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Photodynamic cross-linking of proteins V. Nature of the tyrosine–tyrosine bonds formed in the FMN-sensitized intermolecular cross-linking of *N*-acetyl-L-tyrosine

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Abstract

Some types of proteins are intermolecularly cross-linked via the formation of Tyr–Tyr (dityrosine) bonds on illumination in the presence of certain kinds of photosensitizers. The study of reactions of this type is of importance since they appear to be involved in several processes of biomedical importance such as the photosensitized (photodynamic) treatment of tumors, pathological effects of sunlight (skin photoaging, induction of cataracts), etc. The mechanisms involved in the photosensitized formation of dityrosine cross-links are not clear, and the chemical nature of the cross-link(s) is not known. In the present work, these problems have been studied using *N*-acetyltyrosine as a model substrate. This compound was illuminated under aerobic conditions at pH 6.0 with FMN as the photosensitizer. The Tyr–Tyr cross-linked products were isolated and purified by a preparative silica gel 60 column and by preparative reverse phase HPLC. Three main cross-linked products, designated as **1** (C6, C6′-linked di-(*N*-acetyltyrosine)), **2** (C6, O7′-linked di-(*N*-acetyltyrosine)), and **3** (C6, C4′-linked di-(*N*-acetyltyrosine)), were identified, and their chemical structures were determined by MS, two-dimensional NMR spectral methods, and other standard techniques. Detailed reaction pathways for the formation of the carbon–carbon (**1** and **3**) and carbon–oxygen (**2**) coupled products were proposed.

Keywords: Radical pathway; Cross-linking pathways/mechanism

Abbreviations: ACN, acetonitrile; Ac-Tyr, *N*-acetyltyrosine; Ac-Di-Tyr, acetyl-dityrosine; AC, activated charcoal; FMN, flavin mononucleotide; HPMA, *N*-(2-hydroxypropyl)methacrylamide; PDT, photodynamic therapy; TFA, trifluoroacetic acid

1. Introduction

On illumination in the presence of appropriate photosensitizers, many kinds of proteins are covalently cross-linked. Cross-linking can be intermolecular and/or intramolecular, depending on the protein, the photosensitizer and the reaction conditions [1–4]. These reactions are of biomedical importance since they can be involved in some of the pathological effects of sunlight, such as the induction of cataracts [5–8] and the damaging of skin (photoaging) [9]. They may also be involved in the treatment of disease, in particular, the so-called photodynamic therapy (PDT) of tumors [10,11]. Further, the photosensitized cross-linking of collagen-rich tissues can be used to produce biomaterials useful for surgical implants [12].

The mechanisms involved in the photosensitized intermolecular cross-linking of proteins are not well-understood. In some cases, they appear to involve a non-photochemical reaction between a photooxidized amino acid residue (Cys, His, Tyr, Trp) in one protein molecule with a normal or photooxidized amino acid residue in another protein molecule to produce a cross-link [1–4,13–16]. These reactions are difficult to study with proteins because of their complexity, the possibility of different conformations of the protein, and the presence of reactive amino acid residues of several different types. We have recently described the use of water-soluble *N*-(2-hydroxypropyl)methacrylamide (HPMA) copolymers containing 6-carbon side chains terminating in a selected amino acid for studies of photosensitized cross-linking; this

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permits examining the involvement of a selected type of amino acid without interference by other types [3].

Most recently we have examined the kinetics of the flavin-sensitized photooxidation and photocross-linking of a HPMA copolymer with side chains terminating in tyrosinamide groups. Under certain conditions, the Tyr-copolymer is photooxidized and efficiently cross-linked with the formation of Tyr–Tyr (dityrosine) bonds [17]. The chemical nature of the dityrosine bonds formed by photosensitized reactions is not known. We report here the determination of the chemical structures of the dityrosine bonds produced by the FMN-sensitized photocross-linking of *N*-acetyl-L-tyrosine (Ac-Tyr) as a model compound. Tyrosine, tyrosinamide and *N*-acetyl-tyrosinamide were also covalently cross-linked with the formation of dityrosine bonds under the same treatment (data not shown); Ac-Tyr was selected for these studies since its high solubility made it easier to produce the quantities of cross-linked products required for identification.

2. Materials and methods

2.1. Materials and general methods

The Ac-Tyr, from BACHEM Bioscience, was used as received. The other chemicals were of the highest purity commercially available. All of the reactions were carried out in double distilled water. Light absorption and fluorescence measurements were made with a Perkin–Elmer Lambda 9 spectrophotometer and an Aminco-Bowman spectrophotofluorimeter. TLC was performed using an aluminum backed silica gel 250 μM layer from Whatman (Kent, England). HPLC was carried out using a Rainin pumping system equipped with either an analytical or a preparative VYDAC^{TM} C18 column. NMR experiments were performed on a Varian Unity 500 instrument.

