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Lanasol Dyes and Wool Fibres. Part I: Model Studies on the Mechanism of Dye Fixation in a Mixed Solvent System

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ABSTRACT

The mechanism of fixation of Lanasol dyes, which are distinguished by their α-bromoacrylamido reactive group, to the various amino acid side chain sites within wool protein is quite complex. It has been proposed that the reaction could proceed by either a nucleophilic substitution or Michael addition pathway. We have investigated this mechanism through the reaction between model dye compounds which possess either an α-bromoacrylamido or α, β-dibromopropionamido group and wool mimetics that contain amine, thiol and hydroxyl groups. These latter groups are typical of the reactive sites found in wool proteins. The reactions were carried out in an acetone-water solvent system. The reactions were monitored by HPLC and the products were isolated and then characterised by proton and carbon-13 NMR, and mass spectroscopy. The results of the study confirmed that the dibromo form of the dye reactive group is only converted to the monobromo form in the presence of model wool compounds and that both forms react with these model wool compounds to yield the same products. When the nucleophilic moiety was an amine, the reaction terminated with a product containing an aziridine ring. No evidence for the proposed reaction of this aziridine ring with a second nucleophilic moiety to form a cross-link could be detected. The thiol of cystiene and imidazole of histidine were also found to reaction with the model dyes. © *1998 Elsevier Science Ltd. All rights reserved*

Keywords: wool fibres, Lanasol reactive dyes, mechanism of dye fixation.

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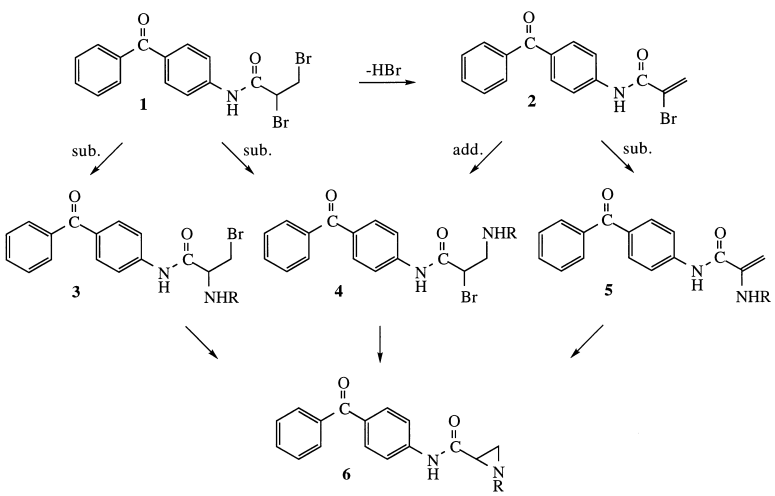
INTRODUCTION

Lanasol dyes (Ciba) were introduced commercially in 1966 and are one of the most successful agents used in the dyeing of wool in terms of their excellent light and wet fastness [1]. The reactivity of these dyes is associated with the α-bromoacrylamido group which can covalently bond with nucleophilic moieties such as amines. Of these reactive sites, the ε-amine group of lysine is thought to be of greatest importance due to its moderate reactivity and high abundance within wool proteins. Other potentially reactive amino functional sites present within the wool fibre include the side chain of histidine and the N-terminal amino groups of the protein chains themselves [2]. Free thiol groups, which are present at low levels in untreated wool on the side chains of cysteine, would be expected to have the highest relative reactivity. In contrast, the hydroxyl groups on the side chains of the amino acids serine, threonine and tyrosine would be the least reactive.

The chemistry proposed for the covalent linkage of the Lanasol dyes to wool is quite complex. This subject has been reviewed by Lewis [3] and is thus only briefly presented here. In the commercial dyestuffs the reactive group can be present as the α-bromoacrylamido form, the α,β-dibromopropionamido form, or a mixture of both forms. Mäusezahl [4] demonstrated that solutions of Lanasol dyes are very stable under dyeing conditions. The dyes studied were found to undergo dehydrobromination only when wool was present. It has been postulated that the α-bromoacrylamido form of the dye reacts with the wool fibre according to Scheme 1



Scheme 1.

which depicts the wool dye as the model compound used in this study. The mechanism put forward can proceed via either nucleophilic substitution of the bromine atom and/or Michael addition across the double bond of the α-bromoacrylamido group. Regardless of the pathway involved, the intermediates can cyclise to form an aziridine ring incorporating the α and β-carbons of the dye reactive moiety. Through measurements of bromide ion liberation during the reaction of Lanasol dyes with wool, Mäusezahl [4] obtained evidence supporting the Michael addition pathway, but could not rule out nucleophilic substitution. Using model compounds, he also demonstrated that the aziridine ring structure was the favoured product. It should be noted, however, that in the only reaction involving an amino acid carried out, the products were not isolated and characterised [4].

In the reaction scheme depicted in Scheme 1, the aziridine ring of the bound dye molecule (6) can react with a second nucleophilic group resulting in the formation of a cross-link within the wool protein. The possibility of the Lanasol dyes undergoing such a cross-linking reaction is very interesting in its own right. These cross-links, which could be formed either between two protein chains or within a single chain, have the potential to improve fibre properties such as tensile strength. Several studies have been carried out in this area but the results are somewhat conflicting. Lewis [3] found that for dyes with both one and two reactive groups the wet burst strength increased with increasing depth of shade. This increase was more pronounced for the dyes with two reactive groups. Ball *et al*. [5] used the urea/thioglycollate solubility of the fibre as a measure of cross-linking. Their findings were consistent with those of Lewis. In a similar study, however, Mosimann and Flensberg [6] observed a decrease in solubility only for fibres treated with Lanasol dyes containing two reactive groups.

In this paper we present the results of a detailed investigation into the reaction of model Lanasol dyes with various compounds and amino acids that contain amine, thiol and hydroxyl groups. The reactions were carried out in an acetone–water solvent system and the products were isolated by precipitation or chromatography. For the first time to our knowledge, a comprehensive set of 'state of the art' analytical methods including ^{1}H and ^{13}C-NMR, and mass spectroscopy (both electrospray and electron impact) has been applied to characterise the reaction products. It was felt that through such a study a better understanding of the mechanism of dye fixation to the wool fibre proteins could be obtained. In addition it was anticipated that, through direct observation of the reaction of two model wool compounds, the controversy over whether Lanasol dyes with one reactive group act as cross-linking agents could be resolved.

EXPERIMENTAL

Chemicals and reagents

4-Aminobenzophenone, α,β-dibromopropionic acid, *N,N′*-dicyclohexylcarbodiimide and all amino acids were obtained from Sigma-Aldrich Chemical Company. All other chemicals and solvents were supplied by BDH and Ajax Chemicals. All solvents were used as supplied except for 1,4-dioxan which was dried over 4Å molecular sieves. Silica gel 60 (70–230 mesh) and silica gel 60 F_{254} thin layer chromatography plates were obtained from Merck (Germany). Deuterated solvents (acetone-d_6, $CDCl_3$, D_2O and methanol-d_4) were obtained from Cambridge Isotope Laboratories.

