Chapter 2

Synthesis and Characterisation of 3OHKyn Amino Acid Adducts

2.1 Introduction

Human lenses become more fluorescent with age[93] due to PTM of the structural proteins. Although a number of compounds have been reported as adducts to human lens proteins,[104-107] it is not known which of these is primarily responsible for the increased fluorescence.

At neutral pH, UV filters undergo deamination of the amino acid side chain to produce intermediate compounds that are susceptible to nucleophilic attack via Michael addition.[109] Amino acid residues such as Cys, His, and Lys are known to be the most reactive with UV filters.[110,111,187] 3OHKynG and Kyn, become covalently attached to proteins in the human lens in an age-dependent manner.[101,103] At the same time, the level of free UV filters in the lens decreases.[19] This is especially true after the lens barrier forms at middle age,[88-90] when UV filters reside for longer time periods within the nuclear region of the lens and are able to deaminate to a greater extent.

3OHKyn differs in one very important regard from the other Kyn UV filters, in that, the aromatic portion of 3OHKyn can readily oxidise.[181,184,188] In model systems, numerous dimeric and other coloured products result from oxidation of 3OHKyn under physiological conditions.[181,184] If such processes also occur after 3OHKyn has become linked to proteins, coloured, insoluble and crosslinked proteins could result. It has been proposed that this could be a contributing factor in the development of human ARN cataract,[100,185,186,189,190] but proof has been lacking largely because of the problems associated with analysing the low levels of such a readily oxidised molecule.

As a first step in testing this theory, adducts (Figure 2.1) between 3OHKyn and the nucleophilic amino acids, Cys, His and Lys, were prepared and characterised by mass spectrometry, NMR spectroscopy, UV-visible and 3-D fluorescence spectroscopy. The

stability of each adduct at pH 4.0 and 7.2 was examined, and the stability of each adduct to acid hydrolysis was also examined.

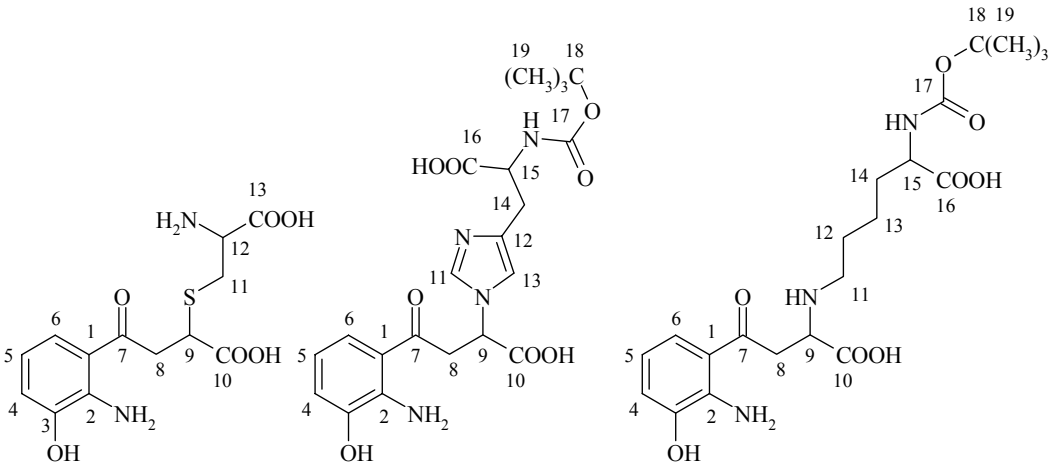


Figure 2.1 Structures of the 3OHKyn amino acid adducts.

2.2 Materials and Methods

2.2.1 Materials

All organic solvents and acids were HPLC grade (Ajax, Auburn, NSW, Australia). Milli-Q® water (purified to 18.2 MΩ/cm^2) was used in the preparation of all solutions. The amino acids (N-α-*t*-Boc-L-His, N-α-*t*-Boc-L-Lys and L-Cys), formic acid, hydrochloric acid (HCl) (6 M, sequencing grade), 3OHKyn, phenol, thioglycolic acid and trifluoroacetic acid (TFA) were obtained from Sigma-Aldrich Chemical Co. (St. Louis, MO, U.S.A.).

2.2.2 Synthesis and Purification of 3OHKyn Modified Amino Acids

3OHKyn (50 mg) was dissolved in 50 mM Na_2CO_3/$NaHCO_3$ buffer, pH 9.5 (30 mL). The amino acids (N-α-*t*-Boc-L-His, N-α-*t*-Boc-L-Lys and L-Cys) were added in 10-fold molar excess. The pH was readjusted to 9.5 with 0.1 M NaOH if required, and then the resulting solution was bubbled with argon, sealed, wrapped in foil and incubated at 37^{0}C for 48 hours.[103] After adjusting the pH to between 4 and 5 with glacial acetic acid, the resulting mixture was separated by semi-preparative or analytical HPLC. The yield, NMR, MS/MS data, and high resolution mass spectra are given below.

***N*-α-*tert*-Butoxycarbonyl-L-lysyl-3-hydroxy-DL-kynurenine (3OHKyn-*t*-Boc-Lys)**: 11.3 mg, 11%. Found: MH^+, 454.2264. Calculated for $C_{21}H_{32}N_3O_8$: MH^+, 454.2189; ESI-MS/MS of *m/z* 454 (MH^+), 354 (100%), 247 (7%), 208 (10%), 203 (96%), 152 (24%), 147 (28%), 128 (12%).

δH 7.54 (1H, d, *J* 8.0, H-6), 7.16 (1H, d, *J* 7.0, H-4), 6.96 (1H, t, *J* 8.0, 8.0, H-5), 4.20 (1H, m, H-9), 4.12 (1H, m, H-15), 3.82 (2H, m, CH_2-8), 3.19 (2H, m, CH_2-11), 1.89 (1H, m, CH_2-14), 1.81 (2H, m, CH_2-12), 1.75 (1H, m, CH_2-14), 1.51 (2H, m, CH_2-13), 1.29 (9H, s, 3xCH_3);

δC 200.2 (CO-7), 172.8 (CO-10), 146.5 (C-3), 134.1 (C-2), 122.8 (C-6), 120.2 (C-4), 120.1 (C-5), 81.8 (C-18), 57.3 (C-9), 53.7 (C-15), 47.1 (C-11), 38.6 (C-8), 30.1 (C-14), 27.7 (C-19), 25.0 (C-12), 22.3 (C-13).

***N*-α-*tert*-Butoxycarbonyl-L-histidyl-3-hydroxy-DL-kynurenine (3OHKyn-*t*-Boc-His)**: 8.6 mg, 9%. Found: MH^+, 463.1771. Calculated for $C_{21}H_{27}N_4O_8$: MH^+, 463.1829;

ESI-MS/MS of *m/z* 463 (MH^+), 407 (52%), 363 (100%), 317 (15%), 209 (10%), 208 (12%), 156 (52%), 110 (5%).

δH 8.75 (1H, s, H-11), 7.42 (1H, d, *J* 8.0, H-6), 7.34 (1H, s, H-13), 7.03 (1H, d, *J* 8.0, H-4), 6.90 (1H, t, *J* 7.5, 8.0, H-5), 5.47 (1H, m, H-9), 4.30 (1H, m, H-15), 3.92 (2H, m, CH_2-8), 3.17 (1H, dd, *J* 15.5, 15.5, CH_2-14), 2.97 (1H, m, CH_2-14), 1.93 (9H, s, 3xCH_3);

δC 199.1 (CO-7), 172.4 (CO-10), 147.0 (C-3), 135.6 (C-11), 135.3 (C-2), 129.7 (C-12), 122.6 (C-6), 122.0 (C-5), 120.7 (C-4), 119.9 (C-13), 119.2 (C-1), 81.8 (C-18), 59.1 (C-9), 52.8 (C-15), 41.9 (C-8), 27.5 (C-19), 26.8 (C-14), 26.8 (C-14).

L-Cysteinyl-3-hydroxykynurenine (3OHKyn-Cys): 12.4 mg, 17%. Found: MH^+, 329.0789. Calculated for $C_{13}H_{17}N_2O_6S$: MH^+, 329.0807; ESI-MS/MS of *m/z* 329 (MH^+), 311 (30%), 240 (10%), 208 (60%), 202 (100%), 190 (65%), 162 (80%), 122 (5%), 110 (12%).

δH 7.69 (1H, d, *J* 7.5, H-6), 7.43 (1H, t, *J* 8.0, 8.5, H-5), 7.32 (1H, d, *J* 8.5, H-4), 4.35 (1H, m, H-12), 3.97 (1H, m, H-9), 3.77 (1H, m, CH_2-8), 3.66 (1H, d, *J* 4.5, CH_2-8), 3.43 (1H, d, *J* 4.5, CH_2-11), 3.33 (1H, d, *J* 5.0, CH_2-11);

δC 200.5 (CO-7), 175.9 (CO-10), 170.8 (CO-13), 150.4 (C-3), 128.3 (C-5), 127.3 (C-1), 122.2 (C-6), 121.5 (C-4), 118.6 (C-2), 52.1 (C-12), 41.7 (C-9), 40.9 (C-8), 31.6 (C-11), 31.3 (C-11).

2.2.3 High Performance Liquid Chromatography (HPLC) for Purification of the 3OHKyn Amino Acid Adducts

Reversed phase high performance liquid chromatography (RP-HPLC) was performed on an ICI HPLC system (ICI Instruments, Australia). For analytical scale separation of the 3OHKyn amino acid adducts, a Phenomenex column (Jupiter C18, 300 Å, 5 μm, 4.6 x 250 mm) was used with the following mobile phase conditions: solvent A (aqueous 0.05% (v/v) TFA) for 5 minutes followed by a linear gradient of 0-50% solvent B (80% (v/v) acetonitrile/H_2O, 0.05% (v/v) TFA) over 20 minutes, followed by a linear gradient of 50-100% B over 15 minutes and re-equilibration in the aqueous phase for 15 minutes. The flow rate was 1 mL/min. Semi-preparative separations were performed using the

same conditions as those for the analytical separations except that a Hypersil® (BDS C18, 5 μm, 10 x 250 mm) column was used at a flow rate of 3 mL/min.

2.2.4 Formation of the 3OHKyn Amino Acid Adducts at pH 7.2

3OHKyn (1.12 mg) and either N-α-*t*-Boc-L-His, N-α-*t*-Boc-L-Lys or Cys (25-fold molar excess) were dissolved in 0.1 M phosphate buffer, pH 7.2 (5 mL), and chloroform (20 μl) was added to inhibit bacterial growth. The pH was readjusted to 7.2 with 4 M NaOH if required, and the resulting solution was bubbled with argon, sealed, wrapped in foil and incubated at 37^0C for 5 days. Aliquots were taken daily and examined by analytical HPLC in order to determine the yield of the adducts. pH remained constant during the entire incubation.

2.2.5 Acid Hydrolysis of 3OHKyn Amino Acid Adducts

Each 3OHKyn amino acid adduct (~0.5 mg) was hydrolysed in an evacuated hydrolysis tube with 6 M HCl (1 mL), thioglycolic acid (5% v/v), and phenol (1% w/v) for 24 hours at 110^0C.[165] Following hydrolysis, the mixture was freeze dried, dissolved in 0.1% (v/v) TFA and purified by HPLC.

2.2.6 Stability of 3OHKyn Amino Acid Adducts at pH 4.0

3OHKyn amino acid adducts (0.2 mg) were dissolved in 0.1 M sodium acetate/acetic acid buffer, pH 4.0 (3 mL), and the resulting solutions were bubbled with either argon or oxygen before being sealed, wrapped in foil, and incubated for 48 hours at 37^0C. Aliquots were taken every 12 hours and examined by HPLC in order to determine the recovery of the adducts.

2.2.7 Stability of 3OHKyn Amino Acid Adducts at pH 7.2

3OHKyn amino acid adducts (0.2 mg) were dissolved in 0.1 M phosphate buffer, pH 7.2 (3 mL), and chloroform (20 μL) was added to inhibit bacterial growth. The pH was adjusted to 7.2 with 4 M NaOH if required. The resulting solutions were bubbled with either argon or oxygen before being sealed, wrapped in foil, and incubated for 120 hours at 37^0C. Aliquots were taken every 24 hours and examined by HPLC.

2.2.8 Incubation of 3OHKyn-Cys in the Presence of Excess N-α-*t*-Boc-His and N-α-*t*-Boc-Lys

3OHKyn-Cys (2 mg) was dissolved in 0.1 M phosphate buffer, pH 7.2 (4 mL) with a 20-fold molar excess of N-α-*t*-Boc-His, and a 20-fold molar excess of N-α-*t*-Boc-Lys. Chloroform (20 μL) was added to inhibit bacterial growth, and the pH was adjusted to 7.2 with 4 M NaOH if required. The solution was bubbled with argon, sealed wrapped in foil and incubated for 48 hours at 37^0C. Aliquots were taken every 12 hours and examined by HPLC.

2.2.9 HPLC for Acid Hydrolysis and Stability Studies

RP-HPLC was performed on a Shimadzu SCL-10A VP system controller, with an LC-10AT VP pump, SIL-10AD VP auto injector with a 500 μL loop, and a SPD-M10AVP diode array detector. Analytical scale separations, were performed on a Phenomenex column (Jupiter C18, 300 Å, 5μm, 4.6 x 250 mm). The following gradient was used: solvent A (aqueous 0.1% (v/v) TFA) for 5 minutes followed by linear gradient of 0-50% solvent B (80% (v/v) acetonitrile/H_2O, 0.1% (v/v) TFA) over 20 minutes followed by a linear gradient of 50-100% B over 15 minutes and re-equilibration in the aqueous phase for 15 minutes. The flow rate was 0.5 mL/min.

2.2.10 Mass Spectrometry

Electrospray ionisation mass spectrometry (ESI-MS) was performed on a Quadrupole Time-of-Flight Q-TOF2 hybrid mass spectrometer (Micromass, Manchester, UK) in positive ion mode. Samples were dissolved in 50% (v/v) aqueous acetonitrile, 0.2% (v/v) formic acid and injected into a Rheodyne injector with a 10 μl loop. Nitrogen was used as both the bath and nebulizing gas. The capillary voltage was 2.8 kV and the cone voltage ranged from 20 eV to 40 eV. The mass spectrometer was calibrated with (Glu^1)-Fibrinopeptide B (0.5 pmol/μl). Typically 10-20 scans were summed to obtain representative spectra. Spectra were acquired with an integration time of 2.4 seconds and a 0.1 second delay.

Nanoelectrospray ionisation mass spectrometry (nanoESI-MS) was performed on a Quadrupole Time-of-Flight Q-TOF2 hybrid mass spectrometer (Micromass,

Manchester, UK) in positive ion mode. Samples were dissolved in 5-10 μL of solvent (50% (v/v) aqueous acetonitrile, 0.2% (v/v) formic acid) and 2 μL loaded into medium NanoES spray capillaries (ES380, Proxeon Biosystems, Denmark), using a 10 μL syringe (SGE, Australia). The capillary voltage was 0.8 kV.

2.2.11 Tandem Mass Spectrometry (MS/MS)

Tandem mass spectrometry (MS/MS) spectra were acquired using the same conditions as above. The collision energy ranged from 20 eV to 30 eV.

2.2.12 High Resolution Mass Spectrometry

High resolution mass spectra were acquired on the Q-TOF2 mass spectrometer. Samples were dissolved in 50% (v/v) aqueous acetonitrile, 0.2% (v/v) formic acid. The molecular ions were calibrated against a lock mass arising from a co-injection of a solution of polyethylene glycol in 50% (v/v) aqueous acetonitrile with 1% (v/v) ammonia.

2.2.13 NMR Spectroscopy

One-dimensional (1-D) and two-dimensional (2-D) ^{1}H and ^{13}C NMR spectra were recorded on a Varian Mercury 500 Fourier Transform spectrometer. The spectrometer operated at 500 MHz for ^{1}H NMR and 125 MHz for ^{13}C NMR respectively. For each compound the following 2-D experiments were performed: ^{1}H-^{1}H correlation spectroscopy (gCOSY), and ^{1}H-^{13}C heteronuclear spectroscopy (gHMBC and gHSQC). All experiments were run in D_2O (1 mL) to which deuterium chloride (DCl) (1 μL) was added to enhance stability. The ^{1}H and ^{13}C NMR data are reported as chemical shifts (δ) in parts per million (ppm). Samples were referenced to HOD at 4.81 ppm and in the case of carbon, acetonitrile was added and referenced at 117 ppm. Coupling constants are given in Hz. The integrated intensity, multiplicity and general assignment of each resonance are described in parentheses. The abbreviations used to describe the multiplicity are as follows: d: doublet, dd: doublet of doublets, m: multiplet, s: singlet, and t: triplet.

