Polypeptide Modification and Cross-Linking by Oxidized 3-Hydroxykynurenine†

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ABSTRACT: 3-Hydroxykynurenine (3OHKyn) is present in the mammalian lens as a UV filter and is formed from kynurenine in the tryptophan metabolic pathway. 3OHKyn is a readily autoxidized *o*-aminophenol which binds to proteins in vitro. The lens, particularly its central region, the nucleus, becomes increasingly oxidized with age. Under such conditions, the oxidation products of 3OHKyn may bind to lens proteins and contribute to nuclear cataract formation. The purpose of this study was to determine the structures of in vitro reaction products of 3OHKyn with model peptides as a general model for 3OHKyn modification of proteins. 3OHKyn was incubated with the dipeptide glycylglycine (GG) and the tetrapeptide tuftsin (sequence TKPR) under oxidizing conditions, and the reaction products were characterized by a variety of spectroscopic techniques. The major 3OHKyn−GG reaction product involves formation of a benzimidazole moiety between the GG N-terminus and the oxidized amino and/or phenol groups of 3OHKyn. In contrast, tuftsin, which has an N-terminal threonine, forms predominantly a cross-linked dimer with oxidized 3OHKyn. This product is analogous in structure to the dimeric reaction product, quinilinobenzoxamine, formed between oxidized 3OHKyn and glycyllysine [Aquilina, J. A., et al. (1999) *Biochemistry 38*, 11455−11464], which contains a benzoxazole moiety. The identification of a tuftsin dimer suggests that 3OHKyn can react with any peptide having a free α-amino group, via a general side chain elimination mechanism. The identification of both benzimidazole and benzoxazole adducts in peptides with a free N-terminus suggests that peptide amino groups can react initially at either the aromatic amino or hydroxyl group of oxidized 3OHKyn. The proportion of each adduct may change, however, depending on the amino acid sequence at the N-terminus.

The human lens contains a group of tryptophan-derived, fluorescent compounds, which act to absorb ultraviolet light in the 300−400 nm region of the electromagnetic spectrum (*1*). These molecules are synthesized along the kynurenine pathway of tryptophan metabolism (Figure 1). At present, five UV-absorbing compounds have been identified. The major component is the glucoside of 3-hydroxykynurenine (3OHKyn),[1] 3-hydroxykynurenine glucoside (3HKG) (*2*). The identification of 3HKG in the lens represents a unique pathway of tryptophan metabolism, since 3OHKyn is not converted to 3-hydroxyanthranilic acid, as in the tryptophan metabolic pathway of other tissues, but instead is conjugated with glucose. Smaller amounts of kynurenine and 3OHKyn (compared with the amount of 3HKG) have been detected in human lens extracts (*3*, *4*). Another tryptophan-derived, UV filter compound was identified by Truscott et al. (*5*) as AHBG [4-(2-amino-3-hydroxyphenyl)-4-oxobutanoic acid *O*-glucoside], which constitutes the second most abundant UV filter in the human lens. More recently, a glutathionyl adduct of deaminated 3HKG was isolated and shown to be a novel fluorophore in aged human lenses (*6*).

Of the five compounds described, only 3OHKyn has a free aromatic hydroxyl group, and as such is an *o*-aminophenol. *o*-Aminophenols are known to readily undergo complex oxidative processes. 3OHKyn reacts with compounds that have a free amine functionality (*7*) and recently was shown to cross-link two peptides containing an N-terminal glycine residue, to form the compound quinilinobenzoxamine (Figure 1) (*8*).

The readiness of 3OHKyn to form a quinonimine in the presence of oxygen leaves it susceptible to nucleophilic Michael addition. This is known to be the case in the formation of xanthommatin, a 3OHKyn dimer which is readily degraded to a quinone product under slightly basic conditions, a process accelerated by hydrogen peroxide (*9*).

3OHKyn can react with lens proteins to give a tanned product resembling cataractous material (*10*) and can react with free glycine in vitro to form fluorescent products (*11*), the structures of which have not been elucidated. Kyn, 3OHKyn, and 3HKG all undergo side chain deamination to yield α, β-unsaturated ketones (*12*); however, since 3OHKyn

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[1] Abbreviations: GG, glycylglycine; QBA, quinilinobenzoxamine; GSH, glutathione; Kyn, kynurenine; 3HKG, 3-hydroxykynurenine *O*-glucoside; AHBG, 4-(2-amino-3-hydroxyphenyl)-4-oxobutanoic acid *O*-glucoside; 3OHKyn, 3-hydroxykynurenine; GK, glycyllysine; GSH−3HKG, glutathionyl 3-hydroxykynurenine *O*-glucoside; 2-DIK, 2-diglycylimidazolekynurenine; LC−MS, in-line HPLC mass spectrometry; MS/MS, tandem mass spectrometry; TOF, time-of-flight; 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.

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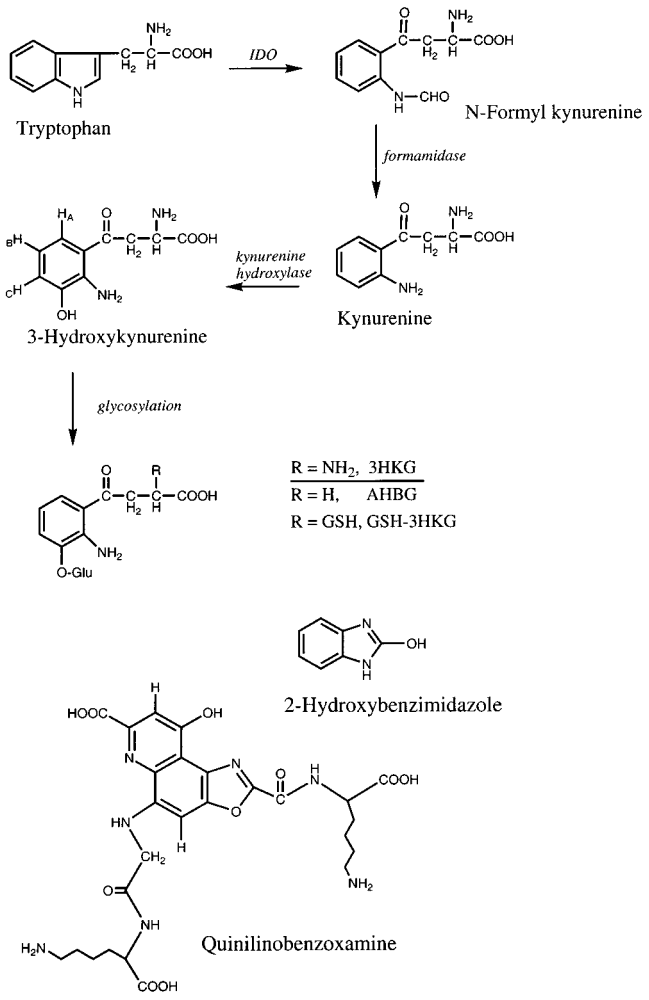

