Detection of α-Dicarbonyl Compounds in Maillard Reaction Systems and in Vivo

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α-Dicarbonyl compounds are of major interest in food chemistry and biochemistry as important precursors of, for example, protein modifications and flavor. Due to their high reactivity most of the published structures were identified and quantitated as stable derivatives after reaction with trapping reagents. However, the present study showed for the first time that the trapping reagents are of dramatic impact on the final qualitative and quantitative α-dicarbonyl spectrum. As important representatives, aminoguanidine and *o*-phenylenediamine were used to compare trapping characteristics and to monitor the dicarbonyl structures arising from the degradation of an Amadori compound. Dicarbonyl structures with a reductone moiety could not be or were only insufficiently detected by slow-reacting reagents such as aminoguanidine. On the other hand, fast-reacting chemicals such as *o*-phenylenediamine imposed high oxidative stress on the investigated system and led to enhanced or false positive formation of dicarbonyl compounds generated by oxidative pathways.

Keywords: *α-Dicarbonyl compounds; Maillard reaction; trapping reagents; o-phenylenediamine; aminoguanidine*

INTRODUCTION

α-Dicarbonyl structures are of major interest in food chemistry and biochemistry. In foods, they are important precursors of color and aroma components but also have negative consequences, for example, leading to protein modifications and thereby to nutritional losses. In vivo, α-dicarbonyl compounds are of direct impact on the formation of protein modifications also termed advanced glycation end products (AGEs). AGEs, in turn, have been associated with the pathology of diabetes and aging. Hence, considerable effort was undertaken in both fields to elucidate the synthesis and nature of the α-dicarbonyl structures formed and to develop methods for their quantitation.

These methods include chromatographic detection of the native dicarbonyl molecules and also of their corresponding alcohols after reduction (*1*, *2*). However, the procedures most widely used employ trapping reagents, such as *O*-aryl or *O*-alkyl hydroxylamines (e.g., *O*-methylhydroxylamine), hydrazines (e.g., Girard T reagent), cysteamine, *o*-diaminobenzene derivatives (e.g., *o*-phenylenediamine), and aminoguanidine to result in oximes, osazones, thiazolidines, quinoxaline derivatives, and 3-amino-1,2,4-triazines, respectively (*3*−*8*) (Figure 1). In foods, the procedures used were optimized by focusing on available short-chained reference dicarbonyl structures (*9*, *10*). The trapping methods were also used to explore the mechanistic pathways of the Maillard reaction, especially with *o*-diaminobenzene derivatives and aminoguanidine (e.g., refs *11* and *12*). It is generally accepted that knowledge of α-dicarbonyl structures is the key to understanding and influencing the product spectrum of the Maillard reaction. The evaluation of the results published is extremely complex because of several unanswered questions. First, besides the use of different model systems and trapping reagents, two totally different approaches were applied in general. One was to perform reactions in the presence of the trapping reagents, the other, to add them at the various time points of sampling. It is obvious that, especially, the first method is prone to artifacts, because the trapping reagent will influence the product spectrum by the trapping action itself, as well as by its redox capabilities and interference as an active amine in the Maillard reaction. Second, concerning the latter method, a wide range of conditions for the final derivatization reaction was used (i.e., temperature, pH, and time). The workup conditions will also significantly influence the results, as the structures so far identified are extremely diverse concerning reactivity and polarity. Finally, despite the many structures published, α-dicarbonyl compounds available as authentic references for detailed studies are very limited. These considerations also count for investigations of α-dicarbonyl structures formed in vivo. Again, two main methods were used, which cannot be directly compared. *O*-diaminobenzene derivatives were added to samples obtained from living subjects, whereas aminoguanidine was directly administered as a pharmaceutical drug (*13*, *14*). In both cases, glyoxal, methylglyoxal, and 3-deoxy-D-*erythro*-2-hexosulose were identified as the major compounds in vivo. However, on the basis of the above considerations, it cannot be excluded that other α-dicarbonyl compounds present were missed because the experimental conditions had not been optimized for these structures.

