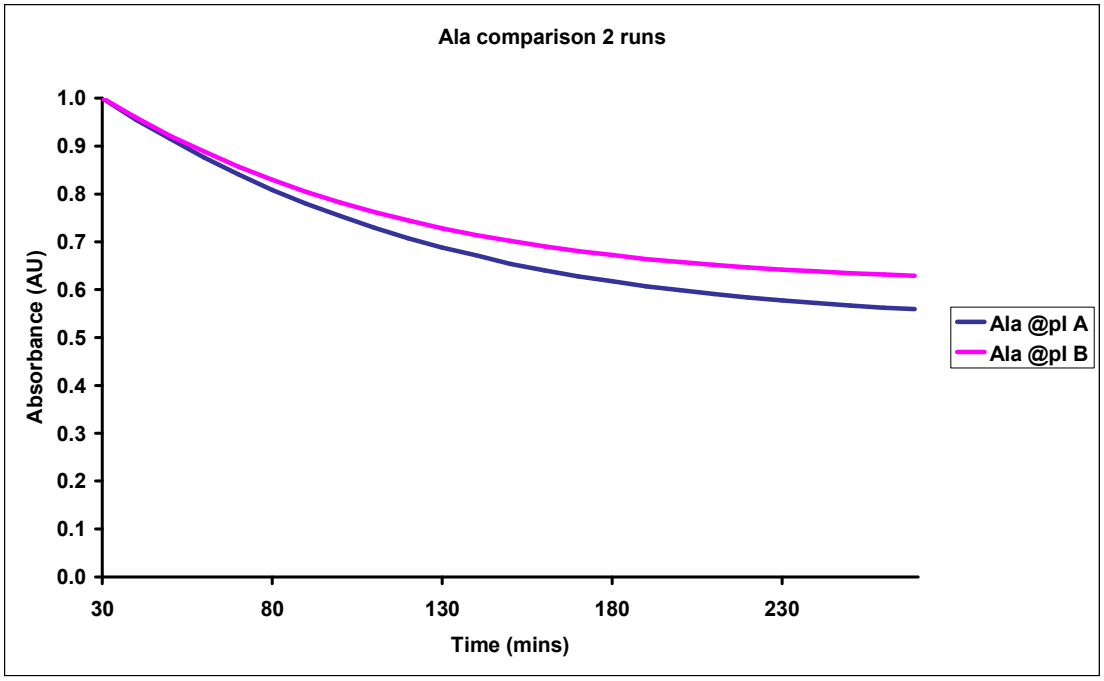


Figure 4.50 Normalised comparison between two sets of kinetics data for Alanine at pI.

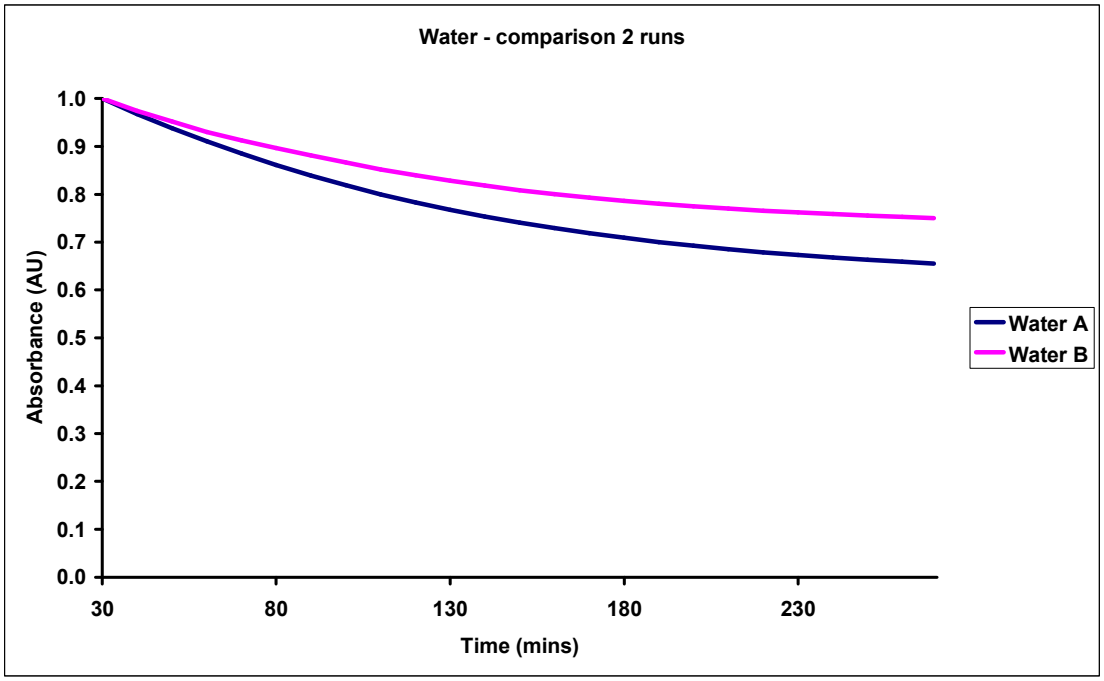


Figure 4.11 Normalised comparison between two sets of kinetics data for water.