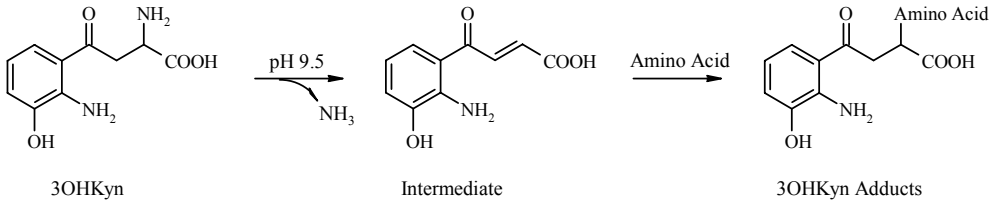
2.2.14 Fluorescence and UV-visible Spectroscopy

Fluorescence spectra were obtained on a Hitachi F-4500 fluorescence spectrometer (Tokyo, Japan) in three-dimensional (3-D) scan mode. Slit widths were routinely 10 nm for excitation and 5 nm for emission, the scan speed was 12 000 nm/min, the sampling interval was 10 nm. UV-visible absorbance spectra were recorded on a Varian CARY 500 Scan UV-Vis-NIR spectrophotometer (Palo Alto, CA, USA).

2.3 Results

2.3.1 Synthesis of the 3OHKyn Amino Acid Adducts

The 3OHKyn amino acid adducts were synthesised by incubation of N-α-*t*-Boc protected His and Lys (to prevent modification of the α-amino group) and free Cys with 3OHKyn at pH 9.5 for 48 hours. Under these high pH conditions, side chains of Kyn derivatives deaminate more readily than at neutral pH.[109] At a pH above neutral pH UV filters undergo deamination to form reactive intermediates (see Scheme 2.1).[109] These deaminated compounds are then highly susceptible to nucleophilic attack via a Michael addition. In the lens, the side chain of amino acid residues, Cys, His and Lys and GSH, are examples of nucleophiles that are known to covalently attach to Kyn and 3OHKynG.[82,103] The aim was to synthesise the corresponding Cys, His and Lys adducts of 3OHKyn (Scheme 2.1).



Scheme 2.1 Synthesis of 3OHKyn amino acid adducts. 3OHKyn is deaminated at pH 9.5, and the intermediate compound is susceptible to nucleophilic attack via a Michael addition. Amino acid side chains of Cys, His or Lys were covalently attached to the 3OHKyn amino acid side chain.

Initially the reaction mixtures were yellow, but after 48 hours of incubation each solution had turned brown in colour. The crude sample mixtures were purified by RP-HPLC (the conditions are described in Section 2.2.3). The products were monitored at 360 nm. A yield of 17% was recorded for the pure 3OHKyn-Cys adduct, 9% for 3OHKyn-*t*-Boc-His, and 11% for the 3OHKyn-*t*-Boc-Lys adduct. Low yields were presumably obtained for each adduct since 3OHKyn readily oxidises under basic conditions with the formation of numerous products.[181,184]

The HPLC chromatogram (Figure 2.2) for the purification of 3OHKyn-Cys exhibited 2 peaks, eluting at 15.6 min and 18.9 min. Each peak was initially examined by mass spectrometry. The mass spectrum for the first peak eluting at 15.6 min had an abundant molecular ion at *m/z* 225, consistent with unreacted 3OHKyn. The mass spectrum of the peak at 18.9 min had a molecular ion at *m/z* 329 (theoretical molecular ion of 3OHKyn-Cys). This product was freeze dried to produce a yellow powder that was further characterised by MS/MS, NMR, UV-visible and 3-D fluorescence spectroscopy.

The HPLC chromatogram for the purification of 3OHKyn-*t*-Boc-His is shown in Figure 2.3. Six major peaks eluted at 14.8, 18.3, 19.8, 22.6, 25.0, and 26.2 min. Each peak was initially examined by mass spectrometry. The peak at 14.8 min was confirmed as unreacted 3OHKyn. The peak at 18.3 min contained unreacted N-α-*t*-Boc-L-His with an *m/z* 256. The peak at 19.8 min could not be identified. The peak at 22.6 min was identified as 3OHKyn-yellow (an intramolecular cyclisation product of 3OHKyn[191]) *m/z* 208 by mass spectrometry. The peak at 25.0 min was identified as xanthommatin (an oxidation product of 3OHKyn[181,184]). Finally the peak at 26.2 min had a molecular ion at *m/z* 463 (theoretical molecular ion of 3OHKyn-*t*-Boc-His). This product was further characterised by MS/MS, NMR, UV-visible and 3-D fluorescence spectroscopy.

The HPLC chromatogram for the purification of 3OHKyn-*t*-Boc-Lys is shown in Figure 2.4. Six major peaks eluted at 14.8, 18.7, 20.1, 22.5, 25.0, and 26.8 min. Each peak was initially examined by mass spectrometry. The peak at 14.8 min was identified as unreacted 3OHKyn. The peak at 18.7 min contained unreacted N-α-*t*-Boc-L-Lys with a molecular ion at *m/z* 247. The peak at 20.1 min could not be identified, the peak at 22.5 min was identified as 3OHKyn-yellow. The peak at 25.0 min was identified as xanthommatin. Finally the peak at 26.8 min had a molecular ion at *m/z* 454 (theoretical molecular ion of 3OHKyn-*t*-Boc-Lys). This product was also further characterised by MS/MS, NMR, UV-visible and 3-D fluorescence spectroscopy.

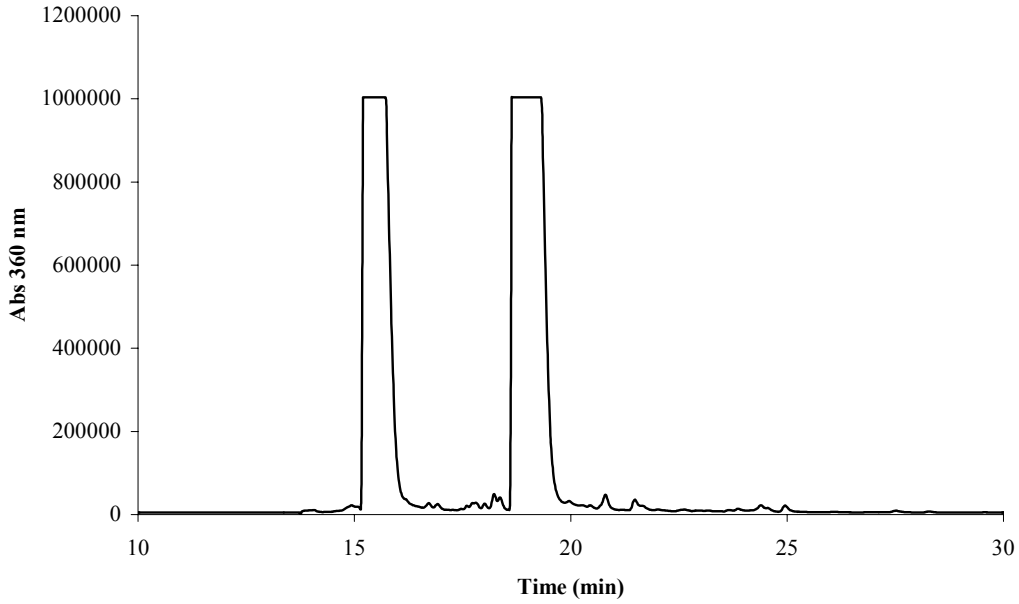


Figure 2.2 HPLC trace of 3OHKyn and L-Cys. 3OHKyn was incubated with L-Cys at pH 9.5, 37^0C for 48 hours (UV detection monitored at 360 nm). The peak at 15.6 min is unreacted 3OHKyn, and the peak at 18.9 min is the 3OHKyn amino acid adduct, 3OHKyn-Cys.

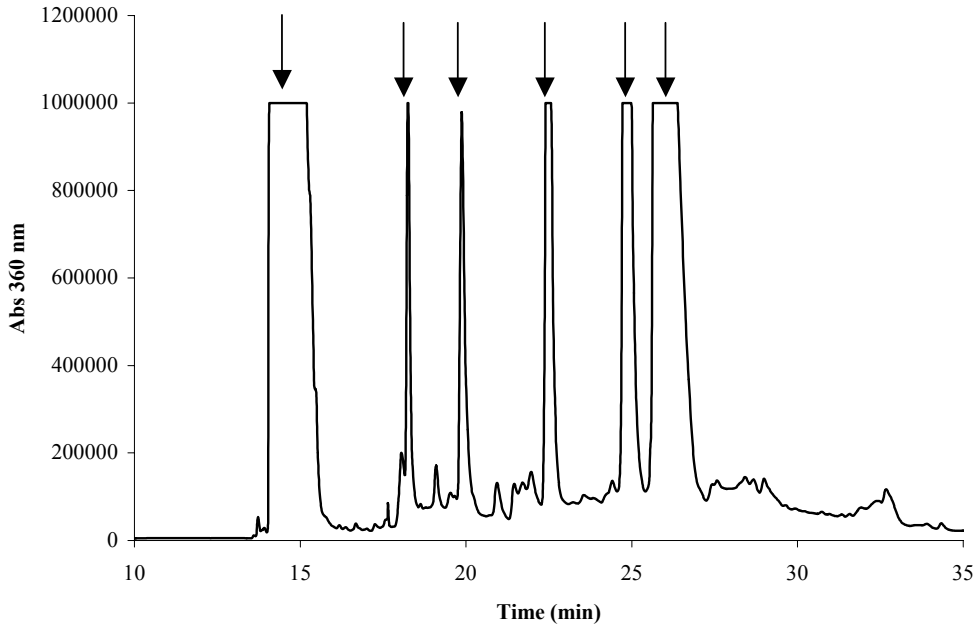


Figure 2.3 HPLC trace of 3OHKyn and N-α-*t*-Boc-L-His. 3OHKyn was incubated with N-α-*t*-Boc-L-His at pH 9.5, 37^0C for 48 hours (UV detection monitored at 360 nm). The peak at 14.8 min is unreacted 3OHKyn, the peak at 18.3 min contained N-α-*t*-Boc-L-His, the peak at 19.8 min is unknown. The peak at 22.6 min is 3OHKyn-yellow, the peak at 25.0 min is xanthommatin, and the peak at 26.2 min is the 3OHKyn amino acid adduct, 3OHKyn-*t*-Boc-His.

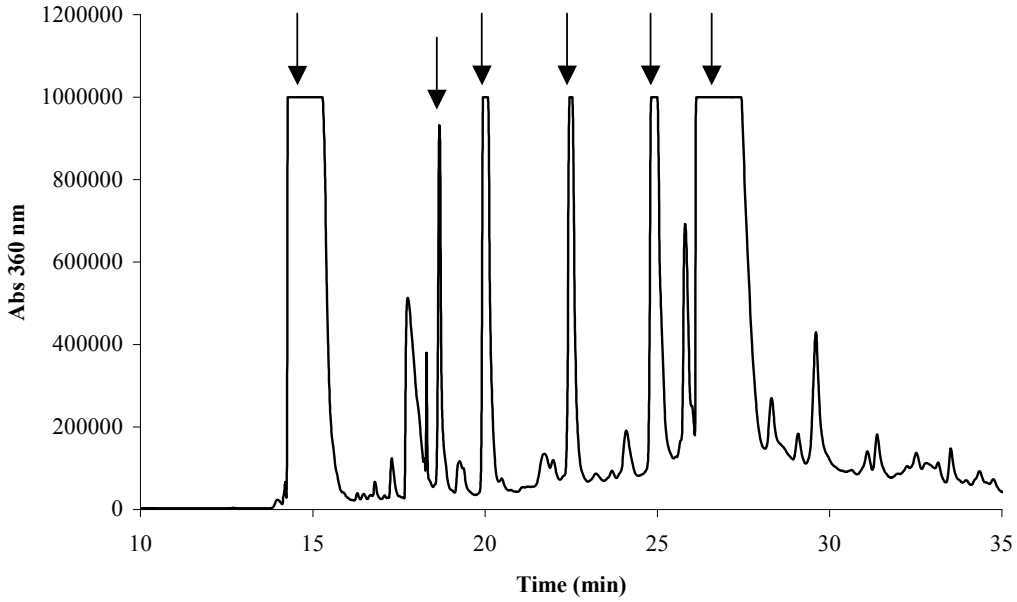


Figure 2.4 HPLC trace of 3OHKyn and N-α-*t*-Boc-L-Lys. 3OHKyn was incubated with N-α-*t*-Boc-L-Lys at pH 9.5 and 37^0C for 48 hours (UV detection monitored at 360 nm). The peak at 14.8 min is unreacted 3OHKyn, the peak at 18.7 min contained N-α-*t*-Boc-L-Lys, the peak at 20.1 min is unknown. The peak at 22.5 min is 3OHKyn-yellow, the peak at 25.0 min is xanthommatin, and the peak at 26.8 min is the 3OHKyn amino acid adduct, 3OHKyn-*t*-Boc-Lys.

2.3.2 Formation of the 3OHKyn Amino Acid Adducts at pH 7.2

The rate of deamination of UV filter side chains increases as the pH increases. 3OHKyn is an *o*-aminophenol compound and is known to be unstable under basic conditions. The 3OHKyn amino acid adducts were synthesised at pH 9.5 based on previous studies[103] to encourage deamination, but to understand the reactivity at physiological pH, the relative formation of these adducts at pH 7.2 was also examined. 3OHKyn was incubated with N-α-*t*-Boc-L-His, N-α-*t*-Boc-L-Lys and Cys separately at pH 7.2 and 37^0C, for 5 days. Figure 2.5 shows the relative rate of formation of the 3OHKyn amino acid adducts over a 5-day incubation.

The initial amount of the adducts formed after 1 day of incubation was 3OHKyn-*t*-Boc-His, greater than 3OHKyn-Cys and greater than 3OHKyn-*t*-Boc-Lys. The 3OHKyn-Cys adduct curve showed that the yield decreased after 3 days of incubation. After 2 days of incubation, a yield of 12% was observed for 3OHKyn-Cys but on day 3, the yield of 3OHKyn-Cys had dropped to 4%, and this continued to drop to 2% by day 5.

The 3OHKyn-*t*-Boc-His adduct plateaued after 1 day. On day 1 the yield of this adduct was 8%, on day 2 this increased to 9%. Then the amount slowly decreased over the next three days. On day 5 a yield of 6% of pure 3OHKyn-*t*-Boc-His was recovered.

The 3OHKyn-*t*-Boc-Lys adduct curve showed that the yield decreased after 4 days. After one day of incubation, 4% of pure 3OHKyn-*t*-Boc-Lys was produced. The amount of this adduct increased to 7% on day 3, and then dropped to 3% by day 5.

This study shows that the relative amounts of adduct at 1 day of incubation are a result of adduct formation, whereas the amounts of adduct at the end of the 5 day incubation are a result of both adduct formation and stability.

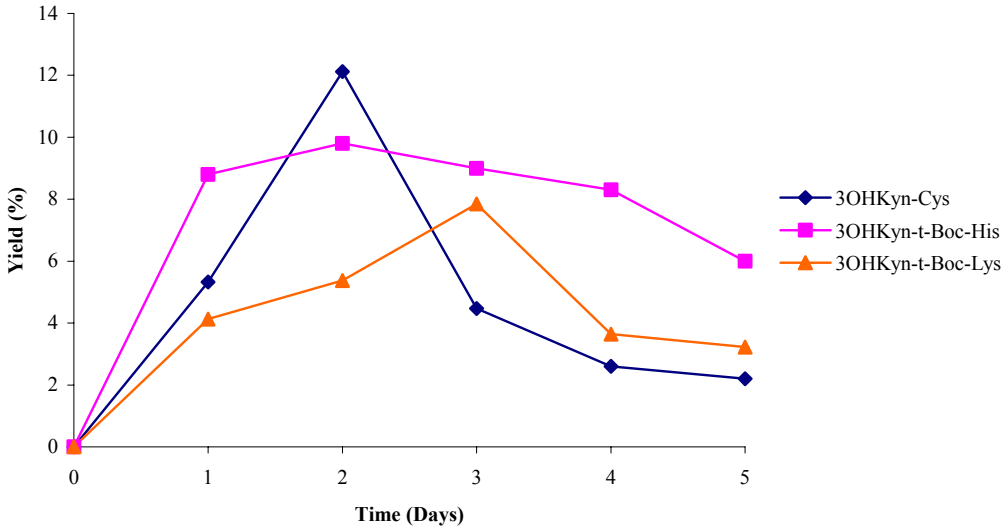


Figure 2.5 Formation of 3OHKyn amino acid adducts over a 5 day incubation. 3OHKyn was incubated with a 25-fold molar excess of N-α-*t*-Boc-L-His, N-α-*t*-Boc-L-Lys, or Cys at pH 7.2, 37^0C.