Thus, we critically investigated the two major methods for the detection of Maillard-derived α-dicarbonyl structures by trapping reagents, using *o*-phenylenediamine and aminoguanidine, respectively. The various

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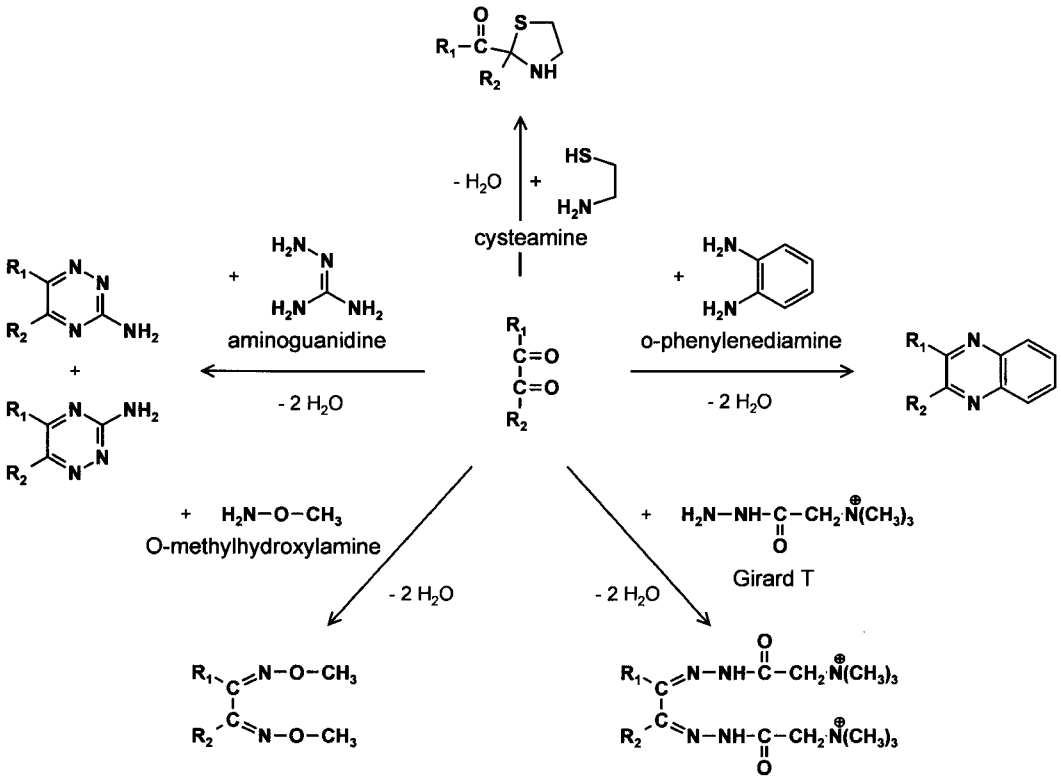

Figure 1. Trapping reagents for the detection of α-dicarbonyl compounds.

influences were studied on a simple model system following the degradation of the Amadori product of glucose and N^{α}-*t*-BOC-lysine. On the basis of our recent successful synthesis of 1-deoxy-D-*erythro*-hexo-2,3-diulose, detailed results were also obtained for the first time for this major Maillard intermediate.

MATERIALS AND METHODS

Materials. Chemicals of the highest quality available were obtained from Aldrich (Steinheim, Germany) and Fluka (Neu-Ulm, Germany), unless otherwise indicated.