FIGURE 1: Structures of UV filter compounds in the kynurenine pathway of tryptophan metabolism and compounds mentioned in the text. IDO is indoleamine-2,3-dioxygenase, and Glu is glucose.

is readily autoxidized at neutral pH, its reactions with proteins and peptides in the presence of oxygen would not be expected to yield products similar to that of Kyn and 3HKG, which do not undergo autoxidation. Deamination of the aliphatic side chain is a nonoxidative process (*12*) which occurs relatively slowly compared to the oxidation of 3OHKyn in the presence of excess oxygen.

The availability of oxygen in the lens and the presence of antioxidants such as glutathione (GSH) and ascorbate may dictate whether this autoxidation of 3OHKyn occurs in vivo. In the majority of experimentally induced cataracts, and in the center of nuclear cataractous lenses, a large decrease in the level of lenticular GSH is observed (*13*, *14*). Stutchbury and Truscott (*10*) found that both the autoxidation of 3OHKyn and its ability to tan proteins were inhibited by the inclusion of GSH in in vitro reactions, and it was not until most of the GSH had oxidized that 3OHKyn became reactive (*15*).

3OHKyn is continuously biosynthesized in the lens with concentrations in the region of 5 μM (*3*). Oxygen tension in the nucleus is thought to be lower than other tissues; however, the availability of even catalytic amounts of molecular oxygen would be sufficient to propagate reactive autoxidation products. It is feasible then, that over time, the presence of micromolar concentrations of 3OHKyn in an increasingly oxidative environment such as the aged lens nucleus may lead to covalent modification of the crystallins by 3OHKyn autoxidation products.

Our previous studies of the dipeptide glycyllysine (GK) reacting with 3OHKyn suggested that the charged ϵ-amino side chain of lysine may have influenced the mechanism of formation of quinilinobenzoxamine (QBA) (*8*). In this study, the dipeptide glycylglycine (GG) was incubated with 3OHKyn to ascertain if the ϵ-amino group of GK played a significant role in the formation of a product involving the α-amino group of glycine. Glycine is a unique amino acid in that it has a single proton as its side chain, and this was initially considered necessary for the mechanism of formation of QBA. Thus, the tetrapeptide tuftsin (sequence TKPR) was also incubated with 3OHKyn to examine the effect of an extended side chain on the nature of the reaction products. It is concluded that the steric effects of the lysine side chain do alter the nature of the reaction product, but the only requirement for the reactivity of a peptide with 3OHKyn is the presence of a free N-terminal amino group.

EXPERIMENTAL PROCEDURES

Materials and Methods. 3OHKyn, tuftsin (sequence TKPR), GG, 2-hydroxybenzimidazole, and DMSO-d_6 were purchased from Sigma (St. Louis, MO). D_2O was supplied by Cambridge Isotope Laboratories. All other chemicals were of analytical quality.

3OHKyn was reacted with GG or tuftsin in a 5.0 mL solution of 100 mM phosphate buffer (pH 7.0) in the following ratios: 2.0:50 mg/mL for 3OHKyn and GG and 0.5:10 mg/mL for 3OHKyn and tuftsin. The reaction mixtures were held at 37 °C in a circulating water bath for 96 h. A gentle stream of humidified oxygen (20 mL/min) was bubbled into the solution to provide mixing and constant saturation with oxygen.

Separation and Purification of the Major Reaction Products. The reaction mixtures were centrifuged to remove any precipitated 3OHKyn oxidation products prior to HPLC purification. The tuftsin−3OHKyn crude reaction mixture was separated by semipreparative HPLC on a Brownlee C8 column (250 mm × 10 mm, 7 μm) using an acetonitrile gradient containing 20 mM phosphate buffer (pH 7.0). UV detection was carried out at 365 nm. A yellow product was collected and further purified by analytical HPLC on a Brownlee C8 column (250 mm × 4.6 mm, 5 μm) to yield 7.0 mg of material from pooled multiple runs.

Analytical HPLC of the GG reaction mixture was initially carried out on a Beckman Microsorb MV 100 Å C18 column (250 mm × 4.6 mm, 5 μm) with a 0 to 40% acetonitrile gradient over the course of 30 min at a flow rate of 1 mL/min. UV detection was carried out at 365 and 229 nm using a Waters 440 UV detector with an extended wavelength module. Fluorescence detection was carried out 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 mm × 10 mm, 7 μm) with a 0 to 40% acetonitrile gradient over the course of 50 min. Twenty milligrams of material was loaded per run, and collected fractions were pooled and lyophilized for subsequent spectroscopic analysis. Three-dimensional fluorescence spectra were acquired on a Hitachi

F4500 fluorimeter at 25 °C. Samples were prepared in 100 mM phosphate buffer (pH 7.0) at a concentration of ~10 μM.

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 and 0.1% formic acid and delivered by syringe pump at a rate of 5−20 μL/min, depending on sample concentration. Electrospray conditions were as follows: nitrogen bath gas flow rate of 350 L/h and a rate of flow of nebulizing gas to the probe of 10 L/h. The capillary probe tip potential was 3.2 kV; the HV lens potential 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 PEG 1000.