2.3.3 Mass Spectrometric Characterisation of the 3OHKyn Amino Acid Adducts

The structures of the 3OHKyn amino acid adducts were investigated initially by mass spectrometry, since NMR characterisation was difficult due to the low yields produced. MS/MS was found to be an important tool for characterising these adducts structurally. ESI mass spectra were initially acquired for each of the adducts. The theoretical molecular ions for each of the adducts (3OHKyn-Cys, *m/z* 329; 3OHKyn-*t*-Boc-His, *m/z* 463 and 3OHKyn-*t*-Boc-Lys, *m/z* 454) were present in the ESI mass spectra (spectra not shown). MS/MS of the molecular ions were performed to confirm the structures.

MS/MS of ion at *m/*z 329 (3OHKyn-Cys) is shown in Figure 2.6, and resulted in fragment ions at *m/z* 311, 240, 208, 202, 190, 162, 122 and 110. The proposed structures for these major fragment ions are shown in Table 2.1, and these are comparable to the structures of the fragment ions of Kyn-Cys[192] whereby 3OHKyn-Cys has the addition of an oxygen atom. A loss of water from the molecular ion generated the fragment ion at *m/z* 311.

MS/MS of ion *m/*z 463 (3OHKyn-*t*-Boc-His) is shown in Figure 2.7, and resulted in fragment ions *m/z* 407, 363, 317, 211, 208, 190, 162, 156, 136 and 110. The proposed structures for these major fragment ions are shown in Table 2.2, and the structures are comparable to the structures of the fragment ions of Kyn-*t*-Boc-His.[192] A loss of the *t*-Boc group generated the product ion *m/z* 363.

MS/MS of ion *m/*z 454 (3OHKyn-*t*-Boc-Lys) is shown in Figure 2.8, and resulted in fragment ions *m/z* 398, 354, 247, 208, 203, 152, 147 and 128. The proposed structures for these major fragment ions are shown in Table 2.3, and the structures are comparable to the structures of the fragment ions of Kyn-*t*-Boc-Lys.[192] A loss of the *t*-Boc group generated the product ion *m/z* 354.

A fragment ion present in all adducts was *m/z* 208 and this corresponds to 3-hydroxykynurenine yellow (3OHKyn-yellow)[192] (Tables 2.1, 2.2 and 2.3), a known intramolecular cyclisation product of 3OHKyn at neutral pH.[191] Two other fragment ions present in all three MS/MS spectra were *m/z* 162 and *m/z* 110. Therefore these

three ions (*m/z* 208, 162 and 110) are 'characteristic' fragment ions for all three 3OHKyn amino acid adducts.

Fragment ions *m/z* 122, 147 and 156 were present in 3OHKyn-Cys (Figure 2.6), 3OHKyn-*t*-Boc-Lys (Figure 2.8) and 3OHKyn-*t*-Boc-His (Figure 2.7) respectively, corresponding to the molecular masses of the amino acid residues Cys, Lys and His respectively.

MS/MS confirmed that the amino acids were attached to the 3OHKyn amino acid side chain. 3OHKyn-Cys had a fragment ion *m/z* 202 (Table 2.1), which was derived from α-cleavage at the carbonyl (C-7) on the 3OHKyn side chain (see Figure 2.1 for the numbering of the atoms). This fragment ion shows that the Cys has attached through C-9 on the 3OHKyn amino acid side chain. 3OHKyn-*t*-Boc-Lys had a fragment ion *m/z* 203 (Table 2.3), which was derived from cleavage at C-9 and C-17 (minus the *t*-Boc) (Figure 2.1). Similarly this fragment ion shows that the Lys has attached through C-9 on the 3OHKyn amino acid side chain. These two ions where abundant ions in the MS/MS spectra, and could be used as markers for identifying these two adducts *in vivo*.

High resolution mass spectrometric data were also obtained for the molecular ions of each of the three adducts to confirm the elemental compositions: 3OHKyn-Cys 329.0789 (calculated for $C_{13}H_{17}N_2O_6S$, 329.0807), 3OHKyn-*t*-Boc-His 463.1771 (calculated for $C_{21}H_{27}N_4O_8$, 463.1829), and 3OHKyn-*t*-Boc-Lys 454.2264 (calculated for $C_{21}H_{32}N_3O_8$, 454.2189). To further confirm the structure of the 3OHKyn amino acid adducts NMR spectroscopy was undertaken.

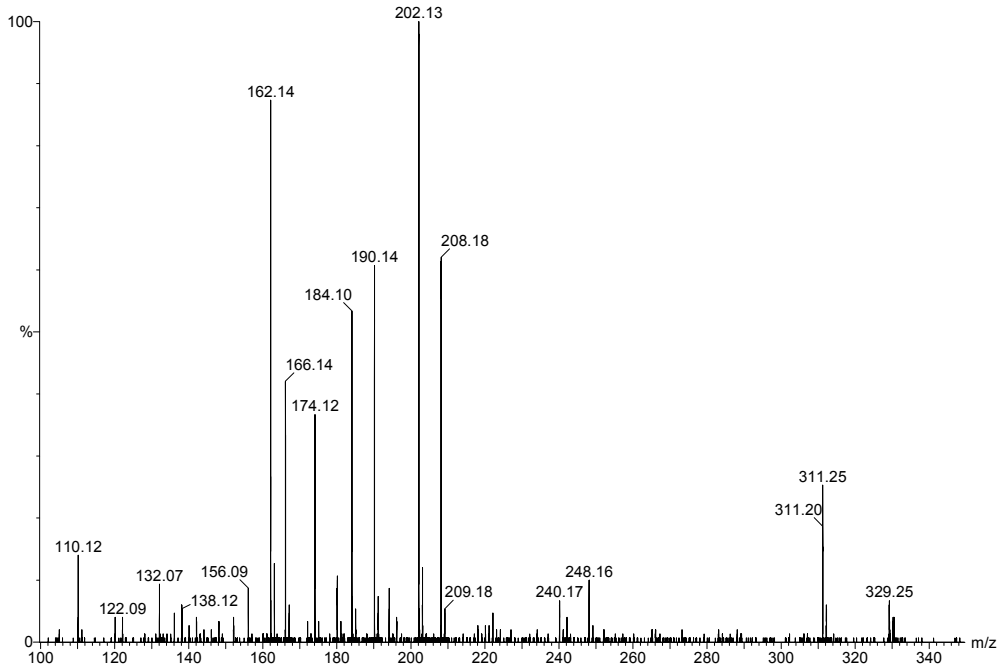
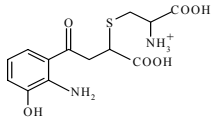
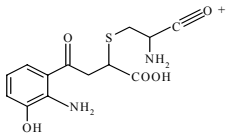
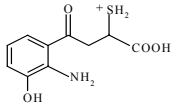
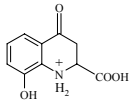
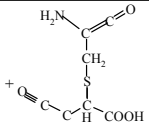
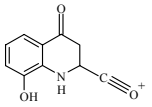
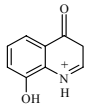
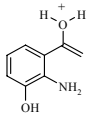
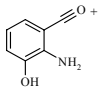
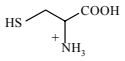


Figure 2.6 MS/MS spectrum of the protonated molecular ion of 3OHKyn-Cys *m/z* 329.

Table 2.1 Proposed structures of the major fragment ions of 3OHKyn-Cys observed in the MS/MS spectrum.

Observed Ion (*m/z*)	**Proposed Structure**
329	
311	
240	
208	
202	
190	
162	
152	
136	
122	
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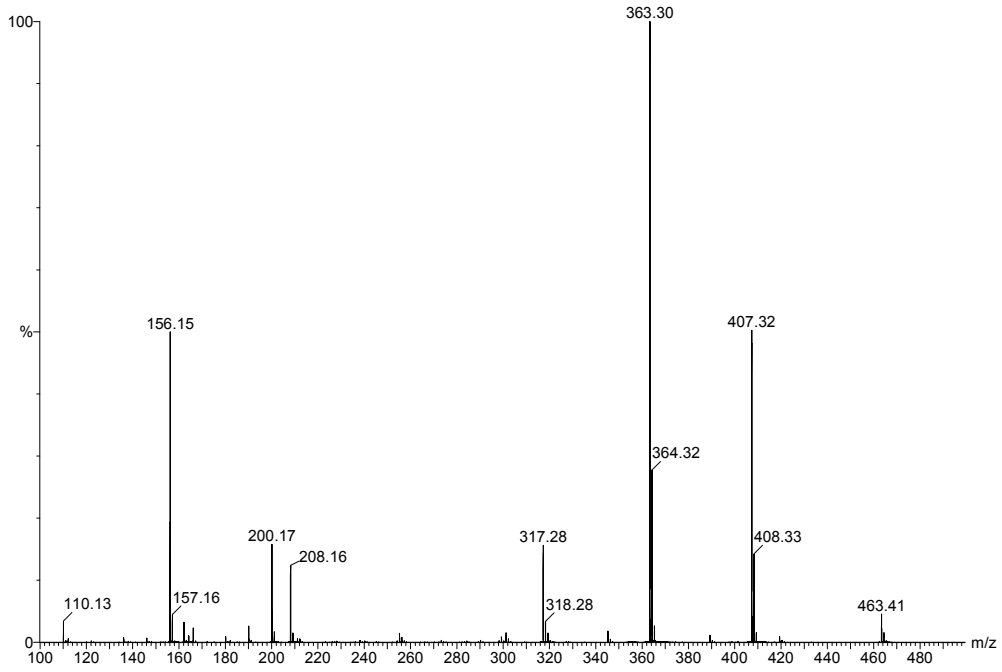
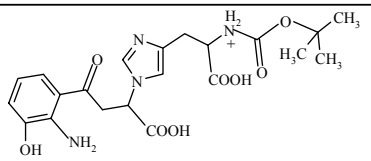
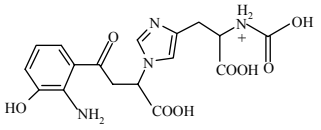
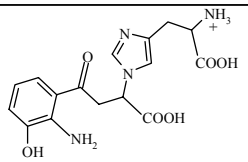
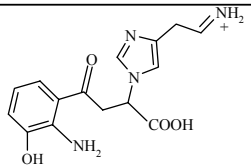
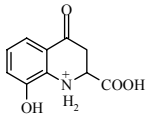
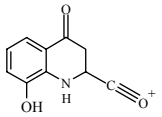
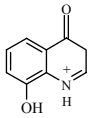
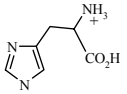
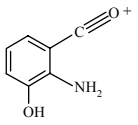
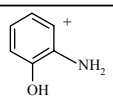


Figure 2.7 MS/MS spectrum of the protonated molecular ion of 3OHKyn-*t*-Boc-His *m/z* 463.

Table 2.2 Proposed structures of the major fragment ions of 3OHKyn-*t*-Boc-His observed in the MS/MS spectrum.

Observed Ion (*m/z*)	**Proposed Structure**
463	
407	
363	
317	
208	
190	
162	
156	
136	
110	

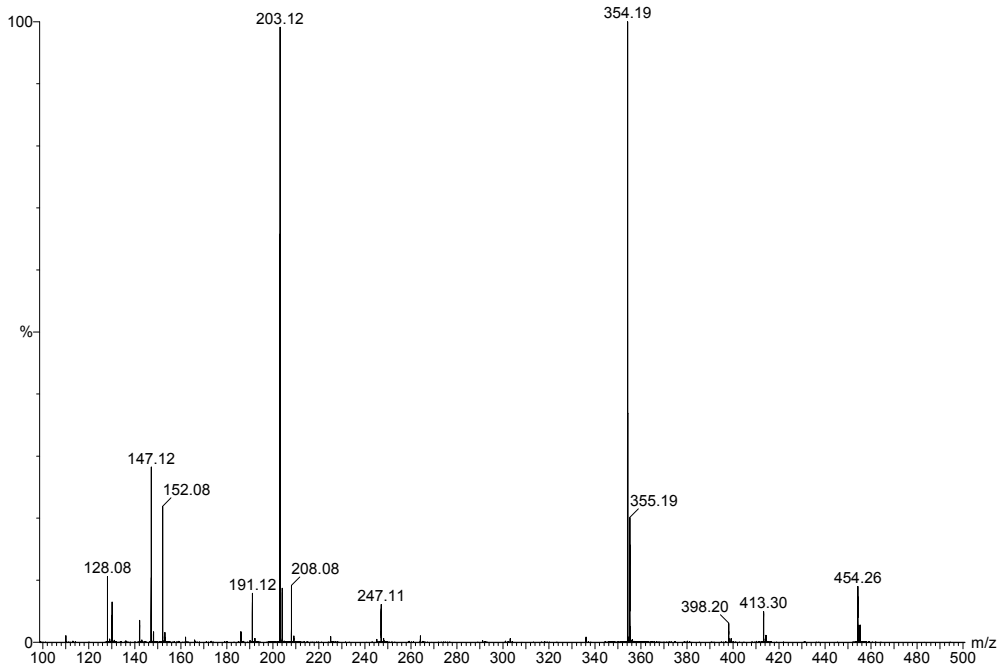
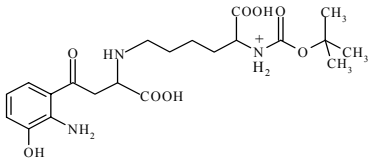
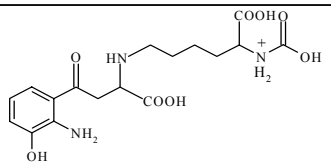
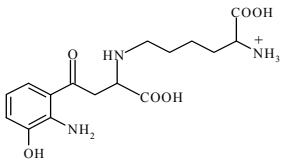
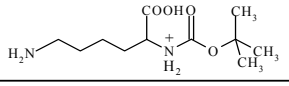
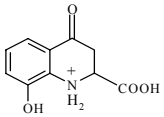
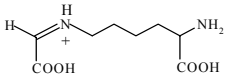
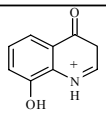
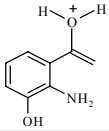
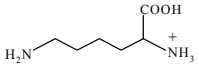
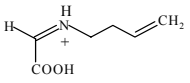


Figure 2.8 MS/MS spectrum of the protonated molecular ion of 3OHKyn-*t*-Boc-Lys *m/z* 454.

Table 2.3 Proposed structures of the major fragment ions of 3OHKyn-*t*-Boc-Lys observed in the MS/MS spectrum.

Observed Ion (*m/z*)	**Proposed Structure**
454	
398	
354	
247	
208	
203	
162	
152	
147	
128	
110	

2.3.4 NMR Characterisation of 3OHKyn Amino Acid Adducts

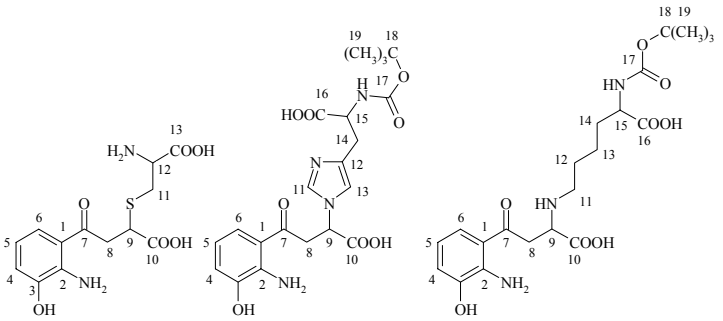
NMR studies were undertaken to characterise the 3OHKyn amino acid adducts. 1-D and 2-D spectra were acquired for each adduct. Samples were dissolved in acidified D_2O, to stabilise each adduct, since *o*-aminophenols readily oxidise at neutral pH.[184] The adducts were not separated into individual diastereoisomers since the resolution by HPLC was inadequate. The chemical shifts for the ^{1}H and ^{13}C NMR spectra are listed in Table 2.4. The chemical shifts of the protons and carbons for each 3OHKyn amino acid adduct was similar to the chemical shifts reported for the Kyn amino acid adducts.[103] In addition, the chemical shifts for 3OHKyn-*t*-Boc-Lys were similar to those reported by Staniszewska, *et al*. who synthesised an antigen using 3OHKyn and *N*-α-acetyl-Lys, for immunohistochemical studies.[193] All protons could be assigned from the ^{1}H NMR spectra (Table 2.4). 2-D NMR experiments (gCOSY, gHSQC and gHMBC) were acquired to demonstrate the proton coupling and to characterise the carbon atoms. The chemical shifts for CH-9 and CH_2-8 were deshielded[103] with reference to 3OHKyn since highly nucleophilic amino acid residues are attached at C-9. gHMBC experiments confirmed that each amino acid was covalently attached to the 3OHKyn amino acid side chain at C-9. Since the adducts were not very soluble, and there was a limited amount of sample available, the quaternary carbons could not be resolved, and therefore structural characterisation of the 3OHKyn amino acid adducts by NMR could not be successfully completed. However the NMR experiments undertaken did confirm that the amino acids were attached at C-9 on the 3OHKyn amino acid side chain as predicted.