The difficulty here is that this is a very sensitive and dynamic system, and there are a number of different factors which may influence the results on a day to day basis. However, the experimental design is very effective at limiting these differences by monitoring a large number of samples in the same run. The platewell reader used is capable of real-time analysis of up to 96 samples simultaneously. The inter-run variability was found to be very low, with standard deviation of between 1 and 4% (see data in Table 4.6 and error bars on Figures 4.12, 4.13). Therefore, the key is to test as many samples as possible in the same run in order to reliably compare the results. The inter-day results show the same general pattern, but they must be compared with more caution, due to the lower precision.

Table 4.6 A cross section of the average inter-run results (n=5) for water, Ala, Phe and ACA. The average standard deviation, and percentage standard deviation for each are shown at the bottom of the table.

	Water	Alanine			Phenylalanine			Aminocaprylic acid		
Time		Below pI	At pI	Above pI	Below pI	At pI	Above pI	Below pI	At pI	Above pI
0	0.719	0.779	0.836	0.810	0.714	0.807	0.815	0.693	0.630	0.608
10	0.831	0.886	0.893	0.878	0.806	0.895	0.935	0.751	0.707	0.633
20	0.808	0.852	0.858	0.842	0.767	0.854	0.856	0.685	0.636	0.577
30	0.778	0.816	0.824	0.798	0.741	0.820	0.813	0.629	0.581	0.528
40	0.753	0.782	0.787	0.758	0.715	0.787	0.777	0.580	0.533	0.489
50	0.730	0.750	0.754	0.722	0.691	0.755	0.744	0.536	0.492	0.447
60	0.709	0.721	0.721	0.689	0.669	0.726	0.713	0.498	0.457	0.417
61	0.707	0.718	0.718	0.686	0.666	0.723	0.710	0.495	0.454	0.415
62	0.705	0.715	0.715	0.682	0.664	0.721	0.707	0.491	0.451	0.412
63	0.703	0.713	0.712	0.679	0.662	0.718	0.704	0.488	0.448	0.409
64	0.701	0.710	0.709	0.676	0.660	0.715	0.701	0.485	0.445	0.407
65	0.698	0.707	0.706	0.674	0.657	0.712	0.698	0.481	0.442	0.404
66	0.697	0.704	0.704	0.670	0.656	0.710	0.696	0.478	0.439	0.402
67	0.695	0.702	0.701	0.667	0.653	0.707	0.693	0.475	0.436	0.400
68	0.693	0.699	0.698	0.664	0.652	0.704	0.690	0.472	0.433	0.397
69	0.691	0.697	0.695	0.661	0.650	0.702	0.687	0.469	0.430	0.394
70	0.689	0.694	0.693	0.658	0.647	0.699	0.684	0.466	0.427	0.392
80	0.670	0.669	0.666	0.631	0.629	0.675	0.659	0.438	0.402	0.371
90	0.653	0.646	0.642	0.607	0.611	0.652	0.635	0.415	0.381	0.353
100	0.637	0.625	0.621	0.585	0.596	0.632	0.614	0.396	0.363	0.338
110	0.623	0.607	0.601	0.565	0.581	0.612	0.594	0.379	0.349	0.326
120	0.609	0.590	0.582	0.548	0.568	0.595	0.577	0.365	0.336	0.315
130	0.598	0.574	0.567	0.533	0.557	0.580	0.561	0.353	0.326	0.307
140	0.587	0.560	0.553	0.519	0.546	0.566	0.547	0.342	0.318	0.300
150	0.577	0.548	0.539	0.506	0.536	0.554	0.535	0.335	0.311	0.294
160	0.568	0.537	0.528	0.495	0.527	0.543	0.524	0.328	0.305	0.290
170	0.559	0.527	0.517	0.486	0.520	0.533	0.513	0.322	0.300	0.286
180	0.552	0.518	0.509	0.478	0.512	0.524	0.504	0.316	0.296	0.283
190	0.545	0.511	0.500	0.470	0.506	0.516	0.497	0.312	0.293	0.280
200	0.539	0.504	0.493	0.463	0.500	0.509	0.490	0.309	0.290	0.278
210	0.533	0.497	0.486	0.457	0.495	0.502	0.483	0.306	0.288	0.276

220	0.528	0.491	0.481	0.452	0.489	0.496	0.477	0.304	0.286	0.275
230	0.524	0.486	0.476	0.448	0.486	0.491	0.472	0.302	0.285	0.273
240	0.520	0.482	0.471	0.445	0.482	0.487	0.468	0.300	0.283	0.272
250	0.516	0.478	0.467	0.441	0.479	0.482	0.464	0.299	0.283	0.271
260	0.513	0.473	0.463	0.437	0.475	0.478	0.460	0.298	0.282	0.271
Average Std Dev	**0.012**	**0.010**	**0.010**	**0.011**	**0.010**	**0.013**	**0.015**	**0.012**	**0.008**	**0.014**
% Std Dev	**1.8**	**1.4**	**1.6**	**1.8**	**1.5**	**1.9**	**2.1**	**2.5**	**1.8**	**3.7**