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 a rate of 5 μL/min in 50% methanol/H_2O and 1% acetic acid. The spectrometer was calibrated with polyethylene glycol 600.

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

NMR Spectroscopy. NMR spectra were acquired at 400 (^{1}H) and 100 MHz (^{13}C) on a Varian Unity-400 NMR spectrometer at 25 °C except for the tuftsin dimer heteronuclear correlation spectra, which were acquired on a Bruker DRX-500 NMR spectrometer. 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 as follows: 13 mM 2-diglycylimidazolekynurenine (2-DIK), 8.7 mM tuftsin dimer, and 230 mM 2-hydroxybenzimidazole.

Two-dimensional ^{1}H NMR spectra (DQF-COSY, TOCSY, and NOESY) were acquired in the phase-sensitive mode using time-proportional phase incrementation (*16*). The mixing time 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 two-dimensional ^{1}H−^{13}C correlation experiments (HSQC and HMBC), gradients were used for coherence selection (*17*). Up to 1024 t_1 increments with up to 128 scans per increment were acquired over 2048 data points. The data were processed as described for the two-dimensional ^{1}H 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

The analytical HPLC chromatogram for the GG−3OHKyn reaction mixture is shown in Figure 2. The 365 nm absorbance profile (Figure 2a) exhibited three large colored peaks labeled P1−P3. Of these peaks, only P2 and P3 were matched by corresponding fluorescent peaks (Figure 2b). P1 was analyzed by mass spectrometry and shown to be unreacted 3OHKyn. Of peaks P2 and P3, P2 gave rise to a much larger peak when monitored at 229 nm (chromatogram not shown), implying that the amount of product eluting was substantially greater than that of P3. Fractions corresponding to P2 and P3 were collected for initial analysis by ESI-MS. The collected peak P2 had an ion at *m*/*z* 335.2, while the molecular ion of P3 was observed at *m*/*z* 462.1. These masses suggested that P2 may be a simple adduct of 3OHKyn and GG, but that P3 may be a dimer of 3OHKyn (224.2 Da) and GG moieties (132.1 Da).

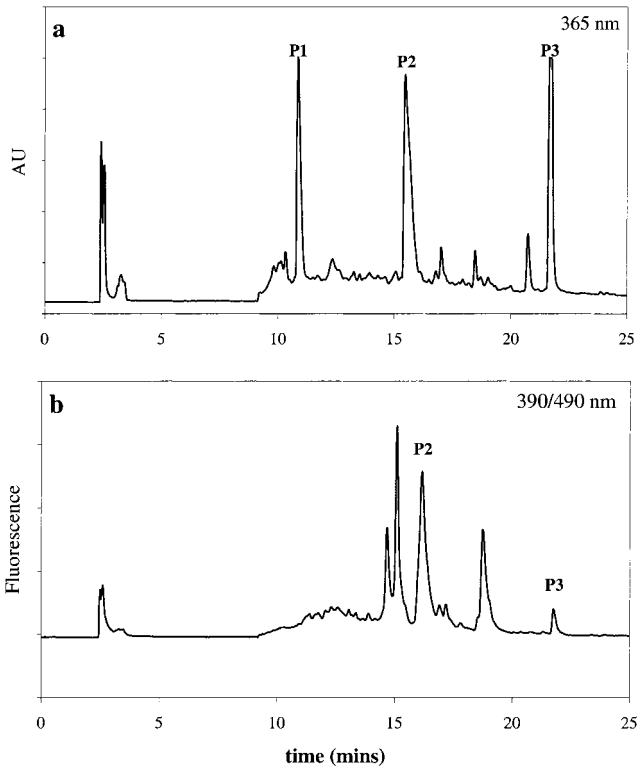

FIGURE 2: UV and fluorescence elution profiles of the GG−3OHKyn reaction mixture following incubation for 96 h at 37 °C: (a) absorbance at 365 nm and (b) fluorescence with excitation at 390 nm and emission at 490 nm. The labels refer to 3OHKyn (P1), 335 *m*/*z* product (P2), and the 462 *m*/*z* product (P3).

To collect sufficient material for structural analysis by NMR spectroscopy, semipreparative HPLC was carried out on the remaining reaction mixture. The major 229 nm absorbing peak (P2) was collected over multiple runs and pooled to give a total of 3.9 mg of product. This represented a 26% yield with respect to 3OHKyn.

NMR Spectroscopy of the Major Reaction Product P2. The one-dimensional ^{1}H NMR spectrum of P2 exhibited three aromatic protons, two doublets at 7.50 and 7.85 ppm, and one triplet (doublet of doublets) at 7.80 ppm. This pattern of resonances is consistent with a 3OHKyn-like ring configuration (Figure 1) and indicated that no addition reaction had occurred at the aromatic proton positions of 3OHKyn. A single α-NH resonance at 9.80 ppm had a triplet appearance (doublet of doublets), suggesting that it arose from a glycine residue. Thus, it appeared that a reaction had occurred between the GG dipeptide and 3OHKyn, but only

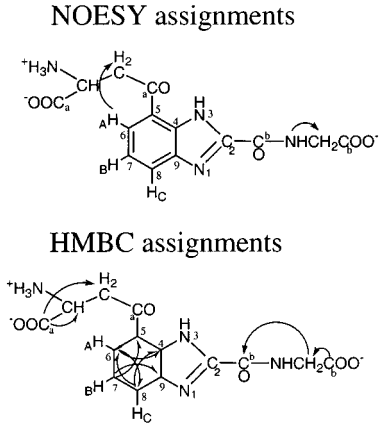

FIGURE 3: Summary of the $^1H-^1H$ NOESY correlations and two-dimensional $^1H-^{13}C$ HMBC correlations for P2.

one glycine residue was intact after reaction, implying that the N-terminal amino group had reacted with 3OHKyn. The CH−CH_2 side chain resonances of 3OHKyn were clearly observed in the one-dimensional ^1H NMR spectrum at around 4 ppm, indicating that cyclization of this aliphatic chain, which has been observed in the oxidative reaction of 3OHKyn with GK (*8*), had not occurred in this case.

