The Attachment of Dyes To Amino Acids

A Thesis Submitted Towards the Degree of

Doctor of Philosophy

by

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Contents

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Statement

This work contains no material which has been accepted for the award of any other degree or diploma in any university or other tertiary institution and, to the best of my knowledge and belief, contains no material previously published or written by another person, except where due reference has been made in the text.

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Abstract

Reactive dyes are a class of dyes where the dye is attached to the fibre (eg. wool) through a covalent bond. To form such a bond they require an active group capable of undergoing reaction with the nucleophilic sites in the fibre. The active group is normally either a carbon with a leaving group which can undergo nucleophilic substitution reactions or an alkene activated by either a carbonyl or a sulfonyl group, which can undergo Michael addition reactions. The search for new types of reactive dyes with better properties, such as increased wet and light fastness, higher fixation, and greater stability to dye bath conditions, continues. Accordingly the Michael addition of the side chain functional groups of cysteine, lysine and serine with a series of alkynes, both terminal and non-terminal, attached to carbonyl groups, from a representative sample of functional group types was examined. The relative reactivities of both the alkynes and the amino acid side chains were determined. The most reactive alkynes were found to be those conjugated to ketones and esters. Mild reaction conditions were found under which the Michael addition reactions occur efficiently and in good yield.

Linker arms terminating in conjugated alkynes were attached to four dyes, fluorescein; Sudan 1; 7-hydroxy coumarin; and a dansyl sulfonamide adduct. The types of conjugated alkynes represented by these compounds were terminal alkynyl esters and alkynyl amides, and non-terminal alkynyl ketones. The reactions of these derivatised dyes with the amino acid side chains were studied. These additions were found to occur easily and in reasonable yield.

The applicability of a conjugated alkyne reactive group for the attachment of dyes to proteins was examined. Sudan 1 functionalised by the addition of a linker arm ending in a terminal alkynyl ester was found to react readily with the nucleophilic moieties present in keratin to yield orange dyed wool. The colour was found to have a better than expected fastness to treatment with washing powders.

Abbreviations

Cbz	carbobenzyloxy
COSY	correlated spectroscopy
dansyl	5-dimethylamino-1-naphthalenesulfonyl
DBU	1,8-diazabicyclo[5.4.0]undec-7-ene
DCC	dicyclohexylcarbodiimide
DCU	dicyclohexylurea
DEAD	diethylazodicarboxylate
DMAP	*N*,*N*-dimethyl-4-aminopyridine
DMF	dimethylformamide
DMPU	dimethylpropylene urea
DMSO	dimethylsulfoxide
EI	electron impact
EtOAc	ethyl acetate
FAB	fast atom bombardment
FABMS	fast atom bombardment mass spectrometry
HMPA	hexamethylphosphoramide
LSIMS	liquid secondary ion mass spectrometry
NHS	*N*-hydroxysuccinimide
NOE	nuclear Overhauser effect
NOESY	nuclear Overhauser effect spectroscopy
PPTS	pyridinium *p*-toluene sulfonate
ROESY	rotational Overhauser effect spectroscopy

RT	room temperature
THF	tetrahydrofuran
THP	tetrahydropyranyl
tlc	thin layer chromatography

Introduction

1.1 The Dyeing of Wool

Processes to dye wool have existed for thousands of years. For the majority of this time the only colours available were those derived from natural sources. The advent of synthetic organic chemistry within the last two hundred years has dramatically increased the range of chemical dyes, and perhaps more importantly allowed the invention of new processes and methods for the dyeing of wool.[1]

1.1.1 The Structure of Wool [2]

Wool is composed of the protein keratin, which consists of eighteen L-α-amino acids. The proportions of the constituent amino acids vary depending on factors such as the breed of the sheep,[3,4] the sheep's diet,[5] and even the time of the year.[6] The nature of the amino acid side chains and the percentages of the individual amino acids present in the keratin are very important to the dyeing process, as acidic and basic side chains provide sites with which to bind dyes.[7,8] The keratin fibres contain several types of crosslinks,[9] such as hydrogen bonds, van der Waals forces, hydrophobic interactions, electrostatic interactions and disulfide linkages, all of which combine to hold the individual peptide strands together and so determine many of the physical and mechanical properties of the wool. These crosslinks and the polypeptide structure of wool must be taken into account during the dyeing process as harsh

conditions, such as prolonged boiling or highly acidic dyebaths can cause alterations of these crosslinks and / or hydrolysis of the peptide bonds, resulting in degradation of the fibre.

On a macroscopic scale wool consists of two main segments, these being the cuticle and the cortex, (**Figure 1**).[2] The cuticle, which is composed of the epicuticle, the exocuticle and the endocuticle, has an overlapping scalar structure and is of prime importance in dyeing. The epicuticle, which is the outer layer of scales, covers the whole of the fibre with the exception of the tip where it has been removed by weathering. The epicuticle is a thin, hydrophobic layer which can provide a barrier to hydrophilic dye molecules and therefore could lead to uneven dyeing. This layer is generally removed by chemical treatment, normally chlorination, before the dyeing process takes place. The second major part of wool, the cortex, consists of more than 90% of the entire fibre and is made up of spindle shaped cortical cells which are easily penetrated by dye molecules.

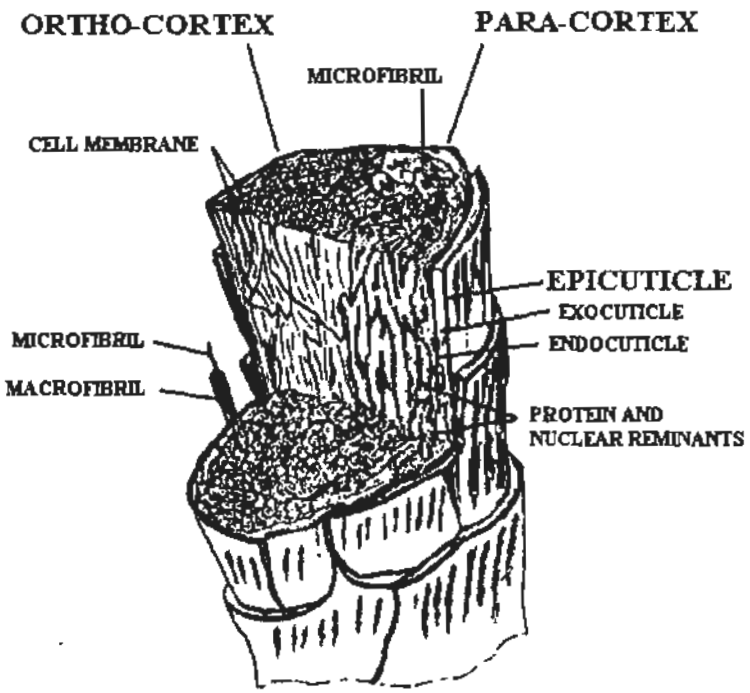
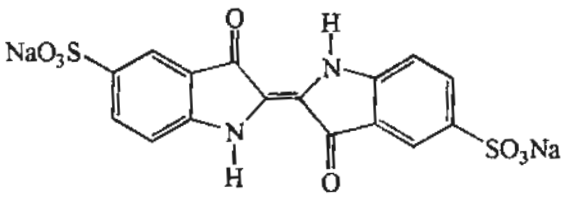


Figure 1 : The Structure of Wool.

There are three main classes of dyes which are used to dye wool; acid dyes, mordant dyes, and reactive dyes.[10]

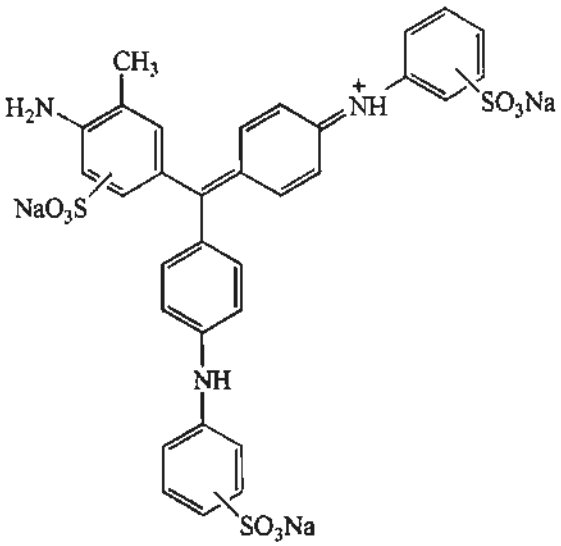
1.1.2 Acid Dyes

Acid dyes are water soluble anionic dyes which are used mainly to dye wool and other fabrics from acidic or neutral dyebaths. The water solubility of these dyes is normally derived from the presence of sulfonic acid groups, commonly as the sodium salt. The name 'acid dye' comes from the acidic dyebath conditions which were originally required for their application to wool and silk. The first acid dye CI Acid Blue 74, **1** was prepared by Barth in 1740 by the sulfonation of indigo.[11]



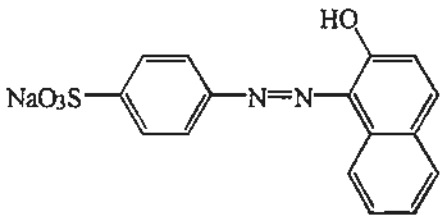
1

The first true preparation of a synthetic acid dye was carried out by Nicholson in 1862. Sulfonation of the sparingly water soluble Aniline Blue (CI Solvent Blue 3) yielded Soluble Blue, **2** (CI Acid Blue 22).[12]



2

The acid dyes fall into several subgroups according to their chemical structures. The class which constitutes the largest number of acid dyes are the azo dyes, such as CI Acid Orange 7, **3** and CI Acid Red 138, **4**. The azo dyes are responsible for the majority of the red, yellow, orange, brown and black colours seen in dyed wool.



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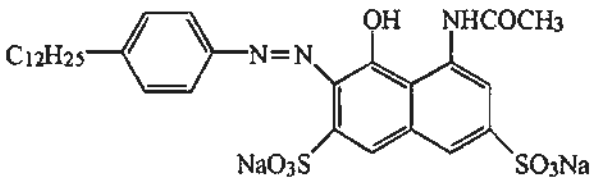
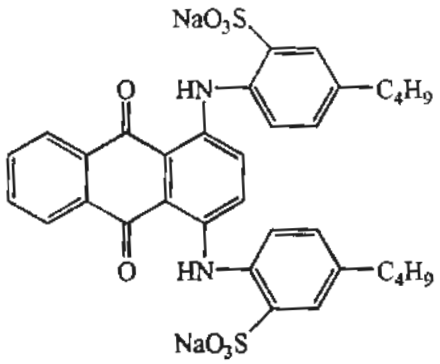


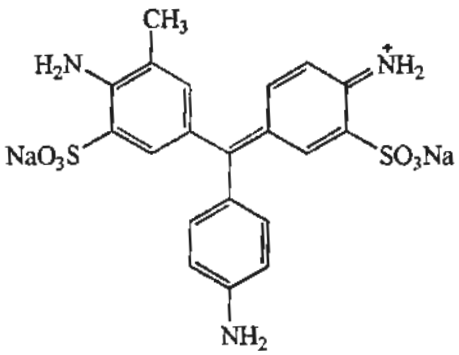
4

The second major class of acid dyes is the anthraquinoids, for example CI Acid Green 27, **5**. The anthraquinoids provide the bright blues, violets and green hues. In general these types of dyes have only low to moderate wet 'fastness'. The term fastness describes how well the dyes bind to the wool during various treatments, such as washing or exposure to light.



5

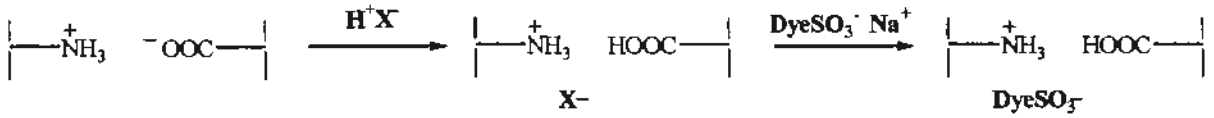
The other major subgroup of the acid dyes is the triphenylmethane related dyes such as CI Acid Violet 19, **6**. This class provides the brilliant violets, blues and greens, all of which have relatively low all round fastness. There are several more classes of acid dyes, but for reasons of hue and economics these dyes have only limited use.[13,14]



6

Small molecular weight dyes such as CI Acid Orange 7, **3**, generally require strongly acidic conditions (pH 2.5-4.0) to give good dyeing of wool, but show only moderate fastness to washing.[15] Also, they require prolonged boiling during the dyeing process or otherwise display uneven dyeing. Immersion of the wool into an acidic dyebath allows the protonation of free carboxyl groups of the zwitterion carboxylate-ammonium ion pairs. The positive charge is

balanced initially by the small acid anions. The slowly diffusing anionic dye molecules later displace the acid anions due to their greater affinity for the keratin, resulting in dyed wool, (**Scheme 1**).[15] Hence for low molecular weight dyes the predominant contributor to dye binding is electrostatic forces which may be augmented by van der Waals forces, depending on the dye.[15] In general these dyes have only poor fastness to wet treatments and are only used when high fastness to these aqueous conditions is not required, eg. knitting yarn.[8]



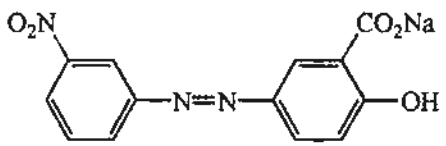
Scheme 1

The acid dyes with large molecular weights, such as CI Acid Red 138, **4**, are known as 'acid milling dyes', and derive their name from their much greater fastness on wool to the milling process. Dyes of this type are applied from less acidic (pH 4.5-6.5) or even neutral dyebaths, normally in the presence of a levelling agent to promote even (or level) dyeing.[16] The mechanism of binding of the acid milling dyes is different to that of the lower molecular weight dyes described above, as in this case van der Waals forces play a much greater part, resulting in a much higher overall affinity of the dye for the keratin.[16] These dyes find application in the dyeing of carpet yarns, machine knitting yarns, and yarns for weaving where high fastness to aqueous conditions is required.[8]

1.1.3 Mordant Dyes

The second major class is mordant dyes which comprises approximately 30-40% of the dyes used to colour wool.[17] The term 'mordant dye' refers to a dye which is applied to the keratin along with a metallic mordant (often containing chromium). A mordant is required because many dyes have very low natural affinities for wool and a pretreatment with tannins or a metallic salt (the mordant) is necessary to increase the affinity of the dye to the extent where a useful degree of binding takes place. Mordant dyes provide generally dull shades with good to very good fastness.[18]

Like the acid dyes, mordant dyes also consist of several subgroups. Again the azo dyes are responsible for the majority of dyes in this class and give a large range of colours with generally very high fastness to wet and light treatments. This subgroup can be divided into the o-hydroxy carboxyazo dyes, eg. CI Mordant Yellow 1, **7**; the *o,o'*-dihydroxyazo dyes, eg. CI Mordant Blue 13, **8**; the *o*-amino, *o'*-hydroxyazo dye, eg. CI Mordant Brown 13, **9**; and the *o*-hydroxy, *o'*-carboxyazo dyes, eg. CI Mordant Red 9, **10**.



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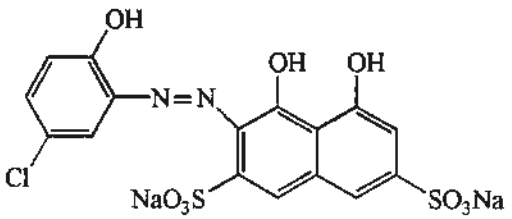

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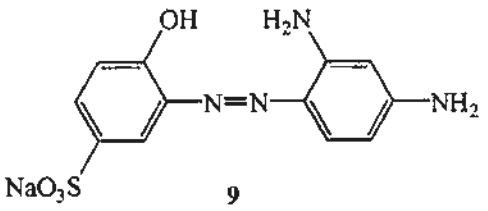

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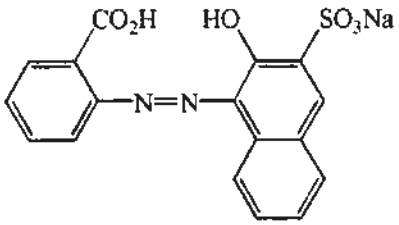
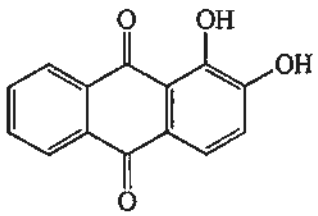


10

The second subgroup is made up of the anthraquinone dyes such as Alizarin **11** (CI Mordant Red 11).[19] Alizarin **11** is a polygenetic dye, in that different mordants produce different hues, eg. red-violet with tin and rose-red with an aluminium mordant. In general this group provides mostly blues, reds and browns.[18]



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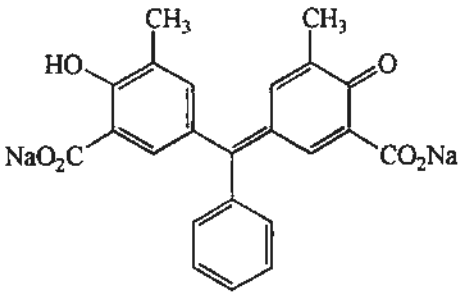
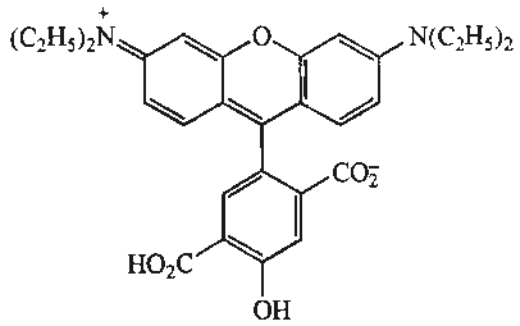


12

The triphenylmethane derived dyes are also used in mordant dyeing, ie. CI Mordant Blue 3, **12**. They give mostly bright violets and blues and generally have only moderate fastness to light.[18]

The last subgroup of mordant dyes is the xanthenes. This is only a very small group and consists of brilliant, mostly red dyes such as CI Mordant Red 27, **13**.



13

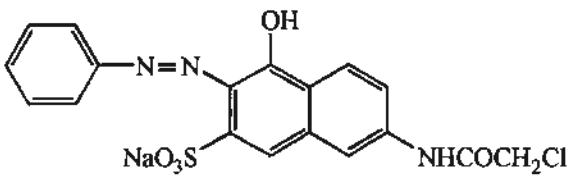
A number of chromium salts have been applied to mordant dyeing[20] with the most common being sodium and potassium dichromate or chromate. The chromium (VI) species is used preferentially to chromium (III) due to its rapid adsorption and desorption allowing even distribution of the metal throughout the wool. The chromium (VI) ion, as either the dichromate or the chromate, forms an electrostatic bond to the protonated amine groups of keratin. The adsorption of the chromium salts increases with decreasing pH. The chromium(VI) ions are then reduced *in situ* to chromium(III), resulting in the oxidation of the cystine groups[21] and a rise in pH.[22] All of the mordant dyes have either a hydroxyl, carboxyl or amino group, through which chromium can co-ordinate to form stable complexes. Evidence for the formation of a 2:1 dye - chromium (III) complex has been obtained by several groups.[22-25] It is believed that this large structure is attached to the wool through a combination of electrostatic and van der Waals forces, with the latter the most important. The high fastness of mordant dyes has been attributed to both the strength of these forces and also the very low diffusional behaviour of this large complex within the fibre.

The chromium salts are applied to the fibre at one of three times: before dyeing (chrome mordant or prechrome method); together with the dye (metachrome method); or after the dyeing process (afterchrome method), of which the afterchrome method is the most popular.

In recent years many efforts have been made reduce the contamination of water supplies with toxic heavy metals by reducing the amounts of chromium in both the wool and the effluent from the dyeing process.[17]

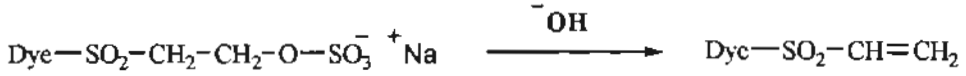
1.1.4 Reactive Dyes [26]

Reactive dyes are unique in that they are the only class of dyes in which the dye is bound to the fibre through a formal covalent bond. Accordingly the reactive dyes have excellent all-round fastness, as the energy needed to remove the dye from the fibre is very high (of the order of that needed to break the carbon-carbon bonds in the dye itself). The first reactive dye, CI Acid Orange 30 **14** was marketed in 1930 by IG Farben. However at the time the lability of the chlorine atom was not recognised.[26]



14

The first reactive dye patent was in 1949 by Heyna and Schumacher.[27, 28] This work led to the introduction of the Remalan range of reactive dyes by Hoechst in 1952. The Remalan dyes contained a β-sulfatoethylsulfone group which was converted to a reactive vinyl sulfone by treatment with hydroxide, (**Scheme 2**).



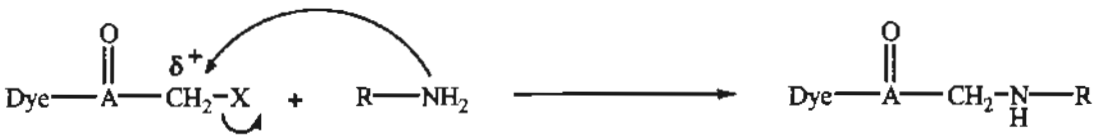
β-sulfatoethylsulfone

Scheme 2

The reactive dyes did not enjoy an initial success with wool, but were more successful in the dyeing of cellulose. The early reactive dyes showed uneven or skittery dyeing, resulting from fast reaction with the weathered tip, and little or no reaction with, or later migration of the dye to, the less reactive roots of the fibre. Many of the original dyes were very prone to hydrolysis, especially in alkaline solutions, and to some extent in mildly acidic solutions. As both of these conditions could be encountered during the dyeing process it was necessary for the hydrolysed dye to be readily removable after dyeing. With cellulose this was easily achieved, but the hydrolysed dyes were found to have very high affinities for wool, making the clearing procedure extraordinarily difficult. In addition low reactivity of the active functional group in the dyes necessitated severe conditions in the dyeing procedure which resulted in partial degradation of the fibre. Most of these problems have been largely conquered by the development of ancillary products to achieve even dyeing, the modification of the dyeing process, and / or the modification of the reactive site of the dyes.[29-33] There are a number of factors which need to be taken into consideration for the development of new reactive dye systems. A high degree of covalent dye-fibre bonding needs to be achieved during dyeing to minimise the clearing process and so obtain the maximum fastness. The rate of the adsorption of the dye and the rate of the reaction between the dye and the fibre must be balanced so as to avoid skittery dyeing. New techniques, such as the Pad-Batch method have been developed which avoid these problems and allow the dyeing of wool at much lower temperatures with dyes which could not be used in the standard processes.[34,35]

In practice restrictions are imposed on the types of reactive groups which can be used in reactive dyeing based on functional group reactivity, the stability of the covalent bond to hydrolysis, the stability of the dye-fibre bond, and the cost and ease of manufacture. The commercially available reactive dyes can thus be divided into two groups, those which react by nucleophilic substitution and those that react *via* a Michael addition.

Dyes which react by nucleophilic substitution generally contain a carbon activated by an adjacent electron withdrawing group (ie. C=O or SO_2). This carbon is bound to a group such as a halogen, sulfato, or a quaternary amine which acts as a leaving group. Reactive groups in keratin, including the amine of a lysine residue, can attack the activated carbon *via* what is believed to be exclusively an S_N2 mechanism to produce the dyed fibre, **(Scheme 3)**.

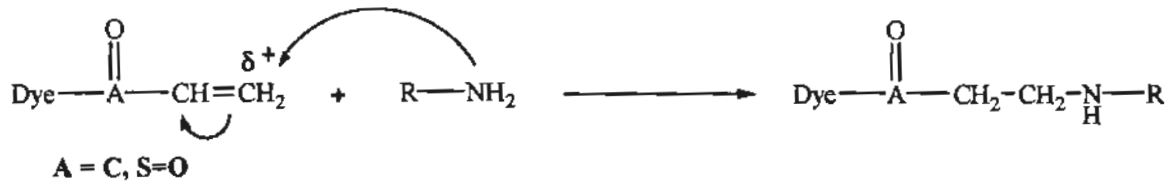


A = C, S=O

X = Halogen, Sulfato, $^{+}_{-NR_4}$

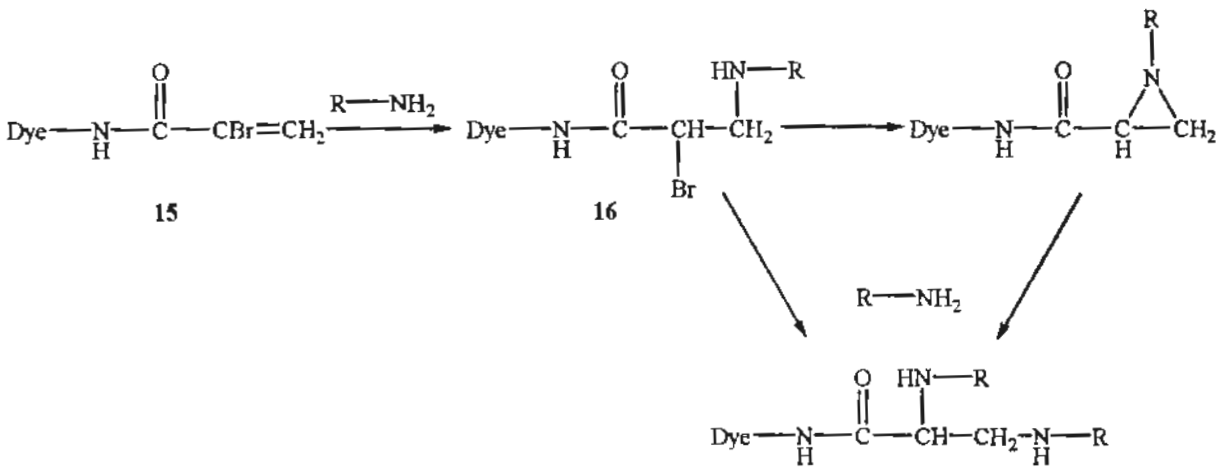
Scheme 3

The reactive dyes which undergo Michael addition with the nucleophilic groups present in the side chains of the amino acid residues in wool generally contain a carbon-carbon double bond activated to 1,4-attack by an electron withdrawing group, **(Scheme 4)**. The electron withdrawing groups most commonly used are carbonyl and sulfonyl groups.



Scheme 4

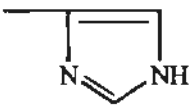
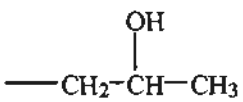
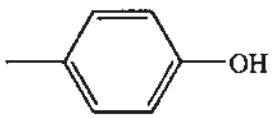
There has been a trend towards bifunctional dyes for both the statistical increase in the probability of finding a binding site in the keratin and for their ability to form a crosslink by reacting with two side chain groups from different peptide strands. The Lanasol dyes **15** produced by Ciba-Geigy exhibit bifunctionality and also reactivity by both Michael addition and nucleophilic substitution.[36-40] The α-bromoacrylamide reacts with the free amines of lysine residues in the keratin in a 1,4 sense (Michael addition) to yield the intermediate **16**. This intermediate can now either ring close by displacement of the bromine to produce an aziridine, followed by reaction with a second lysine residue, or alternatively react directly with the second lysine in a nucleophilic substitution reaction, (**Scheme 5**).



Scheme 5

Techniques have been developed which enable the identification of the reactive sites in the keratin.[41] **Table 1** shows the side chains involved in reacting with the dyes in descending order of importance. Investigations by Shore and others using model compounds have shown that their relative order of reactivity is as follows: cysteine thiol > N-terminal amino > histidine imidazolyl > lysine ε-amino > serine hydroxyl > tryosine phenolic > arginine guanidino > threonine hydroxyl.[42] It has also been suggested that the ratio of the reactivity for the thiols : amines : hydroxyl groups is 10^4:10^2:1.[43] **Table 1** shows lysine as the most important of the amino acids rather than the more reactive cysteine thiol, N-terminal amino, or histidine imidazolyl due to the relatively greater abundance of lysine in the keratin fibre.

Table 1: Side Chains involved in the dye-fibre reaction in decreasing order of importance.

Amino Acid	Reactive Side Chain
Lysine	—CH_2–CH_2–CH_2–CH_2–NH_2
Cysteine	—CH_2–SH
Histidine	
Threonine	
Serine	—CH_2–OH
Tyrosine	
Methionine	—CH_2–S—CH_3
N-terminal amino	

The high reactivity of acrylamido and vinylsulfone groups used in reactive dyes, towards the cysteine thiol groups was confirmed by Baumgarte[44] and Corbett[45] respectively. The major problem in identifying the actual site of reaction in the fibre is that during degradation of the protein into its individual amino acid components many of the dye-amino acid links are also hydrolysed.

The reactive dyes provide a range of brilliant colours with very high fastness to machine washing and other wet treatments due to the covalent bond between the dye and the wool fibre. This class of dyes has made a large contribution to the dyeing of wool. The development of new dyes and better methods to achieve level dyeing, greater stability of both the dye and the dye-fibre bond towards hydrolysis, and to maximise the colour fastness is continuing.

1.2 The Labelling of Proteins

The attachment of dyes to amino acids *via* a covalent bond is also applicable to the labelling of molecules for studies in biology, biomedicine and also for analytical procedures.[46] Fluorescent labels (probes) are extremely useful tools for the investigation of protein structure and function.[47] Alterations in fluorescence quantum yields, or spectral shifts can give information on : i) the microenvironment of an active site,[48] ii) the mechanism of action of a protein,[49] iii) conformational changes in a protein[50] and iv) the proximity of two specific sites from multiple labelling experiments.[51]

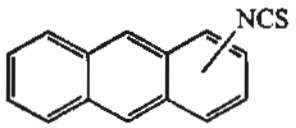
A label is a molecule capable of emitting a signal which is used for labelling proteins and other molecules.[52] A label must possess a number of features: it must specifically bind to a particular target molecule (analyte) under mild conditions, generally *via* a covalent bond; the properties of both the label and the biomolecule should not be significantly altered by the covalent attachment; and the product of the reaction between the label and the analyte must be detectable and generate a signal which can be quantified. Labels consist of three segments: a signal generating group; a reactive site, or anchor group with which to bind to the protein; and a spacer unit or linker arm to reduce or eliminate distortion of the biological molecule due to the signal group, (**Figure 2**).

Figure 2

1.2.1 The Signal Generating Group

Signal generating groups are luminescent compounds (dyes) which exhibit sufficient quantum yields in aqueous solution, are stable under the reaction conditions and can be functionalised by the addition of an anchor group, or otherwise modified to alter their properties. In order to minimise loss of sensitivity due to background fluorescence, light scattering and quenching effects, it is desirable for the signal group to have i) emissions at long wavelengths (500 - 700 nm), ii) a large Stokes shift (the separation of the absorption and emission maxima), and iii) a long fluorescence lifetime ($\tau > 20$ns).[53] An isocyanate derivative of anthracene **17** was the first compound introduced for the fluorescent labelling of biological materials.[54] It was later

superseded by fluorescein isothiocyanate **18**, which was a more efficient label.[55] Fluorescein isothiocyanate **18** remains the most commonly used label due to its high quantum yield and stability.[56, 57]



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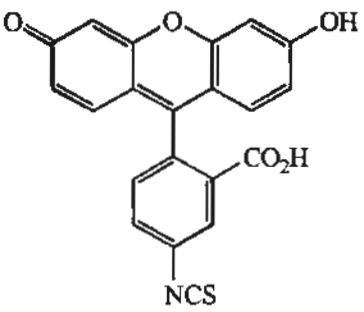

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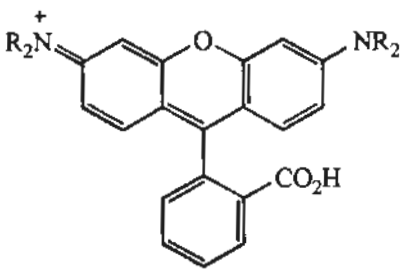
Other commonly used labels are the rhodamines **19**, which exhibit similar properties to fluorescein isothiocyanate **18**, erythrosine **20**, derivatives of resorufin **21**, dansyl chloride **22**, and pyrene derivatives such as **23**. The fluorescein **18**, rhodamine **19**, and resorufin **21** derivatives are used for fluorescence activated cell sorting, fluorescence microscopy and fluorescence immunoassays.[58] Their most important uses are perhaps the labelling of antibodies for direct immunofluorescence detection and the labelling of streptavidin for indirect immunofluorescence.[59] The hydrophobic labels anthracene **17** and pyrene **23** have been used to study aspects of membrane dynamics.[60, 61] Dansyl chloride **22** has been used for sequence analyses of proteins and for the detection of small quantities of peptides and amino acids.[62]



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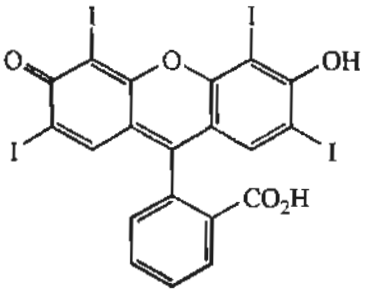

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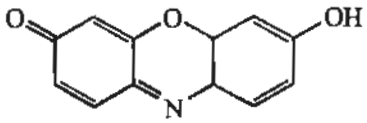

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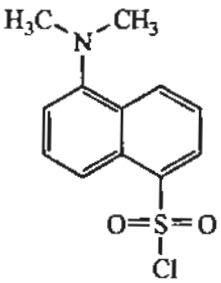

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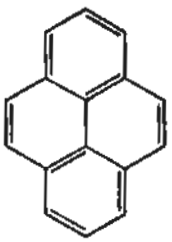
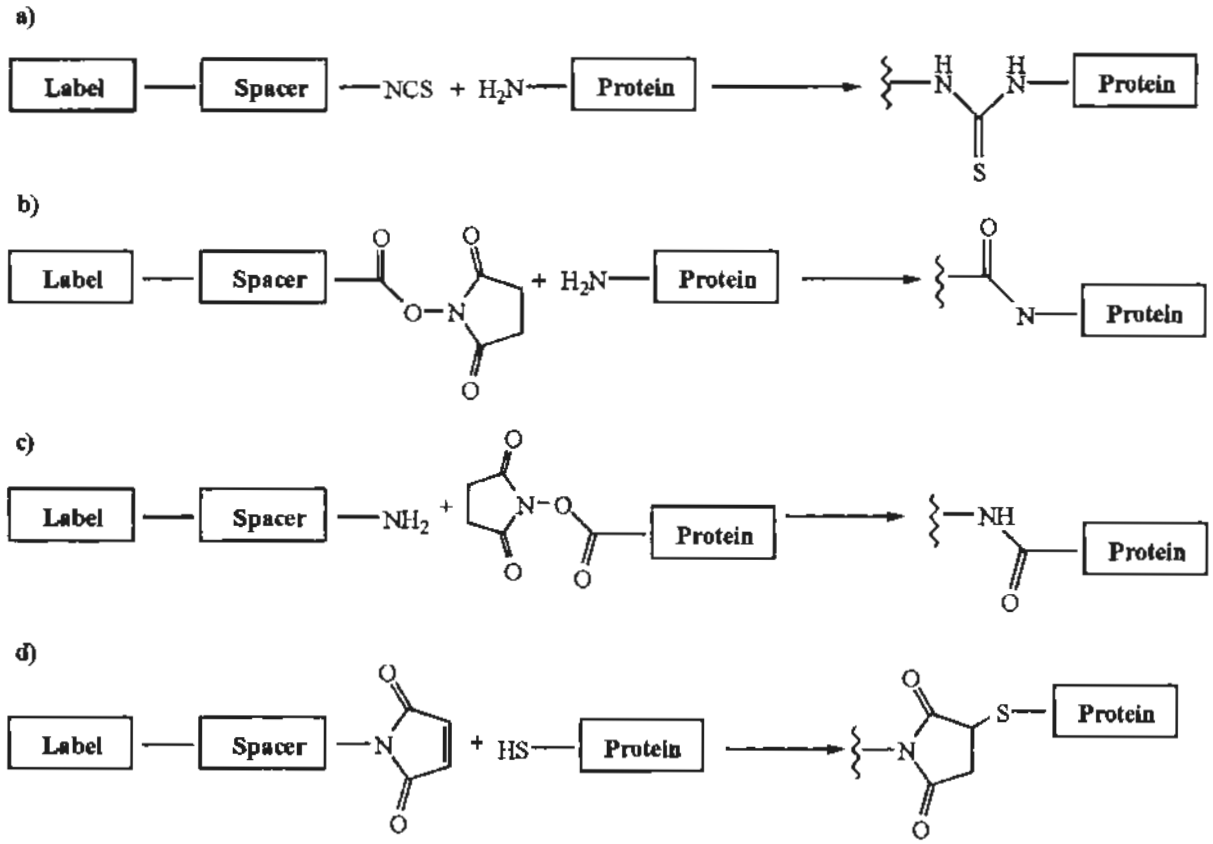


23

1.2.2 The Reactive Group

Proteins contain a large number of freely accessible amino groups to which labels can be bound. Thus many methods for the formation of amide bonds in aqueous solutions under mild conditions are known. The most important of these are summarised in **Scheme 6**.[63] Isocyanate groups on the label will react with an amine to give a urea derivative.[54] The isocyanate group has been replaced by the isothiocyanate group,[55] which yields a thio urea after reaction with an amino group, (**Scheme 6a**). The isothiocyanates suffer from the disadvantage that for coupling with an amine to occur, relatively basic conditions are required, and the thio urea bond formed may not be stable.[59] *N*-Hydroxy succinimide esters do not suffer from this disadvantage and in fact have several advantages over isothiocyanates.[64] They are easily synthesised from carboxylic acid derivatives,[65] and can be readily purified so that

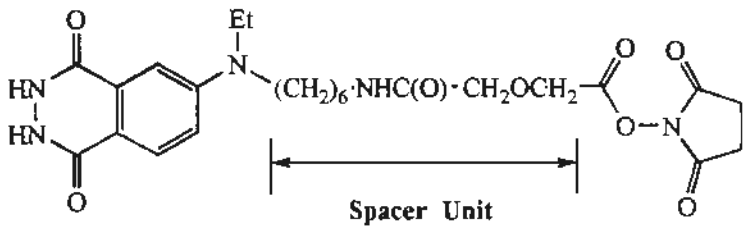
labelling can be carried out in a reproducible manner. Labels with NHS ester functionalities as the reactive group can be stored for long periods of time without alteration of the coupling activity.[64] Coupling between the NHS ester group and amines proceeds at room temperature under mild conditions without affecting other functional groups such as alcohols. The NHS ester may be attached to the label which couples with the free amines in a protein (**Scheme 6b**), or alternatively the reactive group on the label can be an amine for coupling with a NHS ester on the protein (**Scheme 6c**). **Scheme 6d** shows a common method of coupling a maleimide (maleic imido) anchor group to thiol groups present in a protein *via* a Michael type addition.[66] This reaction is of particular importance for the coupling of enzyme labels to proteins.[67]



Scheme 6

1.2.3 The Spacer Unit

The spacer unit is usually a simple, relatively short alkyl chain or a chain containing both aromatic and aliphatic units. Spacer units containing amides and / or ether bridging units, such as that shown in the isoluminol derivative **24**, which confer greater water solubility onto the label - biomolecule conjugate, have also been used.[68]



24

1.2.4 Photophysical Properties and Detection Characteristics

The most important photophysical processes are summarised by the Jablonski diagram, (**Figure 3**).[69] The transitions shown can be used for the production of detectable signals. Luminescence is a general term which describes the emission of a photon by an electron dropping from an electronically excited state to the ground state, and covers both fluorescence and phosphorescence. Luminescence is the property of fluorescent labels most commonly used for detection. When a photon of light is absorbed by a fluorescent label in its ground state, it causes the molecule to be raised to an excited state by promotion of an electron to a higher energy orbital. Generally the electron is promoted to a vibrational level v_n of either the first (S_1) or the second (S_2) electronic states. The time required for the absorption of the photon to occur is approximately 10^{-15} seconds. The molecule then relaxes to the lowest vibrational

energy level (v_0) of the S_1 electronic state by vibrational energy transfer and / or internal conversion from higher electronic states. Fluorescence, which has an average lifetime of 10^{-8} seconds, occurs when the emission of a photon allows an electron to regain S_0. Intersystem crossing to the triplet state T_1 may also occur. The transition of an electron from T_1 to the ground state S_0 is spin forbidden and hence the emission of photons occurs much more slower than emission from the S_1 singlet state (average lifetime of T_1 approximately 10^{-3} to 10^{1} seconds). The emission of photons from the triplet state is known as phosphorescence.

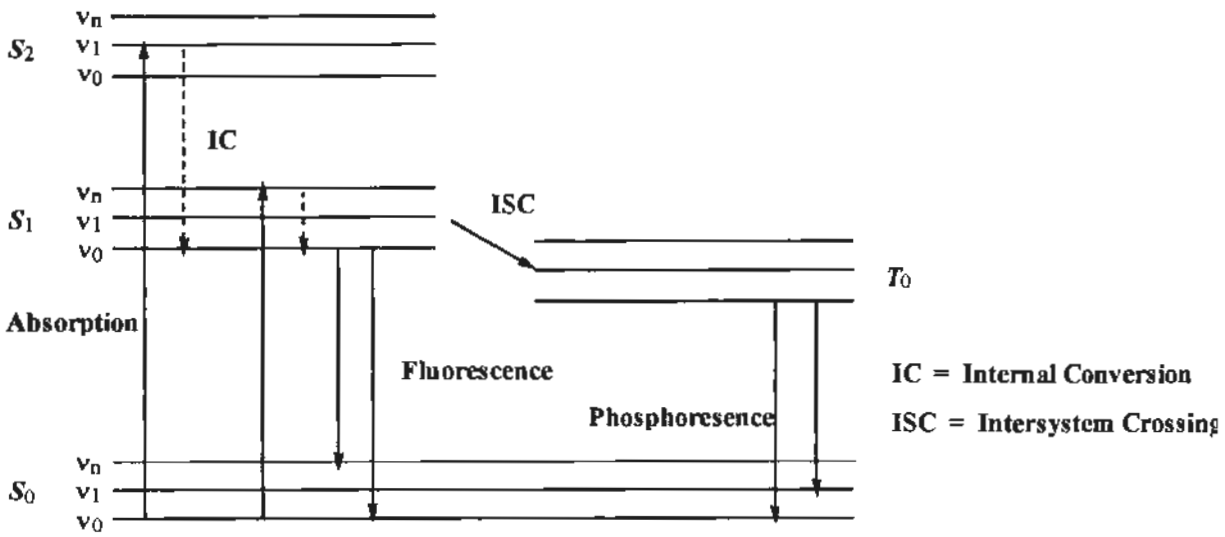


Figure 3

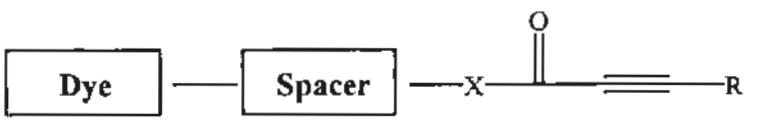
The most important properties of fluorescent molecules are the maximum wavelengths of absorption and emission; the molar extinction coefficient, ε; the lifetimes of the excited states, τ; and the fluorescence quantum yield, ϕ. The quantum yield is the ratio of the number of quanta released to quanta absorbed, and for practical purposes should be greater than 0.4 for fluorescent labels.[70] The quantum yield may be affected by photobleaching, solvent effects, the proximity of quenching species, and the pH of the aqueous solution. The fluorescence

intensity of a given label is determined by the product of the extinction coefficient ε and the fluorescence quantum yield ϕ, and provides a measure of the potential sensitivity of the label.

The specific attachment of fluorescent labels to proteins is a method that has found important uses in immunoassays and for the study of biological processes and mechanisms. It is an area which has great scope for research towards both new molecules with higher fluorescence intensities and better, more specific methods for attaching these molecules to proteins.

1.3 Aims

The aim of this project is to attach dyes to amino acids in a protein. This will be accomplished by the 1,4-addition (Michael type) of the nucleophilic groups present in a protein onto a conjugated alkyne reactive group bound to a dye moiety, (**Figure 4**). Systems of this type provide a reactive group for both dyeing wool and for the fluorescent labelling of proteins for the elucidation of biological structures and mechanisms.



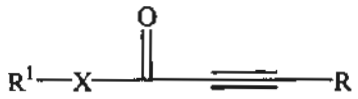
X = C, O, N.

R = H, alkyl

Figure 4

The initial studies will examine the reactivity of individual amino acids with nucleophilic side chains which are common in proteins towards conjugated alkynes. These studies will

concentrate on alkynes activated to Michael-type addition reactions by ketones, esters and amides, (**Figure 5**).



X = C, O, N.

R = H, alkyl

Figure 5

The second stage of this project will require the synthesis of a series of compounds such as those shown in **Figure 4** where a dye is attached to a conjugated alkyne *via* a spacer unit. The reactivity of these compounds with the individual amino acids will be studied to determine the efficacy of the conjugated alkyne as a reactive group. The attachment of compounds of this type to, initially, short peptides, and later a protein such as wool will be examined.

1.3.1 The Advantages of Alkynes

Alkynes have several advantages over the more commonly used conjugated alkenes. Due to the higher electronegativity of sp carbons compared to sp^2 carbons, alkynes are more reactive towards nucleophilic attack than alkenes.[71] Similarly, conjugated alkynes should also be more reactive towards nucleophilic addition than conjugated alkenes. The higher reactivity of conjugated alkynes should enable dyeing at lower temperatures *via* the Pad-Batch method without the inherent degradation of the wool that can result from the prolonged boiling required by the more severe processes for dyeing wool.

