Elucidation of a Novel Polypeptide Cross-Link Involving 3-Hydroxykynurenine

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ABSTRACT: 3-Hydroxykynurenine, a metabolite of tryptophan, is a powerful antioxidant and neurotoxin. The neurotoxicity results from the oxidation of 3-hydroxykynurenine, and hydroxyl radicals, formed via H_2O_2, may also be implicated [Okuda, S., Nishiyama, N., Saito, H., and Katsuki, H. (1996) *Proc. Natl. Acad. Sci. U.S.A. 93*, 12553−12558]. Oxidation of *o*-aminophenols, such as 3-hydroxykynurenine, also results in the formation of highly reactive quinonimines. Thus, one possible consequence of 3-hydroxykynurenine oxidation may be covalent modification of cellular macromolecules. Such a process could contribute to the neurotoxicity and may potentially be important in other tissues, such as the human lens, where 3-hydroxykynurenine functions as a UV filter. In this work, we demonstrate that 3-hydroxykynurenine can bind to protein amino groups and, further, that under oxidative conditions, 3-hydroxykynurenine can function to cross-link polypeptide chains. The structure of the cross-linked moiety, using the peptide glycyllysine, has been elucidated. The cross-link, which is both colored and fluorescent, involves the peptide α-amino groups. Proteins modified by 3-hydroxykynurenine become colored and fluorescent as well as cross-linked. LC−MS studies indicate that the cross-link is also present in γ-crystallin, following incubation of this lens protein in the presence of 3-hydroxykynurenine. Similar posttranslational modifications of lens proteins accompany cataract formation, and knowledge of the precise mode of reaction of 3-hydroxykynurenine with proteins will assist in determining if 3-hydroxykynurenine is involved in degenerative conditions in which oxidation of such aminophenols is implicated.

3OHKyn[1] is a naturally occurring tryptophan metabolite found in a number of biological systems (Figure 1). 3OHKyn has been isolated from the pupae of *Calliphora* and silkworms *(1)* and has been found to be the precursor responsible for the oxidative formation of ommochromes, the eye pigments found in flies, and the body and wing scales of butterflies *(2, 3)*.

Along with other hydroxylated tryptophan metabolites, 3OHKyn has also been found to be an effective scavenger of peroxyl radicals, leading to the proposal that it may act as a local antioxidant in mammalian inflammatory diseases *(4)*. Indeed, it is a major radical scavenger in the respiration of *Aldrichina grahami*, protecting against *tert*-butylhydroperoxide injury to body fat cells *(5)*. Ironically, 3OHKyn has been shown to possess neurotoxic properties due, in part, to the production of hydrogen peroxide and hydroxyl radicals during its autoxidation *(6)* and, along with quinolinic acid, is proposed to be involved in the pathophysiology of several human brain diseases, including Huntington's disease *(7)*, HIV-related dementia *(8)*, and Parkinson's disease *(9)*.

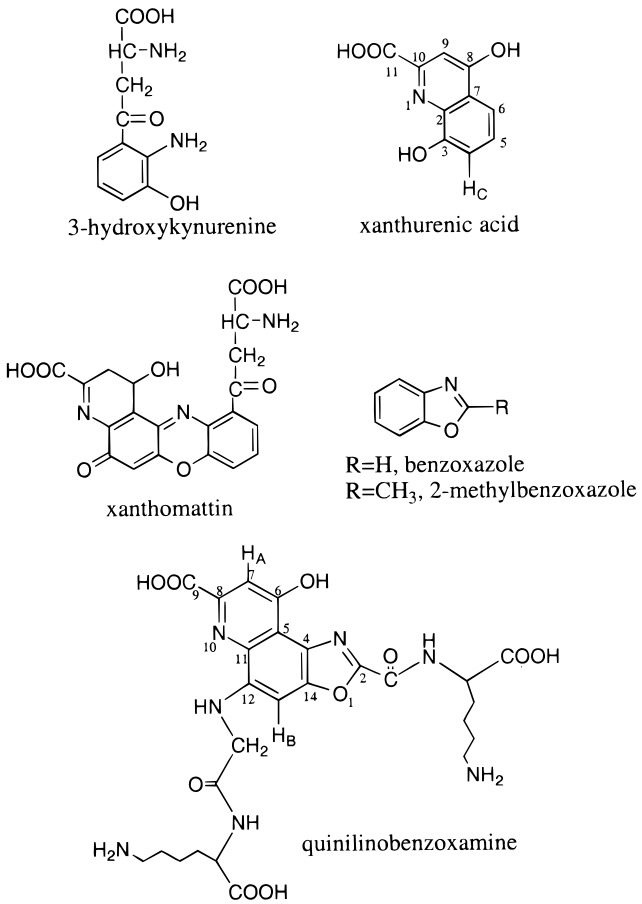

FIGURE 1: Structures of 3OHKyn and related oxidized molecules.

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[1] Abbreviations: QBA, quinilinobenzoxamine; 3OHKyn, 3-hydroxykynurenine; GK, glycyllysine; XA, xanthurenic acid; LC−MS, inline HPLC mass spectrometry; MS/MS, tandem mass spectrometry; TOF, time-of-flight; 2D, two-dimensional; DQF-COSY, double-quantum filtered correlation spectroscopy; TOCSY, total correlation spectroscopy; NOESY, nuclear Overhauser effect spectroscopy; HSQC, heteronuclear single-bond coherence spectroscopy; HMBC, heteronuclear multiple-bond coherence spectroscopy; SDS−PAGE, sodiumdodecylsulfate polyacrylamide gel electrophoresis.

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In the mammalian lens, 3OHKyn is present as one of a number of long-wavelength, UV-light-absorbing compounds *(10)*. It is an *o*-aminophenol and, as such, undergoes facile autoxidation resulting in the formation of products that have been found to react with proteins to produce brown-colored polypeptides with indistinct long-wavelength-absorption characteristics *(11, 12)*. The binding of 3OHKyn oxidation products to lens proteins is accompanied by aggregation to higher molecular weight species and development of non-tryptophan fluorescence *(13)*. Experiments involving reaction of 3OHKyn with various compounds, including glycine, suggested that 3OHKyn reacts with compounds that have a free amine functionality *(14)* and that under oxidative and photolytic conditions this leads to the production of a mixture of blue and green fluorophores *(15)*. The mechanism of formation of these products and the nature of the covalently bound species involved in the evolution of this fluorescence are not known. Furthermore, the use of a single amino acid model system allows for the possibility of Strecker degradation products *(16)*, which complicate the analysis; however, this is not the case with polypeptides, such as the lens crystallins.

Senile nuclear cataracts are characterized by the formation of high-molecular-weight, colored proteins that are insoluble and produce opacity and subsequent light-scattering, leading to a decrease in the transmission of light. These aggregates are formed by posttranslational modification of preexisting polypeptides. A comparison of the levels of blue and green fluorescence in cataractous and normal lenses has revealed that a much higher green-to-blue fluorescence ratio exists in the soluble protein fractions of cataractous lenses compared to the ratio of clear ones *(17)*, indicating that these green products are associated with cataractogenesis.

If 3OHKyn is involved in modification of the proteins in vivo, then valuable markers for this process could be obtained via the elucidation of the structures of 3OHKyn−protein adducts. In the present study, 3OHKyn was reacted, under oxidative conditions, with the dipeptide, glycinelysine (GK), and also with bovine lens γ-crystallins, which possess GK as their first two N-terminal amino acid residues. It was our aim to isolate the major fluorescent product from the GK/3OHKyn reaction mixture and to elucidate its structure. In addition, we proposed to search for evidence of this product in γ-crystallins that had been exposed to 3OHKyn.