Table 2.4 Summary of the ^{1}H and ^{13}C NMR spectral assignments for the 3OHKyn amino acid adducts. The atom numbering adopted is shown below.

Adducts: A, 3OHKyn-Cys; B, 3OHKyn-*t*-Boc-His; C, 3OHKyn-*t*-Boc-Lys

^{1}H NMR	Adducts			^{13}C NMR	Adducts		
(ppm)	A	B	C	(ppm)	A	B	C
H-4	7.32	7.03	7.16	C-1	127.3	119.2	*a*
H-5	7.43	6.90	6.96	C-2	118.6	135.3	134.1
H-6	7.69	7.42	7.54	C-3	150.4	147.0	146.5
H-8	3.77, 3.66	3.92	3.82	C-4	121.5	120.7	120.2
H-9	3.97	5.47	4.20	C-5	128.3	122.0	120.1
H-11	3.43, 3.33	8.75	3.19	C-6	122.2	122.6	122.8
H-12	4.35		1.81	C-7	200.5	199.1	200.2
H-13		7.34	1.51	C-8	40.9	41.9	38.6
H-14		3.17, 2.97	1.89, 1.75	C-9	41.7	59.1	57.3
H-15		4.3	4.12	C-10	175.9	172.4	172.8
H-19		1.93	1.29	C-11	31.6, 31.3	135.6	47.1
				C-12	52.1	192.7	25.0
				C-13	170.8	119.9	22.3
				C-14		26.8, 26.8	30.1
				C-15		52.8	53.7
				C-16		*a*	*a*
				C-17		*a*	*a*
				C-18		81.8	81.8
				C-19		27.5	27.7

[a] Carbons were not visible due to poor resolution



A **3OHKyn-Cys**

B **3OHKyn-*t*-Boc-His**

C **3OHKyn-*t*-Boc-Lys**

2.3.5 UV-visible and Fluorescence Characterisation of the 3OHKyn Amino Acid Adducts

UV-visible spectra were obtained for each adduct at various pH values, to examine the absorbance of each adduct, and to gain information on their relative stability. The UV-visible spectra for the three adducts and 3OHKyn (data not shown) were found to be essentially identical under the various conditions. The solutions used in this study include, 0.1% (v/v) TFA pH 2.1, 6 M guanidine HCl pH 5.5, 0.1 M phosphate buffer pH 7.2, and sodium carbonate/bicarbonate buffer pH 9.5. Figure 2.9 shows the UV-visible spectra for 3OHKyn-Cys at various pH values. As can be seen, broad peaks were observed with wavelength maxima centred at 374 nm and 267 nm (Figure 2.9A, B and C) under acidic and neutral conditions. However at a higher pH (pH 9.5) (Figure 2.9D) there were no visible peaks of absorbance, indicating the compound had been structurally modified under these conditions. Similarly, the UV-visible spectra for 3OHKyn-*t*-Boc-His (Figure 2.11) exhibited broad peaks with wavelength maxima centred at 375 nm and 270 nm (Figure 2.11A, B and C) at acidic and neutral pH. At pH 9.5 (Figure 2.11D) there was again no visible absorbance. The UV-visible spectra for 3OHKyn-*t*-Boc-Lys are shown in Figure 2.13. Broad peaks with wavelength maxima centred at 367 nm and 266 nm were observed (Figure 2.13A, B and C) under acidic and neutral conditions, and there was no absorbance observed at pH 9.5 (Figure 2.13D).

3-D Fluorescence spectra showed that each adduct was fluorescent, but at different excitation (Ex) and emission (Em) wavelengths. 3OHKyn-Cys exhibited maximal fluorescence intensities at Ex 350 nm/Em 520 nm and Ex 420 nm/Em 520 nm at pH 2.1, Ex 340 nm/Em 510 nm and Ex 430 nm/Em 510 nm at pH 5.5, and Ex 330 nm/Em 510 nm and Ex 400 nm/Em 520 nm at pH 7.2 (Figure 2.10A, B and C). The 3-D fluorescence spectrum was significantly different at pH 9.5 with only one contour at Ex 360 nm/Em 520 nm (Figure 2.10D).

3OHKyn-*t*-Boc-His exhibited maximal fluorescence intensities at Ex 370 nm/Em 520 nm at pH 2.1, Ex 350 nm/Em 520 nm at pH 5.5, and Ex 430 nm/Em 530 nm at pH 7.2 (Figure 2.12A, B and C). At pH 9.5, the exhibited maximal fluorescence intensity was Ex 340 nm/Em 510 nm (Figure 2.12D). 3OHKyn-*t*-Boc-Lys was also fluorescent, and

exhibited maximal fluorescence intensities at Ex 420 nm/Em 520 nm at pH 2.1, Ex 350 nm/Em 520 nm and Ex 410 nm/Em 520 nm at pH 5.5, and Ex 360 nm/Em 520 nm at pH 7.2 (Figure 2.14A, B and C). At pH 9.5, the exhibited maximal fluorescence intensity was Ex 330 nm/Em 510 nm (Figure 2.14D).

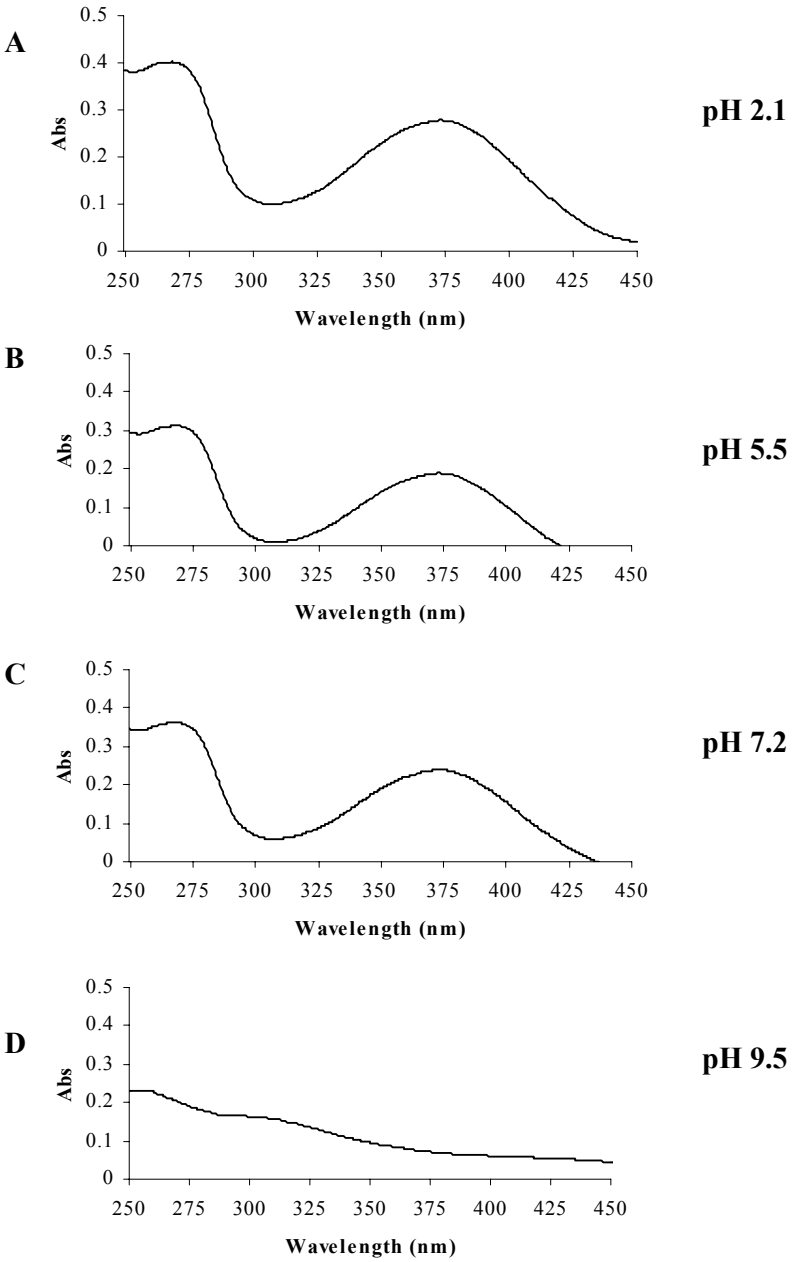


Figure 2.9 UV-visible spectra of 3OHKyn-Cys at various pH values; *A*, pH 2.1; *B*, pH 5.5; *C*, pH 7.2; *D*, pH 9.5.

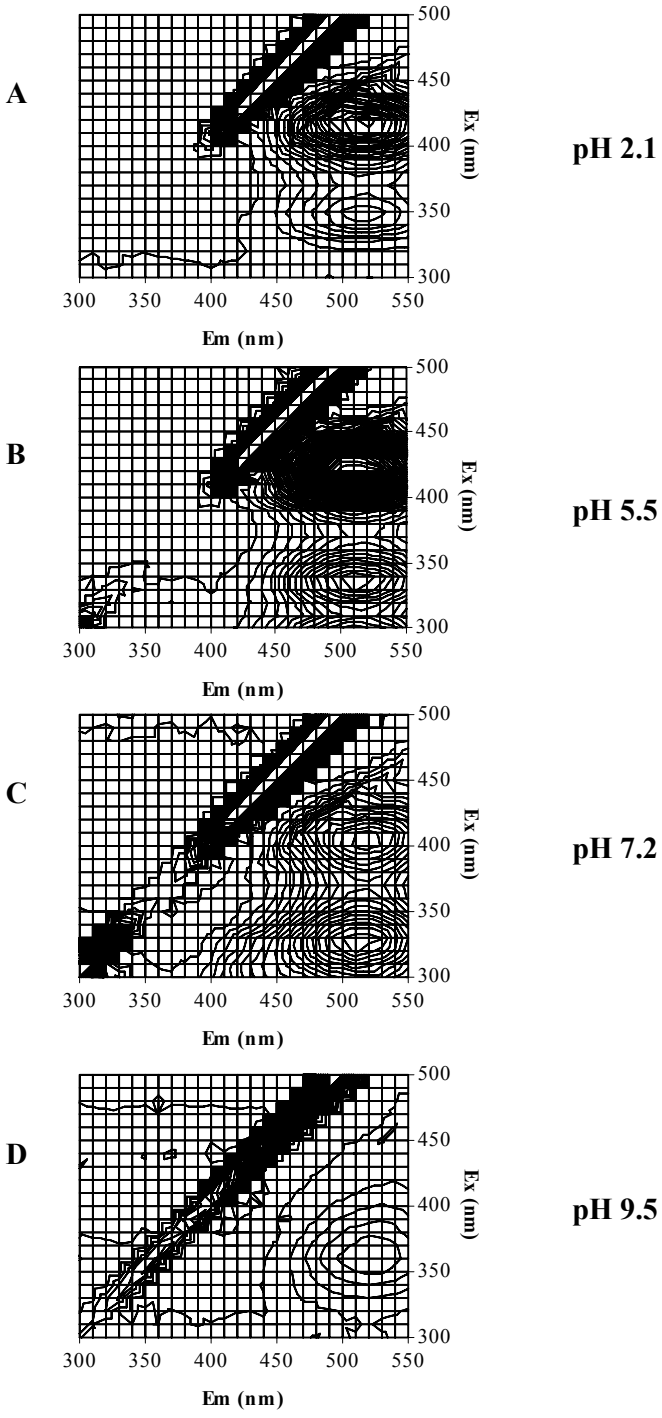


Figure 2.10 3-D Fluorescence spectra of 3OHKyn-Cys at various pH values; *A*, pH 2.1; *B*, pH 5.5; *C*, pH 7.2; *D*, pH 9.5.

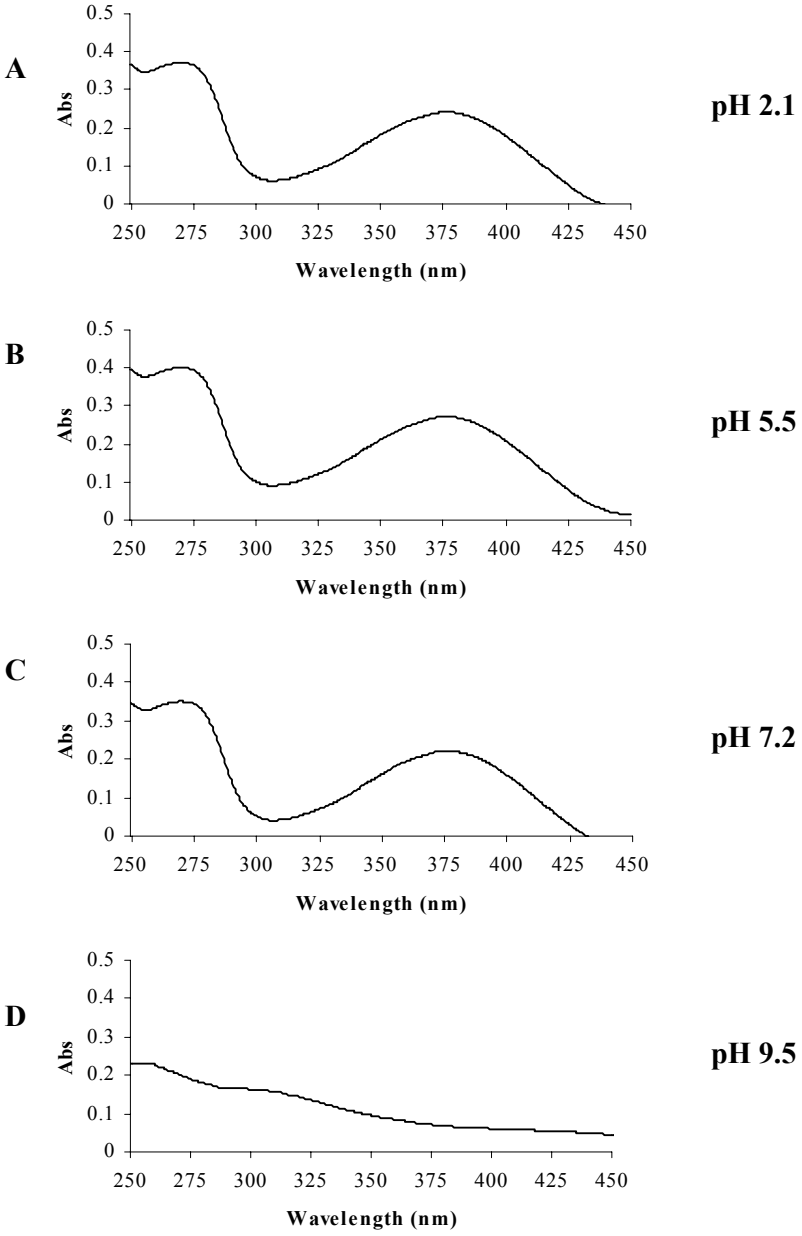


Figure 2.11 UV-visible spectra of 3OHKyn-*t*-Boc-His at various pH values; *A*, pH 2.1; *B*, pH 5.5; *C*, pH 7.2; *D*, pH 9.5.