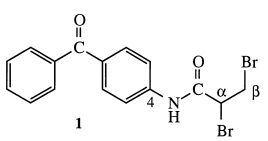
Instrumentation

Electron impact mass spectra (EI–MS) were recorded on a Hewlett–Packard GC–MS System 5988A while electrospray mass spectra (ES–MS) were recorded on a Micromass Platform II single quadrupole mass spectrometer. Nuclear magnetic resonance spectra were recorded on either a JEOL JNM-GX270 spectrometer or a Varian Unity Plus spectrometer. DEPT spectra were recorded on the Varian instrument to assign primary, secondary, tertiary and quaternary carbons. Chemical shifts were referenced to tetramethylsilane. HPLC analyses were performed on a Waters chromatographic system (model 626 dual piston pump, 996 photodiode array detector, 6000S controller and 717 plus autosampler) and data was acquired using Millenium Chromatography Manager software (version 2.1). Samples were either prepared at or diluted to 1 mg/ml with respect to the initial model dye concentration using acetonitrile prior to analysis. Analyte separation (5l injection volume) was achieved using a Waters Delta-Pak C_{18} reversed phase analytical column (150×3.9 mm i.d., 10 μm particle size and 300 pore size) at ambient temperature. The components were eluted using a linear solvent gradient of water/acetonitrile containing 0.1% v/v trifluoroacetic acid. A flow rate of 1.0 ml/min and a detection wavelength of 280 nm were used throughout the analyses. Melting point analyses were performed on an electrothermal melting point apparatus.

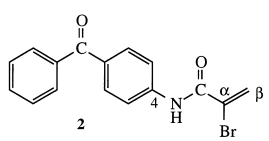
Preparation of model dyes

*4-(α,β-Dibromopropionamido)benzophenone (**1**)*
To a solution of α,β-dibromopropionic acid (2.30 g, 10.0 mmol) in dry 1,4-dioxan (30 ml) at 0°C, *N,N′*-dicyclohexylcarbodiimide (2.06 g, 10.0 mmol) was added and the resulting solution stirred for 20 min. The mixture was

then added to a solution of 4-aminobenzophenone (1.97 g, 10.0 mmol) in dry 1,4-dioxan (20 ml), stirred for 2 h at 0°C and then overnight at room temperature. After filtration to remove the *N,N′*-dicyclohexylurea by-product, the solvent was evaporated *in vacuo* at 30°C. The crude product was dissolved in a minimum volume of ethyl acetate and then precipitated by the addition of hexane. This procedure removed the majority of the unreacted 4-aminobenzophenone while the remainder was removed by column chromatography on silica gel using ethyl acetate/hexane (1:1) as the mobile phase. Like fractions were combined and evaporated under reduced pressure. The remaining white flakes were vacuum dried (0.5 mm Hg for 8 h) resulting in a yield of 3.35 g (82%). The melting point of the product was found to be 153–155°C. The purity and structure of the product was confirmed by HPLC, MS, ^{1}H and ^{13}C-NMR. The HPLC chromatogram contained only a single peak eluting at 17.3 min. The EI–MS contained the expected parent ion for the product at 411 m/z. Isotopic splitting from bromine was observed around the parent ion at 410 and 412 m/z. The resonances from the diastereotopic protons (Hβa and b) in the ^{1}H-NMR spectrum were both split into a doublet by CHBr (α-proton) and each doublet was split into a quartet from the coupling between Hβa and b. Evidence for the formation of an amide bond between 4-aminobenzophenone and α,β-dibromopropionic acid was observed by the presence of the amide signals at 8.15 ppm (NH) in the ^{1}H-NMR spectrum and 164.7 ppm (C = O) in the ^{13}C-NMR spectrum.



1



2

*4-(α-Bromoacrylamido)benzophenone (**2**)*

The preparation of 4-(α-bromoacrylamido)benzophenone was achieved by treating **1** with a warm alcoholic solution of potassium hydroxide, which is a common procedure for the dehydrobromination of vicinal dihalides to form vinylic halides [7]. 4-(α,β-Dibromopropionamido)benzophenone (500 mg, 1.22 mmol) was dissolved in ethanol (35 ml) at 50°C and a solution of potassium hydroxide (69 mg, 1.22 mmol) in ethanol (5 ml) was added. The reaction mixture was stirred at 50°C for 2 h. A sample of the reaction mixture was then collected using a Pasteur pipette and analysed by HPLC and ^{1}H-NMR spectroscopy. Two peaks were observed in the HPLC chromatogram, a minor peak at 17.4 min due to unreacted starting material and a major peak at 16.4 min. The ^{1}H-NMR spectrum of this mixture showed the loss of the original α,β-dibromopropionamido resonances at 3.85–4.05

and 4.43 ppm and the formation of the non-equivalent α-bromoacrylamido resonances at 6.11 and 7.08 ppm (Hβa and b). This indicated that **1** had been dehydrobrominated to form α-bromoacrylamidobenzophenone (**2**) which must have been the major constituent of the HPLC chromatogram.

A sticky polymer-like substance formed during the reaction and continued to form on standing after the heat was removed. Any attempt to concentrate the remaining solution resulted in the formation of more of this substance, making it impossible to isolate enough product for the mechanistic investigations with wool mimetics. The willingness of α-bromoacryl compounds to polymerise, without additional initiators, has been reported previously [8]. In an attempt to avoid this polymerisation, approximately 2 ml of the reaction mixture was taken from the flask and evaporated under reduced pressure to afford crude **2**. After drying under vacuum (0.5 mm Hg, 8 h) the resulting yield was determined to be 6 mg.

Reaction of model dyes with wool mimetics

The molar ratio of dye to wool reactive group for each reaction was calculated based on wool dyed to an approximate depth of 1% o.m.f, and took into account the average content of that particular reactive group in wool and assumed a molecular mass of 650 g/mol for a typical Lanasol dye. The required amount of wool mimetic was dissolved in a mixture of acetone (5 ml) and water (1 ml) in a 10 ml round bottomed flask. The pH of the solution was then adjusted to 5–6 using either HCl or $NaHCO_3$ and determined by spotting on a pH stick. Compound **1** (200 mg, 0.489 mmol) was then added to the solution with stirring and the pH was adjusted to 5 as described above. A micro-condenser was then attached to the flask and the solution was maintained at 65°C for 90 min with stirring. The pH of the solution was maintained at 5 throughout the period of reaction. Reaction mixtures were analysed by HPLC. Reactions involving compound **2** were performed on a reduced scale due to the limited availability of this reagent.