EXPERIMENTAL PROCEDURES

Materials and Methods. 3OHKyn, GK, xanthurenic acid (XA), DMSO-d_6, and trypsin (TPCK-treated) were purchased from Sigma (St. Louis, MO). 2-Methylbenzoxazole was obtained from Fluka Fine Chemicals. D_2O was supplied by Cambridge Isotope Laboratories. All other chemicals were of analytical quality.

3OHKyn (2 mg mL^{-1}) was reacted with GK (50 mg mL^{-1}) in a 5 mL solution of 100 mM phosphate buffer, pH 7.0. A gentle stream of humidified oxygen was bubbled into the solutions to provide mixing and constant saturation with oxygen. The reaction mixture was held at 37 °C in a circulating water bath for 5 days. Lyophilized total calf lens protein was obtained as previously described *(13)*. The crystallins were separated on a Sephacryl S-300 size exclusion chromatography column (1200 × 25 mm) in 100 mM phosphate buffer, 0.02% NaN_3, pH 7.0. The γ-crystallin fractions were collected, dialyzed, and lyophilized and then frozen at −20 °C until required. Reaction of γ-crystallin with 3OHKyn was as for GK except that the concentration of protein was 20 mg mL^{-1}.

Separation and Purification of the Major Reaction Product. The GK reaction mixtures were centrifuged to remove any precipitated 3OHKyn oxidation products prior to HPLC purification. Analytical HPLC was carried out on a Beckman Microsorb MV 100 Å C18 column (250 × 4.6 mm, 5 μm) with a 0−40% acetonitrile gradient over 30 min at a flow rate of 1 mL min^{-1}. UV detection was at 365 and 229 nm, using a Waters 440 UV detector with an extended wavelength module. Fluorescence detection was at excitation and emission wavelengths of 390 and 490 nm, respectively, using an ICI LC1250 in-line fluorescence detector. Purification of the major peak was carried out on the same HPLC system, using a Brownlee C8 semipreparative column (250 × 10 mm, 7 μm) with a 0−40% acetonitrile gradient over 50 min. A 20 mg sample of material was loaded per run, and the collected fractions were pooled and lyophilized for subsequent spectroscopic analysis.

The γ-crystallin reaction mixture was separated on a Pharmacia G-10 FPLC column (250 × 10 mm) with a 10% ethanol eluent. The protein peak was collected, a small amount was taken for fluorescence spectroscopy, and the rest was lyophilized. This protein was boiled for 2 min in 200 mM NH_4HCO_3, 1 mM $CaCl_2$, pH 8.1, prior to digestion for 24 h with trypsin (50:1 w/w).

Fluorescence spectra were acquired on a Hitachi F4500 fluorimeter and UV spectra on a Shimadzu UV-2401-PC spectrophotometer. All samples were dissolved in 100 mM phosphate buffer pH 7.0. SDS-PAGE was performed on 15% homogeneous gels, and gels were scanned with a Biorad densitometer.

Mass Spectrometry. Electrospray ionization mass spectra were acquired on a VG Quattro II triple quadrupole mass spectrometer (VG Biotech Ltd., Altincham, Cheshire, U.K.). Samples were dissolved in 50% aqueous acetonitrile, with 0.1% formic acid, and delivered by a Harvard Apparatus 22 syringe pump at 5−20 μL min^{-1}, depending on sample concentration. Electrospray conditions were as follows: nitrogen bath gas flow at 350 L/h and nebulizing gas to the probe at 10 L/h. Capillary probe tip potential was 3.2 kV, HV lens was 0.2 kV, and skimmer potentials ranged from 25 to 35 V. All spectra were acquired in positive-ion mode at mass unit resolution by multichannel analysis. The mass spectrometer was calibrated with NaI.

LC−MS was carried out using an Applied Biosystems 140B solvent delivery system and 785A UV detector. Separation of the γ-crystallin digest was on an Alltech Alltima 300 Å C18 column (250 × 2.1 mm, 5 μm) at a flow rate of 200 μL min^{-1}, using a 0−80% acetonitrile gradient (1% CH_3COOH) over 40 min and a column oven temperature of 25 °C. All spectra were acquired in centroid mode. Tandem mass spectrometry experiments (MS/MS) were acquired with a collision energy of 25 V and an argon gas collision cell pressure of 3.8×10^{-3} mbar.

The high-resolution TOF mass spectrum was acquired on a VG Autospec orthogonal acceleration TOF mass spectrometer (Micromass, Wythenshawe, U.K.), equipped with an electrospray ion source. The sample was continuously

injected at 5 μL min^{-1} in 50% methanol/H_2O, 1% acetic acid. The machine was calibrated with poly(ethylene glycol) 600.

Deuterium exchange was achieved by adding 500 μL of D_2O and 5 μL of DCl to ~100 μg of lyophilized sample. Exchange was allowed to proceed under nitrogen for 3 h prior to 50% dilution with acetonitrile and subsequent mass spectrometric analysis as above.

NMR Spectroscopy. NMR spectra were acquired at 400 MHz (^{1}H) and 100 MHz (^{13}C) on a Varian Unity-400 NMR spectrometer at 25 °C. Samples were dissolved in DMSO-d_6, and the spectra were referenced to the residual DMSO methyl resonances at 2.6 ppm for ^{1}H spectra and 43.5 ppm for ^{13}C spectra. Sample concentrations were quinilinobenzoxamine (QBA), 11 mM, and XA and 2-methylbenzoxazole, 30 mM.

^{1}H experiments (DQF-COSY, TOCSY, and NOESY) were acquired in the phase-sensitive mode, using time-proportional phase incrementation *(18)*. The mixing for the NOESY experiment was 300 ms. Typically, 512 t_1 increments, with up to 96 scans per increment, were acquired over 2048 data points, which were zero-filled to 2048 data points in both dimensions and multiplied by a Gaussian window function prior to Fourier transformation.

For the ^{1}H−^{13}C correlation experiments (HSQC and HMBC), gradients were used for coherence selection *(19)*. Up to 1024 t_1 increments with up to 128 scans per increment were acquired over 2048 data points. The data were processed as for the ^{1}H 2D experiments. The HSQC and HMBC spectra were acquired in the phase-sensitive and absolute-value modes, respectively. The delays in the HSQC and HMBC spectra were set for J_{CH} values of 140 and 9 Hz, respectively.

RESULTS

UV−vis and Fluorescence Spectroscopy. 3OHKyn, as with other *o*-aminophenols, is readily autoxidized at neutral pH to form numerous products *(20, 21)*. When 3OHKyn was allowed to oxidize at pH 7.0 in the presence of the dipeptide GK, several new peaks were observed. The analytical HPLC chromatograms for the reaction mixture of GK and 3OHKyn, with detection at 365 and 229 nm, and fluorescence (ex 390/em 490 nm, where ex is excitation and em is emission) are shown in Figure 2. The major product absorbing at 365 nm, corresponding to the peak at 18 min, which was not present in oxidized samples of either of the reactants, was collected for spectroscopic analysis. Subsequent preparative HPLC purification of this product for NMR spectroscopy enabled a yield to be calculated. From a total reaction mixture of 10 mg of 3OHKyn and 250 mg of GK, 4.8 mg of purified compound was collected. This represents an 18% yield with respect to 3OHKyn. Several other fluorescent peaks were observed by HPLC but, when collected, were found to be minor products and were not investigated further.