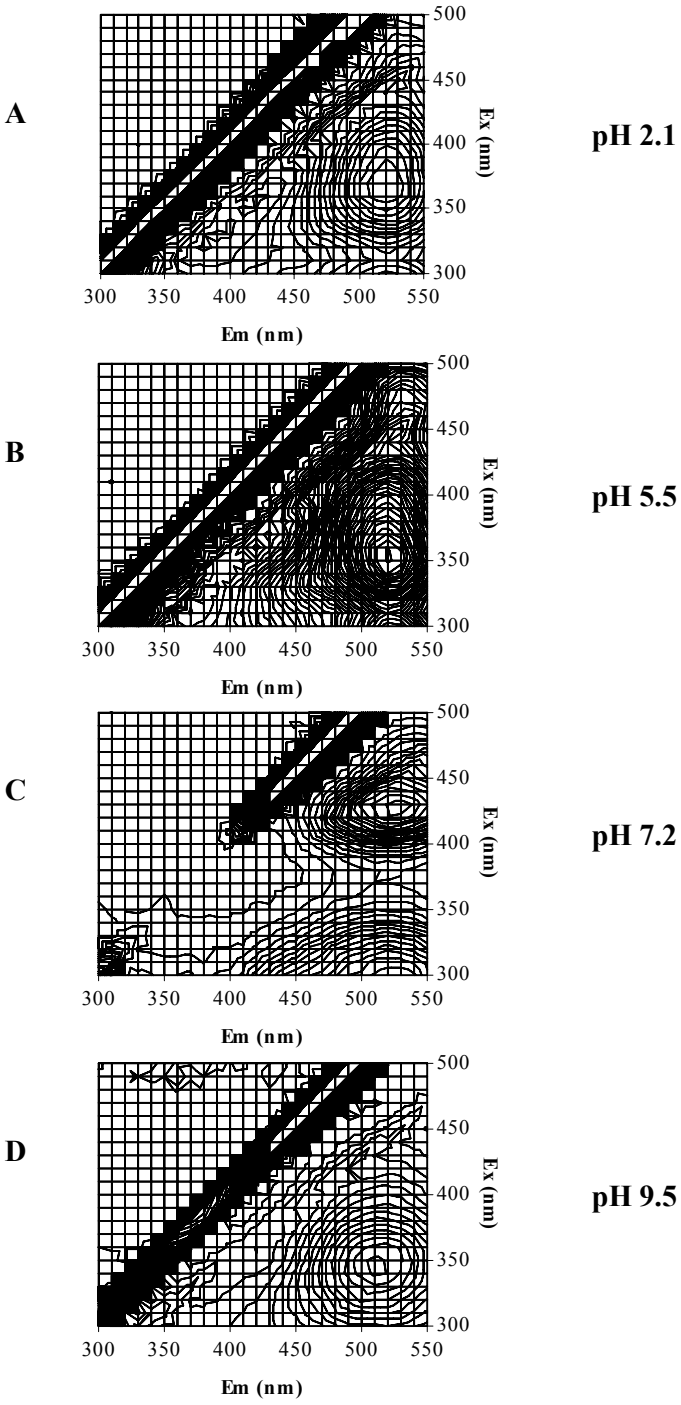


Figure 2.12 3-D Fluorescence spectra of 3OHKyn-*t*-Boc-His at various pH values; *A*, pH 2.1; *B*, pH 5.5; *C*, pH 7.2; *D*, pH 9.5.

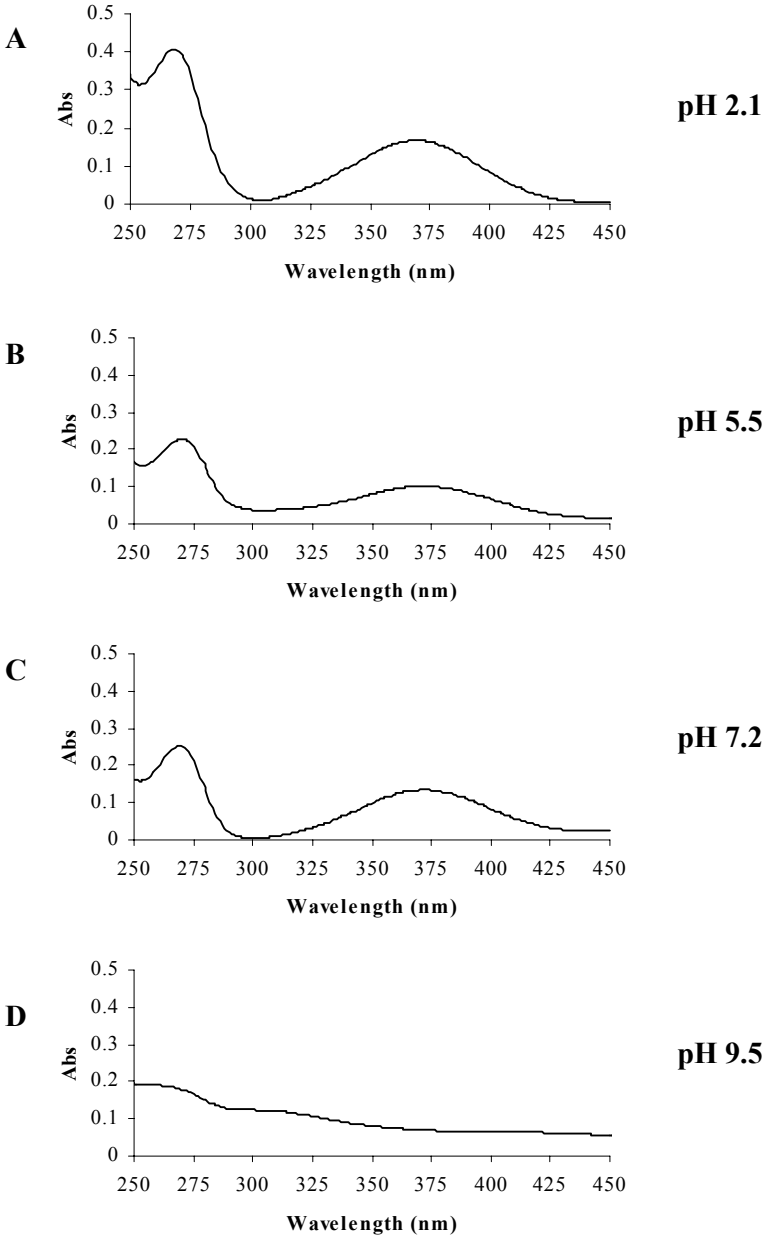


Figure 2.13 UV-visible spectra of 3OHKyn-*t*-Boc-Lys at various pH values; *A*, pH 2.1; *B*, pH 5.5; *C*, pH 7.2; *D*, pH 9.5.

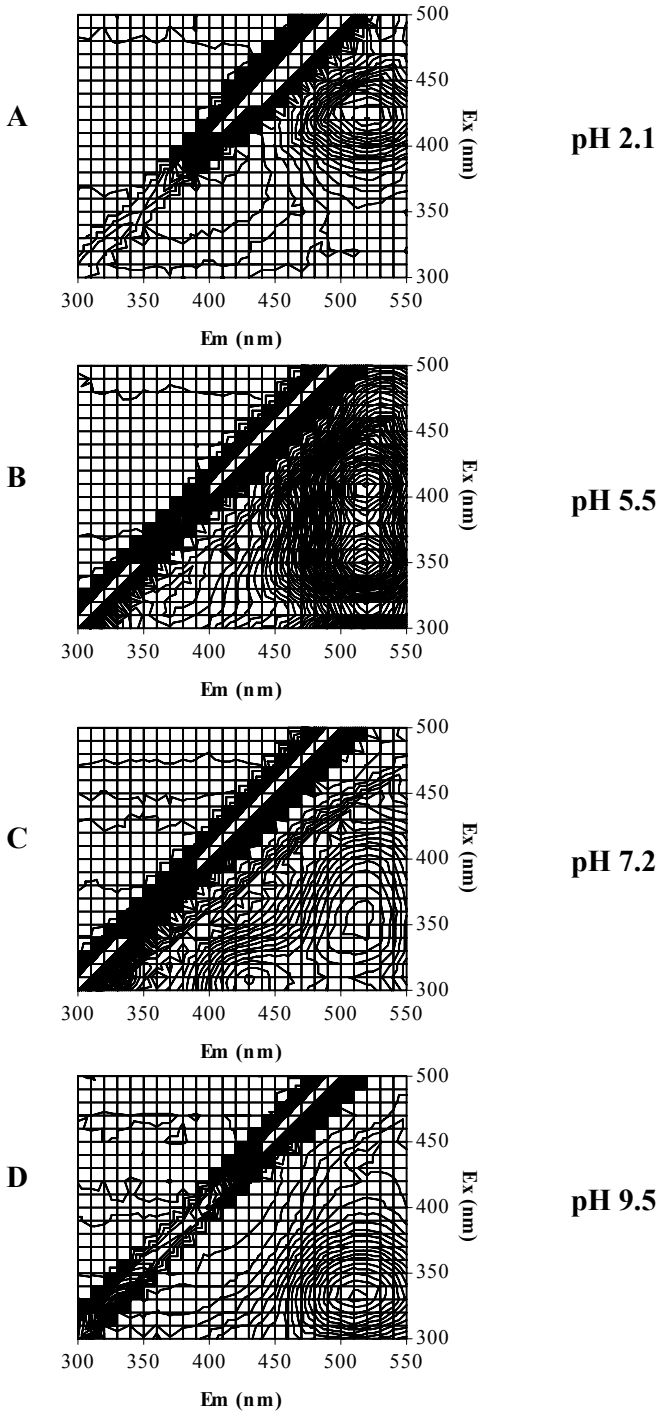


Figure 2.14 3-D Fluorescence spectra of 3OHKyn-*t*-Boc-Lys at various pH values; *A*, pH 2.1; *B*, pH 5.5; *C*, pH 7.2; *D*, pH 9.5.

2.3.6 Stability of 3OHKyn Amino Acid Adducts

3OHKyn is known to autoxidise readily at neutral pH with the production of H_2O_2 and numerous other products.[181,184] It was of interest to discover if the 3OHKyn amino acid adducts were also unstable under physiological conditions.

Stability experiments were performed at pH 7.2 both in the presence, and in the absence, of oxygen. The results are shown in Figure 2.15. In the absence of oxygen, only 15% of 3OHKyn (Figure 2.15A) remained after 5 days of incubation and these results are comparable to those obtained by Taylor, *et al*. whose incubations were done in 25 mM carbonate buffer (pH 7.0).[109] 3OHKyn-Cys and 3OHKyn-*t*-Boc-Lys (Figure 2.15B and D) adducts were found to be less stable than 3OHKyn, resulting in yields of 3% and 7% respectively after 5 days. By contrast, under the same conditions, the His adduct (Figure 2.15C) was recovered in 51% yield.

In the presence of oxygen, the adducts were markedly less stable. There was, for example, no 3OHKyn recovered after 96 hours at pH 7.2 (Figure 2.15A), and no detectable peak for the Cys adduct after only 24 hours of incubation (Figure 2.15B). No trace of the Lys adduct was observed after 48 hours of incubation (Figure 2.15D). Again the His adduct was most stable but even this adduct showed a recovery of 11% after 120 hours (Figure 2.15C).

These data illustrate that all of the 3OHKyn amino acid adducts are unstable under physiological conditions and that this instability is exacerbated by exposure to oxygen. The Cys and Lys adducts are, more labile, and more readily oxidised, than 3OHKyn itself.

One objective of the current study was to develop a method for detecting 3OHKyn if it is attached to proteins. Because of their instabilities, and susceptibility to oxidation, coupled with the fact that they are likely to be present in low levels, isolation of 3OHKyn adducts presents a technical challenge.

On the basis of the studies with 3OHKyn at neutral pH detailed above, and previous investigations, which showed that high pH promoted autoxidation,[181] we investigated the use of acidic pH to see if this would lead to stabilising the adducts. If 3OHKyn adducts, were found to be stabilised under such acidic conditions, these could then be used for their isolation from modified proteins. Stability studies were therefore performed on each of the adducts at pH 4.0 for 48 hours since this time would be required by for example, enzymatic digestion. As described for the incubations at neutral pH, two sets of experiments were performed; one in the absence of oxygen, and one in the presence of oxygen. The results are shown in Figure 2.16 and illustrate a marked effect of acidic pH on adduct stability. In the absence of oxygen 3OHKyn was recovered in 90% yield (Figure 2.16A) after 48 hours; whereas the Cys and Lys (Figure 2.16B and D) adducts showed a 70% recovery, and 3OHKyn-*t*-Boc-His (Figure 2.16C) was detected in 53% recovery after this time. In the presence of oxygen the recoveries of all of the compounds were diminished. That of 3OHKyn dropped to 76%, and the Cys, His and Lys adducts were recovered in yields of 20%, 11% and 36% respectively. On the basis of these data (Figures 2.15 and 2.16), in order to maximise recoveries of 3OHKyn-containing amino acids, acidic pH is required and efforts would need to be taken to exclude oxygen during sample isolation. The only exception may be 3OHKyn-His which displayed reasonable stability at pH 7.2 and pH 4.0 both in the presence and absence of oxygen.

The 3OHKyn amino acid adducts are unstable at pH 7.2. The formation of the unknown breakdown products was exacerbated in the presence of oxygen. Figure 2.17 shows the rate of formation of the unknown breakdown products that formed at pH 7.2 in the absence of oxygen for each of the adducts. The majority of the unknown breakdown compounds could not be structurally identified from the mass spectral data alone. There were seven breakdown products formed from the instability of 3OHKyn-Cys that eluted at 27.3, 28.3, 29.9, 31.2, 32.9, 33.4 and 35.7 min on the HPLC chromatogram (chromatogram not shown), and the levels of these unknown peaks have been graphed in Figure 2.17A over the 5 day incubation period.

There were four breakdown products formed from the instability of 3OHKyn-*t*-Boc-His that eluted at 30.8, 31.8, 38.1 and 39.0 min on the HPLC chromatogram (chromatogram not shown). The levels of these unknown peaks have been graphed in Figure 2.17B. There were three breakdown products formed from the instability of 3OHKyn-*t*-Boc-Lys that eluted at 30.7, 33.1 and 38.1 min on the HPLC chromatogram (chromatogram not shown). The levels of these unknown peaks have been graphed (Figure 2.17C). Figure 2.17 shows that over the 5-day period the quantity of these unknown breakdown products continuously increases as the level of the 3OHKyn amino acid adduct decreases, due to the instability of these adducts at pH 7.2.

The product eluting at 27.3 min in Figure 2.17A, 30.8 min in Figure 2.17B and 30.7 min in Figure 2.17C was identified as xanthurenic acid, a breakdown product of 3OHKyn, by MS/MS. Although the compound eluted at different times in each HPLC chromatogram, co-elution with standard xanthurenic acid confirmed the compound as one of the breakdown products. The remaining breakdown products could not be identified from the mass spectral data, however the breakdown product eluting at 39.0 min in Figure 2.17B showed a prominent ion *m/z* 664, and the proposed structure of this compound has been derived in Section 2.3.8.

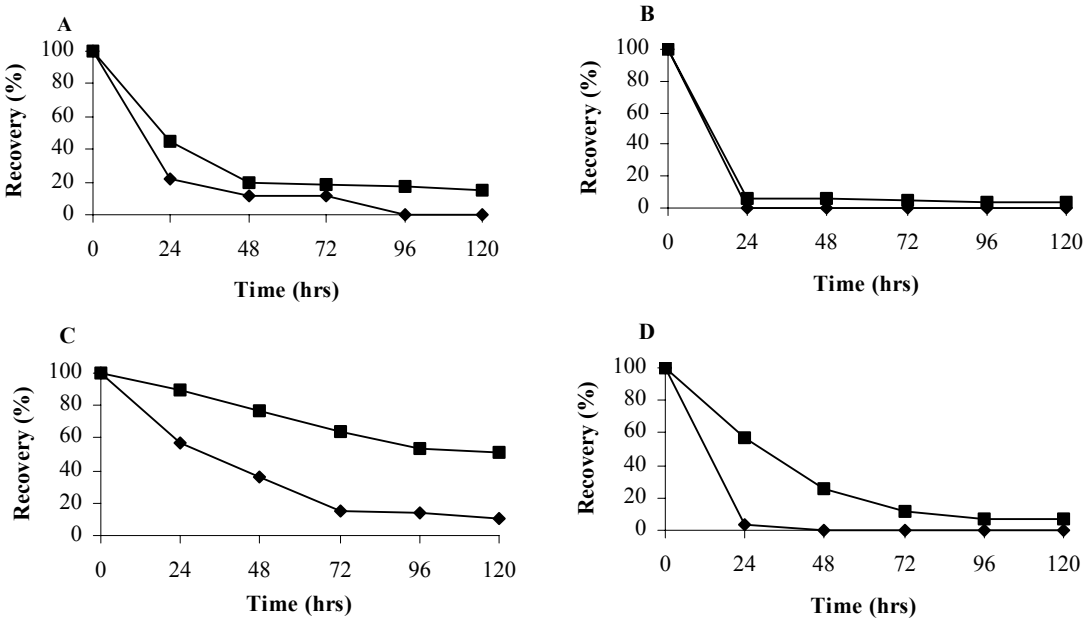


Figure 2.15 Stability of the 3OHKyn amino acid adducts and 3OHKyn at pH 7.2 in the absence (■) and presence (◆) of oxygen. *A*, 3OHKyn; *B*, 3OHKyn-Cys; *C*, 3OHKyn-*t*-Boc-His; *D*, 3OHKyn-*t*-Boc-Lys.