The reactivity of the conjugated alkyne shown in **Figure 4** should be 'tuneable' by the alteration of both the X group and also the R group (from H to alkyl), such that a specific selectivity for different functional group types can be achieved. The relative activating ability of the group adjacent to the alkyne has been correlated with its ability to stabilize an adjacent carbanion,[72] such that

$$NO_2 > RCO > CO_2R > SO_2R > CN \sim CONR_2$$

Thus in proceeding from a ketone to an ester to an amide the reactivity of the conjugated alkyne towards 1,4-addition will be reduced, allowing the possibility of selective addition of the more reactive nucleophiles. The increase in steric hindrance at the β-carbon of the conjugated alkyne also results in a reduction of the reactivity towards conjugate addition, as can be seen from a reactivity series presented by Krief for the analogous enone system, (**Figure 6**).[73]

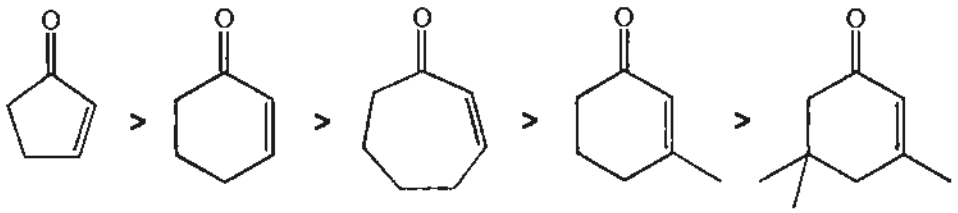
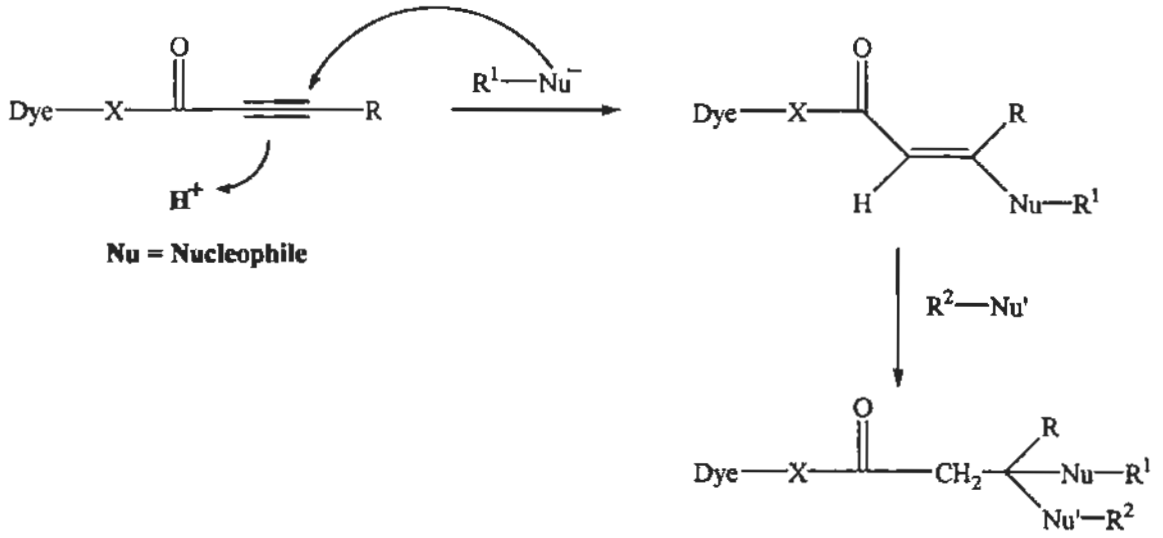


Figure 6

The adduct from the first Michael addition onto the conjugated alkyne is an activated alkene, which can potentially undergo a second conjugate addition, **Scheme 7**. Thus the conjugated alkynes exhibit the possibility for bifunctionality and can therefore act as crosslinking agents by reaction with two amino acid side chains from separate peptide strands.



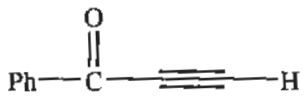
Scheme 7

Due to the high use of acetylene in industrial processes the starting materials for the preparation of dyes of this type should be relatively inexpensive and accessible, and hence the preparation of reactive dyes with conjugated acetylenes should be economically feasible.

Chapter 2: The Model Studies

2.1 The Reactions Of The Model Alkynes.

In order to probe the conditions for the attachment of dyes to proteins *via* a Michael-type addition, the reactions of a series of six model conjugated alkynes were studied. These model alkynes consisted of both terminal and non-terminal alkynes attached to carbonyl groups from a representative sample of functional group types, **25** - **30**.



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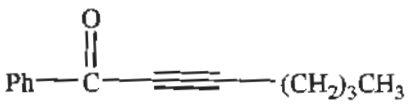

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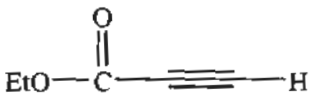

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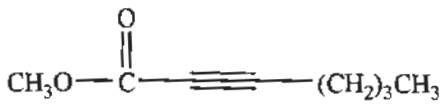

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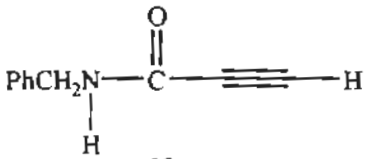

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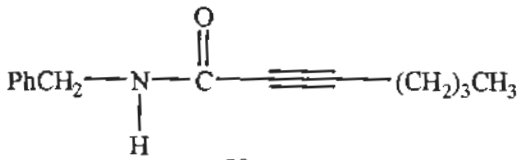
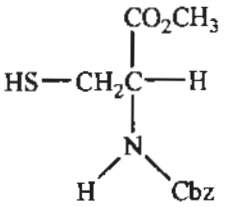


30

Since the main side-chain functional groups found in proteins are sulfhydryl, hydroxyl and amino, the three amino acids cysteine, serine and lysine were examined. These amino acids were protected as the N_{α}-carbobenzyloxy (Cbz) methyl esters, **31** - **33** in order to better examine the reactivity of the side-chain functional group.



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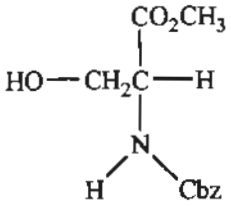

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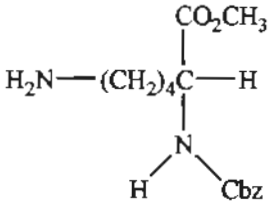

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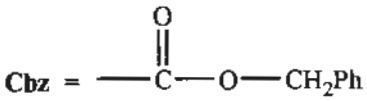
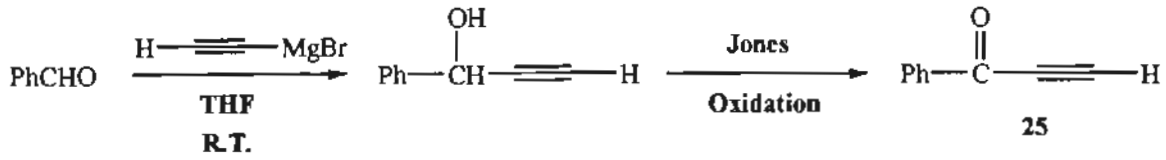


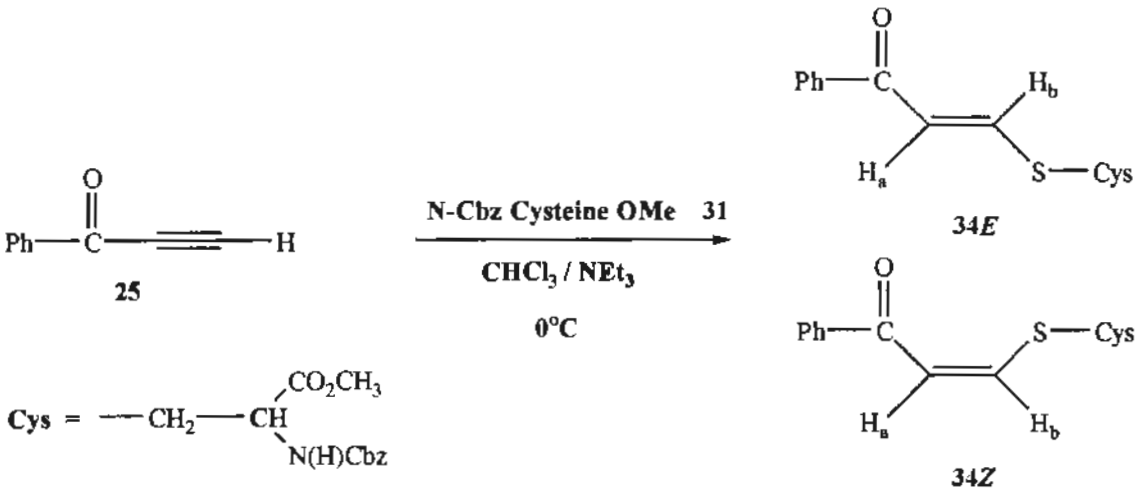
2.1.1 Terminal Acetylenic Ketones

The acetylenic ketone **25** was expected to be the most reactive of the alkynes towards nucleophilic attack, (see page 24), as a result of it having a lesser electron density at the β-carbon of the acetylene than the corresponding conjugated acetylenic esters and amides **27** - **30**. This is due to no atom (ie. oxygen or nitrogen) with available lone pairs which can further delocalise the partial positive charge on the carbonyl carbon, and so increase the electron density at the β-carbon of the acetylene. The alkyne of **25** is terminal and does not have an alkyl substituent to hinder the approach of the nucleophile and so reduce the reactivity, as does the non-terminal acetylenic ketone **26**. Hence the reactions of the terminal acetylenic ketone with the protected amino acids **31** - **33** were the first investigated. Compound **25** was prepared by the reaction of benzaldehyde with ethynylmagnesium bromide under standard Grignard conditions, to yield the corresponding benzyl alcohol derivative.[74] Jones oxidation of this alcohol, followed by Kugelrohr distillation, gave the required terminal acetylenic ketone **25** as a pale yellow solid with an odour similar to that of the starting aldehyde in an overall yield of 30%, (**Scheme 8**).[75]



Scheme 8

The addition of the cysteine derivative **31** to the terminal acetylene **25** was found to proceed rapidly in chloroform when catalysed by a small amount of triethylamine (reaction time ~5 minutes), and generated both the *E* (trans) **34*E***, and the *Z* (cis) **34*Z***, isomers, in a 1.2:1 ratio (determined from the integration of the vinylic protons in the crude ^{1}H NMR spectrum) in a total yield of 71%, (**Scheme 9**). This is consistent with literature results on the studies of the addition of thiols to acetylenic ketones, in which both the *E* and the *Z* isomer were observed.[76]



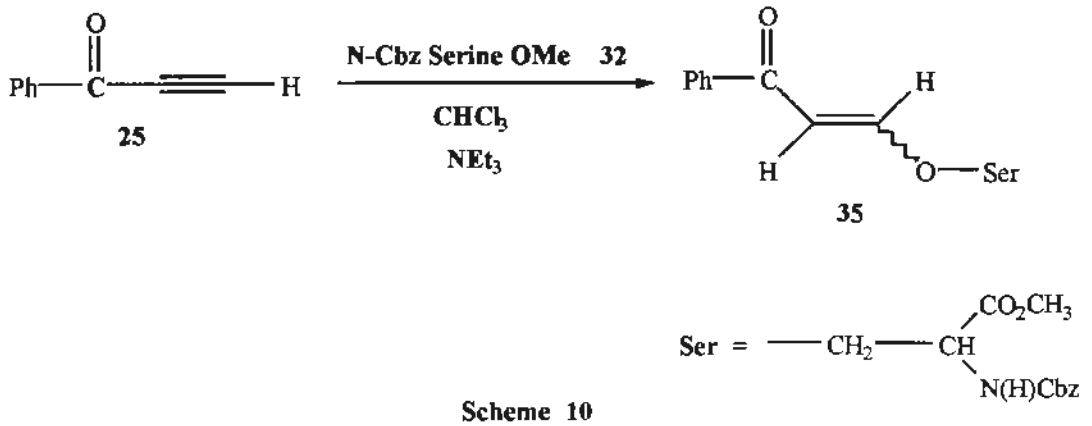
Scheme 9

When triethylamine was added to chloroform containing the terminal acetylenic ketone **25** in the absence of the nucleophile **31**, the solution quickly turned dark brown in colour, possibly due to the base catalysed polymerisation of the terminal alkyne. To minimise this unwanted side reaction the addition of the thiol **31** to the acetylenic ketone **25** was carried out at 0°C and was quenched after approximately ten minutes by the addition of 10% hydrochloric acid to

remove the triethylamine. The addition products were determined to be stable to hydrochloric acid for longer than the time required to complete this procedure. The two isomers, **34*E*** and **34*Z*** had similar R_f's, and were only partially separable by silica gel chromatography. The structures of the two isomers were confirmed by ^{1}H NMR, where the characteristic vinylic protons appeared at δ 7.06 (H_a) and δ 7.80 (H_b) for **34*E*** (J = 15 Hz) and at δ 6.99 (H_a) and δ 7.93 (H_b) for **34*Z*** (J = 9.5 Hz). FAB mass spectral data (molecular ion at 400) also supported the structures shown below. The stereochemistry of the two isomers was determined from the size of the coupling constants between the vinylic protons in the ^{1}H NMR as shown above, (an *E* (trans) isomer has a larger coupling constant than a *Z* (cis) isomer).[77] The addition products were stable at room temperature, showing only minor decomposition after several weeks, but demonstrated *Z* to *E* isomerization if left in solution, to yield a final *E* to *Z* ratio of 3.5 to 1.

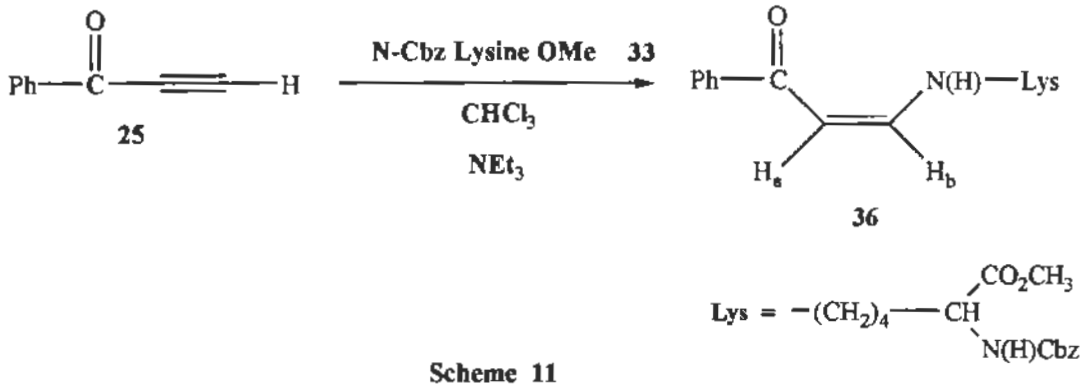
A rapid reaction between the serine derivative **32** and the acetylenic ketone **25** also took place in the presence of triethylamine, (**Scheme 10**). When the procedure from the above reaction was monitored by ^{1}H NMR, two doublets at δ 6.38 and δ 7.66 (J = 12 Hz) and two doublet of doublets at δ 3.21 (J = 8, 16 Hz) and δ 5.92 (J = 2, 8 Hz) were observed. If the reaction was not quenched with hydrochloric acid, but rather the solvent removed *in vacuo* then the two doublets disappeared. This suggests the compound responsible for the doublets was formed by the action of acid. Purification of the crude reaction mixture by flash chromatography on silica yielded 80% of an oil which was confirmed to be the expected addition product **35** by a molecular ion at 384 in the LSIMS spectrum. Extra resonances not attributable to the addition product **35** were observed in the ^{1}H NMR spectrum, indicating that **35** was coincident on silica with an another material. Further chromatography proved unable to remove this impurity. A COSY spectrum of the impure **35** showed coupling between a doublet of doublets at δ 5.92 (*J*

= 2 Hz, 8 Hz), and a doublet of doublets at δ 3.21 (J = 8 Hz, 16 Hz), suggesting these resonances were due to the two vinylic protons. Attempts to determine the stereochemistry of **35** were undertaken using NOE difference spectra, NOESY spectra and ROESY spectra, all of which were unsuccessful, possibly due to the presence of the above mentioned impurity.



Scheme 10

The addition of the N_α-carbobenzyloxy lysine methyl ester **33** to the terminal ketone **25** (**Scheme 11**) occurred over approximately 30 minutes, and was noticeably slower than the additions of the cysteine derivative **31** and the serine derivative **32**, both of which reacted in less than 15 minutes. The slower rate of reaction of the lysine derivative **33** suggests that when triethylamine (pK_a 11) is present, it is the anion of the cysteine (pK_a of **31** ~ 10-11) and serine (pK_a of **32** ~ 16) derivatives which react, whereas for **33**, (pK_a ~ 38 for RNH_2), due to the much higher pK_a, it is the less reactive free amine that undergoes the Michael addition.[78] The addition of the lysine derivative **33** produced only the *Z* isomer **36**, in 50% yield. The stereochemistry of **36** was determined by NOE difference experiments, (**Figure 7**). The ^1H NMR spectrum of purified **36** showed the two vinylic protons as a doublet at δ 5.69 (J = 7.5 Hz) for H_a and a doublet of doublets at δ 6.90 (J = 7.5, 13 Hz) for H_b, where the second coupling is to the enamine proton on the adjacent nitrogen atom.



Scheme 11

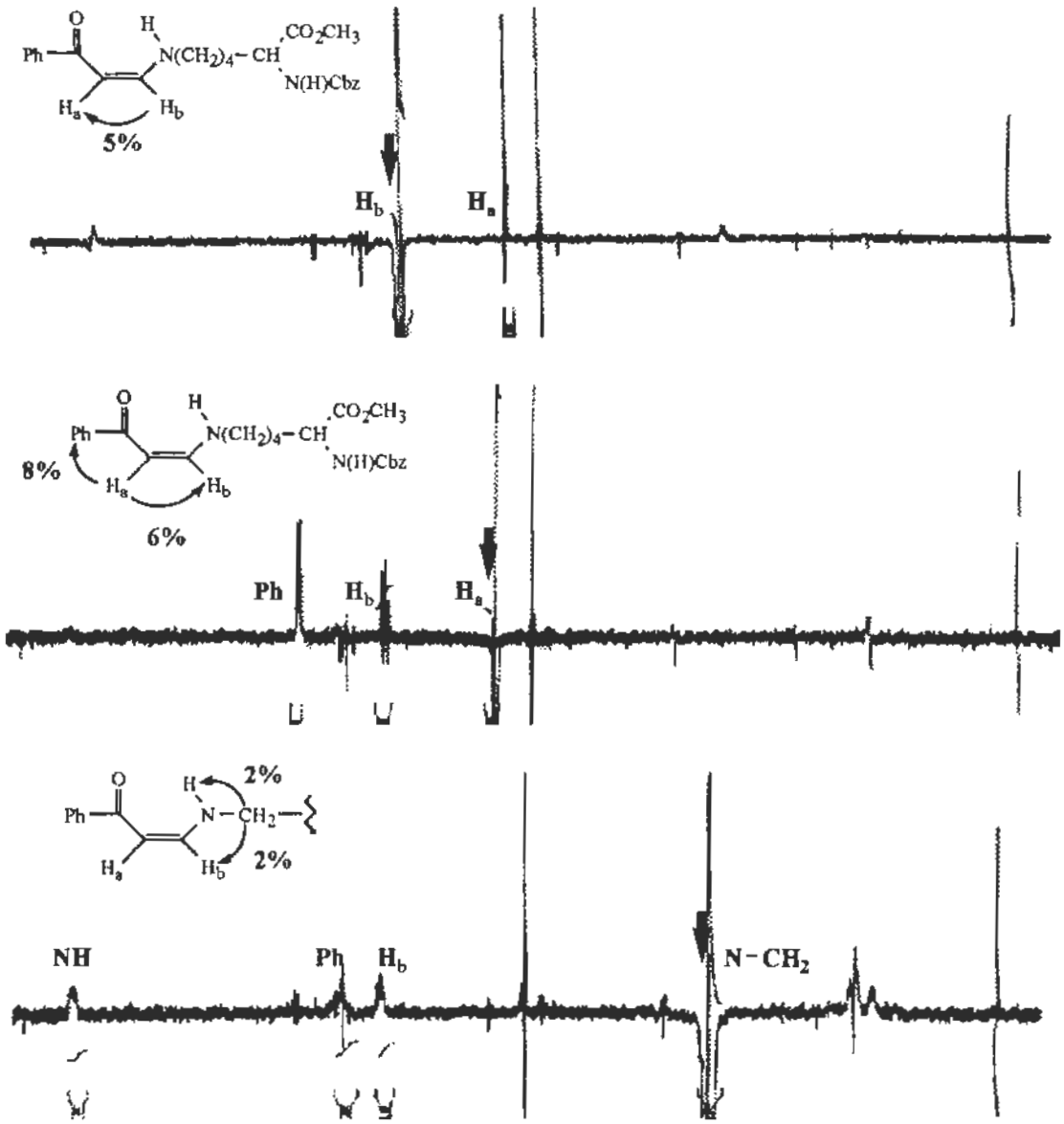


Figure 7: NOE difference spectra

The reaction of the primary amine **33** with the terminal acetylenic ketone **25** forms only a single isomer due to hydrogen bonding of the amine proton to the carbonyl oxygen. This arrangement holds the intermediate anion in the *Z* configuration, which after protonation gives **36**, **(Figure 8)**.[79] Initially, 1.1 equivalents of triethylamine were used to catalyse the reaction (**33** was prepared as the hydrochloride salt). The addition was also found to occur in the absence of added base after the initial removal of the hydrochloride salt by washing with sodium bicarbonate solution. This produced a slower but cleaner reaction due to a reduction in the amounts of side products formed. The yield for the latter procedure was slightly improved (~ 60%).

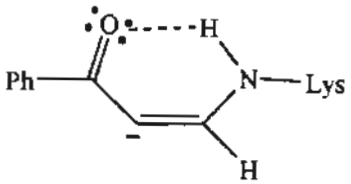
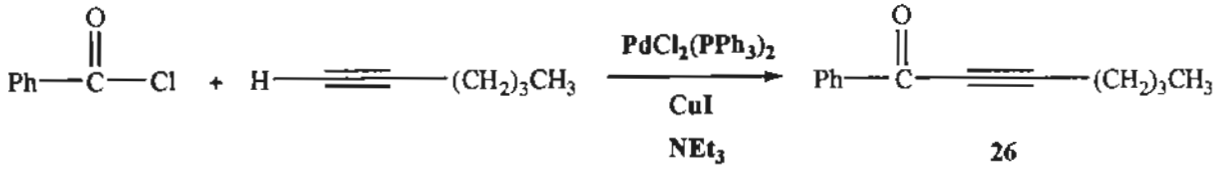


Figure 8

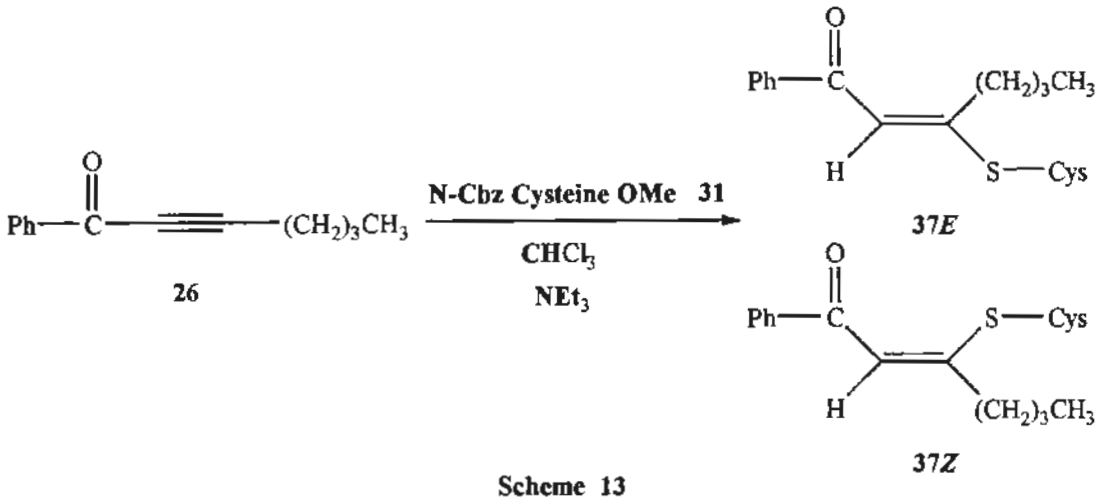
2.1.2 Non-Terminal Acetylenic Ketones

It was expected that the non-terminal acetylenic ketone **26**, like the terminal ketone **25**, would undergo Michael-type additions readily,[80] and that due to the non terminal nature associated with the alkyne, side reactions would be minimised . To test this, compound **26** was prepared, **(Scheme 12)**. Palladium mediated coupling of benzoyl chloride to 1-hexyne in triethylamine with a catalytic amount of copper (I) iodide, gave the conjugated alkyne **26** as a yellow oil in 79% yield after purification by flash chromatography.[81]



Scheme 12

The addition of the protected cysteine derivative **31** to the non-terminal acetylenic ketone **26** was slower than the addition of **31** and the terminal acetylenic ketone **25**, hence this reaction was carried out at room temperature rather than at 0°C. The conjugate addition gave rise to the expected two products, **37*E*** and **37*Z***, in a ratio of 1:2 (from the integration of the vinylic peaks in the crude ^{1}H NMR), (**Scheme 13**).



Scheme 13

The addition products were formed in an overall yield of 53%. The two isomers were separable by chromatography on silica gel. The products were confirmed to be the expected addition products, **37*E*** and **37*Z***, by mass spectral data (molecular ion at 455), and ^{1}H NMR, where the vinylic protons were observed as singlets at δ6.74 (**37*E***) and δ7.01 (**37*Z***). The stereochemistry of the isomers was determined by NOE difference measurements, (**Figure 9**).

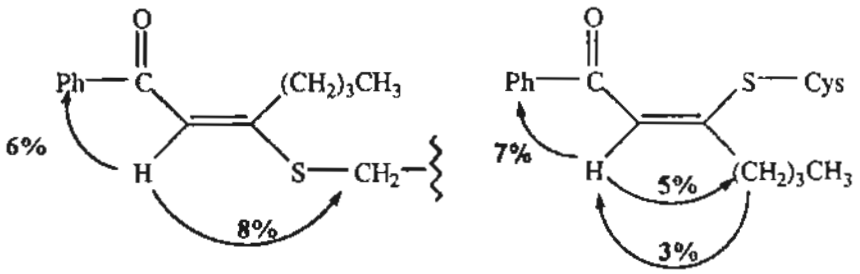
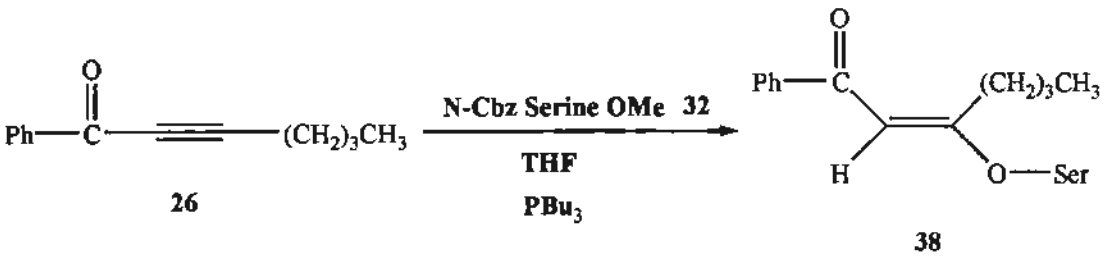


Figure 9

Interestingly the *Z* isomer **37*Z*** was a yellow oil, whereas the *E* isomer **37*E*** was colourless. The *Z* isomer **37*Z*** has a maximum absorbance, (λ_{max} = 327nm), slightly higher than the maximum absorbance of the *E* isomer **37*E***, (λ_{max} = 320nm), and a greater extinction coefficient, (ε = 17120 for **37*Z***, cf. ε = 12310 for **37*E***). This suggests the yellow colour of **37*Z*** results from a shoulder in the visible region which is not apparent in the *E* isomer **37*E***, (ie. **37*E*** has a smaller extinction coefficient and absorbs further into the ultraviolet region). The ^{1}H NMR spectra of the two isomers also showed distinct differences. The methylene protons in the Cbz group occurred as an AB quartet in **37*E***, but was observed as a singlet in **37*Z***. Like **34*E*** and **34*Z***, **37*E*** and **37*Z*** were relatively stable at room temperature, but did show isomerization over a period of one month to favour the *Z* isomer.

When the serine derivative **32** and the non terminal ketone **26** were subjected to the conditions used above in the Michael addition reactions, (ie. chloroform as a solvent with a catalytic amount of triethylamine) no addition product was found after stirring the reaction for one week at room temperature. Heating the reaction to reflux for several days was also unsuccessful in promoting the Michael addition. Previously, a 20% molar ratio of tributylphosphine in THF has been used by Endo *et al* to catalyse the reaction of diols and dithiols with bifunctional acetylenes for the formation of polymers.[82, 83] Following their

procedure for the reaction of the protected serine **32** and the non-terminal acetylenic ketone **26** caused the addition to occur within three hours, generating almost entirely one isomer **38** in 40% isolated yield, (**Scheme 14**).



Scheme 14

The vinylic proton of **38** was observed as a singlet at δ 6.10 in the ^{1}H NMR. The stereochemistry of **38** was demonstrated to be *E* by NOE difference measurements, (**Figure 10**). The reaction occurs *via* an addition-elimination of the tributylphosphine catalyst. The initial addition of the phosphine to the conjugated alkyne is followed by deprotonation of the nucleophile by the vinyl anion to yield a charged intermediate, (**Scheme 15**). The nucleophile, now in close proximity to the alkene, can eliminate the phosphine to provide the addition product. A second compound, possibly another isomer, was also formed in very low yield (>5%), with a vinylic proton appearing as a singlet at δ 6.00 in the ^{1}H NMR. On the scale at which the reaction was undertaken insufficient compound was recovered for complete characterisation. The yield was low due to the formation of other side products, possibly from the further reaction of the anion formed from the initial attack of the tributylphosphine with the alkyne, as suggested by the final brown colour of the reaction.

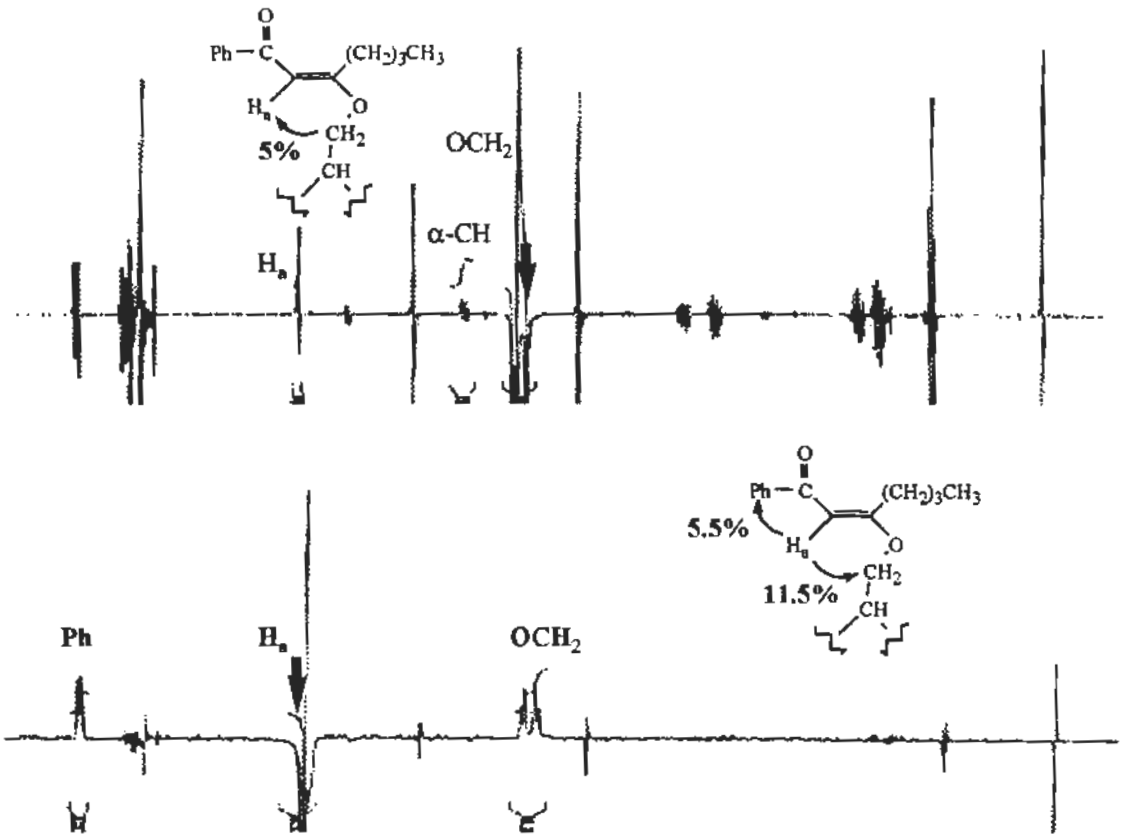
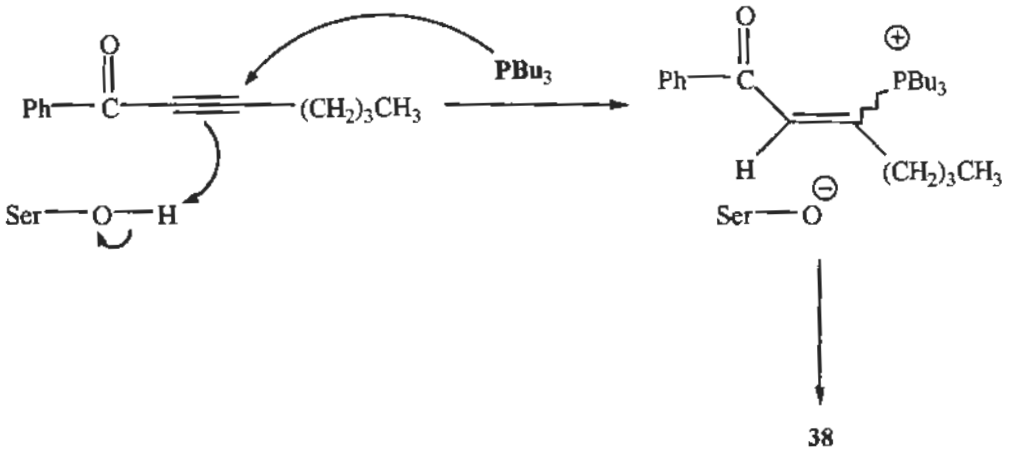
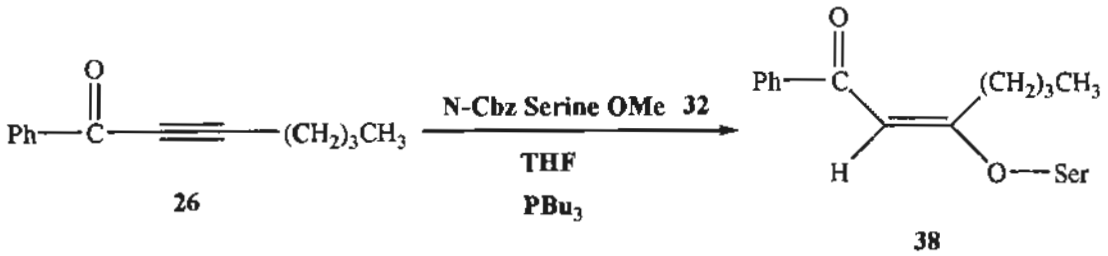


Figure 10: NOE difference spectra



Scheme 15

The amino group of the protected lysine derivative **33** was found to react readily with the non terminal alkyne **26** when using a catalytic amount of triethylamine at room temperature, to generate one isomer **39**, in an isolated yield of 63%, **(Scheme 16)**.



Scheme 14

Only a single isomer was formed for the same reasons as those outlined above for the preferential formation of **36**. The structure of **39** was confirmed by NOE difference measurements (**Figure 11**), mass spectral data (molecular ion at 480), and ^{1}H NMR spectra, where the vinylic proton was observed as a singlet at δ 5.66, and the enamine NH at δ 11.55. Like compound **37Z**, **39** was also yellow in colour. This was not suprising as the two compounds are expected to have similar orbital overlaps and electron distributions due to the positioning of the heteroatom cis to the ketone in both cases. The maximum absorbance in the ultraviolet-visible spectrum of **39** was observed at 348 nm (ε = 28215), (cf. 327, ε = 17120 for **37*Z***), suggesting that **39** is yellow in colour due to a shoulder in the visible region.

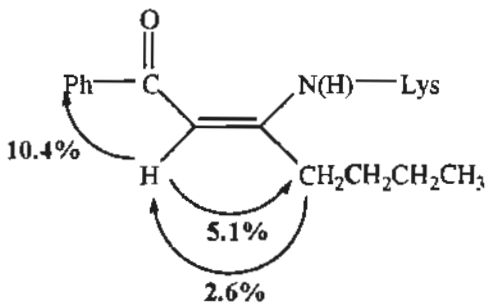
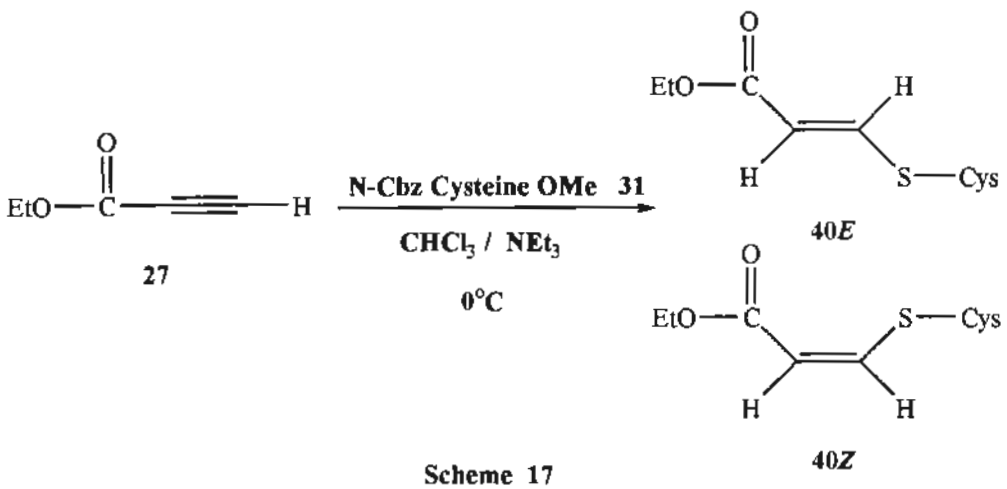


Figure 11

2.1.3 Terminal Acetylenic Esters

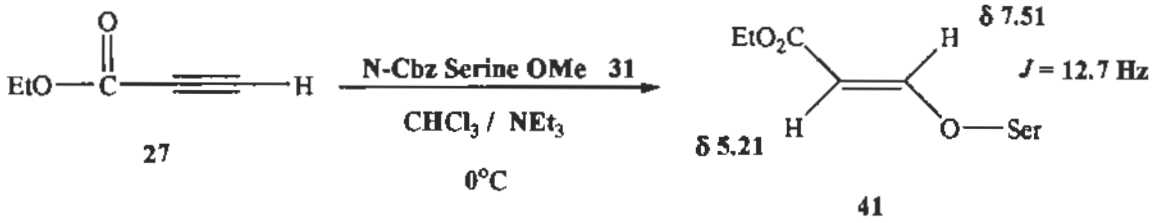
The reactions of the commercially available terminal acetylenic ester ethyl propiolate **27** were next investigated. It was anticipated that the addition of the cysteine derivative **31** to the terminal alkyne **27** would be slower than the corresponding reaction of **31** with the terminal acetylenic ketone **25**. This is a result of the increased electron density at the β-carbon of the alkyne due to the added delocalisation of the partial positive charge on the carbonyl carbon over the oxygen of the ester moiety. The reaction of the thiol **31** with the conjugated ester **27** occurred readily under basic conditions in an ice bath (**Scheme 17**) and was indeed slower than the reaction of **31** with the terminal ketone **25**, (10-15 minutes cf. ~5 minutes). The addition generated two isomers, **40*E*** and **40*Z***, in an total isolated yield of 75%. The ratio of the two isomers was determined from the integration of the vinylic protons in the crude ^{1}H NMR to be 4:1, for **40*E*** and **40*Z*** respectively. The stereochemistry of the two isomers was again determined from the size of the coupling between the vinylic protons in the ^{1}H NMR, with the *E* isomer, (doublets appearing at δ 5.87 and δ 7.57, J = 15 Hz), having a greater coupling constant than the *Z* isomer, (doublets at δ 5.78, and δ 6.92, J = 10 Hz).[77] Some side products, possibly due to polymerisation of the alkyne were also observed, as indicated by a yellow-brown colour formed during the course of the reaction.



Scheme 17

The protected serine derivative **32** also underwent rapid conjugate addition to ethyl propiolate **27** in chloroform at 0°C with a catalytic amount of triethylamine, (**Scheme 18**). The reaction was quenched by the addition of dilute hydrochloric acid after approximately 10 minutes. Flash chromatography yielded only one isomer **41** as a colourless oil in 75%. This result agrees with literature studies which report that the base catalysed addition of alcohols to methyl propiolate gives almost exclusively the *E* isomer.[84] The product showed the characteristic vinylic protons as doublets at δ 5.22 and δ 7.51 (J = 12.7 Hz) in the ^{1}H NMR and a molecular ion at 337 m/z units in the mass spectrum, confirming that Michael addition had occurred. In this reaction only the *E* isomer was observed after acid workup, indicating that the product is less acid labile than the product **35** of the addition of the serine derivative to the terminal alkynyl ketone **25**. The coupling constants (J = 12.7 Hz) for the vinylic protons in the ^{1}H NMR were intermediate between the previously observed coupling constants for *E* (J ~ 15 Hz) and *Z* (J ~ 9-10 Hz) isomers, hence the stereochemistry of **41** was not readily apparent. Thus the stereochemistry of **41** was determined by two methods, the first being rotational Overhauser effect spectroscopy (ROESY), which showed a correlation between the vinyl proton, H_a and the methylene protons adjacent to the oxygen of serine, (**Figure 12**). The second method for determining the stereochemistry of **41** was by comparison with the products from the base catalysed addition of methanol to ethyl propiolate **27**, which resulted in **42*E*** and **42*Z***, (**Scheme 19**). The methyl ester was produced by transesterification from the ethyl ester of ethyl propiolate **27**. In compound **41**, the vinylic protons appeared as doublets at δ 5.21 and δ 7.51 with a coupling constant of J = 12.7 Hz in the ^{1}H NMR, which compared very favourably with doublets at δ 5.22 and δ 7.66 and a coupling constant of J = 12.6 Hz for the *E*

addition product **42*E***. The corresponding Z isomer (**42*Z***) showed the vinylic protons as two doublets at δ 4.86 and δ 6.48 with a coupling constant of J = 7 Hz.



Scheme 18

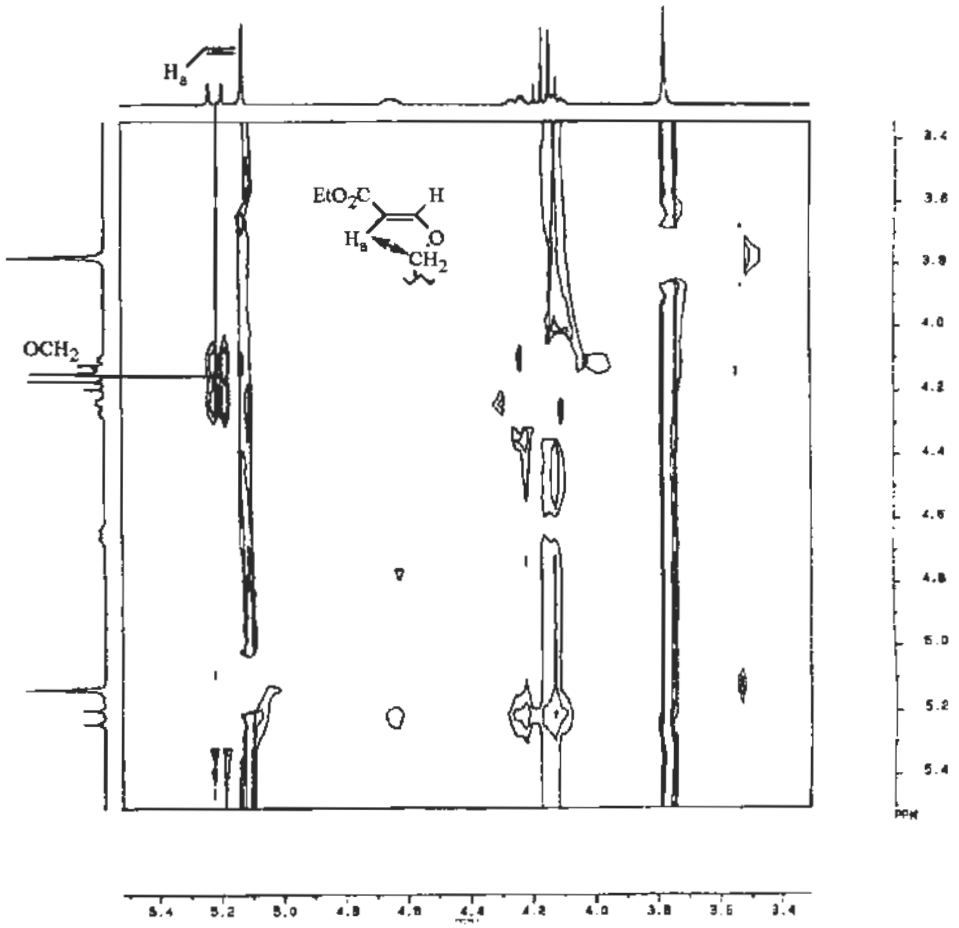
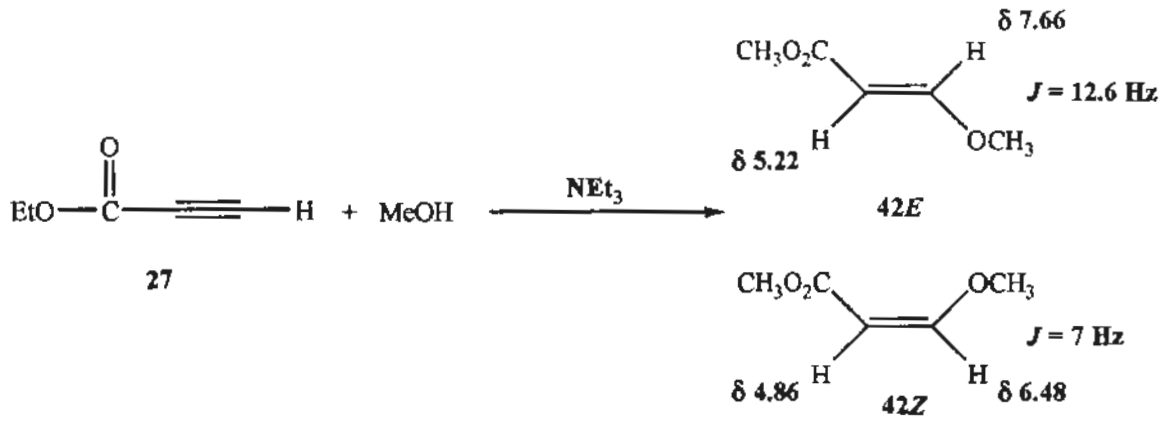
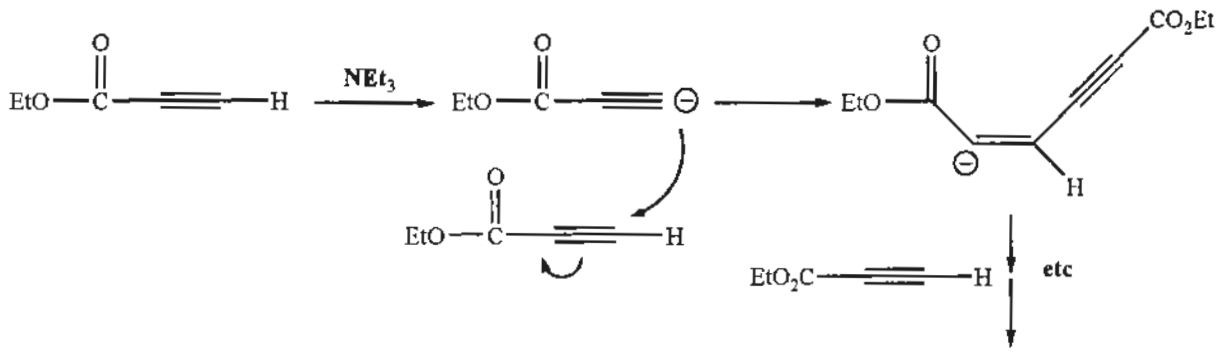


Figure 12: ROESY spectrum of **41**



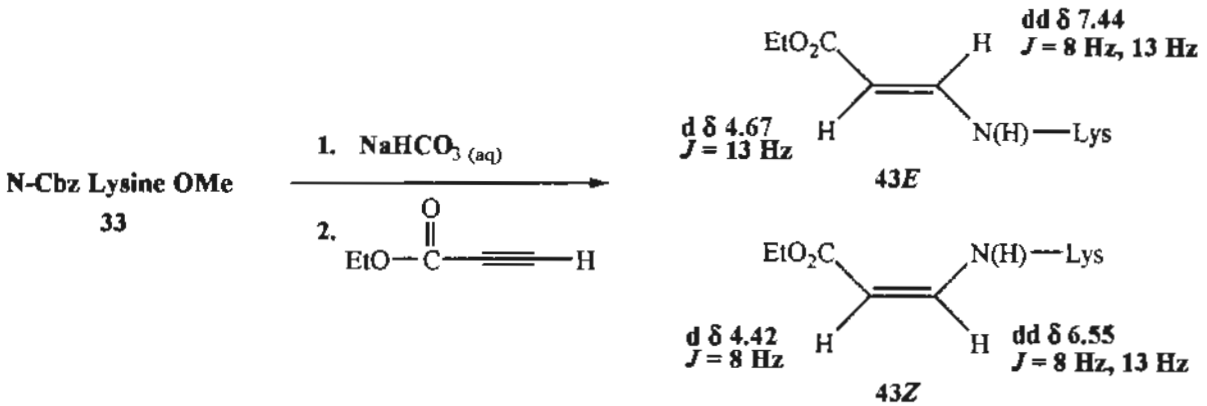
Scheme 19

The addition of triethylamine to a solution of the terminal acetylenic ester **27** and N_{α}-carbobenzyloxy lysine methyl ester **33** in chloroform at 0°C caused a colour change from colourless to dark brown. The crude ^1H NMR was almost identical to the ^1H NMR from the same reaction where the lysine derivative had been omitted; and no addition products were recovered after repeated flash chromatography. This suggests that triethylamine catalysed the self condensation of the ethyl propiolate **27** rather than the Michael addition of the amine **33**, (**Scheme 20**). This indicates the rate of the Michael addition to the terminal acetylenic ester **27** had decreased to a greater extent than the rate of the competitive condensation reaction when compared to the rates of the similar reactions of the lysine derivative **33** with the terminal acetylenic ketone **25**.



