Novel Dehydrofuroimidazole Compounds Formed by the Advanced Maillard Reaction of 3-Deoxy-D-hexos-2-ulose and Arginine Residues in Proteins

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Similar unknown peaks were detected on an amino acid chromatogram of the acid hydrolysates in 3-deoxyglucosone (3DG)-lysozyme as well as glucose−lysozyme reaction systems. Unknown peaks were also detected in the acid hydrolysates of 3DG and N^{α}-benzoylarginine amide ($BzArgNH_2$) reaction system. By this system, 2-(N^{α}-benzoyl-N^{δ}-ornithylamide)-5-(2,3,4-trihydroxybutyl)-4-imidazolone (S17) and 2-(N^{α}-benzoyl-N^{δ}-ornithylamide)-5-(2,3,4-trihydroxybutyl)-2-imidazoline-4-one (S12) were identified as major products in a previous paper (Hayase et al. *Biosci. Biotechnol. Biochem*. **1995**, *59*, 1407−1411). In addition, minor products were also formed from one molecule of $BzArgNH_2$ and two molecules of 3-DG. The minor product was identified as 2-(N^{α}-benzoyl-N^{δ}-ornithylamide)-5,6a-di(2,3,4-trihydroxybutyl)-5,6-dihydroxydehydrofuro[2,3-*d*]imidazole (S11). Periodic changes in the amounts of compounds S17, S12, and S11 suggest the formation of compound S17 via compound S12 by dehydration and oxidation from the $BzArgNH_2$-3DG reaction system to be the major pathway, the formation of compound S11 by the addition of 3DG to compound S12 being the other pathway.

Keywords: *Maillard reaction; arginine; 3-deoxyglucosone; glycation; imidazolone compounds*

INTRODUCTION

In an advanced stage of the Maillard reaction, the intermediate dicarbonyls result in damage to proteins in biological systems and in foods, such as the impairment of amino acid residues, reduction of digestibility, loss of solubility, formation of fluorescent compounds, browning, polymerization, and degradation (Mohammad et al., 1949; Cho et al., 1986; Shin et al., 1988; Ledl and Schleicher, 1990; Wolff et al., 1991). Among these changes, glucose-mediated advanced glycation end products (AGEs) have been hypothesized to be responsible for the aging of tissues or diabetic complications (Monnier et al., 1991; Cerami, 1994). Therefore, much attention has been focused on elucidation of chemical structures of the AGEs.

Among intermediate dicarbonyls, glyoxal was recently identified as a fragmentation product of autoxidation of glucose and by oxidation of the Schiff base to form N^{ϵ}-(carboxylmethyl)lysine (CML) (Well-Knecht et al., 1995a; Baynes, 1991). Such oxidative reactions lead to generation of AGEs, including the glycoxidation products, such as an imidazolium compound (GOLD) identified as a lysine−lysine cross-link (Well-Knecht et al., 1995b), as well as CML. Pentosidine identified as a fluorescent lysine−arginine cross-link is an AGE which is considered to form *in vivo* through glycoxidation of Amadori compounds (Sell and Monnier, 1989).

Nonoxidative reactions without fragmentation of Amadori compounds also lead to formation of AGEs. The crossline (Nakamura et al., 1992) was recently identified as fluorescent lysine−lysine cross-link.

3-Deoxy-D-hexos-2-ulose, also called 3-deoxyglucosone (3DG) has been reported to be generated from Amadori compounds by nonoxidative reactions *in vitro* (Shin et al., 1988) and *in vivo* (Hayase et al., 1991; Knecht et al., 1992; Niwa et al., 1993; Yamada et al., 1994). 3DG was also formed by fructose 3-phosphate in the lens of diabetic rats (Szwergold et al., 1990), and by the Maillard reaction in some foods (Hayase and Kato, 1994). 3DG is known to be a highly reactive and cytotoxic compound (Shinoda et al., 1994). Previous work (Shin et al., 1988; Kato et al., 1987a,b) has revealed that 3DG liberated by the degradation of ϵ-deoxyfructosyllysine residues, attacked amino acids such as arginine, lysine, and tryptophan to cross-link proteins in the solid state at 50 °C and 75% relative humidity and under physiological conditions at 37 °C and pH 7.4.