*Reaction of 4-(α,β-dibromopropionamido)benzophenone (**1**) with cyclohexylamine*

Four reactions of 4-(α,β-dibromopropionamido)benzophenone (200 mg, 0.489 mmol) were performed using varying amounts of cyclohexylamine, which resulted in model dye to amine molar ratios of 3:1, 1:1, 1:3 and 1:16. In the case of the 1:16 ratio, the addition of water caused the product to precipitate from the reaction mixture. The product was collected by vacuum filtration and washed with water (3×2 ml). After drying under vacuum (0.5 mm Hg, 8 h) the yield was 159 mg (94%); mp = 158–160°C; ^{1}H-NMR

($CDCl_3$) δ 1.14–1.51 (m, 3×cyclohexyl CH_2), 1.60 (br s, 1H, Hβ), 1.72–1.88 (m, 5H, 2×cyclohexyl CH_2, Hβ), 2.01 (d, 1, Hα), 2.18 (dd, 1H, cyclohexyl CH), 7.40–7.85 (m, 9H, ArH), 8.63 (s, 1H, NH); ^{13}C-NMR (CDCl3) δ 24.4, 26.0, 32.4, 34.1, 38.3, 67.2, 118.5, 128.2, 129.8, 131.6, 132.1, 132.8, 137.9, 141.5, 169.2, 195.5.

*Reaction of 4-(α-bromoacrylamido)benzophenone (**2**) with cyclohexylamine*
Cyclohexylamine (33 μl, 0.29 mmol) was dissolved in a mixture of acetone (0.3 ml) and water (0.1 ml) and the pH of the solution was adjusted to 5 with 1M HCl. 4-(α-bromoacrylamido)benzophenone (6 mg, 1.8 mmol) dissolved in acetone (0.2 mL) was then added (molar ratio of **2** to cyclohexylamine of 1:16). A micro-condenser was connected to the flask and the solution stirred at 65°C for 90 min, after which the reaction mixture was analysed by HPLC and ES mass spectrometry.

*Reaction of 4-(α,β-dibromopropionamido)benzophenone (**1**) with* Nα-*acetyl*-L-*lysine*-N-*methylamide*
4-(α, β-Dibromopropionamido)benzophenone (200 mg, 0.489 mmol) was reacted with *N*α-acetyl-L-lysine-*N*-methylamide (295 mg, 1.47 mmol), resulting in a model dye to wool mimetic molar ratio of 1:3. The product could not be isolated in a pure form by precipitation. As a result, column chromatography using acetone/water (10:1) as an eluent was used to purify the product; ^{1}H-NMR (CD_3OD) δ 1.35–1.93 (m, 3×lysCH_2), 1.99 (s, 3H, $COCH_3$), 2.16 (s, 1H, Hβ), 2.18 (s, 1H, Hβ), 2.35–2.58 (m, 2H, lysCH_2), 2.63 (s, 1H, Hα), 2.71 (s, 3H, N-CH_3), 4.26 (dt, 1H, lysCH), 7.50–7.82 (m, 9H, ArH); ^{13}C-NMR (CD_3OD) δ 22.6, 24.6, 26.3, 29.9, 32.9, 35.2, 40.4, 55.0, 60.6, 120.2, 129.5, 130.8, 132.4, 133.6, 134.0, 139.1, 143.7, 171.1, 173.6, 175.0, 197.5.

*Reaction of 4-(α,β-dibromopropionamido)benzophenone (**1**) with ethylamine*
4-(α, β-Dibromopropionamido)benzophenone (200 mg, 0.489 mmol) was reacted with a 70% aqueous solution of ethylamine (0.50 ml, 7.8 mmol) resulting in a model dye to amine molar ratio of 1:16 (the ethylamine solution was initially dissolved in acetone/water 5 ml/0.5 ml to allow for the additional water contained in the model compound solution). The product was precipitated from the reaction mixture by the addition of water. Collection by vacuum filtration on a Hirsch funnel, washing with water (3×2 ml) and drying under vacuum (0.5 mm Hg, 8 h) afforded a pure white solid (130 mg, 90%); mp = 126–128°C; ^{1}H-NMR ($CDCl_3$) δ 1.20 (t, 3H, CH_3), 1.73 (d, 1H, Hβ), 2.03 (d, 1H, Hβ), 2.16 (d, 1H, Hα), 2.44 (q, 2H, CH_2), 7.43–7.83 (m, 9, ArH), 8.61 (br s, 1H, NH); ^{13}C-NMR ($CDCl_3$) δ 14.2, 35.1, 39.3, 53.8, 118.5, 128.2, 129.8, 131.6, 132.1, 132.8, 137.9, 141.5, 169.0, 195.5.

*Reaction of 4-(α,β-dibromopropionamido)benzophenone (**1**) with* Nα*-acetyl-*L*-histidine*

4-(α, β-Dibromopropionamido)benzophenone (200 mg, 0.489 mmol) was reacted with *N*α-acetyl-L-histidine (453 mg, 2.30 mmol). This corresponded to a model dye to wool mimetic molar ratio of 1:4.7. Column chromatography using acetone:water (5:1) afforded the pure product; ^{1}H-NMR (CD_3OD) δ 1.83-2.01 (m, 5H, CH_3, Hβ), 2.75-3.18 (m, 2H, hisCH_2), 4.38–4.54 (m, 2H, hisCH, Hα), 7.00 (s, 1H, hisCH), 7.42–7.82 (m, 9H, ArH), 7.91 (s, 1H, hisCH); ^{13}C-NMR (CD_3OD) δ 23.5, 28.4, 32.3, 46.4, 54.6, 121.5, 122.0, 130.5, 131.8, 133.3, 134.9, 137.3, 139.7, 143.7, 169.0, 173.4, 174.4, 199.7.

*Reaction of 4-(α,β-dibromopropionamido)benzophenone (**1**) with* Nα*-acetyl-*DL*-tryptophan*

4-(α, β-Dibromopropionamido)benzophenone (200 mg, 0.489 mmol) was reacted with *N*α-acetyl-DL-tryptophan (349 mg, 1.42 mmol), resulting in a model dye to wool mimetic molar ratio of 1:2.9. No product peaks was observed by HPLC.

*Reaction of 4-(α,β-dibromopropionamido)benzophenone (**1**) with* N*-acetyl-*L*-cysteine*

4-(α, β-Dibromopropionamido)benzophenone (200 mg, 0.489 mmol) was reacted with *N*-acetyl-L-cysteine (206 mg, 1.27 mmol), resulting in a model dye to wool mimetic molar ratio of 1:2.6. Pure product was separated by column chromatography (silica gel) using acetone:water (10:1) as the mobile phase; ^{1}H-NMR (CD_3OD) δ 2.02 (s, 3H, CH_3), 2.70–2.81 (m, 2H, CH_2), 2.84–3.02 (m, 2H, CH_2), 2.86–3.18 (m, 2H, cysCH_2), 4.46 (br s, 1H, NH), 7.42–7.86 (m, 9H, ArH); ^{13}C-NMR (CD_3OD) δ 21.5, 27.5, 33.8, 37.0, 54.6, 118.0, 128.1, 129.4, 131.0, 132.1, 132.3, 137.7, 142.8, 171.6, 172.0, 177.1, 196.1.