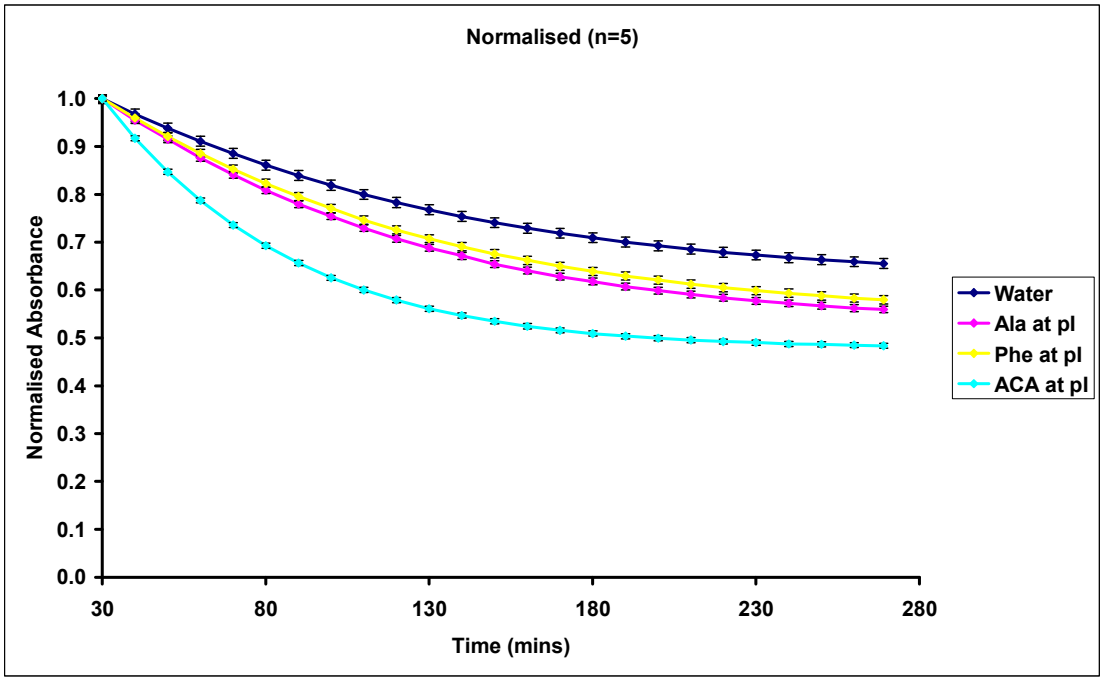


Figure 4.12 Normalised plot comparing rate of decay of merocyanine in the presence of water, Ala, Phe and ACA. The rate for ACA can be seen to be much faster than the other samples. Error bar = Standard Deviation (n=5).

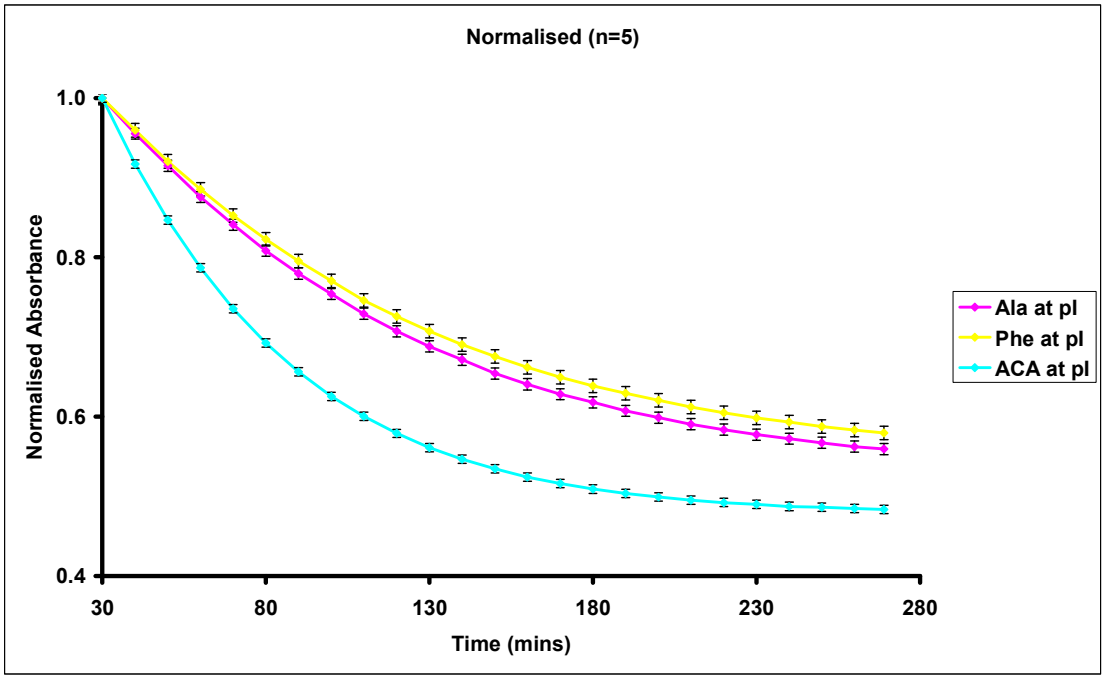
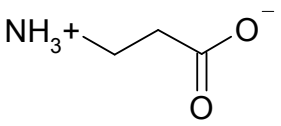
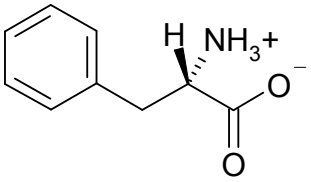
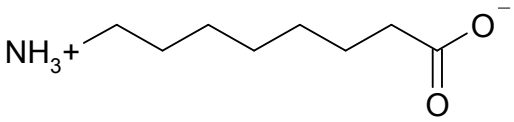