UV−vis and fluorescence spectra of the major HPLC fraction are shown in parts A and B of Figure 3, respectively. The UV−vis spectrum of GK/3OHKyn shows two absorption maxima at 245 and 367 nm. These maxima are consistent with a kynurenine absorbance (λ_{max} 370 nm) *(22)*, or 3OHKyn-like absorbance (λ_{max} 365 nm) *(23)*, and benzoxazole, which has a λ_{max} at 247 nm *(24)*. The 3D fluorescence spectrum exhibits a single sharp peak at

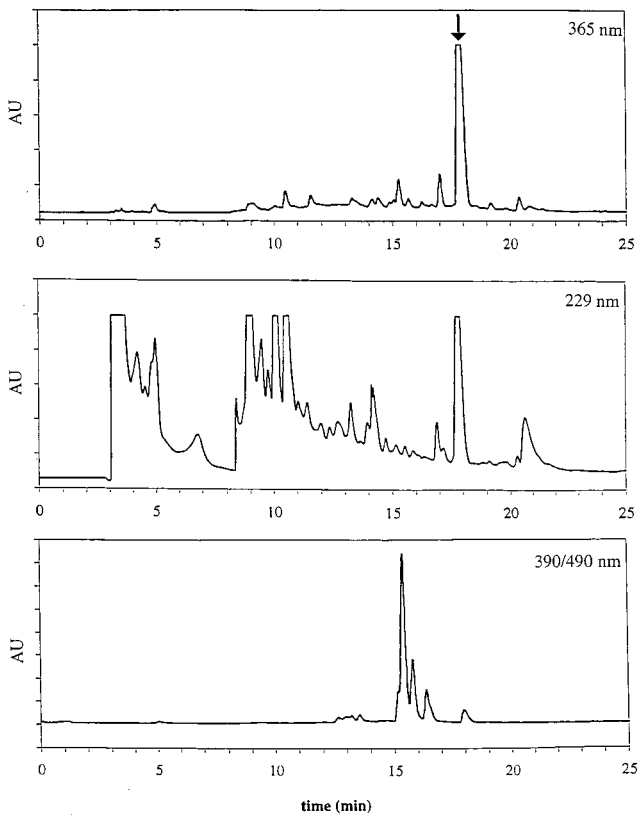

FIGURE 2: HPLC trace and fluorescence (ex 390 nm/em 490 nm) of the GK/3OHKyn reaction mixture. The eluant was monitored for absorbance at 365 and 229 nm. The arrow indicates the major peak absorbing at 365 nm that was collected for structural analysis.

excitation and emission maxima of 390 and 490 nm, respectively. Kynurenine has fluorescence maxima of ex 370/em 490 at pH 11.0 *(25)*, indicating that kynurenine and the unknown product may contain structural similarities.

Mass Spectrometry. The mass spectrum of the major reaction product collected from HPLC (Figure 4A) revealed a singly charged species at *m*/*z* 604 and a doubly charged species at 303 *m*/*z*. A molecular mass of 603 Da was significantly higher than that expected for a simple combination of GK (203 Da) and 3OHKyn (224 Da), suggesting that the adduct may be a trimer. This was supported by the TOF high-resolution MS that yielded a mass of 603.228891 Da and a corresponding calculated empirical formula of $C_{26}H_{33}N_7O_{10}$. Such a formula could best be rationalized by a structure consisting of two GK and one 3OHKyn moieties.

An MS/MS spectrum of the singly charged molecular ion (*m*/*z* 604) was acquired and is shown in Figure 4B. Ions at *m*/*z* 84, 101, 130, and 147 suggest the presence of at least one relatively unmodified GK moiety since they were also found in the MS/MS spectrum of GK (not shown). The base peak at *m*/*z* 84 is indicative of loss of ammonia from the lysine immonium ion. A fragmentation scheme based on the MS/MS spectrum of the structure referred to as QBA is shown in Figure 5. Assignment of some of the product ions is indicated by bracketing of the bond(s) involved. The product ion of largest mass, at *m*/*z* 476, is attributed to cleavage on the carboxyl side of either the Lys_1 or Lys_2 α-NH groups. Loss of CO and water appeared to be responsible for the ions at *m*/*z* 458 and 430. This was suggested by the MS/MS of a standard sample of XA, which exhibited this characteristic loss under identical electrospray conditions

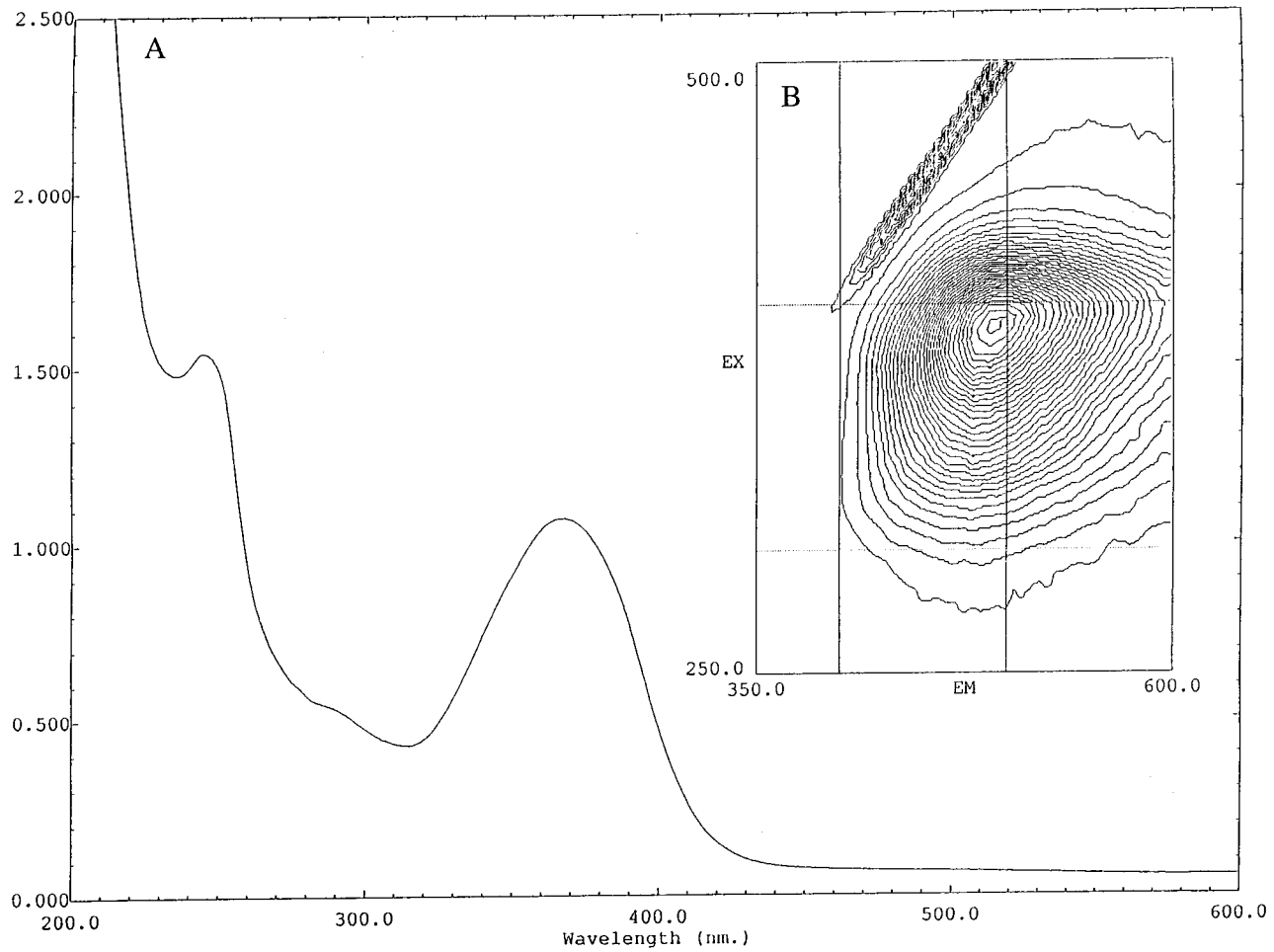

FIGURE 3: UV and fluorescence spectra of the major 365 nm absorbing HPLC peak indicated in Figure 2. (A) Two maxima at 245 and 367 nm are evident. (B) The 3D fluorescence spectrum of the same compound. Excitation and emission maxima of 390 and 490 nm were obtained.