Syntheses. 3-Deoxy-D-*erythro*-2-hexosulose (3-DG), 1-deoxy-D-*erythro*-2,3-hexodiulose (1-DG), and D-*arabino*-2-hexosulose (glucosone) were synthesized mainly according to the methods of Madson and Feather (*15*), Glomb and Pfahler (*16*), and Bayne (*17*), respectively. The purity of these α-dicarbonyl compounds was tested as follows: a solution of 2 mg of 3-DG or 1-DG and 1.3 mg (0.008 mmol) of 2-deoxy-D-glucose in 200 μL of water was stirred with 7 mg (0.184 mmol) of $NaBH_4$ for 1 h; the reaction was quenched with HOAc, and solvents were evaporated. Three times the still moist residue was taken up in 1% methanolic HCl and evaporated again. The resulting residue was dried under high vacuum, dissolved in MeOH, and filtered, and the solvents were removed. The final residue was derivatized with 100 μL of *N*,*O*-bis(trimethylsilyl)acetamide in anhydrous pyridine (1:1, v/v) and subjected to HRGC/FID (temperature program for 3-DG, 160 °C isothermal; for 1-DG, injection at 100 °C, followed by ramp of the oven temperature to 200 °C at 5 °C·min^{-1}, then to 270 °C at 10 °C·min^{-1}, and a 10 min hold; for glucosone, 195 °C isothermal). Following the same workup procedure, 2.5 mg of glucosone were reduced in the presence of 2.5 mg (0.014 mmol) D-galactose. In all cases, the purity was calculated on the basis of equal molar detector response for the respective trimethylsilylated sugar alcohols to be 75% for 3-DG, 95% for 1-DG, and 68% for glucosone.

N^{α}-*t*-*BOC*-N^{ϵ}-*(1-deoxy-D-fructos-1-yl)lysine* (Amadori product of D-glucose and N^{α}-*t*-BOC-lysine) was synthezised and quantified after reduction by high-performance liquid chromatography, mainly according to the method of Glomb and Monnier (*18*).

3-Amino-5-methyl-6-ethyl-1,2,4-triazine and 3-amino-5-ethyl-6-methyl-1,2,4-triazine (ISTD-triazines). A solution of 400 mg (4 mmol) of 2,3-pentanedione and 545 mg (4 mmol) of aminoguanidine bicarbonate in 300 mL of phosphate buffer (70 mM, pH 7.0) was stirred overnight and extracted with EtOAc, and the combined organic layers were dried and evaporated. The resulting residue was subjected to column chromatography (EtOAc/hexane 9:1). Fractions with material having R_f 0.22 (TLC, same solvent) were combined and evaporated to give yellowish crystals (502 mg, 91%, HRGC-FID analysis revealed a ratio of 5:6 for the 5-methyl- and 6-methyl isomers).

HRGC-FID/MS (after trimethylsilylation): t_R 27.26/28.96 min; *m*/*z* 210 ($M^{\bullet+}$, 38%), 195 (30), 167 (6), 115 (100), 99 (18), 73 (33), 69 (6).

HR-MS: *m*/*z* 210.3532 (210.3540 calcd for $C_9H_{18}N_4Si$).

^{1}H NMR (CD_3OD) 6-methyl isomer: δ 1.25 (t, 3 H, J = 7.6 Hz), 2.42 (s, 3 H), 2.70 (q, 2 H, J = 7.4 Hz).

^{1}H NMR (CD_3OD) 5-methyl-isomer: δ 1.26 (t, 3 H), 2.40 (s, 3 H), 2.80 (q, 2 H).

^{13}C NMR (CD_3OD) 6-methyl isomer: δ 10.8, 17.9, 28.3, 143.1, 163.1, 167.2.

^{13}C NMR (CD_3OD) 5-methyl isomer: δ 12.8, 21.4, 25.8, 153.9, 162.7, 162.9.

3-Amino-1,2,4-triazine (glyoxal-triazine) was synthesized as for ISTD-triazine, but starting from a 40% aqueous glyoxal solution. EtOAc was the eluent for TLC (R_f 0.29) and column chromatography: yield 83%; spectroscopic data compliant with those of Erikson (*19*).

HRGC-FID/MS (after trimethylsilylation): t_R 14.57 min; *m*/*z* 168 ($M^{+\bullet}$, 35%), 153 (33), 139 (15), 114 (15), 99 (100), 73 (18), 69 (9).