Figure 4.13 Normalised plot comparing rate of decay of merocyanine in the presence of the three amino acids tested. It can be clearly seen than ACA decays at a much faster rate than Ala or Phe. Error bar = Standard Deviation (n=5).

Water showed greater MC stabilisation than any of the amino acids tested. Water is an extremely polar solvent, with an E_T^N value of 1[65], compared to ethanol's E_T^N of 0.654. Therefore the merocyanine is stabilised to a great extent in water. The small size of the water molecule may give it an advantage over the bulkier amino acid molecules, giving it easier access to the charge sites on the merocyanine. We have already discussed the strong nature of hydrogen bonding, and indeed these kinetics results suggest that hydrogen bonding plays an important role in this system.

However, the strong stabilisation of the MC by water cannot mask the fact that there are significant differences observed between the amino acids tested. These differences can only be explained by the different properties of the three amino acids studied leading to a variation in interactions with the MC. This is backed up by the literature, where amino acid – merocyanine interactions are reported[50-53]. The structures and charge separations of the three amino acids are shown in Table 4.7.

Table 4.7 The structures and charge separations of the three amino acids studied.

AA	**Structure**	**Charge Separation**
Ala		4.924 Å
Phe		2.147 Å
ACA		11.364 Å

What remains is to elucidate the nature and cause of these interactions. There are several potential explanations to be considered.

One possibility is that the difference in absorbance and rate of MC decay between the different amino acids may be a solvatochromic effect due to the variations in polarity between the different spikes added to the spiropyran solution and the effect these

variations have on the spiropyran merocyanine equilibrium. The lower intensity colour observed for the MC in the presence of ACA could possibly be due to the decreased polarity of the environment due to the non-polar long chain moiety of the molecule. This would be a very simple effect related to the relative stabilities of open and closed forms in polar and non-polar solvents. The MC is stabilised more in polar solvents, the SP is stabilised in non-polar solvents. However, there is no real solvatochromic shift observed in the absorbance maximum for ACA, which is at odds with this theory.

The variation in absorbance of the merocyanine in the presence of water and different amino acids could possibly be due to electrostatic interactions between complementarily oriented zwitterions, or could be a charge stabilisation effect. In order to investigate this, the kinetic experiment was carried out on the merocyanine in the presence of sodium chloride, an electrolyte, at two different concentrations: 10^{-3} and 10^{-1} M. The results are displayed in Fig. 4.14. It can be seen that the merocyanine is again most stable in the presence of water, suggesting that it is not a dielectric effect merely relying on the polarity of the solution.

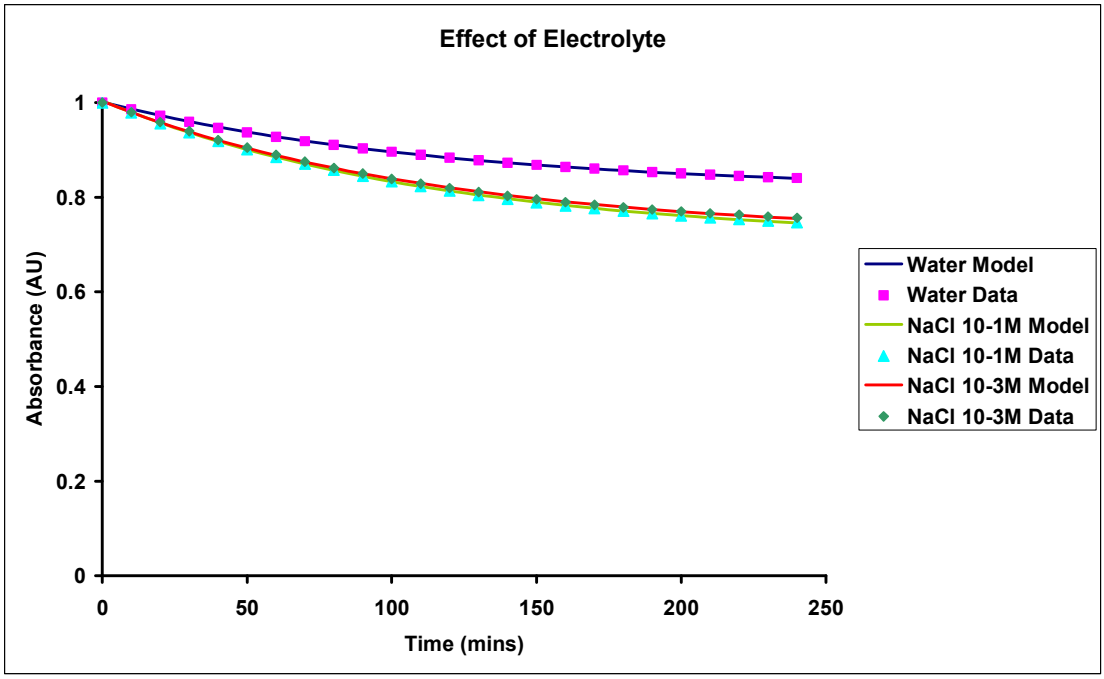


Figure 4.14 Decaying absorbance of merocyanine over time, in the presence of water and different concentrations of electrolyte – NaCl. The data was fitted to a model using Microsoft Excel Solver, and the rate constants determined.