(spectrum not shown). Cleavage of the second lysine, again on the carboxyl side of the α-NH group, gave rise to the fragment ion *m*/*z* 301. Consecutive losses of the remaining amide moieties would account for the peaks at *m*/*z* 257 and 214.

Further evidence to support the proposed structure was obtained from the mass spectrum of a sample of the compound that had been incubated in D_2O to allow exchangeable protons to be replaced by deuterium atoms. There are 13 exchangeable protons in the fully protonated (doubly charged) molecule and 12 such protons in the singly charged species (Figure 5). Complete proton−deuteron exchange would realize an increase in mass of 13 and 12 Da, respectively. The spectrum of the deuterated molecule contained two major peaks at *m*/*z* 309 and 616, which is consistent with a structure containing 13 exchangeable protons in the doubly charged species and 12 such in the molecular ion.

NMR Spectroscopy. Purification of a sufficient quantity of material for NMR spectroscopic characterization involved multiple semipreparative HPLC separations. Detailed information on the structure of this product was obtained from 1D and 2D ^1H and ^{13}C NMR spectra. The aromatic region of the 1D ^1H NMR spectrum showed only two singlet aromatic protons, H_A and H_B, at 7.86 and 6.98 ppm, respectively, three α-NH protons at 8.17, 8.55, and 9.16 ppm, and four broad, exchangeable NH resonances from 7.1 to 7.8 ppm (Figure 6). The two downfield α-NH doublet resonances had $J_{\alpha NH}$ values of 7.7 and 7.9 Hz, respectively, which was consistent with them arising from lysine residues. The upfield α-NH resonance had a triplet appearance with $J_{\alpha NH}$ values of 6.0 and 5.6 Hz, suggesting that it arose from a glycine residue.

3OHKyn has three aromatic protons (Figure 1). Thus, the presence of H_A and H_B in the spectrum of the reaction product suggested that an addition reaction had occurred para to the aromatic amino group of 3OHKyn. The $CH-CH_2$ spin system of the aliphatic side chain of 3OHKyn was absent from the ^1H spectrum of the product, possibly indicating some form of cyclization, such as that found in the 3OHKyn dimer, xanthomattin *(2)*, and also in the transamination product of 3OHKyn, xanthurenic acid (XA) (Figure 1).

Cross-peaks in the TOCSY spectrum from the α-NH protons arose from two lysine residues, referred to as Lys_1 and Lys_2, and one glycine residue (Gly_1). The four broad resonances from 7.1 to 7.8 ppm correlated to the ϵ-CH_2 resonances of Lys_2, indicating that they arose from the ϵ-NH_3^+ group of this lysine. In the NOESY spectrum, no exchange cross-peaks were observed between these resonances. The DMSO solvent contained some water, as judged by the presence of a large, broad resonance at 3.58 ppm. In the NOESY spectrum, the four Lys_2 ϵ-NH_3^+ resonances each exhibited an exchange cross-peak with this water resonance. Normally the ϵ-NH_3^+ protons of a lysine residue give rise

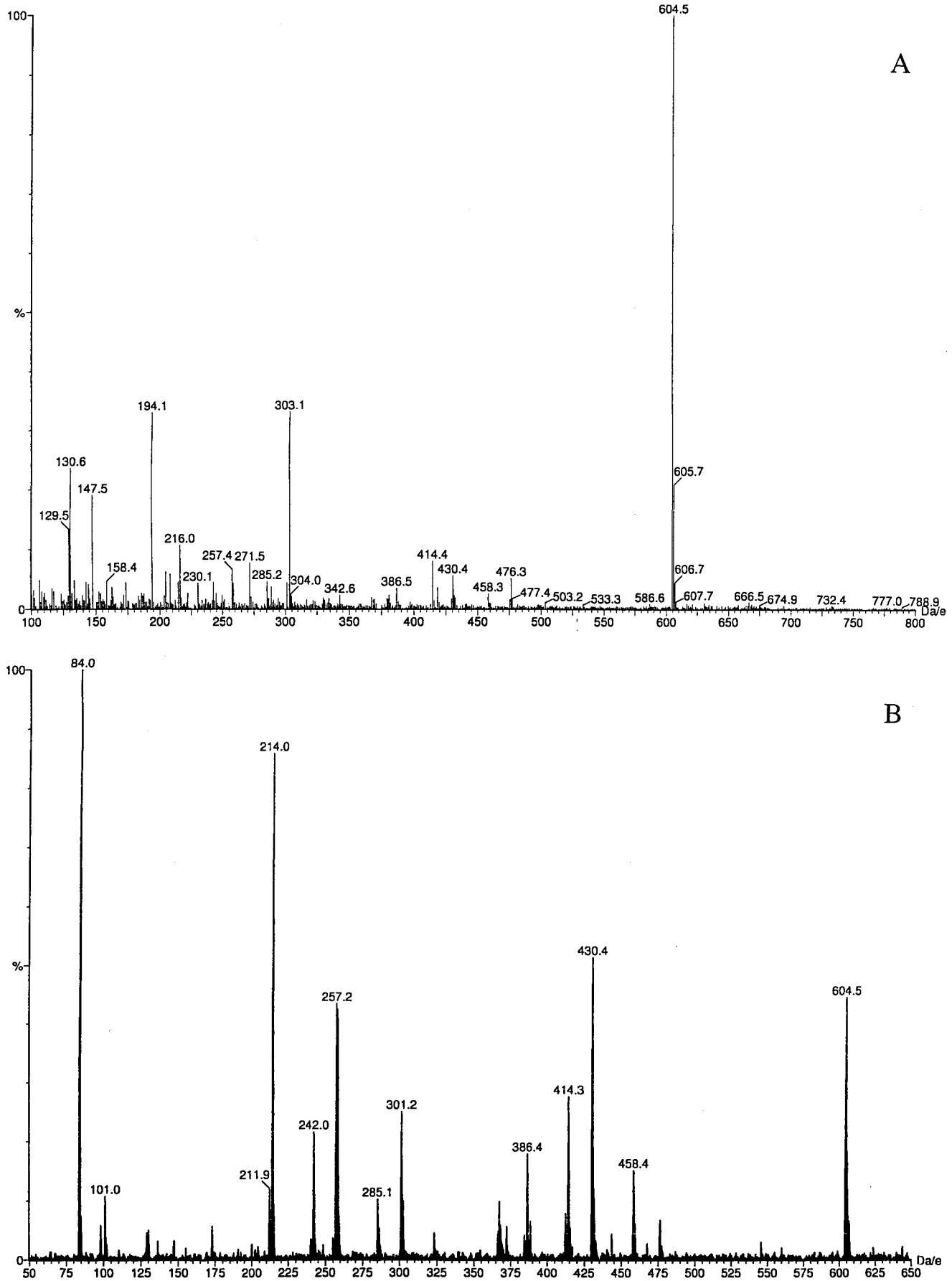

FIGURE 4: Mass spectra of the major GK/3OHKyn reaction product. (A) Mass spectrum revealing a singly charged ion at *m*/*z* 604.5 and a doubly charged ion at *m*/*z* 303.1. (B) Tandem mass spectrum of the *m*/*z* 604.5 peak and resultant fragment ions. This fragmentation pattern indicated that at least one GK moiety was incorporated into the compound and formed the basis of the fragmentation mechanism proposed in Figure 5.