*Reaction of 4-(α,β-dibromopropionamido)benzophenone (**1**) with* N*-acetyl-*L*-tyrosine*

4-(α, β-Dibromopropionamido)benzophenone (200 mg, 0.489 mmol) was reacted with *N*-acetyl-L-tyrosine (2.62 g, 11.7 mmol), resulting in a model dye to wool mimetic molar ratio of 1:24. No product peaks were observed in the HPLC chromatogram.

*Reaction of 4-(α,β-dibromopropionamido)benzophenone (**1**) with* Nε*-carbobenzoxy-*L*-lysine*

4-(α, β-Dibromopropionamido)benzophenone (200 mg, 0.489 mmol) was reacted with *N*-carbobenzoxy-L-lysine (178 mg, 0.636 mmol), resulting in a model dye to wool mimetic molar ratio of 1:1.3. The reaction mixture was analysed by HPLC and ES mass spectrometry.

*Reaction of 4-(α,β-dibromopropionamido)benzophenone (**1**) with S-carboxymethyl-L-cysteine*

4-(α,β-Dibromopropionamido)benzophenone (200 mg, 0.489 mmol) was reacted with S-carboxymethyl-L-cysteine (114 mg, 0.636 mmol) resulting in a model dye to wool mimetic molar ratio of 1:1.3. The reaction mixture was analysed by HPLC and EI mass spectrometry.

RESULTS AND DISCUSSION

4-(α,β-Dibromopropionylamino)benzophenone as a model dye

The compound chosen to simulate a Lanasol dye molecule for the preliminary studies was 4-(α,β-dibromopropionamido)benzophenone (**1**). The use of compound **1** has a number of advantages for mechanistic investigations of this type; (i) it is easily prepared using literature methodology [9], (ii) it possesses a UV chromophore (benzophenone) which allows easy detection in TLC and HPLC and (iii) the α and β-protons and carbons are easily assigned in the NMR spectra. However, since 4-(α,β-dibromopropionamido)benzophenone exhibited low water solubility, all reactions with model wool compounds were performed in acetone/water (5:1 v/v) which was the most polar aqueous solvent mixture in which it was soluble. A disadvantage of this solvent mixture is the low boiling point (65°C), which resulted in the reactions being performed at lower temperatures than those used for commercial dyeing (100°C).

The current investigation has several advantages over previous studies [2,4] in that the reaction conditions more closely resemble those used in commercial Lanasol dyeing. Virtually all dyeing of wool with Lanasol dyes is performed under mild acidic conditions [10], so each reaction solution was maintained at pH 5. Particular attention was paid to the molar ratio of model dye (compound **1** or **2**) to wool mimetic (amine or protected amino acid) used, ensuring that it was similar to the molar ratio of a typical Lanasol dye (average molecular mass of approx. 650 g/mol [3]) to the particular amino acid present in wool fibre that was under investigation. All calculations were based on a depth of shade of 1% (omf) and the amino acid concentration of wool fibre. All products were definitively characterised using modern instrumentation (HPLC, EI and ES-MS, FT-NMR).

The effect of the solvent on 4-(α,β-dibromopropionamido)benzophenone (1)

To test the stability of the α,β-dibromopropionamido group of the model dye in the acetone/water solvent mixture used in this investigation, 4-(α,β-dibromopropionamido)benzophenone (**1**) was dissolved in acetone-d_6:D_2O

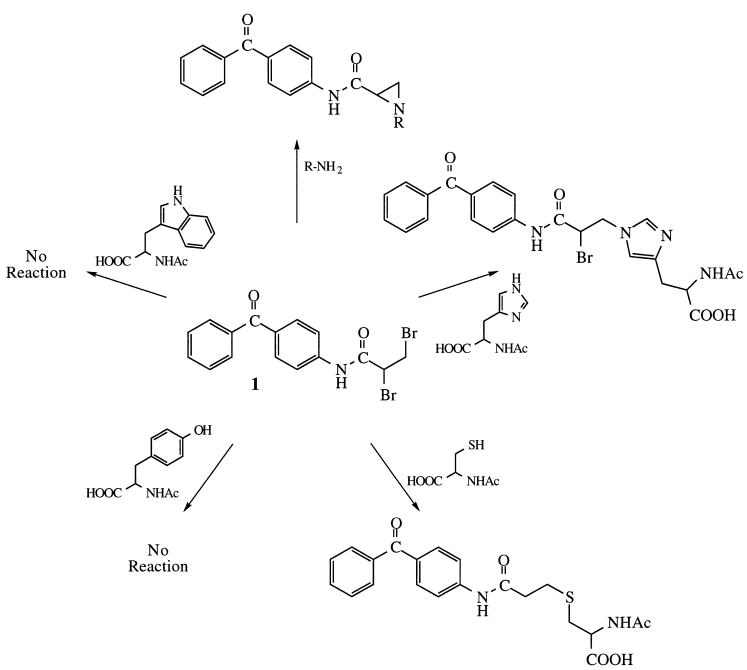
(5:1) in an NMR tube and maintained at 65°C and pH 5 for 90 min. If any 4-(α-bromoacrylamido)benzophenone (**2**) had formed, the α-bromoacrylamido resonances (in place of the α,β-dibromopropionamido resonances) would be clearly apparent in the ^{1}H-NMR spectrum. However, after 90 min no resonances attributable to compound **2** were observed. This result implies that any **2** formed during reactions between **1** and wool mimetics is not due to the acetone/water solvent system.

Reactions of 4-(α,β-dibromopropionamido)benzophenone (1) and wool mimetics

A summary of the results obtained from this study of the chemical interactions between 4-(α,β-dibromopropionamido)benzophenone and compounds that simulate the amino acid side chains of wool protein is presented as Scheme 2.

*Reaction of 4-(α,β-dibromopropionamido)benzophenone (**1**) with wool lysine mimetics*

The ε-amino side chain of lysine has been the most studied of all the amino acid functional groups believed to be involved in reactive wool dyeing. Wool



Scheme 2.

fibre contains approximately 193–277 mol/g of lysine residues [2], making it relatively abundant compared to the other amino acid residues considered to be involved in reactive dyeing of wool. In studies using model compounds, the side chain amino group of lysine is the only functional group that has been studied with Lanasol type dyes [4,11,12]. Aside from compounds containing the α-bromoacrylamido and α,β-dibromopropionamido groups, lysine has been found to be reactive with monochlorotriazine [13], chlorodifluoropyrimidyl [14], vinylsulphone [15], acrylamide [16,17] and chloroacetamide reactive dyes [18].