3-Amino-5-methyl-1,2,4-triazine and *3-amino-6-methyl-1,2,4-triazine (methylglyoxal-triazines).* Synthesis as for the ISTD-triazine, but starting from a 40% aqueous methylglyoxal solution. EtOAc was the eluent for TLC (R_f 0.24) and column chromatography. Yield 79%, spectroscopic data compliant with Lo et al. (*20*). HRGC-FID revealed a ratio of 13:1 for the 5-methyl- and the 6-methyl-isomer.

HRGC-FID/MS (after trimethylsilylation): t_R 17.59/17.88 min; *m*/*z* 182 ($M^{\bullet+}$, 33%), 167 (27), 154 (6), 139 (9), 114 (15), 99 (100), 73 (18), 69 (9).

(1R,2S,3R)-1-(3-Amino-1,2,4-triazin-5-yl)-1,2,3,4-tetrahydroxybutane and (1R,2S,3R)-1-(3-Amino-1,2,4-triazin-6-yl)-1,2,3,4-tetrahydroxybutane (Glucosone-triazines). A solution of 450 mg (1.7 mmol) of glucosone and 346 mg (2.5 mmol) of aminoguanidine bicarbonate in 200 mL of phosphate buffer (70 mM, pH 7.0) was stirred overnight at 40 °C. Water was evaporated, the resulting residue was taken up in MeOH, 30 mL of silica gel was added, and the volatiles were evaporated again. The resulting dry powder was subjected to column chromatography and eluted with 300 mL of EtOAc/MeOH (1:1). Solvents were evaporated. and the resulting material was subjected to column chromatography with EtOAc/MeOH (2:1). Material having R_f 0.31 (TLC, same solvents) was combined, and solvents were evaporated to yield yellowish crystals [175 mg, 47%, spectroscopic data compliant with those of Hirsch et al. (*8*)]. HRGC-FID revealed a ratio of 4:1 for the 5- and 6-isomers).

HRGC-FID/MS (after trimethylsilylation): t_R 66.23/71.14 min; *m/z* 576 ($M^{\bullet+}$, 2%), 561 (1), 342 (55), 270 (66), 217 (33), 147 (30), 103 (27).

(2S,3R)-1-(3-Amino-1,2,4-triazin-5-yl)-2,3,4-trihydroxybutane and (2S,3R)-1-(3-Amino-1,2,4-triazin-6-yl)-2,3,4-trihydroxybutane (3-DG-triazines). Synthesis was the same as for glucosone-triazine, but 3-DG was employed instead of glucosone. The eluent for TLC (R_f 0.25) and column chromatography was EtOAc/MeOH (4:1) [yield 40%, spectroscopic data compliant with those of Hirsch et al. (*8*)]. HRGC-FID revealed a ratio of 1:1 for the 5- and 6-isomers.

HRGC-FID/MS (after trimethylsilylation): t_R 65.84/67.65 min; *m/z* 473 (M − 15, 5%), 398 (9), 385 (4), 309 (12), 295 (24), 283 (30), 254 (9), 217 (100), 182 (24), 147 (21), 103 (9), 73 (61).

(1S,2R)-1-(3-Amino-6-methyl-1,2,4-triazin-5-yl)-1,2,3-propanetriol (1-DG-triazine) was synthesized according to the method of Glomb and Pfahler (*16*) with major modifications. (4*R*,5*S*)-5-(3-Amino-6-methyl-1,2,4-triazin-5-yl)-2,2-dimethyl-1,3-dioxolan-4-yl]methanol (125 mg, 0.52 mmol) was dissolved in 10 mL of methanolic HCl (5%). After 1 h, the solution was neutralized with NaOH and the volatiles were evaporated. The residue was taken up in 1 mL of water and subjected in 200 μL portions to Envicarb columns (Supelco, Bellefonte, PA; 0.5 g, 6 mL), activated with 6 mL of MeOH and 12 mL of water. After addition of the samples, columns were first eluted with 12 mL of water, and 1-DG-triazine was then eluted with 12 mL of MeOH. The methanolic fractions were combined, solvents were evaporated, and the residue was subjected to column chromatography with $CHCl_3$/MeOH (8:2). Fractions with material having R_f 0.18 were combined and volatiles evaporated to yield yellowish crystals [63 mg, 61%, spectroscopic data compliant with those of Hirsch et al. (*21*)].