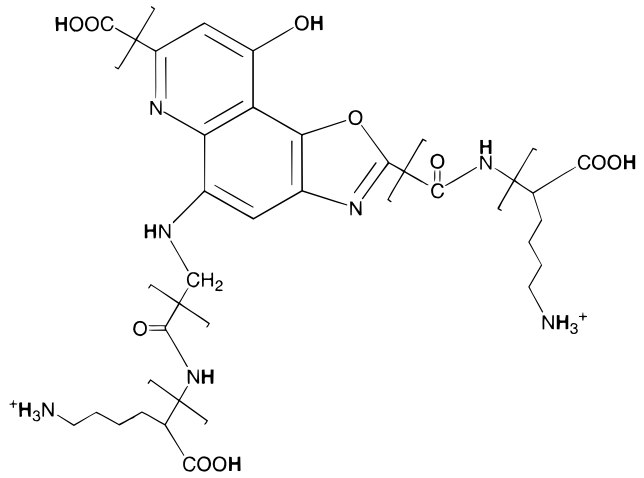

FIGURE 5: Proposed structure and fragmentation pattern of the major colored product from the reaction between 3OHKyn and GK. The compound is referred to as QBA. The 13 exchangeable protons are indicated in bold.

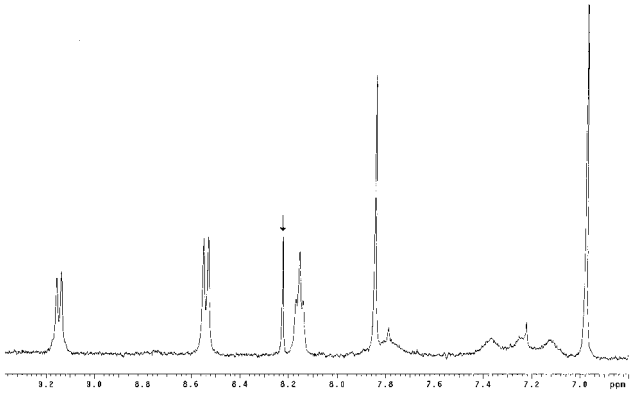

FIGURE 6: Aromatic and NH region of the 1D ^{1}H NMR spectrum of QBA. Two singlet (isolated) protons, H_A and H_B, are shown at 7.86 and 6.98 ppm, respectively. Three α-NH protons at 9.16, 8.55, and 8.17 ppm, correspond to two Lys residues and one Gly residue, based on the observed coupling constants (see text). Four broad, exchangeable NH resonances can be seen from 7.1 to 7.8 ppm. The arrow indicates an impurity.

to a single broad resonance due to rapid chemical and solvent exchange. The observation of four individual nonchemically exchanging ϵ-NH_3^+ resonances for Lys_2 suggests that the side chain of Lys_2 is restrained, i.e., the ϵ-NH_3^+ protons experience different environments. One possibility is that this group is restricted in conformation due to salt bridge interaction with the carboxyl group at C9, although it was not possible to confirm this via nuclear Overhauser effect (nOe) interactions because of the absence of protons in the vicinity of C9. In contrast, Lys_1 produced no ϵ-NH_3^+ to ϵ-CH_2 cross-peaks in the TOCSY spectrum, probably because of broadening due to chemical exchange with the water. A cross-peak was observed, however, between the α-NH and ϵ-CH_2 resonances of Lys_1. Thus, it appeared that two GK moieties had been incorporated into the structure with a second glycine residue (Gly_2) either being cleaved or involved in the formation of a ring. The former process, however, was unlikely due to the preserved mass of the reactants.

The ^{1}H NMR spectrum was assigned via a combination of through bond (DQF-COSY and TOCSY) and through-space (NOESY) spectra. Further insight into the structure (Figure 1) was obtained from the NOESY spectrum, the NH and aromatic region of which is depicted in Figure 7. An nOe from Gly_1 NH to Lys_1 NH was present, indicating that these two residues were linked. A strong nOe from Lys_1 NH to Gly_1 α-CH_2 was also observed (not shown), which confirmed this conclusion. The downfield aromatic proton (H_A) had no cross-peaks arising from it, suggesting that it was isolated. Its reduced intensity compared to H_B in the 1D ^{1}H NMR spectrum (Figure 6) implies that H_A has a longer T_1 value than H_B, which is also consistent with it not having many protons nearby.

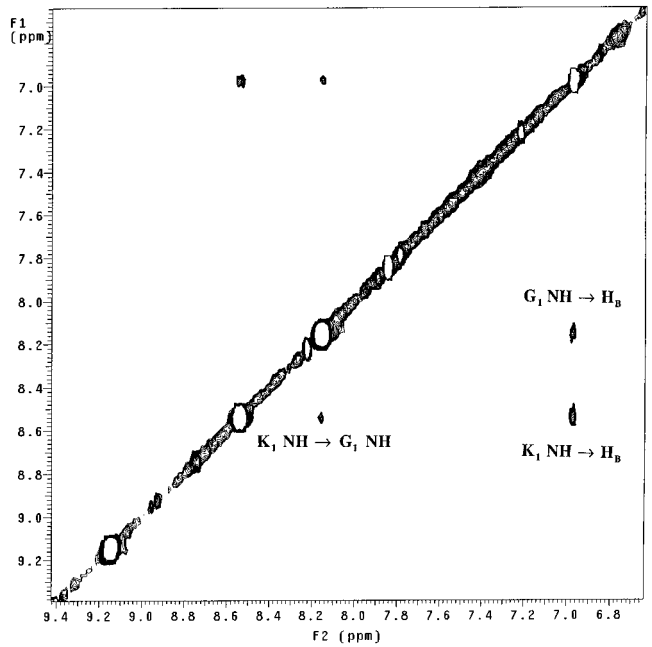

FIGURE 7: Aromatic and NH region of the 2D ^{1}H NOESY spectrum of QBA. An nOe from Lys_1 to Gly_1 indicated that the residues were linked. Observed nOe's from H_B to the α-NH groups of Lys_1 and Gly_1 suggested that the dipeptide had attached to C12 of the structure (see Figure 1), isolating it from the ortho aromatic proton and remaining in close proximity to the α-NH groups. No cross-peaks were observed for H_A, indicating that it was isolated from both the peptide residues and other aromatic protons.

In contrast to H_A, H_B had three correlations in the NOESY spectrum: to Gly_1 NH and α-CH_2 (not shown), and the Lys_1 α-NH. This suggested that only one aromatic proton, i.e., H_B, remained on the 3OHKyn ring after reaction and that a GK dipeptide had attached para to the aromatic amino group of 3OHKyn. The observed nOe correlations are summarized in Figure 8a.