Scheme 20

If the hydrochloride salt of the protected lysine **33** was removed by washing with sodium bicarbonate solution and no extra base was added, the conjugate addition reaction proceeded as expected, and two isomers **43*E*** and **43*Z*** were isolated in 45% yield, in a 1:1 ratio, (**Scheme 21**). These isomers were inseparable by flash chromatography, due to streaking on both silica and alumina. A COSY spectrum of the mixture showed a correlation between a doublet at δ 4.67 (J = 13 Hz) and a doublet of doublets at δ 7.44 (J = 8 Hz, 13 Hz), and also between a doublet at δ 4.42 (J = 8 Hz) and a doublet of doublets at δ 6.55 (J = 8 Hz, 13 Hz), indicating these are the vinylic protons for **43*E*** and **43*Z*** respectively, (**Figure 13**). Some self condensation products were also formed in this reaction, but in much reduced quantities.



Scheme 21

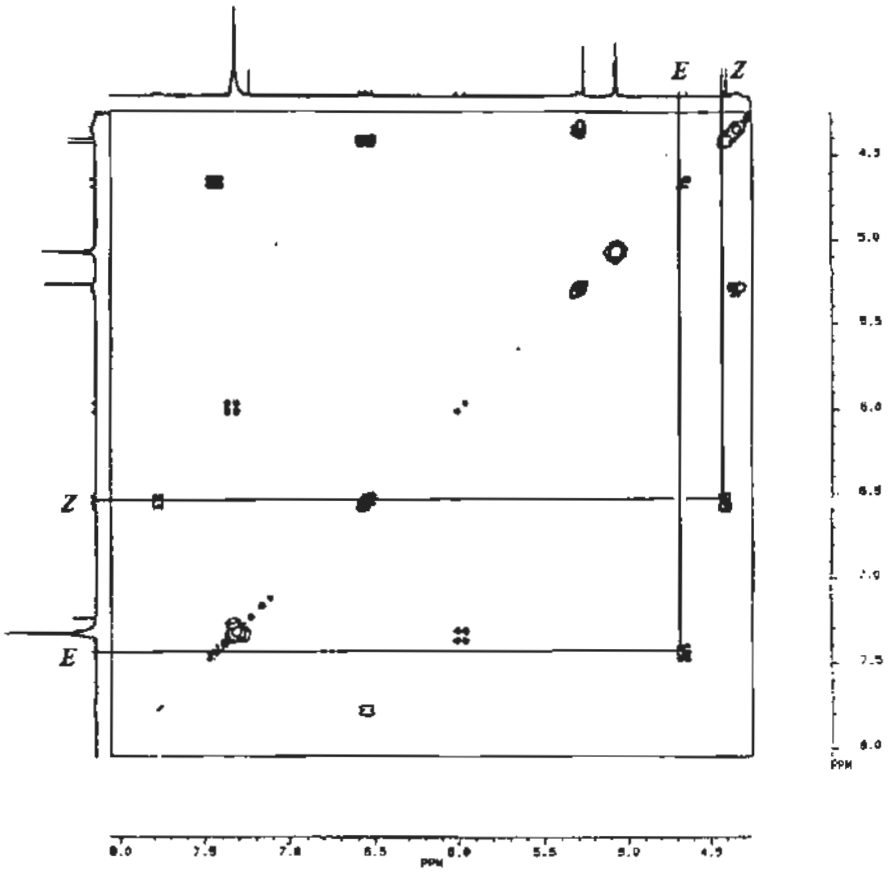
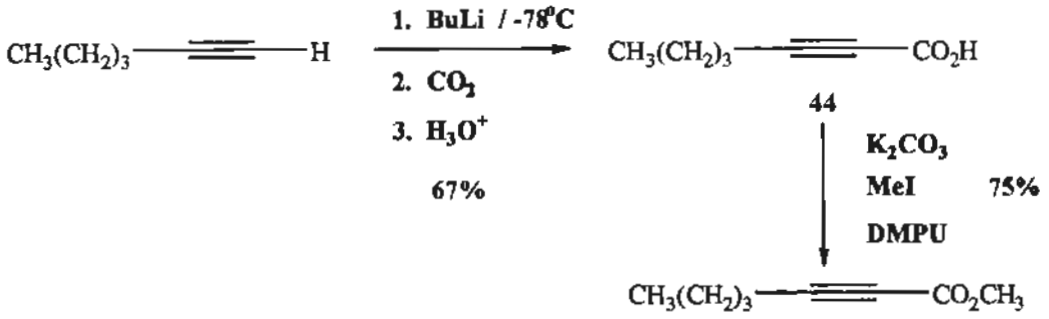


Figure 13: COSY spectrum of the mixture of **43*E*** and **43*Z***

2.1.4 Non-Terminal Acetylenic Esters

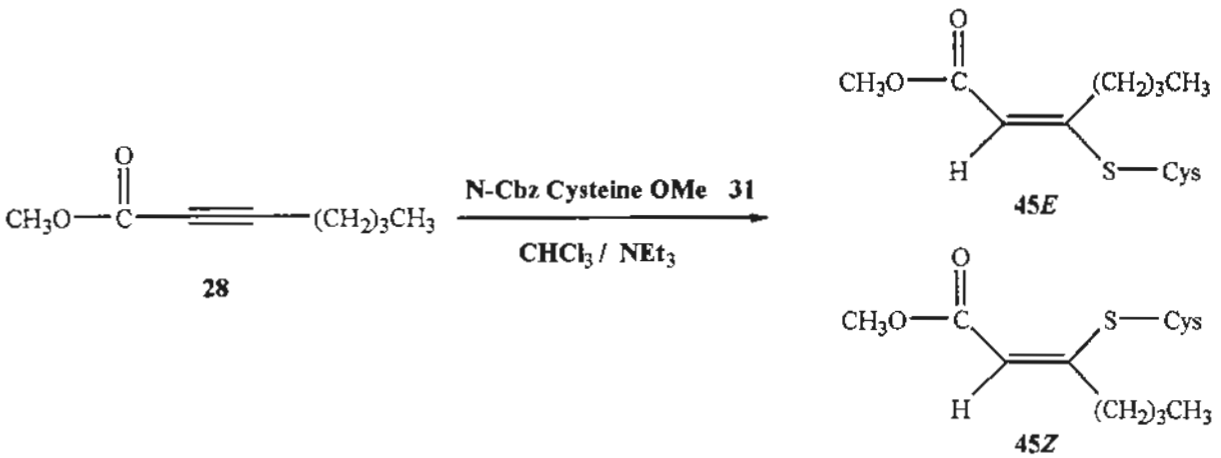
As different reaction pathways due to the presence of the terminal acetylene were again arising, the next model compound studied was the non terminal acetylenic ester **28**, where the terminal proton was replaced by an alkyl chain. The methyl ester was used to simplify characterisation. The conjugated acetylene **28** was produced by the route outlined in **Scheme 22**. Reaction of 1-hexyne with butyllithium produced the lithium acetylide, which was added to carbon dioxide (solid), which, after acidic workup, produced the alkynyl acid **44**. This was followed by

treatment with potassium carbonate and methyl iodide in DMPU, to form the required acetylene **28** in 50% overall yield.[85]



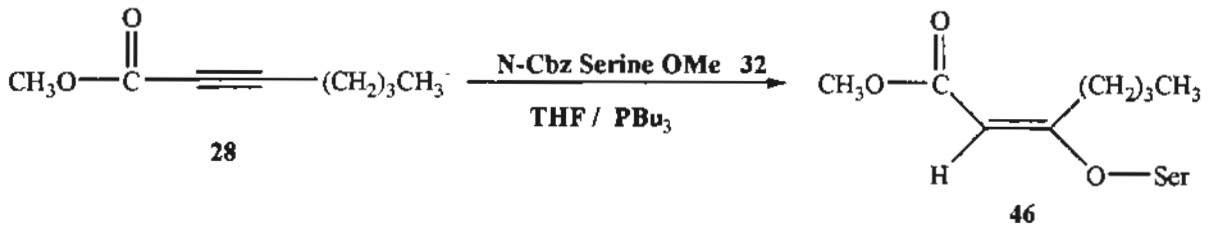
Scheme 22

Reaction of the protected cysteine derivative **31** with the non terminal conjugated ester **28** in chloroform with a catalytic amount of triethylamine gave, after 24 hours, the expected addition products **45*E*** and **45*Z*** in a ratio of 2:1, in 40% isolated yield, (**Scheme 23**). The stereochemistry of the products was determined by comparison with the ^{1}H NMR spectra of polymers prepared by Endo *Et al*, which contained similar units.[83] The characteristic vinyl protons appeared as singlets at δ 5.57 for **45*E*** and at δ 5.83 for **45*Z***. If triethylamine was replaced with tributylphosphine as the reaction catalyst the addition proceeded to completion within 4 hours.



Scheme 23

The non terminal conjugated ester **28** was expected to be less reactive than the non terminal conjugated ketone **26** due to greater electron density at the β-carbon of the alkyne resulting from delocalisation of the partial positive charge on the carbonyl carbon over the ester moiety. Thus it was not suprising that the addition of the alcohol **32** to the ester **28** did not occur with triethylamine as the catalyst, even after stirring for two weeks. The use of a catalytic amount of tributylphosphine caused the reaction to occur when stirred overnight at ambient temperature. Only a single isomer of the product **46** was formed in a yield of 35%, (**Scheme 24**).



Scheme 24

Possibly the yield was low due to further reaction of the anion formed from the first addition of the tributylphosphine to the acetylenic ester **28**. This could occur because of the relatively slower addition of the hydroxyl group of **32** when compared to the rate of attack of the phosphine. Compound **46** was characterised by ^{1}H NMR, where the vinyl proton appeared as a singlet at δ 4.97 and by FAB mass spectrometry, (molecular ion at 394). The stereochemistry was determined by NOE difference measurements, (**Figure 14**). Like the tributylphosphine catalysed addition of the serine derivative **32** to the non terminal acetylenic ketone **26**, this reaction also showed traces of another product, possibly a second isomer, which was again formed in very low yield.

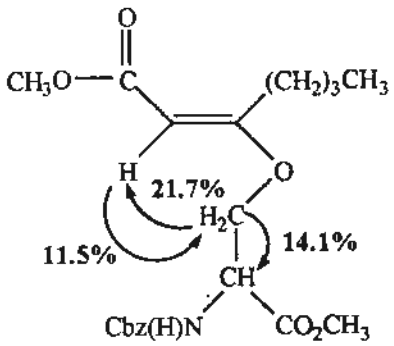


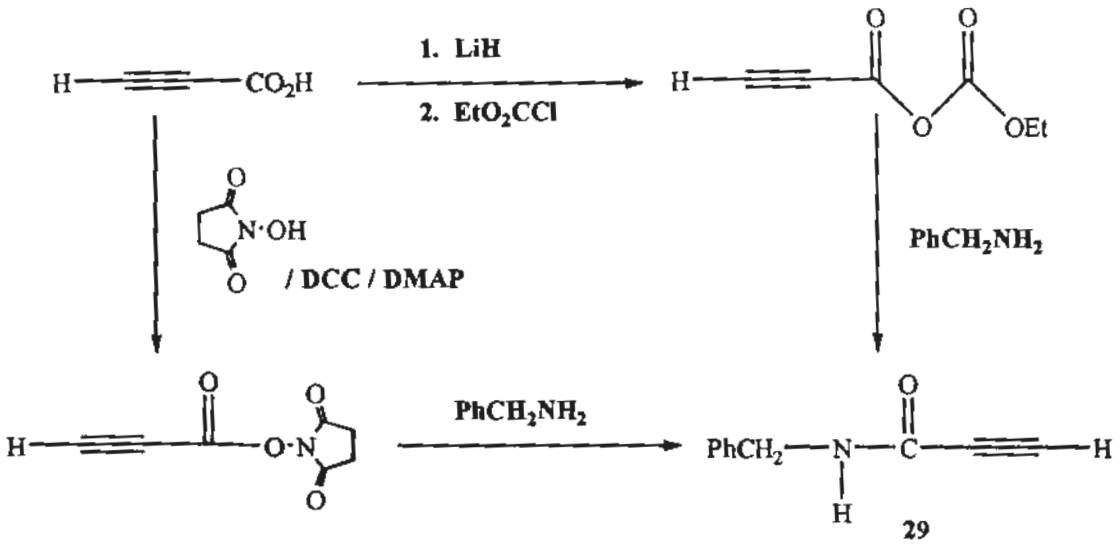
Figure 14

The addition of the protected lysine **33** to the conjugated ester **28** was attempted using both triethylamine and tributylphosphine as the catalyst, but in each case none of the expected Michael product was found. When triethylamine was used the starting materials were recovered unchanged, whilst addition of tributylphosphine resulted in the recovery of products from the further reaction of the initial phosphine-alkyne adduct. To determine if tributylphosphine could be used as a catalyst for the addition of nitrogen nucleophiles to conjugated alkynes, the reaction of the lysine derivative **33** with the non terminal acetylenic ketone **26**, which was known to occur under basic conditions, was attempted. When the phosphine was added to a solution of the nitrogen nucleophile **33** and the conjugated alkyne **26** the reaction turned dark brown after a short period of time (~20 minutes). Tlc analysis of the solution showed a number of new spots, none of which appeared at the same R_f as the addition product. No peak due to the vinyl proton of the predicted adduct was observed in the crude ^{1}H NMR, suggesting the materials formed were from the reaction of the alkyne with the phosphine only. The reaction was attempted again after initial cooling of the solution to 0°C in an effort to reduce the phosphine initiated reactions of the alkyne, while still allowing the conjugate addition to occur, but the results obtained were identical to those from the first experiment.

These results suggest that for a phosphine catalysed addition to occur, an intermediate must be formed where there is an interaction between the nucleophile and the phosphorus atom. This may happen when the nucleophile is sulfur or oxygen, such as for the cysteine derivative **31** or the serine derivative **32**, but would not be expected to occur for a nitrogen nucleophile, as was found to be the case for the tributylphosphine catalysed additions of the protected lysine derivative **33**.

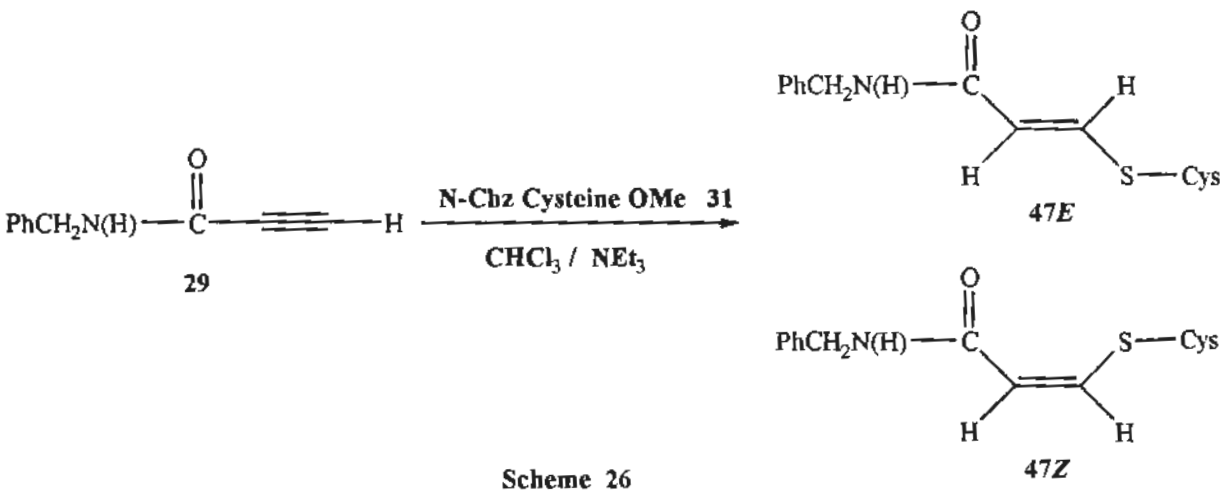
2.1.5 Terminal Acetylenic Amides

The reactions of the three protected amino acids with the terminal alkynyl amide **29** were next studied. Compound **29** was prepared from propiolic acid *via* a mixed anhydride, using the procedure of Coppola and Damon.[86] The acid in tetrahydrofuran at 0°C was treated with lithium hydride, followed by the addition of ethylchloroformate at -10°C, which gave the intermediate anhydride. The anhydride was then reacted with benzylamine at 0°C to produce the amide **29** as yellow crystals in 11% yield. The low yield is probably due to the lithium hydride catalysed self condensation of the propiolic acid, (**Scheme 20**). Alternatively the alkynyl amide **29** was produced by the initial preparation of the N-hydroxy succinimide ester of propiolic acid, using DCC and DMAP in dichloromethane, and the subsequent displacement of this ester with benzylamine, in a slightly better yield (35%), (**Scheme 25**). The moderate yield is probably due to the addition of the DMAP to the propiolic acid.[87]



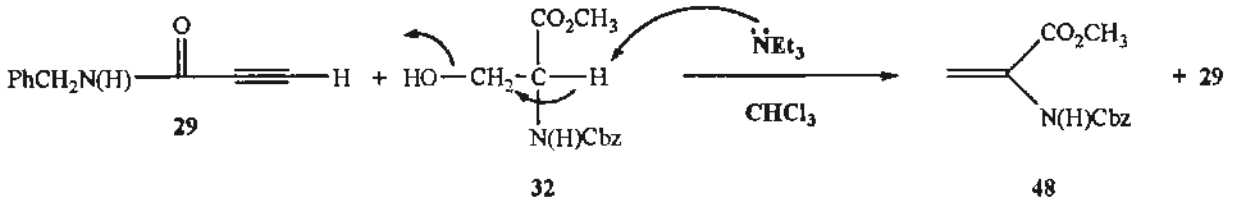
Scheme 25

The thiol containing amino acid **31** was added to the terminal acetylenic amide **29** in chloroform with a catalytic amount of triethylamine. The reaction was stirred for three hours, after which time tlc showed the reaction to be complete. This reaction produced two isomers **47*E*** and **47*Z***, which were isolated as white solids by flash chromatography on silica in 58% combined yield, in a ratio of 1.4:1 (**Scheme 26**). Their structures were determined from mass spectral data, (molecular ion at 428), and ^{1}H NMR, where the characteristic vinylic protons were observed as doublets at δ 5.88 and δ 7.48, (J = 15Hz) for **47*E*** and doublets at δ 5.69 and δ 6.73, (J = 10Hz) for **47*Z***.



Scheme 26

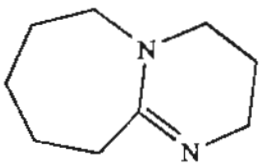
When triethylamine was added to the amide **29** and the serine derivative **32** in chloroform, the major products recovered after stirring the reaction for 24 hours were the dehydroalanine derivative **48**, formed by the elimination of water from the alcohol **32**, and unreacted **29**, **(Scheme 27)**.



Scheme 27

As the rates of conjugate addition reactions are known to increase with increasing solvent polarity,[88] it was hoped that by using a more polar solvent for the above addition the Michael reaction would become the dominant pathway. Accordingly the above reaction was undertaken using acetonitrile as the solvent. Again only the dehydroalanine **48** and the amide **29** were recovered, showing the elimination reaction was still the major pathway. The reaction was also attempted with one equivalent of a stronger base, 1,8-diazabicyclo[5.4.0]undec-7-ene, (DBU) **49**, as the rate of the Michael addition should increase with increasing anion concentration. The conjugate addition was again not rapid enough to preclude elimination. It was apparent that the base catalysed addition of the serine derivative **32** to the terminal acetylenic amide **29** was so slow that side reactions predominated, hence the reaction was attempted with a catalytic quantity of tributylphosphine. Using Endo's conditions for the tributylphosphine catalysed addition, the reaction was attempted twice and in each case the reaction rapidly turned dark brown, and no identifiable products were recovered. To

determine if the use of tributylphosphine was indeed feasible, the reaction of the protected serine **32** and ethyl propiolate **27**, which was known to occur rapidly under base catalysis, was attempted using these conditions. This reaction also quickly changed colour to brown, but some of the expected product was observed in the crude ^{1}H NMR. Flash chromatography on the crude material enabled recovery of an impure sample containing the expected addition product **41**, in less than 25% yield. This sample was brown, showed several spots on a tlc plate and would have been difficult and time consuming to purify. A blank reaction carried out in the absence of the serine derivative demonstrated that most of the resonances in the ^{1}H NMR spectrum of the crude material were due to reactions between the ethyl propiolate **27** and the phosphine and did not involve the amino acid **32**. The above experiments suggest that except in the case where a simple method for purification of the product is available, (such as direct precipitation of the polymers in Endo's studies), the phosphine catalysed conjugate addition of alcohols to terminal alkynes is not an effective method. The addition of the serine derivative **32** to the acetylenic amide **29** was expected to be less efficient than the reaction of **32** and the ester **27**.



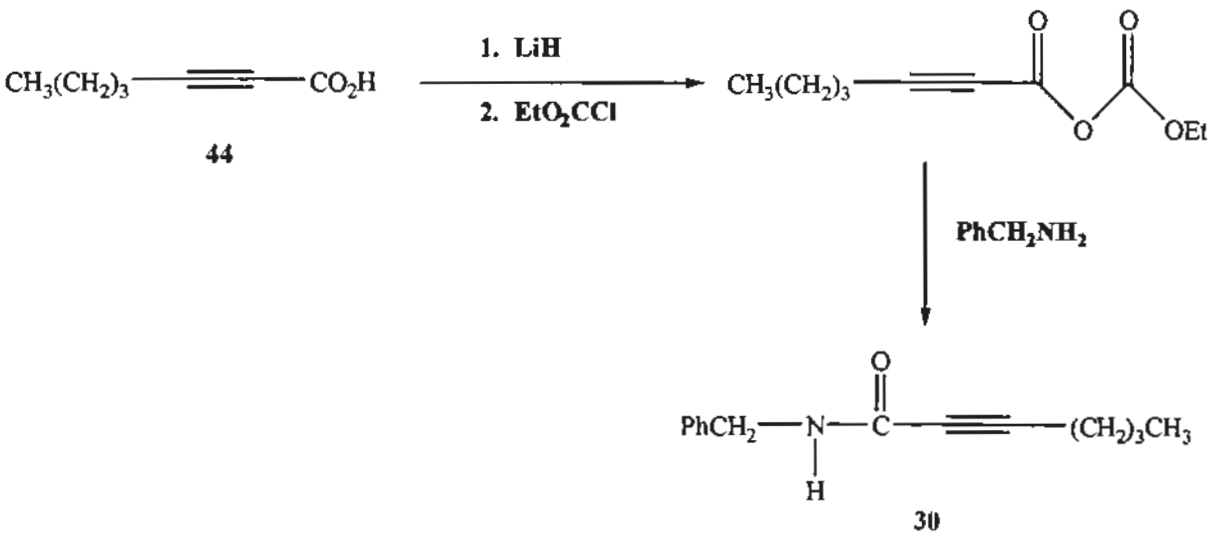
49

The reaction of the protected lysine **33** and the terminal conjugated amide **29** was carried out in acetonitrile to increase the rate of the conjugate addition, with triethylamine as the catalyst. After stirring for several days at room temperature, analysis by tlc showed only the presence of starting materials in the reaction mixture. This indicates the electron density on the β-carbon of the alkyne is high enough (due to delocalisation of the partial positive charge on the

carbonyl carbon over the nitrogen atom of the amide) that the Michael addition of the lysine derivative **33** will not occur under these conditions.

2.1.6 Non Terminal Acetylenic Amides

To complete the series of reactions on terminal and non-terminal acetylenic ketones, esters and amides, a non-terminal acetylenic amide **30** was prepared *via* an analogous method to that used for the formation of the terminal acetylenic amide **29**. Lithium hydride was added to hept-2-ynoic acid **44**, followed by ethylchloroformate, to form the mixed anhydride. The anhydride was displaced with benzylamine, giving the required amide **30** in 67% yield, (**Scheme 28**).

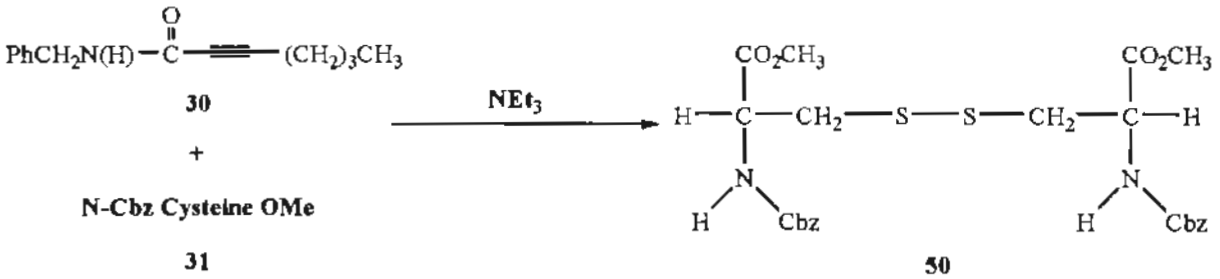


44 **30**

Scheme 28

Using the conditions previously found to be effective, a catalytic amount of triethylamine was added to the protected cysteine derivative **31** and the amide **30** in acetonitrile at room temperature and left stirring for 24 hours. Upon workup the major isolated product was found to be *N,N'*-bis(carbobenzyloxy) cystine dimethyl ester **50**, resulting from oxidation of the thiol side chain in the cysteine derivative **31**, to the disulfide, (**Scheme 29**). The acetylenic amide **30**

was not recovered from the reaction mixture. This unwanted reaction suggests the rate of conjugate addition of the thiol **31** to the non terminal amide **30** was very slow, most likely due to the combined effects of the steric hindrance of the carbon chain and the increase in the electron density on the β-carbon of the alkyne afforded by the delocalisation of the partial positive charge over the amide group. The oxidation of the thiol to the disulfide was favoured by the basic conditions and unexpectedly by the presence of the amide **30**, as shown by the greatly decreased rate of the oxidation in acetonitrile and triethylamine in the absence of **30**, (several days produced only minor amounts). The initial reaction was carried out in air, suggesting that oxygen may have been involved in the oxidation, but after degassing the solvent and repeating the reaction under a stream of nitrogen, *N,N'*-bis(carbobenzyloxy) cystine dimethyl ester **50** was again isolated as the major product. This suggests that oxygen was not directly involved, but rather that the oxidation proceeds by some other mechanism.



Scheme 29

The addition was then carried out in the polar protic solvent methanol, which should favour a relatively fast addition. This was found not to be the case, as the disulfide **50** was again the major product.

When catalytic amounts of triethylamine are used only small amounts of the cysteine derivative **31** exist as the thiolate anion. To increase the rate of addition of the thiol **31** to the acetylenic amide **30** larger amounts of the thiolate anion must be present. In order to achieve this triethylamine was replaced with tetrabutylammonium hydroxide (1 equivalent) (pK_a ~15-16). The tetrabutylammonium hydroxide was added to a solution of the thiol **31** and the amide **31** in acetonitrile under a nitrogen atmosphere. This reaction resulted in the formation of two products. Dehydroalanine **48** was produced by the base initiated loss of hydrogen sulfide from **31**, and a second product, **51** from the Michael addition of the hydroxide anion to the acetylene **30**. The addition product **51** showed resonances in the ^1H NMR spectrum from both of the tautomeric forms, at δ 5.48, due to the vinylic proton in **51a** and at δ 4.06 due to the methylene protons on carbon 2 in **51b**.

51a **51b**

This result indicated the need for a strong base with little nucleophilic character, and so DBU was chosen. When DBU was added to a solution of the protected cysteine **31** and the amide **30** in acetonitrile and left stirring for 24 hours only starting materials were recovered. In this reaction no *N*-Cbz cystine methyl ester **50** was isolated. suggesting that triethylamine plays an important role in the oxidation of the thiol **31** to the disulfide **50**.

As it had already been determined that tributylphosphine was suitable for catalysing the reactions of the cysteine derivative **31** to conjugated alkynes, (cf the addition of **31** to the non terminal acetylenic ester **28**), the use of the phosphine catalyst in this reaction was examined. After stirring a mixture of the amide **30**, the thiol **31** and tributylphosphine in THF overnight there was no sign of any addition product, only starting materials and products that were presumably from the reaction of the phosphine with the conjugated alkyne. These results indicate that in combination, the delocalising effect of the amide bond and the steric hindrance afforded by the carbon chain attached to the acetylene are such as to lower the reactivity of the non terminal acetylenic amide **30** to the extent where it will not undergo any conjugate additions with the cysteine derivative **31** when subjected to the relatively mild conditions discussed above.

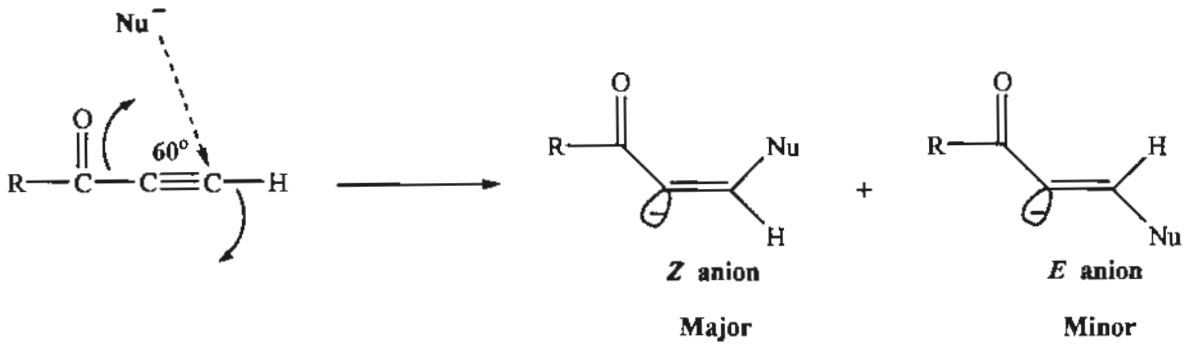
Given that the *N*-Cbz cysteine methyl ester **31** has been seen to be the most reactive of the three protected amino acid used in this investigation, no additions of either the serine derivative **32** or the lysine derivative **33** with the non terminal acetylenic amide **30** were attempted.

2.2 Solvent Effects

A study of how the Michael addition reaction is affected by the reaction solvent was undertaken by examining the reactions of the three most reactive conjugated alkynes, the terminal and non terminal acetylenic ketones **25** and **26**, and the terminal acetylenic ester **27**, with the most reactive of the amino acids, the cysteine derivative **31**. The solvent dependence of the isomeric ratio and the reaction time was investigated and the information obtained compared with the expected results from the mechanism proposed below.

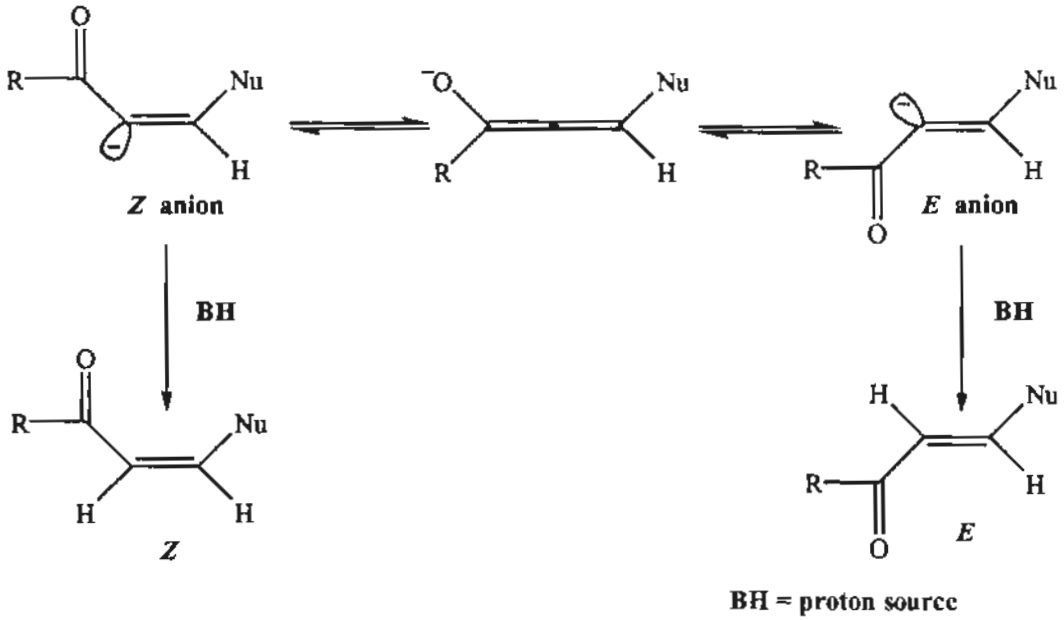
2.2.1 The Mechanism Of the Conjugate Addition Reaction. [89]

Theoretical studies have shown that the nucleophile approaches the β carbon of the alkyne along a carbon-carbon-nucleophile angle of approximately 60°.[90] The approach of the nucleophile is accompanied by the trans motion of the substituents on the acetylene, and is such as to minimise the activation energy required for the formation of the vinyl anion. The addition of the nucleophile to the alkyne initially generates the *Z* anion, and only minor amounts of the *E* anion, due to the favoured trans bending of the acetylene (**Scheme 30**).[91, 92]



Scheme 30

Two processes can now occur i) fast protonation of the anions to give the *Z* isomer as the major product or ii) isomerisation *via* an allene intermediate, followed by protonation, to generate a thermodynamic mixture of isomers, (**Scheme 31**).[93]



Scheme 31

Therefore the ratio of the isomers observed in the product depends on the proton donating ability of the solvent, such that in solvents where protonation is fast, ie protic solvents such as methanol, the kinetic or *Z* isomer should predominate, whereas in non protic solvents such as THF and chloroform where protonation is much slower, equilibration can occur, and the more stable isomer should be formed preferentially.[94] The ratio of the isomers is also dependent on how well the carbonyl group can stabilise the allene intermediate, which is determined by the ability of the activating group to stabilise an α anion by resonance (ie. ketones > esters > amides).[95] Hence, the better the carbonyl group is able to stabilise the allene, the faster the equilibrium is established and the greater the proportion of the thermodynamically more stable ion formed before the protonation can occur.

The information obtained from the reactions of the *N*-Cbz cysteine methyl ester **31** with the conjugated alkynes **25** - **27** is shown in **Table 2**.

Table 2 : A study of how solvent affects the Michael addition reactions of *N*-Cbz cysteine methyl ester **31** with the conjugated alkynes **25-27**.

Solvent	Ph(C=O)C≡CH			Ph(C=O)C≡C(CH$_2$)$_3$CH$_3$			EtO(C=O)C≡CH		
	E	*Z*	Time	*E*	*Z*	Time	*E*	*Z*	Time
Chloroform	**1.2**	**1**	**~5**	**1**	**2**	**5hrs**	**4**	**1**	**>15**
THF	**2.5**	**1**	**15**	**1**	**1.8**	**24hrs**	**2**	**1**	**120**
Methanol	**1**	**2**	**>5**	**1**	**3**	**>15**	**1**	**3.5**	**>5**
Acetonitrile	**3**	**1**	**>5**	**7.5**	**1**	**>15**	**-**	**-**	**-**

The results shown in **Table 2** agree with the above mechanism in most instances. The reactions carried out in methanol all favour the kinetic *Z* isomer as predicted. Those performed in chloroform or THF form the more stable *E* isomer,[93] as the major product in the reactions of **31** with the terminal alkynes and the *Z* isomer predominantly in the addition of the thiol **31** and the non terminal ketone **26**. This suggests that for the products of this addition, **37*E*** and **37*Z*,** the *Z* isomer is the more stable. The experiments undertaken in acetonitrile do not seem to fit into the proposed mechanism, so other factors may be having an effect on the course of the reaction. Acetonitrile is a dipolar solvent and as such should stabilise the more polar anion in preference to the less stabile anion. The results in **Table 2** show that the *E* isomer of the addition product is formed preferentially to the *Z* isomer. These results suggests that the *E* anions, (**Figure 15**), are more polar than the *Z* anions and hence better stabilised by the dipolar solvent.

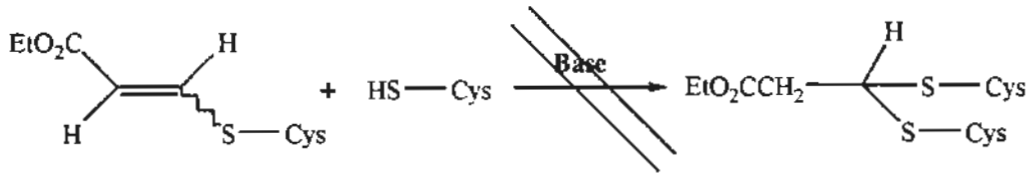
Figure 15

No results were obtained when the reaction of the cysteine derivative **31** and the ethyl propiolate **27** was carried out in acetonitrile. The reaction quickly changed colour to dark brown upon the addition of the triethylamine. This is presumably due to the self condensation of the terminal ester **27**, and probably occurs because the acetonitrile is favouring this process over the addition. Lowering the reaction temperature to -20°C gave the same result. The fact that this occurs with the terminal alkynyl ester **27** and not with the terminal alkynyl ketone **25** suggests that the ester is involved in, or is necessary for this process.

When methanol was used as the solvent, competing addition of the methoxide anion to the terminal alkynes **25** and **27** was found to occur. As **34*E*** and **34*Z***, from the reaction of the thiol **31** with the terminal acetylenic ketone **25** and **40*E*** and **40*Z***, from the addition of **31** to the terminal acetylenic ester **27**, were still formed in the presence of far greater molar quantities of methanol, this demonstrated quite clearly that the sulfur of the cysteine derivative **31** is a much better nucleophile than the oxygen of methanol, and by analogy the oxygen of the serine derivative, **32**.

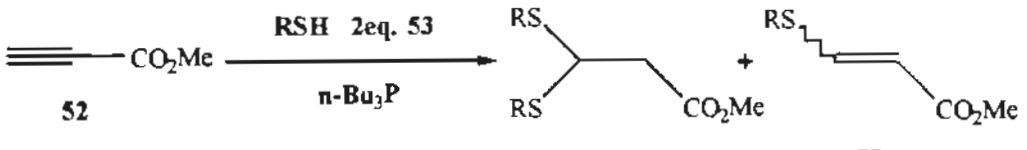
2.3 Second Additions

In all of the reactions discussed above no products were observed from a second Michael addition onto the conjugated alkene formed from the first addition. To determine if a second addition could occur under basic conditions, the product of the addition of the cysteine derivative **31** to ethyl propiolate **27** was purified and added to another equivalent of the thiol **31** in chloroform with a catalytic amount of base, (**Scheme 32**). This reaction was attempted using both triethylamine and the stronger base, DBU to catalyse the reaction. In each case, after stirring the reactions for over 24 hours no trace of a second addition product was apparent by either tlc or in the ^{1}H NMR spectra.



Scheme 32

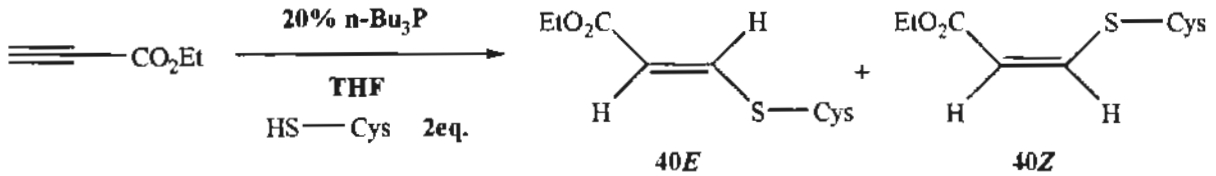
A recent report by Endo *et al* discusses the tributylphosphine catalysed addition of sulfur nucleophiles to methyl propiolate **52**, (**Scheme 33**).[96] It was reported that the ratio of the di-addition product **54** to the monoaddition product **55** could be controlled by varying the number of equivalents of both the thiol **53** and the tributylphosphine catalyst such that they could generate either product selectively. These studies also determined that the first addition of the thiol to the unsaturated acetylene is fast, (kinetic coefficient for the reaction of **52** and **53a** is 2.0 $L^2.mol^{-2}.s^{-1}$) whereas the addition of the thiol to the conjugated alkene formed from the first reaction is much slower, (kinetic coefficient for the reaction of **53a** and **55a** is $2.0x10^{-3}$ $L^2.mol^{-2}.s^{-1}$).



R = $PhCH_2$- (53a)
Ph- (53b)
-CH_2CH_2- (53c)

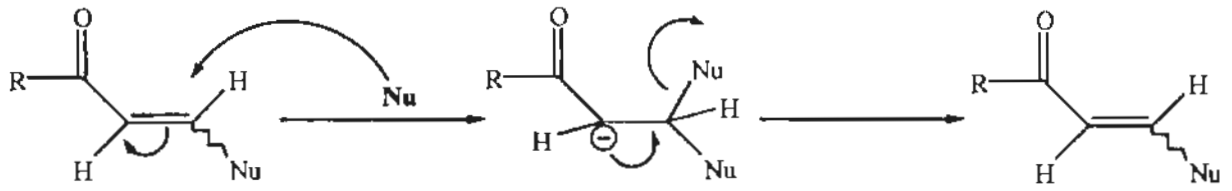
Scheme 33

To discover if such a di-addition was possible for the more sterically bulky protected amino acids used in this study, a 20% molar equivalent of tributylphosphine was added to ethyl propiolate **27** and two equivalents of *N*-Cbz cysteine methyl ester **31** in a solution of tetrahydrofuran, (**Scheme 34**). After stirring the solution for 48 hours tlc showed only the two monoaddition products **40*E*** and **40*Z***. A further equivalent of the thiol **31** was added to the mixture and the solution left stirring several days. Again tlc showed only the monoaddition products. This suggests that the second addition does not occur under these mild conditions due to the steric bulk of the protected amino acid blocking further attack onto the conjugated alkene.



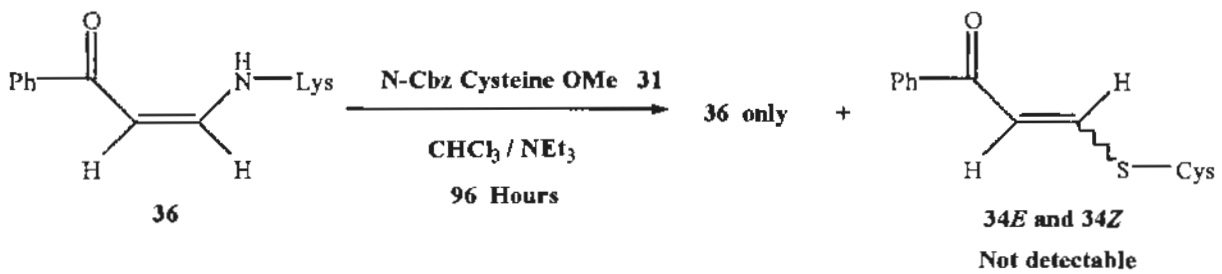
Scheme 34

The possibility of a second, reversible addition followed by a later elimination was also considered, (**Scheme 35**). It this process can occur it should effect the isomeric ratios in the final product.



Scheme 35

To determine the likelihood of the above process occurring a catalytic amount of triethylamine was added to a solution of **36** (the product of the addition of the lysine derivative **33** to the terminal acetylenic ketone **25**) and the thiol **31**, (**Scheme 36**). If the reversible addition took place then a mixture of the two addition products **34** and **36** should be obtained from the elimination of the lysine **33** and serine **31** derivatives respectively. The reaction was allowed to stir for 96 hours, after which time the solvent was removed and the crude ^{1}H NMR taken. The ^{1}H NMR showed no evidence of for the presence of either **34*E*** or **34*Z*** (doublets at δ 7.06 and δ 7.80 for **34*E*** and at δ 6.99 and δ 7.93 for **34*Z***) demonstrating that a reversible addition had not occurred. This indicates that if a reversible addition is possible then the time scale of this addition is such as to leave unaffected the isomeric ratios determined in the studies described in this Chapter.

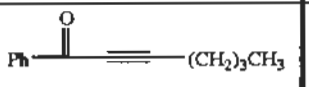
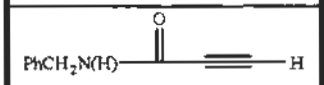
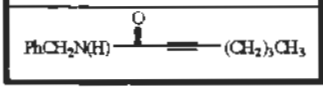


Scheme 36

2.4 Conclusions

Several conclusions can be drawn from the studies of the reactions of the model conjugated alkynes, **25 - 30** with the protected amino acids, **31 - 33**, as outlined in this chapter and summarised in **Table 3**.