The reactions of several compounds representing the ε-amino group of lysine (i) cyclohexylamine, (ii) *N*α-acetyl-L-lysine-*N*-methylamide and (iii) ethylamine with **1** and **2** were investigated. The expected products are shown in Scheme 1. Taking the average ε-amino content of wool to be 246 mmol/g the molar ratio of dye to ε-amine groups was calculated to be 1:16. From the reactions containing lower molar ratios of amine (3:1 and 1:1) it is most likely that only one molar equivalent of the amine would react with **1**. It should be pointed out that there are three possible nucleophilic substitution routes for the attachment of the amine to model dye. Two of which, that to our knowledge have not been noted in the literature, involve the direct reaction with 4-(α,β-dibromopropionamido)benzophenone (**1**) and do not require the formation of **2**. Michael addition requires the α, β-unsaturated moiety to form in solution prior to covalent reaction with the amine. It is possible that any of the products formed (**3–5**) could rearrange to a structure containing an aziridine ring (**6**). For the higher amine molar ratios (1:3 and 1:16), there may be sufficient amine present in solution for two cyclohexylamine molecules to react with each molecule of **1**. This type of reaction would result in the cross linking of the protein chains.

(i) Cyclohexylamine. The 4-(α,β-dibromopropionamido)benzophenone-cyclohexylamine reaction mixtures (ratios of 3:1, 1:1, 1:3 and 1:16) were analysed by HPLC at 10 min intervals for a total reaction time of 90 min. No change was observed in the chromatograms regardless of the reactant molar ratios after a reaction time of 10 min. For the reaction with the lowest molar ratio of cyclohexylamine (3:1), there were only two peaks present, which had the same retention times as **1** (17.5 min) and **2** (16.5 min). This suggested that the amine caused dehydrobromination of **1**, but did not react covalently with either compounds **1** or **2**. When one equivalent of 4-(α,β-dibromopropionamido)benzophenone was reacted with one equivalent of cyclohexylamine, most of it was converted to the α-bromoacrylamido form. The chromatogram of the reaction mixture in which a three fold molar excess of cyclohexylamine was used contained a small peak due to **2**, no peak corresponding to starting material, and a large peak at 12.0 min which can be

attributed to the formation of a new product. A sixteen fold molar excess of cyclohexylamine resulted in a chromatogram containing only the peak at 12 minutes. These results suggest **1** was initially dehydrobrominated by the amine which then reacted with the α-bromoacrylamido component so formed. The elimination of HBr from **1** was not caused by the solvent mixture, nor was it base catalysed as the reaction mixture was maintained at pH 5.

The product was precipitated from the reaction mixture, dried and analysed. The ES–MS of the precipitate showed a parent ion at 348 m/z. This ion is consistent with either a product containing a cyclohexylamine molecule that had substituted the α-bromine of **2**, or a product containing an aziridine ring **6**. Parent ions were not observed for the other possible products depicted in Scheme 1. The structure of the product was unambiguously assigned to that containing the aziridine ring by NMR spectroscopy. It was immediately obvious from the ^{1}H-NMR spectrum of the product that it was free of resonances in the region expected for acryl protons (approx. 5–7 ppm) [19] which eliminated the α-bromine substituted structure **5**. Resonances due to aziridine protons were assigned to Hα (2.01 ppm) and Hβa (1.60 ppm). The resonance for Hβb were assumed to reside within the multiplet, which also contained resonances due to the two methylene groups at the *ortho* positions of the covalently attached cyclohexylamine unit at 1.72–1.88 ppm (based on the integration of this region). The structure of the 4-(α,β-dibromopropionamido)benzophenone/cyclohexylamine product suggested by ^{1}H-NMR spectroscopy was further supported by the ^{13}C-NMR spectrum. Resonances due to the α and β-carbons of the aziridine ring were observed at 38.3 and 34.1 ppm, respectively. Resonances were not observed for acryl carbons which would be expected at approximately 100–150 ppm [19]. All of the resonances in this region were assigned to aromatic carbons due to the benzophenone moiety of the product. Both the ^{1}H and ^{13}C-NMR assignments for the aziridine resonances observed in this investigation are consistent with those of Yazhen *et al.* [11], who investigated the reaction of *p*-(α-bromoacrylamide)benzenesulphonic acid with glycine, alanine, phenylalanine and β-aminopropionic acid in DMSO-d_6/D_2O.

It would be worthwhile to compare the reactions of wool mimetics with the two forms of the Lanasol reactive group (the α,β-dibromopropionamido form and the α-bromoacrylamido form) to investigate whether both forms would produce the same reaction products. This comparison would be best made if the two model dyes had identical chromophores. Mäusezahl carried out such an experiment using cyclohexylamine and Nα-acetyl-lysine-N-methylamide as the model wool compounds [4]. For both the monobromo and dibromo forms of the dye, the aziridine product was produced, however the reactant molar ratios and other products formed, if any, were not reported. A small quantity of 4-(α-bromoacrylamido)benzophenone (**2**) was

reacted with a sixteen fold molar excess of cyclohexylamine. The reaction mixture was analysed by HPLC, which showed only a single peak, which proved through characterisation by ES–MS and NMR to be identical to the product of the 4-(α,β-dibromopropionamido)benzophenone-cyclohexylamine (1:16 molar ratio) reaction. The absense of any **2** in the chromatogram indicated that it had reacted completely with cyclohexylamine.

(ii) Nα-*Acetyl*-L-*lysine*-N-*methylamide*. *N*α-L-lysine-*N*-methylamide is a good representation of a lysine residue bound as part of a wool protein strand, since the Nα and carboxyl groups of lysine are not available for reaction with the dye. The only part of this blocked lysine molecule available for reaction with a Lanasol dye is the *N*ε amine of its side chain. As was cyclohexylamine, *N*α-acetyl-L-lysine-*N*-methylamide was used as a wool mimetic by Mäusezahl [4], which allows a comparison of the results between both investigations.

4-(α,β-Dibromopropionamido)benzophenone was reacted with a three fold molar excess of *N*α-acetyl-L-lysine-*N*-methylamide under the standard reaction conditions used throughout this work. The HPLC chromatogram of the reaction mixture contained a major peak at 8.2 min due to the formation of a new product and a minor peak at 16.3 minutes due to compound **2**. This indicated that the 4-(α,β-dibromopropionamido)benzophenone/*N*α-acetyl-L-lysine-*N*-methylamide molar ratio of 1:3 was sufficient to dehydrobrominate **1** and covalently react with most of the **2** which formed. The reaction mixture was separated by column chromatography on silica gel using acetone/water (10:1 v/v) as the mobile phase. The purified product had a positive ion ES-MS parent ion at 451 m/z, which is consistent with either a product containing an *N*α-acetyl-L-lysine-*N*-methylamide molecule that had substituted the α-bromine of **2** or a product that contained an aziridine ring. The exact structure of this product was determined by NMR spectroscopy. The ^{1}H-NMR spectrum of the product was free of resonances in the region expected for acryl protons (approx. 5–7 ppm) [19], which eliminated the α-bromine substituted structure. Resonances due to aziridine protons were assigned to Hα (2.35–2.58 ppm), Hβ (2.18 and 2.16 ppm). Confirmation of the aziridine structure was obtained from the ^{13}C-NMR spectrum obtained from the isolated product. From these results it can be concluded that the reaction of **1** with *N*α-acetyl-L-lysine-*N*-methylamide is analogous to that of cyclohexylamine, resulting in the formation of the aziridine ring compound **6** as the only major product.