Previous work (Hayase et al., 1989) indicating the ability of ϵ-deoxyfructosyllysine and 3DG to form a protein-bound pyrrole aldehyde, called pyrraline, suggested a synthetic pathway whereby ϵ-deoxyfructosyllysine was first generated from glucose and subsequently degraded into 3DG, and then 3DG reacted with free amino groups to form pyrraline. We also identified pyrropyridinium compound named pyrropyridine (Hayase et al., 1994) as a feasible fluorescent lysine−lysine cross-link generated in a butylamine−3DG or glucose reaction system. Tryptophan residues have also been speculated to be modified with α-dicarbonyl compounds (Saito et al., 1986).

Moreover, several nonfluorescent imidazolone compounds were identified as arginine adducts with 3DG (Konishi et al., 1994; Hayase et al., 1995). 2-(N^{α}-Benzoyl-N^{δ}-ornithylamide)-5-(2,3,4-trihydroxybutyl)-4-imidazolone (S17) was identified as a stable end product. However, S11, purified as a minor product formed from one molecule of N^{α}-benzoylarginine amide ($BzArgNH_2$) and two molecules of 3DG, is speculated to have participated in further reactions such as the formation of cross-links (Konishi et al., 1994; Hayase et al., 1995).

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In this paper, we describe the isolation and identification of S11 formed in a model reaction between 3DG and $BzArgNH_2$.

MATERIALS AND METHODS

Materials. Lysozyme from egg white (crystallized six times) was obtained from Seikagaku Co. (Tokyo, Japan), and N^{α}-benzoyl-L-arginine amide ($BzArgNH_2$) was obtained from Nacalai Chemical Co. (Kyoto, Japan). 3DG was synthesized according to the method mentioned in a previous paper (Kato et al., 1990), all the other chemicals used in this study being analytical reagent grade. Water was used after purification by reverse osmosis, using Milli RO 10 PLUS and Milli-Q Jr. (Millipore) membranes, and ion exchange.

Incubation System. $BzArgNH_2$ (0.1 M) and 3DG (0.2 M) were dissolved in 0.1 M sodium phosphate buffer at pH 7.4 and incubated at 50 °C for 96 h. 10 mg/mL of lysozyme was incubated with 200 mM 3DG or D-glucose in a 0.2 M sodium phosphate buffer at pH 7.4 and 50 °C for 96 h or 2 weeks, respectively. After incubation, the reaction mixture was extensively dialyzed against water. The non-dialyzable fraction was reduced with $NaBH_4$ (Kato et al., 1987b) and extensively dialyzed against 0.1 M NaCl for 24 h, followed by water for 24 h. The non-dialyzable fraction was then lyophilized.

S12, 2-(N^{α}-benzoyl-N^{δ}-ornithylamide)-5-(2,3,4-trihydroxybutyl)-2-imidazoline-4-one, was isolated and purified according to the method mentioned in a previous paper (Hayase et al., 1995). S12 (13.3 mM) and 3DG (13.3 mM) were dissolved in a 0.1 M sodium phosphate buffer at pH 7.4 and incubated at 50 °C for up to 120 h.

Amino Acid Analysis. The products of lysozyme and $BzArgNH_2$ incubated with 3DG or D-glucose were hydrolyzed with 6 M HCl at 110 °C for 24 h. The hydrolysates were subjected to amino acid analysis with Hitachi model 835 equipment.

Chromatography for Purification. The products from the $BzArgNH_2$ and 3DG reaction mixture were purified by HPLC (Shimadzu LC-10AS), performed by monitoring at 235 or 275 nm with a Shimadzu SPD-AV detector under the following conditions:

(1) Reversed phase HPLC with a solvent system of 20% methanol used an ODS 4251-N column (Senshu Kagaku, 250 mm length × 10 mm i.d.) for separation and isolation. With a solvent system of 15% or 10% methanol, an ODS 1251-N (Senshu Kagaku, 250 mm length × 4.6 mm i.d.) column was used for verification of the purity. Flow rates were 1.0 and 0.5 mL/min, respectively.

(2) Gel permeation HPLC was done with a solvent system of 10% methanol−0.1 M NaCl and an Asahipak GS-220 column (Asahi Chemicals, 500 mm length × 7.6 mm i.d.) for verification of the purity (flow rate, 0.7 mL/min).

Spectrometry. UV absorption spectra were recorded with a Hitachi 220A spectrophotometer and mass spectra were recorded with a JEOL SX102 mass spectrometer. The ionization mode was set to fast-atom bombardment (FAB)-(+) with glycerol as a matrix. The ^{13}C-NMR and ^{1}H-NMR spectra were recorded with a JEOL GSX-500 (500 MHz) instrument, and the NMR data were recorded in CD_3OD.