2.2. Photosensitized cross-linking of Ac-Tyr

Illumination of the Ac-Tyr solution with stirring in the presence of FMN was carried out either in a cuvette placed in a thermostatted holder at 25°C, or in a double-walled reaction vessel maintained at 25°C with circulating water. Illumination was provided by a 500 W incandescent lamp with a heat-absorbing filter and a Corion LL-400 long-wave length pass filter to remove UV radiation. The lamp was placed 20 cm away from the reaction vessel, and gave an incident light energy flux of ∼3 W/cm^2. Illumination lasted for 1.5 h. Preliminary experiments were performed at pH values ranging from 4.0 to 11.5 to determine the optimal pH for photocross-linking. The reaction systems were 300 μM in FMN, 0.1 M in Na phosphate buffer, 0.22 mM in oxygen (air-saturated), and 0.5 wt.% in Ac-Tyr. The optimal pH for the photocross-linking of Ac-Tyr was 6 (data not shown). This pH was also the optimum for the FMN-sensitized photocross-linking of Tyr-copolymer [17]. To determine the effect of O_2 on cross-linking, reaction mixtures in a cuvette were bubbled with N_2 for 15 min, and then the cuvette was sealed. Acetyl-dityrosine (Ac-Di-Tyr) was detected by its characteristic fluorescence emission at ∼405 nm with excitation at 315 nm [18]. Based on the preliminary experiments, 10 batches (Ac-Tyr (335 mg), FMN (4 mM) 5 ml, buffer (0.1 M, pH 6.0) 60 ml) of illuminated reaction mixture were prepared for isolation of the cross-linked product(s).

2.3. Isolation and characterization of the photocross-linked product(s) of Ac-Tyr

The photocross-linked reaction mixtures were first treated with activated charcoal (0.3 g/60 ml illuminated mixture), shaken for 10 min, filtered to remove the charcoal (which adsorbed the FMN) and lyophilized. The resulting solid was dissolved in absolute EtOH, shaken for 5 min, and filtered to remove the buffer salt. The EtOH was removed by rotoevaporation, giving a viscous residue. The above procedure was repeated for each batch. All of the residues were combined. One gram of residue was dissolved in a minimal amount (10 ml) of isopropanol:H_2O (7:3), applied to a silica gel 60 column (55 cm×4.5 cm) which had been equilibrated with the same solvent system, and eluted with the same solvent system. The collected fractions from the same peak were combined, concentrated by rotoevaporation at room temperature to remove isopropanol, and lyophilized. The cross-linked products were isolated by repetitive preparative reverse phase HPLC on a 22 mm×250 mm VYDAC^{TM} C18 column. A linear gradient from 0.1% TFA/88% H_2O/12% ACN (or MeOH) to 0.1% TFA/21.6% H_2O/78.4% ACN (or MeOH) over 95 min at a flow rate of 2 ml/min was used with detection at 214/250 nm. Fractions from each product peak were combined and lyophilized. Three main product peaks were identified; **1** (C6, C6′-linked di-(*N*-acetyltyrosine), 12.5 mg, 52%), **2** (C6, O7′-linked di-(*N*-acetyltyrosine), 5.4 mg, 23%) and **3** (C6, C4′-linked di-(*N*-acetyltyrosine), 6.0 mg, 25%). The overall conversion to cross-linked products was about 1% (yield of the three products combined). After the photodynamic treatment, a very small amount of non-cross-linked oxidized product(s) of Ac-Tyr was detected by TLC; approximately 99% of the Ac-Tyr was unreacted.

Product **1** was obtained as an amorphous white solid; TLC (silica gel/isopropanol:H_2O (7:3)), bright blue-violet spot under UV lamp, R_f=0.50; $[\alpha]_D^{25}$=37° (*c*=0.432, H_2O); UV (H_2O) λ_{max}=284 nm, ε=3200; IR (film) ν_{max} 3695–2167 br (COOH), 3284 (OH), 1732 (C=O), 1714 (C=O) cm^{-1}; ^{1}H NMR and ^{13}C NMR (DMSO-d_6, 500 MHz), see Table 1; ESMS (50:50 H_2O:CH_3OH with 1% CH_3COOH) observed $(M+H)^+$ *m/z* (relative intensity) 445 (100), $(M+Na)^+$ 467 (6), $(M–H_2O+2H)^{2+}$ 214 (20); LRCIMS DCI(NH_3) observed $(M+H)^+$ *m/z* 445; HRCIMS (NH_3)

Table 1
NMR assignments for **1**[a]

Atom No.	^{13}C δ	^{1}H δ	HMBC (8 Hz)
1	173.3		
2	53.8	4.34 (m, 1H)	C1, C3, C4, C10
3	36.1	2.95 (dd, 1H, *J*=4.8, 14.0)	C1, C2, C4, C9
		2.75 (dd, 1H, *J*=9.0, 14.0)	C1, C2, C4, C9
4	127.8		
5	128.6	6.98 (s, 1H)	C3, C6′/C6, C7, C9
6	125.6		
7	152.9		
8	115.7	6.78 (d, 1H, *J*=8.2)	C4, C6, C7,C9
9	132.2	6.98 (d, 1H, *J*=8.2)	C3, C5, C7, C8
10	169.3		
11	22.4	1.79 (s, 3H)	C2, C10
NH		8.10 (d, 1H, *J*=8.0)	C2, C3, C10
OH		9.10 (br s, 1H)	
1′	173.3		
2′	53.8	4.34 (m, 1H)	C1′, C3′, C4′, C10′
3′	36.1	2.95 (dd, 1H, *J*=4.8, 14.0)	C1′, C2′, C4′, C9′
		2.75 (dd, 1H, *J*=9.0, 14.0)	C1′, C2′, C4′, C9′
4′	127.8		
5′	128.6	6.98 (s, 1H)	C3′, C6/C6′, C7′, C9′
6′	125.6		
7′	152.9		
8′	115.7	6.78 (d, 1H, *J*=8.2)	C4, C6, C7, C9
9′	132.2	6.98 (d, 1H, *J*=8.2)	C3, C5, C7, C8′
10′	169.3		
11′	22.4	1.79 (s, 3H)	C2′, C10′
NH′		8.10 (d, 1H, *J*=8.0)	C2′, C3′, C10′
OH′		9.10 (br s, 1H)	

[a] All the spectra were acquired in DMSO-d_6 at 500 MHz. *J* values were reported in hertz (Hz). Assignments were aided by HMQC, GCOSY, DEPT, GHMBC and reference NMR spectra of Ac-Tyr.