Table 3 : A summary of the model addition reactions

Conjugated Alkyne	*N*-Cbz Cysteine OMe		*N*-Cbz Serine OMe		N_{α}-Cbz Lysine OMe	
	E	*Z*	*E*	*Z*	*E*	*Z*
	1.2	1	1	0	0	1
	1	2	97[A]	>3[A]	0	1
	4	1	1	0	1.5	1
	2	1	97[A]	>3[A]	N.R.	N.R.
	1.4	1	N.R.	N.R.	N.R.	N.R.
	N.R.	N.R.	-	-	-	-

N.R. = No Reaction.
A = Reaction only occurs with tributylphosphine as the catalyst.

The use of two catalysts has been established that allow the reaction of the three amino acids with the conjugated alkynes under mild conditions at room temperature or below. For the additions of the cysteine derivative and for the reaction of the lysine and serine derivatives with the more reactive alkynes, the terminal ketone **25**, the non terminal ketone **26** (serine excepted) and the terminal ester **27**, catalytic amounts of triethylamine in chloroform suffice to give a relatively clean and reasonably fast reaction. The reactions of the alcohol **32** with both the non

terminal ketone **26** and the non terminal ester **28** were better suited to the use of tributylphosphine in either THF or chloroform to catalyse the addition, resulting in a slightly lower yielding reaction than when the catalyst was triethylamine. The rate of the reaction of the protected cysteine **31** with the non terminal ester **28** was also substantially increased by the use of the phosphine catalyst. Perhaps due to the mildness of the conditions used and the steric bulk of the protected amino acids no products from a second conjugate addition were ever found in any of the above reactions.

When the additions were carried out under these conditions the reactions of the cysteine derivative **31** produced both the *E* and the *Z* isomers in ratios that were dependent on both the solvent and the carbonyl group of the particular conjugated acetylene used, and in most instances the results obtained agreed with the proposed mechanism. The reactions of the serine derivative **32** produced almost entirely the *E* isomer in each case. The additions of the protected lysine **33** to the conjugated acetylenic ketones **25** and **26** produced only the *Z* isomer as a result of hydrogen bonding in the anion intermediate, whereas the amine **33** produced both isomers when added to the ethyl propiolate **27**.

The reactions discussed in this chapter have allowed the establishment of orders of reactivity for both the model alkynes, such that **25** > **27** > **26** ≥ **29** ≥ **28** > **30**, and also for the amino acids, where **31** > (**32**, **33**). The thiol **31** is clearly the most reactive of the amino acids. The serine derivative **32** reacts faster than the protected lysine **33** (anion vs neutral amine), but the amine **33** is more nucleophilic than the serine **32** (it reacts with the terminal acetylenic ketone under basic conditions where the alcohol **32** does not). This order and the results in **Table 3** suggest that the selective reaction of cysteine residues in a peptide in the presence of serine and lysine residues is possible by the choice of a suitable conjugated alkyne and catalyst, (ie. **28** or

29 with triethylamine). Also a lysine or a serine residue could be labelled selectively in the presence of the other by the choice of the catalyst, using triethylamine to label lysine (with the non terminal acetylenic ketone **26**) or tributylphosphine to label serine (with the ketone **26** or the non terminal acetylenic ester **28**).

These model studies demonstrate that for the attachment of dyes to amino acids, terminal and non-terminal acetylenic ketones and terminal acetylenic esters, would be the most useful. To a lesser extent, and specifically for the labelling of cysteine residues conjugated terminal acetylenic amides would also be useful, (**Figure 16**).

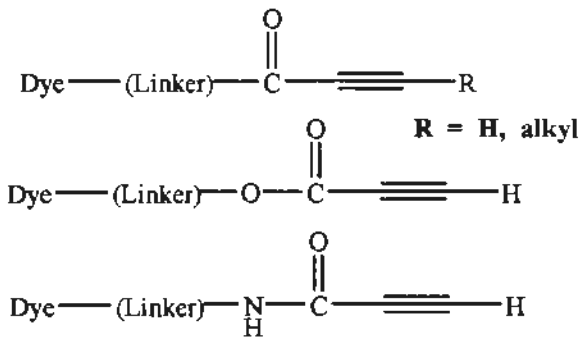
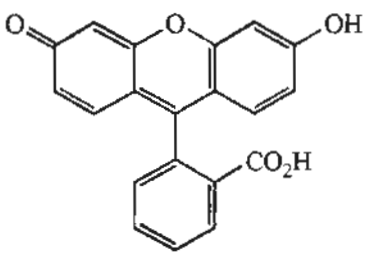


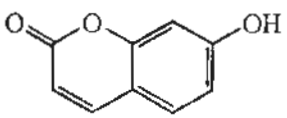
Figure 16 : The types of conjugated alkynes useful for attachment to dyes

Chapter 3: The Functionalisation Of Dyes

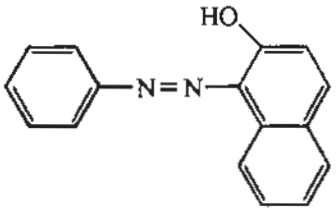
Fluorescent labels and reactive dyes consist of three sections: the dye, a spacer unit and a reactive group. To attach dyes to amino acids *via* a Michael type addition onto an activated alkyne group requires the functionalisation of the dyes with a spacer unit or linker arm terminating in a conjugated alkyne. Four dyes with different types of functionalities were chosen to act as models in order to probe the most appropriate method for the attachment of such linker arms. Fluorescein **56** is a representative of the xanthene dyes which have found extensive use in both wool dyeing and due to their excellent photophysical properties as fluorescent probes for biological systems. 7-Hydroxycoumarin **57** is a fluorescent dye which serves as an example for other classes of dyes, such as the resorufins which are commonly used in fluorescent labelling experiments. Sudan 1 **58** is a member of the family of azo dyes which are used extensively in wool dyeing. Dansyl chloride **22** is used as a fluorescent label to probe the local environment of the label in biological systems. Fluorescein **56**, 7-hydroxycoumarin **57** and Sudan 1 **58** contain the reactive phenolic hydroxyl for the attachment of the linker arm. Dansyl chloride **22** contains a sulfonyl chloride, through which a linker arm can be affixed.



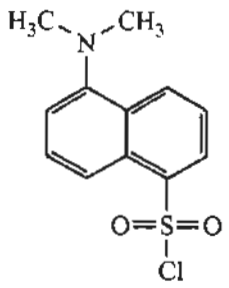
56



57



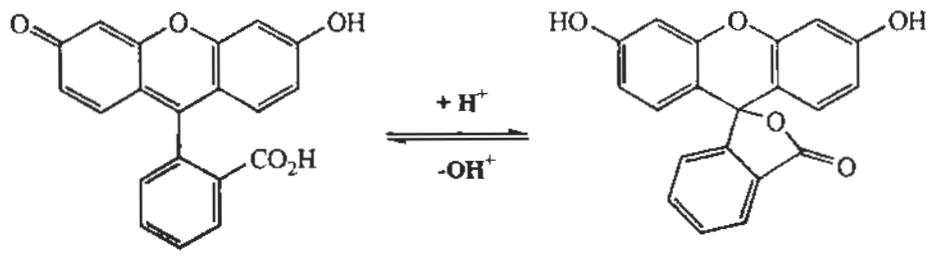
58



22

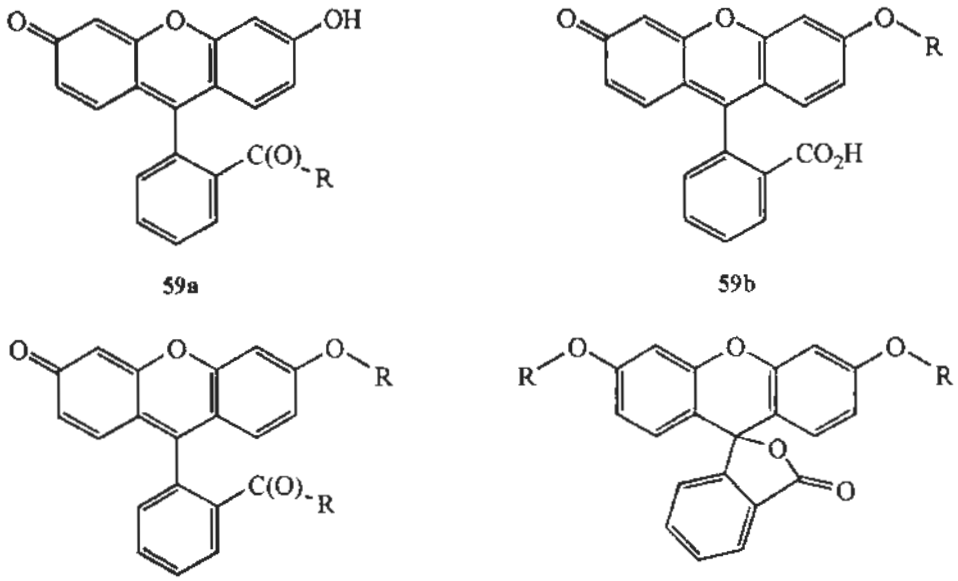
3.1 Probing the Method of Attachment of the Linker Arm to the Dye

In order to probe the method of attachment of the linker arm to the dye the reactivity of fluorescein **56** towards esterification and alkylation was examined. Initially the direct attachment of a spacer unit to fluorescein *via* an ester linkage through the phenolic hydroxyl was examined. Fluorescein exists in two forms, the fully conjugated **56a**, which is orange in colour, and the colourless lactone **56b**, which is the favoured form under acidic conditions, (**Figure 17**).[97] In each state fluorescein has two reactive functionalities; the phenolic hydroxyl group and the carboxylic acid in **56a**; and the two phenolic hydroxyls of the lactone form **56b**. Therefore reaction of fluorescein with an electrophilic alkylation or acylation reagent can give rise to four products, (**Figure 18**); the mono - ester **59a**; the mono - ether **59b**; and the ether - ester **59c** which are all derived from **56a**, and the diether **59d** from the lactone **56b**. The mono ether type compound **59b** has previously been formed exclusively by silver oxide mediated alkylations in a mixed THF - benzene solvent system.[97]



56a **56b**

Figure 17



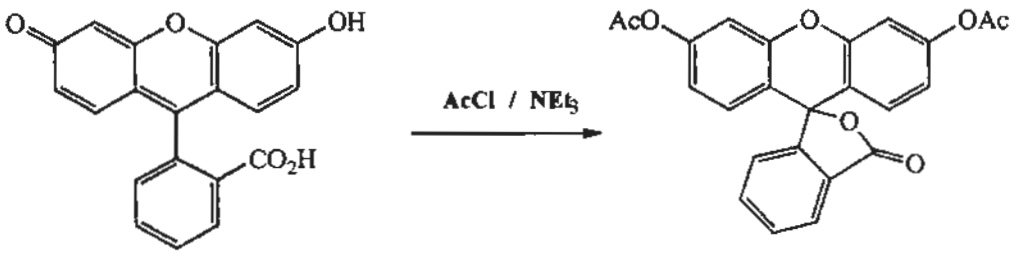
59a **59b**

59c **59d**

Figure 18

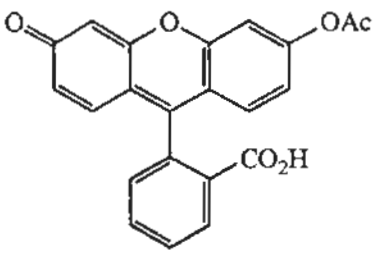
Initially the reaction of fluorescein with acetyl chloride was investigated under three different sets of conditions. Triethylamine was added to a solution of fluorescein and acetyl chloride (1 equivalent) at 0°C in either acetone, THF or DMF, and in each case complete reaction occurred within 10 minutes to give the diester **60** along with starting material **56**, (**Scheme 37**). As **60** exists in the lactone form it is colourless and therefore of no use as a dye. Lowering the reaction temperature to -78°C in an attempt to stop the reaction before the second addition of acetyl chloride could occur was unsuccessful and also produced only **60**. In each of these reactions none of the mono ester **61** was isolated. Attempts to quench the

reaction by the addition of methanol before the reaction proceeded to completion were unsuccessful and demonstrated that the second addition was rapid as the diaddition product **60**, fluorescein and only very minor amounts of the mono adduct **61** were recovered.



56 **60**

Scheme 37



61

Silver oxide with a mixed THF, benzene solvent system has been applied previously to the selective alkylation of fluorescein **56**.[97] In order to probe the applicability of these conditions towards the selective esterification of fluorescein, the reaction with acetyl chloride was undertaken using these conditions. The diacetoxy product **60** was again the only product observed, showing silver oxide mediated alkylations are not applicable to selective esterifications of this sort.

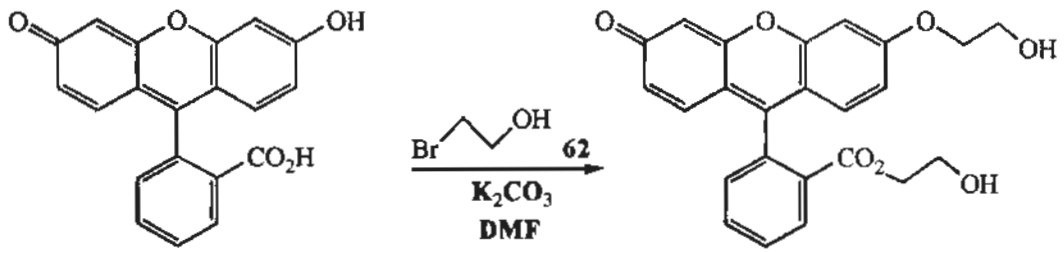
Fluorescein **56** was subsequently reacted with acetyl chloride under classic Schotten-Baumann conditions, using a two phase 10% aqueous sodium hydroxide-dichloromethane mixture.[98]

After 24 hours tlc showed only fluorescein and no acylation products. Given the ease of formation of diacetylfluorescein **60** seen previously this suggests that the ester was formed during the reaction but was later hydrolysed by the hydroxide present. The reaction was repeated with the time reduced to 2 hours with again only starting materials being recovered, showing that the hydrolysis by the sodium hydroxide occurs almost immediately after the formation of the diester **60**. The sodium hydroxide was replaced with a weaker base, sodium bicarbonate, and the reaction repeated, however only fluorescein **56** was recovered. To examine the stability of the diacetoxy compound **60**, a sample prepared by the previously described method (**Scheme 37**) was subjected to the Schotten - Bauman conditions. Substantial hydrolysis of the ester linkage, as indicated by the formation of an intense yellow - green colour in the aqueous layer, was seen to occur within a short period of time (20 minutes). This demonstrates that esters of fluorescein bonded through the phenolic hydroxyl groups are not stable under alkaline conditions. Such esters are unstable as fluorescein is a good leaving group due to the extensive delocalisation of the phenolic anion afforded by the highly conjugated aromatic system.

3.2 The Attachment of Linkers Terminated With An Amine Or Hydroxyl Group

As a direct ester linkage through the phenolic hydroxyl groups of fluorescein **56** and by analogy other dyes with large aromatic systems (eg. **57**, **58**) was too unstable to be practical, an ether linkage was considered. For this methodology the dye needed to be attached to a linker arm terminating in a functionality capable of binding to another unit containing a conjugated alkyne, the simplest of which is either an amine or a hydroxyl group. Accordingly

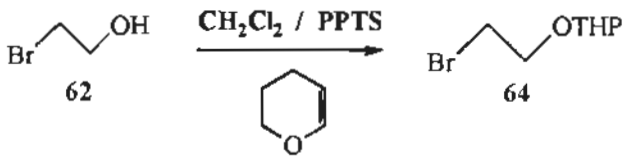
fluorescein **56** and 2-bromoethanol **62** were heated to 60°C in DMF for 12 hours, (**Scheme 38**). After flash chromatography on silica **63** was isolated as the major product in 54% yield.



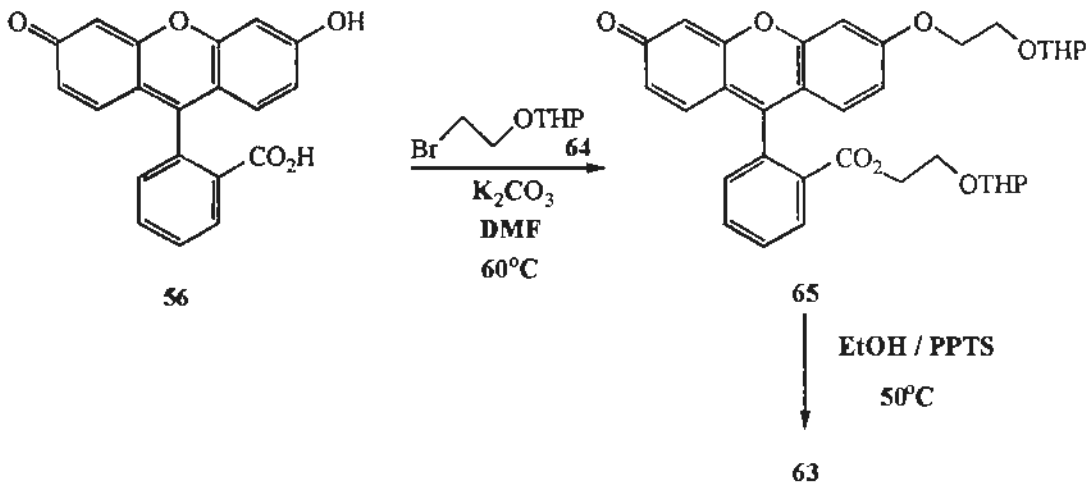
56 **63**

Scheme 38

The diol **63** was a very polar compound and as such was difficult to separate from the minor products present in the crude reaction mixture. To rectify this problem 2-bromoethanol **62** was protected with dihydropyran using a mild acid catalyst, pyridinium *p*-toluene sulphonate (PPTS), to form the corresponding tetrahydro-2*H*-pyranyl (THP) ether **64**, see (**Scheme 39**).[99] Fluorescein was alkylated with **64** using the same conditions as previously (**Scheme 38**), to generate **65** (R = THP), the THP protected equivalent to **63**, in 95% yield. This compound was substantially less polar than **63** and readily purified. Subsequent removal of the THP protecting groups by heating to 50°C for 3 hours in ethanol with PPTS present gave **63** as an orange solid in 63% yield, (**Scheme 40**).[99]

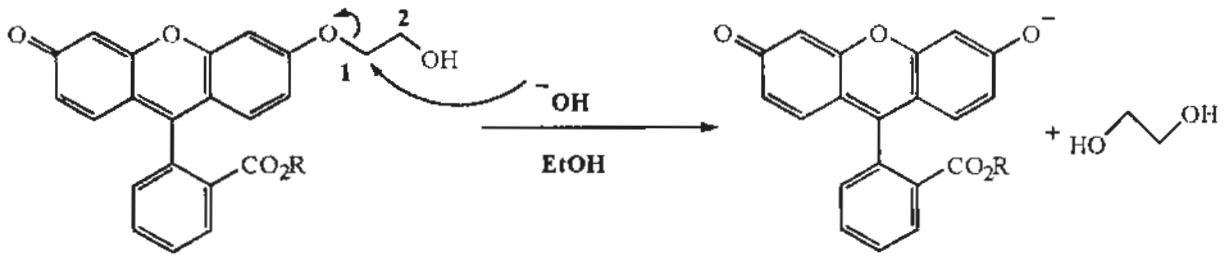


Scheme 39



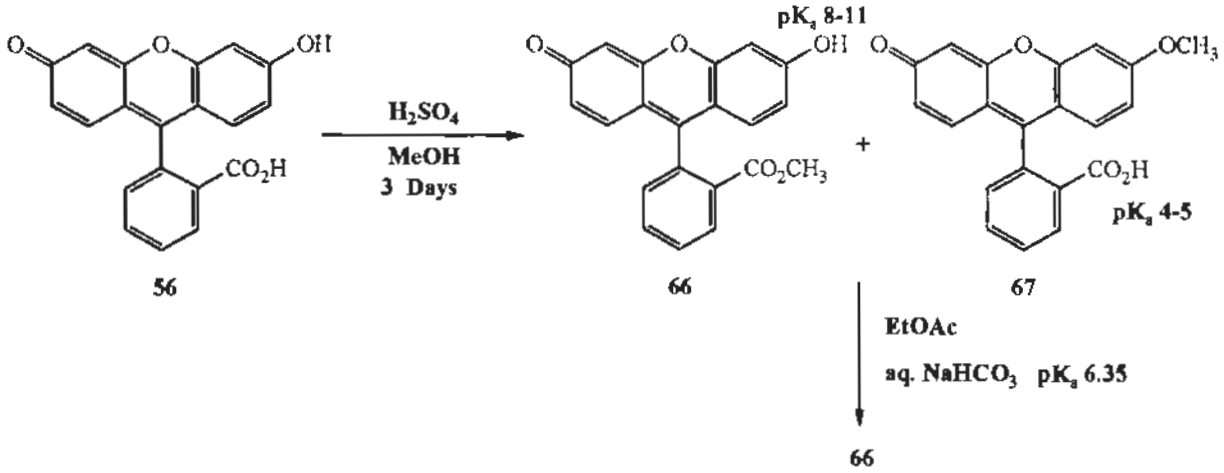
Scheme 40

Compound **63** is a dialkylated derivative of fluorescein which on coupling of the hydroxyl groups to suitable conjugated alkyne containing compounds will form a bis conjugated-acetylene. This compound should be capable of reacting with the side chain functionalities of two amino acids and thus crosslinking a peptide chain. While this is a useful compound, a mono-alkylated fluorescein derivative was also desired. To prepare this compound base catalysed hydrolysis of **63** was undertaken to remove the ester linker arm and liberate the free acid.[100] To achieve the hydrolysis of the ester required heating to 60°C in aqueous ethanolic sodium hydroxide for 2-3 hours. The severity of these conditions also resulted in the partial hydrolysis of the required ether linker arm, giving only a very low yield (~10%) of the monoalkylated product. Attempts to increase the yield of the required material by reducing the hydrolysis time and by stirring at lower temperatures were unsuccessful as mixtures of fluorescein **56** and the dialkylation product **63** were always isolated, with at best small amounts of the required material. The ether was presumably hydrolysed by the direct attack of the hydroxide anion onto carbon 1 with the resultant loss of the highly stabilised phenoxide anion of fluorescein as the leaving group (**Scheme 41**).



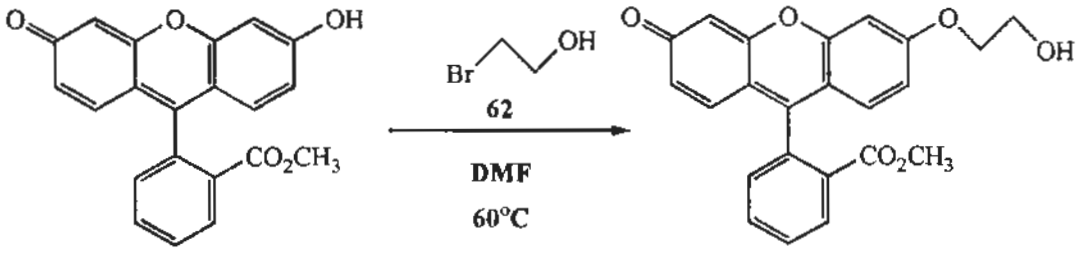
Scheme 41

To overcome the difficulties in the above method for the production of mono-alkylated derivatives of the difunctional compound **56**, the preparation of esters of fluorescein was undertaken. The methyl ester **66** was prepared by heating fluorescein **56** to reflux in methanol for 3 days with a catalytic amount of sulfuric acid, (**Scheme 42**).[101] After this time the reaction was allowed to cool to ambient temperature and a mixture of the methyl ester **66** along with some of the methyl ether **67**, precipitated from the solution. The other two possible products from the methylation reaction (**Figure 18**), the dimethyl ether - ester (eg. **59c**, R = Me) and the dimethyl ether (eg. **59d**, R = Me) were sufficiently soluble to remain in solution. The difference in the acidity of the phenol proton in **66** (pK_a 8-11) and the carboxylic acid proton (pK_a 4-5) in **67** was made use of for the purification of the methyl ester **66**.[78] The mixture of **66** and **67** was dissolved in ethyl acetate and the methyl ether **67** was selectively removed by washing the solution with sodium bicarbonate (pK_a 6.35)[78], leaving only the ester **66**. Due to the high polarity of the methyl ester **66**, this procedure was difficult and relatively large amounts of solvent were needed for the purification process. The yield of the esterification was also highly variable (15%-95%). Fluorescein methyl ester **66** was then alkylated with 2-bromoethanol **62** and potassium carbonate in DMF to produce the desired mono-alkylated fluorescein derivative **68** in 52% yield, **Scheme 43**).



56 **66** **67** **66**

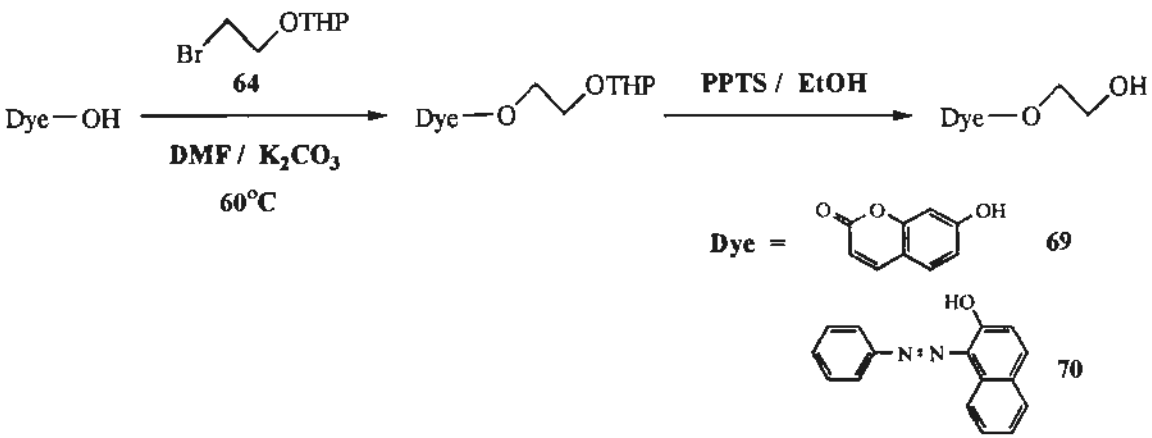
Scheme 42



66 **68**

Scheme 43

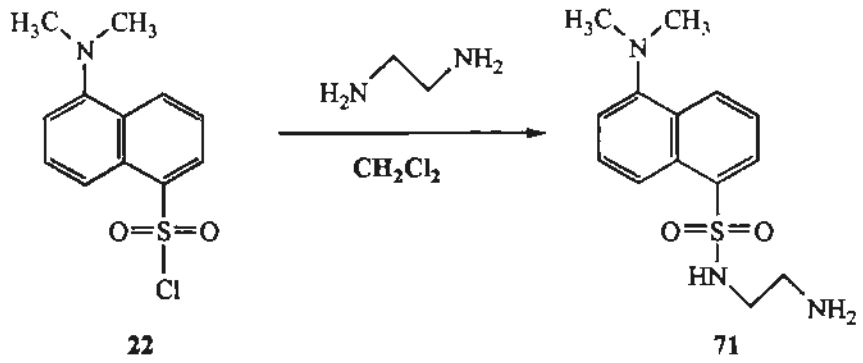
The inability of fluorescein to react with acetyl chloride to form stable esters suggests that 7-hydroxycoumarin **57** and Sudan 1 **58** would suffer from the same problem. Accordingly linker arms terminating in hydroxyl groups were attached *via* ether linkages to **57** and **58**. Initially **57** was stirred with 2-bromoethanol **62** and potassium carbonate in DMF at 60°C, to form the expected product **69**. The product had an almost identical R_f to the starting dye, so purification of **69** was extremely difficult. To combat this problem **57** was alkylated with **64**, the THP protected 2-bromoethanol, using the standard conditions. After purification, the THP protecting group was removed by heating with PPTS in ethanol, to give **69** in an overall yield of 99%, (**Scheme 44**).



Scheme 44

When the alkylation of Sudan 1 with 2-bromoethanol **62** was attempted using the conditions described above no reaction occurred after heating for 1 week. The base induced elimination of hydrogen bromide from the 2-bromoethanol **62** under the basic conditions prevented the alkylation. When the protected alcohol **64**, was used the alkylation proceeded smoothly, probably due to the increased steric bulk of the protecting slowing the competing elimination reaction. Removal of the THP protecting group gave the required alcohol 70 in a 99% yield over the two steps, **(Scheme 44)**.

The fourth dye, dansyl chloride **22**, has a sulfonyl chloride functionality rather than a phenolic hydroxyl moiety, and so a different method of functionalisation was used. Compound **22** was reacted with an excess of ethylenediamine in dichloromethane to produce the dansyl chloride derivative **71**, (**Scheme 45**). This material was not stable at room temperature, partially decomposing within 1 week. To overcome this problem the amine 71 was prepared immediately before use, the solvent and excess ethylenediamine removed under vacuum and the crude material added as a solution to further reactions (see section **3.3**).



22 **71**

Scheme 45

Five compounds were prepared from the four dyes **56**, **57**, **58** and **22** which are suitable for attachment to conjugated alkynes. These were the mono-alkylated derivatives of fluorescein, 7-hydroxycoumarin, and Sudan 1, **68**, **69**, and **70** respectively, the dialkylated fluorescein derivative **63**, all of which have a linker arm terminated with an alcohol. The dansyl derivative **71** where the linker arm terminates with an amine was also prepared. The structures of each of these compounds was confirmed by ^{1}H NMR, ^{13}C NMR and mass spectral data.

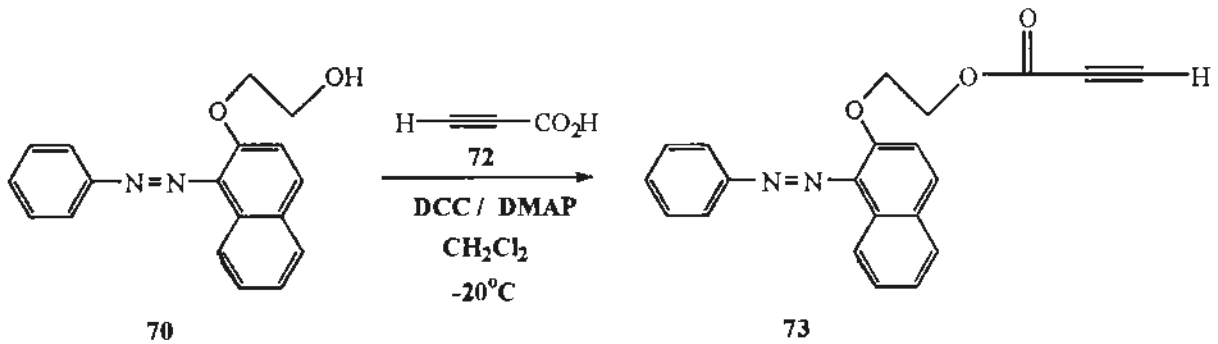
3.3 The Attachment Of Conjugated Alkynes To The Linker Arms

The initial method chosen to attach conjugated alkynes to the functionalised dyes prepared in the previous section was *via* a coupling of the alcohol or amine of the derivatised dyes to propiolic acid **72**, to form the terminal alkynyl ester or amide.

3.3.1 The Preparation of Conjugated Terminal Alkynyl Esters

When the coupling of propiolic acid **72** to the alkylated Sudan 1 derivative **70** under the standard conditions using DCC and DMAP in dichloromethane was attempted,[102] the solution

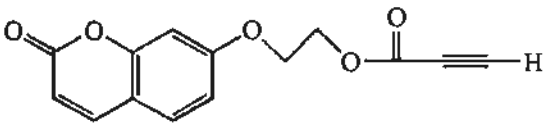
boiled upon the addition of the DCC and rapidly turned black. Very little of the expected coupling product **73** was recovered from this reaction. The reaction was repeated and cooled to 0°C prior to the addition of the DCC, and then allowed to warm to room temperature while stirring. Again the solution turned black and little product was recovered. Literature results[103] suggested that DMAP was adding to the propiolic acid **72** to initiate polymerisation of the alkyne and caused the decomposition observed, and that this problem could be avoided by cooling the solution to -20°C prior to the addition of the DCC and the DMAP. When this modification was made to the above procedure the expected product **73** was isolated in 73% yield, along with a 14% recovery of the starting alcohol **70**, (85% yield based on reacted starting material), (**Scheme 46**). The structure of **73** was confirmed by ^{1}H NMR, where the terminal acetylene proton was observed at δ 2.86 and by IR, in which an absorption at 2125 cm^{-1} due to the terminal alkyne was observed.



70 **73**

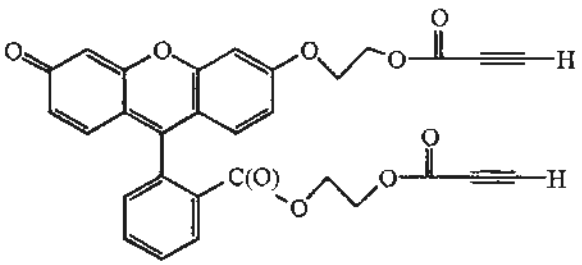
Scheme 46

The alkynyl ester derivative **74** of the alkylated 7-hydroxycoumarin **69** was also prepared by this method in a 31% yield. The lower yield was possibly due to a slower reaction allowing more polymerisation of the propiolic acid **72** to occur. The terminal acetylene proton was observed at δ 2.96 in the ^{1}H NMR, and the alkyne at 2125 cm^{-1} in the IR spectrum.

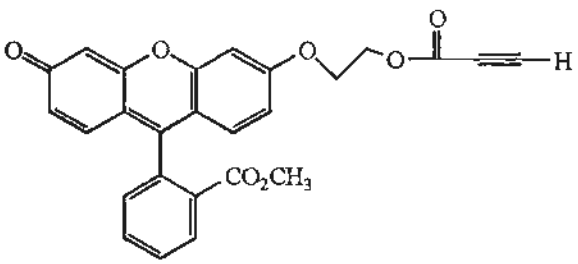


74

The DCC mediated coupling reactions of the di- and mono-alkylated fluorescein derivatives, **63** and **68** with propiolic acid **72** proved to be unsuccessful when attempted under the conditions described above. The reactions were repeated several times and in each case generated several products as indicated by tlc analysis of the reaction mixtures. No materials corresponding to the expected alkynyl esters **75** and **76** were isolated after flash chromatography on silica.

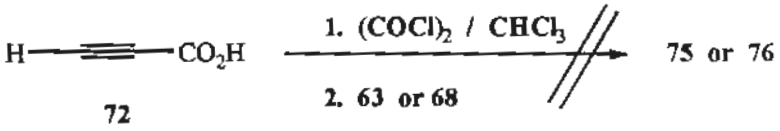


75



76

The preparation of the esters **75** and **76** was also attempted *via* the initial formation of the acid chloride of propiolic acid **72** with oxalyl chloride, followed by the addition of the alcohol **63** or **68**, (**Scheme 47**). Tlc analysis of the reaction mixtures again showed several products had formed in each case. No product corresponding to the expected terminal alkynyl esters **75** and **76** was isolated after repeated flash chromatography in either the esterification of the dialkylated fluorescein derivative **63** or in the esterification of the mono-alkylated derivative **68**.

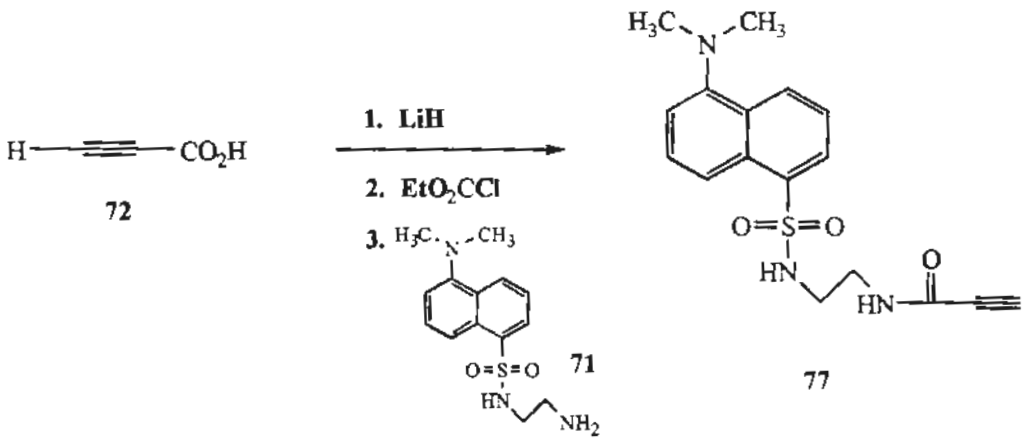


Scheme 47

Two compounds, **73** and **74**, in which a dye is attached to a terminal alkynyl ester *via* a linker arm have been prepared. These compounds are suitable for attachment to amino acids with nucleophilic side chains using the conjugate addition reaction discussed in the previous chapter.

3.3.2 The Preparation of the Conjugated Terminal Alkynyl Amide

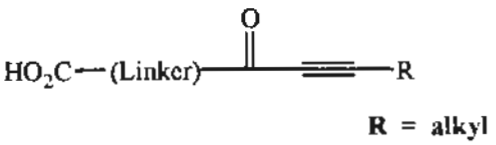
To prepare a compound in which a dye is attached to a conjugated terminal acetylenic amide the dansyl derivative **71** was used. The amide bond was formed by reacting the terminal amine with the mixed anhydride of propiolic acid **72** as shown in **Scheme 48**, to yield the required conjugated amide **77** in 58% overall yield from dansyl chloride **22**.



Scheme 48

3.3.3 The Preparation of Conjugated Non-Terminal Alkynyl Ketones

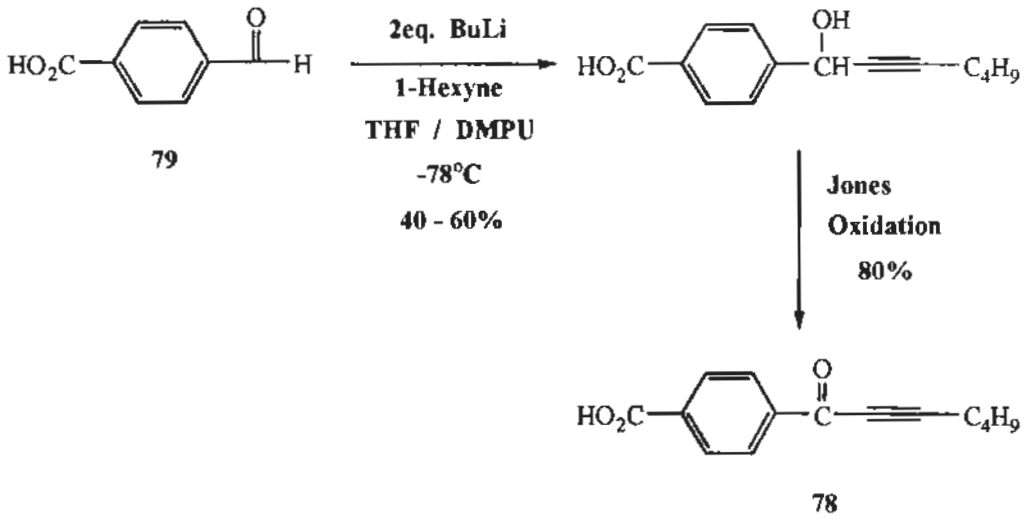
To form dyes with linker arms containing conjugated non-terminal acetylenic ketones from the previously derivatised dyes, **63**, **68**, **69**, **70**, and **71**, a difunctional compound was required which contained both a conjugated non-terminal acetylenic ketone and also another group, such as a carboxylic acid, capable of forming a bond to an alcohol or amine, (**Figure 19**).



R = alkyl

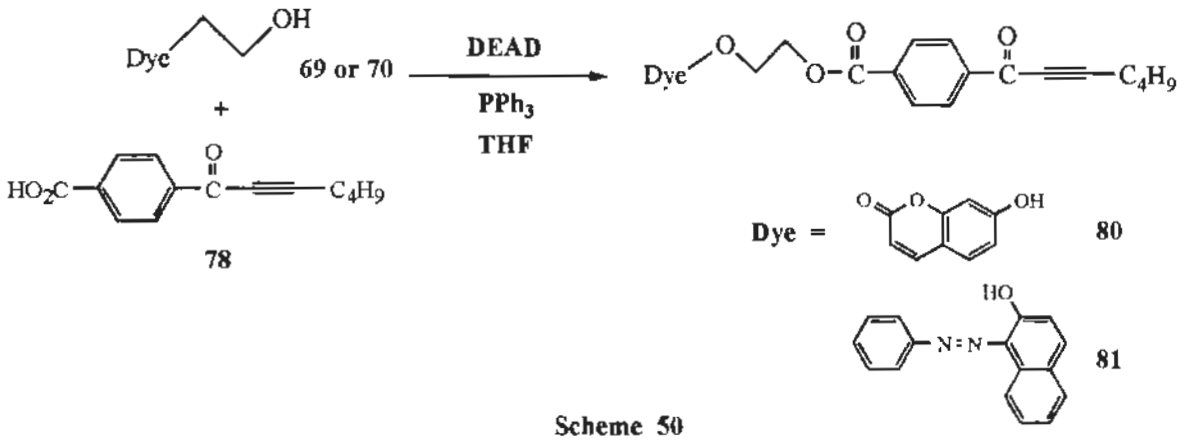
Figure 19

Compound **78**, where the linker between the two necessary moieties is an aromatic ring, was chosen as the initial target. To prepare the difunctional **78**, two equivalents of butyllithium were added to 1-hexyne at -78°C in THF with two equivalents of DMPU. This was followed by 4-carboxybenzaldehyde **79** to give the benzyl alcohol derivative shown. The yield for this reaction was generally low to moderate (40-60%) and the purification procedure difficult due to incomplete reaction and the production of several other side products as indicated by tlc analysis. The low to moderate yield was probably due to the necessity of a nucleophilic attack onto a species bearing a negative charge (ie. the carboxylate anion) as a crucial part of the reaction. Jones oxidation of the intermediate benzyl alcohol produced **78** as a cream coloured solid in 80% isolated yield, (**Scheme 49**). The difunctional compound **78** exhibited a broad IR absorption at 2600 - 3000 cm^{-1} due to the carboxylic acid and a narrower absorption at 2200 cm^{-1} due to the alkyne. The FAB mass spectrum showed a molecular ion at 231.



Scheme 49

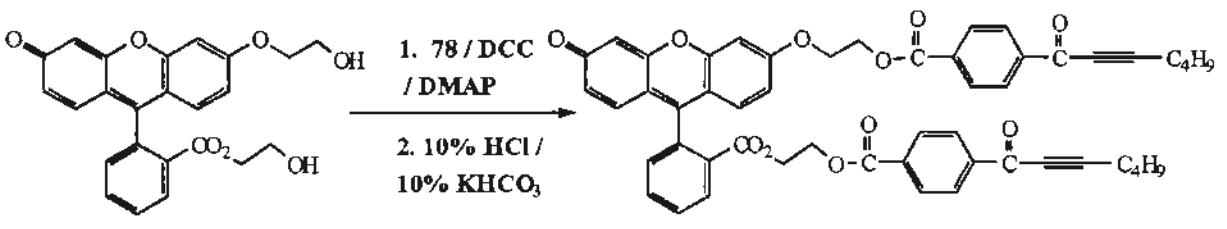
The acid **78** was coupled to both the 7-hydroxycoumarin derivative **69** and the Sudan1 derivative **70** using a standard Mitsunobu coupling.[104] The corresponding products, **80** and **81**, were isolated in 54% and 58% yield respectively, (**Scheme 50**). Both **80** and **81** showed absorbances at 2200 cm^{-1} due to stretching of the acetylene, confirming they were the expected coupling products. Although Mitsunobu coupling reactions involve a phosphine they do not give Michael addition to acetylenic ketones due to the low nucleophilicity of the triphenylphosphine.[104]



Scheme 50

In an attempt to increase the yields of the couplings, the reaction of the Sudan 1 derivative 70 and the acid **78** was carried out under standard DCC / DMAP conditions, resulting in a similar yield of the alkynyl ketone **81**, (51%). The moderate yield in this case was probably due to the reaction of the DMAP with the conjugated alkyne, as evidenced by the darkening in colour of the reaction with time.

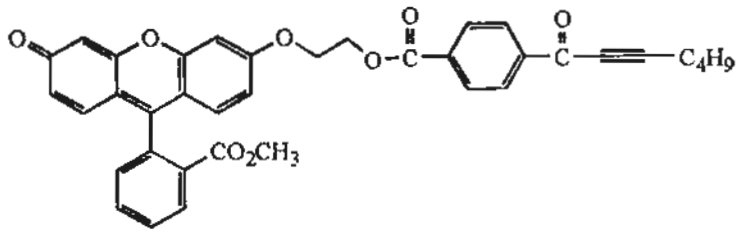
When the DCC mediated coupling of the dialkylated fluorescein **63** and the conjugated alkyne **78** was initially attempted, the reaction was allowed to stir overnight before filtration to remove DCU and removal of the solvent. The solution had changed from orange to a dark brown in colour. Tlc analysis of the resulting material showed a large number of spots, most of which streaked on silica. The coupling reaction was repeated and only stirred for 3 hours, but when the solvent was removed using warm water (~30-40°C) the solution again turned a dark brown colour with the same results on tlc. When the reaction was stirred for only two hours, filtered and washed with dilute hydrochloric acid and 10% potassium bicarbonate solution the expected coupling product **82** was recovered in 50% yield, (**Scheme 51**). Compound **82** showed the characteristic absorbance at 2200 cm^{-1} in the infra-red spectrum confirming the presence of the alkyne in the molecule.



63 **82**

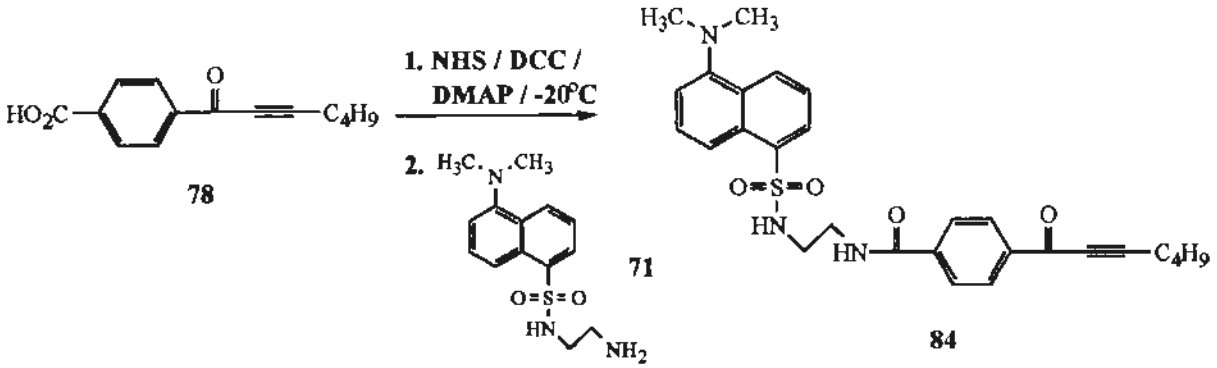
Scheme 51

When the mono-alkylated fluorescein methyl ester **68** and the difunctional conjugated alkyne-carboxylic acid **78** were subjected to the DCC mediated coupling conditions described above, several new products were formed as indicated by tlc analysis of the crude reaction mixture. Attempts at purification by chromatographic methods were unable to isolate any of the expected addition product **83**.