The protonated ^{13}C resonances were assigned from an HSQC spectrum and are presented in Table 1 along with the ^{1}H assignments. The nonprotonated ^{13}C resonances were assigned from HMBC spectra. Some of the HMBC correlations are presented in Figure 8b. On the basis of the lack of nOes from H_A, cyclization to an XA-like structure was most likely. Thus, a standard sample of XA (Figure 1) was prepared and HSQC, HMBC, and ^{13}C 1D NMR spectra were acquired to assist with the assignments of the nonprotonated carbons of QBA (i.e., C4, C5, C6, C11, C12, and C14). Cross-peaks from the aromatic and NH protons in the HMBC spectra of the reaction product (QBA) and XA are shown in parts A and B of Figure 9, respectively. In combination with the HSQC spectrum, a definitive assignment of all the carbon

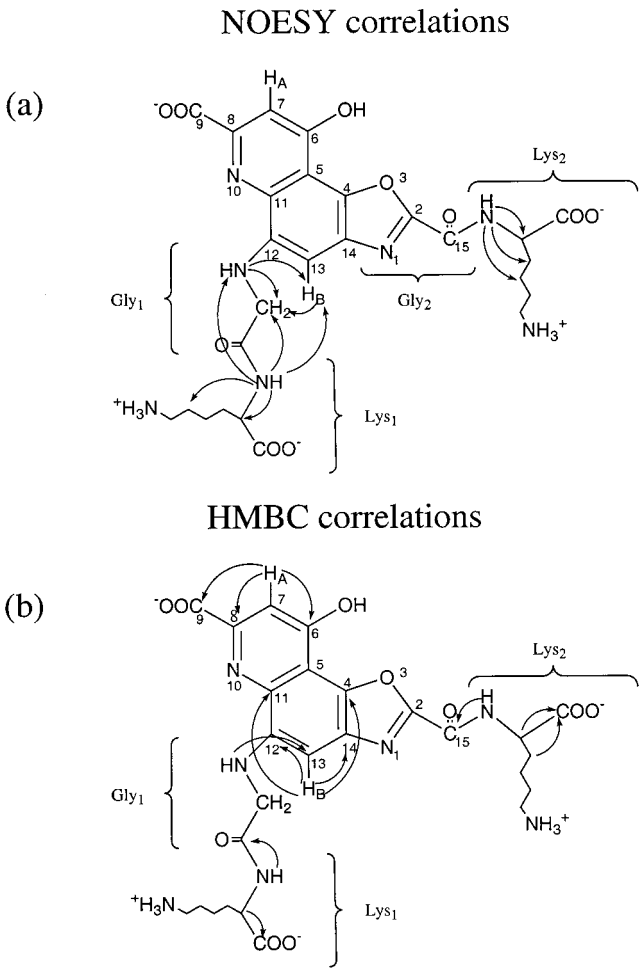

FIGURE 8: Summary of the (a) 2D ^{1}H NOESY correlations and (b) 2D ^{1}H-^{13}C HMBC correlations for QBA.

resonances of XA was determined. Interestingly, the pattern of the C2−C6 ring spin system in the HMBC spectrum of XA revealed a correlation in which cross-peaks across three bonds were stronger than those across two bonds. This characteristic was used to assign the ^{13}C shifts of C4 and C11−14 in QBA. The chemical shift for C5 was assigned to a ^{13}C resonance at 129.0 ppm by analogy with the XA HMBC results, as no cross-peak was observed to it from H_A or H_B in the HMBC spectra.

To propose a structure for the cyclic N-terminal attachment of Gly_2 at C4 and C14, we referred to the empirical formula $C_{26}H_{33}N_7O_{10}$ from the high-resolution MS experiment. On the basis of the number of nitrogens in the formula, the most likely structure was deemed to be a heterocyclic benzoxazole ring. The comparable ^{13}C chemical shifts of C4 and C14 assigned for 2-methylbenzoxazole (Figure 1, spectrum not shown), 145.1 and 154.3 ppm, respectively, are in close agreement with those assigned from the HMBC spectrum of QBA, thus supporting the structure shown in Figure 1.

Interestingly, no ^{13}C resonance was observed for C2 in our compound or the model compound, 2-methylbenzoxazole, even in ^{13}C 1D spectra acquired with a very long delay (15 s) between scans. It is unlikely, therefore, that the absence of this resonance is due to it having a long T_1 value. More likely, it is broadened because of a resonance structure being present about the N, C, and O atoms at positions 1, 2, and 3, respectively.

Table 1: Summary of ^{1}H Assignments and the Protonated and Nonprotonated ^{13}C Resonances Assigned for the Major Reaction Product, QBA, from a Combination of HSQC and HMBC Experiments

^{1}H assignments[a]							
	α-NH	α-CH	β-CH_2	γ-CH_2	δ-CH_2	ϵ-CH_2	ϵ-NH_3^+
Gly_1	8.17	4.19					
Lys_1	8.55	4.35	1.73	1.47, 1.85	1.62	2.86	
Lys_2	9.16	4.53	1.98	1.52	1.66	2.89	7.14, 7.26, 7.38, 7.78

^{13}C assignments						
	C=O	α-CH	β-CH_2	γ-CH_2	δ-CH_2	ϵ-CH_2
Gly_1	173.3	49.9				
Lys_1	177.4	55.4	34.0	26.4	30.5	43.1
Lys_2	176.9	56.1	34.4	26.6	30.5	43.1

C2	n/o[b]	C9	169.5
C4	142.0	C11	126.1
C5	129.0	C12	155.5
C6	118.7	C13	93.1
C7	110.8	C14	150.2
C8	166.0	C15	159.3

[a] H_A and H_B are 7.86 and 6.98 ppm, respectively. [b] Not observed, possibly due to broadening associated with resonance stabilization (see text).

SDS-PAGE and Comparative Reactions Involving γ-Crystallins. The dipeptide GK was chosen for these studies as it represents the two amino acids at the N-terminus of human and bovine γ-crystallins, one of the major classes of lens structural proteins. Thus, γ-crystallin was incubated with 3OHKyn under the conditions used for the reaction with GK. The reaction produced a colored product that was initially examined by SDS-PAGE (Figure 10). Lane 1 shows a control sample of γ-crystallin, which has an average mass of approximately 20 kDa, oxidized in the absence of 3OHKyn. Lanes 2−4 show decreasing loadings of the γ-crystallin/3OHKyn reaction mixture. In these lanes, it is evident that a species of molecular weight greater than 40 kDa has been produced as a result of the reaction with 3OHKyn (cf. ovalbumin, 45 kDa). This suggests that dimerization had occurred between two γ-crystallin subunits, possibly through an oxidation product of 3OHKyn.

The reaction product was desalted to remove 3OHKyn autoxidation products and digested with trypsin. The 3D fluorescence spectrum of this γ-crystallin digest (Figure 11) exhibited an ex 390/em 490, which when compared with the fluorescence spectrum of the GK/3OHKyn reaction mixture (Figure 3B), implies that a similar chromophore is present in both samples. Since the γ-crystallin digest contained no free 3OHKyn autoxidation products, it appears likely that the fluorophores are a result of a covalent interaction between an oxidized form of 3OHKyn and a protein functional group.