The TOCSY spectrum exhibited a cross-peak from the single glycine α-NH to its α-CH_2. Interestingly, there was no evidence of a second α-CH_2 from the other Gly, which implied that this portion of the N-terminal Gly was involved in the reaction with 3OHKyn.

Using the notation for 3OHKyn in Figure 1, assignments for H_A and the side chain CH_2 were made from the NOESY experiment, where a cross-peak was observed between these two groups. A cross-peak was also observed between the Gly α-NH and its α-CH_2, reinforcing the finding from the TOCSY spectrum. The remaining proton assignments, i.e., the aliphatic side chain CH proton and the aromatic protons H_B and H_C, were made from the through-bond DQF-COSY spectrum.

The protonated ^{13}C resonances were assigned from one-bond correlations to their attached protons via an HSQC spectrum, whereas nonprotonated ^{13}C resonances were assigned from the HMBC spectrum. The HMBC correlations, along with the NOESY correlations, are presented in Figure 3. Using the numbering in this proposed structure, the cross-peaks from the H_B and H_C protons to their corresponding ortho carbons revealed shifts of 128.3 (C9), 143.3 (C4), and 158.0 ppm (C5). The ^{13}C chemical shifts for C4 and C9 suggested that they remained as quaternary carbons after oxidative reaction with GG (*18*). The absence of resonances, however, from the N-terminal Gly α-NH_3^+ and CH_2 groups implied that they were involved in the reaction with 3OHKyn. Since the NMR data showed that the aliphatic side chain and the three aromatic protons of 3OHKyn were not involved in the reaction, then the most likely site of reaction was through the aromatic amino and/or hydroxyl group(s). If this were so, and considering that both the N-terminal Gly α-NH_2 and CH_2 groups had reacted, then a likely product of this reaction was a five-membered heteroaromatic ring structure.

A molecular mass of 334.098 380 Da obtained from the high-resolution MS experiment was consistent with a structural formula $C_{14}H_{14}N_4O_6$. The most likely configuration based on this formula was a substituted benzimidazole molecule with the N-terminal Gly α-NH_3^+ and CH_2 groups forming part of the imidazole ring (Figure 3).

A ^{13}C one-dimensional NMR spectrum of our sample exhibited no resolved peaks due to a lack of material. The HBMC spectrum revealed no cross-peak to C2 in this proposed structure; thus, a standard sample of 2-hydroxybenzimidazole was examined to see if a C2 resonance could be observed at higher concentrations. At a concentration of 230 mM in DMSO-d_6, a cross-peak was observed between the imidazole NH and C2 of 2-hydroxybenzimidazole in the HMBC spectrum, i.e., between 10.67 and 159.3 ppm (not shown). The imidazole NH resonance was not observed in the ^1H NMR spectra of P2. It is proposed that proton exchange with water was responsible for the absence of an imidazole NH resonance in P2 and, thus, the absence of a cross-peak between the imidazole NH and C2. Indeed, the imidazole NH resonance is never observed in ^1H spectra of imidazole derivatives (e.g., histidine peptides) in protic solvents (*19*). Similarly, the kynurenine side chain NH_3^+ was not observed due to rapid exchange with water in the DMSO solvent.

Mass Spectrometry of the Major Reaction Product P2. The MS/MS spectrum of the singly charged P2 molecular ion at *m*/*z* 335.1 is shown in Figure 4a. The first major fragment appears at *m*/*z* 289.5, corresponding to a possible loss of formic acid, equivalent to the 179.1 *m*/*z* fragment in the MS/MS spectrum of 3OHKyn (Figure 4b). The peak at *m*/*z* 262.4 corresponds to the loss of 73 mass units from the molecular ion. Similarly, in Figure 4b, a loss of 73 mass units from the 3OHKyn molecular ion gave rise to a *m*/*z* 152.0 fragment, which corresponds to a structure with an acetyl group as the side chain of 3OHKyn. The fragment ion at *m*/*z* 74.1 correlates with formation of iminoacetic acid from the side chain. The peak at *m*/*z* 216.3 may indicate a loss of formic acid from the Gly side chain of the 262.4 *m*/*z* fragment.

Evidence for the presence of a glycylglycine at C2 is provided by the equivalent losses of 103 from the 289.5 and 262.4 *m*/*z* peaks. If the N-terminal Gly α-NH_2 and CH_2 groups were involved in imidazole ring formation as proposed, cleavage next to the carbonyl with a loss of the remaining portion of the glycylglycine would account for the 103 amu difference described herein. This portion of the GG molecule gave rise to a peak at 104.0 *m*/*z* in the MS/MS spectrum of P2 (Figure 4a). Rationalization of the smaller fragments provided no further insight into the structure. This is not surprising considering the fact that benzimidazoles have a high level of resistance to fragmentation (*20*). The proposed molecular ion structure for P2, represented in Figure 3, is henceforth termed 2-diglycylimidazolekynurenine (2-DIK).

To further examine the proposed structure, a sample of the compound was incubated in D_2O and the exchangeable protons were replaced with deuterium atoms. After exchange for 90 min under acidic conditions, the molecular ion had gained 7 mass units, indicative of a molecule containing seven exchangeable protons. With respect to the structure (Figure 3), this would be the case: three protons of the charged free amino group, two carboxylic acid protons, the Gly α-NH, and the single proton on the imidazole ring. This result provided additional evidence for the presence of an imidazole moiety in the structure.