Table 2
NMR assignments for **2**[a]

Atom No.	^{13}C δ	^{1}H δ	HMBC (8 Hz)
1	173.0		
2	53.6	4.32 (m, 1H)	C1, C3, C4, C10
3	35.8	2.68 (dd, 1H, *J*=9.5, 14.0)	C1, C2, C4, C5, C9
		2.89 (dd, 1H, *J*=4.8, 14.0)	C1, C2, C4, C5, C9
4	128.9		
5	122.7	6.80 (s, 1H)	C3, C6, C7, C9
6	141.8		
7	147.8		
8	116.9	6.85 (d, 1H, *J*=6.0)	C4, C6, C7
9	125.8	6.86 (d, 1H, *J*=6.0)	C3, C5, C7
10	169.1		
11	22.2	1.74 (s, 3H)	C10
NH		8.10 (d, 1H, *J*=8.0)	C2, C10
OH		9.30 (s, 1H)	
1′	173.1		
2′	53.7	4.33 (m, 1H)	C1′, C4′, C10′
3′	35.9	2.76 (dd, 1H, *J*=5.0, 14.0)	C1′, C2′, C4′, C5′, C9′
		2.96 (dd, 1H, *J*=9.7, 14.0)	C1′, C2′, C4′, C5′, C9′
4′	130.9		
5′	130.0	7.12 (d, 1H, *J*=8.5)	C3′, C7′
6′	115.7	6.72 (d, 1H, *J*=8.5)	C5′, C7′, C8′
7′	156.6		
8′	115.7	6.72 (d, 1H, *J*=8.5)	C6′, C7′, C9′
9′	130.0	7.12 (d, 1H, *J*=8.5)	C3′, C7′
10′	169.2		
11′	22.3	1.77 (s, 3H)	C10′
NH′		8.16 (d, 1H, *J*=8.0)	C2′, C10′
OH′		9.30 (s, 1H)	

[a] All spectra were recorded in DMSO-d_6 at 500 MHz. *J* values were reported in hertz (Hz). Assignments were supported by HMQC, GCOSY, DEPT, and GHMBC.

observed $(M+H)^+$ *m/z* 445.1599 (calculated for $C_{22}H_{25}N_2O_8$, 445.1611).

Product **2** was also obtained as an amorphous white solid; TLC (silica gel/isopropanol:H_2O (7:3)), gray spot under UV lamp, R_f=0.57; $[\alpha]_D^{25}$=41° (*c*=0.045, H_2O); UV (H_2O) λ_{max}=274 nm, ε=1340; IR (film) ν_{max} 3680–2166 br (COOH), 3319 (OH), 1732 (C=O), 1714 (C=O) cm^{-1}; ^{1}H NMR and ^{13}C NMR (DMSO-d_6, 500 MHz), see Table 2; ESMS (50:50 H_2O:CH_3OH with 1% CH_3COOH) observed $(M+H)^+$ *m/z* (relative intensity) 445 (100), $(M+Na)^+$ 467 (5), $(M–H_2O+2H)^{2+}$ 214 (9); LRFABMS (MeOH/NBA) observed $(M+Na)^+$ *m/z* 467, $(M–H+2Na)^+$ 489; HRFABMS (MeOH/NBA) observed $(M+Na)^+$ *m/z* 467.1408 (calculated for $C_{22}H_{24}N_2O_8Na$, 467.1430).

Product **3** was obtained as an amorphous white solid; TLC (silica gel/isopropanol:H_2O (7:3)), gray spot under UV lamp, R_f=0.57; $[\alpha]_D^{25}$=82° (*c*=0.447, H_2O); UV (H_2O) λ_{max}=284 nm, ε=1780; IR (film) ν_{max} 3683–2163 br (COOH), 3283 (OH), 1732 (C=O), 1714 (C=O), 1690 (C=O) cm^{-1}; ^{1}H NMR and ^{13}C NMR (DMSO-d_6, 500 MHz), see Table 3; ESMS (50:50 H_2O:CH_3OH with 1% CH_3COOH) observed $(M+H)^+$ *m/z* (relative intensity) 445 (100), $(M+Na)^+$ 467 (13), $(M–H_2O+2H)^{2+}$ 214 (13); LRFABMS (MeOH/NBA) observed $(M+H)^+$ *m/z* 445, $(M+Na)^+$ 467, $(M–H+2Na)^+$ 489; HRFABMS (MeOH/NBA/PEG) observed $(M+H)^+$ *m/z* 445.1589 (calculated for $C_{22}H_{25}N_2O_8$, 445.1611).