83

The preparation of the compound in which the non-terminal conjugated acetylene is attached to the dansyl group **84** required the initial preparation of the N-hydroxy succinimide (NHS) ester of the acid **78**. This was carried out in chloroform using DCC and DMAP to couple the alcohol and the acid. After stirring for 5 hours tlc analysis of the reaction mixture showed complete consumption of the acid **78**, so the solution was filtered and added to the crude amine **71**, (**Scheme 52**). The overall yield for this procedure was 30% from dansyl chloride **22**. The dansyl derivative **84** showed the characteristic alkyne absorbance at 2200 cm^{-1}, indicating the alkyne had coupled to the amine **71**. This compound was unstable and decomposed within one week at room temperature.



Scheme 52

3.3.4 The Preparation of Conjugated Terminal Alkenyl Ketones

A compound exhibiting difunctionality such as that shown in **Figure 20** would be useful for the preparation of conjugated terminal alkynyl ketones. Accordingly, the initial synthetic target **85** possessed both a terminal acetylenic ketone and a carboxylic acid functionality.

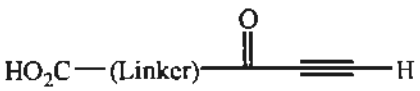
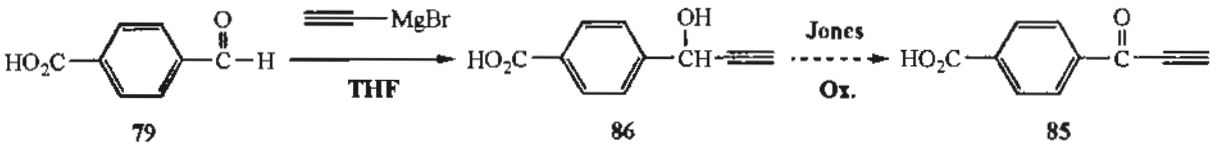


Figure 20

3.3.4.1 Via a Grignard Reaction

The preparation of the difunctional compound **85** was attempted *via* the reaction of 4-carboxy benzaldehyde **79** with ethynyl magnesium bromide in THF to yield the corresponding benzyl alcohol derivative **86**. This was to be followed by Jones oxidation to the required ketone, **(Scheme 53)**.



79 86 85

Scheme 53

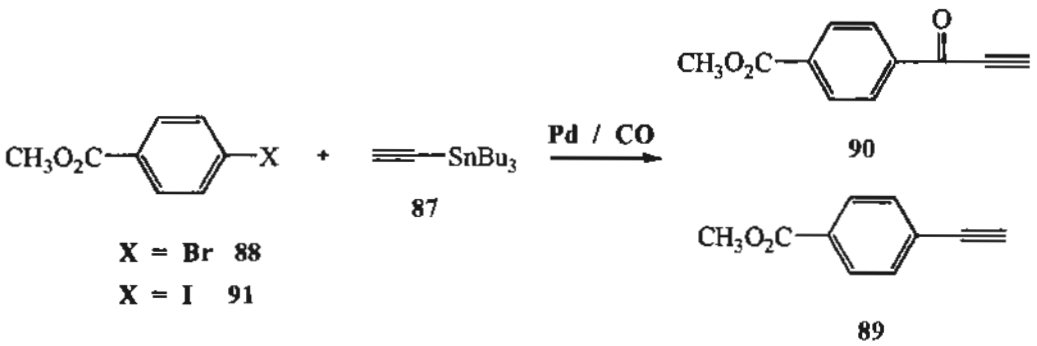
The addition of ethynyl magnesium bromide to 4-carboxy benzaldehyde **79** produced a number of new products as shown by tlc analysis of the crude reaction mixture. After repeated attempts at purification by flash chromatography on silica none of the expected alcohol **86** was isolated. The starting material **79** was consumed in the reaction but none of the products formed from **79** were identifiable. The reaction of benzaldehyde with ethynyl magnesium bromide described in Chapter 2 yielded the corresponding benzyl alcohol derivative, which upon oxidation gave ethynylphenyl ketone **25**. This indicates that the carboxylic acid group in 4-carboxy benzaldehyde **79** is causing a change in the reaction pathway. The reaction was initially attempted with two equivalents of the Grignard reagent, one to act as the base and the other as the nucleophile. This procedure possibly led to further additions of the Grignard reagent and to products from the reaction of the carboxy group. To stop further additions from occurring the anion of the acid **79** was formed with sodium hydride before the addition of the Grignard reagent (1 equivalent). Tlc analysis of the reaction mixture showed this method produced a cleaner reaction. However after quenching with acid and chromatographic purification the major product recovered was shown by ^{1}H NMR to be mainly 4-carboxy benzaldehyde **79** with only traces of what appeared to be the expected addition product **86**. As these two materials were coincident on tlc no further separation was possible. It was thought that low solubility of the sodium anion of **79** in THF may have been responsible for the reaction not going to completion. The reaction was repeated with lithium hydride with DMPU added to solubilise the lithium anion. After stirring the 4-carboxy benzaldehyde **79** with

lithium hydride for several hours a precipitate formed and no product was recovered on addition of the Grignard reagent, showing that lithium hydride is also unsuitable for this reaction. The difficulty of achieving a successful result in this reaction indicates that this route to conjugated terminal acetylenic ketones is not practical.

3.3.4.2 Via a Palladium Catalysed Carbonylative Insertion Reaction

An alternate approach for the preparation of conjugated terminal acetylenes was therefore required. A palladium catalysed carbonylative insertion reaction between *p*-halobenzoate esters and tributylethynyl tin **87** was examined, **(Scheme 54)**, where the alkylated dyes discussed in Section **3.2** will be attached through the ester group. The initial studies were carried out on methyl *p*-bromobenzoate **88** which was chosen as a model for the compounds containing the dyes. Methyl *p*-bromobenzoate **88** and tributylethynyl tin **87** were added to a solution of tetrakistriphenylphosphine palladium(0) (5% molar equivalent) in DMPU and the mixture heated to 80°C for 12 hours under a carbon monoxide atmosphere (1 atm). The solution turned black almost immediately after the addition of the tin reagent **87** to the reaction mixture. DMPU was used as the solvent as a replacement for HMPA, which has been reported to be an efficient solvent for carbonylation reactions.[105] Tlc analysis of the crude solution showed only starting materials to be present. The reaction was repeated in acetonitrile, another common solvent for carbonylation reactions.[106] The reaction mixture again changed colour to black soon after the addition of the tributylethynyl tin **87**. Tlc analysis of the solution after heating at 80°C overnight under a carbon monoxide atmosphere, showed starting materials along with a very faint spot at a slightly lower R_f than the starting aryl bromide **88**. This spot was possibly due to a small amount of the coupling product **89** where carbon

monoxide had not inserted, (the ketone **90** was expected to have a much lower R_f than the starting material **88**). In an attempt to force the carbonylation reaction to occur, the coupling was repeated under a carbon monoxide pressure of 4 atmospheres. Heating the black solution for twelve hours again resulted in the recovery of the bromide **88** and the tin reagent **87**. Traces of a second material with a lower R_F, possibly the expected product **90** were observed by tlc analysis.

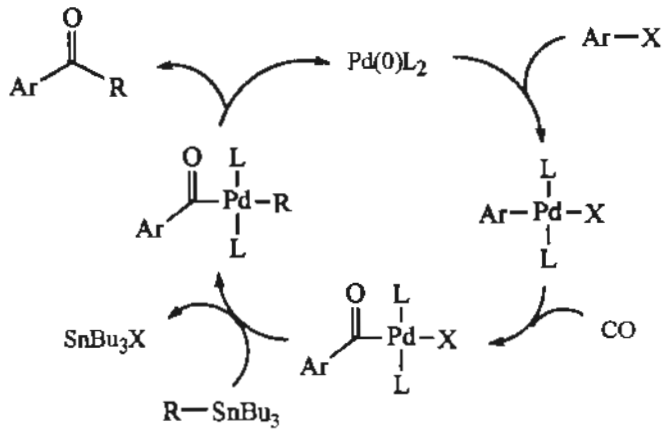


Scheme 54

As aryl iodides are more reactive towards palladium catalysis than the aryl bromides,[107] the coupling reaction was attempted with methyl *p*-iodobenzoate **91**. Bis(1,3-diphenylphosphino)propane palladium(0) prepared *in situ* from bis(acetonitrile) palladium(II) dichloride and 1,3-diphenylphosphinopropane was used in place of tetrakistriphenylphosphine palladium(0) as literature results indicate that for carbonylative couplings it is a more efficient catalyst.[108] This reaction also turned black within 5 minutes of the addition of the tributylethynyl tin **87** and prolonged heating of the solution returned only starting materials.

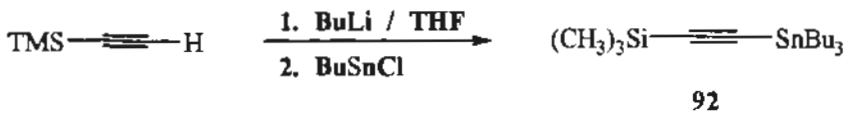
The rapid formation of the black colour upon the addition of the tin reagent **87** suggests the possibility that the terminal acetylene is binding to the palladium in such a way as to reduce its catalytic ability and prevent the reaction. The traces of the other materials seen by tlc analysis in the above reactions suggest that the palladium is at most able to undergo only a small

number of catalytic cycles (**Scheme 55**) before the catalyst is poisoned and the reaction is halted.



Scheme 55

To determine if the terminal acetylene was having an adverse effect on the carbonylation reaction a non terminal acetylene, tributyl(trimethylsilylethynyl) tin **92** was prepared by reaction of the lithium anion of trimethylsilyl acetylene with tributyltin chloride, (**Scheme 56**).



92

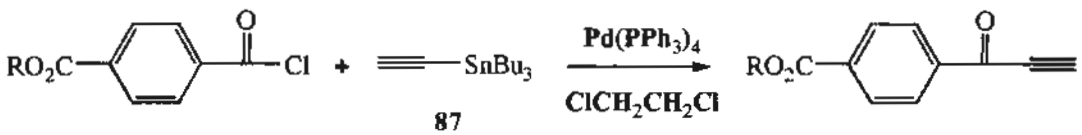
Scheme 56

When the coupling of the protected acetylene **92** to methyl *p*-bromobenzoate **88** was attempted using dichlorobis(1,3-diphenylphosphino)propane palladium(II) / acetonitrile as the catalyst / solvent system the reaction rapidly turned black in colour. After heating at 80°C overnight only starting materials were observed by tlc analysis of the crude reaction mixture. Workup of the solution and purification by flash chromatography confirmed that no reaction had occurred.

This result indicates that the terminal hydrogen of the tributylethynyl tin **87** was not interfering with the palladium to prevent the reaction and suggests that the phosphine ligands used in the above reaction may not be suitable for the carbonylative addition of aryl halides and alkynyl tin reagents. Studies by Stille and Echavarren have shown that dichlorobis(1,3-diphenylphosphino)propane palladium(II) is at best a poor catalyst for coupling reactions between alkynyl tin reagents and aryl triflates,[109] which are slightly less reactive than aryl bromides.[107] Their work suggests that dichloro [1,1'-bis(diphenylphosphino)ferrocene] palladium(II) is a better catalyst for carbonylative coupling reactions.[109]

3.3.4.3 Via Palladium Catalysed Coupling of Tin Reagents to Acid Chlorides

Another approach to the preparation of conjugated terminal acetylenic ketones is *via* the palladium catalysed coupling of functionalised acid chlorides with the previously prepared tributylethynyl tin **87**, **(Scheme 57)**.

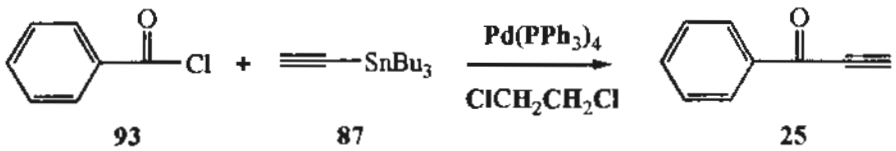


R = Dye—(Linker Arm)—

Scheme 57

This approach is similar to the previously discussed palladium catalysed carbonylative coupling reactions, but a specific catalyst is not required. Like the earlier approach this method also requires the initial preparation of the ester before the formation of the conjugated acetylene can

occur. The initial reactions were undertaken on benzoyl chloride **93**, a model for the functionalised acid chloride shown in (**Scheme 57**). Tributylethynyl tin **87** was added to a solution of freshly distilled benzoyl chloride **93** in 1,2-dichloroethane containing tetrakis(triphenylphosphine) palladium(0) and the mixture was heated to reflux for 90 minutes,[111] (**Scheme 58**), at which time tlc analysis indicated the reaction had proceeded to completion. The major product formed in this reaction had an identical R_f to the expected coupling product phenylethynyl ketone **25**, however repeated attempts at chromatographic purification on silica were unable to isolate a clean sample of the ketone **25** from the reaction mixture. This suggested that the terminal alkynyl ketone may have been decomposing on silica to provide the impurities observed by tlc analysis.

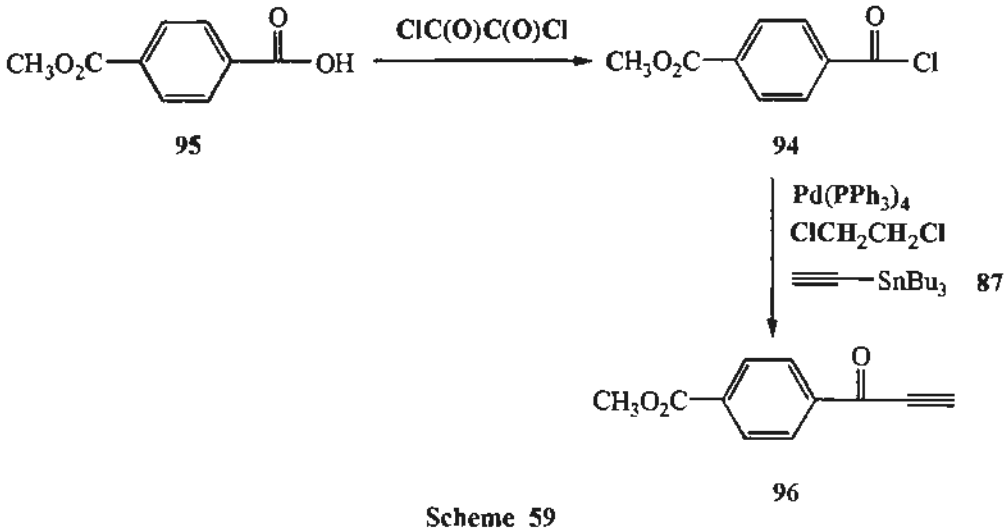


93 **87** **25**

Scheme 58

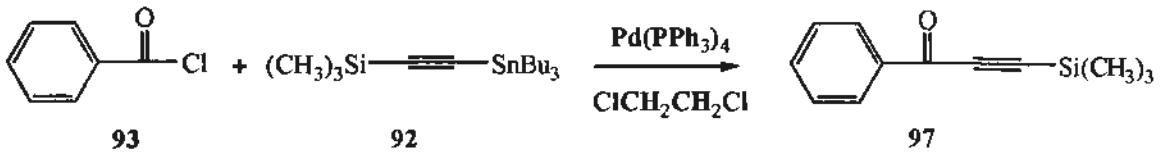
To further model the functionalised acid chloride shown in **Scheme 57**, and to probe the stability of terminal alkynyl ketones, the coupling of *mono*-methyl teraphthaloyl chloride **94** to tributylethynyl tin **87** under the palladium mediated conditions described above was undertaken (**Scheme 59**). *Mono*-methyl teraphthaloyl chloride **94** prepared from *mono*-methyl teraphthalate **95** and oxalyl chloride. Tlc analysis of the reaction mixture showed the disappearance of the starting material and the formation of a new product, presumably the expected product **96**. When tlc indicated the reaction to be complete the palladium catalyst was removed by squat chromatography and the purification of the resulting material attempted by flash chromatography. The separation of the major product from impurities once again

proved to be problematic and a pure sample was unable to be obtained after repeated chromatography. This again suggests the terminal alkyne may have been decomposing on silica. The purification of the model compounds by distillation was not deemed a suitable method in this case as the high molecular weights of alkynyl ketones attached to dyes would preclude this technique. The purity of labelled compounds is essential to ensure the reproduciblity of the biological studies and assays in which they are used.



Scheme 59

The high reactivity of terminal alkynyl ketones, as exhibited by their tendency to decompose on silica suggested that a protected alkyne would be a better synthetic target than a terminal alkyne. The protected alkyne could be deprotected *in situ* immediately before the Michael addition reaction was to be undertaken. The coupling of tributyl(trimethylsilylethynyl) tin **92** to benzoyl chlorides has been reported to proceed to form the trimethylsilyl protected alkynyl ketone **97**, (**Scheme 60**).[110] This suggests the coupling of the acid chlorides of *mono*-teraphthalate esters with the protected alkynyl tin reagent **92** should also proceed providing an efficient route to protected alkynyl ketones. This pathway was not attempted due to time constraints.



93 **92** **97**

Scheme 60

3.4 Conclusions

The investigations described in this Chapter determined that the attachment of the linker arms to the dyes *via* an ether bridge to the phenolic hydroxyl was the preferred method for fluorescein **56**, 7-hydroxycoumarin **57** and Sudan 1 **58**. The attachment *via* an ester linkage was found to be unstable under alkaline conditions. Hence four compounds with linker arms terminating in a hydroxyl group capable of later attachment to a conjugated alkyne were prepared. These compounds were a dialkylated fluorescein derivative **63**, a monoalkylated fluorescein methyl ester **68** and the 7-hydroxycoumarin and Sudan 1 derivatives **69** and **70**.

Dansyl chloride **22** was functionalised by the addition of ethylenediamine to yield the derivative **71** where the dansyl group is attached to a linker arm terminated in an amine which is suitable for attachment to a conjugated alkyne.

Two conjugated terminal alkynyl esters, **73** and **74**, were prepared by the coupling of the Sudan 1 and 7-hydroxycoumarin derivatives, **69** and **70** with propiolic acid **72**.

A conjugated terminal alkynyl amide 77 was prepared from the reaction of propiolic acid **72** with the dansyl derivative **71** *via* a mixed anhydride.

A convergent synthesis for the preparation of non-terminal alkynyl ketones was developed *via* the synthesis of the difunctional compound **78** which contains both a conjugated non-terminal alkynyl ketone and also a carboxylic acid capable of forming ester or amide bonds. Four compounds were prepared by the coupling of the derivatised dyes with linker arms terminating in alcohols or an amine (**63**, **69**, **70**, and **71**) to the carboxylic acid of **78**. These were the 7-hydroxycoumarin **80**, Sudan 1 **81**, dialkylated fluorescein **82** and dansyl **84** derivatives.

The preparation of conjugated terminal acetylenic ketones was attempted by several methods but the required compounds were not synthesised due to time constraints.

Overall, seven compounds consisting of a conjugated alkyne joined to a dye through a linker arm were prepared from fluorescein **56**, 7-hydroxycoumarin **57**, Sudan 1 **58** and dansyl chloride **22** in moderate to good yields. These compounds are suitable for attachment to amino acids with nucleophilic side chains *via* a Michael type addition reaction onto the conjugated alkyne.

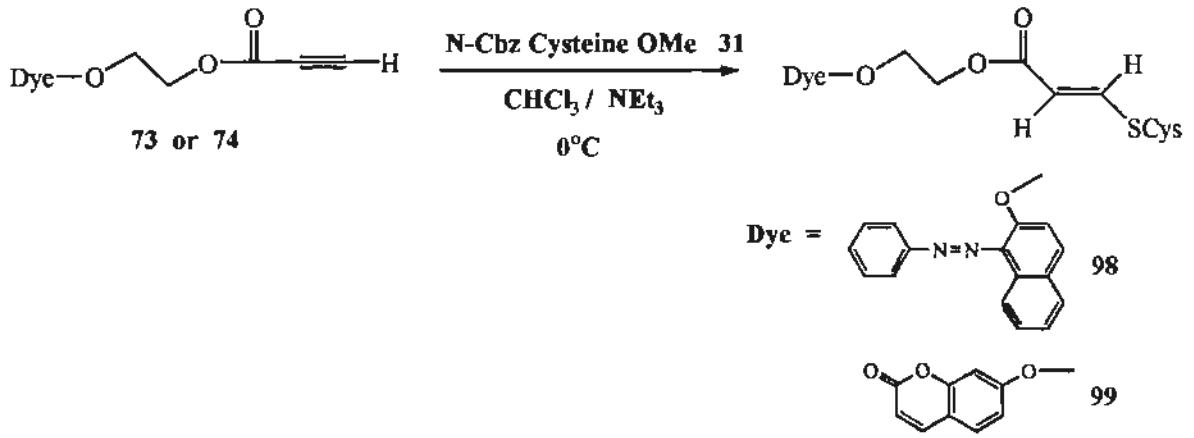
Chapter 4: The Reactions of the Protected Amino Acids with the Derivatised Dyes

To further probe the Michael addition of nucleophiles to activated acetylenes the reactions of the three protected amino acids *N*-Cbz cysteine methyl ester **31**, *N*-Cbz serine methyl ester **32**, and N_{α}-Cbz lysine methyl ester **33**, with some of the conjugated acetylenes attached to dyes *via* linker arms prepared in Chapter 3 were examined. These additions were expected to proceed in a similar manner to the model reactions discussed in Chapter 2 and this was generally found to be the case, however in some of the reactions the presence of the dye moiety had a marked impact.

4.1 The Reactions with the Conjugated Terminal Alkynyl Esters

The studies described in Chapter 2 determined that conjugated acetylenic ketones undergo rapid reactions with each of the three amino acids. Hence, the reactions of the alkynyl ester derivatives of Sudan 1 **73** and 7-hydroxycoumarin **74** with the amino acids **31** - **33** were examined. When the addition of the cysteine derivative **31** to the Sudan 1 derivative **73** was undertaken in chloroform at 0°C with a catalytic amount of triethylamine, only the *E* isomer of the expected addition product **98** was isolated in 73% yield, (**Scheme 61**). Traces of another product, possibly the *Z* isomer, were observed in the crude ^{1}H NMR and by tlc, but due to the minor amount formed this material was not isolated. The FAB mass spectrum of **98** confirmed that this was the expected addition product (molecular ion at 614). The ^{1}H NMR spectrum and a COSY spectrum showed two resonances due to the vinyl protons as doublets at δ 5.79

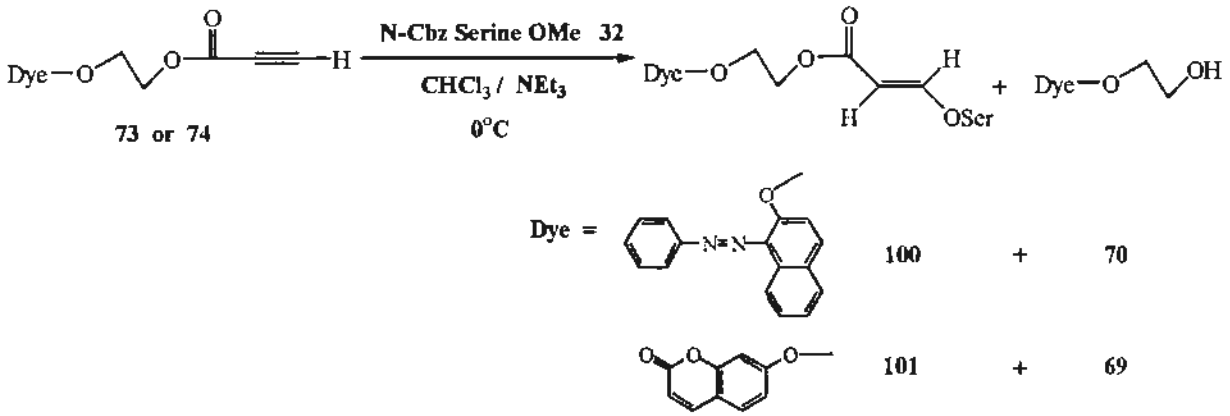
and δ 7.35 with a coupling constant of J = 15 Hz. This indicates that, by analogy with the trans addition product **40*E*** from the model reaction described in Chapter 2, **98** was the *E* isomer. The isomeric ratio observed in the product was unexpected, as in the model reaction where the thiol **31** was added to ethyl propiolate **27** under the same conditions both the *E* and the *Z* isomer were generated in a 4 to 1 ratio. This suggests that the bulky dye moiety is affecting the relative stabilities of the two intermediate anions, such that the *E* anion is greatly preferred over the less stable *Z* anion, where steric interactions between the dye and the protected amino acid group are more likely to occur. This results in the formation of the *E* isomer as the major product.



Scheme 61

The reaction under basic conditions in chloroform of the protected cysteine **31** with the coumarin derivative **74** gave a similar result to the addition of the thiol **31** to the Sudan 1 derivative **73**, discussed above, (**Scheme 61**). Again the addition produced mainly the *E* isomer **99** (in 66% isolated yield), as indicated by a coupling constant of J = 15 Hz for the two vinylic resonances which were observed at δ 5.88 and δ 7.63, (cf. **40*E***). LSIMS showed a molecular ion at 528 indicating the addition product **99** had the predicted structure. Traces of a possible second isomer were again observed by ^{1}H NMR.

In an analogous manner the alcohol **32** was added to the Sudan 1 derivative **73** to give two products after purification by chromatography, (**Scheme 62**). The identity of the major product (44% isolated yield) was confirmed to be the expected conjugate addition product **100** by FABMS (molecular ion at 597). The characteristic vinylic protons were observed at δ 5.07 and δ 7.33 in the ^{1}H NMR. The value of the coupling between the vinylic protons was J = 12 Hz, indicating that **100** had *E* stereochemistry (cf. the product **41** of the corresponding model reaction between the serine derivative **32** and ethyl propiolate **27** where J = 12.7 Hz). The second product, recovered in approximately 30% yield, proved to be the alcohol **70**, which was presumably formed by a 1,2-addition of the alcohol **32**, rather than the expected 1,4-addition. The 1,2-addition was not observed in the model reaction which suggests that the presence of the dye is causing the reaction to adopt this second, normally less favoured pathway. As the nucleophile approaches the β-carbon of the alkyne along a carbon-carbon-nucleophile angle of 60° (see Chapter 2.2.1) it is likely that the large bulk of the dye is partially blocking this route (**Figure 21**), thus making the normally less accessible 1,2-addition possible. This was not seen for the reactions of the cysteine derivative **31** discussed above due to the thiol having a higher reactivity compared to the hydroxyl group.



Scheme 62

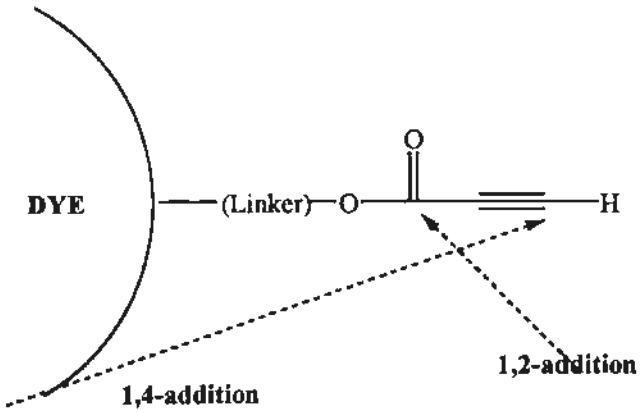
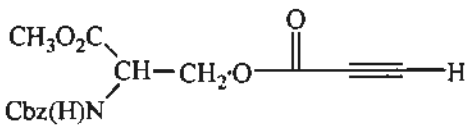
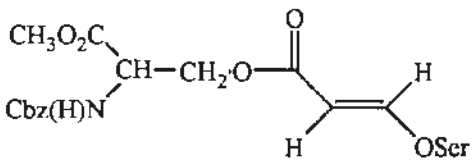


Figure 21

The reaction of the 7-hydroxycoumarin derivative **74** with the alcohol **32** in chloroform with a catalytic amount of triethylamine produced the same results as for the addition to the Sudan 1 derivative **73**. Again both the expected 1,4-addition product **101** (29%) and the alcohol **69** (~30%) were formed, (**Scheme 62**). The Michael addition product was characterised by ^{1}H NMR and FABMS (molecular ion at 512). The vinylic protons were observed as two doublets at δ 5.26 and at δ 7.55, (J = 12.5 Hz), indicating that **101** was of *E* stereochemistry. In the two reactions described in **Scheme 62** only the alcohols **69** and **70** were isolated as evidence of the 1,2-addition due to their ease of visualisation on tlc. In each case some low R_f materials were formed during the reaction which probably contained the second product from the 1,2-addition **102** and / or its derivative **103**.



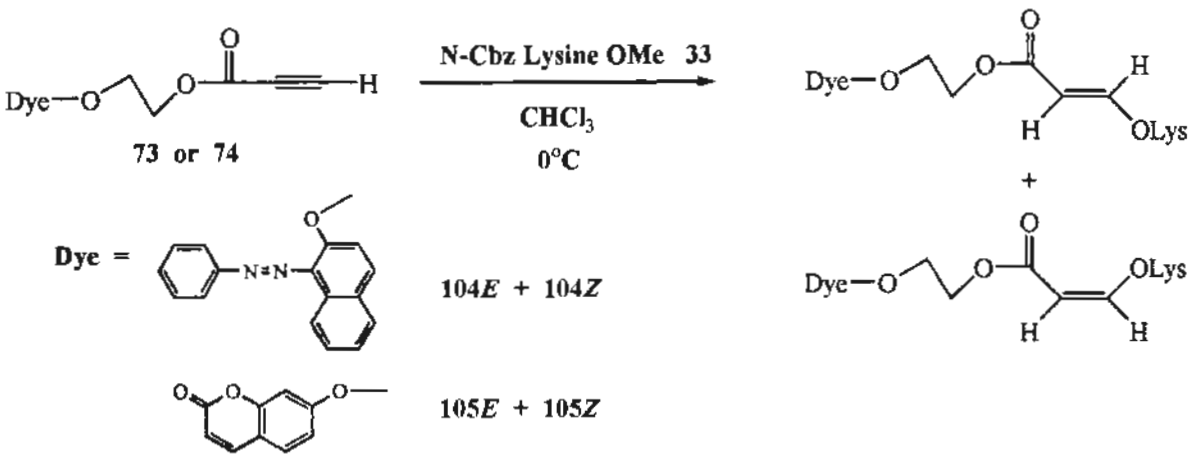
102



103

The reactions of N_α-Cbz lysine methyl ester **33** with the two alkynyl esters **73** and **74** in chloroform, (**Scheme 63**), produced almost identical results to the model reaction between

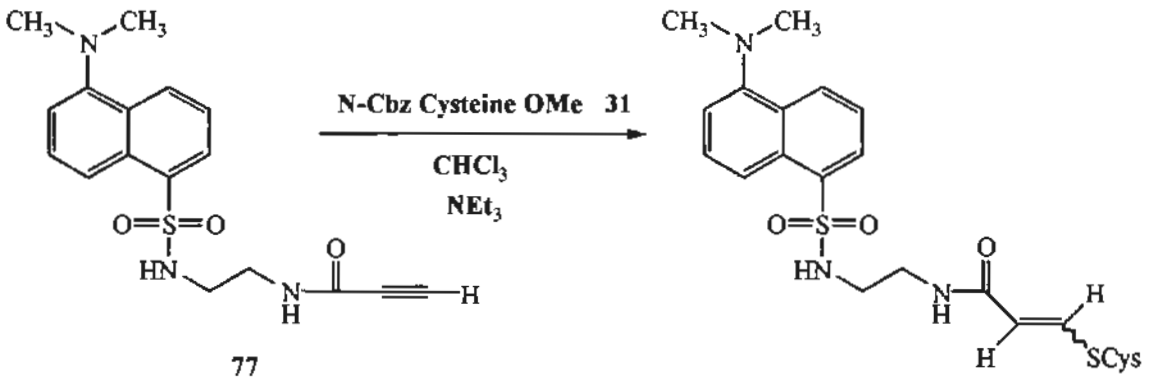
ethyl propiolate **27** and the lysine derivative **33**. In each case the addition reaction produced both the *E* and the *Z* isomers as an inseparable mixture. The addition products **104*E*** and **104*Z***, from the reaction of the amine **33** with the Sudan 1 derivative **73** were produced in a ratio of 1.1:1 in 51% overall yield and showed a molecular ion at 639 (FABMS). Resonances due to the vinylic protons were observed in the ^{1}H NMR at δ 4.60 (d) (J = 13 Hz) and δ 7.62 (dd) (J = 8, 13 Hz) for **104*E*** and at δ 4.49 (d) (J = 8 Hz) and δ 6.54 (dd) (J = 8, 13 Hz) for **104*Z***. The products **105*E*** and **105*Z*** from the addition of the lysine derivative **33** to the functionalised 7-hydroxycoumarin 74 were formed in 55% yield, (1.5:1). The structure was confirmed by LSIMS (molecular ion at 553) and by ^{1}H NMR, where the vinylic protons were observed at δ 4.73 (d) (J = 13 Hz) and δ 7.54 (dd) (J = 8, 13 Hz) for **105*E*** and at δ 4.51 (d) (J = 8 Hz) and δ 6.63 (dd) (J = 8, 13 Hz) for **105*Z***. These reactions could be carried out in the presence of triethylamine, albeit with reduced yields, indicating that to at least some extent the large dye moiety was hindering the self condensation observed in the model reaction. However, in order to achieve optimum results it was necessary to remove the hydrochloride salt from the amine **33** by washing with sodium bicarbonate prior to the reaction.



Scheme 63

4.2 The Reactions with the Terminal Alkynyl Amide

The reaction of the compound where a dansyl group was attached to a terminal acetylenic amide **77** was only carried out with the *N*-Cbz cysteine methyl ester **31**, as the model reactions had demonstrated that this was the only amino acid of the three studied which was reactive towards the deactivated conjugated terminal acetylenic amide moiety. The thiol **31** was added to the amide **77** in chloroform with a catalytic amount of triethylamine. As expected, after stirring the reaction overnight, two products **106*E*** and **106*Z***, were recovered, in 55% overall yield, in a 1.6:1 ratio, **(Scheme 64)**. The FAB mass spectrum showed a molecular ion at 615 and the ^{1}H NMR spectrum showed the vinylic resonances as doublets at δ 5.66 and δ 7.39 with a coupling constant of J = 15 Hz for **106*E*** and at δ 5.57 and δ 6.67 with a coupling of J = 10 Hz for **106*Z***.



77 **106*E* + 106*Z***

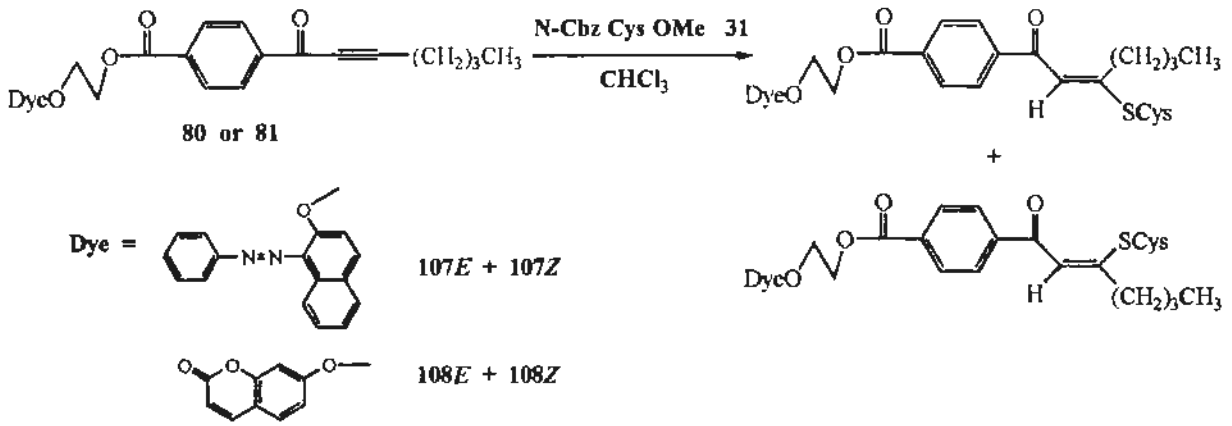
Scheme 64

4.3 The Reactions with the Non Terminal Alkynyl Ketones

The reactions of the three protected amino acids **31** - **33** with the Sudan 1 **81**, 7-hydroxycoumarin **80** and the dialkylated fluorescein derivative **82** attached to a linker arm terminated in a non terminal conjugated acetylenic ketone are described below. The reactions of the three amino acids with the dansyl non terminal conjugated alkynyl ketone **84** were not undertaken due to the decomposition of this material.

4.3.1 The Sudan 1 and 7-Hydroxycoumarin Derivatives

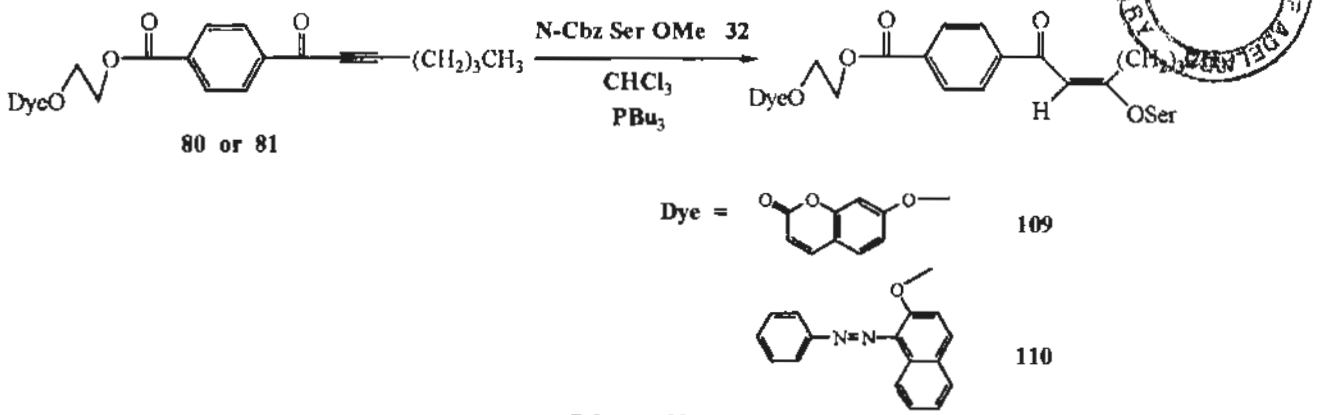
The model addition reaction between the non-terminal acetylene **26** and the protected cysteine **31** was carried out in chloroform with triethylamine as the catalyst and gave rise to both the *E* and the *Z* isomers in a 1 : 2 ratio, suggesting that the *Z* isomer was the more stable of the two. When the addition of the protected cysteine **31** to the Sudan 1 non-terminal ketone **81** was carried out under the same reaction conditions as above, again both isomers, **107*E*** and **107*Z***, were formed, (78% isolated yield), (**Scheme 65**). However in this case the isomers were formed in a ratio of 2.5 to 1 for **107*E*** to **107*Z***, showing that **107*E*** rather than **107*Z*** is the more stable isomer in this case. This reversal in stability is almost certainly due to steric interactions by the bulky dye destabilizing the intermediate *Z* anion. The stereochemistry of the isomers was assigned by comparison with the ^{1}H NMR spectra from the products of the model reaction, **37*E*** and **37*Z***. The vinylic protons were observed as singlets at δ 6.74 in **37*E*** and at δ 7.01 for **37*Z***, which compare favourably with resonances at δ 6.69 for **107*E*** and δ 6.95 for **107*Z***.



Scheme 65

The reaction of the 7-hydroxycoumarin derivative **80** with the cysteine derivative **31** occurred in much the same manner as the above reaction, producing the two isomers **108*E*** and **108*Z*** in a ratio of 2 : 1, (66% isolated yield), (**Scheme 65**). The slightly lower ratio of *E* to *Z* isomers may be a reflection of the less bulky nature of the 7-hydroxycoumarin dye when compared to Sudan 1. The vinylic protons of **108*E*** and **108*Z*** were observed as singlets at δ 6.74 and δ 7.00 respectively, giving excellent agreement with the values from the model compounds **37*E*** and **37*Z***.

The reaction of *N*-Cbz serine methyl ester **32** with the 7-hydroxycoumarin conjugated non terminal acetylenic ketone **80** was undertaken in chloroform with a 20% molar ratio of tributylphosphine present to catalyse the addition, (**Scheme 66**). The reaction produced the expected Michael addition product **109**, as indicated by a molecular ion of 672. The close correspondence of the observed resonance due to the vinylic proton of **109** at δ 6.07 (s) with the vinylic resonance for the corresponding model addition product **38** at δ 6.10 (s) demonstrates that **109** is also the *E* isomer. The addition product **109** had an almost identical R_f to the starting amino acid **32**, and hence could only be isolated as a mixture with **32**. Integration of the ^{1}H NMR spectrum showed that **109** was formed in a yield of 31%.

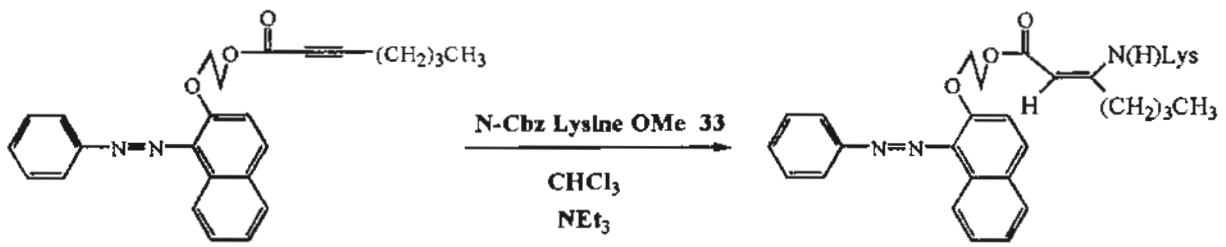


Scheme 66

Tributylphosphine was added to a solution of the Sudan 1 derivative **81** and the protected serine **32** in chloroform and allowed to stir until tlc analysis of the mixture indicated the reaction had proceeded to completion, (**Scheme 66**). After chromatographic purification one isomer **110** was recovered in 43% yield. Compound **110** was confirmed to be the expected addition product by FABMS, where a molecular ion of 758 units was observed. This isomer was shown to be the *E* isomer by comparison of the ^{1}H NMR spectrum with that of the corresponding model addition product **38**, (the vinylic proton of **110** was observed as a singlet at δ 6.04, cf. δ 6.10 for **38**).

The reaction of the protected lysine **33** with the Sudan 1 derivative **81** was undertaken in chloroform in the presence of a catalytic amount of triethylamine, (**Scheme 67**). Tlc analysis of the reaction mixture after stirring for 48 hours at room temperature showed only starting materials to be present. The reaction was heated to 60°C for a further 48 hours, at which time tlc analysis showed mainly the starting material as well as a very faint spot at lower R_f. The red-orange colour of this new spot suggested it to be the expected addition product **111**. Purification of the crude reaction mixture by chromatography allowed the isolation of this material which was confirmed to be the expected addition product by ^{1}H NMR (the vinylic proton was observed at δ 5.63 (s) and the enamine proton at δ 11.67 (m); cf. the vinyl proton

at δ 5.66, and the enamine NH at δ 11.55 for the product of the model addition, **39**). The close correspondence with the ^{1}H NMR of the product **39** from the model reaction between the protected lysine **33** and the conjugated non terminal alkynyl ketone **26** indicates that **111** is also the *Z* isomer. On the scale at which this reaction was undertaken insufficient compound was isolated for complete characterisation. The time scale required for this reaction to go to completion renders it an impractical process for the dyeing of wool, hence this reaction was not investigated further. The reaction of the lysine derivative **33** with the functionalised 7-hydroxycoumarin **80** was not undertaken for the same reasons.



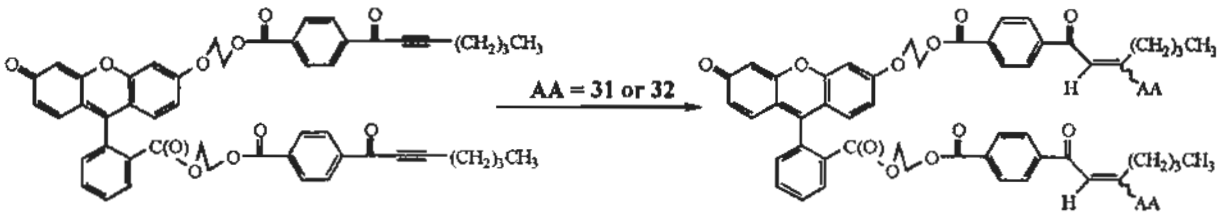
81 **111**

Scheme 67

4.3.2 The Dialkylated Fluorescein Derivative

The addition of the protected cysteine **31** to the dialkylated fluorescein derivative **82** was undertaken using the standard conditions of a catalytic amount of triethylamine in chloroform, (**Scheme 68**). Tlc analysis of the crude mixture indicated that all of the four possible isomers of the addition product **112** had formed in this reaction in 48% combined yield. The addition products streaked on both silica and alumina and hence purification by flash chromatography was not possible. The separation of the four isomers by HPLC was undertaken using a semi preparative reverse phase column, (**Figure 22**). The insolubility of the relatively non polar

addition products **112** in the polar solvents required to adsorb the material onto the reverse phase column precluded the isolation of suitable quantities of material for characterisation purposes. The structure of compound **112** was confirmed by LSIMS where a molecular ion at 1382 was observed. The complexity of the ^{1}H NMR spectrum of the mixture prevented the identification of the resonances due to the vinylic protons.



82

AA = N-Cbz Cysteine OMe 112

= N-Cbz Serine OMe 113

Scheme 68

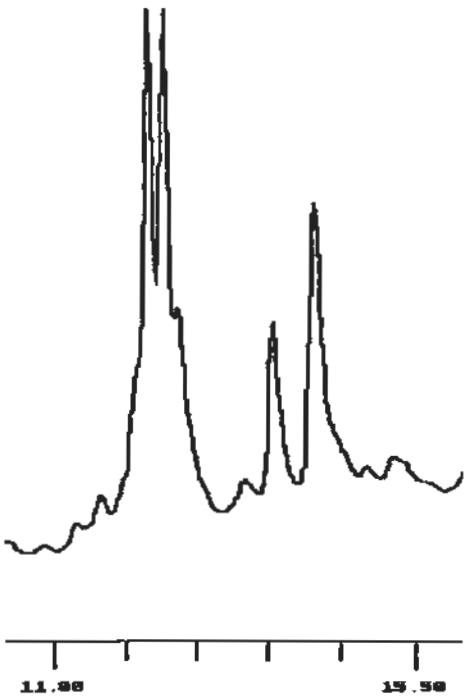


Figure 22 - HPLC trace of **112** showing the four isomers.