RESULTS

Elucidation of Arginine Adduct (S11) Modified with Two Molecules of 3-Deoxyglucosone. Since it is considered that 3DG affects cross-linking by first attacking the arginine residues in a protein (Kato et al., 1987a,b), we compared the reaction products between 3DG and lysozyme with those between 3DG and $BzArgNH_2$ as a model reaction system of the arginine residues in proteins and with those between glucose and lysozyme. The amino acid chromatogram of the acid hydrolysate of lysozyme incubated with 3DG at 50 °C and pH 7.4 shows four characteristic unknown peaks as described in a previous paper (Konishi et al., 1994). These peaks are identical to those detected in the 3DG−$BzArgNH_2$ reaction system, as well as in the lysozyme−glucose reaction system. These results strongly indicate that arginine residues modified with 3DG are the source of the unknown peaks in the chromatogram of the hydrolysate of lysozyme incubated with glucose.

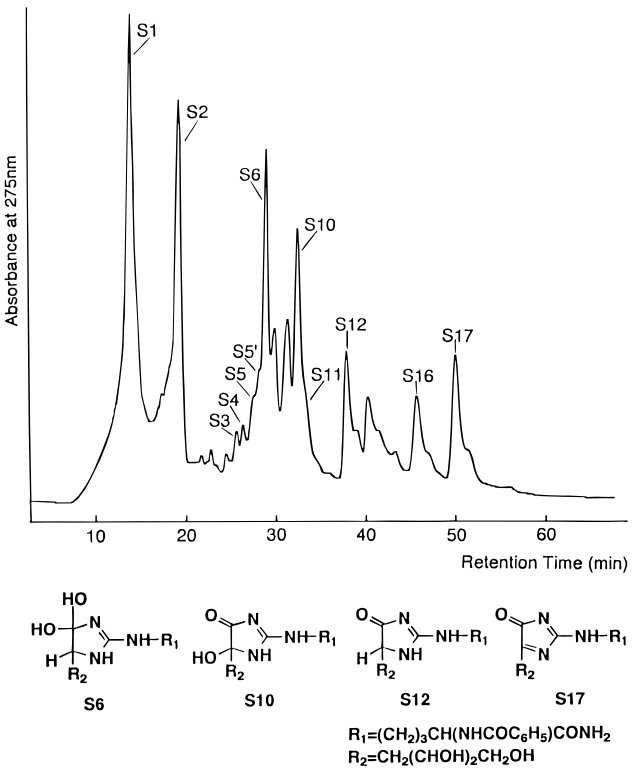

Figure 1. HPLC pattern from an ODS column for $BzArgNH_2$ (100 mM) incubated with 3DG (200 mM) in a 0.1 M sodium phosphate buffer (pH 7.4) at 50 °C for 4 days. S6, S10, S12, and S17 were identified in a previous paper [see Konishi et al. (1994) and Hayase et al. (1995)].

After $BzArgNH_2$ was incubated with 3DG at pH 7.4 and 50 °C for 96 h, the mixture was analyzed by reversed phase HPLC. Figure 1 shows that several peaks were generated from the 3DG−$BzArgNH_2$ reaction system, identified as S1−S17. S1 and S2 were derived from salt and $BzArgNH_2$, respectively, while S6, S10, S12, and S17 were identified in previous papers (Konishi et al., 1994; Hayase et al., 1995) as major products. S3, S4, S5, S5′, and S11 were also purified as minor products.

FAB-MS data for all these minor isolated compounds revealed an $[M + 1]^+$ ion at $m/z = 584$, indicating that these compounds are considered to have been formed from one molecule of $BzArgNH_2$ and two molecules of 3DG by dehydration.

S11 was isolated with 99.5% purity by preparative HPLC repeated at two or three times until single peak of S11 has been obtained. Approximately 2 mg of S11 was obtained from 5 mL of reaction solution. The high-resolution FAB-MS data for S11 revealed an $[M + H]^+$ ion at m/z 584.2571, the molecular formula of S11 was estimated to be $C_{25}H_{37}N_5O_{11}$ (calcd for $C_{25}H_{38}N_5O_{11}$, 584.2568). The UV spectrum of S11 showed one peak at 221 nm with a weak shoulder peak (270 nm), resembling the UV spectrum of $BzArgNH_2$ ($\lambda_{max} = 227$ nm with shoulder peak at 272 nm). These results indicate that S11 contained the aromatic ring derived from $BzArgNH_2$.