HRGC-FID/MS (after trimethylsilylation): t_R 58.18 min; *m/z* 488 ($M^{\bullet+}$, 23%), 473 (5), 284 (55), 269 (10), 187 (2), 147 (12), 117 (8), 73 (100).

Quinoxaline (glyoxal-quinoxaline) and 2-methylquinoxaline (methylglyoxal-quinoxaline) are commercially available.

2-Ethyl-3-methylquinoxaline (ISTD-quinoxaline) and (1S,2R)-1-(3-methyl-2-quinoxalinyl)-1,2,3-propanetriol (1-DG-quinoxaline) were prepared mainly according to the method of Glomb and Pfahler (*16*).

HRGC-FID/MS (glyoxal-quinoxaline): t_R 13.81 min; *m/z* 130 ($M^{\bullet+}$, 68%), 103 (67), 75 (100), 61 (6), 59 (6), 53 (33), 50 (39).

HRGC-FID/MS (methylglyoxal-quinoxaline): t_R 17.40 min; *m/z* 144 ($M^{\bullet+}$, 88%), 117 (100), 103 (8), 90 (22), 75 (55), 63 (12), 50 (50).

HRGC-FID/MS (ISTD-quinoxaline): t_R 29.97 min; *m/z* 172 ($M^{\bullet+}$, 100%), 157 (5), 144 (13), 130 (15), 117 (8), 103 (8), 89 (3), 76 (18), 63 (5), 50 (14).

HRGC-FID/MS (1-DG-quinoxaline, after trimethylsilylation): t_R 59.72 min; *m/z* 450 ($M^{\bullet+}$, 3%), 435 (10), 345 (2), 271 (6), 246 (42), 231 (3), 205 (8), 172 (2), 147 (30), 133 (4), 117 (18), 103 (5), 73 (100).

(2S,3R)-1-(2-Quinoxalinyl)-2,3,4-trihydroxybutane (3-DG-quinoxaline). A solution of 350 mg (1.62 mmol) of 3-DG and 270 mg (2.50 mmol) of *o*-phenylenediamine in 35 mL of MeOH was stirred overnight at ambient temperature in the presence of 3 Å molecular sieves under argon atmosphere. After filtration, solvents were evaporated and the residue was subjected to column chromatography with EtOAc. Fractions with material having R_f 0.15 (TLC, EtOAc) were combined and volatiles evaporated to yellowish crystals [250 mg, 66%, spectroscopic data compliant with those of Morita et al. (*22*)].

HRGC-FID/MS (after trimethylsilylation): t_R 69.46 min; *m/z* 450 ($M^{\bullet+}$, 1%), 435 (4), 360 (6), 345 (6), 270 (10), 245 (26), 217 (15), 181 (5), 147 (10), 133 (4), 103 (6), 73 (100).

(1R,2S,3R)-1-(2-Quinoxalinyl)-1,2,3,4-tetrahydroxybutane (Glucosone-quinoxaline). A solution of 400 mg (1.53 mmol) of glucosone and 306 mg (2.83 mmol) of *o*-phenylenediamine in 40 mL of MeOH was stirred overnight at 40 °C in the presence of 3 Å molecular sieves under argon atmosphere. After filtration, volatiles were evaporated. The residue was suspended in 5 mL of EtOAc/MeOH (9:1) and filtered, and the insoluble material was washed several times with small amounts of EtOAc/MeOH (9:1) to give yellowish crystals [182 mg, 47%, spectroscopic data compliant with those of Morita et al. (*23*)].

HRGC-FID/MS (after trimethylsilylation): t_R 70.78 min; *m/z* 538 ($M^{\bullet+}$, 2%), 523 (3), 355 (2), 307 (5), 277 (3), 245 (9), 217 (25), 189 (3), 147 (8), 103 (15), 73 (100).