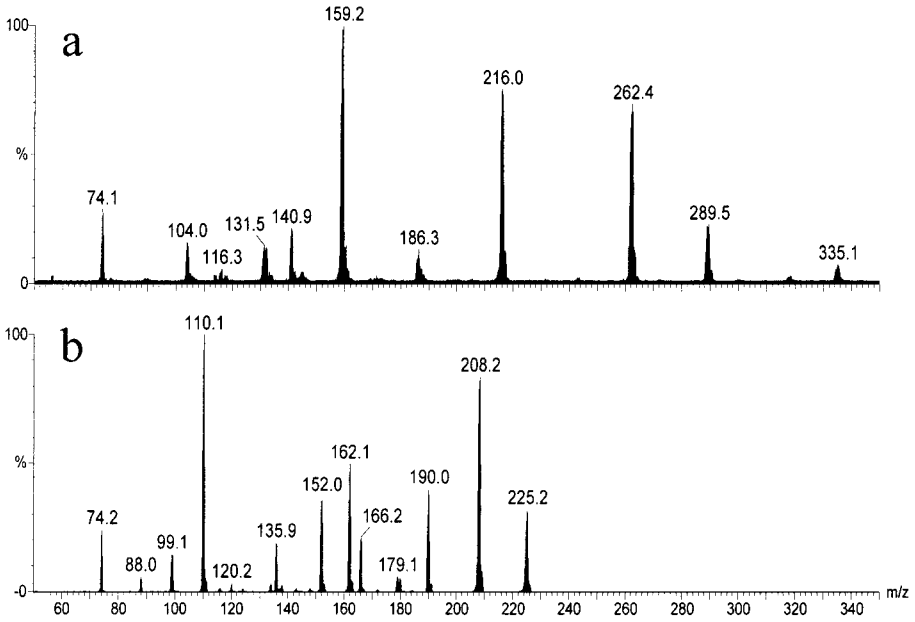

FIGURE 4: Tandem electrospray mass spectra of (a) the major reaction product P2 (*m*/*z* 335.1) and (b) a standard sample of 3OHKyn.

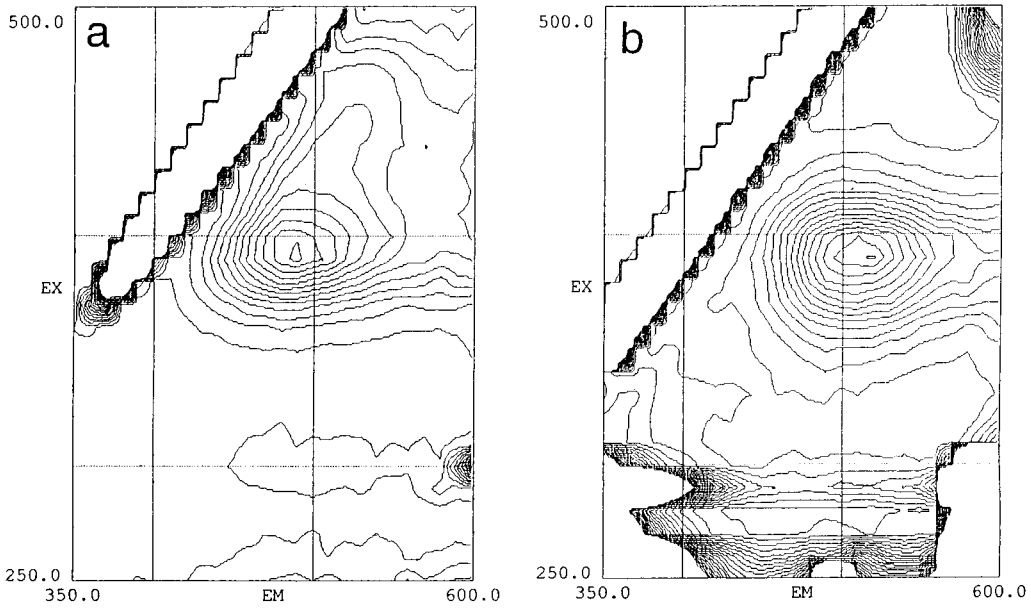

FIGURE 5: Three-dimensional fluorescence spectra of (a) the proposed structure, 2-DIK, and (b) a standard of benzimidazole. 2-DIK exhibits a single sharp peak at excitation and emission maxima of 390 and 490 nm, respectively. Benzimidazole, when prepared in the same buffer solution as 2-DIK, exhibits similar fluorescence maxima, the excitation of 390 nm being identical, and slightly higher emission maximum out to 510 nm.

Fluorescence Spectroscopy. Of the collected peaks, P2 had the greatest fluorescence with excitation at 390 nm and emission at 490 nm (Figure 2b). These wavelengths were chosen on the basis of the three-dimensional fluorescence spectrum of the P2 peak from the analytical HPLC separation, which is shown in Figure 5a. The spectrum exhibits a single sharp peak at excitation and emission maxima of 390 and 490 nm, respectively. It was interesting to compare this fluorescence, i.e., of the proposed structure, 2-DIK, to the standard of benzimidazole whose three-dimensional fluorescence spectrum is shown in Figure 5b. It can be seen that benzimidazole, when prepared in the same buffer solution as 2-DIK, exhibits similar fluorescence maxima, the excitation of 390 nm being identical, with a slight increase in the emission maximum from 490 to 510 nm.

Reaction of Tuftsin with 3-Hydroxykynurenine. Semipreparative HPLC of the tuftsin−3OHKyn reaction mixture gave rise to four distinct peaks when monitored at 360 nm. The largest of these peaks, which represented ~70% of the total peak area, was collected and rechromatographed to yield a single, sharp peak which was purified over successive runs until approximately 7 mg of material was collected. Insufficient amounts of material could be practicably obtained from the other peaks for detailed structural analyses by NMR spectroscopy.