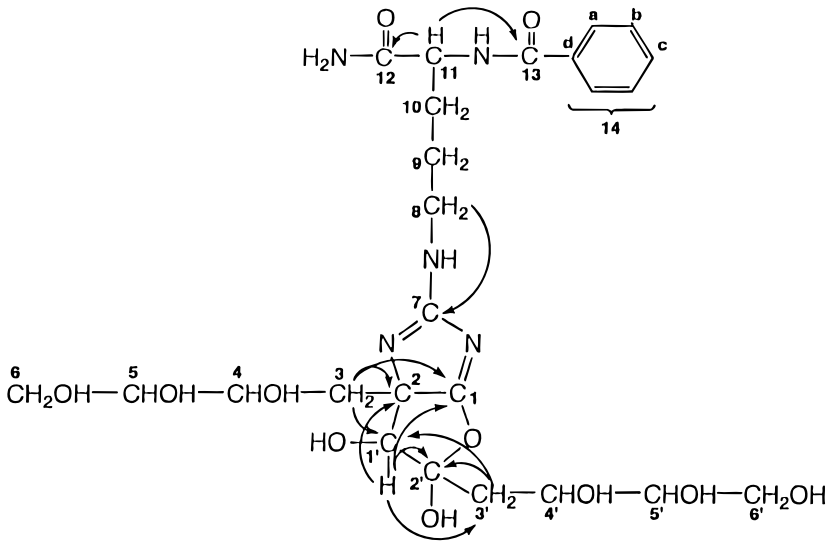

Figure 2. Identified structure of S11. Arrows indicate the carbon−proton long-range coupling between quaternary carbons and protons detected in the HMBC spectrum of S11.

Table 1. ^{13}C- and ^{1}H-NMR Spectral Data for S11

position no.	δC (ppm)	δH (ppm)	spin−spin
9	27.8	1.62−1.80 (2H, m)	8, 10
10	30.2	1.82−2.00 (2H, m)	9, 11
3	36.8	1.73−1.77, 1.88−1.97 (2H, m)	4
3′	39.3	1.87−1.90, 1.99−2.04 (2H, m)	4′
8	43.0	3.37−3.42 (2H, m)	9
11	54.8	4.54−4.57 (1H, m)	10
6′	64.2	3.52−3.58, 3.62−3.65 (2H, m)	5′
6	64.4	3.52−3.58, 3.66−3.70 (2H, m)	5
4	67.6	3.99−4.05 (1H, m)	3, 5
2	68.7		
4′	69.8	4.08−4.14 (1H, m)	3′, 5′
1′	72.7	3.59 (1H, s)	
5	75.2	3.55−3.57 (1H, m)	4, 6
5′	76.3	3.46−3.48 (1H, m)	4′, 6′
2′	100.2		
14(a)	128.6	7.84 (2H, d)	14(b)
14(b)	129.6	7.48 (2H, t)	14(a), (c)
14(c)	132.9	7.57 (1H, t)	14(b)
14(d)	135.2		
13	170.4		
7	171.9		
12	177.0		
1	192.4		

^{1}H-NMR and ^{13}C-NMR measurements (completely decoupled and DEPT with $^{3/4}J$ spectra) of S11 gave the following data. ^{13}C-NMR δC(CD_3OD): (numbers in parentheses show the proton position) position 9(9), 27.8 (1C, t); 10 (10), 30.2 (1C, t); 3 (3a, 3b), 36.8 (1C, t); 3′ (3′), 39.3 (1C, t); 8 (8), 43.0 (1C, t); 11 (11), 54.8 (1C, d); 6′ (6′), 64.2 (1C, t); 6 (6), 64.4 (1C, t); 4 (4), 67.6 (1C, d); 2 (no H), 68.7 (1C, s); 4′ (4′), 69.8 (1C, d); 1′ (1′), 72.7 (1C, d); 5 (5), 75.2 (1C, d); 5′ (5′), 76.3 (1C, d); 2′ (no H), 100.2 (1C, s); 14a (14a), 128.6 (2C, d); 14b (14b), 129.6 (2C, d); 14c (14c), 132.9 (1C, d); 14d (no H); 135.2 (1C, s); 13 (no H), 170.4 (1C, s); 7 (no H), 171.9 (1C, s); 12 (no H); 177.0 (1C, s); 1 (no H), 192.4 (1C, s). ^{1}H-NMR δH(CD_3OD): 9, 1.62−1.80 (2H, m); 3, 1.73−1.77 and 1.88−1.97 (2H, m); 10, 1.82−2.00 (2H, m); 3′, 1.87−1.90 and 1.99−2.04 (2H, m); 8, 3.37−3.42 (2H, m); 5′, 3.46−3.48 (1H, m); 6′, 3.52−3.58 and 3.62−3.65 (2H, m); 6, 3.52−3.58 and 3.66−3.70 (2H, m); 5, 3.55−3.57 (1H, m); 1′, 3.59 (1H, s); 4, 3.99−4.05 (1H, m); 4′, 4.08−4.14 (1H, m); 11, 4.54−4.57 (1H, m); 14b, 7.48 (2H, t); 14c, 7.57 (1H, t); 14a, 7.84 (2H, d).