The rate constants were determined using Microsoft Excel Solver as described above, and are summarised in Table 4.8. There is a difference between the rate constant value for water obtained in this experiment and in the two previous experiments, meaning that the k values for sodium chloride cannot be directly compared to the amino acid k values obtained previously.

Table 4.8 Rate constants determined using MS Excel Solver

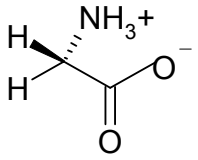
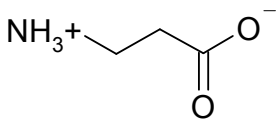
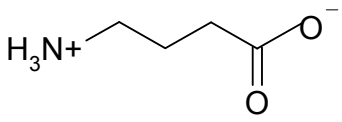
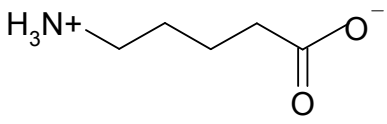
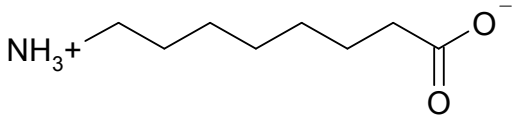
	rate constant k (s^{-1})
Water	8.28E-03
NaCl 10^{-1} M	8.58E-03
NaCl 10^{-3} M	8.60E-03

These results show that the stabilisation of the merocyanine cannot simply be explained by the dielectric constant of its environment. The difference in results between different amino acids indicates that the addition of an amino acid to the SP-MC equilibrium does have an effect on the system whose strength seems to depend on the structure and properties of the amino acid used.

One possible explanation for what is taking place is an acid-base interaction. This theory is suggested by the yellow colour observed in the presence of ACA. A yellow colour is also observed when the phenolate anion of the merocyanine is protonated (see UV-Vis spectra in Figure 1.6), suggesting that in these experiments interactions with the phenolate may have occurred. The colour visible to the eye in the samples containing ACA is effectively orange – a mixture of pink and yellow. This is confirmed by the UV-Vis absorbance spectrum obtained. This suggests that there could be a mixture of protonated and unprotonated MC in the solution. If the merocyanine is being protonated, the only sources of hydrogen ions are the zwitterionic ACA itself or the water solvent. One possibility is that the merocyanine acts as a base and deprotonates the basic NH_3^+ group of the amino acid. ACA is a very weak acid, with poor proton-donating capability, as can be seen by examining the pK_a value. Although the pK_{a2} for ACA cannot be found in the literature, it can be estimated to be above 11 by examining the trend in pK_{a2} for amino acids with increasing spacer length, as demonstrated in Table 4.9. As can be seen, the extremely high pK_{a2} for aminocaprylic acid would indicate that a very strong base would be needed for deprotonation to occur. It seems unlikely that the merocyanine could act as such a strong base to deprotonate this weak acid. The pK_a of the particular spiropyran

used in this study could not be found in the literature, but studies on similar spiropyrans (see Figure 1.6) have found their pK_a to be approximately 4 or 5[27].

Table 4.9 Comparison of pK_{a2} values of amino acids. There is a general increase in pK_{a2} as the chain length increases.

Amino Acid	**Structure**	**pK_{a2}[66]**
Glycine		9.78
β-Alanine		10.19
γ-Aminobutyric acid		10.43
5-Aminovaleric acid		10.77
8-Aminocaprylic acid		>11 (estimate)

Another possible explanation for the protonation of the merocyanine involves the dielectric effect. Ethanol has quite a high dielectric constant, which may be affected by the addition of the aminocaprylic acid. This long chain zwitterion has a large lipophilic moiety, which could possibly decrease the effective dielectric constant of the solution. This in turn could enhance the basicity of the merocyanine, enabling it to scavenge protons from the water. In his book "Reactions of Acids and Bases in Analytical Chemistry"[67] Hulanicki states that for acids and bases in non aqueous

solvents, if there is a large dielectric constant, the interactions between ions will be weak, whereas in a solvent with a small dielectric constant the interactions between the ions will be enhanced. This is due to the varying degrees of solvation power, according to Williams and Hale, who described how the reduction of the dielectric constant leads to increased acid-base reactivity as the solvation energy becomes less important[68]. In order to test this concept in relation to the SP-MC system, a simple experiment was carried out to vary the dielectric constant of the solution. Toluene has a very low dielectric constant, and therefore it was added in varying ratios (10:1 and 5:1) to the spiropyran solution in ethanol. A blank solution for each ratio was prepared by adding the relevant amount of ethanol instead of toluene. Water was then added to each solution, and the samples left in the dark overnight to investigate whether or not protonation would occur.