Model Reactions. In general, experiments were conducted in 0.1 M phosphate buffer (pH 7.4) at 37 °C in a shaker incubator after sterile filtration. Deaerated conditions were achieved by the presence of 1 mM diethylenetriaminepentaacetic acid and gassing with argon. The concentrations of the reactants are given in the respective figure legends. Aliquots (2.5 mL) of the incubations were taken at various time points and worked up as follows. For triazines, samples were diluted with 2.5 mL of water and 50 μg of ISTD-triazine was added; 25 mL of a 1:1 mixture of EtOAc with MeOH was added, and the emulsion was vigorously shaken for 30 min. After centrifugation, the volatiles of the organic layer were evaporated, and the residue was taken up in 2 mL of water and passed on an Envicarb column (0.5 g, 6 mL), activated with 6 mL of MeOH and 24 mL of H_2O. The column was washed with 12 mL of water, and triazines were then eluted with 6 mL of MeOH. Volatiles were evaporated, and the final residue was derivatized with 200 μL of *N*,*O*-bis(trimethylsilyl)acetamide with 5% trimethylchlorosilane in anhydrous pyridine (1:1, v/v) and subjected to HRGC/FID. For quinoxalines, samples were diluted with water and 100 μg of ISTD-quinoxaline was added. Twenty-five milliliters of EtOAc was added, and the emulsion was vigorously shaken for 30 min. The organic layer was separated by centrifugation, dried with 1.6 g of $CaSO_4$, and filtered. After evaporation (30 °C, 110 mbar), the final residue was derivatized with 200 μL of *N*,*O*-bis(trimethylsilyl)acetamide with 5% trimethylchlorosilane in anhydrous pyridine (1:1, v/v) and subjected to HRGC/FID.

Chromatography. Thin-layer chromatography (TLC) was performed on Silica Gel 60 F_{254} plates (Merck, Darmstadt, Germany). Visualization of separated material was achieved with UV. Preparative column chromatography was performed on Silica Gel 60, 63−200 μm (Merck). Solvents were all of chromatographic grade. From the individual chromatographic fractions solvents were removed under reduced pressure.

Nuclear Magnetic Resonance Spectroscopy (NMR). NMR spectra were recorded on a Bruker AC 400 instrument (Rheinstetten, Germany). Chemical shifts are given in parts per million relative to residual nondeuterated solvent as the internal reference.

High-Resolution Mass Spectrometry (HR-MS). HR-MS was run on a VG 7070 (VG, Manchester, U.K.) with heptacosane as the internal standard.

High-Resolution Gas Chromatography (HRGC-FID/MS). HRGC-FID was performed on a VEGA 6000 (Fisons Instruments, Mainz, Germany), quartz capillary column (triazine/quinoxaline analysis: 60 m, inner diameter = 0.32 mm, DB-5, 1.0 μm, He, 35 cm/s) (α-dicarbonyl purity analysis: 30 m, inner diameter = 0.32 mm, DB-5, 1.0 μm, He, 33 cm/s) (both columns were from J&W Scientific, Cologne, Germany; injection port = 270 °C, detector = 270 °C). The temperature program for triazine analysis was as follows: after the samples were injected at 120 °C, the temperature of the oven was raised

Table 1. Recovery of Triazines/Quinoxalines during the Respective Workup Procedures

	% recovery	
	triazine	quinoxaline
glyoxal	101	62
methylglyoxal	98	60
ISTD	105	91
1-DG	57	99
3-DG	33	92
glucosone	16	98

at 5 °C·min^{-1} to 180 °C and held for 15 min, then raised at 5 °C·min^{-1} to 245 °C and held for 35 min, then raised at 15 °C·min^{-1} to 280 °C, and held for 15 min. The temperature program for quinoxaline analysis was as follows: after the samples were injected at 120 °C, the temperature of the oven was raised at 5 °C·min^{-1} to 170 °C and held for 20 min, then raised at 5 °C·min^{-1} to 250 °C and held for 24 min, then raised at 15 °C·min^{-1} to 280 °C, and held for 15 min. The temperature program for α-dicarbonyl purity analysis is given under Synthesis. For HRGC-MS analysis the HRGC was connected to a MAT ITD 700 (Finnigan, Bremen, Germany): transfer line, 280 °C; EI at 70 eV.