The mass spectrum of the purified reaction product revealed a doubly charged species at *m*/*z* 577.8 corresponding to a molecular mass of 1153.6 Da. Tuftsin is a tetrapeptide which has a monoisotopic mass of 500.6 Da, and 3OHKyn has a mass of 224.2 Da. The mass of 1153.6 Da suggested

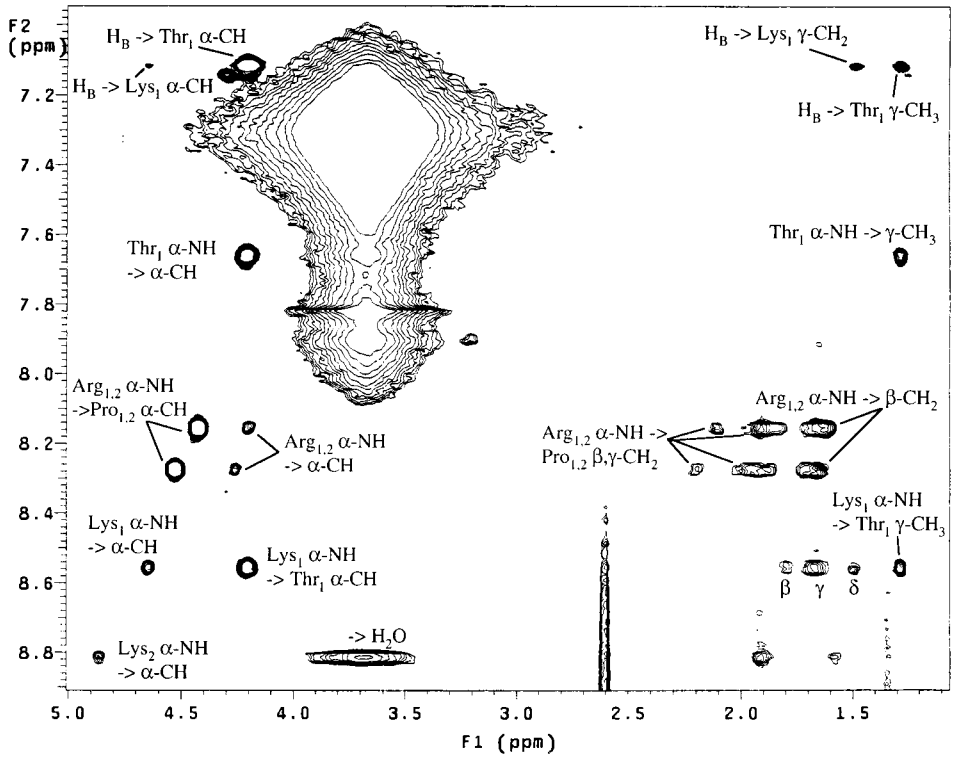

FIGURE 6: NOEs from the aromatic and NH region to the aliphatic region in the two-dimensional $^1H-^1H$ NOESY spectrum of the tuftsin−3OHKyn reaction product. Cross-peaks between adjacent residues (sequential NOEs) permitted a distinction between the two tuftsin chains numbered 1 and 2. The large cross-peak arises from exchange between the side chain lysine and arginine NH_3^+ and NH groups and the water resonance at ~3.7 ppm.

that two peptides had been cross-linked by an oxidation product of 3OHKyn. An MS/MS spectrum acquired on the *m*/*z* 577.8 peak revealed ions which corresponded to those observed in a standard tuftsin MS/MS spectrum. The presence of these ions suggested that tuftsin had been incorporated into the compound. An ion at *m*/*z* 301.3 was considered significant, as it was also present in the MS/MS spectrum of QBA (*8*), where it was proposed to arise from cleavages on the carboxyl side of the α-NH groups of both lysine residues that had been incorporated into QBA from the GK dipeptide.

NMR Spectroscopy. More detailed structural information was obtained from NMR spectroscopy. In the TOCSY spectrum (not shown), cross-peaks from the α-NH protons arose from two lysine residues, two arginine residues, and one threonine residue, indicating that two tuftsin peptides were present in the sample. Residues with a subscript 1 belong to the tuftsin molecule which is proximal to H_B and contains an intact threonine residue (Figure 7). Cross-peaks were also observed from Arg_1 ϵ-NH but not from Arg_2 ϵ-NH. Proline, which is the third residue in tuftsin, does not have an α-NH proton; thus, cross-peaks from it were not observed in this region of the spectrum. In the aliphatic region of the TOCSY spectrum (not shown), correlations were observed for seven amino acids: Thr, Lys_1, Lys_2, Pro_1, Pro_2, Arg_1, and Arg_2. The presence of only one Thr residue in the TOCSY spectrum implied that the N-terminus of one of the tuftsin peptides had been modified, as was observed for an N-terminal glycine residue in QBA (*8*). In the aromatic and α-NH region of the NOESY spectrum, cross-peaks were observed from an aromatic proton of 3OHKyn (H_B) to the Thr and Lys_1 α-NH protons (not shown). 3OHKyn has three aromatic protons, but only one was observed in the NOESY spectrum; i.e., H_B is close in space to these two α-NH protons. The other aromatic proton, H_A, gave rise to no cross-peaks in the NOESY spectrum, indicating that it was isolated from other protons.

Assignments were made for all cross-peaks observed in the aromatic and NH region to the aliphatic region in the two-dimensional ^1H NOESY spectrum (Figure 6). These NOEs allowed an unequivocal assignment of which tuftsin peptide each residue belonged to, and a summary of the observed correlations is presented in Figure 7a.

The protonated ^{13}C resonances were assigned from an HSQC spectrum (not shown). The HMBC spectrum (not shown) revealed the identity of only four nonprotonated ^{13}C resonances, C12, C4, C11, and the threonine carbonyl. The three former resonances gave rise to cross-peaks with H_B (Figure 7b). A complete assignment of the quaternary carbons was therefore not possible.