The results showed that the protonation of the MC was not observed, as there was no change in the colour of each solution. The UV-Vis spectra of the samples and blanks show that there was very little difference observed between the blanks and the solutions containing toluene (Fig. 4.15), indicating that the low dielectric constant of the toluene did not have a significant effect on the merocyanine.

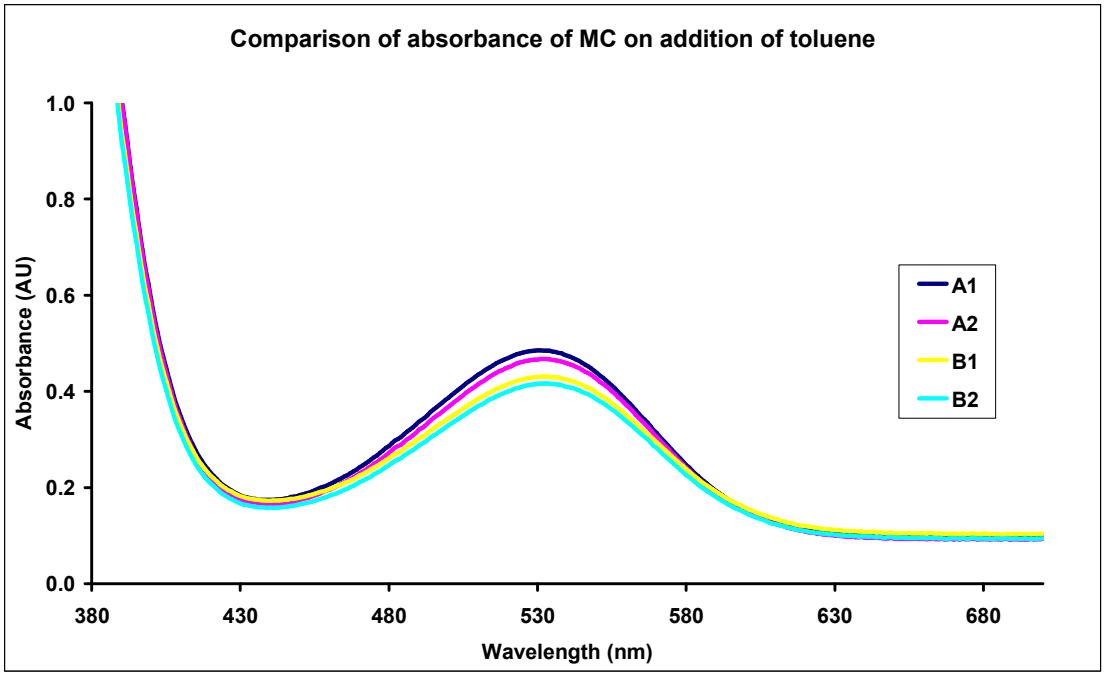


Figure 4.6 UV-Vis spectra comparing the absorbance of merocyanine in the presence and absence of toluene. The graphs overlaid are as follows: A1 is a 10:1 ratio of ethanol to toluene, A2 is its corresponding blank; B1 is a 5:1 ratio of ethanol to toluene, B2 is its corresponding blank. There is no shift observed in the ~400 nm region, as noted in the presence of aminocaprylic acid, suggesting that an acid-base interaction could be taking place, which is not reproduced with the addition of the non-polar toluene.

Therefore, this experiment does not back up the theory that the merocyanine is protonated in the presence of aminocaprylic acid due to the effective dielectric constant of the solution. It would be necessary to carry out further investigations into this matter. Ion migration and gel permeation studies could clarify whether or not protonation is taking place.

A further possible explanation for the varying degree of interaction relates to the spacer length between the charges on the amino acids. It was proposed in the introduction to this chapter that the amino acids with spacer lengths which approached that of the charged merocyanine would have stronger interactions with the MC than

an amino acid with a much larger spacer length. This would mean that the merocyanine would be more stable, i.e. have a slower decay rate, in the presence of amino acids with shorter spacer lengths. This theory is backed up by the significantly faster rate of decay observed in the presence of ACA, which has a much longer spacer length than the other two amino acids. However, one difficulty with this hypothesis is the yellow colour observed in the MC-ACA solutions, which cannot be explained by the basic spacer length theory. It appears that the system is more complex than the simple spacer length model, and this merits further research.

4.4. Conclusions

From these results we can see that there are differences in the rates of decay of MC back to SP over time in the presence of different amino acids and water. It was found that ACA had a significantly faster rate of decay than the other amino acids, indicating that the structure of the amino acid has an affect on the SP-MC equilibrium.

The inter-run and inter-day reproducibility were compared, and it was found that while the dynamic nature of the system led to large inter-day variations, the inter-run precision was very high. The instrumentation used capitalised on this by facilitating the monitoring of up to 96 samples in one run, enabling many samples to be tested and reliably compared.