RESULTS AND DISCUSSION

The dicarbonyl compound formation was followed during the degradation of the Amadori product of glucose and N^{α}-*t*-BOC-lysine to study the various influences of aminoguanidine (AG) and *o*-phenylenediamine (OPD) as trapping reagents. In general, to keep the product spectrum simple and reproducible, incubations were conducted in 0.1 M phosphate buffer at pH 7.4 and 37 °C. The monitored dicarbonyl spectrum was focused on glyoxal, methylglyoxal, 3-deoxy-D-*erythro*-2-hexosulose (3-DG), 1-deoxy-D-*erythro*-hexo-2,3-diulose (1-DG), and D-*arabino*-2-hexosulose (glucosone). Although this paper was not aimed at elucidating mechanistic pathways within the Maillard reaction, these known carbonyl structures were chosen as important representatives of fragmented and still full carbon-chained intermediates of amine-catalyzed hexose degradation. Generation of glyoxal and glucosone requires an oxidation step, whereas 3-DG and 1-DG are formed nonoxidatively. Thus, the relative ratios of these α-dicarbonyl compounds allow for estimating the extent of oxidation processes in the course of Maillard reactions. The respective triazine and quinoxaline derivatives were synthesized and two efficient workup procedures for the isolation and detection with coupled gas chromatography−mass spectrometry established. Triazines were separated from incubation mixtures by solid phase extraction on graphitized carbon material and quinoxalines by liquid−liquid extraction with ethyl acetate. Purified target material was derivatized as trimethylsilyl ethers prior to gas chromatography. In both cases, quantitation was performed on the basis of an internal standard of triazine or quinoxaline, which were synthesized from 2,3-pentanedione. Nevertheless, reproducible results could be achieved only when each step of the respective work up protocol was kept rigorously constant (relative standard deviation: triazines, 9%; quinoxalines, 4%; both $n = 10$). One reason was the wide range in polarity within the two groups of target structures. Especially for the triazines this became obvious by comparing the recovery of the standards during the workup procedure (Table 1). A second reason was the relatively high volatility of the unpolar low molecular weight derivatives; this resulted in a loss of, especially, the glyoxal and methylglyoxal quinoxalines, which is difficult to control.

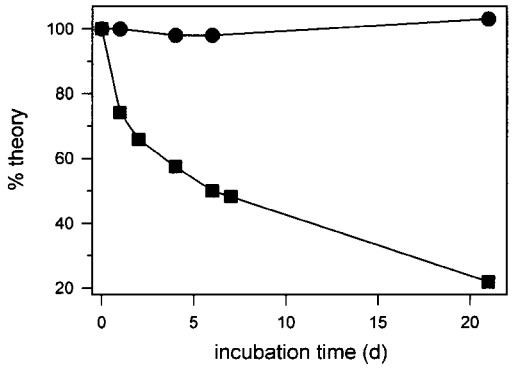

Figure 2. Stability of the triazine (■) and quinoxaline (●, both 100 μM) derivatives from 1-DG under incubation conditions (0.1 M phosphate buffer, pH 7.4, 37 °C, 5 mM AG/OPD).

Triazines and quinoxalines are generally accepted to be stable derivatives of α-dicarbonyl compounds. As detailed studies have never been performed, target structures were incubated under the above reaction conditions in the presence of AG and OPD, respectively. The quinoxalines could be confirmed to be stable; only for the glyoxal and methylglyoxal derivatives were slight losses of 5 and 7% recorded after 1 week. In contrast, only the triazines of glyoxal, methylglyoxal, and 3-DG were found to be stable, whereas only 47 and 49% of the triazines of 1-DG and glucosone could be recovered after 1 week, respectively. As an example, Figure 2 depicts the degradation of the quinoxaline versus the triazine of 1-DG within 3 weeks of incubation. Whereas the quinoxaline persisted, almost 80% of the triazine was degraded. Compared to the stable triazine of 3-DG, the only obvious difference from the ones of 1-DG and glucosone is an additional hydroxyl function at the α-carbons next to the triazine ring, which might facilitate fragmentation reactions. However, the exact mechanism of degradation remains unclear, as there were no extra products detectable with the workup procedures used.