DISCUSSION

When the dipeptide glycylglycine was reacted oxidatively at neutral pH with the tryptophan metabolite, 3OHKyn, a number of colored and fluorescent products were formed. Using a combination of mass spectrometry and NMR spectroscopy, sufficient information was obtained to propose a structure for the major isolated product, P2. From the one-dimensional ^1H NMR spectrum, it was clear that the aliphatic side chain of 3OHKyn remained unreacted, and the three existing aromatic protons of 3OHKyn were present. Reaction therefore appeared to have occurred through the amino and/or phenol ring substituents of 3OHKyn. The inclusion of GG in the reaction product was determined from distinct α-NH and α-CH_2 resonances from a glycine residue in the

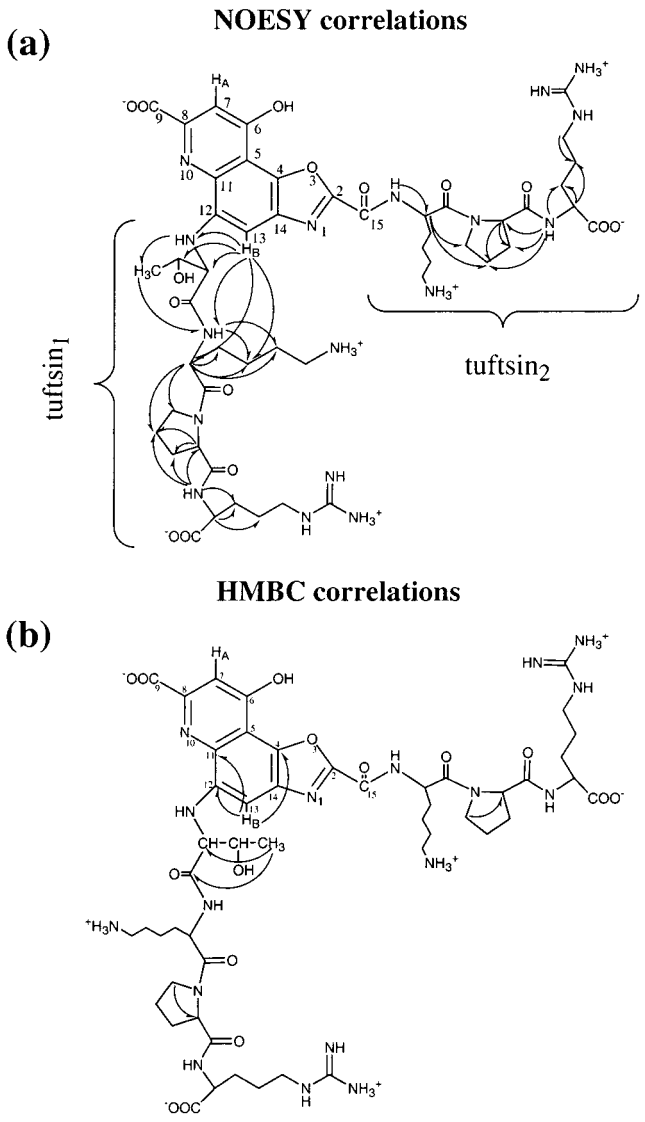

FIGURE 7: Summary of the (a) two-dimensional $^1H-^1H$ NOESY correlations and (b) two-dimensional $^1H-^{13}C$ HMBC correlations of the tuftsin−3OHKyn reaction product.

one-dimensional ^1H NMR spectrum. The absence of second α-NH and α-CH_2 resonances from a glycine residue, however, implied that the amino-terminal glycine was involved in the cross-link. It was suggested that an imidazole ring had formed, which was in agreement with the mass from the high-resolution mass spectrum and the number of exchangeable protons from the deuterium exchange experiment.

This result is in contrast to the major product observed when 3OHKyn was reacted with glycyllysine (GK), where a benzoxazole moiety was formed to produce QBA, a dimer incorporating two GK dipeptides. A monomeric substituted benzimidazole species (*m*/*z* 406), homologous in mass to 2-DIK, was also observed in this reaction; however, the proportion of this species to the dimer was only 18% (*21*).

Figure 8 represents a possible mechanism for the formation of the imidazole-based product. The preferential formation of a benzimidazole cross-link (rather than benzoxazole) may result from the initial nucleophilic attachment of the α-amino group of GG at the carbonyl of oxidized 3OHKyn **2**. A loss of water to give **3** and subsequent tautomerization result in

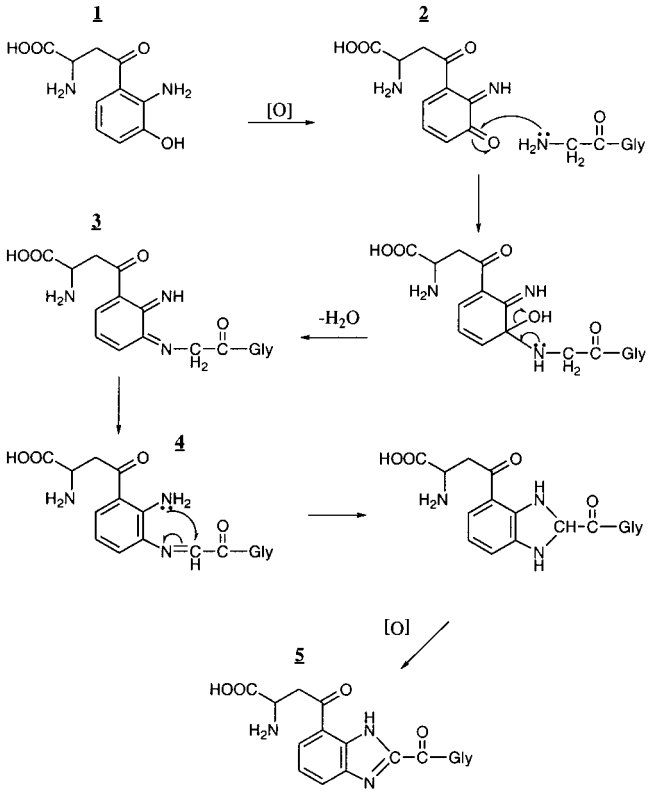

FIGURE 8: Proposed mechanism for the formation of 2-DIK from the oxidative reaction of 3OHKyn and GG.

the ortho-substituted glycylglycine kynurenine Schiff intermediate **4**. A nucleophilic attack of the aromatic amine at the α-CH group of glycine results in ring closure. Further oxidation of this intermediate leads to the formation of a substituted benzimidazole, the proposed product of the reaction between 3OHKyn and glycylglycine **5**.