Moreover, ^{1}H-detected ^{1}H−^{13}C COSY (HMQC) (Summers et al., 1986) and DEPT (distortionless enhancement by polarization transfer) spectra enabled assignment of the carbons bound with protons.

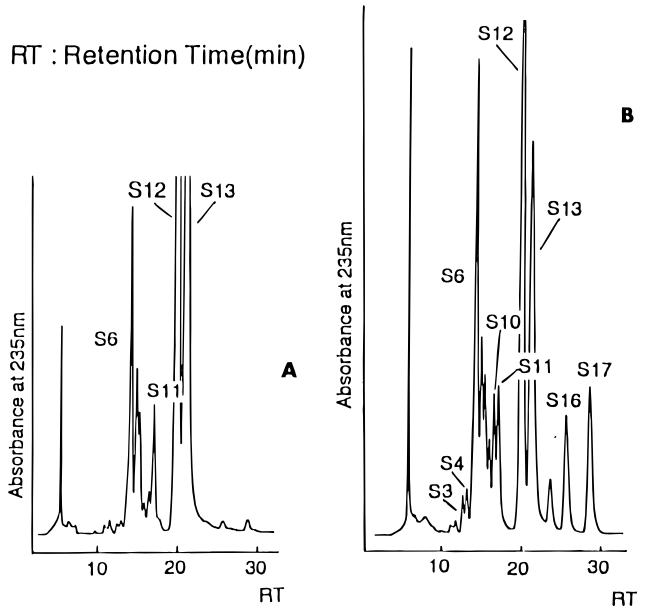

Figure 3. HPLC patterns on an ODS column of S12 (13.3 mM) incubated with 3-deoxyglucosone (13.3 mM) in a 0.1 M sodium phosphate buffer at 50 °C and pH 7.4 for 24 h (A) and 120 h (B).

The ^{1}H-NMR and ^{1}H−^{1}H COSY spectra of S11 in CD_3OD showed the presence of the ^{1}H network on C-3 to C-6 and on C-3′ to C-6′ of two molecules of 3DG and on the α, β, γ, and δ carbons of $BzArgNH_2$. Moreover, the carbons bound with protons could be assigned by HMQC, and these results are summarized in Table 1.

The long-range coupled quaternary carbons could be assigned by the ^{1}H-detected long-range ^{1}H−^{13}C COSY (HMBC) (Bax et al., 1986) spectrum. The HMBC spectrum for S11 gave cross peaks with carbon−proton long-range coupling within two or three bonds. Figure 2 shows carbon−proton long-range coupling around the quaternary carbon detected in the HMBC spectrum of S11. In addition, the assignment of the carbons, except for the quaternary carbons assigned by the HMQC spectrum, was also supported by the HMBC spectrum. The ^{1}H−^{1}H COSY and HMBC spectra for S11 indicate that S11 contained part of the native structure of both