3. Results

3.1. Isolation and characterization of the photocross-linked product(s) of Ac-Tyr

The illuminated reaction mixture consisted of FMN and its photobleached product(s), Ac-Tyr and its photooxidized and photocross-linked products, and Na phosphate buffer. A preliminary series of experiments of using activated charcoal (AC) to remove FMN was performed to estimate the minimum amount of AC needed to remove FMN. It was found that after AC (15 mg) was added to the lyophilized reaction mixture (50 mg containing approximately 0.5 mg FMN) dissolved in 1 ml double distilled water and shaken for 10 min, all of the yellow color arising from FMN was removed, except for a small amount of bleached product(s), detectable under a UV lamp, that remained unmoved at the baseline of the TLC plate. The same AC treatment procedure (Ac-Tyr/H_2O/AC (17 mg/1 ml/15 mg)) was used to treat the starting material Ac-Tyr, and approximately 95% of the Ac-Tyr was recovered owing to its good solubility

Table 3
NMR assignments for **3**[a]

Atom No.	^{13}C δ	^{1}H δ	HMBC (8 Hz)
1	173.1		
2	53.7	4.32 (m, 1H)	C1, C3, C4, C10
3	36.3	2.96 (dd, 1H, *J*=4.8, 14.0)	C1, C2, C4, C5
		2.76 (dd, 1H, *J*=9.0, 14.0)	C1, C2, C4, C5
4	130.6		
5	124.8	7.22 (s, 1H)	C3, C7, C8, C9, C4′
6	128.7		
7	157.5		
8	109.2	6.73 (d, 1H, *J*=8.2)	C4, C5, C6, C7, C4′
9	130.2	7.05 (d, 1H, *J*=8.2)	C3, C5, C6, C7
10	169.0		
11	22.2	1.75 (s, 3H)	C2, C10
NH		8.11 (d, 1H, *J*=8.0)	C2, C3, C10
1′	173.8		
2′	59.5	4.76 (dd, 1H, *J*=8.5, 9.0)	C1′, C3′, C4′, C9′
3′	39.6	2.70 (dd, 1H, *J*=8.5, 14.0)	C1′, C4′, C5′, C9′
		2.50 (dd, 1H, *J*=9.0, 14.0)	C1′, C2′, C4′, C5′, C6
4′	52.0		
5′	86.7	4.99 (dd, 1H, *J*=3.0, 3.5)	C3′, C4′, C7′, C8′, C9′, C7
6′	40.2	2.90 (dd, 1H, *J*=3.5, 18.5)	C4′, C5′, C7′
		2.67 (dd, 1H, *J*=3.0, 18.5)	C4′, C5′, C7′, C8′
7′	206.1		
8′	37.7	3.30 (dd, 1H, *J*=3.5, 17.0)	C4′, C6′, C7′, C9′
		1.94 (dd, 1H, *J*=3.0, 17.0)	C4′, C7′, C9′
9′	63.8	3.94 (dd, 1H, *J*=3.0, 3.5)	C4′, C5′, C7′, C8′, C10′, C6
10′	169.9		
11′	22.8	1.83 (s, 3H)	C2′, C10′

[a] All experiments were performed in DMSO-d_6 at 500 MHz. *J* values were reported in hertz (Hz). Assignments were aided by HMQC, GCOSY, DEPT, and GHMBC.

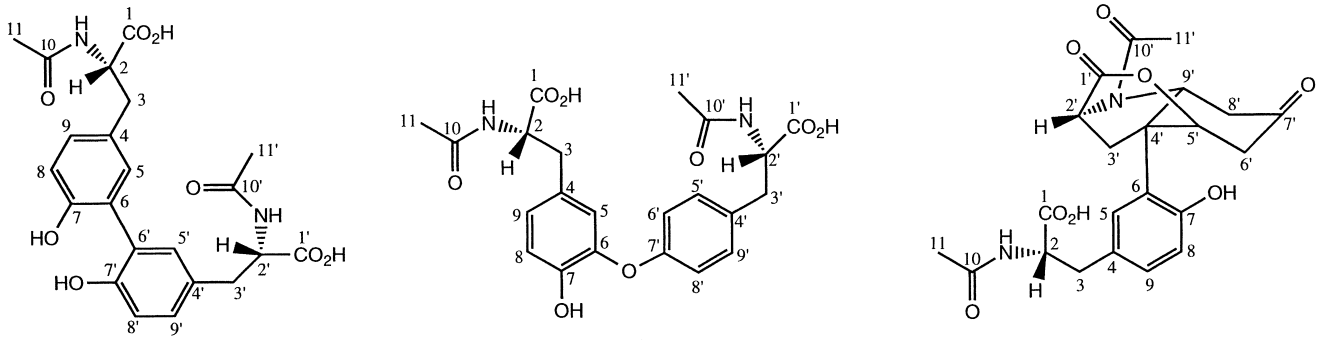
in H_2O (50 mg Ac-Tyr/ml H_2O). The same AC treatment procedure was also used for one controlled illuminated mixture of FMN (300 μM) in 0.1 M Na phosphate buffer (pH 6.0) without adding Ac-Tyr; TLC results (isopropanol:H_2O (8:1)) showed that all of the yellow color arising from FMN was removed except the bleached product at the baseline of TLC. It was, therefore, expected that this AC treatment would retain the majority of the cross-linked products. Four spots (Ac-Tyr, cross-linked product(s) of Ac-Tyr, bleached product of FMN and a small amount of brownish oxidized product(s) of Ac-Tyr) appeared on TLC after AC treatment of the illuminated mixture containing Ac-Tyr.