(iii) Ethylamine. Products containing only one molecule of amine were observed from the simulated Lanasol dyeing reactions studied using cyclohexylamine and *N*α-acetyl-L-lysine-*N*-methylamide, even when there

was a sixteen fold excess of amine. The reason for this may have been due to steric hindrance of the reasonably large cyclohexylamine and *N*α-acetyl-L-lysine-*N*-methylamide molecules. To minimise this possible steric hindrance, a small model wool compound containing a primary amine adjacent to a hydrocarbon moiety, ethylamine, was chosen for reaction with **1**.

Compound **1** was reacted with a sixteen fold excess of ethylamine. The HPLC of the reaction mixture contained only one peak at 7.9 min. The product was precipitated from the reaction mixture by the addition of water and dried under vacuum. This material had an EI–MS parent ion of 294 m/z, which was consistent with either a product in which ethylamine has substituted for bromine, or a product containing an aziridine ring. Again, NMR spectroscopy supported the second possibility. The ^{1}H-NMR spectrum of the product contained resonances which could be assigned to the aziridine protons (Hα: 2.16 ppm and Hβ: 2.03 and 1.73 ppm). This was confirmed by the ^{13}C-NMR spectrum, which was consistent with those obtained from the cyclohexylamine and *N*α-acetyl-L-lysine-*N*-methylamide reaction products. These results suggest that the size of the group attached to the amine is not a contributing factor in preventing two amine molecules from covalently bonding with the α and β-carbons of **1**.

*Reaction of 4-(*α,β*-dibromopropionamido)benzophenone (**1**) with* Nα*-acetyl-*L*-histidine*

Histidine contains an imidazole functional group as its side chain which incorporates a secondary amine as a possible reaction site for Lanasol dyes. Wool contains approximately 58–82 μmol/g [2] of histidine residues, which makes it approximately three and a half times less abundant than lysine, but more than twice as abundant as cysteine. Histidine has been found to be reactive towards monochlorotriazine [13,20], vinylsulphone [15,20], acrylamide [17], chlorodifluoropyrimidine [14] and chloroacetamide dyes [18,20]; however, no similar investigations have been reported for reactive dyes containing the α-bromoacrylamido or α,β-dibromopropionamido groups. The reaction of histidine with a Lanasol reactive group is very interesting, since the imidazole group contains an aromatic amine rather than a primary amine. It is unlikely that the product would contain an aziridine ring, since ring formation would require the formation of a quaternary ammonium species, which would destroy the aromaticity of the imidazole ring [7].

In this investigation, histidine residues were simulated using *N*α-acetyl-L-histidine which contains the protected α-amine group, leaving the imidazole side chain as the only available reaction site. Compound **1** was reacted with a 4.5 fold molar excess of *N*α-acetyl-L-histidine. The HPLC chromatogram of the reaction mixture contained only one significant peak at 10.5 min. The absence of peaks due to **1** and **2** indicated that the simulated Lanasol dye had

been completely converted into a different form. The reaction mixture was separated by column chromatography on silica gel using acetone/water (5:1 v/v) as the mobile phase. The purified product had a ES-MS parent ion which displayed bromine isotopic peaks at 527 and 529 m/z. A parent ion at this position was only expected for a product that contained an $N\alpha$-acetyl-L-histidine molecule that had covalently bound to the β-carbon and bromine remained bonded to the α-carbon. Parent ions were not observed for a product containing an $N\alpha$-acetyl-L-histidine molecule that had substituted the α-bromine of dehydrobrominated **1** (expected at 447 m/z), or for a product containing two molecules of $N\alpha$-acetyl-L-histidine (expected at 644 m/z).

The structure of the 4-(α,β-dibromopropionamido)benzophenone/$N\alpha$-acetyl-*L*-histidine product was confirmed by NMR spectroscopy. As anticipated, the ^{1}H-NMR spectrum of the isolated product contained no acryl resonances (expected at 5–7 ppm), while the resonance at 7.00 ppm was assigned to a methyne imidazole proton. The region from 4.38 to 4.54 ppm integrated as two protons, which were assigned to the CH resonance of the amino acid backbone of covalently bound histidine and to the resonance due to CH-Br. This assignment compared well with the CH-Br resonance (α-position) of **1** observed at 4.65 ppm. Acryl carbons were not observed in the expected region of the ^{13}C-NMR spectrum of the product (100–150 ppm). The resonances in this region were assigned to benzophenone aromatic carbons and imidazole carbons of the product. The resonance at 46.4 ppm was more characteristic of a CH-Br carbon than an aziridine CH carbon as it corresponded well to the CH-Br resonance (α-position) observed in the ^{13}C-NMR spectrum of **1** at 45.7 ppm. The presence of an aziridine ring within the structure of the product was discounted, since the difference between the α and β-carbon resonances was too large (14 ppm) based on the amine model wool compound reactions previously studied in this investigation and on the work of Yazhen et al [11] (3–8 ppm).

There are two possible mechanisms that can account for the major product identified for the reaction of $N\alpha$-acetyl-*L*-histidine with 4-(α,β-dibromopropionamido)benzophenone (**1**). The histidine compound could react in an analogous manner to the primary amines observed in this investigation, initially forming **2** by dehydrobromination and then reacting across the vinylic bromide via Michael addition. However, with a pK_a of only 6.0 [21] the imidazole secondary amine of Nα-acetyl-*L*-histidine may not be basic enough to facilitate the dehydrobromination of **1**. The other possibility is for the histidine compound to react directly with compound **1** via nucleophilic substitution of the β-bromine atom. Nucleophilic substitution of alkyl bromides by aromatic amines is a common procedure for the preparation of tertiary amines [22].

*Reaction of 4-(α,β-dibromopropionamido)benzophenone (**1**) with* Nα-*acetyl-*DL-*tryptophan*

Apart from histidine, the only other amino acid within the wool protein that contains a heterocyclic side chain is tryptophan. Wool contains approximately 35–44 μmol/g of tryptophan [2] which is similar to the cysteine content of the fibre. The most likely point of reaction with a Lanasol dye when tryptophan is present as an amino acid residue is at the amine of the indole side chain group. This amine was found to have little or no reactivity towards monochlorotriazine [20] dyes. The greater reactivity of the histidine side chain (imidazole) compared with the side chain of tryptophan (indole) was attributed to the greater participation of the unshared pair of electrons on the nitrogen atom in the aromatic system of the indole group [20].