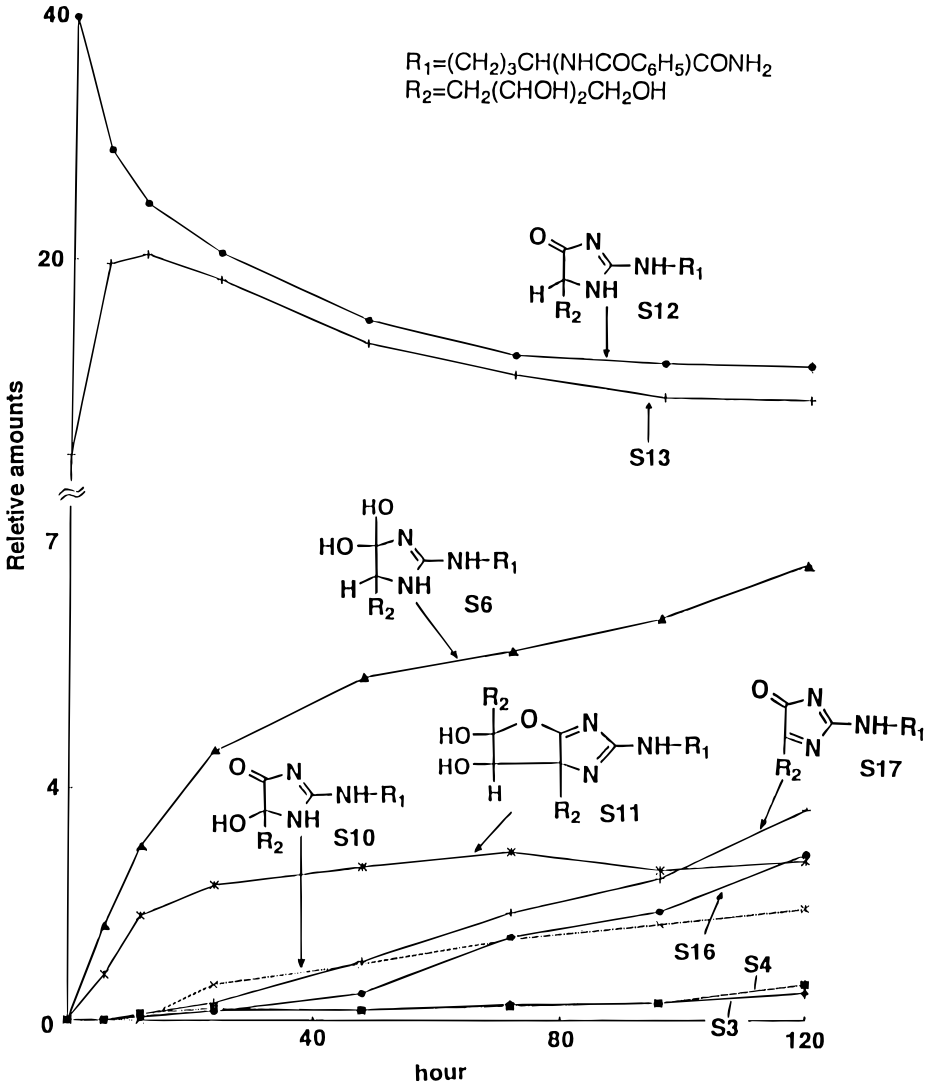

Figure 4. Changes in the amounts of products formed by the reaction of 13.3 mM S12 incubated with 13.3 mM 3-deoxyglucosone in a 0.1 M sodium phosphate buffer at 50 °C and pH 7.4.

molecules of 3DG and one molecule of $BzArgNH_2$, and that the C-1 carbon of 3DG reacted with the guanidyl amino group. As S11 was not detected by spraying a saturated solution of 2,4-DNPH reagent (2,4-dinitrophenylhydrazine saturated in 2 M HCl), no carbonyl carbons existed in S11.

On the basis of these NMR and high-resolution FAB-MS data, S11 was identified as 2-(N^{α}-benzoyl-N^{δ}-ornithylamide)-5,6a-di(2,3,4-trihydroxybutyl)-5,6-dihydroxydehydrofuro[2,3-*d*]imidazole.