Several different solvent systems for silica gel TLC were investigated for the isolation of the cross-linked products from the illuminated mixture. The Ac-Tyr and reaction products were efficiently separated by two solvent systems (propanol:H_2O (7/3) and isopropanol:H_2O (7/3)). The products appeared as blue-violet spots under illumination with UV. This color was later found to be generated solely by **1** after isolation and purification of all three products (**1**, **2**, and **3**), which overlapped on TLC for the above two solvent systems. The amount of Ac-Di-Tyr was estimated by fluorescence measurement. Increasing the pH from 6.0 to 11.5 decreased the fluorescence intensity of the Ac-Di-Tyr approximately by 70%. Although all of the illuminated mixtures at different pH values showed a bright blue-violet spot under a UV lamp, it was unknown whether the same types of cross-linked products were produced. No cross-linked products of Ac-Tyr (**1**, **2** or **3**) were produced with illumination in a N_2 atmosphere, as determined by TLC and fluorescence studies.

The above solvent systems were used for preparative silica gel 60 column purification of the cross-linked products. The products were further purified on a reverse phase HPLC column with two different solvent systems. All of the dimers had the same molecular formula of $C_{22}H_{24}N_2O_8$, with 12 units of unsaturation, as determined by HRFABMS, HRCIMS and ESMS in conjunction with NMR methods (Tables 1, 2 and 3). They were formed by three different modes of covalent cross-linking of two Ac-Tyr molecules. None of the products were racemized. The presence of phenol groups in **1**, **2** and **3** was established by their positive reaction with diazotized sulfanilamide, diazotized 4-nitroaniline (buffered), and silver nitrate. The keto group present in **3** was confirmed by its positive reaction with 2,4-dinitrophenylhydrazine.

The UV spectrum of **1** demonstrated that it absorbed at a longer wavelength than the starting material, indicating its increased aromaticity, probably through the conjugation of the two benzene rings. The IR spectra of **1** and Ac-Tyr were very similar. The IR absorption peaks at 3284, 1732 and 1714 cm^{-1}, and the ^{13}C chemical shifts of δ 173.3 (C1/C1′), 152.9 (C7/C7′), 169.3 (C10/C10′) were assigned to the functional groups of hydroxyl, the carbonyls of carboxylic acid and amide bond, respectively.

The ^{1}H NMR spectrum of **1** had the same number of proton resonances as that of Ac-Tyr with slightly different proton chemical shifts. This indicated that the molecule had a plane of symmetry with identical fragments of $C_{11}H_{12}NO_4$ positioned on each side of the plane. The integrations of the aromatic signals were consistent with one less proton in each half of the molecule relative to that of Ac-Tyr. Analysis of the vicinal coupling constants and proton chemical shifts indicated that the two units of Ac-Tyr were connected by a bond between C6 and C6′. Carbons C1, C2, C3, C4, C10 and C11 were assigned by comparison with the NMR spectra of Ac-Tyr as a reference, and this was confirmed by HMQC, COSY, DEPT and HMBC experiments. The signal at δ 152.9 was assigned at the most deshielded carbon C7 and C7′, while δ 115.7 and 125.6 were assigned to the adjacent carbons C8/C8′ and C6/C6′, with their expected higher field shifts. A HMQC experiment showed that the four protons resonating at δ 6.98 were directly connected to carbons at δ 132.2 (C9/C9′) and 128.6 (C5/C5′). The carbon signals at δ 132.2 and 128.6 were assigned as C5/C5′ and C9/C9′, respectively, using correlations observed from the proton at δ 6.78 in a HMBC experiment. The two quaternary carbons C6 and C6′ (δ 125.6) connected the two rings. Thus, on the basis of NMR data, the structure of **1** was established (Fig. 1).



1 **2** **3**

Fig. 1. The chemical structures of the three dimerized products of Ac-Tyr produced by illumination with FMN as a sensitizer at pH 6.0, as determined by NMR methods.

The UV spectrum of **2** showed a maximum absorption peak at 274 nm at the same wavelength as Ac-Tyr, implying that the two benzene rings were not directly conjugated. The IR absorption peaks at 3319, 1732, and 1714 cm^{-1} were assigned to the hydroxyl, the carbonyls of carboxylic acid and the amide bond functionalities, respectively.

The ^{1}H NMR and COSY spectra of **2** revealed the presence of five spin systems, two of which were identical. Assignment of most of the protons and carbons was facilitated by the NMR data and in comparison to **1**. Carbon resonances at δ 128.9 and 130.9 were assigned to the two quaternary carbons C4 and C4′, respectively, using a HMBC experiment, and the resonances at δ 147.8 and 156.6 were assigned to the two most deshielded carbons C7 and C7′, respectively. Two proton signals at δ 7.12 and 6.72 were assigned to two identical vicinally coupled pairs of protons in a *para*-disubstituted phenol ring (C5′, C6′, C8′ and C9′). The two vicinally coupled protons at δ 6.85 and 6.86 and the singlet at δ 6.80 were assigned as part of a ring identical in structure to half of **1**. The quaternary carbon resonating at δ 141.8 (C6) was assigned as being connected to the other ring via an ether linkage with C7′, by virtue of its characteristic downfield chemical shift in comparison with the carbon–carbon linked products. All of the assignments were established and confirmed by HMQC, COSY, HMBC, their chemical shifts, and coupling constants. The structure of **2** was thus established (Fig. 1).