Tryptophan residues were simulated in this investigation using *Nα*-acetyl-DL-tryptophan, which has the indole side chain as the only available reaction site. Due to the very low tryptophan content of wool, the dye to tryptophan molar ratio calculation was based on the maximum tryptophan content instead of the average content. In this way the possibility of reaction is maximised. 4-(α,β-Dibromopropionamido)benzophenone (**1**) was reacted with a 2.9 fold molar excess of *Nα*-acetyl-DL-tryptophan. The HPLC chromatogram of the reaction mixture contained peaks at 4.5 and 17.4 min. On the basis of comparison with the starting materials, the former was assigned to *Nα*-acetyl-DL-tryptophan, while the latter was assigned to compound **1**. As there were no other peaks observed in the chromatogram, it can be concluded that *Nα*-acetyl-DL-tryptophan did not react with **1** under the experimental dyeing conditions used. A reaction may occur under alkaline conditions, but such conditions are not used in the application of Lanasol dyes to wool.

*Reaction of 4-(α,β-dibromopropionamido)benzophenone (**1**) with* N-*acetyl-*L-*cysteine*

Cysteine contains a thiol functional group in the β-position of its side chain. Wool contains approximately 20–40 μmol/g [2] of cysteine residues and hence is, on average, eight times less abundant than lysine. The thiol side chain of cysteine has been found to react with reactive dye groups, including monochlorotriazine [13], acrylamide [16], chloroacetamide [18] and chlorodifluoropyrimidine [14]. It has been observed that the side chain of cysteine is more reactive than the ε-amine group of lysine [13,15,18]. In fact, for vinyl sulphone dyes, thiols were found to be approximately one hundred times more reactive than amines [15].

In this investigation, cysteine residues were simulated using *N*-acetyl-L-cysteine in which the α-amino group is protected, leaving the thiol side chain available for reaction. As the cysteine content of wool is very low, comparable

to that of histidine, the dye to cysteine molar ratio used was calculated in a similar manner. 4-(α,β-Dibromopropionamido)benzophenone (**1**) was reacted with a 2.6 fold molar excess of *N*-acetyl-L-cysteine. The HPLC chromatogram of the reaction mixture contained only one significant peak at 10.2 min and there was no evidence of unreacted **1** or **2**. This is consistent with the greater reactivity of the cysteine-thiol group compared with the side chain amine of lysine, since a three fold molar excess of *N*α-acetyl-L-lysine-*N*-methylamide did not react completely under the same conditions.

The major product was isolated from the reaction mixture by column chromatography on silica gel using acetone:water (10:1) as the mobile phase, and the purified product was found to have a positive ion ES–MS parent ion at 415 m/z. Unlike the product isolated from the reactions using amines as model wool compounds, this ion did not correspond to any of the expected structures. The parent ion of a product containing an *N*-acetyl-L-cysteine molecule that had substituted the α-bromine of the model dye would be expected at 413 m/z, while a product containing a thiirane ring would be expected at 414 m/z. It is unlikely that the structure containing a thiirane ring would be stable due to the positively charged sulphur atom. Thiiranium cations are quite reactive towards nucleophiles [7] and these model compound reactions were performed in a nucleophilic solvent system. Parent ions were not observed for a product containing an *N*-acetyl-L-cysteine molecule that had reacted across the double bond of dehydrobrominated **1** (expected at 493 m/z) or for a product containing two molecules of *N*-acetyl-L-cysteine (expected at 576 m/z).

A clearer picture of the structure of the isolated 4-(α,β-dibromopropionamido)benzophenone/N-acetyl-L-cysteine product was gained from NMR spectroscopy. The ^{1}H-NMR spectrum of the product contained no resonances due to acryl protons (approx. 5–7 ppm) [19] which eliminated the α-bromine substituted structure. Resonances due to methine-bromine and methylene-bromine groups (3.5–5.5 ppm) were also absent from the spectrum. Thus, it was most likely that both of the original bromine atoms of **1** were eliminated during the reaction with *N*-acetyl-L-cysteine. Aside from the methylene resonance of the bound *N*-acetyl-L-cysteine molecule (2.86–3.18 ppm), the spectrum of the product contained resonances due to two new methylene groups (2.74 and 2.89 ppm) which were not present in the spectra of the reactants. These new methylene groups were also observed in the ^{13}C-NMR spectrum of the product and verified from the corresponding DEPT spectrum.

The structure consistent with the spectral data has the *N*-acetyl-L-cysteine attached to the β carbon and has lost bromine from the α-carbon (see Scheme 2).

The expected parent ion of this molecule calculated to 415 m/z which matches that of the + ES mass spectrum of the isolated product. This structure

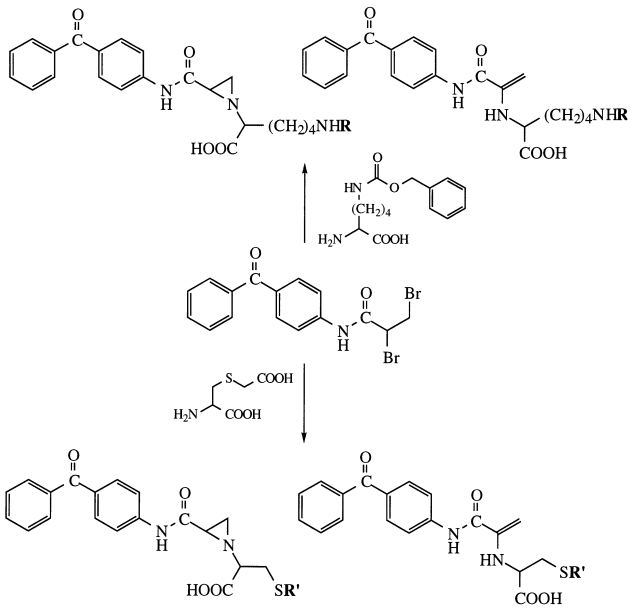
also explains the two adjacent methylenes (one attached to sulphur) which were observed in the ^{1}H and ^{13}C-NMR spectra. The formation may have resulted from the thiol of the cysteine initially substituting for the β-bromine (in a similar fashion to the NH of histidine) to form α-bromo-β-substituted intermediate. The subsequent loss of the bromine may have resulted from direct reduction of this intermediate or, alternatively, from elimination of HBr followed by transfer hydrogenation of the resultant double bond. Both possibilities seem plausable in the presence of a reducing agent such as a thiol.