Inline HPLC−MS of the γ-crystallin digest gave rise to a chromatogram that showed a species of *m*/*z* 604 that eluted at 12.1−12.2 min. This corresponds to the elution time, and mass, of QBA (Figure 12). Considering that the two N-terminal residues of γ-crystallin are GK, this result indicates that the two molecules in both profiles may be the same; i.e., QBA was present in the tryptic digest of the

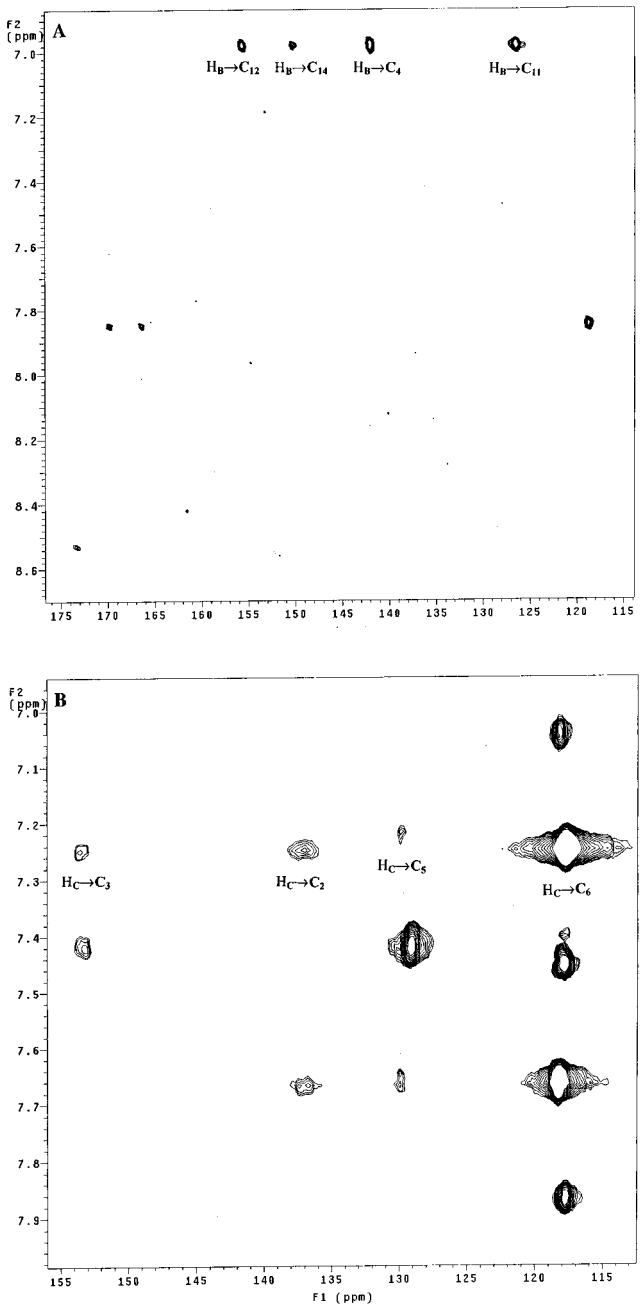

FIGURE 9: HMBC spectra of the $^1H-^{13}C$ cross-peaks from the aromatic and NH regions of (A) QBA and (B) XA. In both spectra, the ortho and meta carbon cross-peaks to the equivalent aromatic protons in each molecule (H_B and H_C) have been labeled. It is clear that in both spectra, the cross-peaks across three bonds (meta) are stronger than those across two (ortho). This pattern was more easily assigned for XA and by analogy was used to assign the ^{13}C shifts for C4, C11, C12, and C14 in the spectrum of QBA.

γ-crystallin/3OHKyn reaction. Therefore, with respect to the SDS-PAGE profile (Figure 10), cross-linking of the γ-crystallin subunits may have occurred through their N-terminal α-amino groups.

DISCUSSION

3OHKyn has been shown to be a powerful antioxidant, which may be utilized as a local defense against oxidative stress in inflammatory diseases through the up-regulation of indoleamine 2,3-dioxygenase *(4)*. This antioxidative property, however, means that 3OHKyn itself is oxidized to products

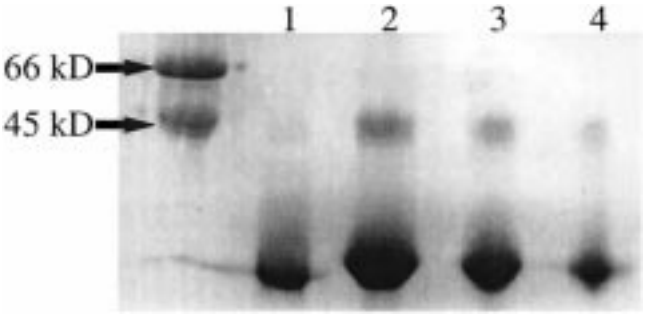

FIGURE 10: 15% homogeneous SDS-PAGE gel of γ-Crystallin (20 mg mL^{-1}) reacted for 48 h with 3OHKyn (1 mg mL^{-1}). Lane 1 is a control sample of γ-crystallin reacted in the absence of 3OHKyn, and lanes 2−4 are decreasing loads of the γ-crystallin reacted with 3OHKyn. With reference to the ovalbumin standard (45 kD), it is clear that a product has been formed with a molecular weight slightly less than 45 kD. This product presumably was a result of a dimerization reaction between the γ-crystallin subunits ($\sim$ 20 kD) and at least one 3OHKyn moiety.

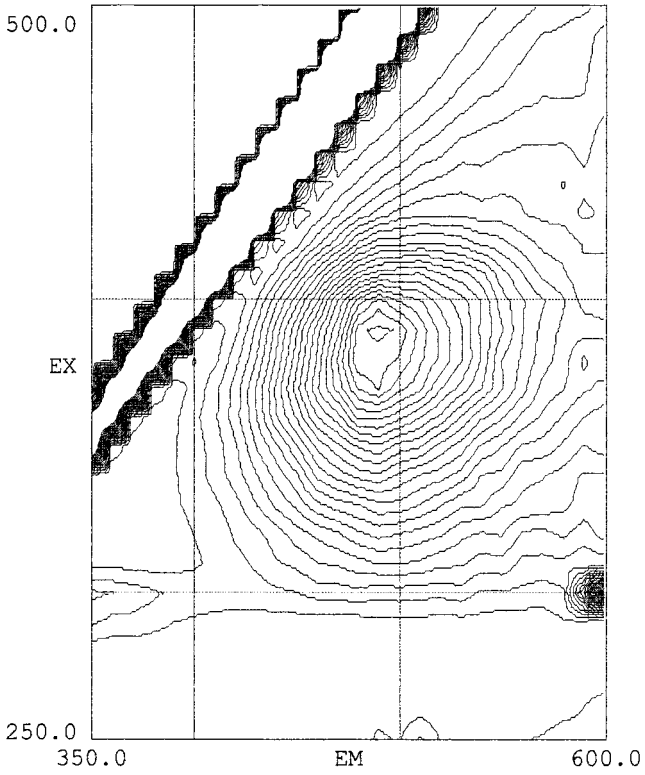

FIGURE 11: 3D fluorescence spectrum of a tryptic digest of γ-crystallin after 48 h reaction with 3OHKyn.

that have been shown to bind readily to proteins *(11)*. Furthermore, a byproduct of 3OHKyn oxidation, hydrogen peroxide, is thought to be largely responsible for cell death observed in neuronal cell cultures and, possibly, neurodegenerative disorders *(6)*.