The dimerized molecule, **3**, also showed a UV absorption peak (λ_{max}=283 nm) similar to that of **1**. The IR spectrum of **3** had absorption peaks at 3283, 1732, and 1690 cm^{-1} that were assigned to the hydroxyl group, and the carbonyl groups of carboxylic acid and the amide bond, respectively, as with the other two products. A further absorption was observed at 1714 cm^{-1} and this was assigned to a keto carbonyl group in conjunction with the observance of a resonance at δ 206.1 in the ^{13}C NMR spectrum.

All protons in the ^{1}H NMR spectrum of **3** were magnetically distinct; thus **3** lacked a symmetry plane. The proton resonances of half of the new molecule were similar to that of Ac-Tyr, while the other half of the molecule contained no aromatic structure since there was a lack of further downfield signals in the ^{13}C NMR spectrum. There was only one amide resonance δ 8.11 in the ^{1}H spectrum of **3**. The ^{1}H, COSY and HMQC NMR experiments allowed the assignments of five different spin systems.

For the first half of **3**, carbons C1, C2, through C11 were assigned based on the reference NMR spectra of Ac-Tyr, HMQC, COSY and HMBC NMR data, and analogous interpretation of NMR data for **1**.

The three spin systems on the other half of the molecule were separated by the keto group C7′ (δ 206.1) and the quaternary carbon C4′ (δ 52.0). C9′ (δ 63.8) was assigned as being directly connected to N′ because of the observance of correlations between the protons resonating at δ 3.94 and C10′ (δ 169.9), and δ 4.76 and C9′ (δ 63.8) in a HMBC experiment. C5′ was assigned as the carbinol carbon in an ester linkage with C1′ based on its distinctive chemical shifts for H5′ (δ 4.99) and C5′ (δ 86.7). The two quaternary carbons C6 (δ 128.7) and C4′ (δ 52) connected the two partial structures, supported by the correlations between the proton δ 7.22 and C4′, δ 2.50 and C6, and δ 3.94 and C6 observed in HMBC experiments. All other assignments were established from HMQC, and HMBC NMR data. Therefore, the chemical structure of **3** was established (Fig. 1).

4. Discussion

The treatment of Tyr, Tyr derivatives, and some proteins with peroxidase and hydrogen peroxide, oxidants, or UV or gamma radiation produces **1**, which has been characterized by chemical analysis, chromatographic methods, ultraviolet absorption and fluorescence studies (its chemical structure has been deduced mainly by chemical analysis) [16,19–22]. The carbon–oxygen linked dimer **2** has been found to be formed by the UV irradiation of *p*-cresol [23]. Product **3** has apparently not been reported previously.

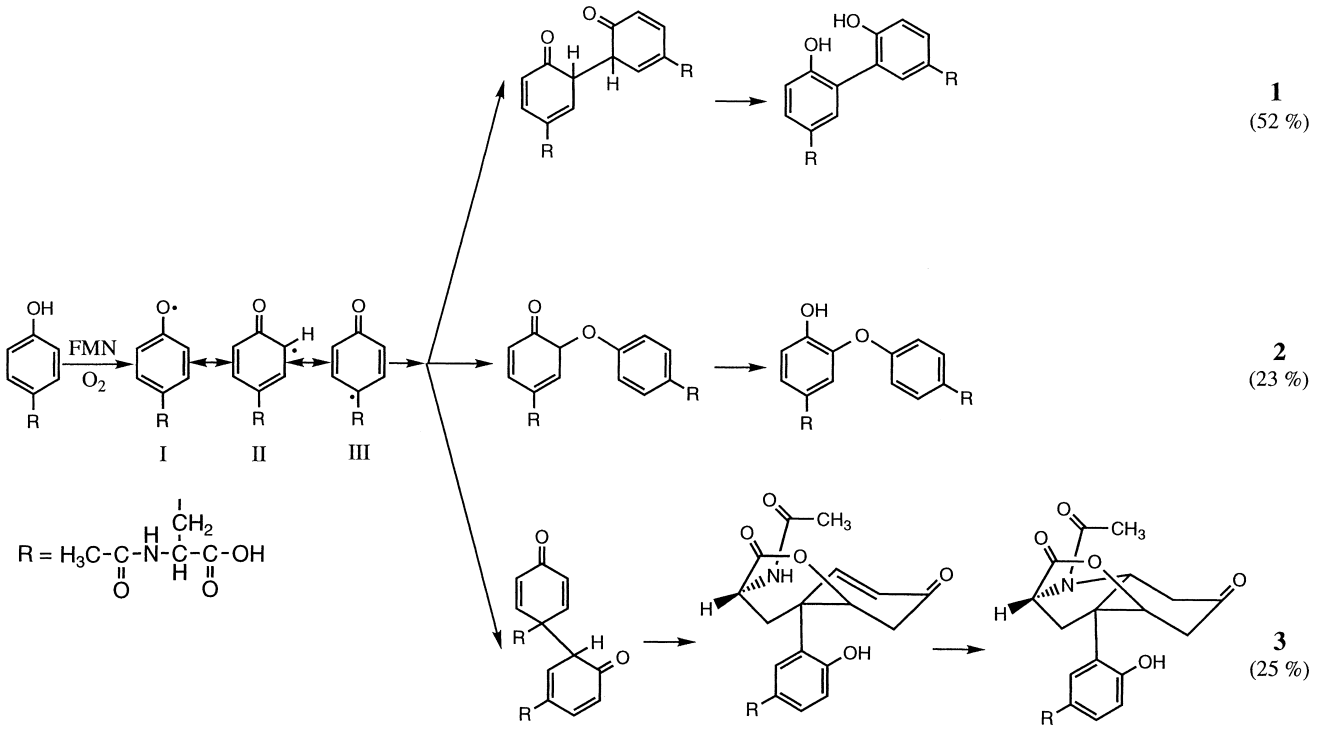


Fig. 2. Possible cross-linking pathways for the formation of the three Ac-Tyr conjugated products, **1**, **2**, and **3** resulting from the FMN-sensitized photooxidation of Ac-Tyr.