*Reaction of 4-(α,β-dibromopropionamido)benzophenone (**1**) with a wool tyrosine mimetic*

Tyrosine contains a phenolic hydroxyl side chain and has a concentration in wool of approximately 350–380 μmol/g [2] making it the most abundant of all of the amino acids explored in this investigation. The phenolic hydroxyl group of tyrosine has also been found to react with monochlorotriazine [13,20], chloroacetamide [18], chlorodifluoropyrimidine [14] and acrylamide reactive dyes [16]. It was also observed to react very slowly with vinyl sulphone dyes [15]. In this investigation tyrosine residues were simulated using *N*-acetyl-L-tyrosine. 4-(α,β-Dibromopropionamido)benzophenone (**1**) was reacted with a twenty-four fold molar excess of *N*-acetyl-L-tyrosine. The HPLC chromatogram of the reaction mixture contained only one peak at 17.4 min, which was assigned to **1**. The absence of any other peaks in the chromatogram indicated that, under the reaction conditions employed, *N*-acetyl-L-tyrosine did not react with the model dye.

Reactions of 4-(α,β-dibromopropionamido)benzophenone (1) and wool N-terminal mimetics

A summary of the results obtained from this study of the chemical interactions between **1** and model compounds that simulate the N-terminal groups of wool proteins is presented as Scheme 3. The concentration of N-terminal groups within wool protein is extremely low (10–20 μmol/g) [2] compared with the total amount of amino acid reactive side chains available for covalent bonding with Lanasol dyes. Since this investigation has shown no evidence that two amino acid side chains will covalently bind with one molecule of 4-(α,β-dibromopropionamido)benzophenone (**1**), regardless of their functional group (i.e. amine, imidazole, indole, thiol or hydroxyl), it was necessary to determine whether the N-terminal amine groups were capable of reacting. If this is the case, then it would be possible for cross links between the N-terminal amino groups of wool protein chains to form. N-terminal amine groups (normally simulated using glycine) have been found to react with monochlorotriazine [13,20], acrylamide [17], chloroacetamide [20] and



Scheme 3. (R = Carbobenzoxy, R′ = S-carboxymethyl).

vinylsulphone [15,20] reactive dyes. The N-terminal amines can be simulated using any amino acid containing a free amine in the α position and either a side chain that was unreactive towards Lanasol type dyes or a side chain blocked by a protecting group (e.g. carbobenzoxy or carboxymethyl groups). Glycine is the obvious example as it contains no side chain, but could not be used in this study as it was not soluble in the reaction solvent (acetone:water 5:1). Two amino acids containing blocked side chains and free α-amine groups were found to be soluble, namely *N*ε-carbobenzoxy-L-lysine (*N*ε-CBZ-lys) and *S*-carboxymethyl-L-cysteine. The reaction of these compounds with 4-(α,β-dibromopropionamido)benzophenone (**1**) was investigated using a 1:1.3 molar ratio of dye to N-terminal amine.

*Reaction of 4-(α,β-dibromopropionamido)benzophenone (**1**) with* Nε-*carbobenzoxy*-L-*lysine*

The HPLC chromatogram of the 4-(α,β-dibromopropionamido)benzophenone/*N*ε-CBZ-lys reaction solution contained a major peak at 13.6 min and a minor peak at 8.5 min. No residual **1** (or **2**) was present. The negative ion ES–MS of the reaction solution contained a molecular ion at 528 *m*/*z* which corresponded to either a structure where the α-bromine of dehydrobrominated **1** had been substituted for an Nε-CBZ-lys molecule or a structure containing an aziridine ring. Parent ions were not observed for any of

the other possible products. Two peaks were present in the mass spectrum of higher mass to charge ratio than the peak at 528 m/z (32% relative abundance). These were observed at 559 m/z (8% relative abundance) and 582 m/z (5% relative abundance). A logical explanation for the existence of either peak could not be found; however, they may be related to the minor peak at 8.5 min observed in the HPLC chromatogram of the reaction mixture.

Reaction of 4-(α,β-dibromopropionamido)benzophenone with S-carboxymethyl-L-cysteine

The HPLC chromatogram of the 4-(α,β-dibromopropionamido)benzophenone/*S*-carboxymethyl-L-cysteine reaction solution contained one major peak at 14.5 min and minor peaks at 9.9 and 10.0 min. Peaks corresponding to **1** and **2** were absent. The negative ion ES–MS of the reaction solution contained a molecular ion at 427 m/z which, similar to the *N*ε-CBZ-lys reaction product, could correspond to either to a substitution or an aziridine ring containing structure. Similarly, parent ions were not observed for any of the other possible products.

CONCLUSIONS

The investigations under simulated dyeing conditions involving 4-(α,β-dibromopropionamido)benzophenone (**1**) in solution allowed several conclusion about the Lanasol dyeing process to be drawn. Results were obtained indicating that the α,β-dibromopropionamido group was initially dehydrobrominated by the amine, forming the α-bromoacrylamido group which then covalently reacted with the amine. This is supported by the products formed during the reaction of the monobromo model dye compound **2**. This investigation does not support the assumptions that a Lanasol type dye containing one reactive group is capable of covalently reacting with two functional groups of wool which would result in a cross-link between two wool protein chains. Using a range of compounds containing functional groups considered to be involved in the wool dyeing process, including primary amines, heterocyclic amines, a thiol and a hydroxyl group, for those compounds where a reaction occurred, a single product containing one molecule of model dye to one molecule of model wool compound was observed. With the primary amines, the products contained an aziridine ring, while the products from *N*α-acetyl-L-histidine and *N*-acetyl-L-cysteine contained the amino acid attached to the β-carbon of **1**. Under the simulated dyeing conditions used in this study, *N*α-acetyl-DL-tryptophan and *N*-acetyl-L-tyrosine were not reactive towards 4-(α,β-dibromopropionamido)benzophenone. Steric hindrance was excluded as a factor in preventing a second model compound reacting

with a molecule of Lanasol type dye, as the reaction of the model dye **1** with ethylamine formed an aziridine ring only.

Although the products from the reactions of the model compounds simulating the N-terminal amine groups of wool protein were not isolated, several conclusions about these reactions could be made from the HPLC and mass spectral analyses. It was shown that the $N\alpha$-amine of amino acids covalently reacted with **1**. The major products observed in the analyses were consistent with structures where the amino acid either substituted the α-bromine of dehydrobrominated **1** and/or formed a product containing an aziridine ring. Evidence for a product containing two molecules of a compound representing the N-terminal groups was not observed which suggests that these groups are probably not capable of forming cross-links between protein chains within wool.

No products or intermediates could be detected by the methods of analysis used in this study that would distinguish between the two postulated pathways to the formation of the aziridine ring, namely the nucleophilic substitution-aziridine formation or the Michael addition-aziridine formation pathways. It was shown by HPLC that the reactions of cyclohexylamine with **1** had concluded in 10 min or less regardless of the reactant molar ratios. Therefore, ring closure of these intermediates probably occurred too quickly for evidence of their existence to be observed.

In Part II of this study we will report on the reaction of a sulfonated version of the model dye compound with amino acids and small peptides, as well as on the results of reactions with an actual Lanasol dye. As this work was carried out in a totally aqueous system, the reaction conditions mimic those used in industrial dyeing more closely than any other study currently in the literature.

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