The reaction of the dialkylated fluorescein derivative **82** with the protected serine **32** in the presence of a 20% molar ratio of tributylphosphine produced a single isomer **113** in only 12% isolated yield, (**Scheme 68**). Tlc analysis of the crude reaction mixture showed large amounts of material at low R_f, possibly from the further reaction of the initially formed phosphine-alkyne adduct. The presence of this material accounts for the low yield of the Michael addition product. The structure of the addition product **113** was confirmed by a molecular ion at 1352 by LSIMS. Vinylic resonances were observed as singlets at δ 6.07 and δ 6.16 in the ^{1}H NMR spectrum. This indicates that **113** possesses *E* stereochemistry about both double bonds by analogy with the product **38** of the corresponding model reaction (vinylic proton at δ 6.10). Similarly to the product **112** of the addition of *N*-Cbz cysteine methyl ester **31** to the dialkylated fluorescein derivative **82**, compound **113** streaked on both silica and alumina, and hence was difficult to separate from other side products.

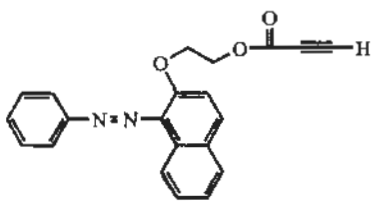
4.4 The UV - Visible Spectra

The maximum wavelengths of absorption and the corresponding molar extinction coefficients for the derivatised dyes (**73**, **74**, **77**, **80**, and **81**) and for the addition products from the reactions described in this Chapter are shown in **Tables 4 - 8**. The UV-visible spectra for the products (**112** and **113**) of the additions of the protected cysteine **31** and serine derivatives **32** to the dialkylated fluorescein **82** were not measured due to irremovable impurities present in the samples.

Table 4 shows the maximum absorption wavelengths (λ_{max}) and the corresponding molar extinction coefficients (ε) for the Sudan 1 conjugated terminal acetylenic ester **73** and the three amino acid derivatives **98**, **100**, and **104**. **Table 5** shows the absorptions and extinction

coefficients for the compounds derived from the Sudan 1 conjugated non terminal acetylenic ketone **81**. The wavelengths of the major absorptions change only slightly with the change in structure of the prepared compounds showing that Sudan 1 is relatively insensitive to its environment. The weaker absorption above 300 nm does move with changes in the structure, suggesting that it is due to the conjugated alkyne or alkene. This absorption is thus affected by the substituents, especially those with a lone electron pair (ie. sulfur, nitrogen and oxygen). The orange - red colour of the Sudan 1 dye results from a much weaker absorption at 466 nm, ($\varepsilon \sim 1600$), which is not shown in the tables below. The molar extinction coefficients do alter significantly with changes in the structure, (cf. for **73** $\lambda_{max} = 236$, $\varepsilon = 30155$ with for **110** $\lambda_{max} = 232$, $\varepsilon = 127050$).

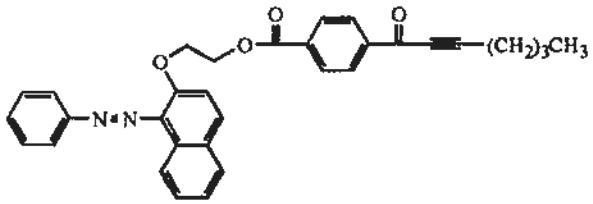
Table 4: Sudan 1 derivative **73** and reaction products.



73

73		+ Cysteine → **98**		+ Serine → **100**		+ Lysine → **104**	
λ_{max}	ε	λ_{max}	ε	λ_{max}	ε	λ_{max}	ε
236	30155	230	43660	234	90335	233	56110
278	14495	272	34890	279	29325	278	45910
372	8155	-	-	368	17790	-	-

Table 5: The Sudan 1 derivative **81** and reaction products

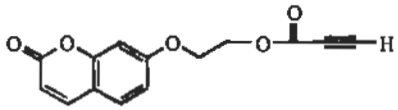


81

81		+ Cysteine → **107*E***		+ Cysteine → **107*Z***		+ Serine → **110**	
$\lambda_{max.}$	ε	$\lambda_{max.}$	ε	$\lambda_{max.}$	ε	$\lambda_{max.}$	ε
235	47790	236	47925	238	46960	232	127050
268	41735	256	36385	260	35065	285	75070
364	11317	328	24835	342	25925	303	14130

The compounds derived from 7-hydroxycoumarin do show some environmental sensitivity. The products from the reaction of the conjugated terminal ester **74** with the cysteine **99** and lysine derivatives **105** show large shifts from the parent compound **74**, whereas the serine derivative **101** does not, (**Table 6**). The compounds **108*E*, 108*Z*** and **109**, derived from the addition of cysteine and serine to the 7-hydroxycoumarin non terminal alkynyl ketone **80**, all have substantially different UV-visible spectra to **80**, (**Table 7**). The molar extinction coefficients are generally small when compared to those obtained for the other dyes used in this study which is a reflection of the less conjugated aromatic system of 7-hydroxycoumarin. The highest wavelength absorption which is most likely due to the conjugated alkyne or alkene system, if it is observable, again shows alterations with changes in the structure.

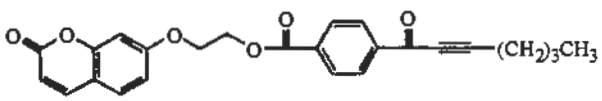
Table 6: The 7-hydroxycoumarin derivative **74** and reaction products



74

74		+ Cysteine → **99**		+ Serine → **101**		+ Lysine → **105**	
λ_{max}	ε	λ_{max}	ε	λ_{max}	ε	λ_{max}	ε
233	7360	280	55695	236	30100	277	40810
319	4520	322	39035	322	27318	321	31120

Table 7: The 7-hydroxycoumarin derivative **80** and reaction products



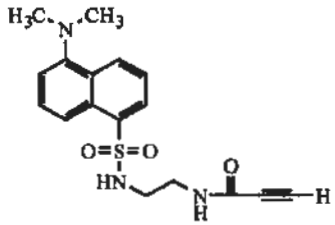
80

80		+ Cysteine → **108*E***		+ Cysteine → **108*Z***		+ Serine → **109**	
λ_{max}	ε	λ_{max}	ε	λ_{max}	ε	λ_{max}	ε
263	25260	230	34840	231	12880	230	13420
311	16610	254	33810	254	13550	253	15620
		324	49960	324	16660	293	17505

Table 8 shows the maximum wavelengths of absorption and the corresponding molar extinction coefficients for the compounds derived from dansyl chloride **22**. The two compounds in which the dye is attached to a protected cysteine residue, **108*E*** and **108*Z***, show marked alterations in the values obtained for the λ_{max} when compared to the conjugated non

terminal alkyne 77, illustrating the excellent environmental sensitivity exhibited by the dansyl group.

Table 8: The dansyl derivative 77 and reaction products



77

77		+ Cysteine → **108*E***		+ Cysteine → **108*Z***	
λ_{max}	ε	λ_{max}	ε	λ_{max}	ε
232	19690	264	107482	263	46576
257	25075	336	23032	342	11260
347	9395	-	-	-	-

4.5 Conclusions

The studies described in this Chapter illustrate the applicability of systems where a reactive conjugated acetylene is joined to a dye *via* a linker arm to the attachment of labels to amino acids. A number of labelled single amino acids have been prepared in generally moderate yields by the Michael type addition of the protected amino acid to the activated alkyne system of the functionalised dyes whose synthesis was discussed in Chapter 3. The ultra violet - visible spectra of these compounds reflect both the environmental sensitivity of the dyes and to a lesser extent the structure of the newly formed conjugated alkenes.

Chapter 5: Dyeing Wool

The studies discussed in the previous Chapter describe the attachment of dyes to single amino acids *via* a Michael type addition onto an activated alkyne. In order to determine the applicability of this method towards both the labelling of peptides for the purpose of biological studies and to the dyeing of wool with reactive dyes, the reaction of a peptide with a dye functionalised by the attachment of a conjugated alkyne needed to be examined. For this purpose wool was chosen as the peptide to be studied.

5.1 The Preparation of the Wool

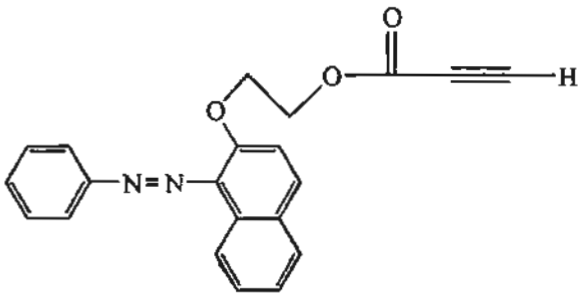
The sample of wool which was obtained was 'straight off the sheep's back', and as such was heavily contaminated with dirt, grass seeds, lanolin and other natural oils and products from the sheep. (Merino fleece, micron 23.5, yield 66%, tyte 74, length 90 mm). In order to remove these unwanted materials the wool was initially washed with hexane to remove the oils, followed by washing with ethanol and then with water. After drying the wool was pale cream, almost white in colour.

Two samples of wool were prepared for dyeing, the first as described above while the second sample was further processed. In wool the majority of the cysteine residues exist as the disulfide cystine,[2] which are unreactive to Michael type additions. In order to increase the number of reactive sites present in the peptide the second sample of wool was stirred in a 0.1 M solution of thioglycolic acid in water for a 72 hour period. This results in the reduction of

approximately 12 - 20% of the disulfide linkages to yield the free thiols.[111] At the end of this period the wool was washed with water and ethanol to remove the thioglycolic acid and allowed to dry. The wool treated by this procedure was pure white in colour.

5.2 Dyeing the wool

The azo dye Sudan 1 functionalised by the addition of a linker arm terminated in a conjugated terminal alkynyl ester **73** was the dye of choice for this study due to its relative ease of preparation.



73

The derivatised dye **73** is relatively non polar and so is not soluble in water, hence a solution of the dye in DMF was prepared. The two samples of wool were each added to separate solutions and allowed to stir for 2 hours to ensure that the dye achieved even penetration of the fibre. After this time one equivalent of triethylamine (based on the dye) was added to initiate the Michael addition and the solution was left to stir at ambient temperature for 24 hours. A small portion of the wool from each sample was removed from the solution and washed with ethanol to remove the DMF. The samples were then washed with dichloromethane until no more colour appeared in the solvent, to remove unbound dye from

the fibre. The remaining wool was allowed to stir for a further 72 hours before removal from the DMF solution and washing by the above procedure. The samples of wool taken from the dyebath after 24 hours were identical in colour with the samples removed after 96 hours, indicating that the dyeing process had gone to completion within 24 hours.

The two samples of wool, **Sample A** where the wool was simply washed before the dyeing process and **Sample B** where some of the disulfide linkages were reduced prior to dyeing are shown below.

Sample A: The untreated wool.

Sample B: Wool treated with 0.1 M thioglycolic acid

Sample A is a pale orange whereas **Sample B** where more sites existed for the attachment of the dye, is a much darker orange, almost red in colour.

5.3 The Stability of the Dye

As a result of the ester linkages present in the functionalised dye **73** the expectation was that the colour would have a poor fastness to aqueous treatments due to hydrolysis of the esters. However when samples of the dyed wool were added to two solutions containing commercial laundry detergents: 'Drive™' an all purpose detergent; and 'Softly™' a wool wash; very little colour was lost after stirring at room temperature for a period of one week, (**Sample C**). The solutions initially turned very pale orange after several hours but changed little for the remaining time. This suggests the detergent was removing any remaining unbound dye rather than hydrolysing the ester linkages of the bound dye. This unexpected colour fastness suggests that the non polar dye is sitting in, or is responsible for a hydrophobic cavity in the fibre and so is blocking the approach of the water and the detergent to the vulnerable ester bonds.

Sample C: Wool after treatment with Drive™ at room temperature.

5.4 Conclusions

The studies discussed in this Chapter illustrate the applicability of systems where a dye is joined to an activated alkyne to the attachment of dyes to proteins. The ease with which the wool was dyed at relatively low temperatures to yield highly coloured samples with better than expected fastness demonstrates the usefulness of this method for the reactive dyeing of wool.

Chapter 6: Conclusions

Conjugated alkynes activated by an adjacent electron withdrawing group have been examined as a potential reactive group for the attachment of dyes to amino acids for the purpose of either labelling peptides for biology, biomedicine and for analytical procedures, or for the dyeing of wool.

The initial studies described in Chapter 2 determined the conditions which best promoted the Michael addition and demonstrated the use of two catalysts which allowed the reaction of the nucleophilic side chains of amino acids with conjugated alkynes under mild conditions. The model studies described in this Chapter established an order of reactivity for both the amino acids and the terminal and non-terminal conjugated alkynes. This order showed that terminal alkynes activated by ketones, esters and to a lesser extent amides were potential reactive groups, while for non terminal alkynes only those activated by ketones were suitable.

Accordingly a series of compounds which contained a conjugated alkyne attached to a dye *via* a linker arm were prepared. Initially four dyes were taken and functionalised by the addition of a linker arm terminated with either an alcohol or an amine, which was followed by the later attachment of the conjugated alkyne. Dyes connected to terminal alkynyl esters were prepared by direct coupling with propiolic acid. Non terminal alkynyl ketones attached to dyes by a linker arm were formed *via* the initial preparation of a difunctional compound containing both the activated acetylene and a carboxylic acid moiety with which to couple to the alcohols or amine.

The reactions of the functionalised dyes containing the activated acetylenes with the amino acids cysteine, lysine and serine are described in Chapter 4. These reactions illustrated the applicability of systems of this types as reactive groups.

The usefulness and success of the conjugated alkyne as a reactive group was further emphasised by the successful dyeing of wool described in Chapter 5. The wool was dyed under mild conditions which did not require boiling, to yield orange coloured wool with a better than expected colour fastness.

Experimental

Melting points were recorded on a Reichhert hot stage apparatus and are uncorrected. Proton and carbon NMR spectra were recorded on a Bruker ACP-300 or a Varian Gemini 200 spectrometer with $CDCl_3$ as the solvent unless otherwise stated, and tetramethylsilane as an internal standard. Mass spectra were recorded on a VG ZAB 2HF mass spectrometer with either electron impact (EI) or fast atom bombardment (FAB) ionisation, or on an AEI-GEC MS 3074 instrument with EI ionisation. Accurate mass determinations using EI or liquid secondary ion mass spectroscopy (LSIMS) were made by the Organic Mass Spectrometry Facility at the University of Tasmania. Ultra violet spectra were recorded on a PYE Unicam SP8-100 Ultraviolet spectrophotometer. Infra red spectra were recorded on either a Hitachi 270-30 spectrometer and data processor or a ATI Mattson Genesis Series FTIR™ spectrometer as either liquid films (neat), nujol mulls or in solution, as stated.

Triethylamine, chloroform and dichloromethane were distilled from calcium hydride under nitrogen and stored over 4Å molecular sieves. DMF was distilled from calcium hydride (*ca.* 80°C at 20 mmHg) and stored over 4Å molecular sieves. THF was freshly distilled from sodium and benzophenone under nitrogen. Other reagents were purified according to literature procedures.[112] All organic extracts were dried over anhydrous magnesium sulfate unless otherwise specified.

Analytical thin layer chromatography was carried out using Merck aluminium sheets precoated with kieselgel 60 F_{254} and visualised using either a 254 nm or a 365 nm lamp, or with a 5%

solution of phosphomolybdic acid in ethanol. Flash chromatography[113] was carried out using Merck kieselgel 60 (230-400 mesh) and solvents were distilled before use.

Pyridinium *p*-toluene sulfonate[99], and ethynyltributyl tin[114] were synthesised according to literature procedures. *N*-Carbobenzyloxy serine and *N*-Carbobenzyloxy lysine were purchased from Aldrich chemical company. A sample of methyl *p*-iodobenzoate was a gift from the research group of Professor Michael Bruce, University of Adelaide.

The following abbreviations have been used in defining peak shape for the various spectra: br = broad, d = doublet, m = multiplet, q = quartet, s = singlet, t = triplet. Unless otherwise stated $J = J^3$ for ^{1}H NMR. Mass spectral data are reported as m/z ratio (% relative abundance). UV data are reported as λ_{max} (ε).

When a reaction produced more than one isomer the mass spectrum and analytical data were determined on the mixture of isomers.

Experimental Described in Chapter 2

Ethynyl phenyl ketone 25

Ethynylmagnesium bromide (39.4 ml, 0.5 M in THF, 23.6 mmol) was added slowly to benzaldehyde (2.0 ml, 19.7 mmol) in freshly distilled dry THF (12 ml) at room temperature. After the addition of the Grignard reagent was completed the reaction was warmed with a hot water bath for 60 minutes, then left stirring for a further 3 hours at room temperature. The THF was removed on a rotary evaporator and 10% sulfuric acid added to the residue until pH paper indicated the solution was acidic. This solution was extracted with ethyl acetate (4x30 ml) and the organic layer washed with water (20 ml) and brine (20 ml). Kugelrohr distillation (~125°C at 15 mmHg) gave the intermediate alcohol. Jones oxidation[75] of this alcohol followed by a standard work up gave the required conjugated alkyne (0.78 g) in an overall yield of 30%.

Mp 48 - 49 °C (Lit. Mp 50 - 51 °C)[75]; ^{1}H NMR δ 3.59 (s, 1H, ≡C-H), 7.4-7.6 (m, 3H, Ar), 8.13 (m, 2H, Ar); ^{13}C NMR δ 81.1, 128.4, 128.8, 129.3, 134.2, 135.8, 177.1; IR ν_{max} ($CHCl_3$) 3300, 3025, 2100, 1660, 1600, 1575, 1450, 1320, 1260, 1245, 1175, 1010, 910, 730, 660 cm^{-1}.

1-Hexynyl phenyl ketone 26 [81]

Copper(I) iodide (5 mg, 0.03 mmol) and bis(triphenylphosphine)palladium(II) dichloride (5 mg, 0.007 mmol) were added to a solution of 1–hexyne (0.57 ml, 5.0 mmol) and benzoyl chloride (0.58 ml, 5.0 mmol) in triethylamine (10 ml), and left stirring at room temperature for 16 hours. Methanol (1 ml) was then added to destroy any remaining benzoyl chloride, followed by the removal of the solvent under reduced pressure. The remaining oil was extracted with hexane, the organic layer dried and the hexane removed. Flash chromatography (2.5% EtOAc / 97.5% hexane) gave the title compound as an oil, 0.81g, 87%. ^{1}H NMR δ 0.95 (t, J = 7 Hz, 3H, CH_3), 1.47 (m, 2H, CH_2), 1.65 (m, 2H, CH_2), 2.49 (t, J = 7 Hz, 2H, CH_2), 7.4-7.6 (m, 3H, Ar), 8.1 (m, 2H, Ar); IR ν_{max} ($CDCl_3$) 2960, 2870, 2220, 1730, 1650, 1600, 1590, 1450, 1215, 1270, 1180, 1115, 910, 710 cm^{-1}; MS (EI) m/z 186 ($M^{+\cdot}$), 144 (95), 105 (100), 77 (60).

2-Heptynoic acid 44

Butyl lithium (3.67 ml, 9.1 mmol, 2.5M solution in ether) was added slowly to 1-hexyne (1.0 ml, 8.7 mmol) in dry THF (30 ml) at -78°C under nitrogen. After stirring for 30 minutes the mixture was carefully poured onto powdered dry ice and allowed to warm to room temperature. The solvent was then removed and 10% hydrochloric acid added to the resulting white solid until pH paper indicated the solution was acidic. The aqueous layer was extracted with dichloromethane (4x25 ml). The organic layer was dried and the solvent removed, to yield the title compound[115, 116] as a colourless oil, 0.74g, 67%. ^{1}H NMR δ 0.93 (t, J = 7 Hz, 3H, CH_3), 1.4-1.6 (m, 4H, 2xCH_2), 2.37 (t, J = 7 Hz, 2H, CH_2), 11.30 (br s, 1H, CO_2H); IR

ν_{max} (neat) 3600-2400 (broad), 2225, 1690, 1470, 1410, 1280 cm^{-1}; MS (EI) m/z 126 (M^+, 100), 110 (67), 108 (54), 83 (50), 80 (54).

Methyl-2-heptynoate 28

Methyl iodide (0.30 ml, 4.8 mmol) and potassium carbonate (0.66g, 4.8 mmol) were added to a solution of the acid **44** (0.5g, 4.0 mmol) in DMPU (10 ml) under nitrogen and stirred for 12 hours. Water (30 ml) was added and the solution extracted with ether (3x20 ml). The extracts were washed with water (3x20 ml), then saturated sodium bicarbonate solution (20 ml). The solution was dried and the solvent removed to yield the title compound[116, 117] in good purity as an oil in 75% yield (0.42g). ^{1}H NMR δ 0.92 (t, J = 7 Hz, 3H, CH_3), 1.44 (m, 2H, CH_2), 1.54 (m, 2H, CH_2), 2.34 (t, J = 7 Hz, 2H, CH_2), 3.76 (s, 3H, OCH_3); ^{13}C NMR δ 13.5, 18.4, 22.0, 29.6, 52.5, 72.9, 89.8, 154.3; IR ν_{max} (neat) 2950, 2860, 2230, 1715, 1435, 1255, 1075, 1040, 930, 750 cm^{-1}; MS (EI) m/z 109 (M-OCH_3, 30), 97 (M-C_3H_7, 50), 83 (M-C_4H_9, 60), 69 (90), 57 (100).

***N*-Benzyl-2-propynamide 29**

Method A [86]

Lithium hydride (0.12g, 15 mmol) was added to a solution of propiolic acid, (0.88 ml, 14.3 mmol) in THF (10 ml) under nitrogen and stirred for 2 hours. The mixture was then cooled to -10°C and ethyl chloroformate (1.43 ml, 15 mmol) in THF (5 ml) was added slowly. The solution was allowed to warm to room temperature and then stirred for a further 30 minutes before being cooled to approximately 5°C. Benzylamine (1.64 ml, 15 mmol) in THF (5 ml) was added, and the mixture left to stir overnight. The solvent was then removed,

dichloromethane added, and the solution washed with 10% sodium bicarbonate solution (30 ml), and 10% hydrochloric acid (30 ml) followed by drying and removal of the dichloromethane. The resulting material was purified by flash chromatography (35% EtOAc / 65% hexane) followed by recrystallization from EtOAc / hexane, to yield the title compound, 0.24g (11%) as yellow crystals.

Method B

Dicyclohexylcarbodiimide (0.66g, 3.2 mmol) and 4-dimethylaminopyridine (0.02g, 0.2 mmol) were added slowly to a solution of propiolic acid (0.20 ml, 3.2 mmol) and *N*-hydroxy succinimide (0.37g, 3.2 mmol) in chloroform (10 ml) at -20°C, and the reaction was left stirring to warm to room temperature overnight. The precipitated DCU was removed by filtration and benzylamine (0.35 ml, 3.2 mmol) was added to the solution. The reaction was stirred for 5 hours. Removal of the solvent and flash chromatography (as above) yielded 0.18g of the expected product (35% yield). Mp 90-93°C (lit. Mp 89-92°C)[86]; ^{1}H NMR δ 2.81 (s, 1H, ≡C-H), 4.41 (d, J = 6 Hz, 2H, CH_2N), 7.01 (br s, 1H, NH), 7.3 (m, 5H, Ph); IR ν_{max} (nujol) 3200, 3060, 2950, 2920, 2840, 2100, 1620, 1550, 1450, 1430, 1290, 1240, 750, 690 cm^{-1}; MS (EI) m/z 159 ($M^{+\cdot}$, 100), 116 (50), 115 (60), 91 (40), 79 (30), 77 (35), 53 (60).

N-Benzyl-2-heptynamide 30

Lithium hydride (0.016g, 2.0 mmol) was added to a solution of the acid **44** (0.25g, 1.98 mmol) in THF (10 ml) under nitrogen and stirred for 2 hours. The mixture was then cooled to -10°C and ethyl chloroformate (0.19 ml, 1.98 mmol) in THF (3 ml) was added slowly. The solution

was allowed to warm to room temperature and stirred for a further 30 minutes before being cooled to approximately 5°C, at which time benzylamine (0.22 ml, 1.98 mmol) in THF (3 ml) was added, and the mixture left to stir overnight. Workup as for the preparation of *N*-Benzyl-2-propynamide **29** (Method A) yielded the expected product in 67% (0.28g). Mp 32-34.5°C; ^{1}H NMR δ 0.90 (t, J = 7 Hz, 3H, CH_3), 1.39 (m, 2H, CH_2), 1.51 (m, 2H, CH_2), 2.28 (t, J = 7 Hz, 2H, CH_2), 4.45 (d, J = 6 Hz, 2H, CH_2N), 6.20 (br s, 1H, NH), 7.30 (m, 5H, Ph); ^{13}C NMR δ 13.5, 18.3, 29.7, 43.8, 75.4, 88.0, 127.7, 127.8, 128.7, 137.6, 153.6; IR ν_{max} (nujol) 3270, 3060, 2950, 2860, 2240, 1700, 1640, 1600, 1540, 1500, 1450, 1360, 1290, 1075, 1025, 830, 700 cm^{-1}; MS (EI) m/z 215 ($M^{+\cdot}$), 179 (50), 178 (30), 171(25), 170 (15), 149 (100), 105 (100), 91 (45).

_N,N'_-Bis(carbobenzyloxy) cystine dimethyl ester 50

Thionylchloride (1.52 ml, 20.8 mmol) was added slowly to methanol (50 ml) at 0°C, followed by the addition of cystine (2.0g, 8.4 mmol) after 10 minutes, and the mixture was left stirring overnight. The solvent was removed under reduced pressure and the resulting sticky solid recrystallized from methanol / isopropanol to give 2.08g (73%) of cystine dimethyl ester dihydrogenchloride as a white solid. ^{1}H NMR δ 3.27 (d, J = 6 Hz, 2H, CH_2S), 3.79 (s, 3H, CH_3), 4.37 (m, 1H, α-CH); IR ν_{max} (nujol) 3300, 1720 cm^{-1}.

Benzyl chloroformate (4.2 ml, 29.4 mmol) in chloroform (20 ml) was slowly added to cystine dimethyl ester dihydrogenchloride (2.0g, 5.8 mmol), dissolved in aqueous sodium carbonate (31.0g, 29.4 mmol, in 80 ml of water) and stirred for 24 hours. The organic layer was separated and pyridine (3.0 ml) added to remove excess chloroformate, then washed with 10%

hydrochloric acid (2x30 ml) followed by 10% sodium carbonate (30 ml). Removal of the solvent and chromatography (40% ethylacetate / 60% hexane) gave 1.02g (33%) of the title compound[118] as a colourless oil. ^{1}H NMR δ 3.13 (m, 2H, CH_2S), 3.74 (s, 3H, CH_3), 4.66 (m, 1H, α-CH), 5.11 (s, 2H, $OC\underline{H}_2Ph$), 5.73 (d, J = 8 Hz, 1H, NH), 7.33 (s, 5H, Ph).

N–Carbobenzyloxy cysteine methyl ester 31

The protected cystine derivative **50** (1.0g, 1.86 mmol) was dissolved in a mixture of chloroform (40 ml) and methanol (10 ml). Concentrated hydrochloric acid (1.2 ml) was added, followed by zinc dust (0.56g, 8.6 mmol), and the solution was left stirring for 30 minutes. The organic layer was separated and washed with water (2x40 ml), then brine (40 ml), dried and the solvent removed. Chromatography (30% ethylacetate / 70% hexane) gave 0.75g (75%) of **31**[119] as a colourless oil. ^{1}H NMR δ 1.39 (t, J = 9 Hz, 1H, SH), 3.00 (m, 2H, CH_2S), 3.78 (s, 3H, CH_3), 4.68 (m, 1H, α-CH), 5.12 (s, 2H, $OC\underline{H}_2Ph$), 5.73 (br d, J = 7 Hz, 1H, NH), 7.36 (s, 5H, Ph); ^{13}C NMR δ 27.1, 52.7, 55.3, 67.2, 128.1, 128.2, 128.5, 136.0, 155.7, 170.4; IR ν_{max} (neat) 3400, 3000, 2225, 1720, 1500, 1200, 1050 cm^{-1}; MS (EI) m/z 269 ($M^{+\cdot}$), 208 (100), 152 (45), 138 (80), 102 (65), 92 (100), 52 (100).

***N*–Carbobenzyloxy serine methyl ester 32**

Thionylchloride (0.61 ml, 8.36 mmol) was added dropwise to methanol (10 ml) at 0°C. After 10 minutes *N*–carbobenzyloxy serine (1.0g, 4.18 mmol) was added, and the mixture was left stirring overnight. The solvent was removed under reduced pressure and the resulting solid recrystallized from methanol / ether to give 1.05g (90%) of the expected product[120, 121] as

white crystals. Mp 28-33°C; ^{1}H NMR δ 3.71 (s, 3H, CH_3), 3.88 (d, J = 11.5 Hz, 2H, CH_2O), 4.40 (br s, 1H, α-CH), 5.07 (s, 2H, $OC\underline{H}_2Ph$), 6.10 (br s, 1H, NH), 7.31 (s, 5H, Ph); IR ν_{max} (nujol) 3550, 3300, 1720, 1540, 1210, 1060 cm^{-1}; MS (EI) m/z 253 ($M^{+\cdot}$,7), 209 (10), 180 (7), 161 (8), 149 (10), 107 (35), 90 (100).

N–Carbobenzyloxy lysine methyl ester 33

Thionylchloride (0.52 ml, 5.2 mmol) was added slowly to methanol (10 ml) at 0°C, followed by the addition of N-carbobenzyloxy lysine (1.0g, 3.57 mmol) after 10 minutes, and the mixture was left stirring overnight. The solvent was removed under reduced pressure and the resulting solid recrystallized from methanol / ether to give 1.04g (94%) of the title compound[119, 122] as white crystals. Mp 105-108°C; ^{1}H NMR δ 1.39 (m, 2H, CH_2), 1.60 (m, 4H, 2xCH_2), 2.72 (t, J = 7 Hz, 2H, CH_2), 3.63 (s, 3H, OCH_3), 4.00 (m, 1H, CH), 5.04 (s, 2H, $OC\underline{H}_2Ph$), 7.36 (s, 5H, Ph), 7.79 (d, J = 8 Hz, 1H, NHCbz); ^{13}C NMR δ 22.2, 26.7, 31.3, 39.4, 52.3, 53.7, 66.8, 127.8, 128.0, 128.3, 136.1, 156.3, 172.9; IR ν_{max} (neat) 3300 (br), 3030, 2950, 2830, 1740, 1615, 1530, 1455, 1395, 1340, 1290, 1265, 1220, 1170, 1030, 740, 700 cm^{-1}; MS (EI) m/z 295 ($M^{+\cdot}$, 70), 142 (40), 91 (100).

General Procedure for the base catalysed reactions between the protected amino acids and the conjugated alkynes (Procedure A)

Triethylamine (~5 drops, catalytic) was added to a mixture of the protected amino acid (50 mg) and the conjugated alkyne (1.2 molar equivalents) in the reaction solvent (normally chloroform, unless otherwise stated) at either room temperature or 0°C. The reaction was

stirred until tlc analysis indicated the reaction had proceeded to completion, at which time either the solvent was removed or the reaction was quenched with dilute hydrochloric acid followed by normal workup. Flash chromatography on silica then yielded the purified product(s).

General Procedure for the tri-*n*-butylphosphine catalysed reactions between the protected amino acids and the conjugated alkynes (Procedure B)

Tri-*n*-butylphosphine (0.2 molar equivalents) was added to the amino acid (50mg) and the conjugated alkyne (1.2 molar equivalents) in either THF or chloroform at room temperature. The solution was stirred until tlc analysis of the mixture showed the disappearance of the protected amino acid, then the solvent was removed and the resulting material chromatographed on silica to yield the addition product.

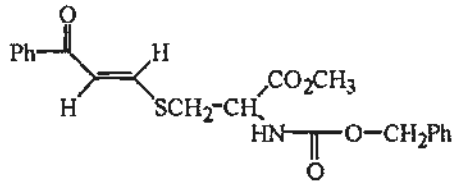
1-Phenyl-3-(*N*-carbobenzyloxy-*O*-methyl cysteinyl) propenones 34*E* and 34*Z*

The addition of the alkyne **25** and the protected cysteine **31** was undertaken at 0°C following Procedure A. After an acidic workup, purification of the crude material by flash chromatography (30% EtOAc / 70% hexane) yielded 53 mg, (71%) of the expected addition products **34*E*** and **34*Z*** in a 1.2 : 1 ratio..

34*E* / 34*Z*

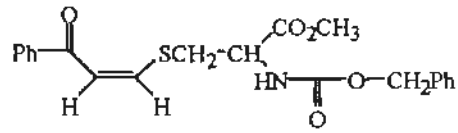
MS (FAB) m/z 400 (MH^+, 15), 163 (25), 154 (15), 136 (15), 105 (60), 91 (M-$PhCH_2$, 100), 77 (15), 57 (23); Anal. Calcd for $C_{21}H_{21}NO_5S$: C, 63.14; H, 5.30; N, 3.51. Found C, 62.98; H, 5.12; N, 3.30.

34*E*



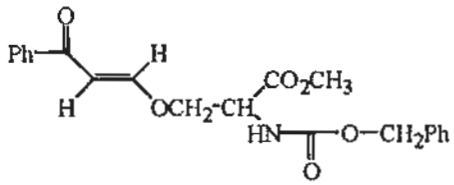
^{1}H NMR δ 3.46 (m, 2H, CH_2S), 3.79 (s, 3H, OCH_3), 4.76 (m, 1H, α-CH), 5.12 (m, 2H, $OC\underline{H}_2Ph$), 5.70 (br d, J = 7 Hz, 1H, NH), 7.06 (d, J = 15 Hz, 1H, HC=), 7.3-7.6 (m, 8H, Ar), 7.80 (d, J = 15 Hz, 1H, HC=), 7.94 (d, J = 7 Hz, 2H, Ar); ^{13}C NMR δ 35.4, 53.0, 53.5, 67.3, 119.5, 128.0, 128.1, 128.3, 128.4, 128.5, 128.6, 132.7, 132.7, 135.8, 137.7, 147.4, 155.5, 170.1, 186.6; IR ν_{max} ($CHCl_3$) 3425, 2950, 1720, 1640, 1600, 1550, 1500, 1450, 1340, 1260, 1060, 1020, 950 cm^{-1}; UV λ_{max} (CH_2Cl_2) 262 (11150), 316 (22750).

34*Z*



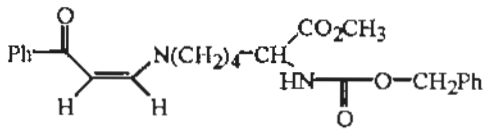
^{1}H NMR δ 3.32 (d, J = 4.5 Hz, 2H, CH_2S), 3.79 (s, 3H, OCH_3), 4.71 (m, 1H, α-CH), 5.14 (m, 2H, $OC\underline{H}_2Ph$), 5.68 (br d, J = 7 Hz, 1H, NH), 6.99 (d, J = 9.5 Hz, 1H, HC=), 7.2-7.5 (m, 8H, Ar), 7.93 (d, J = 9.5 Hz, 1H, HC=), 7.94 (d, J = 7 Hz, 2H, Ar); ^{13}C NMR δ 39.3, 53.0, 54.2, 67.2, 117.1, 128.0, 128.2, 128.3, 128.4, 128.56, 128.6, 132.6, 136.0, 137.6, 151.7, 155.6, 170.2, 189.0; IR ν_{max} ($CHCl_3$) 3460, 2975, 1735, 1650, 1610, 1590, 1515, 1460, 1390, 1360, 1330, 1080, 1030, 920, 900 cm^{-1}; UV λ_{max} (CH_2Cl_2) 260 (4995), 324 (24465).

1-Phenyl-3-(*N*-carbobenzyloxy-*O*-methyl serinyl) propenone 35



The reaction between the alkyne **25** and the serine derivative **32** was undertaken using Procedure A at 0°C. The solvent was removed and the resulting material purified by flash chromatography (35% EtOAc / 65% hexane) to yield 61 mg (80%) of the title compound as a mixture with an unidentified impurity. ^{1}H NMR δ 3.21 (dd, J = 8, 16 Hz, 1H, =CH), 3.58 (s, 3H, OCH_3), 4.13 (m, 2H, CH_2O), 4.31, (m, 2H, CH_2O), 4.49 (m, 2H, CH_2O), 5.18 (m, 2H, $OC\underline{H}_2Ph$), 5.92 (dd, J = 2, 8 Hz, 1H, =CH), 7.3-7.6 (m, 8H, Ar), 7.99 (d, J = 7 Hz, 2H, Ar); MS (LSIMS) m/z 384 (MH^+, 72), 264 (100), 254 (37), 248 (20), 220 (78), 210 (32); Exact Mass Calcd for $C_{21}H_{21}NO_6$ 383.13689. Found 383.13705.

1-Phenyl-3-(*N*-carbobenzyloxy-*O*-methyl lysinyl) propenone 36



The addition reaction between the terminal alkyne **25** and the protected lysine **33** was undertaken at room temperature following Procedure A. Purification of the crude material by flash chromatography (50% EtOAc / 50% hexane) yielded 36 mg, (50%) of the expected addition product. ^{1}H NMR δ 1.42 (m, 2H, CH_2), 1.63 (m, 2H, CH_2), 1.87 (m, 2H, CH_2), 3.25 (m, 2H, NCH_2), 3.74 (s, 3H, OCH_3), 4.39 (m, 1H, α-CH), 5.10 (s, 2H, $OC\underline{H}_2Ph$), 5.34 (d, J = 8 Hz, 1H, NH), 5.69 (d, J = 7.5 Hz, 1H, HC=), 6.90 (dd, J = 7.5, 13 Hz, 1H, HC=), 7.35 (m, 8H, Ar), 7.85 (m, 2H, Ar), 10.33 (m, 1H, enamine NH); ^{13}C NMR δ 22.2, 29.7, 30.5, 32.3, 48.8, 52.5, 53.6, 67.0, 90.2, 127.0, 128.1, 128.2, 128.5, 130.9, 136.2, 139.7,

154.2, 155.8, 172.7, 189.9; IR ν_{max} ($CHCl_3$) 3435, 3030, 3010, 2950, 1720, 1630, 1600, 1580, 1540, 1500, 1480, 1255, 1235 cm^{-1}; MS (LSIMS) m/z 425 (MH^+, 100), 214 (10); Exact Mass (EI) Calcd for $C_{24}H_{28}N_2O_5$ 424.19982. Found 424.19934.

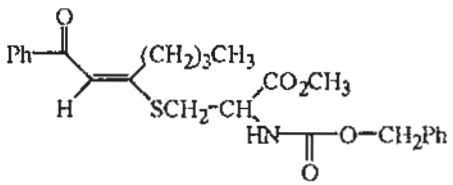
1-Phenyl-3-(*N*-carbobenzyloxy-*O*-methyl cysteinyl) heptenones 37*E* and 37*Z*

The reaction between the non terminal alkynyl ketone **26** and the cysteine derivative **31** was undertaken at room temperature by Procedure A. Flash chromatography (25% EtOAc / 75% hexane) produced 53% (45 mg) of the two alkenes in a ratio of 1:2 for ***37E*** and ***37Z***.

37E / 37Z

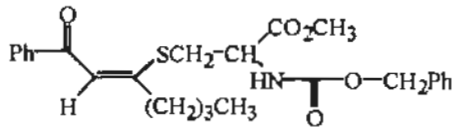
MS (EI) m/z 455 (M^+), 378 (10), 364 (15), 269 (15), 232 (15), 219 (100), 187 (25), 105 (100), 91 (100); Anal. Calcd for $C_{25}H_{29}NO_5S$: C, 65.91; H, 6.41; N, 3.08; Found C, 66.06; H, 6.46; N, 2.95.

37E



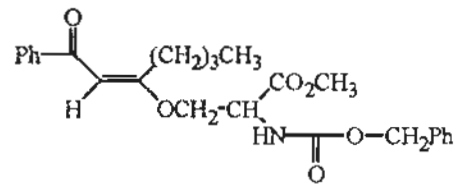
^{1}H NMR δ 0.93 (t, J = 7 Hz, 3H, CH_3), 1.43 (m, 2H, CH_2), 1.61 (m, 2H, CH_2), 2.85 (m, 2H, CH_2), 3.37 (m, 2H, CH_2S), 3.77 (s, 3H, OCH_3), 4.78 (m, 1H, α-CH), 5.10, 5.01 (AB pattern, J = 12 Hz, 2H, $OC\underline{H}_2Ph$), 5.59 (d, J = 7.5 Hz, 1H, NH), 6.74 (s, 1H, HC=), 7.3-7.6 (m, 8H, Ar), 7.94 (d, J = 7 Hz, 2H, Ar); ^{13}C NMR δ 13.8, 22.7, 32.0, 33.6, 34.9, 52.8, 53.0, 67.3, 113.7, 127.9, 128.1, 128.3, 128.5, 128.6, 132.2, 139.2, 155.5, 165.4, 170.4; IR ν_{max} ($CDCl_3$) 3650, 3045, 2985, 1720, 1420, 1240, 890, 760, 660 cm^{-1}; UV λ_{max} (EtOH) 257 (4955), 320 (12310).

37*Z*



^{1}H NMR δ 0.96 (t, J = 7 Hz, 3H, CH_3), 1.43 (m, 2H, CH_2), 1.60 (m, 2H, CH_2), 2.57 (t, J = 8 Hz, 2H, CH_2), 3.38 (m, 2H, CH_2S), 3.78 (s, 3H, OCH_3), 4.69 (m, 1H, α-CH), 5.11 (s, 2H, $OC\underline{H_2}Ph$), 5.67 (d, J = 8 Hz, 1H, NH), 7.01 (s, 1H, HC=), 7.3-7.5 (m, 8H, Ar), 7.92 (m, 2H, Ar); ^{13}C NMR δ 13.9, 22.3, 32.0, 32.5, 36.8, 53.0, 53.7, 67.2, 117.9, 128.0, 128.1, 128.2, 128.4, 128.5, 128.6, 132.2, 138.6, 155.7, 165.8, 170.2; IR ν_{max} ($CDCl_3$) 3600, 3400, 3050, 2980 1720, 1530, 1500, 1420, 1250, 1040, 890, 720 cm^{-1}; UV λ_{max} (EtOH) 256 (7560), 327 (17130).

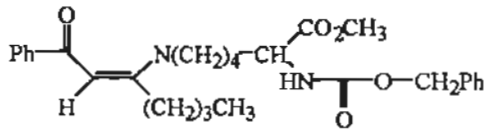
1-Phenyl-3-(*N*-carbobenzyloxy-*O*-methyl serinyl) heptenone 38



The addition of the ketone **26** and the alcohol **32** was undertaken following Procedure B. The crude material was purified by flash chromatography on silica (25% EtOAc / 75% hexane) to yield 28 mg, (40%) of **38**. ^{1}H NMR δ 0.90 (t, J = 7 Hz, 3H, CH_3), 1.35 (m, 2H, CH_2), 1.53 (m, 2H, CH_2), 2.68 (m, 1H, CH), 2.91 (m, 1H, CH), 3.80 (s, 3H, OCH_3), 4.26 (m, 2H, CH_2O), 4.74 (m, 1H, α-CH), 5.15 (s, 2H, $OC\underline{H_2}Ph$), 5.67 (d, J = 7.5 Hz, 1H, NH), 6.09 (s, 1H, HC=), 7.3-7.5 (m, 8H, Ar), 7.86 (d, J = 7 Hz, 2H, Ar); ^{13}C NMR δ 13.9, 22.4, 29.4, 32.2, 52.9, 53.4, 67.3, 68.1, 97.1, 127.7, 128.1, 128.3, 128.4, 128.6, 132.0, 135.9, 140.1, 155.7, 169.6, 176.4, 189.8; IR ν_{max} ($CHCl_3$) 3450, 2950, 1750, 1725, 1660, 1600, 1590, 1510, 1460, 1380, 1350, 1310, 1180, 1110, 1070, 910 cm^{-1}; MS (FAB) m/z 340 (MH^+, 30),

382 (10), 332 (12), 320 (10), 154 (10), 105 (20), 95 (20), 91 (100), 69 (20), 57 (20), 54 (30); Exact Mass Calcd for $C_{25}H_{29}NO_6$ 483.19949. Found 439.20043.

1-Phenyl-3-(*N*-carbobenzyloxy-*O*-methyl lysinyl) heptenone 39



The reaction between the alkyne **26** and the protected lysine **33** was undertaken using Procedure A. Flash chromatography (25% EtOAc / 75% hexane) produced 63% (51 mg) of **39**. ^{1}H NMR δ 0.95 (t, J = 7 Hz, 3H, CH_3), 1.4-1.7 (m, 10H, 5xCH_2), 2.30 (t, J = 7.5 Hz, 2H, CH_2C=), 3.30 (m, 2H, CH_2N), 3.74 (s, 3H, OCH_3), 4.45 (m, 1H, α-CH), 5.10 (s, 2H, OCH_2Ph), 5.51 (d, J = 8 Hz, 1H, NH), 5.66 (s, 1H, HC=), 7.37 (m, 8H, Ar), 7.85 (m, 2H, Ar), 11.56 (br s, 1H, enamine NH); ^{13}C NMR δ 13.8, 22.6, 29.6, 30.2, 32.2, 42.5, 52.4, 53.7, 67.0, 85.0, 91.0, 126.9, 128.1, 128.3, 128.5, 128.6, 130.3, 137.1, 136.3, 140.6, 168.9, 172.8, 187.7; IR ν_{max} ($CDCl_3$) 3440, 2960, 2865, 1730, 1600, 1550, 1520, 1460, 1340, 1290, 1210, 1100, 1060, 930, 870, 790, 700 cm^{-1}; UV λ_{max} (CH_2Cl_2) 246 (15530), 348 (28215); MS (EI) m/z 480 ($M^{+\cdot}$, 60), 463 (15), 451 (15), 438 (75), 333 (20), 258 (20), 216 (100), 202 (25), 132 (70); Anal. Calcd for $C_{28}H_{35}N_2O_5$: C, 69.97; H, 7.55; N, 5.83. Found C, 69.83; H, 7.52; N, 5.93.

Ethyl 3-(*N*-carbobenzyloxy-*O*-methyl cystinyl) propenoates 40*E* and 40*Z*

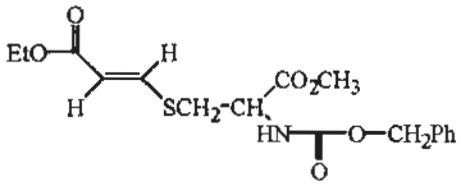
The addition reaction between the non terminal conjugated alkynyl ester **27** and the cysteine derivative **31** was undertaken at 0°C following Procedure A. Acid workup, followed by

purification of the crude material by flash chromatography (30% EtOAc / 70% hexane) yielded 51 mg, (75%) of the expected addition products in a ratio of 4:1 in favour of the *E* alkene.

40*E* / 40*Z*

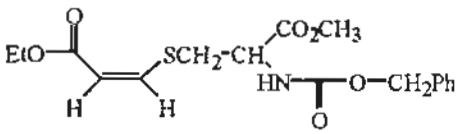
MS (EI) m/z 367 ($M^{+\cdot}$), 216 (90), 91 (M-CH_2Ph, 100); Anal. Calcd for $C_{17}H_{21}NO_6S$: C, 55.56; H, 45.76; N, 3.81. Found C, 55.45; H, 5.61; N, 4.21.