A large amount of evidence indicates that intermediate free radicals of monophenolic compounds are involved in the formation of dimeric products [19,24]. Dityrosine formation by peroxidase-hydrogen peroxide [19], UV irradiation [16], and FMN-sensitized photooxidation [25] has been suggested to be mediated via a free radical pathway. Phenoxy radicals can isomerize into three different forms. Coupling of these radicals can produce different dimers. In the present work, three main dimers were isolated and purified from the products resulting from the FMN-sensitized photocross-linking of Ac-Tyr. The proposed mechanisms for the formation of the three cross-linked products are summarized in Fig. 2. We find that oxygen is required for the FMN-sensitized photocross-linking of Ac-Tyr; however, as shown in Fig. 2, oxygen does not appear to be incorporated into the cross-linked products. Similarily, as we reported earlier, oxygen is required for the FMN-sensitized photocross-linking of a Tyr-copolymer via the formation of Tyr–Tyr bonds [17]. It has been reported that the rate of H atom abstraction from the Tyr phenolic group by triplet FMN to give the Tyr radicals involved in cross-linking is very slow under anaerobic conditions, and increases with increasing oxygen concentration [26,27]. Thus, oxygen is required to give a useful rate of Ac-Tyr cross-linking by some unknown mechanism that does not involve its incorporation into the cross-linked products of Ac-Tyr.

From the proposed reaction pathways, six possible products may be produced by pairing the three types of radical intermediates. However, the pairings between two Type I radicals, a Type I and Type III radical, and two Type III radicals (Fig. 2) are expected to be unlikely due to the instability of the intermediates formed or because of steric hindrance preventing the formation of dimers.

The first and the second cross-linked dimers, **1** and **2** are presumably formed by the direct pairing of two Type II radicals, and a Type I with a Type II radical, respectively, followed by enolization. Product **3** is probably generated by the coupling of a Type II and a Type III radical, followed by the electrophilic addition of the carboxylic acid group to the carbon–carbon double bond at positions C5′ and C6′, and then the nucleophilic addition of the secondary amine (amide bond) to the C8′ and C9′ positions. This is in agreement with the reaction sequence for the electrophilic and nucleophilic addition of α,β-unsaturated carbonyl compounds [28].

In biological situations, it is possible that Tyr residues in proteins could be conjugated by the two phenol groups in two ways analogous to **1** and **2** upon illumination in the presence of riboflavin, FMN or other similar types of photosensitizers, since the phenol group is the only reactive group involved in the formation of these products. However, a residue analogous to **3** cannot be produced in a protein except for the Tyr residue at the C-terminal end, since both the amide bond and the carboxylic group participate in the reaction. Other types of cross-links have little likelihood of being produced because of their very low yields in the

photocross-linking of the model compound, Ac-Tyr, under the reaction conditions used.

The previous study with Tyr-containing HPMA copolymers and this study show that the extent of cross-linking of the Tyr-copolymer decreased much more significantly than that of Ac-Tyr with increasing pH [17]. This suggests that steric hindrance by the macromolecule backbone could significantly influence the collision probability of the radical intermediates, probably resulting in the pairing of two Tyr radicals. It can be imagined that the position of Tyr residues in the three-dimensional structures of protein molecules determines whether two Tyr residues can be conjugated. It is expected that spacially adjacent Tyr residues in the three-dimensional structures of proteins could form intramolecular cross-links, while Tyr residues at the periphery of proteins could form intermolecular cross-links.

The photodynamic cross-linking of proteins involving Tyr residues modeled by Ac-Tyr is our first step in the study of the flavin-sensitized cross-linking of Tyr residues in proteins. Our next step is to model the photocross-linking reaction using HPMA copolymers with side chains terminating in Tyr-NH_2, and to isolate, purify and characterize the cross-linked products formed, and compare them with the cross-linked products formed from the model compound, Ac-Tyr. Eventually, we plan to study the chemical nature of the photocross-linking of proteins involving Tyr using model proteins (natural or genetically engineered), and to evaluate the influence of Tyr localization and the microenvironment on the course and efficacy of the photocross-linking reaction.

Photocross-linking of proteins involving Tyr residues may be involved in catarogenesis, skin photoaging, and other biological situations [5–9]. This study may be helpful in developing strategies to prevent pathological pathways. Understanding the chemical nature and detailed mechanism [5] of the photocross-linking of Tyr residues in proteins may allow more efficient exploitation of this type of cross-linking reaction for biomedical applications. The knowledge obtained may permit the design of genetically engineered proteins with cross-linking points at predetermined spacial positions. Such materials could be used as biodegradable scaffolds for tissue engineering. Also, it is well-known that dityrosine is present in resilin, silk fibroin, collagen, elastin and other structural proteins to form a stable three-dimensional structure with elastic properties [22,24,29].