The major reaction product between GG and 3OHKyn (2-DIK) was shown to be monomeric; i.e., only one dipeptide was incorporated. Two less abundant colored products were observed by HPLC (Figure 2), one of which, P3, was shown to have a mass of *m*/*z* 462 which would correspond to a dimeric structure homologous to QBA, only with GG rather than GK as the cross-linked peptide. Tandem mass spectral data supported such an analogous structure (*21*). This product was present at approximately 44% with respect to 2-DIK; thus, the proportion of monomer (benzimidazole based) to dimer (benzoxazole based) varied, depending upon the type of dipeptide involved in the oxidation reaction. The reason for this preferential formation of one product over the other is not clear; however, it would appear to be dictated by the initial site of nucleophilic attack on the 3OHKyn aromatic ring. Possibly, GK is more likely to form the benzoxazole due to the Michael addition at C7 being the favored initial site of reaction. Our reasoning for this is that the ϵ-NH_3^+ of one of the Lys residues of QBA was observed by NMR to be restricted in its conformation, possibly due to ionic interaction with the carboxyl group of 3OHKyn (*8*). The fact that the Lys side chain can interact with charged groups on the molecule may explain the preferential initial attack at C7 on the 3OHKyn aromatic ring, leading to the formation of a benzoxazole rather than benzimidazole.

A product of the oxidative reaction of tuftsin and 3OHKyn was found to have a mass of 1153.6 Da and to include two

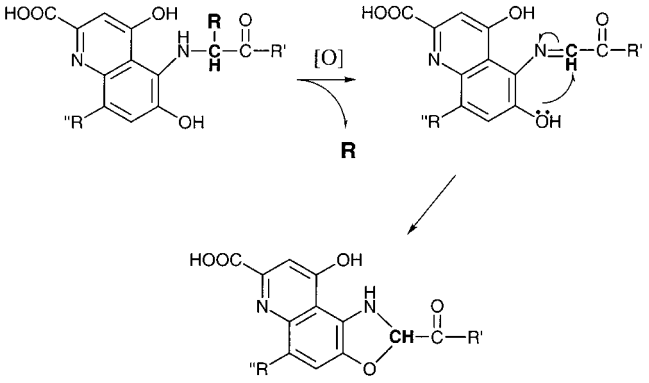

FIGURE 9: General reaction mechanism for the formation of 3OHKyn-cross-linked peptides.

tuftsin peptides. The NMR data suggested that a threonine residue of one of the peptides was either absent or modified and that, as observed for QBA, the compound was essentially composed of two peptides and one 3OHKyn molecule.

For QBA, the combined masses of two GK moieties and one 3OHKyn moiety equal 630.4 Da; thus, the net result of the dimerization reaction was the loss of approximately 27.2 Da. If this net loss is applied to the combined units of a theoretical tuftsin−3OHKyn dimer, the expected product mass would be

$$2 \times 500.3 \text{ Da (tuftsin)} + 224.2 \text{ Da (3OHKyn)} - 27.2 \text{ Da} = 1197.6 \text{ Da}$$

The mass of the 3OHKyn−tuftsin reaction product was 1153.6 Da, i.e., 44.0 Da lower than the calculated mass for the equivalent benzoxazole-linked dimer, QBA. The side chain of glycine has one proton, while that of threonine is composed of $CH(OH)CH_3$, with a mass of 45 Da. The net difference in the masses of the two side chains is 44 Da, the same mass difference observed between QBA and a theoretical tuftsin−3OHKyn dimer. This difference implies that the side chain of threonine has been eliminated on formation of the tuftsin−3OHKyn complex via a mechanism that is the same as that for formation of QBA from GK and 3OHKyn. A reaction mechanism for the formation of QBA has been proposed (*8*) which incorporates the α-NH and α-CH_2 of a glycine into the oxazole ring. Initially, it was thought that this mechanism may be unique to glycine due to the absence of a side chain in this amino acid; i.e., elimination of either of the protons of the α-CH_2 group would permit the formation of the oxazole ring. However, the determination of an analogous product to QBA for the reaction of tuftsin with 3OHKyn suggests that this is not a prerequisite.

The result presented in this work for the reaction of tuftsin with 3OHKyn has led to the proposal of a general mechanism of oxidative cross-linking through free N-terminal amino groups. This mechanism is illustrated in Figure 9. Essentially, the first oxidation step results in the loss of any R group (side chain), presumably in preference to that of the α-CH proton. The resulting Schiff intermediate is then prone to intramolecular attack by the aromatic hydroxyl group as previously described (*8*).

In conclusion, the major product of the oxidative reaction between the dipeptide GG and 3OHKyn has been shown to be a monomeric adduct, which involves formation of a benzimidazole moiety. This result differs from that observed previously for the GK−3OHKyn dimer, in which the major product was a benzoxazole-linked dimer. In each reaction, however, both the benzimidazole and benzoxazole adducts were observed.

Glycine is unique among the amino acids in that it has a single proton as its side chain. We considered that this property may have made glycine particularly prone to nucleophilic attack during formation of the oxazole and imidazole rings of QBA and 2-DIK. To test this hypothesis, the major reaction product of the tetrapeptide tuftsin (TKPR) and 3OHKyn was examined. The mass of this product combined with NMR spectroscopic data suggested that a cross-linked dimer of tuftsin had been produced and that a threonine residue of one of the peptides was either absent or modified. A comparison of the mass of this product with the mass of QBA suggested that the side chain of one of the threonine residues had been eliminated to form an oxazole ring, as observed in the formation of QBA.

Thus, tuftsin, a peptide with threonine as the N-terminal residue, was able to form a dimer via the same mechanism as QBA. This result suggests that 3OHKyn can react with any peptide that has a free N-terminus, regardless of the identity of the amino acid (with the exception of proline), to form both benzimidazole and benzoxazole adducts. These benzimidazole and benzoxazole compounds could represent biomarkers of oxidative modification by 3OHKyn in age-related nuclear cataract, and other disease states where damage by 3OHKyn oxidation products is implicated.

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