The results showed that water has a very strong stabilisation effect on the merocyanine. This however could not mask the differences in rate between the amino acids. There were several possible reasons proposed for the observed variations in rate. One suggestion was that the different rates of decay were due to the dielectric constant of the environment, which is of course linked to the ionic strength of the solution. An electrolyte was added to the MC to investigate this theory, but the MC was found to still be most stable in water, indicating that the stabilisation of the zwitterionic MC is not simply due to the polarity of the solution.

Another proposal was that an acid-base reaction was taking place. This was backed up by the yellow colour observed in MC in the presence of ACA. However, the fact that ACA is a very weak acid does not support this theory. Then it was suggested that the

MC was becoming protonated due to a decrease in the dielectric constant of the solution, because of the lipophilic moiety in the ACA molecule. However an attempt to replicate this effect by lowering the dielectric constant of the MC by adding toluene failed. Therefore, this proposition is also unsubstantiated.

Finally, the theory that the strength of MC-AA interactions depends on the relative zwitterionic spacer length was put forward. This is supported by the rates of decay found experimentally, with the MC-ACA solution decaying much more rapidly than the other two amino acids, indicating that ACA stabilises the MC to a lesser extent. However, this theory does not explain the yellow colour observed in the MC-ACA solution. A more detailed study is required to elucidate this matter. Perhaps the variations of MC behaviour in the presence of different amino acids is due to a combination of some of these factors, and maybe the interactions are weak due to the fact that the AA and MC zwitterions are strongly hydrated, and therefore a much higher concentration of AA relative to MC is needed to drive the process. This is something that will have to be taken into account in further studies.

5. Overall Discussion and Conclusions

Although the photochromism, thermochromism and solvatochromism of spiropyran have been studied in detail for more than 50 years, the spiropyran-merocyanine equilibrium is still a delicate system which is subject to many factors that prevent it from acting in a predictable way Any investigation into the potential use of spiropyran as a molecule for use in chemical or biological sensing must take into account the multiple variables which can alter the equilibrium of the system. The effect that variables such as light, heat, polarity, acidity have on the equilibrium, must be properly understood and regulated, to ensure a full and thorough investigation of amino acid – spiropyran interactions.

The results of this study have demonstrated the various interactions taking place to be quite complex, with many factors potentially involved. The experiments carried out demonstrated that there is a difference in MC-AA interactions depending on the structure of the amino acid used. However, further work is needed to determine the nature and cause of these interactions.

From the results observed it seems that hydrogen bonding and the solvation of the MC by water is very energetically favourable. This may have caused difficulties in trying to observe and analyse MC-AA interactions, considering that the amino acids used were only soluble in water. This led to either a two-phase system or a mixture of solvents, thus adding further variables to affect the SP-MC equilibrium. It could be that these interactions are weak due to the fact that the AA and MC zwitterions are

strongly hydrated, and therefore a much higher concentration of AA relative to MC is needed to drive the process. It could be helpful to reduce the system to a single solvent. One way of doing this would be to carry out a kinetics study on the MC-AA interactions in water. We have seen from the two phase experiments carried out that the MC crossed very easily into the aqueous phase in a high enough concentration to observe a substantial colour. Even with the strong hydrogen bonding when the MC was dissolved in water, variations in absorbance were demonstrated for the different amino acids. This would indicate that a more in-depth study of MC-AA interactions in water could prove very fruitful, and provide valuable information showing how the differences in structure and polarity of the amino acid could affect absorbance and even rate of decay. This would be an interesting piece of research.

Another alternative would be to put a membrane between the spiropyran and the aqueous layer, as described by Sunamoto[50]. It is evident that amino acid – spiropyran interactions do take place, as reported in the literature. This study has merely been an observation of these interactions in a very simple solvent system, and more work is needed to further characterise the interactions taking place. The use of a two-phase system separated by a membrane could be the key to understanding and characterising MC-AA interactions.

An additional way to optimise the MC-AA interactions could be to modify the spiropyran used. The basic spiropyran used throughout this project may not be the optimum spiropyran molecule to act as a ligand to an amino acid. The addition of some side chain could drastically alter the host-guest properties of the molecule, and make electrostatic interactions with amino acids a lot more favourable. This theory is

supported by the spiropyran used by Tsubaki and co-workers[52] for the recognition of amino acid enantiomers.