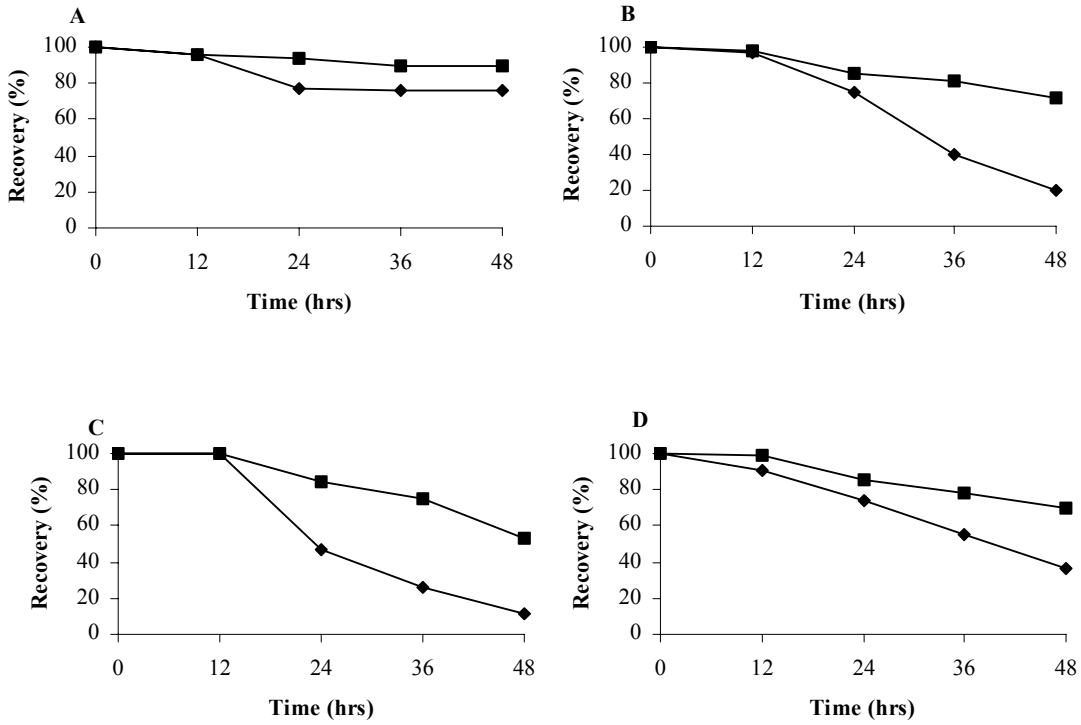


Figure 2.16 Stability of the 3OHKyn amino acid adducts and 3OHKyn at pH 4.0 in the absence (■) and presence (◆) of oxygen. *A*, 3OHKyn; *B*, 3OHKyn-Cys; *C*, 3OHKyn-*t*-Boc-His; *D*, 3OHKyn-*t*-Boc-Lys.

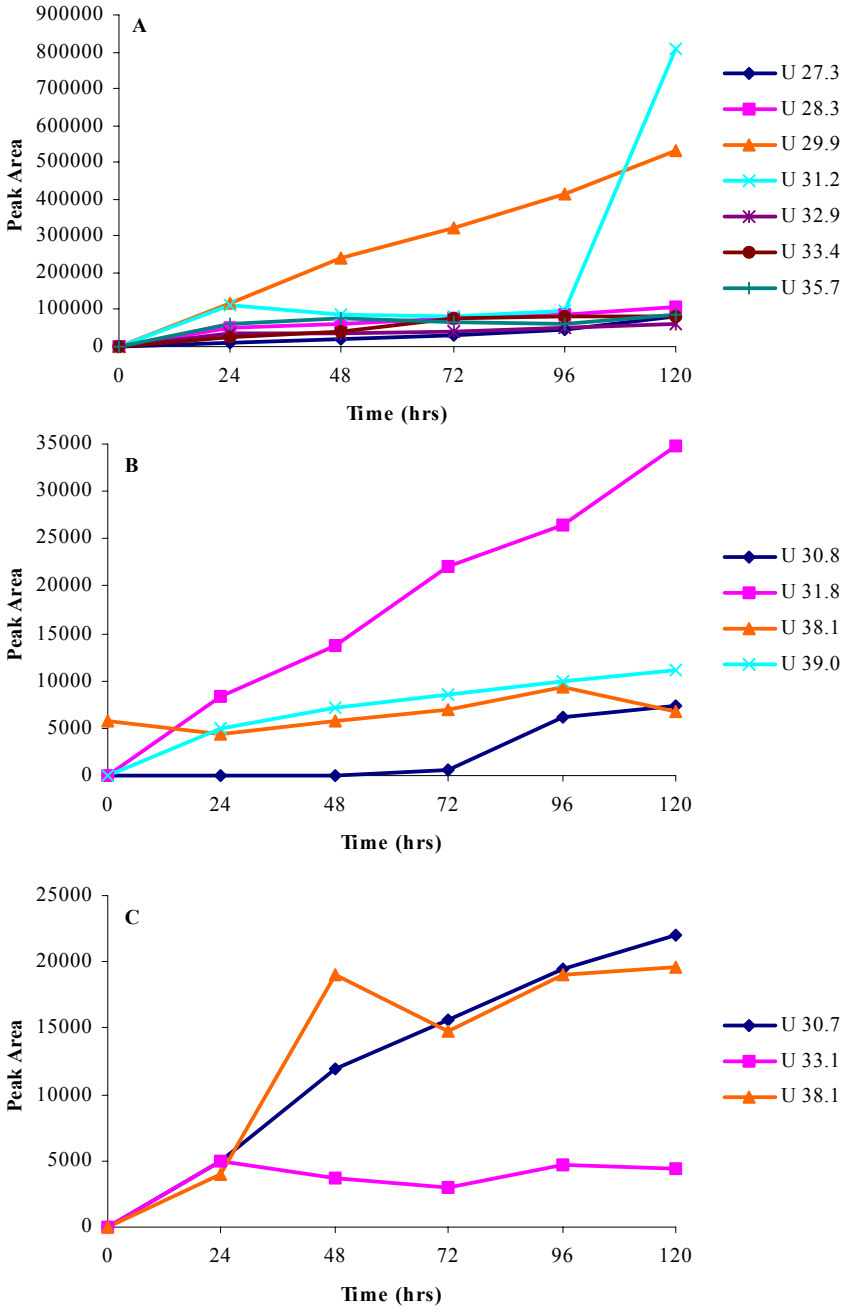


Figure 2.17 Formation of breakdown products from stability study of 3OHKyn amino acid adducts at pH 7.2 in the absence of oxygen. *A*, 3OHKyn-Cys; *B*, 3OHKyn-*t*-Boc-His; *C*, 3OHKyn-*t*-Boc-Lys.

2.3.7 Acid Hydrolysis of 3OHKyn Amino Acid Adducts

The stability of each adduct under conditions used for the hydrolysis of proteins was determined by hydrolysing with 6 M HCl in the presence of antioxidants,[165] since low yields were recovered initially for each adduct in the absence of such antioxidants (3OHKyn-Cys 17%, 3OHKyn-His, 20% and 3OHKyn-Lys, 24%). If antioxidants were added the recoveries were markedly improved; 3OHKyn-Cys, 87%; 3OHKyn-His, 78% and 3OHKyn-Lys, 95% (Table 2.5). Under the same conditions, the recovery of 3OHKyn was 58%. The HPLC profiles (not shown) showed the presence of other minor compounds in addition to the major adduct peak, but these minor peaks were not examined further.

Table 2.5 Recovery of 3OHKyn amino acid adducts after acid hydrolysis for 24 hours at 110^0C in the presence of antioxidants.

Adduct	Recovery (%)
3OHKyn	58%
3OHKyn-Cys	87%
3OHKyn-His	78%
3OHKyn-Lys	95%

2.3.8 Incubation of 3OHKyn-Cys in the Presence of Excess N-α-*t*-Boc-His and N-α-*t*-Boc-Lys

This experiment was undertaken to further examine the reactivity of the UV filter compounds. The aim was to see if the 3OHKyn attached to Cys could cleave and form deaminated 3OHKyn, and covalently attach to either N-α-*t*-Boc-His or N-α-*t*-Boc-Lys. Synthetic 3OHKyn-Cys was incubated with excess N-α-*t*-Boc-His and N-α-*t*-Boc-Lys in phosphate buffer at pH 7.2 for 48 hours. Aliquots were taken at 12 hourly intervals and analysed by HPLC. Figure 2.18 is the HPLC chromatogram for the aliquot at 48 hours of incubation. There were seven major peaks eluting, and mass spectrometry was utilised to analyse these compounds. In Figure 2.18 a doublet peak eluted at 28.4 min. MS/MS confirmed that this peak was 3OHKyn-Cys. Mass spectrometry of the second peak eluting at 30.6 min showed that the molecular ion was *m/z* 256. MS/MS of this ion confirmed that the compound contained unreacted N-α-*t*-Boc-His. MS/MS confirmed that the peak at 32.4 min contained unreacted N-α-*t*-Boc-Lys with a molecular ion of *m/*z 247. The peak eluting at 33.5 min was confirmed by mass spectrometry to be 3OHKyn-yellow (an intramolecular cyclisation product of 3OHKyn at neutral pH).[191] The large peak eluting at 35.1 min had a molecular ion of *m/z* 463, MS/MS confirmed that the compound was 3OHKyn-*t*-Boc-His. The peak at 36.0 min was confirmed as 3OHKyn-*t*-Boc-Lys. The peak that eluted at 39.5 min could not be identified, but had a relatively abundant ion at *m/z* 664 in the ESI mass spectrum.

In Figure 2.19, the 3OHKyn amino acid adducts as well as 3OHKyn-yellow and the unknown compound were quantified over the 48 hour incubation period. After 12 hours of incubation, the amount of 3OHKyn-Cys had decreased to 0.47 mM, and 3OHKyn-*t*-Boc-His, 3OHKyn-*t*-Boc-Lys and 3OHKyn-yellow had formed at concentrations of 0.25, 0.13 and 0.36 mM, respectively. The level of 3OHKyn-Cys continued to decrease to 0.11 mM at 48 hours of incubation, whereas 3OHKyn-*t*-Boc-His continued to increase to 0.48 mM, and 3OHKyn-*t*-Boc-Lys increased to 0.19 mM. The maximum amount of 3OHKyn-yellow was formed at 12 hours of incubation, following that, the amount of 3OHKyn-yellow decreased to 0.19 mM at 48 hours of incubation. The unknown compound that eluted at 39.5 min (U39.5), detected after 24 hours of incubation, continued to form over the following 24 hours.

The unknown compound eluting at 39.5 min (U39.5) in the HPLC chromatogram had an apparent molecular ion at *m/z* 664 in the ESI mass spectrum (Figure 2.20A). MS/MS of this molecular ion yielded spectrum Figure 2.20B. The major fragment ions are *m/z* 608 (loss of 56 Da), *m/z* 564 (further loss of 44 Da) and *m/z* 409 (further loss of 155 Da). Figure 2.21 shows the proposed structures for these ions. Molecular ion *m/z* 664 appears to be due to a phenoxazone compound made up from 3OHKyn-*t*-Boc-His attached to an oxidised 3OHKyn molecule. The fragment ion *m/z* 564 is a loss of 100 Da from the molecular ion *m/z* 664, and is typical of the loss of a *t*-Boc group. A further loss of 155 Da is indicative of a loss of His yielding *m/z* 409.

Theoretically this compound could form from autoxidation of 3OHKyn alone. In order to prove that *m/z* 409 could be derived from autoxidation of 3OHKyn, 3OHKyn was incubated in phosphate buffer at pH 7.2 and 37^0C for several days, and aliquots were taken and monitored by mass spectrometry for the formation of ion at *m/z* 409. A *m/z* 409 ion was identified after 3 hours of incubation, and the ESI mass spectrum is shown in Figure 2.22A. The *m/z* 409 ion could not be detected after 2 days of incubation, indicating that it is unstable. The MS/MS spectrum of ion *m/z* 409 is shown in Figure 2.22B and a common fragment ion also seen in Figure 2.20B is *m/z* 391, loss of water.

The chemical name of U39.5 is, 1,3,4,5-tetrahydro-11-(2-(N-α-*tert*-butyloxycarbonyl-histidyl)-(4-hydroxy-1,4-dioxo-butanyl)-1,5-dioxo-2H-pyrido(3,2-a)phenoxazine-3-carboxylic acid, and to further confirm the proposed structure of this compound, high resolution mass spectrometric data was obtained for the molecular ion 664.2438 (calculated for $C_{31}H_{30}N_5O_{12}$, 664.2496).

In addition, the UV-visible spectrum (Figure 2.23) further confirmed that U39.5 contains a phenoxazone moiety since a broad peak with a maximum absorbance centred at 437 nm is shown. Phenoxazones exhibit a broad UV-visible peak centred at 440 nm.[184]

To confirm the structure of U39.5, more material would need to be collected for NMR.

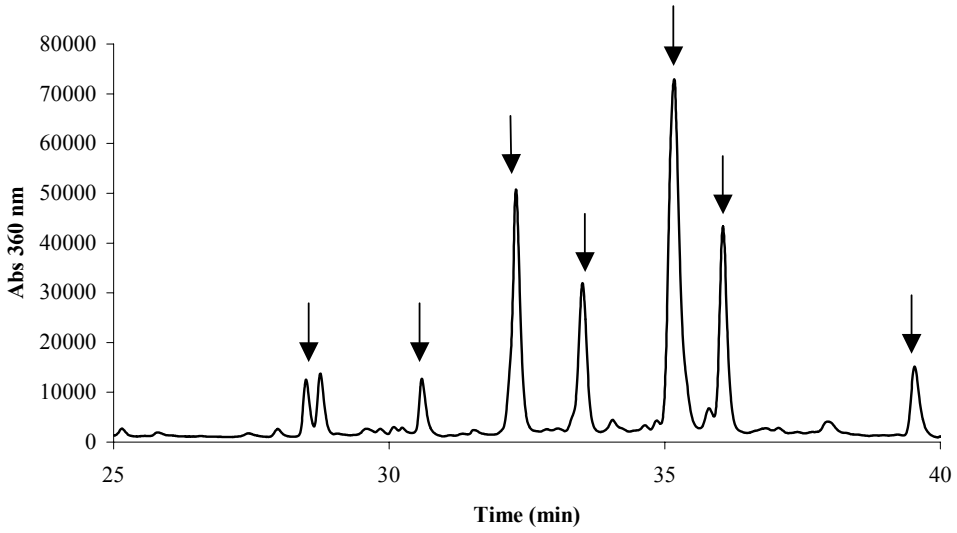


Figure 2.18 3OHKyn-Cys was incubated with excess N-α-*t*-Boc-His and N-α-*t*-Boc-Lys at pH 7.2 for a total of 48 hours. Shown is the 48 hour time sample (aliquot). The peak at 28.4 min is unreacted 3OHKyn-Cys. The peak at 30.6 min contained unreacted N-α-*t*-Boc-His, and the peak at 32.4 min contained unreacted N-α-*t*-Boc-Lys. The peak at 33.5 min is 3OHKyn-yellow, the peak at 35.1 min is 3OHKyn-*t*-Boc-His and the peak at 36.0 min is 3OHKyn-*t*-Boc-Lys. The peak at 39.5 min is unknown (U39.5).