Reaction between S12 and 3-Deoxyglucosone. In the previous paper, S17 was identified as 2-(N^{α}-benzoyl-N^{δ}-ornithylamide)-5-(2,3,4-trihydroxybutyl)-4-imidazolone and S12 was identified as 2-(N^{α}-benzoyl-N^{δ}-ornithylamide)-5-(2,3,4-trihydroxybutyl)-2-imidazoline-4-one. The formation of S17 via S12 is considered to be the major pathway with dehydration and subsequent oxidation. In order to clarify the formation mechanism of S11, the reaction mixture incubated with S12 and 3DG at pH 7.4 and 50 °C for 120 h was analyzed by reversed phase HPLC. Figure 3 indicates that several compounds identified as described in the previous paper were also generated from an S12−3DG reaction system. S13 is estimated to be an isomer of S12 as FAB-MS data revealed an $[M + 1]^+$ ion at $m/z = 422$ and S16 to be an isomer of S17 from an $[M + 1]^+$ ion at $m/z = 420$. Figure 4 shows the changes in the relative amounts of the products, the profile indicating that S13, S11, and S6 were major products, and S16 and S17 were minor products after incubation for 24 h. S6 has been identified as 2-(N^{α}-benzoyl-N^{δ}-ornithylamide)-5-(2,3,4-trihydroxybutyl)-4-dihydroxy-2-imidazoline (Hayase et al., 1995). S10, identified as 2-(N^{α}-benzoyl-N^{δ}-ornithylamide)-5-(2,3,4-trihydroxybutyl)-5-hydroxy-2-imidazoline-4-one, was formed after 24 h, indicating that S10 is generated by the hydration of S17 (Hayase et al., 1995). S3 and S4 ($M_w = 583$) were generated after 24 h of incubation.

DISCUSSION

Although arginine and its derivatives are known to react readily with 2-oxoaldehydes, such as phenylglyoxal (Takahashi, 1968, 1977), *p*-hydroxyphenylglyoxal (Eun and Miles, 1984), and *p*-nitrophenylglyoxal (Soman et al., 1985), in order to modify specifically the arginine residues in proteins, the reaction mechanisms for arginine and its derivatives with 2-oxoaldehyde compounds are not yet completely clear. Proposed here is a modification of the structure of arginine via reaction with 2-oxoaldehydes. The reaction of arginine and 3DG is extremely complex (Konishi et al., 1994). S11 was already formed after 6 h of incubation of S12 and 3DG (Figure 4). Therefore, S11 is speculated to have been

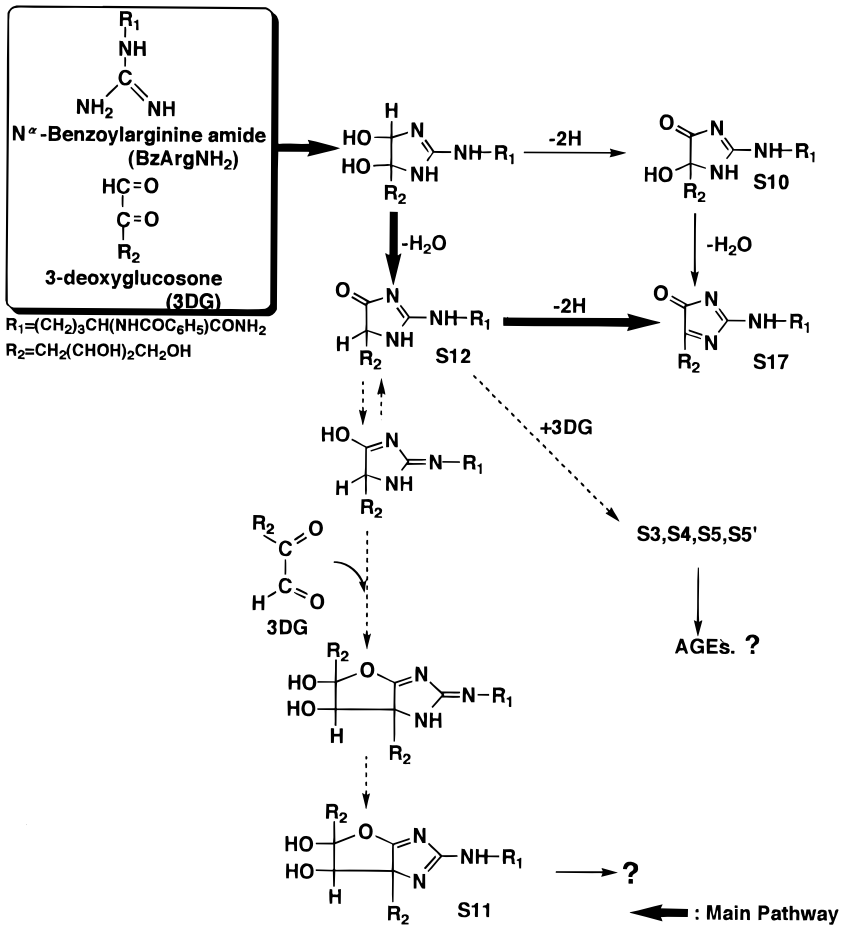

Figure 5. Proposed formation pathway for products from the reaction of 100 mM N^{α}-benzoylarginine amide incubated with 200 mM 3-deoxyglucosone in a 0.1 M sodium phosphate buffer at 50 °C and pH 7.4.