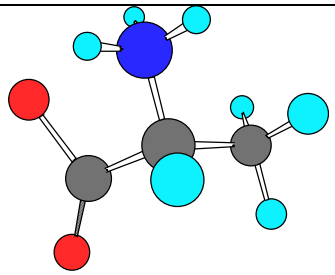
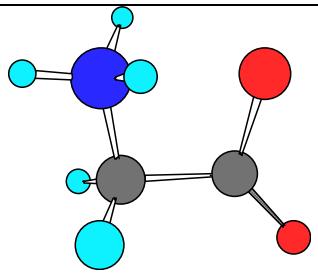
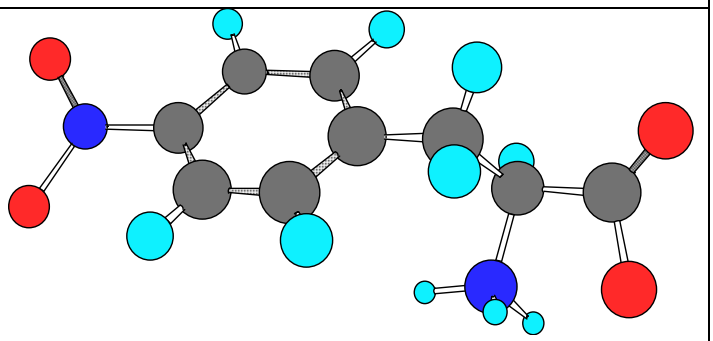
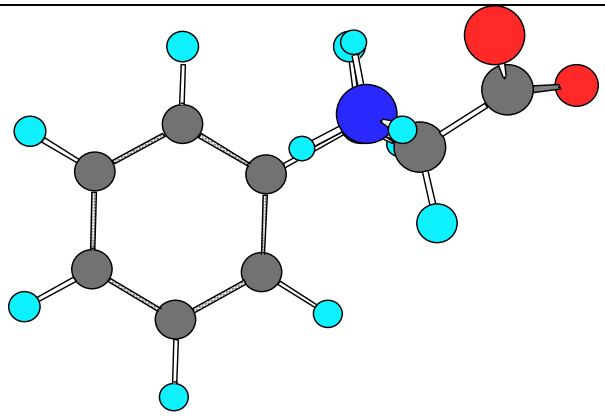
The possibility that an acid-base reaction is taking place in the MC-AA solution is another area that requires further investigation. Initial tests to investigate whether this was a dielectric constant effect proved inconclusive, and more work is needed to clarify this issue. The modification of the dielectric constant by the addition of toluene did not yield the same yellow solution observed with ACA. Again a more comprehensive study of the various factors involved in the amino acid interactions is needed.

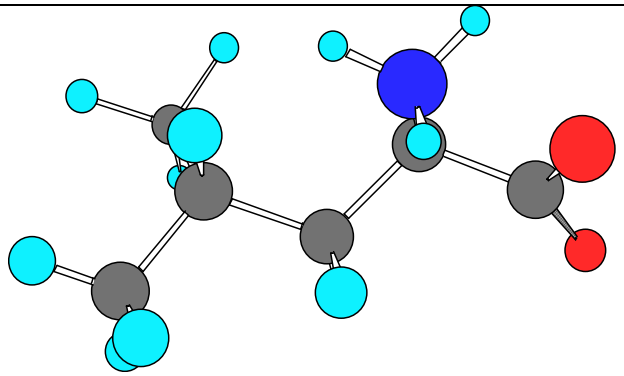
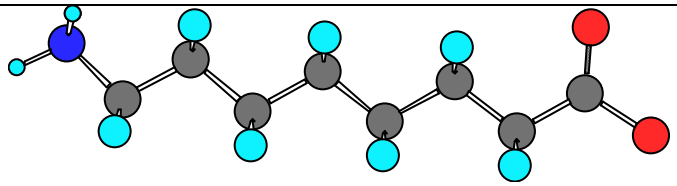
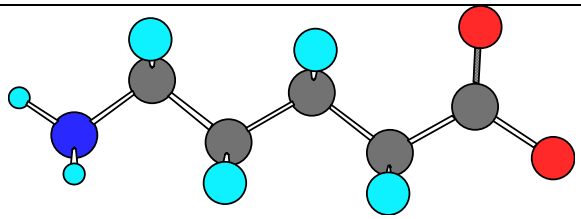
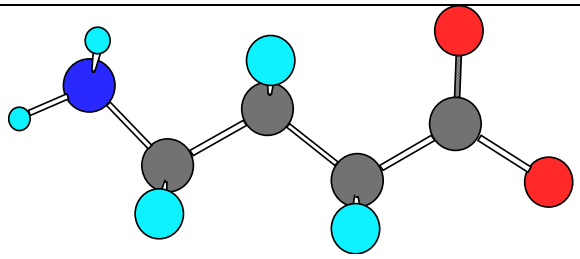
Another exciting area of future work would be the immobilisation of the spiropyran onto a surface or onto beads. This would enable light-modulated activation of the surface/bead, and could provide a platform for selective amino acid binding. This immobilisation could eliminate a lot of the solvent effects that have been observed thus far, and could potentially clarify the nature and strength of interactions between amino acids and merocyanine. This field of study has already been commenced in the research group.

Therefore, in order to optimise interactions between amino acids and spiropyran, intelligent system design is needed to minimise the influence of water and hydrogen bonding on the merocyanine and to optimised interactions with amino acid. This is a very exciting area of research with potentially far-reaching consequences in the biological sensing field. However, in order to fully exploit the potential of spiropyran as a selective self-indicating biological sensing agent, further research is needed. This

study confirms that the photochromic equilibrium between spiropyran and merocyanine is indeed affected differently in the presence of a range of amino acids, showing that the area merits additional work. This body of research has also highlighted some of the key factors involved in the intermolecular interactions between the solvent, spiropyran and amino acid. Due to time constraints, a comprehensive analysis of each of these factors was not possible, and this study merely provides an overview which can stimulate further discussion and the development of focussed research into the areas highlighted.

6. Appendix

β-Alanine	
Glycine	
L-Tyrosine	
L-Phenylalanine	

L-Leucine	
8-Aminocaprylic acid	
5-Aminovaleric acid	
γ -Aminobutyric acid	

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