It was, however, an interest in the direct covalent modification of proteins by the oxidation products of 3OHKyn that formed the basis of this study. At present, we have no way of detecting such posttranslational modifications, and since tryptophan metabolites have been suspected of being cataractogenic *(11, 12, 26−30)*, the role of the endogenous UV-filter 3OHKyn in the lens is of particular interest. 3OHKyn has been shown to bind to lens crystallins *(11, 13)* to produce colored proteins similar to those observed in the nuclear cataract. Thus, to determine if 3OHKyn is involved

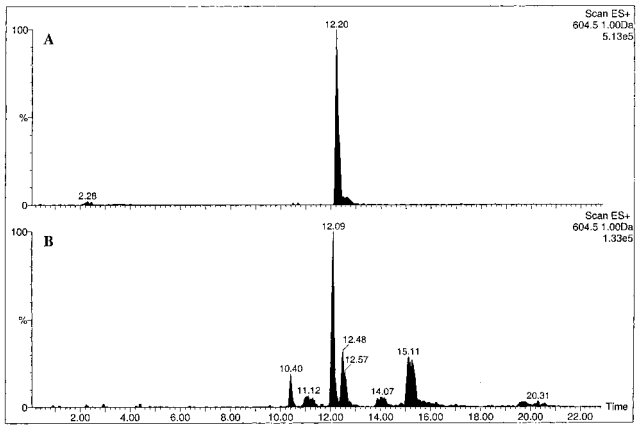

FIGURE 12: Single mass, total ion chromatograms scanning for 604 *m*/*z* ion in (A) standard QBA sample and (B) tryptic digest of γ-crystallin.

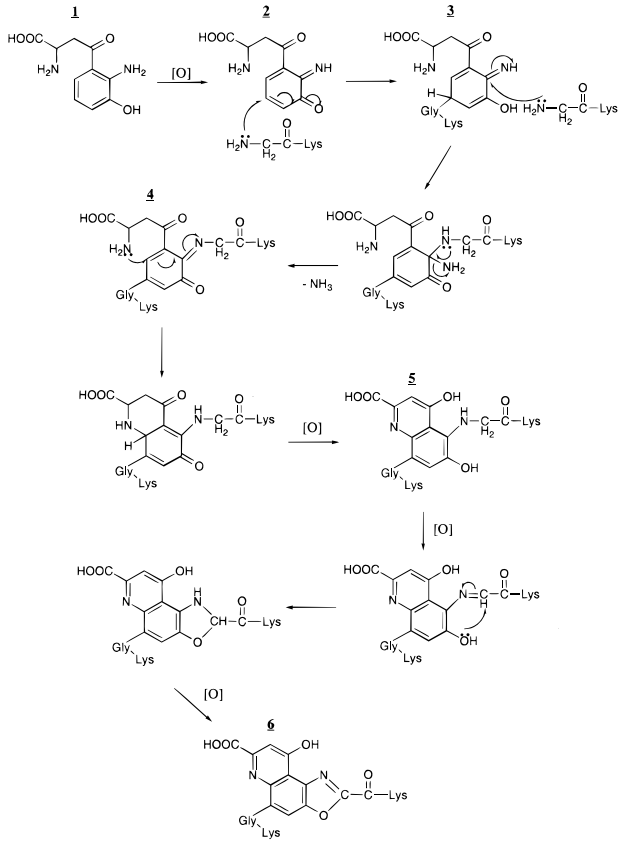

FIGURE 13: Possible mechanism of reaction of 3OHKyn with the dipeptide GK, leading to the formation of the product QBA (***6***).

in this process, biochemical markers are required. This study involved the reaction of the dipeptide GK with 3OHKyn to produce a covalent adduct and, consequently, is the first identification of such a biomarker. It was found that a product absorbing strongly at 365 nm, which also exhibited fluorescence, was formed during the oxidative reaction of GK and 3OHKyn. The yield of this compound was 18% relative to 3OHKyn.

Mass spectrometry and NMR spectroscopy were used to elucidate the structure of a novel polypeptide cross-link incorporating 3OHKyn. We have named the cross-linked major product, QBA. QBA is a colored and fluorescent compound that contains two GK moieties and arises from the in vitro reaction between the α-amino groups of GK and 3OHKyn. Under neutral conditions, the reactivity of α-amino groups is greater than that of the ϵ-amino groups due to the latter's higher pK_a values, and this may explain the lack of apparent involvement of the lysine side-chain in the adduct. Any product that results from Lys ϵ-NH_2 addition may also be less stable. This represents the first structure elucidated for the reaction between 3OHKyn and an amino acid. Several pathways for the formation of QBA are possible, one of which is depicted in Figure 13. Initially, 3OHKyn **1** is oxidized to the corresponding *o*-quinonimine **2**, which is susceptible to a nucleophilic Michael addition by the glycine α-amino group. The resultant iminophenol **3** is attacked by a second glycine α-amino group to form a Schiff base intermediate **4**. The vinyl form of **4** again allows for an intramolecular Michael addition that, via a keto−enol tautomerization (not shown), followed by oxidation, gives a substituted quinoline carboxylic acid **5**. Compound **5** is further oxidized to a Schiff intermediate, which is subject to an intramolecular nucleophilic attack on the imine by the aromatic hydroxyl group, resulting in ring closure and formation of the benzoxazole moiety, which upon oxidation yields QBA **6**.

The reactivity of oxidized 3OHKyn toward amino acid functional groups is similar to that of DOPA, catecholamines, catechol, and other aminophenols *(31)*. Hegedus and Nayak (32) found that when these compounds were incubated with human plasma samples, melanins of identical fluorescence characteristics to those found in vivo were produced in vitro. The fluorescence properties of QBA are similar to those described by many researchers as being characteristic of the aging human lens *(10*, *17*, 33−36). This lens fluorescence has been shown to increase with age and is accompanied by a concomitant decrease in lens transmittance (37), which, over time, may lead to the development of senile nuclear cataract.

In concordance with our results, Spector, et al (38) detected blue fluorescence in the soluble and insoluble fractions of older human lenses, which resulted exclusively from a 43 kDa polypeptide and suggested that it consists of two crystallin subunits that have been cross-linked. This protein was shown to lack N-terminal amino groups, and hydrolysis yielded a fluorescent, low-molecular-weight compound. Furthermore, Casey et al. (39) found that the most reactive site for glycation of γB-crystallin was the N-terminal α-amino group and that this played an important role in the glycation-mediated cross-linking of this protein. SDS-PAGE of our 3OHKyn-modified γ-crystallin showed that a cross-link had occurred between two subunits to yield a polypeptide of approximately 43 kDa in mass. A tryptic digest of the modified γ-crystallin exhibited the same fluorescence maxima as QBA, suggesting that a similar fluorescent moiety was in the digest, possibly as a result of a cross-link between the N-terminal glycines of two γ-crystallin subunits. This hypothesis was further confirmed by LC−MS of a tryptic digest, as a peak of *m*/*z* 604 coeluted with a standard of QBA.

In conclusion, a novel fluorescent product consisting of a peptide cross-linked by a highly modified 3OHKyn derivative has been isolated and characterized. The structure of this product, which was elucidated by 2D-NMR spectroscopy and mass spectrometry, contained a substituted benzoxazole moiety, presumably generated via attack of the 3OHKyn phenolic group to an imine-linked GK. A second GK was

attached via its α-amino group to the aromatic ring of 3OHKyn. Cyclization of the 3OHKyn aliphatic side chain yielded the final product. Examination of a tryptic digest of 3OHKyn-modified γ-crystallin suggested that 3OHKyn can act as a protein cross-linking agent under oxidative conditions via the formation of QBA. The isolation of this product from cataractous lens protein would strongly suggest that 3OHKyn is indeed involved in cataract formation and is the aim of future research in our laboratory.

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