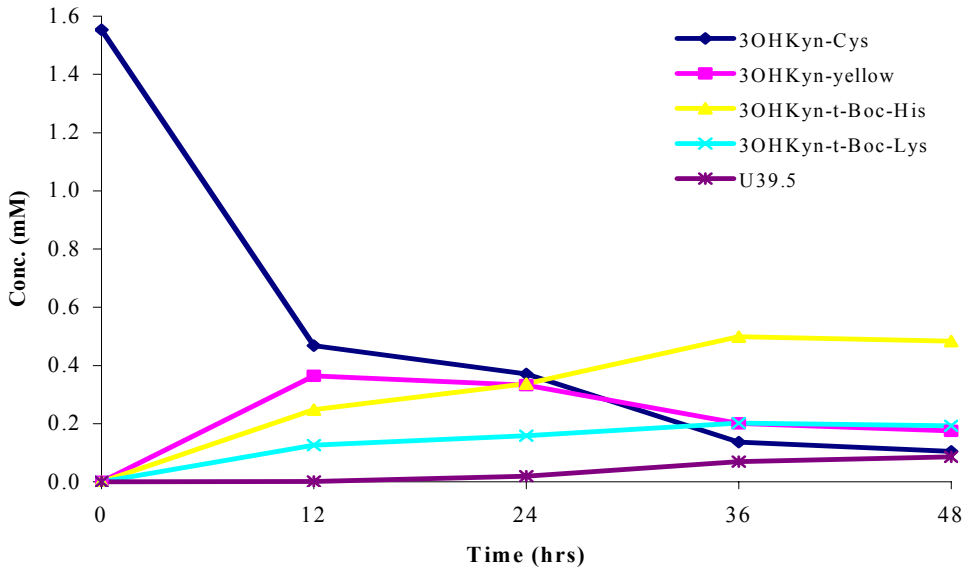


Figure 2.19 The rate of loss of 3OHKyn-Cys in relation to the rate of formation of 3OHKyn-yellow, 3OHKyn-*t*-Boc-His, 3OHKyn-*t*-Boc-Lys and unknown compound eluting at 39.5 min (U39.5) on the HPLC chromatogram (Figure 2.18).

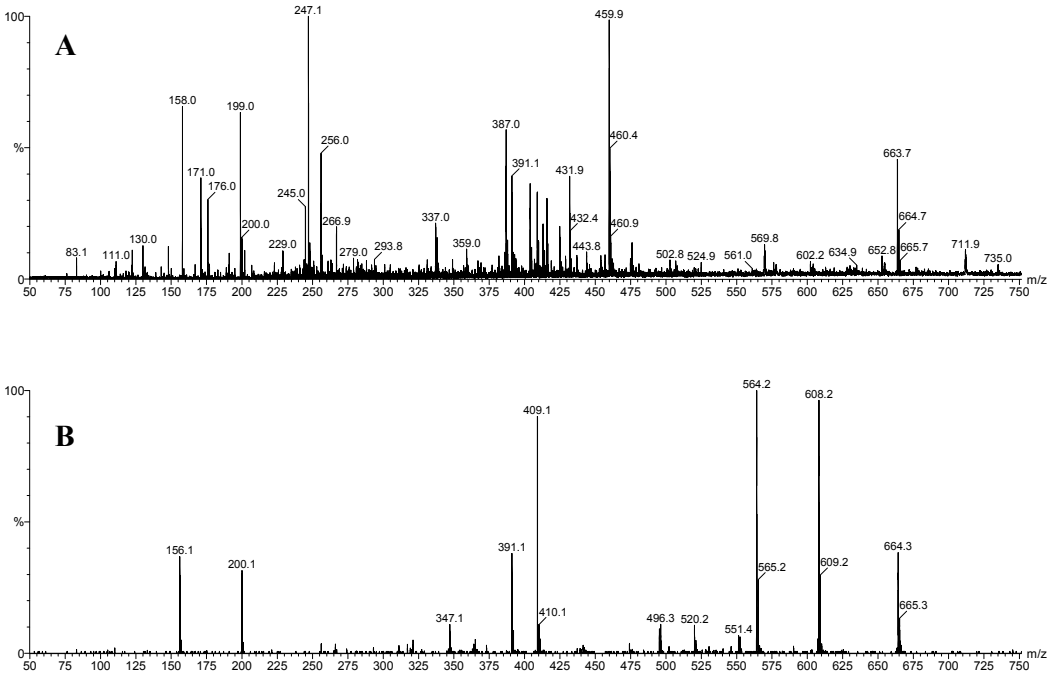


Figure 2.20 Mass spectra of U39.5. *A*, ESI mass spectrum; *B*, MS/MS spectrum of *m/z* 664 ion.

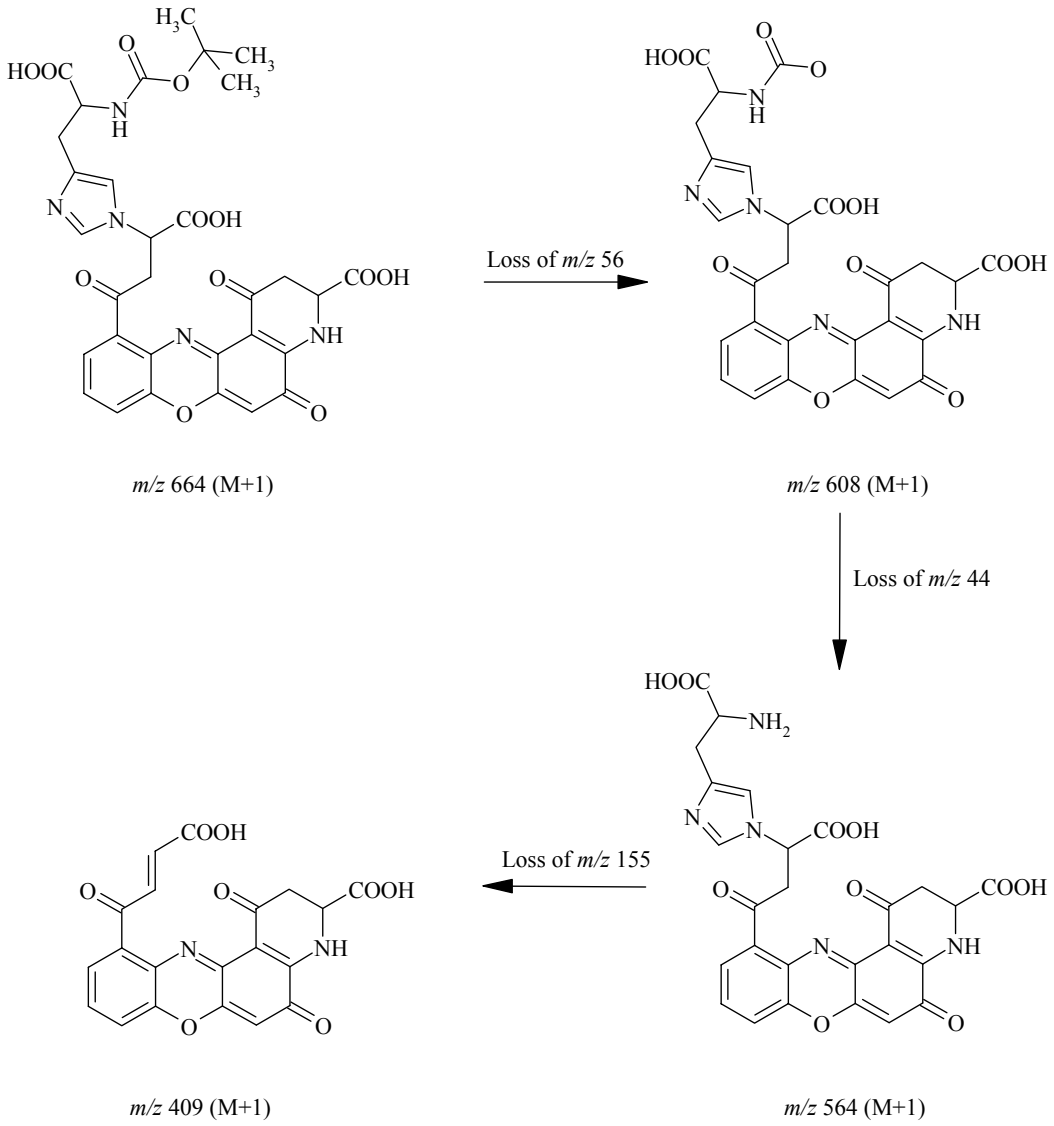


Figure 2.21 Proposed structures of molecular ion *m/z* 664, and fragment ions *m/z* 608, 564 and 409.

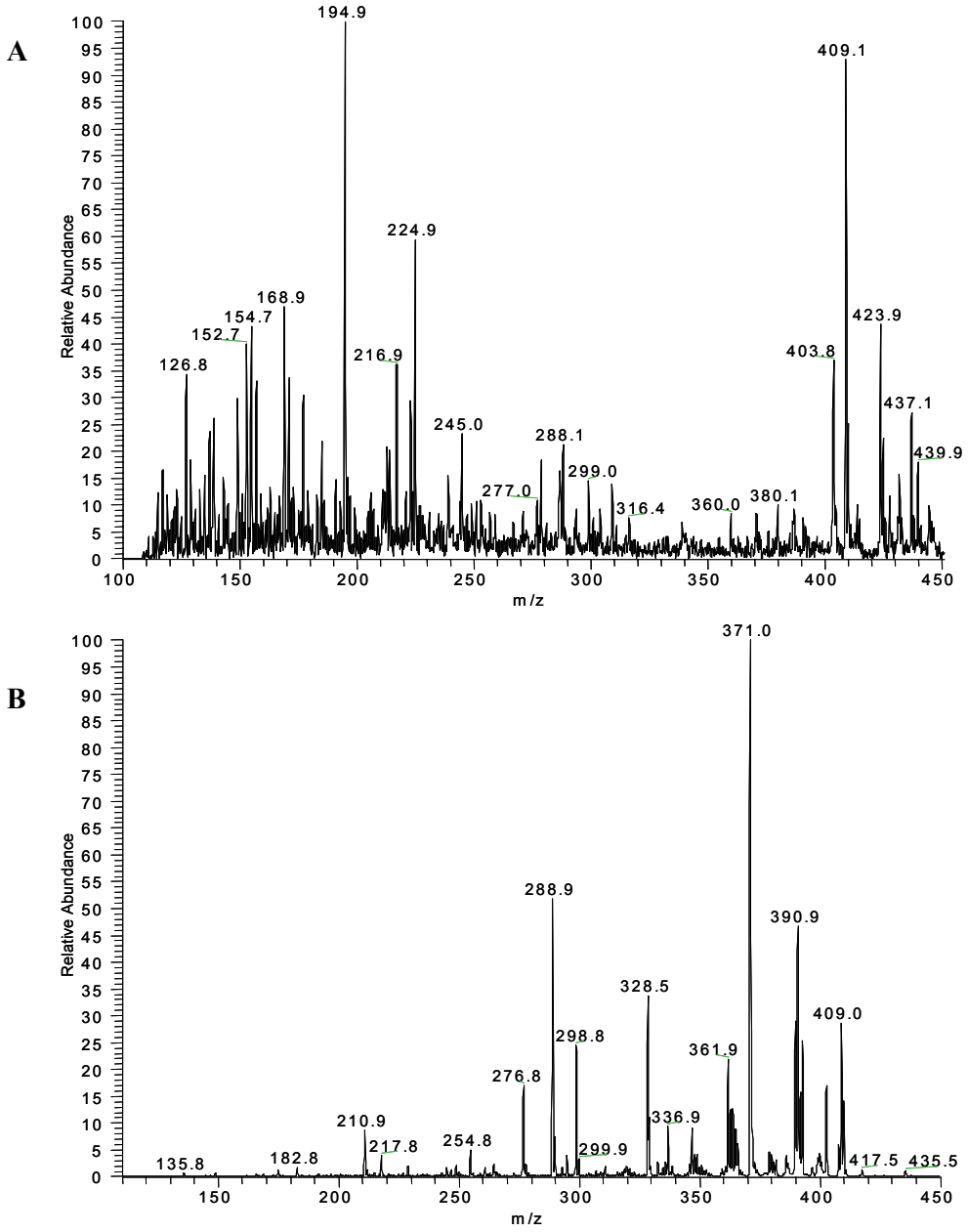


Figure 2.22 Mass spectra of aliquot from autoxidation of 3OHKyn at pH 7.2. *A*, ESI mass spectrum of aliquot after 3 hours of incubation; *B*, MS/MS spectrum of ion *m/z* 409.

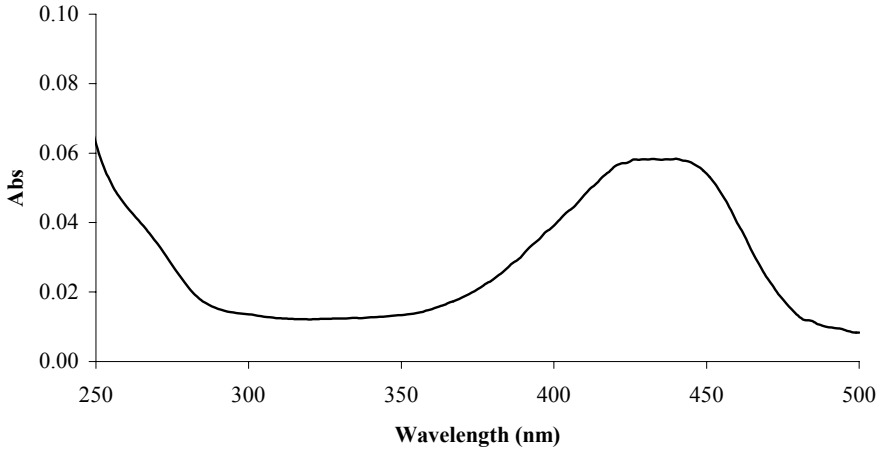


Figure 2.23 UV-visible spectrum of U39.5.

2.4 Discussion

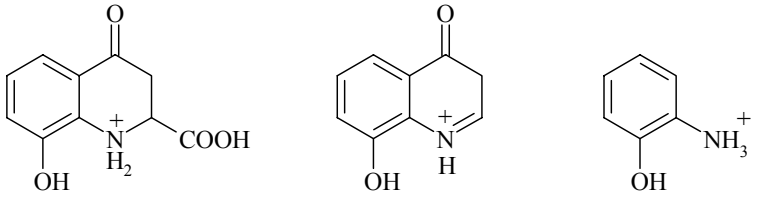
It is known that the human lens UV filter compounds, 3OHKynG and Kyn, attach covalently to lens proteins, particularly after middle age.[101,103] This is, at least in part, responsible for the age-dependent yellowing of the human lens. In the case of Kyn, attachment is via Cys, His and Lys residues.[103,110-112] The covalent attachment of these compounds arises because of the spontaneous deamination of the UV filters at pH 7 (Scheme 2.1).[109] The current study examined 3OHKyn, which is the third kynurenine UV filter compound known to be present in the lens. It deaminates more rapidly than the other two UV filters.[109] Unlike 3OHKynG and Kyn, 3OHKyn is an *o*-aminophenol, and is therefore likely to be much less stable at neutral pH, particularly under oxidative conditions.[181,184] In order to investigate the properties of such compounds, the 3OHKyn amino acid adducts of Cys, His and Lys were synthesised and characterised, by NMR spectroscopy, mass spectrometry, UV-visible and 3-D fluorescence spectroscopy. The stability of each 3OHKyn amino acid adduct at pH 4.0 and 7.2 was also examined. In addition, the stability towards acid hydrolysis was also examined.

The 3OHKyn amino acid adducts were synthesised using the method of Vazquez, *et al*. for Kyn amino acid adducts.[103] The reaction was undertaken at a high pH (*i.e.* pH 9.5) since it increases the rate of deamination of the UV filter amino acid side chain. However, unlike Kyn, 3OHKyn is unstable under basic conditions,[181,184] and therefore, the yields for the reaction were low in comparison. The yields for the 3OHKyn adducts were 17% for 3OHKyn-Cys, 9% for 3OHKyn-*t*-Boc-His, and 11% for the 3OHKyn-*t*-Boc-Lys adduct, compared to 49% for Kyn-Cys, 30% for Kyn-*t*-Boc-His, and 56% for the Kyn-*t*-Boc-Lys.[103] Characterisation of the 3OHKyn amino acid adducts by NMR spectroscopy was difficult since low yields were obtained, and the adducts were rather insoluble. The chemical shifts for the ^{1}H and ^{13}C of each 3OHKyn amino acid was similar to the chemical shifts reported for the Kyn amino acid adducts.[103] In addition, the chemical shifts for 3OHKyn-*t*-Boc-Lys were similar to those reported by Staniszewska, *et al*.[193] who have synthesised an antigen using 3OHKyn and *N*-α-acetyl Lys, for immunohistochemical studies. The carbons and hydrogens could all be assigned except for the quarternary carbons, which could not be resolved. The gHMBC

experiments confirmed that in each case, attachment of the amino acid was through C-9 of the UV filter amino acid side chain.