formed by the reaction of an additional molecule of 3DG with S12 (Hayase et al., 1995). Figure 5 shows the proposed formation scheme for S11 from an S12−3DG reaction system. Since S11 increased and then decreased in a $BzArgNH_2$−3DG reaction system during incubation for 7.5 days, and for 120 h in S12−3DG reaction system, it is speculated that S11 participated in further reactions such as the formation of cross-links. However, S11 was stable in an aqueous solution. In the previous paper (Hayase et al., 1995), the formation of S17 from S12 via dehydration and subsequent oxidation is considered to be the major pathway. The formation of S17 from S10 is less important. The first step in formation of S12 is presumed to be condensation of one molecule of 3DG with the guanidino group to give 3,4-dihydroxy-2-imidazoline compound (Figure 5). This imidazoline compound seems then to be dehydrated to give S12. This scheme was also speculated by Takahashi (1977) who investigated the reaction between phenylglyoxal and N^{α}-benzyloxycarbonyl-L-arginine.

S11 is different from basic structure of the 2:1 diphenylglyoxal−arginine adducts proposed by Takahashi (1968). However, it cannot be denied on the structural similarity between S3, S4, S5, or S5′ and the 2:1 diphenylglyoxal arginine adducts.

Our reaction system at 50 °C is considered to simulate reaction under physiological conditions, because the difference in the reaction between 37 and 50 °C was only that of rate (Shin et al., 1988; Kato et al., 1987b; Konishi et al., 1994; Hayase et al., 1995).

Glucosone has been reported to be formed by the decomposition of Amadori compounds catalyzed by copper ion (Cheng et al., 1994). Glucosone, which is a 2-oxoaldehyde compound, is also able to modify the arginine residues to produce imidazo compounds, in the same manner as 1-deoxyglucosone, these being Maillard reaction intermediates (Beck et al., 1988).

Methylglyoxal and glyoxal have been reported to be formed *in vivo* as well as in food systems. Recent studies on the reaction of arginine residues with methylglyoxal have revealed that the major products are imidazolone compounds, 5-methylimidazol-4-one and 5-hydro-5-methylimidazol-4-one, corresponding to S12 and S17 in the present paper, respectively (Henle et al., 1994; Lo et al., 1994). Imidazole compounds formed from arginine residues and two molecules of 2-oxoaldehydes have not been previously reported.

CML, which is well-known as an AGE, was originally described as a product of the cleavage of the Amadori compound formed during glycation of protein under oxidative conditions (Ahmed et al., 1986). CML has been recently reported to be also formed by direct modification of lysine residues with glyoxal generated by autoxidation of glucose (Well-Knecht, 1995a) and by lipid peroxidation (Fu et al., 1996). Glyoxal is also able to react with arginine residues.

Recently, Niwa et al. (1996) have been reported that 3DG accumulating in uremic serum may be involved in the AGE modification of β_2 microglobulin isolated from the amyloid deposits in patients with dialysis-related amyloidosis. Although a potential pathobiological role for such imidazole compounds is presently speculative, one possibility is that arginine modification by 3DG may be closely related to AGE modification of β_2 micro-

globulin-amyloid as well as the formation of pyrropyridine (Hayase et al., 1994). Studies on the physiological properties of imidazolones, imidazolines, and imidazoles *in vivo* will be necessary to address the question of whether the imidazo compounds play a role in the pathology of diabetes and aging.

ABBREVIATIONS

3DG and 3-deoxyglucosone, 3-deoxy-D-hexos-2-urose; $BzArgNH_2$, N^{α}-benzoyl-L-arginine amide; AGEs, advanced glycation end products; CML, N^{ϵ}-(carboxymethyl)lysine; GOLD, 1,3-bis-N^{α}-hippuryl−lysine−imidazolium salt or glyoxal−lysine dimer; pyrropyridine, fluorescent pyrrolopyridinium compound or 6,8-disubstituted 3,3a,8,8a-tetrahydro-3a-hydroxy-2-(1,2-dihydroxyethyl)-5-hydroxymethyl-2*H*-furo[3′,2′:4,5]pyrrolo-[2,3-*c*]pyridinium.

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