40*E*



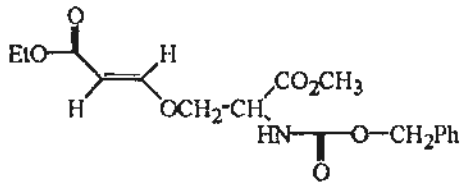
^{1}H NMR δ 1.29 (t, *J* = 7 Hz, 3H, CH_3), 3.35 (m, 2H, CH_2S), 3.80 (s, 3H, OCH_3), 4.18 (q, *J* = 7 Hz, 2H, CH_2O), 4.72 (m, 1H, α-CH), 5.15 (s, 2H, $OC\underline{H}_2Ph$), 5.66 (br d, *J* = 8 Hz, 1H, NH), 5.87 (d, *J* = 15 Hz, 1H, HC=), 7.36 (s, 5H, Ph), 7.57 (d, *J* = 15 Hz, 1H, HC=); ^{13}C NMR δ 14.4, 36.2, 53.0, 53.5, 60.5, 67.5, 115.8, 128.2, 128.4, 128.7, 136.0, 145.5, 155.4, 165.2, 170.3; IR ν_{max} ($CDCl_3$) 3450, 2950, 1720, 1580, 1510, 1440, 1340, 1300, 1260, 1210, 1180, 930, 790 cm^{-1}.

40*Z*



^{1}H NMR δ 1.28 (t, *J* = 7 Hz, 3H, CH_3), 3.29 (m, 2H, CH_2S), 3.79 (s, 3H, OCH_3), 4.18 (q, *J* = 7 Hz, 2H, OCH_2), 4.70 (m, 1H, α-CH), 5.11 (m, 2H, $OC\underline{H}_2Ph$), 5.65 (br d, *J* = 7.5 Hz, 1H, NH), 5.78 (d, *J* = 10 Hz, 1H, HC=), 6.92 (d, *J* = 10 Hz, 1H, HC=), 7.37 (s, 5H, Ph); ^{13}C NMR δ 14.6, 35.3, 52.6, 53.7, 60.6, 67.6, 116.0, 128.4, 128.5, 128.8, 136.2, 145.7, 155.8, 165.2, 170.4; IR ν_{max} ($CDCl_3$) 3450, 2950, 1720, 1600, 1500, 1260, 930, 780 cm^{-1}.

Ethyl 3-(*N*-carbobenzyloxy-*O*-methyl serinyl) propenoate 41



The addition of the alkyne **27** and the protected serine **32** was undertaken at 0°C using Procedure A. Acidic workup was followed by purification of the crude material by flash chromatography on silica (30% EtOAc / 70% hexane) to yield 39 mg, (75%) of **41**. ^{1}H NMR δ 1.27 (t, J = 7 Hz, 3H, CH_3), 3.79 (s, 3H, OCH_3), 4.2 (m, 4H, 2xOCH_2), 4.68 (m, 1H, α-CH), 5.15 (s, 2H, $OC\underline{H}_2Ph$), 5.22 (d, J = 12.7 Hz, 1H, HC=), 5.68 (br d, J = 8 Hz, 1H, NH), 7.37 (s, 5H, Ph), 7.51 (d, J = 12.7 Hz, 1H, HC=); ^{13}C NMR δ 14.5, 53.1, 53.7, 60.1, 67.5, 70.6, 98.0, 126.3, 128.5, 128.7, 136.1, 155.9, 161.3, 167.3, 169.6; IR ν_{max} ($CDCl_3$) 3420, 3040, 2975, 1710, 1620, 1500, 1430, 1420, 1270, 1240, 1200, 1140, 1050, 880, 760, 670 cm^{-1}; MS (EI) m/z 337 (M^+, 3), 260 (10), 198 (10), 90 (20), 91 (100), 42 (50), 41 (70), 39 (35); Anal. Calcd for $C_{17}H_{21}NO_7$ C, 60.52; H, 6.28; N, 4.15. Found C, 57.80; H, 6.39; N, 3.83.

Ethyl 3-methoxy propenoates 42*E* and 42*Z*

Triethylamine (6 drops, catalytic) was added to a solution of ethyl propiolate **27** (0.25 ml, 2.5 mmol) in methanol (10 ml). After stirring for 30 minutes the solvent was removed and the resulting oil purified by flash chromatography (10% EtOAc / 90% hexane) to yield the two alkenes in a combined yield of 58%, (0.17 g) in a ratio of 4.5:1 in favour of the *E* alkene.

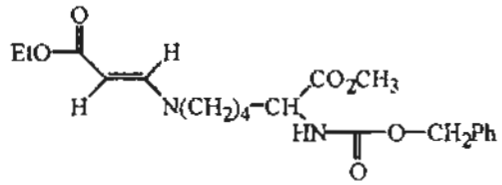
42*E*

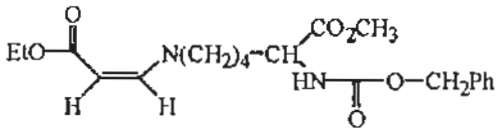
^{1}H NMR δ 3.89 (s, 3H, OCH_3), 5.22 (d, J = 12.6 Hz, 1H, =CH), 7.66 (d, J = 12.6 Hz, 1H, =CH).

42*Z*

^{1}H NMR δ 3.89 (s, 3H, OCH_3), 4.86 (d, J = 7 Hz, 1H, =CH), 6.48 (d J = 7 Hz, 1H, =CH).

Ethyl 3-(*N*-carbobenzyloxy-*O*-methyl lysinyl) propenoates 43*E* and 43*Z*





The addition reaction between ethyl propiolate **27** and the lysine derivative **33** was undertaken at room temperature after the initial removal of the hydrochloride salt by washing the protected lysine with 10% sodium bicarbonate solution. Purification of the crude material by flash chromatography (50% EtOAc / 50% hexane) yielded 45% of the two isomers as an inseparable mixture in a 1:1 ratio. ^{1}H NMR δ 1.2 (m, 3H, CH_3), 1.4-1.9 (m, 6H, 3xCH_2), 2.98 (m, 2H, CH_2N (*E*)), 3.10 (m, 2H, CH_2N (*Z*)), 3.71 (s, 3H, OCH_3), 3.72 (s, 2H, OCH_3), 4.09 (2x q, J = 7 Hz, 4H, 2xOCH_2), 4.40 (m, 1H, α-CH), 4.42 (d, J = 8 Hz, 1H, HC= (*Z*)), 4.55 (m, 1H, α-CH), 4.67 (d, J = 13 Hz, 1H, HC= (*E*)), 5.08 (s, 4H, 2xOCH_2Ph), 5.31 (t, J = 8 Hz, 1H, NH), 6.55 (dd, J = 8, 13 Hz, 1H, HC= (*Z*)), 7.32 (s, 5H, Ph), 7.33 (s, 5H, Ph), 7.44 (dd, J = 8, 13 Hz, 1H, HC= (*E*)), 7.8 (m, 1H, NH enamine); IR ν_{max} ($CHCl_3$) 3425, 3025, 2950, 1720, 1650, 1600, 1500, 1350, 1200, 1150, 1040 cm^{-1}; MS (FAB) m/z 393 (MH^+, 73), 347 (100), 306 (16), 295 (20), 232 (12); Exact mass calcd for $C_{20}H_{29}N_2O_6$ (MH^+) 393.20256. Found 393.20220.

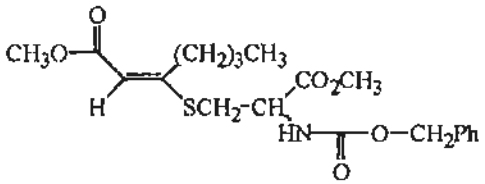
Methyl 3-(*N*-carbobenzyloxy-*O*-methyl cystinyl) heptenoates 45*E* and 45*Z*

The addition reaction between the conjugated methyl ester **28** and the cysteine derivative **31** was undertaken at room temperature following Procedure A. Purification of the crude material by flash chromatography (20% EtOAc / 80% hexane) yielded 40% of the expected addition products **45*E*** and **45*Z*** in a ratio of 2:1.

45*E* / 45*Z*

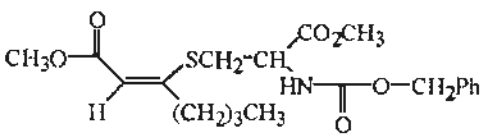
MS (FAB) m/z 410 ($M^{+\cdot}$), 378 (53), 270 (28), 107 (40), 91 (100); Anal. Calcd for $C_{20}H_{27}NO_6S$: C, 58.66; H, 6.65; N, 3.42. Found C, 58.59; H, 6.64; N, 3.36.

45*E*



Mp 86.5-88.5°C; ^{1}H NMR δ 0.91 (t, J = 7 Hz, 3H, CH_3), 1.38 (m, 2H, CH_2), 1.57 (m, 2H, CH_2), 2.77 (t, J = 7 Hz, 2H, CH_2), 3.24 (m, 2H, CH_2S), 3.66 (s, 3H, OCH_3), 3.77 (s, 3H, OCH_3), 4.71 (m, 1H, α-CH), 5.12 (s, 2H, OCH_2Ph), 5.57 (s, 1H, HC=), 5.64 (d, J = 7.5 Hz, 1H, NH), 7.34 (s, 5H, Ph); ^{13}C NMR δ 13.9, 22.6, 32.0, 33.5, 34.0, 51.0, 52.7, 52.9, 67.3, 108.8, 128.1, 128.3, 128.6, 136.1, 155.7, 162.7, 165.0, 170.5; IR ν_{max} ($CHCl_3$) 3425, 3030, 2950, 1750, 1715, 1600, 1500, 1425, 1340, 1215, 1175, 1125, 1060, 1000 cm^{-1}.

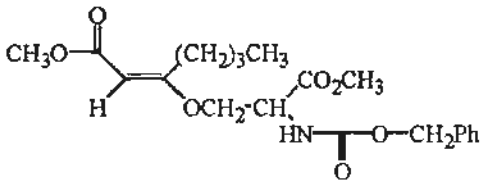
45*Z*



^{1}H NMR δ 0.92 (t, J = 7 Hz, 3H, CH_3), 1.34 (m, 2H, CH_2), 1.52 (m, 2H, CH_2), 2.39 (t, J = 7 Hz, 2H, CH_2), 3.34 (m, 2H, CH_2S), 3.70 (s, 3H, OCH_3), 3.77 (s, 3H, OCH_3), 4.67 (m,

1H, α-CH), 5.11 (s, 2H, OC<u>H</u>$_2$Ph), 5.64 (d, J = 8 Hz, 1H, NH), 5.83 (s, 1H, HC=), 7.34 (s, 5H, Ph); ^{13}C NMR δ 13.8, 22.0, 31.2, 32.3, 36.2, 51.1, 52.9, 53.8, 67.2, 113.5, 128.1, 128.2, 128.5, 136.0, 155.6, 159.4, 166.3, 170.3; IR ν_{max} ($CHCl_3$) 3410, 3025, 2950, 2870, 1720, 1580, 1505, 1450, 1435, 1340, 1320, 1210, 1185, 1060, 1020, 905, 830 cm^{-1}.

Methyl 3-(*N*-carbobenzyloxy-*O*-methyl serinyl) heptenoate 46



The addition of the alkynyl ester **28** and the serine derivative **32** was undertaken following Procedure B. Purification of the crude material by flash chromatography on silica (25% EtOAc / 75% hexane) yielded 14 mg, (35%) of **46**. ^{1}H NMR δ 0.90 (t, J = 7 Hz, 3H, CH_3), 1.31 (m, 2H, CH_2), 1.44 (m, 2H, CH_2), 2.57 (m, 1H, CHC=), 2.86 (m, 1H, CHC=), 3.67 (s, 3H, OCH_3), 3.77 (s, 3H, OCH_3), 4.09 (m, 2H, OCH_2), 4.68 (m, 1H, α-CH), 4.97 (s, 1H, HC=), 5.14 (s, 2H, OC<u>H</u>$_2$Ph), 5.63 (d, J = 8 Hz, 1H, NH), 7.36 (s, 5H, Ph); ^{13}C NMR δ 13.8, 22.2, 29.4, 31.2, 50.9. 52.8, 53.3, 67.3, 67.9, 91.6, 128.2, 128.3, 128.6, 135.9, 155.7, 167.5, 169.7, 175.1; IR ν_{max} ($CHCl_3$) 3430, 2940, 1750, 1710, 1620, 1500, 1455, 1435, 1370, 1350, 1300, 1140, 1110, 1050, 905 cm^{-1}; MS (FAB) m/z 394 (MH^+, 20), 362 (20), 308 (10), 236 (20), 154 (15), 136 (15), 107 (12), 91 (75), 77 (43), 69 (40), 57 (100), 55 (45); Anal. Calcd for $C_{20}H_{27}NO_7$: C, 61.05; H, 6.92; N, 3.56. Found C, 60.84; H, 7.12; N, 3.43.

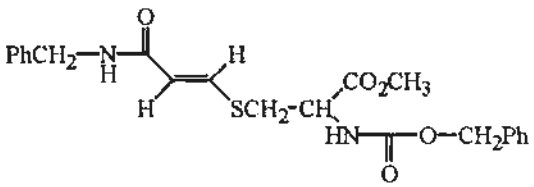
_N_-Benzyl-3-(_N_-carbobenzyloxy-_O_-methyl cystinyl) propenamides 47*E* and 47*Z*

The addition reaction of the protected cysteine **31** to the alkynyl amide **29** was undertaken at room temperature by Procedure A. Purification of the crude material by flash chromatography (50% EtOAc / 50% hexane) yielded 47 mg, (58%) of the two isomers **47*E*** and **47*Z*** in a ratio of 1.4:1.

47E / 47Z

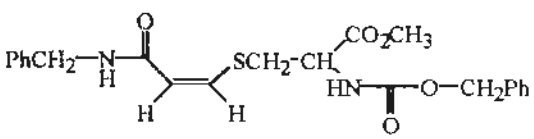
MS (EI) m/z 428 ($M^{+\cdot}$), 337 (15), 206 (50), 192 (60), 160 (90), 106 (100); Anal. Calcd for $C_{22}H_{24}N_2O_5S$: C, 61.66; H, 5.64; N, 6.54. Found C, 61.90; H, 5.69; N, 6.26.

47E



Mp 134-137°C; ^{1}H NMR δ 3.27 (m, 2H, CH_2S), 3.77 (s, 3H, OCH_3), 4.47 (d, J = 6 Hz, 2H, CH_2N), 4.67 (m, 1H, α-CH), 5.12, 5.08 (AB pattern, J = 12 Hz, 2H, $OC\underline{H}_2Ph$), 5.65 (br d, J = 7.5 Hz, 1H, NH), 5.73 (m, 1H, NH), 5.87 (d, J = 15 Hz, 1H, HC=), 7.25-7.35 (m, 5H, Ph), 7.49 (d, J = 15 Hz, 1H, HC=); ^{13}C NMR δ 35.2, 43.7, 53.0, 53.3, 67.3, 118.0, 127.5, 127.8, 128.1, 128.3, 128.6, 128.7, 138.1, 141.5, 155.9, 164.3, 170.6; IR ν_{max} ($CDCl_3$) 3675, 3440, 3045, 2980, 1730, 1660, 1590, 1510, 1420, 1270, 1240, 890, 760, 740, 670 cm^{-1}.

47Z



Mp 109-112°C; ^{1}H NMR δ 3.23 (m, 2H, CH_2S), 3.76 (s, 3H, OCH_3), 4.48 (d, J = 6 Hz, 2H, CH_2N), 4.67 (m, 1H, α-CH), 5.11 (s, 2H, $OC\underline{H}_2Ph$), 5.6 (m, 2H, 2xNH), 5.70 (d, J =

10 Hz, 1H, HC=), 6.73 (d, J = 10 Hz, 1H, HC=), 7.2-7.4 (m, 5H, Ph); ^{13}C NMR δ 38.6, 43.5, 52.9, 54.3, 67.1, 115.9, 127.55, 127.6, 127.9, 128.0, 128.1, 128.2, 128.5, 128.7, 136.1, 136.9, 138.2, 145.1, 155.6, 165.8, 170.3; IR ν_{max} ($CDCl_3$) 3430, 3140, 2950, 1710, 1640, 1570, 1500, 1460, 1370, 1240, 1210, 1170, 1090, 925, 860, 760, 650 cm^{-1}.

_N_-Carbobenzyloxy dehydroalanine methyl ester 48

_N_-Carbobenzyloxy dehydroalanine methyl ester[123, 124] **48** was formed as a by product from the reaction of triethylamine with either _N_-Cbz cysteine methyl ester **31** or _N_-Cbz serine methyl ester **32**, in the attempted reactions of these amino acids with _N_-benzyl-2-heptynamide **30** and also in the attempted reaction of **32** with _N_-benzyl-2-propynamide **29**. ^{1}H NMR δ 3.84 (s, 3H, OCH_3), 5.17 (s, 2H, $OC\underline{H}_2Ph$), 5.80 (d, J = 1.4 Hz, 1H, HC=), 6.26 (s, 1H, HC=), 7.38 (s, 5H, Ph).

Experimental Described in Chapter 3

Diacetylfluorescein 60

Method A:

Triethylamine (1.0 ml, 7.5 mmol) was added to a solution of acetylchloride (0.40 ml, 6.0 mmol) and fluorescein (1.0g, 3.0 mmol) in acetone (50 ml). The mixture was stirred for 20 minutes, at which time tlc analysis showed the reaction to be complete. The solvent was removed under reduced pressure and the solid residue purified by chromatography (5% methanol / 95% dichloromethane) to yield 1.12 g (83%) of the title compound as a white solid.

Method B:

A mixture of fluorescein (1.0 g, 3.0 mmol), acetylchloride (0.40 ml, 6.0 mmol) and silver oxide (0.035g, 0.15 mmol) in a THF (40 ml) / benzene (120 ml) solvent system was refluxed for two hours. After this time the solvent was removed and the residue purified by flash chromatography (as above) to yield 1.16 g (80%) of diacetylfluorescein. Mp 202-203°C, (lit. Mp 205°C)[125]; ^{1}H NMR δ 2.32 (s, 6H, 2xCH_3), 6.82 (s, 4H, Ar), 7.10 (s, 2H, Ar), 7.21 (m, 1H, Ar), 7.66 (m, 2H, Ar), 8.04 (m, 1H, Ar); IR ν_{max} ($CDCl_3$) 3150, 2950, 1760, 1610, 1590, 1495, 1420, 1370, 1220, 1210, 1150, 1110, 1010, 930, 870, 760, 660 cm^{-1}.

O-[2′-(Tetrahydro-2*H*-pyranyl)ethyl] fluorescein 2′-(tetrahydro-2*H*-pyranyl)ethyl ester 65

2-(2′-Bromoethoxy)tetrahydro-2*H*-pyran **64** (1.40g, 6.62 mmol) was added to fluorescein (1.0g, 3.01 mmol) and potassium carbonate (0.21g, 1.51 mmol) in DMF (50 ml) and the solution heated to 60°C for 12 hours. After this period the mixture was cooled and water (150 ml) was added. The solution was extracted with ethyl acetate (5x30 ml) and the combined organic layers washed with water (4x30 ml) and brine (30 ml). Drying, removal of the solvent and flash chromatography (5% EtOAc / 95% hexane) on the residue yielded the THP protected alcohol **65**, (1.61g, 91%). ^{1}H NMR δ 1.45-1.75 (m, 12H, 12xCH_2), 3.4-3.6 (m, 3H, 3xCH-O), 3.70 (m, 2H, CH_2O), 3.87 (m, 2H, CH_2O), 4.1-4.3 (m, 5H, CH-O), 4.43 (m, 1H, OCHO), 4.71 (m, 1H, OCHO), 6.45 (d, J = 2 Hz, 1H, Ar), 6.54 (dd, J = 2, 10 Hz, 1H, Ar), 6.7-6.9 (m, 2H, Ar), 7.0 (d, J = 2 Hz, 1H, Ar), 7.31 (d, J = 7 Hz, 1H, Ar), 7.68-7.75 (m, 2H, Ar), 8.01 (s, 1H, Ar), 8.29 (dd, J = 1, 7.5 Hz, 1H, Ar).

O-(2′-Hydroxy)ethyl fluorescein (2′′-hydroxy)ethyl ester 63

The tetrahydropyranyl protected alcohol **65** was dissolved in ethanol (100 ml) with PPTS (50 mg, 0.2 mmol) and warmed to 50°C for 3 hours. Removal of the solvent and flash chromatography (10% methanol / 90% dichloromethane) produced the title compound in 63% (0.72g) yield from **65**. ^{1}H NMR δ (d^6-DMSO) 3.76 (m, 2H, 2xCH-O), 3.97 (t, J = 5 Hz, 2H, 2xCH-O), 4.16 (m, 2H, 2xCH-O), 4.73 (t, J = 5.5 Hz, 1H, CH-O), 4.97 (t, J = 5 Hz, 1H, CH-O), 6.23 (d, J = 1.5 Hz, 1H, Ar), 6.38 (d, J = 9.5 Hz, 1H, Ar), 6.86 (m, 3H, Ar), 7.22 (d, J = 1.8 Hz, 1H, Ar), 7.50 (d, J = 7 Hz, 1H, Ar), 7.80 (m, 2H, Ar), 8.26 (d, J = 7 Hz, 1H, Ar); ^{13}C NMR δ (d^6-DMSO) 58.6, 59.3, 66.6, 70.7, 100.9, 104.5, 113.9, 114.3,

116.7., 128.9, 129.3, 129.8, 129.9, 130.4, 130.6, 130.7, 133.1, 133.8, 150.1, 153.6, 158.4, 163.4, 164.9, 183.9; IR ν_{max} ($CDCl_3$) 3300(br), 2950, 2920, 2850, 1715, 1600, 1500, 1460, 1415, 1375, 1345, 1270, 1255, 1220, 1110, 1070, 1050 cm^{-1}; UV λ_{max} (EtOH) 204 (50590), 233 (70490), 256 (29430), 439 (35315), 460 (49330), 488 (40640) nm; MS (EI) m/z 420 ($M^{+\cdot}$, 50), 377 (10), 333 (15), 305 (15), 287 (20), 259 (20), 203 (12), 105 (100); Exact Mass (EI) Calcd for $C_{24}H_{20}O_7$ 420.12090. Found 420.12212.

2-(2′-Bromoethoxy)tetrahydro-2*H*-pyran 64 [126]

Dihydropyran (29.0 ml, 0.32 mol) was added to 2-bromoethanol (15 ml, 0.22 mol) in dichloromethane (125 ml) with a catalytic amount of PPTS (0.25 g, 1.0 mmol), and the mixture was stirred overnight. The solution was washed with brine (2x50 ml), dried, the solvent removed and the resulting oil distilled, (Bp. 45-46°C at 0.06 mmHg). The title compound was recovered as a colourless oil in 81% yield (35.8g). ^{1}H NMR δ 1.5-1.95 (m, 6H, 3xCH_2), 3.5 (m, 3H, CH_2Br and 1H of CH_2O), 3.7-4.1 (m, 3H, O$\underline{CH_2}CH_2$Br and 1H of CH_2O), 4.68 (t, J = 3 Hz, 1H, OCHO); ^{13}C NMR δ 19.2, 25.3, 30.4, 30.8, 62.2, 67.5, 98.9; IR ν_{max} ($CDCl_3$) 3025, 2950, 2870, 2850, 1450, 1435, 1350, 1260, 1230, 1130, 1080, 1035, 980, 905, 870, 810 cm^{-1}.

Fluorescein methyl ester 66 [101]

Fluorescein methyl ester **66** was prepared by heating fluorescein (5.0g, 15.0 mmol) in methanol (150 ml) with concentrated sulfuric acid (5 ml) to reflux for 72 hours. The solution was then allowed to cool and the solid that precipitated was collected and dissolved in ethyl acetate (200 ml). The ethyl acetate was washed with 10% sodium bicarbonate solution (100 ml), the

organic layer dried and the solvent removed under vacuum. The resulting solid was recrystallised from methanol. The yield of this procedure was extremely variable with yields from 0.8 g (15%) up to 4.97g (95%). Mp 273-275°C. (Lit. Mp 274-5°C).

_O_-(2′-Hydroxy)ethyl fluorescein methyl ester 68

Potassium carbonate (0.60g, 4.3 mmol) was added to a solution of fluorescein methyl ester (1.0 g, 2.9 mmol) and 2-bromoethanol (0.41 ml, 5.8 mmol) in dimethylformamide (40 ml) and the mixture heated for 12 hours at 80°C. The solution was then allowed to cool followed by filtration and removal of the solvent and excess 2-bromoethanol under high vacuum. Purification of the resulting material by flash chromatography (7.5% methanol / 92.5% dichloromethane) gave the expected product as an orange solid in 52% yield (0.59 g). Mp 205-209°C; ^{1}H NMR δ (d^6-DMSO) 3.62 (s, 3H, OCH_3), 3.85 (m, 2H, CH_2O), 4.18 (t, J = 4.5 Hz, 2H, OCH_2), 4.96 (t, J = 5 Hz, 1H, OH), 6.29 (d, J = 1.5 Hz, 1H, CH), 6.40 (d, J = 11 Hz, 1H, Ar), 6.85 (m, 3H, Ar), 7.10 (d, J = 2 Hz, 1H, Ar), 7.38 (d, J = 7 Hz, 1H, Ar), 7.77 (m, 2H, Ar), 8.24 (d, J = 8 Hz, 1H, Ar); ^{13}C NMR δ (d^6-DMSO) 52.3, 59.3, 70.7, 101.0, 104.5, 113.9, 114.2, 116.6, 128.8, 129.3, 129.5, 130.0, 130.3, 130.7, 133.1, 133.8, 150.1, 153.5, 158.3, 163.4, 165.2, 183.8; IR ν_{max} ($CDCl_3$) 3300(br), 2950, 1720, 1635, 1595, 1440, 1335, 1305, 1295, 1285, 1230, 1190, 1125, 1085, 800, 670 cm^{-1}; UV λ_{max} (EtOH) 205 (108525), 232 (84710), 257 (36500), 441 (36695), 460 (49185), 488 (39430) nm; MS (EI) m/z 390 ($M^{+\cdot}$, 25), 129 (17), 97 (22), 81 (30), 83 (30), 73 (30), 69 (66), 57 (51), 55 (72), 43 (83), 41 (100); Exact Mass (EI) Calcd for $C_{23}H_{18}O_6$ 390.11033. Found 390.11126.

7-(2′-Hydroxy)ethoxy coumarin 69

A mixture of potassium carbonate (1.3 g, 9.3 mmol), 2-(2′-bromoethoxy)tetrahydro-2*H*-pyran **64** (2.6 g, 12 mmol) and 7-hydroxycoumarin (1.0 g, 6.2 mmol) was heated to 60°C overnight in DMF (50 ml). After cooling to room temperature water (150 ml) was added to the solution, followed by extraction with ethyl acetate (4x30 ml). The organic layer was washed with water (3x30 ml) and brine (30 ml), dried and the solvent removed on a rotary evaporator. Flash chromatography (30% EtOAc / hexane) gave the THP protected derivative of **69** as a white solid (1.78 g, 99%). ^{1}H NMR δ 1.5-1.8 (m, 6H, 3xCH_2), 3.58 (m, 1H, CH-O), 3.87 (m, 2H, OCH_2), 4.1-4.3 (m, 3H, 3xCH-O), 4.72 (m, 2H, OCHO), 6.26 (d, J = 9.5 Hz, 1H, =CH), 6.87 (m, 2H, Ar), 7.39 (d, J = 8.5 Hz, 1H, Ar), 7.65 (d, J = 9.5 Hz, 1H, =CH).

The tetrahydropyranyl derivative of **69** (1.78 g, 6.1 mmol) was dissolved in dichloromethane (10 ml) and added to a solution of PPTS (50 mg, 0.2 mmol) in ethanol (30ml). The mixture heated at 50°C until tlc analysis showed no starting material remaining. At this time the solvent was removed under vacuum and the resulting material purified by flash chromatography (70% EtOAc / 30% hexane) to yield the title compound as a white solid in quantitative yield (1.26 g).

Mp 92.5-93.5°C; ^{1}H NMR δ 4.02 (m, 2H, OCH_2), 4.15 (m, 2H, OCH_2), 6.25 (d, J = 9.5 Hz, 1H, =CH), 6.85 (m, 2H, Ar), 7.35 (d, J = 8.5 Hz, 1H, Ar), 7.64 (d, J = 9.5 Hz, 1H, =CH); ^{13}C NMR δ 58.3, 68.6, 99.8, 110.9, 111.0, 111.1, 127.6, 142.4, 154.0, 159.0. 160.6; IR ν_{max} ($CDCl_3$) 3600, 3430, 3010, 2940, 2875, 1710, 1620, 1560, 1510, 1405, 1350, 1295, 1280, 1230, 1200, 1125, 1080, 1040, 840, 670 cm^{-1}; UV λ_{max} (CH_2Cl_2) 232 (23801), 323 (21476)

nm; MS (EI) m/z 206 ($M^{+\cdot}$, 50), 162 (40), 134 (100), 105 (15), 89 (15), 69 (12), 43 (8); Exact Mass Calcd for $C_{11}H_{10}O_4$ 206.05791. Found 206.05872.

7-(2′-Hydroxy)ethoxy Sudan 1 70

Sudan 1 **58** (1.0 g, 4.0 mmol), 2-(2′-bromoethoxy)tetrahydro-2*H*-pyran **64** (1.7 g, 8.0 mmol) and potassium carbonate (1.1 g, 8.0 mmol) were heated to 80°C in DMF (50 ml) for twelve hours. After cooling, water (150ml) was added and the solution extracted with ether (4x40 ml). The organic layer was washed with water (3x30 ml), dried and the solvent removed. Flash chromatography (20% EtOAc / 80% hexane) on the crude material yielded 1.51 g (99.5%) of the THP protected derivative of the title compound as a red oil. ^{1}H NMR δ 1.3-1.8 (m, 6H, 3xCH_2), 3.38 (m, 1H, CH-O), 3.75 (m, 2H, 2xCH-O), 4.00 (m, 1H, CH-O), 4.31 (t, J = 5 Hz, 2H, OCH_2), 4.62 (m, 1H, OCHO), 6.9-7.1 (m, 1H, Ar), 7.35-7.55 (m, 5H, Ar), 7.80 (m, 2H, Ar), 8.01 (2x d, J = 8, 8 Hz, 2H, Ar), 8.32 (d, J = 7 Hz, 1H, Ar).

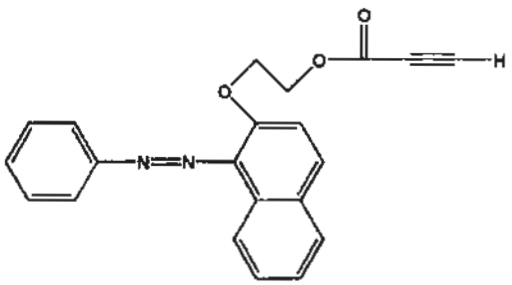
PPTS (50 mg 0.2 mmol) was added to a solution of the THP protected alcohol (1.51 g, 4.0 mmol) in ethanol (30 ml) with dichloromethane (10 ml) to solubilise the protected alcohol. The solution was heated at 50°C for 5 hours before cooling and removal of the solvent under vacuum. Purification by chromatography (40% EtOAc / 60% hexane) gave the expected alcohol in 99% yield (1.17 g) as a red oil. ^{1}H NMR δ 3.88 (m, 2H, CH_2O), 4.35 (m, 2H, CH_2O), 7.35-7.6 (m, 6H, Ar), 7.84 (m, 2H, Ar), 8.01 (m, 2H, Ar), 8.45 (d, J = 8 Hz, 1H, Ar); ^{13}C NMR δ 60.9, 72.7, 117.5, 122.5, 123.1, 124.9, 127.5, 127.8, 129.2, 129.6, 130.4,

131.2, 131.5, 136.6, 145.8, 153.1; IR ν_{max} ($CHCl_3$) 3400, 3015, 2950, 2840, 1620, 1600, 1590, 1500, 1450, 1430, 1340, 1280, 1245, 1240, 1150, 1090, 1070, 1040, 1020, 810, 690 cm^{-1}; UV λ_{max} (CH_2Cl_2) 236 (30695), 282 (11985), 378 (7855) nm; MS (EI) m/z 292 (M^+, 22), 248 (17), 246 (13), 170(13), 159 (17), 143 (20), 136 (25), 115 (59), 106 (40), 103 (68), 85 (100); Exact Mass Calcd for $C_{18}H_{16}N_2O_2$ 292.12177. Found 292.12074.

2´-Aminoethyl dansylsulfonamide 71

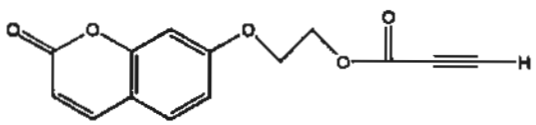
A solution of dansyl chloride (1.0 g, 3.7 mmol) in dichloromethane (10 ml) was slowly added to ethylene diamine (0.99 ml, 1.5 mmol) in dichloromethane (50 ml). After stirring for 30 minutes the solvent and the excess ethylene diamine were removed to yield the title compound as a pale green solid along with a small amount of the dimer. Further purification was not carried out as this was mixture added directly to further reactions. ^1H NMR δ 2.89 (s, 6H, 2xCH_3), 3.03 (m, 2H, CH_2), 3.19 (m, 2H, CH_2), 7.19 (d, J = 7 Hz, 1H, CH), 7.55 (m, 2H, 2xCH), 8.17 (d, J = 7 Hz, 1H, CH), 8.34 (d, J = 8.5 Hz, 1H, CH), 8.53 (d, J = 8.5 Hz, 1H, CH); MS (EI) m/z 293 (M^+, 52), 235 (100), 171 (60), 170 (80), 168 (28), 154 (20), 127 (18).

Sudan1 terminal alkynyl ester 73



DCC (0.72 g, 3.5 mmol) and 4-dimethylaminopyridine (50 mg, 0.4 mmol) were added to a solution of the alcohol **70** (1.0 g, 3.4 mmol) and propiolic acid **72** (0.22 ml, 3.5 mmol) in ether (50 ml) at -20°C. After stirring at room temperature overnight the reaction was filtered, the solvent was removed and the resultant oil chromatographed (30% EtOAc / 70% hexane) to yield 0.86 g (75%) of the title compound as a red oil. ^{1}H NMR δ 2.86 (s, 1H, ≡-H), 4.24 (m, 2H, CH_2O), 4.41 (m, 2H, CH_2O), 7.22 (d, J = 9 Hz, 1H, Ar), 7.35-7.53 (m, 5H, Ar), 7.30 (m, 2H, Ar), 8.00 (d, J = 7 Hz, 2H, Ar), 8.36 (d, J = 8 Hz, 1H, Ar); ^{13}C NMR δ 64.4, 69.1, 74.2, 117.8, 122.5, 122.7, 123.4, 125.0, 127.5, 127.8, 128.6, 128.9, 129.1, 129.9, 130.7, 131.0, 138.0, 146.6, 152.4, 153.3; IR ν_{max} ($CHCl_3$) 3300, 3030, 2125, 1720, 1600, 1515, 1460, 1230, 1090 cm^{-1}; UV λ_{max} (CH_2Cl_2) 236 (30155), 278 (13495), 372 (8155), 466 (1600) nm; MS (FAB) m/z 345 (MH^+, 6), 154 (10), 136 (6), 109 (13), 107 (14), 97 (53), 83 (38), 81 (40), 69 (75), 57 (87), 55 (100); Exact Mass Calcd for $C_{21}H_{16}N_2O_3$ 344.11609. Found 344.11566.

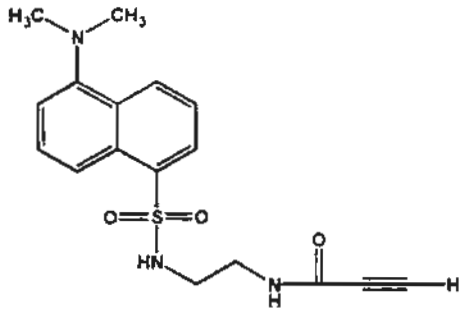
Coumarin terminal alkynyl ester 74



DCC (1.0 g, 4.9 mmol) and DMAP (25 mg, 0.2 mmol) were added slowly to a solution of the coumarin derivative **69** (1.0 g, 4.9 mmol) and propiolic acid **72** (0.30 ml, 4.9 mmol) in dichloromethane (50 ml) at -20°C. After stirring at room temperature overnight the solution was filtered, the solvent removed and the resultant material purified by chromatography (60% EtOAc / 40% hexane) to yield 0.39 g (31%) of the title compound as a white solid. Mp 84-86°C; ^{1}H NMR δ 2.96 (s, 1H, ≡-H), 4.28 (m, 2H, CH_2O), 4.58 (m, 2H, CH_2O), 6.27 (d, J = 9.5 Hz, 1H, =CH), 6.86 (m, 2H, Ar), 7.39 (d, J = 8.5 Hz, 1H, Ar), 7.65 (d, J = 9.5 Hz, 1H,

=CH); ^{13}C NMR δ 63.9, 65.9, 76.0, 101.7, 112.9, 113.0, 113.1, 113.5, 129.0, 143.4, 152.5, 155.8, 161.0, 161.4; IR ν_{max} ($CHCl_3$) 3300, 3030, 2125, 1730, 1615, 1510, 1410, 1280, 1230, 1160, 1140, 1130, 1000, 840 cm^{-1}; UV λ_{max} (CH_2Cl_2) 233 (7359), 319 (4519) nm; MS (EI) m/z 258 ($M^{+\cdot}$, 20), 134 (18), 97 (100), 89 (12), 69 (13), 53 (66), 43 (10); Exact Mass Calcd for $C_{14}H_{10}O_5$ 258.05282. Found 258.05247.

Dansyl terminal alkynyl amide 77



Propiolic acid **72** (0.24 ml, 3.8 mmol) was added slowly to lithium hydride (0.03 g, 4.0 mmol) and THF (50 ml) at ~5°C. After stirring for three hours the solution was cooled to -10°C and ethylchloroformate (0.38 ml, 4.0 mmol) added. The mixture was allowed to warm to room temperature and stirred for a further hour before the addition of the amine **71** (3.7 mmol, see above for preparation). The solution was stirred overnight, the solvent removed and the resulting oil chromatographed (60% EtOAc / 40% hexane) to yield 0.74 g (58%) of the conjugated alkynyl amide **77** as a pale green oil. ^{1}H NMR δ 2.81 (s, 1H, ≡-H), 2.82 (s, 6H, 2xCH_3), 3.05 (m, 2H, CH_2N), 3.33 (m, 2H, CH_2N), 6.40 (t, J = 6 Hz, 1H, NH), 7.10 (d, J = 8 Hz, 1H, Ar), 7.24 (t, J = 6 Hz, 1H, NH), 7.47 (m, 2H, Ar), 8.18 (dd, J = 1, 7 Hz, 1H, Ar), 8.27 (d, J = 8 Hz, 1H, Ar), 8.49 (d, J = 8 Hz, 1H, Ar); ^{13}C NMR δ 39.9, 42.4, 45.4, 74.6, 77.0, 115.4, 118.8, 123.3, 128.7, 129.4, 129.5, 129.9, 130.6, 134.5, 152.0, 153.2; IR ν_{max} ($CHCl_3$) 3430, 3390, 3300, 3025, 2950, 2870, 2835, 2790, 2115, 1660, 1575, 1515, 1480,

1455, 1355, 1230, 1160, 1145, 1090, 570 cm^{-1}; UV λ_{max} (CH_2Cl_2) 232 (19690), 257 (25075), 347 (9395) nm; MS (FAB) m/z 346 (MH^+, 4), 345 (5), 170 (12), 154 (10), 137 (10), 136 (12), 107 (18), 95 (32), 83 (32), 81 (41), 69 (80), 67 (41), 57 (74), 55 (100).

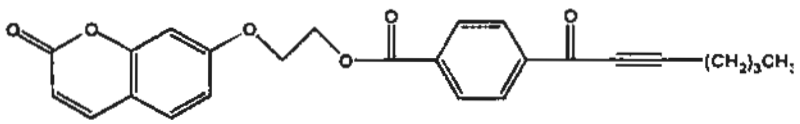
(4′-Carboxyphenyl)-1-hexynyl ketone 78

Butyl lithium (5.46 ml, 2.5 M, 13.7 mmol) was added slowly to a solution of 1-hexyne (1.54 ml, 13.3 mmol) and DMPU (1.6 ml, 13.3 mmol) in THF (50 ml) at -78°C. The solution was stirred for 20 minutes before the addition of 4-carboxy benzaldehyde **79** (1.0 g, 6.66 mmol). After stirring for 3 more hours the excess butyllithium was quenched by the careful addition of water (5 ml) and the THF removed under vacuum. The resulting aqueous solution was acidified with 10% sulfuric acid (as indicated by pH paper), followed by extraction with ethyl acetate (4x40 ml). The organic layer was washed with water (2x40 ml) and brine (40 ml) and then dried. After removal of the solvent the resulting yellow solid was chromatographed (1% AcOH / 30% EtOAc / 69 % hexane) to yield between 0.64 g and 0.96 g (40-60%) of the benzyl alcohol derivative as a white solid. ^1H NMR δ 0.91 (t, J = 7 Hz, 3H, CH_3), 1.38-1.57 (m, 2H, 2xCH_2), 2.28 (t, J = 7 Hz, 2H, CH_2), 5.52 (s, 1H, CH-O), 7.63 (d, J = 7.5 Hz, 2H, Ar), 8.11 (d, J = 7.5 Hz, 2H, Ar); IR ν_{max} ($CHCl_3$) 3250, 2950, 2900, 2850, 2650, 2535, 1690, 1600, 1570, 1500, 1455, 1420, 1370, 1310, 1285, 1115, 990, 860 cm^{-1}.

Jones reagent was added to a solution of the benzyl alcohol derivative (1.0 g, 4.3 mmol) in acetone (125 ml) at 0°C until the reaction remained orange in colour for more than 10 minutes. The solution was then filtered and the acetone removed. The solution was extracted with ethyl acetate (3x40 ml) and the organic layer washed with 10% sulfuric acid (40 ml) and saturated

ammonium chloride (40 ml). After removal of the ethyl acetate the resulting material was purified by flash chromatography (1% AcOH / 35% EtOAc / 64% hexane) to yield 0.79 g (80%) of the title compound as a white solid. Mp 146-149°C; ^{1}H NMR δ 0.98 (t, J = 7 Hz, 3H, CH_3), 1.51 (m, 2H, CH_2), 1.69 (m, 2H, CH_2), 2.54 (t, J = 7 Hz, 2H, CH_2), 8.21, 8.17 (AB pattern, J = 9 Hz, 4H, Ar); ^{13}C NMR δ 13.6, 19.0, 22.2, 29.8, 79.7, 98.6, 129.6, 130.7, 133.7, 140.7, 177.4; IR ν_{max} ($CHCl_3$) 2950, 2860, 2680, 2560, 2200, 1690, 1660, 1610, 1500, 1470, 1430, 1380, 1295, 1265, 1250, 1130, 1110, 920, 725 cm^{-1}; MS (FAB) m/z 231 (MH^+, 12), 185 (30), 149 (30), 105 (10), 93 (100), 75 (30), 57 (30).

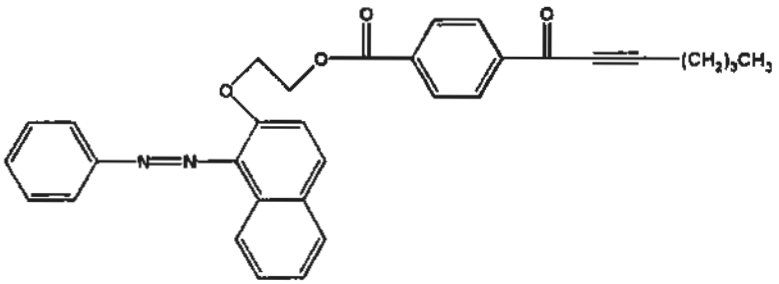
Coumarin non terminal alkynyl ketone 80



Diethylazodicarboxylate (0.38 ml, 2.4 mmol) was added slowly to a solution of triphenylphosphine (0.76 g, 2.9 mmol) in THF (25 ml). When the yellow colour of the DEAD reagent had disappeared, the coumarin alcohol derivative **69** (0.5 g, 2.4 mmol) was added in a THF solution (5 ml) and the mixture stirred for a further 20 minutes. At this time the acid 78 (0.55 g, 2.4 mmol) was added to the reaction. The reaction was stirred for 60 minutes, the solvent removed under vacuum and the resulting orange oil chromatographed (40% EtOAc / 60% hexane) to yield 0.46 g (54%) of the expected ester **80** as a white solid. Mp 106-108°C; ^{1}H NMR δ 0.95 (t, J = 7 Hz, 3H, CH_3), 1.50 (m, 2H, CH_2), 1.67 (m, 2H, CH_2), 4.41 (t, J = 4.5 Hz, 2H, CH_2O), 4.75 (t, J = 4.5 Hz, 2H, CH_2O), 6.26 (d, J = 9.5 Hz, 1H, =CH), 6.88 (m, 2H, Ar), 7.41 (d, J = 8.5 Hz, 1H, Ar), 7.66 (d, J = 9.5 Hz, 1H, =CH), 8.16 (m, 4H, Ar); ^{13}C NMR δ 13.4, 18.8, 21.9, 29.6, 63.2, 66.2, 79.4, 98.2, 101.5, 112.7, 112.8, 113.2, 128.8, 129.2, 129.7, 133.8, 140.0, 143.2, 155.6, 160.9, 161.4, 165.4, 177.2; IR ν_{max} ($CHCl_3$) 3030,

2990, 2960, 2940, 2200, 1730, 1640, 1615, 1505, 1405, 1265, 1230, 1120, 1105, 840 cm^{-1}; UV λ_{max} (CH_2Cl_2) 263 (25260), 311 (16608) nm; MS (EI) m/z 418 ($M^{+\cdot}$, 45), 337 (40), 257 (100), 213 (23), 104 (12), 43 (8); Exact Mass Calcd for $C_{25}H_{22}O_6$ 418.14164. Found 418.14147.

Sudan 1 non terminal alkynyl ketone 81



Method A:

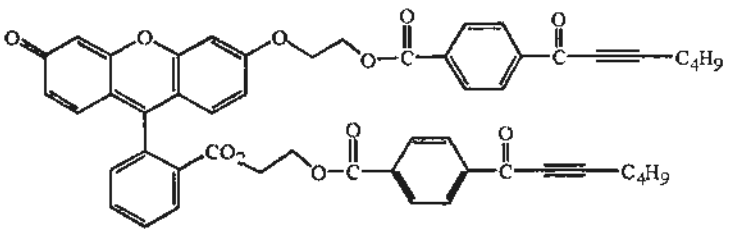
The DEAD reagent (0.27 ml, 1.7 mmol) was added to triphenylphosphine (0.70 g, 2.7 mmol) in THF (25 ml). After stirring until the yellow colour faded, the Sudan 1 alcohol **70** (0.5 g, 1.7 mmol) was added to the solution, followed by after a further 20 minutes the acid **78** (0.39 g, 1.7 mmol). The solvent was removed under vacuum and the residue purified by chromatography (20% EtOAc / 80% hexane) to produce the title compound **81** in 58% yield (0.50 g) as a red oil.