Mass spectrometry was also important for confirming the structure of each 3OHKyn amino acid adduct, and it could be used for later investigations. MS/MS of the molecular ions yielded fragment ions that were comparable to the fragment ions of the Kyn amino acid adducts, since, the difference between them should be 16 Da (the mass of an oxygen atom) higher for 3OHKyn (if the fragments contain the aromatic ring).

MS/MS analysis of all three 3OHKyn amino acid adducts showed that although all three adducts contain the 3OHKyn aromatic moiety, there are only three common fragment ions present in the MS/MS of each adduct. The fragment ion *m/z* 208, (Figure 2.24) corresponds to 3OHKyn-yellow, an intramolecular cyclisation product of 3OHKyn at neutral pH.[191] In addition, the fragment ions *m/z* 162 and 110 (Figure 2.24) are also common fragment ions for all three 3OHKyn amino acid adducts. It is unknown why these three fragment ions are the only common ions amongst all three adducts. However, the collision energy used during MS/MS, together with the stability of each adduct may be responsible for this outcome. Other fragment ions that contain the 3OHKyn aromatic moiety, include, *m/z* 190 and 136, which was observed for 3OHKyn-Cys and 3OHKyn-*t*-Boc-His adducts, and *m/z* 152, which was observed for the 3OHKyn-Cys and 3OHKyn-*t*-Boc-Lys adducts (Tables 2.1, 2.2 and 2.3).



m/z 208 *m/z* 162 *m/z* 110

Figure 2.24 Structures of the common fragment ions for all three 3OHKyn amino acid adducts.

In the MS/MS spectra of 3OHKyn-*t*-Boc-Lys and 3OHKyn-Cys there were two abundant fragment ions at *m/z* 203 and 202 respectively, which further confirm the proposed structures of the 3OHKyn amino acid adducts. These ions are a portion of the amino acid covalently attached to a portion of 3OHKyn at C-9 (Table 2.1 and 2.3). These ions are also characteristic for Kyn-Lys and Kyn-Cys.[103] Therefore, these two ions may be used as markers for detecting UV filter attachment in protein digests.

Since 3OHKynG and Kyn covalently attach to human lens protein after middle age,[101,103] it was assumed that 3OHKyn should also attach. However, the level of free 3OHKyn in the lens is considerably less than 3OHKynG and Kyn.[19] The average amount of 3OHKynG in 20 year old human lenses is approximately 400 nmol/gram of protein, compared to 15 nmol of 3OHKyn per gram of protein, and these concentrations decrease at a rate of ~12% per decade.[19] Characterising the fragment ions of the 3OHKyn adducts (Table 2.1, 2.2 and 2.3) is important, since these will act as 'markers' for identifying these adducts in lens proteins, where it is expected that these levels will be very low.

The UV-visible spectra for each adduct was essentially identical at pH 2.1, 5.5 and 7.2. 3OHKyn-Cys, 3OHKyn-*t*-Boc-His and 3OHKyn-*t*-Boc-Lys all exhibited broad peaks centred at 374, 375 and 367 nm respectively, on the UV-visible spectra. These absorbances are comparable to the UV-visible absorbance of lens UV filters, which are centred at 365 nm.[73] Each of the 3OHKyn amino acid adducts was yellow and therefore they may contribute to age-related lens colouration.[93] The 3OHKyn adducts did not exhibit a broad peak at any wavelength between 250 and 450 nm, at pH 9.5. Once each adduct was added to the sodium carbonate/bicarbonate buffer, pH 9.5, the solution rapidly turned a dark yellow/orange colour. The colour of the solutions when the 3OHKyn adducts were added to TFA, guanidine HCl and phosphate buffer, were all a pale yellow colour by comparison. The darker coloured solution that resulted at pH 9.5, indicates that the 3OHKyn amino acid adducts had begun to oxidise possibly to phenoxazones, but the spectra did not contain the typical broad peak centred at 440 nm, typical for phenoxazones.[184]

The 3-D fluorescence data for each 3OHKyn amino acid adduct has been summarised in Table 2.6. Two fluorophores with Ex 440 nm/Em 520 nm and Ex 340 nm/Em 400 nm increase in intensity with aging of the lens.[97,98] All of the 3OHKyn amino acid adducts fluoresced, and the observed fluorescence excitation/emission wavelengths are similar to those in human lenses.

Table 2.6 Summary of the 3-D fluorescence intensities for each 3OHKyn amino acid adduct.

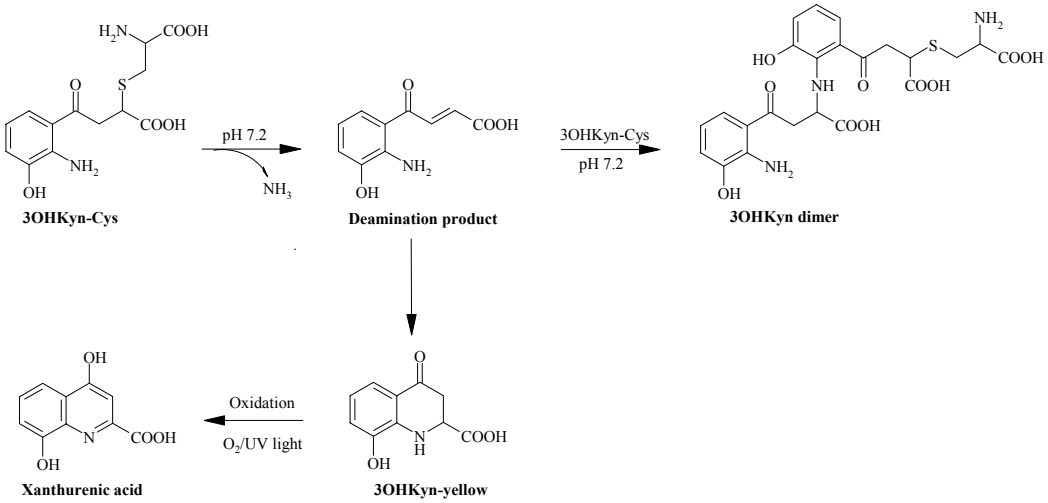
	Ex (nm)/Em (nm)			
3OHKyn Adduct	**pH 2.1**	**pH 5.5**	**pH 7.2**	**pH 9.5**
3OHKyn-Cys	350/520 420/520	340/510 430/510	330/510 400/520	360/520
3OHKyn-*t*-Boc-His	370/520	350/520	430/530	340/510
3OHKyn-*t*-Boc-Lys	420/520	350/520 410/520	360/520	330/510

Stability studies were performed at pH 7.2 and 4.0 under non-oxidative conditions to determine the properties of each 3OHKyn amino acid adduct. At pH 7.2, 3OHKyn-*t*-Boc-His was the most stable adduct. Decomposition of all adducts was accelerated in the presence of oxygen. 3OHKyn-*t*-Boc-Lys and 3OHKyn-Cys were both unstable at pH 7.2. Under oxidative conditions, the Lys and Cys adducts could not be recovered after 48 and 24 hours of incubation respectively. Under non-oxidative conditions, minimum amounts of 3OHKyn-Cys and 3OHKyn-*t*-Boc-Lys could be recovered after 5 days of incubation (Figure 2.15).

At pH 4.0, the stability of each adduct improved. Under non-oxidative conditions, the Cys and Lys adducts were recovered in ~70% yield and His adduct recovered in ~50% yield, after 48 hours (Figure 2.16). Therefore, acidic conditions can be used, for example, for protease digestion of 3OHKyn-modified proteins, in order to isolate modified peptides that can be characterised by MS/MS. EDTA was not added to any of

the reaction mixtures and metals such as Cu^{2+} and Fe^{2+} may play a role in the autoxidation of the adducts. This was not investigated.

A number of additional peaks were identified in the HPLC chromatograms (chromatograms not shown) of each adduct following incubation at pH 7.2 (stability studies). The isolated peaks were examined by mass spectrometry, but only one compound could be identified. Xanthurenic acid was the only compound identified as a breakdown product of all three adducts at pH 7.2. Xanthurenic acid forms from oxidation of 3OHKyn-yellow[86,109,191] (Scheme 2.2). Other breakdown products expected from the autoxidation of 3OHKyn amino acid adducts at pH 7.2 include, deaminated 3OHKyn and 3OHKyn-yellow. For example, 3OHKyn cleaves from the 3OHKyn-Cys adduct and results in the deaminated compound. 3OHKyn-yellow forms from cyclisation of the deaminated compound (Scheme 2.2). Since the ions for deaminated 3OHKyn and 3OHKyn-yellow could not be detected in the mass spectra of any of the isolated peaks from the HPLC chromatogram, this indicates that these two compounds are both unstable. Another compound, which has been identified in Kyn stability studies, is Kyn dimer.[194] At neutral pH, Kyn deaminates and is susceptible to nucleophilic attack by the *ortho* amino group of unreacted Kyn, producing Kyn dimer. Analysis of all the peaks from each 3OHKyn stability study failed to show the presence of a 3OHKyn dimer. An example of a 3OHKyn dimer is shown in Scheme 2.2.



Scheme 2.2 Mechanism for the formation of breakdown products of 3OHKyn-Cys at pH 7.2.

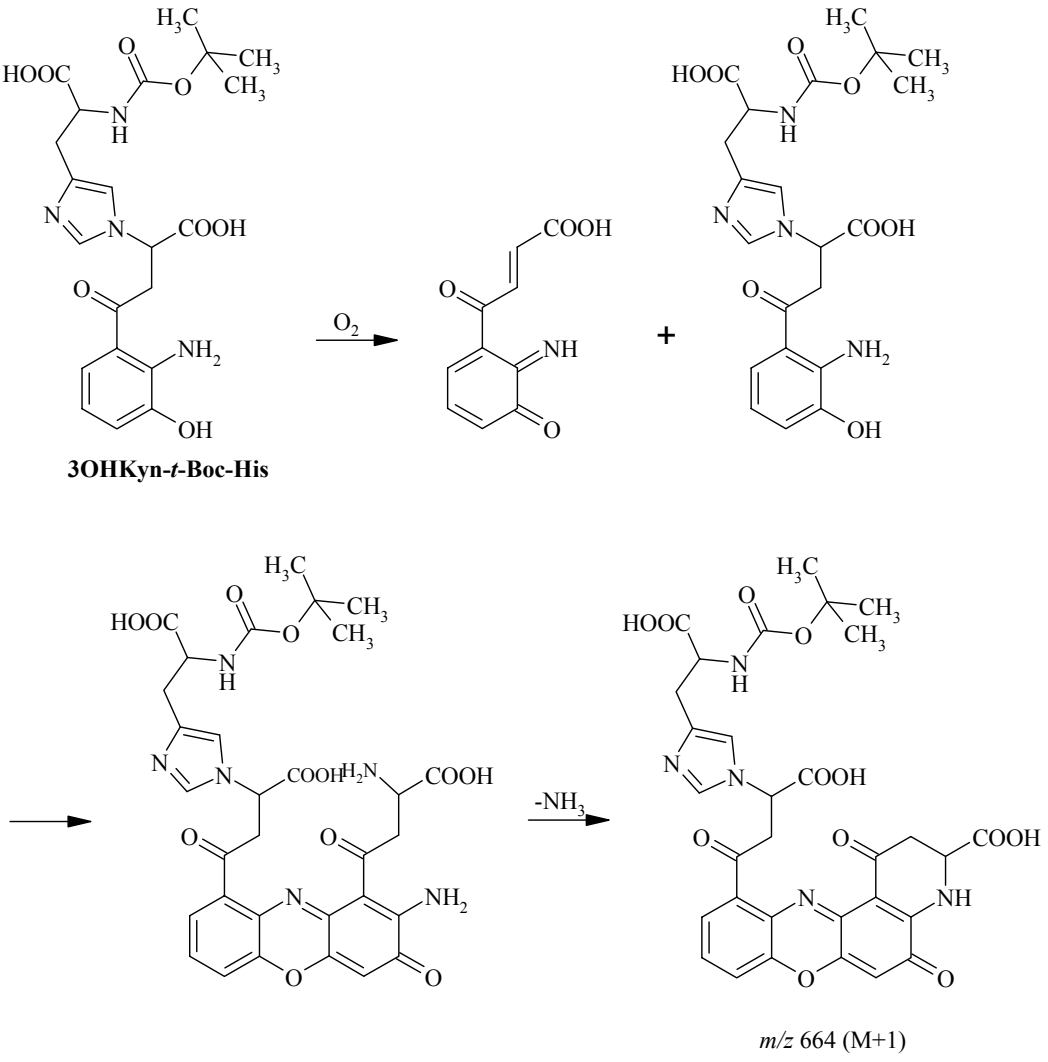
The stability of the 3OHKyn adducts was also determined under conditions used for acid hydrolysis of proteins. Acid hydrolysis was the technique used to identify Kyn adducts in lens proteins.[103] The addition of antioxidants in this procedure increased the recovery of each of the adducts. The protocol (thioglycolic acid 5% (v/v) and phenol 1% (w/v)) was adopted from a paper by Fu, *et al*. where these compounds were used as a reductant and antioxidant respectively to optimise the recovery of DOPA from lens samples.[165] The recovery of the 3OHKyn amino acid adducts from acid hydrolysis with antioxidants was approximately 4-fold higher than without thioglycolic acid and phenol. Although the 3OHKyn adducts are recoverable from acid hydrolysis, these compounds are unstable in comparison to the Kyn adducts. The recovery of Kyn adducts from acid hydrolysis without antioxidants is 96% for Kyn-Cys and Kyn-Lys and 99% for Kyn-His.[103]

Incubation of 3OHKyn-Cys, together with excess N-α-*t*-Boc-His and N-α-*t*-Boc-Lys, at pH 7.2, showed that deaminated 3OHKyn formed from 3OHKyn-Cys, and readily transferred to either N-α-*t*-Boc-His or N-α-*t*-Boc-Lys, forming 3OHKyn-*t*-Boc-His and 3OHKyn-*t*-Boc-Lys. This experiment further demonstrated the intrinsic instability of the 3OHKyn amino acid adducts, and it also suggests how 3OHKyn may react *in vivo*.

The HPLC chromatogram (Figure 2.18) showed that a new product had also formed with a molecular ion at *m/z* 664. This ion was also observed as a breakdown product of 3OHKyn-*t*-Boc-His at pH 7.2. Since this ion was observed reproducibly, it was of interest to try to characterise this compound. A proposed structure of this compound is shown in Figure 2.21. Oxidation of 3OHKyn yields a phenoxazone.[184] The MS/MS spectrum of this unknown compound, which was originally referred to as U39.5, showed a prominent loss of 100 Da, indicative of the loss of a *t*-Boc group. A further loss of 155 Da was indicative of a loss of a His. The proposed structure of the resulting fragment ion, *m/z* 409, consisted of a phenoxazone moiety with a deaminated 3OHKyn amino acid side chain. To confirm that the ion *m/z* 409 was derived from autoxidation of 3OHKyn, 3OHKyn was incubated at pH 7.2, and this ion was present in the reaction mixture. Finally, the UV-visible spectrum of U39.5 exhibited a broad peak with a maximum absorbance centred at 437 nm. An absorbance centred at 440 nm on the UV-

visible spectrum is characteristic of phenoxazones.[184] The data supports the proposed structure of U39.5.

The chemical name of U39.5 is 1,3,4,5-tetrahydro-11-(2-(N-α-*tert*-butyloxycarbonyl-histidyl)-(4-hydroxy-1,4-dioxo-butanyl)-1,5-dioxo-2H-pyrido(3,2-a)phenoxazine-3-carboxylic acid, and the mechanism of formation of this compound (Scheme 2.3) is similar to the formation of xanthommatin.[184]



Scheme 2.3 Mechanism of formation of U39.5, *m/z* 664.

In conclusion, three 3OHKyn amino acid adducts were synthesised and characterised. The characterisation of the fragment ions is important in future work, where these ions could be used as markers in protein digests. Additionally, a new oxidation product of 3OHKyn-*t*-Boc-His has been identified.