The FMN-mediated photodynamic cross-linking process might be applied to cross-link and stabilize the three-dimensional structure of biomaterials such as bovine or porcine pericardial tissues for bioprosthetic heart valves. Information obtained from this study could help to rationally optimize the modification process, which may allow zero-length cross-linking through Tyr residues within the existing matrix while minimizing other types of cross-linking and photooxidation reactions. Previous studies have shown that dye-sensitized photocross-linking mediated through His residues has the potential to improve the mechanical properties, chemical and enzymatic stability, and minimize calcification of tissue-derived heart valves [12,30,31]. The cross-linking process involving Tyr residues may have advantages over photocross-linking involving His residues because of the instability of the His cross-linked bonds [3,4]. Photodynamically-cross-linked pericardial tissue used as prosthetic heart valves is biocompatible and supports the growth of endothelial cells. In contrast, glutaraldehyde-cross-linked tissues used as prostheses are cytotoxic and are not epithelialized in vitro due to aldehyde retained in the tissue [30]. Other modes of oxidative cross-linking, such as treatment with peroxidase/H_2O_2 mixture, might also result in the retention of cytotoxic materials in the pericardial tissue; also, the use of enzyme-mediated cross-linking systems might result in the cross-linking of the enzyme to the pericardial tissue. Pericardial tissue cross-linked by FMN-sensitized photooxidation would not be expected to be cytotoxic since the flavin is a normal constituent of cells.

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References

[1] T.M.A.R. Dubbelman, A.F.P.M. De Goeij, J. Van Steveninck, Biochim. Biophys. Acta 511 (1978) 141–151.
[2] R.C. Straight, J.D. Spikes, in: A.A. Frimer (Ed.), Singlet O_2, Vol. IV, CRC Press, Boca Raton, 1985, pp. 91–143.
[3] H.-R. Shen, J.D. Spikes, P. Kopečková, J. Kopeček, J. Photochem. Photobiol. B: Biol. 34 (1996) 203–210.
[4] H.-R. Shen, J.D. Spikes, P. Kopečková, J. Kopeček, J. Photochem. Photobiol. B: Biol. 35 (1996) 213–219.
[5] J.D. Goosey, J.S. Zigler Jr., J.H. Kinoshita, Science 208 (1980) 1278–1280.
[6] J.S. Zigler Jr., J.D. Goosey, Photochem. Photobiol. 33 (1981) 869–874.
[7] S. Garcia-Castineiras, J. Dillon, A. Spector, Exp. Eye Res. 26 (1978) 461–476.
[8] M.K. McNamura, R.C. Augusteyn, Exp. Eye Res. 30 (1980) 319–321.
[9] M.D. Carbonare, M.A. Pathak, J. Photochem. Photobiol. B: Biol. 14 (1992) 105–124.
[10] B.W. Henderson, T.J. Dougherty (Eds.), Photodynamic Therapy, Marcel Dekker, New York, 1992.
[11] M. Ochsner, J. Photochem. Photobiol. B: Biol. 39 (1997) 1–18.
[12] G.L. Mechanic, U.S. Patent No. 5,147,514, 15 September 1992.
[13] K. Kohn, D. Kessel, Biochem. Pharmacol. 28 (1979) 2465–2470.
[14] A.W. Girotti, Biochim. Biophys. Acta 602 (1980) 45–56.
[15] H. Verweij, J. Van Steveninck, Photochem. Photobiol. 35 (1982) 265–267.
[16] Y. Kato, K. Uchida, S. Kawakishi, Photochem. Photobiol. 59 (1994) 343–349.

[17] J.D. Spikes, H.-R. Shen, P. Kopečková, J. Kopeček, Photochem. Photobiol. 70 (2) (1999) 130–137.
[18] V. Malanic, M. Ledvina, Prep. Biochem. 9 (1979) 273–280.
[19] A.J. Gross, I.W. Sizer, J. Biol. Chem. 234 (1959) 1611–1614.
[20] F. LaBella, P. Waykole, G. Queen, Biochem. Biophys. Res. Commun. 30 (1968) 333–338.
[21] D. Creed, Photochem. Photobiol. 39 (1984) 563–575.
[22] A.J. Kungl, G. Landl, A.J.W.G. Visser, M. Breitenbach, H.F. Kauffmann, J. Fluoresc. 2 (1992) 63–74.
[23] H.-I. Joschek, S.I. Miller, J. Am. Chem. Soc. 88 (1966) 3273–3281.
[24] S.O. Andersen, Acta Physiologica Scandinavica 66 (Supplementum 263) (1966) 46–48.
[25] E. Silva, J. Godoy, Int. J. Vit. Nutr. Res. 64 (1994) 253–256.
[26] P. Byrom, J.H. Turnbull, Photochem. Photobiol. 6 (1967) 125–131.
[27] M.B. Taylor, G.K. Radda, Meth. Enzymol. B 18 (1971) 496–506.
[28] R.T. Morrison, R.N. Boyd, Organic Chemistry, Allyn & Bacon, Boston, 1980, pp. 867–873.
[29] R. Amado, R. Aeschbach, H. Neukom, Meth. Enzymol. 107 (1984) 377–388.
[30] L.A. Bengtsson, R. Phillips, A.N. Haegerstrand, Ann. Thorac. Surg. 60 (1995) S365–S368.
[31] M.A. Moore, W.-M. Chen, R.E. Phillips, I.K. Bohachevsky, B.K. McIlroy, J. Biomed. Mater. Res. 32 (1996) 209–214.