Method B:

Dicyclohexylcarbodiimide (0.35 g, 1.7 mmol) and DMAP (25 mg, 0.2 mmol) were added to a solution of the Sudan 1 alcohol **70** (0.5 g, 1.7 mmol) and the acid **78** (0.39 g, 1.7 mmol) in chloroform (25 ml) and the mixture was stirred overnight. Filtration of the reaction followed by removal of the solvent and chromatography (as above) yielded 0.44 g (51%) of the

expected product **81**. ^{1}H NMR δ 0.97 (t, J = 7 Hz, 3H, CH_3), 1.50 (m, 2H, CH_2), 1.67 (m, 2H, CH_2), 2.53 (t, J = 7 Hz, 2H, CH_2), 4.50 (m, 2H, CH_2O), 4.65 (m, 2H, CH_2O), 7.01 (m, 1H, Ar), 7.45 (m, 5H, Ar), 7.8-8.3 (m, 8H, Ar), 8.32 (d, J = 9 Hz, 1H, Ar); ^{13}C NMR δ 13.4, 18.9, 22.0, 29.7, 63.9, 69.5, 79.6, 97.9, 117.7, 122.6, 123.3, 124.9, 127.5, 127.8, 128.6, 129.0, 129.1, 129.2, 129.4, 129.5, 129.6, 129.8, 130.6, 131.0, 134.0, 138.2, 139.8, 146.5, 153.2, 165.4, 177.3; IR ν_{max} ($CHCl_3$) 3025, 2960, 2935, 2875, 2200, 1720, 1640, 1595, 1505, 1455, 1410, 1270, 1230, 1130, 1105, 1020, 910, 805, 690 cm^{-1}; UV λ_{max} (CH_2Cl_2) 235 (47790), 268 (41735), 364 (11317) nm; MS (FAB) m/z 505 (MH^+, 6), 257 (33), 213 (12), 154 (20), 137 (20), 136 (19), 97 (32), 83 (40), 81 (38), 67 (41), 69 (83), 57 (100), 55 (95); Exact Mass Calcd for $C_{32}H_{28}N_2O_4$ 504.20491. Found 504.20536.

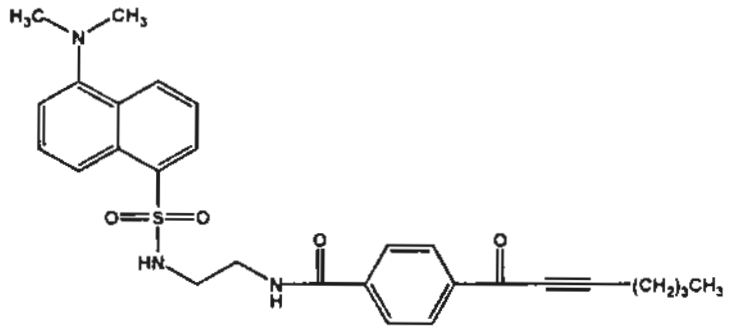
Dialkylated fluorescein non terminal alkynyl ketone 82



A solution of the dialkylated fluorescein derivative **63** (0.5 g, 1.2 mmol), the alkynyl acid **78** (0.82 g, 3.6 mmol), DCC (0.74 g, 3.6 mmol), and DMAP (25 mg, 0.1 mmol) in chloroform (50 ml) was stirred for 2 hours at room temperature. The reaction was then filtered and the organic layer washed with 10% hydrochloric acid (30 ml) and 10% potassium bicarbonate (30 ml), before the solution was dried and the solvent removed. Purification by flash chromatography (100% EtOAc) of the residue yielded 0.51g (50%) of the ester **82** as an orange solid. ^{1}H NMR δ 0.95 (m, 6H, 2xCH_3), 1.50 (m, 4H, 2xCH_20), 1.68 (m, 4H,

2xCH_2), 2.53 (m, 4H, 2xCH_2), 4.30 (m, 2H, 2xCH-O), 4.40 (m, 4H, 4xCH-O), 4.74 (m, 2H, 2xCH-O), 6.36 (d, J = 2 Hz, 1H, Ar), 6.50 (dd, J = 2, 10 Hz, 1H, Ar), 6.72 (dd, J = 2, 9 Hz, 1H, Ar), 6.90 (m, 3H, Ar), 7.32 (dd, J = 1, 7.5 Hz, 1H, Ar), 7.73 (m, 2H, Ar), 8.00 (d, J = 8 Hz, 2H, Ar), 8.17 (m, 6H, Ar), 8.29 (dd, J = 1, 7Hz, 1H, Ar); ^{13}C NMR δ 13.4, 18.8, 22.0, 24.8, 25.5, 29.7, 33.6, 49.2, 62.9, 63.0, 63.1, 66.5, 79.5, 98.3, 98.4, 101.0, 105.5, 114.0, 115.1, 117.6, 129.1, 129.3, 129.4, 129.6, 129.7, 129.9, 130.5, 131.4, 133.0, 133.7, 133.8, 134.1, 134.2, 140.1, 151.6, 154.2, 157.6, 158.9, 163.1, 164.8, 165.1, 165.4, 168.1, 177.2, 185.3; IR ν_{max} ($CHCl_3$) 3010, 2935, 2860, 2340, 2200, 1720, 1640, 1600, 1530, 1500, 1410, 1340, 1260, 1245, 1170, 1150, 1120, 680 cm^{-1}; UV λ_{max} (CH_2Cl_2) 234 (47650), 262 (41735), 434 (13690) nm; MS (FAB) m/z 846 (MH^+, 9), 431 (33), 257 (53), 213 (30), 149 (44), 83 (39), 81 (40), 71 (48), 69 (62), 57 (96), 55 (100).

Dansyl non terminal alkynyl ketone 84



DCC (0.38 g, 1.9 mmol) and DMAP (25 mg, 0.1 mmol) were slowly added to a solution of *N*-hydroxy succinimide (0.21 g, 1.9 mmol) and the acid **78** (0.42 g, 1.9 mmol) in chloroform (25 ml) at -20°C and the reaction allowed to stir for 5 hours while warming to room temperature. At this stage the mixture was filtered and added to the previously prepared dansyl amine derivative **71** (1.9 mmol). After stirring overnight the solvent was removed and the residue

purified by flash chromatography (55% EtOAc / 45% hexane) to produce the title compound as a pale green oil in 30% overall yield (0.27g) from dansyl chloride **22**. ^{1}H NMR δ 0.97 (t, *J* = 7 Hz, 3H, CH_3), 1.51 (m, 2H, CH_2), 1.69 (m, 2H, CH_2), 2.53 (t, *J* = 7 Hz, 2H, CH_2), 2.86 (s, 6H, 2xCH_3), 3.17 (m, 2H, CH_2N), 3.53 (m, 2H, CH_2N), 6.09 (t, *J* = 6 Hz, 1H, NH), 7.09 (s, 1H, Ar), 7.13 (t, *J* = 5 Hz, 1H, NH), 7.49 (m, 2H, Ar), 7.71 (d, *J* = 8.5 Hz, 2H, Ar), 8.04 (d, *J* = 8.5 Hz, 2H, Ar), 8.24 (m, 2H, Ar), 8.50 (d, *J* = 8.5 Hz, 1H, Ar); IR ν_{max} ($CHCl_3$) 3390, 3030, 3010, 2960, 2935, 2200, 1740, 1610, 1530, 1405, 1355, 1320, 1230, 1160, 1090, 1075, 910, 625, 570 cm^{-1}; UV λ_{max} (CH_2Cl_2) 231 (37920), 260 (44062), 333 (13000) nm; MS (FAB) m/z 506 (MH^+, 27), 505 (24), 213 (15), 170 (31), 149 (33), 129 (27), 113 (20), 71 (63), 69 (29), 56 (100), 54 (38).

Methyl *p*-bromobenzoate 88

Potassium carbonate (1.37 g, 9.9 mmol) was added to a solution of *p*-bromobenzoic acid (1.0 g, 5.0 mmol) and methyl iodide (0.46 ml, 7.5 mmol) in DMPU (20 ml) and the mixture stirred overnight at room temperature. Water (50 ml) was then added and the solution extracted with ethyl acetate (4x30 ml). The organic layer was washed with water (2x50 ml), 10% sodium bicarbonate solution (30 ml) and brine (30 ml). This was followed by drying and removal of the solvent to produce 0.96 g (89%) of the expected product **88** as a white solid, without the need for further purification. Mp 77-79°C (Lit. Mp 81°C)[127]; ^{1}H NMR δ 3.88 (s, 3H, OCH_3), 7.51 (d, *J* = 8.5 Hz, 2H, Ar), 7.83 (d, *J* = 8.5 Hz, 2H, Ar); ^{13}C NMR δ 52.2, 128.0, 129.0, 131.1, 131.6, 166.3; IR ν_{max} ($CHCl_3$) 3030, 3010, 2950, 1720, 1590, 1485, 1435, 1400, 1285, 1175, 1115, 1105, 1070, 1015, 850, 825 cm^{-1}; MS (EI) m/z 216 ($M^{+\cdot}$, 38), 214 ($m^{+\cdot}$, 40), 185 (98), 183 (100), 157 (34), 155 (35), 76 (39), 75 (38), 74 (22), 50 (39).

Tributyl(trimethylsilylethynyl) tin 92 [128]

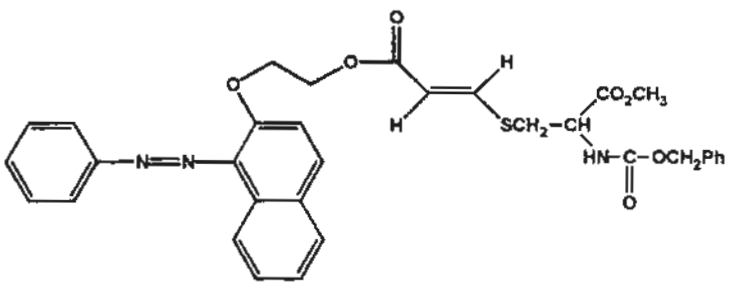
Butyl lithium (3.11 ml, 2.5 M, 7.8 mmol) was added dropwise to trimethylsilylacetylene (1.0 ml, 7.1 mmol) in THF (15 ml) at -78°C. After stirring for twenty minutes tributyltin chloride (1.9 ml, 7.1 mmol) was added and the solution stirred for a further three hours while warming to room temperature. The THF was then removed, hexane (20 ml) added and the solution put through a pad of silica to remove lithium salts. Removal of the hexane, followed by Kugelrohr distillation yielded 2.43 g (89%) of the tributyl(trimethylsilylethynyl) tin **92** as a colourless oil. ^{1}H NMR δ 0.13 (s, 9H, 3xCH_3), 0.87 (t, J = 7 Hz, 9H, 3xCH_3), 0.96 (t, J = 8 Hz, 6H, 3xCH_2), 1.32 (m, 6H, 3xCH_2), 1.54 (m, 6H, 3xCH_2); ^{13}C NMR δ 0.24, 11.1, 13.7, 26.9, 28.8, 113.1, 118.8; IR ν_{max} (neat) 2960, 2930, 2870, 2850, 2360, 2340, 1460, 1250, 855, 840, 760, 700 cm^{-1}.

Experimental Described in Chapter 4

General Procedure for the base catalysed reactions between the protected amino acids and the dyes attached to conjugated alkynes (Procedure C)

Triethylamine (catalytic) was added to the protected amino acid (25 mg) and the conjugated alkyne (1.0 molar equivalent) dissolved in chloroform at either room temperature or 0°C, as stated. The reaction was stirred until tlc analysis revealed none of the derivatised dye remaining, at which time the solvent was removed. Purification of the crude material by flash chromatography then yielded the addition product(s).

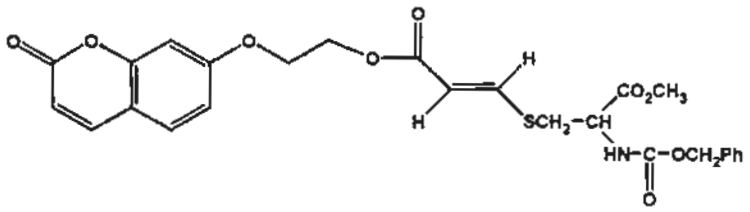
Sudan 1 cysteinyl propenoate 98



The reaction between the alkynyl ester **73** and the thiol **31** was undertaken at 0°C following Procedure C. Flash chromatography (40% EtOAc / 60% hexane) produced 73% (48 mg) of **98**. ^{1}H NMR δ 3.20 (m, 2H, CH_2S), 3.72 (s, 3H, OCH_3), 4.36 (m, 2H, CH_2O), 4.42 (m, 2H, CH_2O), 4.65 (m, 1H, α-CH), 5.09, 5.12 (AB pattern, J = 12 Hz, 2H, OCH_2Ph), 5.62 (d, J = 7.5 Hz, 1H, NH), 5.79 (d, J = 15 Hz, 1H, =CH), 6.89 (m, 1H, Ar), 7.08 (m, 1H, Ar),

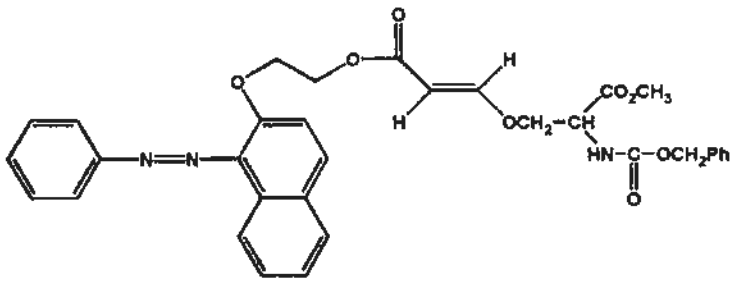
7.3-7.6 (m, 9H, Ar), 7.35 (d, J = 15 Hz, 1H, =CH), 7.82 (m, 2H, Ar), 7.99 (m, 2H, Ar), 8.33 (d, J = 8 Hz, 1H, Ar); IR ν_{max} ($CHCl_3$) 3430, 2950, 1720, 1590, 1500, 1460, 1350, 1310, 1155, 1070, 995, 955 cm^{-1}; UV λ_{max} (CH_2Cl_2) 230 (43660), 272 (34890) nm; MS (FAB) m/z 614 (MH^+, 18), 366 (23), 307 (12), 154 (100), 137 (58), 136 (78), 91 (98), 87 (40); Exact mass calcd for $C_{33}H_{32}N_3O_7S$ (MH^+) 614.19610. Found 614.19782.

Coumarin cysteinyl propenoate 99



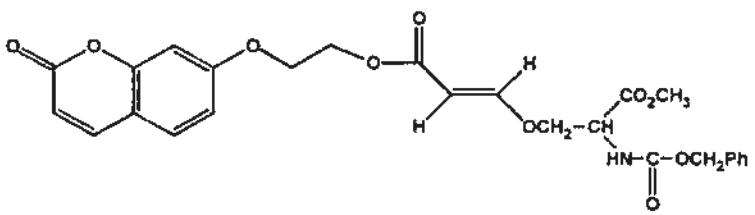
The reaction between the coumarin derivative 74 and the protected cysteine **31** was carried out at 0°C following Procedure C. Flash chromatography (50% EtOAc / 50% hexane) produced 66% (32 mg) of the E alkene. ^1H NMR δ 3.33 (m, 2H, CH_2S), 3.78 (s, 3H, OCH_3), 4.24 (m, 2H, CH_2O), 4.51 (m, 2H, CH_2O), 4.70 (m, 1H, α-CH), 5.10, 5.13 (AB pattern, J = 12 Hz, 2H, OCH_2Ph), 5.68 (d, J = 7.5 Hz, 1H, NH), 5.88 (d, J = 15 Hz, 1H, =CH), 6.26 (d, J = 9 Hz, 1H, =CH (Coumarin)), 6.85 (m, 2H, Ar), 7.33 (m, 6H, Ar), 7.63 (d, J = 15 Hz, 1H, =CH), 7.64 (d, J = 9 Hz, 1H, =CH (Coumarin)); IR ν_{max} ($CHCl_3$) 3420, 2930, 1715, 1605, 1590, 1490, 1450, 1395, 1340, 1300, 1115, 1050, 985, 940, 890, 830 cm^{-1}; UV λ_{max} (CH_2Cl_2) 280 (55695), 322 (39035) nm; MS (LSIMS) m/z 528 (MH^+, 100), 484 (70), 376 (25), 366 (26), 307 (26), 286 (70), 225 (86), 216 (69), 189 (43); Exact Mass (EI) Calcd for $C_{26}H_{25}NO_9S$ 527.12500. Found 527.12419.

Sudan 1 serinyl propenoate 100



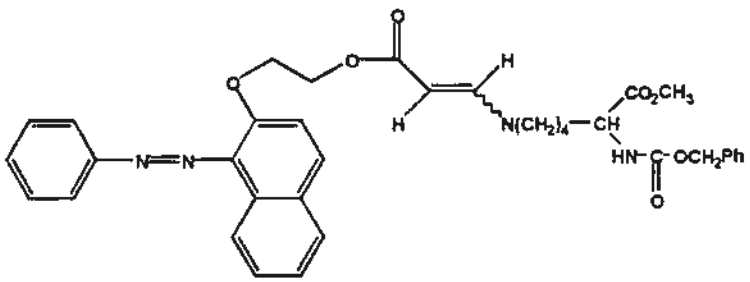
The addition reaction between **73** and **32** was undertaken using Procedure C at 0°C. Purification of the crude material by flash chromatography (40% EtOAc / 60% hexane) yielded 26 mg, (44%) of the expected addition product. ^{1}H NMR δ 3.77 (s, 3H, OCH_3), 3.89 (m, 2H, OCH_2), 4.26 (m, 2H, OCH_2), 4.40 (m, 2H, OCH_2), 4.66 (m, 1H, α-CH), 5.07 (d, J = 12 Hz, 1H, =CH), 5.20 (m, 2H, $OC\underline{H}_2Ph$), 5.75 (m, 1H, NH), 6.9-7.15 (m, 2H, Ar), 7.3-7.65 (m, 9H, Ar), 7.33 (d, J = 12 Hz, 1H, =CH), 7.91 (m, 2H, Ar), 8.05 (m, 2H, Ar), 8.46 (d, J = 8.5 Hz, 1H, Ar); IR ν_{max} ($CHCl_3$) 3450, 2950, 1725, 1630, 1600, 1510, 1465, 1345, 1330, 1280, 1140, 1090, 1070, 980 cm^{-1}; UV λ_{max} (CH_2Cl_2) 234 (90335), 279 (29324), 368 (17790) nm; MS (EI) m/z 597 ($M^{+\cdot}$, 10), 489 (10), 362 (10), 292 (50), 248 (30), 242 (57), 198 (22), 115 (62), 91 (100), 87 (22); Exact Mass (EI) Calcd for $C_{33}H_{31}N_3O_8$ 597.21111. Found 597.21187.

Coumarin serinyl propenoate 101



The addition of the alkyne **74** and the alcohol **32** was undertaken at 0°C using Procedure C. Purification of the crude material by flash chromatography on silica (60% EtOAc / 40% hexane) yielded 15 mg, (29%) of **101**. ^{1}H NMR δ 3.79 (s, 3H, OCH_3), 4.13 (m, 1H, CH-O), 4.28 (m, 3H, 3xCH-O), 4.50 (m, 2H, CH_2O), 4.66 (m, 1H, α-CH), 5.13 (s, 2H, $OC\underline{H}_2Ph$), 5.26 (d, J = 12.5 Hz, 1H, =CH), 5.65 (m, 1H, NH), 6.27 (d, J = 9.5 Hz, 1H, =CH (Coumarin)), 6.85 (m, 2H, Ar), 7.35 (s, 5H, Ph), 7.55 (d, J = 12.5 Hz, 1H, =CH), 7.64 (d, J = 9.5 Hz, 1H, =CH (Coumarin)); IR ν_{max} ($CHCl_3$) 3430, 3000, 2950, 1725, 1610, 1500, 1275, 1230, 1210, 1120, 1060, 790, 740 cm^{-1}; UV λ_{max} (CH_2Cl_2) 236 (30100), 322 (27318) nm; MS (FAB) m/z 512 (MH^+, 100), 468 (50), 391 (20), 307 (37), 289 (43), 259 (60), 225 (65), 207 (60); Exact mass calcd for $C_{26}H_{26}NO_{10}$ (MH^+) 512.15567. Found 512.15433.

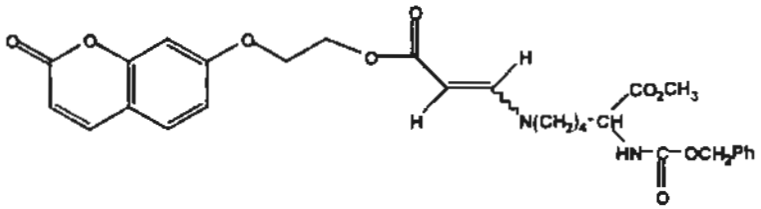
Sudan 1 lysinyl propenoates 104*E* and 104*Z*



The addition reaction between the Sudan 1 derivative **73** and the amine **33** was undertaken at room temperature after the initial removal of the hydrochloride salt by washing the protected lysine with 10% sodium bicarbonate solution. Purification of the crude material by flash chromatography (50% EtOAc / 50% hexane) yielded 51% (30 mg) of the two isomers **104*E*** and **104*Z*** (1.1:1) as an inseparable mixture. ^{1}H NMR δ 1.3-1.85 (m, 6H, 3xCH_2), 2.90 (m, 2H, CH_2N (*E*)), 3.10 (m, 2H, CH_2N (*Z*)), 3.72 (s, 3H, OCH_3), 3.73 (s, 3H, OCH_3), 4.21 (m, 1H, α-CH), 4.38 (m, 4H, 2xCH_2), 4.49 (d, J = 8 Hz, 1H, =CH (*Z*)), 4.60 (d, J = 13 Hz, 1H, =CH (*E*)), 5.10 (s, 4H, 2x$OC\underline{H}_2Ph$), 5.33 (m, 1H, NH), 6.54 (dd, J = 8, 13 Hz, 1H, =CH

(*Z*)), 6.9-7.1 (m, 2H, Ar), 7.25-7.6 (m, 9H, Ar), 7.62 (dd, J = 8, 13 Hz, 1H, =CH (*E*)), 7.83 (m, 2H, Ar), 8.00 (m, 2H, Ar), 8.36 (d, J = 8 Hz, 1H, Ar); IR ν_{max} ($CHCl_3$) 3440, 2940, 1720, 1665, 1615, 1500, 1455, 1345, 1305, 1275, 1140, 1065, 980, 900 cm^{-1}; UV λ_{max} (CH_2Cl_2) 233 (56110), 278 (45910) nm; MS (FAB) m/z 639 (MH^+, 15), 551 (18), 513 (18), 446 (10), 405 (13), 391 (23), 307 (10), 154 (100), 137 (60), 136 (80), 107 (32), 91 (52), 77 (48), 38 (46); Exact mass calcd for $C_{36}H_{39}N_4O_7$ (MH^+) 639.28187. Found 639.28331.

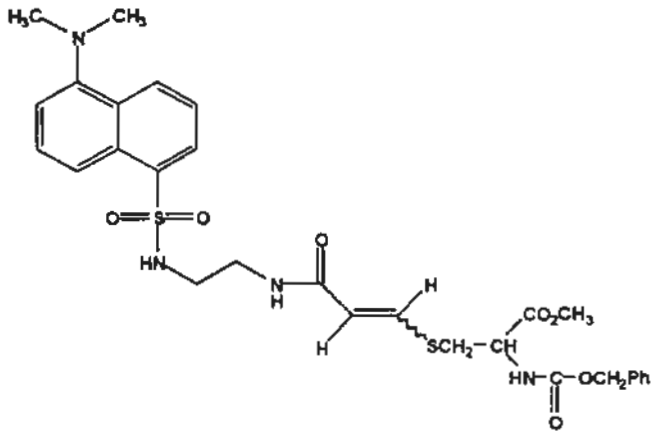
Coumarin lysinyl propenoates 105*E* and 105*Z*



The hydrochloride salt of the protected lysine **33** was removed prior to the reaction by washing with 10% sodium bicarbonate solution, and the reaction of the lysine derivative **33** with the alkyne 74 was undertaken without added base at room temperature. Purification of the crude material by flash chromatography (60% EtOAc / 40% hexane) yielded 27 mg, (55%) of the two alkenes, **105*E*** and **105*Z***, in a 1.5:1 ratio, as an inseparable mixture. ^{1}H NMR δ 1.38 (m, 2H, CH_2), 1.5-1.8 (m, 4H, 2xCH_2), 3.02 (m, 2H, CH_2N (*E*)), 3.15 (m, 2H, CH_2N (*Z*)), 3.75 (s, 6H, 2xOCH_3), 4.25 (m, 2H, CH_2O), 4.42 (m, 2H, CH_2O), 4.51 (d, J = 8 Hz, 1H, =CH (*Z*)), 4.73 (d, J = 13 Hz, 1H, =CH (*E*)), 5.10 (s, 4H, 2xOCH_2Ph), 5.39 (d, J = 8 Hz, 1H, NH), 6.25 (d, J = 9.5 Hz, 1H, =CH (Coumarin)), 6.63 (dd, J = 8, 13 Hz, 1H, =CH (*Z*)), 6.86 (m, 2H, Ar), 7.35 (m, 6H, Ar), 7.54 (dd, J = 8, 13 Hz, 1H, =CH (*E*)), 7.63 (d, J = 9.5 Hz, 1H, =CH (Coumarin)), 7.7 (m, 1H, NH); IR ν_{max} ($CHCl_3$) 3440, 2950, 1725, 1615, 1600, 1555, 1500, 1450, 1395, 1380, 1125, 1060, 995, 835 cm^{-1}; UV λ_{max} (CH_2Cl_2) 277 (40810),

321 (31120) nm; MS (LSIMS) m/z 553 (MH^+, 60), 391 (21), 347 (100), 295 (51); Exact mass (EI) calcd for $C_{29}H_{32}N_2O_9$ 552.21078. Found 552.20958.

Dansyl cysteinyl propenamides 106*E* and 106*Z*



The addition reaction between the conjugated alkynyl amide **77** and the protected cysteine **31** was undertaken at room temperature using Procedure C. Purification of the crude material by flash chromatography (40% EtOAc / 60% hexane) yielded 31 mg, (55%) of the expected addition products in a ratio of 1.6:1 favouring the *E* isomer.

106*E* / 106*Z*

MS (FAB) m/z 615 (MH^+, 22), 614 (21), 506 (10), 446 (22), 170 (45), 154 (95), 137 (53), 136 (74), 91 (100), 57 (37); Exact mass calcd for $C_{29}H_{34}N_4O_7S_2$ 614.18689. Found 614.18611.

106*E*

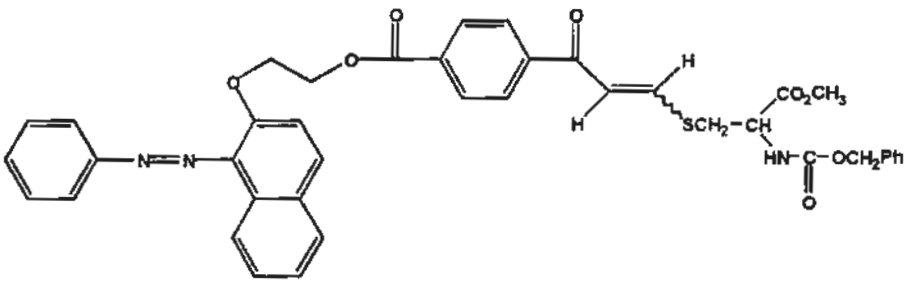
^{1}H NMR δ 2.89 (s, 6H, 2xNCH_3), 3.03 (m, 2H, CH_2N), 3.27 (m, 4H, CH_2N+CH_2S), 3.79 (s, 3H, OCH_3), 4.69 (m, 1H, α-CH), 5.13 (s, 2H, $OC\underline{H}_2Ph$), 5.66 (d, J = 15 Hz, 2H, =CH+NH), 5.79 (d, J = 8 Hz, 1H, NH), 5.93 (t, J = 6 Hz, 1H, NH), 7.18 (d, J = 8 Hz, 1H,

Ar), 7.36 (m, 5H, Ph), 7.39 (d, J = 15 Hz, 1H, =CH), 7.55 (m, 2H, Ar), 8.23 (m, 2H, Ar), 8.54 (d, J = 8.5 Hz, 1H, Ar); IR ν_{max} ($CHCl_3$) 3425, 3030, 2930, 2855, 1720, 1510, 1230, 750, 740, 715, 670 cm^{-1}; IR λ_{max} (CH_2Cl_2) 264 (107482), 336 (23032) nm.

106*Z*

^{1}H NMR δ 2.88 (s, 6H, 2xNCH_3), 3.03 (m, 2H, CH_2N), 3.20 (d, J = 5 Hz, 2H, CH_2S), 3.33 (m, 2H, CH_2N), 3.74 (s, 3H, OCH_3), 4.63 (m, 1H, α-CH), 5.10 (s, 2H, $OC\underline{H}_2Ph$), 5.57 (d, J = 10 Hz, 1H, =CH), 5.73 (t, J = 6 Hz, 1H, NH), 5.83 (d, J = 7.5 Hz, 1H, NH), 6.03 (t, J = 5.5 Hz, 1H, NH), 6.67 (d, J = 10 Hz, 1H, =CH), 7.18 (d, J = 7.5 Hz, 1H, Ar), 7.33 (s, 5H, Ph), 7.51 (m, 2H, Ar), 8.21 (m, 2H, Ar), 8.53 (d, J = 8.5 Hz, 1H, Ar); IR ν_{max} ($CHCl_3$) 3430, 3030, 2945, 2930, 2870, 1720, 1650, 1575, 1510, 1410, 1340, 1320, 1260, 1230, 1145, 1060, 795, 775, 740, 720 cm^{-1}; UV λ_{max} (CH_2Cl_2) 263 (46576), 342 (11260) nm.

Sudan 1 cysteinyl heptenones 107*E* and 107*Z*



The addition of the non terminal conjugated alkyne **81** and the thiol **31** was undertaken at room temperature following Procedure C. Purification of the crude material by flash chromatography on silica (35% EtOAc / 65% hexane) yielded 56 mg, (78%) of the alkenes in a 2.5:1 ratio for **107*E*** : **107*Z***.

107*E* / 107*Z*

MS (FAB) m/z 774 (MH^+, 65), 526 (100), 436 (27), 358 (5), 291 (15), 289 (15), 257 (36), 213 (15); Exact mass calcd for $C_{44}H_{44}N_3O_8S$ (MH^+) 774.28491. Found 774.28507.

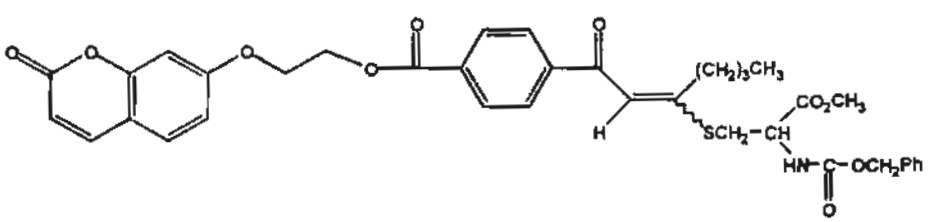
107*E*

^{1}H NMR δ 0.95 (t, J = 7 Hz, 3H, CH_3), 1.45 (m, 2H, CH_2), 1.62 (m, 2H, CH_2), 2.87 (m, 2H, CH_2), 3.37 (m, 2H, CH_2S), 3.76 (s, 3H, OCH_3), 4.51 (m, 2H, CH_2O), 4.66 (m, 2H, CH_2O), 4.77 (m, 1H, α-CH), 5.07 (m, 2H, OCH_2Ph), 5.62 (d, J = 7.5 Hz, 1H, NH), 6.69 (s, 1H, =CH), 7.04 (m, 1H, Ar), 7.05-7.50 (m, 10H, Ar), 7.82 (m, 4H, Ar), 7.96 (m, 4H, Ar), 8.30 (d, J = 8 Hz, 1H, Ar); IR ν_{max} ($CHCl_3$) 3420, 3030, 3015, 2950, 2930, 2870, 1720, 1650, 1550, 1505, 1340, 1280, 1270, 1250, 1245, 1230, 1060, 800 cm^{-1}; UV λ_{max} (CH_2Cl_2) 236 (47925), 256 (36385), 328 (24835) nm.

107*Z*

^{1}H NMR δ 0.97 (t, J = 7 Hz, 3H, CH_3), 1.42 (m, 2H, CH_2), 1.59 (m, 2H, CH_2), 2.59 (m, 2H, CH_2), 3.40 (m, 2H, CH_2S), 3.79 (s, 3H, OCH_3), 4.51 (m, 2H, CH_2O), 4.65 (m, 2H, CH_2O), 4.71 (m, 1H, α-CH), 5.11 (s, 2H, OCH_2Ph), 5.65 (d, J = 7.5 Hz, 1H, NH), 6.85-7.05 (m, 2H, Ar), 6.95 (s, 1H, =CH), 7.3-7.5 (m, 9H, Ar), 7.8-7.95 (m, 8H, Ar), 8.33 (d, J = 8 Hz, 1H, Ar); IR ν_{max} ($CHCl_3$) 3430, 3020, 3300, 2960, 1720, 1650, 1505, 1270, 1235, 1230, 1200, 800 cm^{-1}; UV λ_{max} (CH_2Cl_2) 238 (46960), 260 (35065), 342 (25925) nm.

Coumarin cysteinyl heptenones 108*E* and 108*Z*



The addition reaction between the coumarin derivative **80** and the thiol **31** was carried out by Procedure C at room temperature. Purification of the crude material by flash chromatography (55% EtOAc / 45% hexane) yielded 66% (42 mg) of the expected addition products in a 2:1 ratio in favour of the *E* isomer.

108*E* / 108*Z*

MS (FAB) m/z 688 (MH^+, 8), 644 (5), 552 (8), 526 (9), 451 (14), 337 (100), 257 (15), 219 (28), 149 (83), 105 (76); Exact mass calcd for $C_{37}H_{38}NO_{10}S$ (MH^+) 688.22164. Found 688.22096.

108*E*

^{1}H NMR δ 0.94 (t, *J* = 7 Hz, 3H, CH_3), 1.43 (m, 2H, CH_2), 1.61 (m, 2H, CH_2), 2.87 (m, 2H, CH_2), 3.37 (m, 2H, CH_2S), 3.78 (s, 3H, OCH_3), 4.39 (m, 2H, CH_2O), 4.73 (m, 3H, CH_2O+α-CH), 5.08 (m, 2H, $OC\underline{H}_2Ph$), 5.68 (d, *J* = 7.5 Hz, 1H, NH), 6.27 (d, *J* = 9.5 Hz, 1H, =CH (Coumarin)), 6.74 (s, 1H, =CH), 6.88 (m, 2H, Ar), 7.32 (m, 6H, Ar), 7.64 (d, *J* = 9.5 Hz, 1H, =CH (Coumarin)), 8.00 (d, *J* = 8 Hz, 2H, Ar), 8.13 (d, *J* = 8 Hz, 2H, Ar); IR ν_{max} ($CHCl_3$) 3430, 3040, 2960, 1720, 1620, 1555, 1505, 1410, 1335, 1270, 1230, 1200, 1125, 1105, 1060, 840, 800 cm^{-1}; UV λ_{max} (CH_2Cl_2) 230 (34840), 254 (33810), 324(49960) nm.

108*Z*

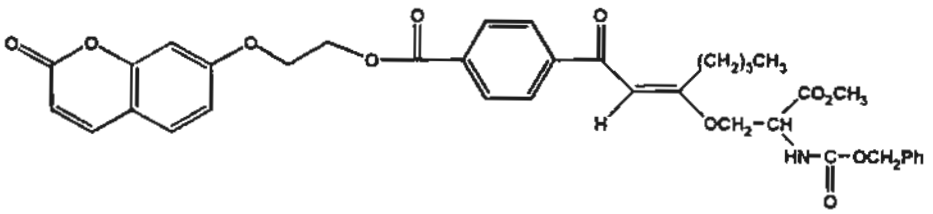
^{1}H NMR δ 0.96 (t, *J* = 7 Hz, 3H, CH_3), 1.43 (m, 2H, CH_2), 1.60 (m, 2H, CH_2), 2.59 (t, *J* = 7 Hz, 2H, CH_2), 3.40 (m, 2H, CH_2S), 3.79 (s, 3H, OCH_3), 4.38 (m, 2H, CH_2O), 4.70 (m, 3H, CH_2O+α-CH), 5.11 (s, 2H, $OC\underline{H}_2Ph$), 5.65 (d, *J* = 7.5 Hz, 1H, NH), 6.27 (d, *J* = 9.5 Hz, 1H, =CH (Coumarin)), 6.88 (m, 2H, Ar), 7.00 (s, 1H, =CH), 7.34 (m, 6H, Ar), 7.65 (d, *J* = 9.5 Hz, 1H, =CH (Coumarin)), 7.97 (d, *J* = 8 Hz, 2H, Ar), 8.12 (d, *J* = 8 Hz, 2H, Ar);

IR ν_{max} ($CHCl_3$) 3430, 3020, 2950, 1725, 1610, 1500, 1270, 1230, 1200, 1105, 910, 840, 800 cm^{-1}; UV λ_{max} (CH_2Cl_2) 231 (12880), 254 (13550), 324 (16660) nm.

General Procedure for the tri-*n*-butylphosphine catalysed reactions between the protected amino acids and the dyes attached to conjugated alkynes (Procedure D)

Tri-*n*-butylphosphine (0.2 molar equivalents) was added to the amino acid (25mg) and the conjugated alkyne (1 molar equivalent) in chloroform at room temperature. The solution was stirred until tlc analysis revealed none of the derivatised dye remaining, at which time the solvent was removed and the resulting material chromatographed to yield the addition product.

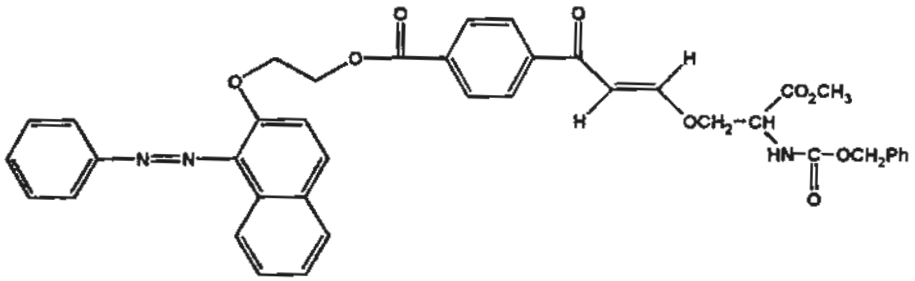
Coumarin serinyl heptenone 109



This reaction between the alkyne **80** and the alcohol **32** was carried using Procedure D. Flash chromatography (55% EtOAc / 45% hexane) allowed the recovery of the addition product as an inseparable mixture with the starting protected amino acid *N*-Cbz serine methyl ester. The yield of the reaction was calculated to be 31% from the integration in the ^1H NMR spectrum. ^1H NMR δ 0.90 (t, J = 7 Hz, 3H, CH_3), 1.33 (m, 2H, CH_2), 1.54 (m, 2H, CH_2), 2.70 (m, 1H, CHC=), 2.95 (m, 1H, CHC=), 3.78 (s, 3H, OCH_3), 4.01 (m, 2H, CH_2O), 4.30 (m, 2H, CH_2O), 4.44 (m, 1H, α-CH), 4.74 (m, 2H, CH_2O), 5.13 (s, 2H, $OC\underline{H}_2Ph$), 5.73 (d, J = 8 Hz, 1H, NH), 6.07 (s, 1H, =CH), 6.27 (d, J = 9.5 Hz, 1H, =CH (Coumarin)), 6.89 (m, 2H, Ar), 7.36 (m, 6H, Ar), 7.64 (d, J = 9.5 Hz, 1H, =CH (Coumarin)), 7.91 (d, J = 8.5 Hz, 2H,

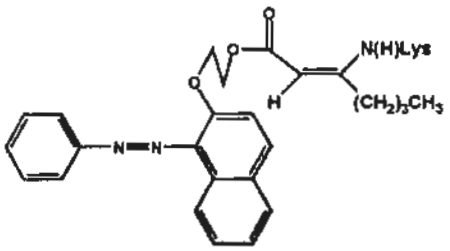
Ar), 8.11 (d, J = 8.5 Hz, 2H, Ar); IR ν_{max} ($CHCl_3$) 3430, 3025, 2960, 1725, 1615, 1580, 1510, 1270, 1235, 1200, 1180, 1120, 1105, 1060 cm^{-1}; UV λ_{max} (CH_2Cl_2) 230 (13420), 253 (15620), 293 (17505) nm; MS (FAB) m/z 672(MH^+, 31), 637 (6), 595 (8), 337 (43), 321 (35), 254 (57), 219 (100); Exact mass calcd for $C_{37}H_{38}NO_{11}$ (MH^+) 672.24448. Found 672.24386.

Sudan 1 serinyl heptenone 110



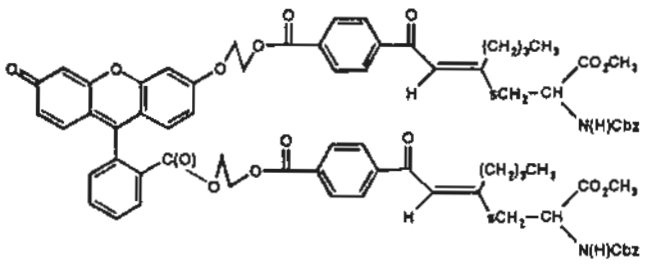
This reaction of the non terminal alkynyl ketone **81** and the protected serine derivative **32** was undertaken following Procedure D. The crude reaction mixture was purified by flash chromatography on silica (35% EtOAc / 65% hexane) to yield 31 mg (43%) of the title compound, **110**. ^1H NMR δ 0.97 (t, J = 7 Hz, 3H, CH_3), 1.35 (m, 2H, CH_2), 1.55 (m, 2H, CH_2), 2.70(m, 1H, CHC=), 2.95 (m, 1H, CHC=), 3.81 (s, 3H, OCH_3), 4.27 (m, 2H, CH_2O), 4.53 (m, 2H, CH_2O), 4.65 (m, 2H, CH_2O), 4.77 (m, 1H, α-CH), 5.16 (m, 2H, $OC\underline{H}_2Ph$), 5.73 (d, J = 8 Hz, 1H, NH), 6.04 (s, 1H, =CH), 7.00 (m, 1H, Ar), 7.35-7.55 (m, 10H, Ar), 7.75-8.00 (m, 8H, Ar), 8.32 (d, J = 8 Hz, 1H, Ar); IR ν_{max} ($CHCl_3$) 3430, 3030, 2960, 1720, 1660, 1580, 1510, 1280, 1265, 1245, 1235, 1230, 1180, 1105, 800 cm^{-1}; UV λ_{max} (CH_2Cl_2) 232 (127050), 285 (75070), 303 (14130) nm; MS (FAB) m/z 758 (MH^+, 55), 510 (100), 420 (20), 275 (45), 267 (25), 230 (18), 219 (20); Exact mass calcd for $C_{44}H_{44}N_3O_9$ (MH^+) 758.30775. Found 758.30826.

Sudan 1 lysinyl heptenone 111



The addition reaction of the amine **33** to the conjugated alkyne **81** was undertaken using Procedure C. The solution was stirred for 48 hours followed by heating at reflux for a further 48 hours. The reaction mixture was allowed to cool and the solvent removed. Purification of the crude material by flash chromatography (40% EtOAc / 60% hexane) produced the expected addition product in very low yield (>5%). ^{1}H NMR δ 0.98 (t, J = 7 Hz, 3H, CH_3), 1.40-1.75 (m, 4H, 2xCH_2), 2.29 (t, J = 7 Hz, 2H, CH_2), 3.33 (m, 2H, NCH_2), 3.75 (s, 3H, OCH_3), 4.45 (m, 1H, α-CH), 4.50 (m, 2H, CH_2O), 4.62 (m, 2H, CH_2O), 5.11 (s, 2H, $OC\underline{H}_2Ph$), 5.46 (d, J = 8 Hz, 1H, NH), 5.63 (s, 1H, =CH), 6.91 (m, 1H, Ar), 7.02 (m, 2H, Ar), 7.3-7.5 (m, 7H, Ar), 7.7-8.0 (m, 9H, Ar), 8.30 (d, J = 7 Hz, 1H, Ar), 11.67 (m, 1H, enamine NH).

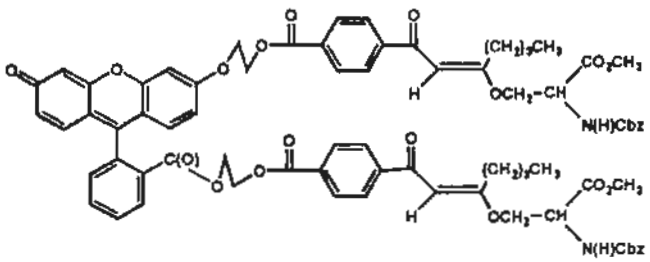
Fluoresceinyl di(cysteinyl heptenone) 112



The addition reaction between the alkyne **82** and the thiol **31** was undertaken following Procedure C. Purification of the crude material by flash chromatography (30% EtOAc / 70%

hexane) yielded 48% of the four alkenes. MS (LSIMS) m/z 1382 (MH^+, 50), 526 (85), 291 (57), 257 (100), 225 (70).

Fluoresceinyl di(serinyl heptenone) 113



The addition of the dialkylated fluorescein derivative **82** and the protected serine **32** was undertaken by Procedure D. The crude material was purified by flash chromatography on silica (25% EtOAc / 75% hexane) to give **113** in 12% yield. ^{1}H NMR δ 0.95 (m, 6H, 2xCH_3), 1.1-2.0 (m, 8H, 4xCH_2), 2.70 (m, 2H, CH_2), 2.92 (m, 2H, CH_2), 3.47 (m, 4H, 2xCH_2S), 3.79 (s, 6H, 2xOCH_3), 4.0-4.5 (m, 10H, 5xCH_2O), 4.72 (m, 4H, CH_2O+2xα-CH), 5.14 (s, 4H, 2xO$C\underline{H}_2$Ph), 5.78 (m, 1H, NH), 5.87 (d, J = 7 Hz, 1H, NH), 6.07 (s, 1H, =CH), 6.16 (s, 1H, =CH), 6.31 (m, 1H, Ar), 6.51 (dd, J = 2, 9.5 Hz, 1H, Ar), 6.75 (m, 1H, Ar), 6.82 (d, J = 1 Hz, 1H, Ar), 6.87 (m, 3H, Ar), 7.37 (m, 10H, Ar), 7.69 (m, 2H, Ar), 7.91 (m, 6H, Ar), 8.11 (m, 2H, Ar), 8.30 (dd, J = 1, 9 Hz, 1H, Ar); MS (LSIMS) m/z 1352 (MH^+, 60), 1144 (85), 510 (15), 494 (10), 390 (60), 349 (13), 303 (20), 275 (17), 273 (15), 225 (85), 219 (100).

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