Another major factor of influence on the final product spectrum is the efficiency of the derivatization reaction. Incubations of the native dicarbonyl compounds with OPD revealed complete reaction within 5 h for all dicarbonyl structures tested, with the short-chained ones reacting much more rapidly (Figure 3). Plausible explanations are that not only the sterical complexity of the condensation reaction increases with longer carbon backbones but also that the required hemiacetal opening represents a slow, rate-limiting step prior to condensation. Within the full carbon-chained α-dicarbonyl structures there seems to be a trend of decreasing reactivity from 3-DG to glucosone and 1-DG. Glucosone and 1-DG represent not only α-dicarbonyls but also reductones due to their isomeric α-oxoenediol structures. Thus, as the α-dicarbonyl moiety is prerequisite for condensation, glucosone and 1-DG, in addition to ring opening, need to shift equilibrium toward the α-dioxo structure. The impact of the individual chemical's characteristics on the trapping efficiency became even more obvious with AG, because the trapping reaction proceeded much more slowly. Glyoxal and methylglyoxal still led to almost complete reaction within 5 h, whereas for 3-DG 24 h was needed. The results are in line with detailed studies published by Thornalley et al. (*24*) focusing solely on the reaction of these three dicarbonyl

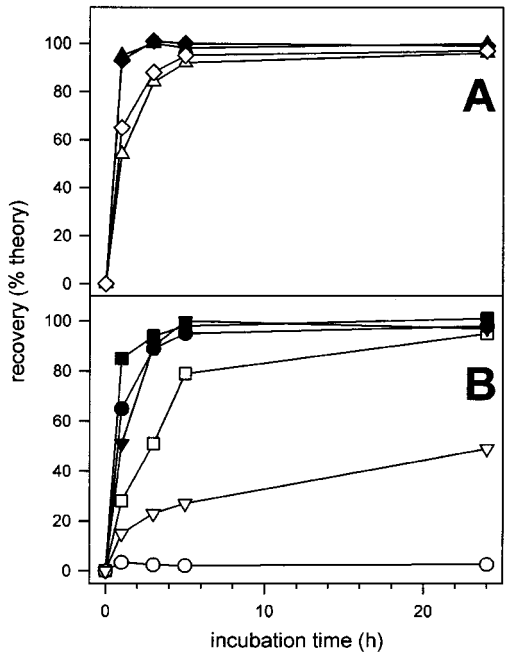

Figure 3. Kinetic studies on the trapping reaction of aminoguanidine and *o*-phenylenediamine (both 5 mM) with α-dicarbonyl compounds (all 300 μM) leading to triazines (open symbols) and quinoxalines (solid symbols), respectively: (A) glyoxal (▲, △) and methylglyoxal (◆, ◇); (B) 1-DG (●, ○), 3-DG (■, □), and glucosone (▼, ▽).

structures with AG. The authors explain the decreasing reactivity with the different actual species reacting in aqueous solution formed by hydration and hemiacetal/ketal ring closure. In contrast, for the reaction of glucosone only a maximum of 50% could be recovered after 24 h as the corresponding triazines. Prolonged incubation did not lead to increased but, finally, again to lower yields, which must be explained by the instability of the triazine structures described above. As a reductone, glucosone readily will be decomposed especially under oxidative conditions by fragmentation reactions. Wells-Knecht et al. (5) have published a half-life for glucosone of 4.9 h in 0.2 M phosphate buffer at 37 °C, generating mainly ribulose. Therefore, the rate of reaction with AG in comparison to OPD is by far too slow to prevent degradation of the native α-dicarbonyl. This discrepancy was even more extreme for 1-DG, because from the reaction with AG only 3% could be recovered as the triazine. When AG or OPD was added to incubations of 3-DG in phosphate buffer after 24 h, the dicarbonyl was recovered completely as the respective trapped derivative (Figure 4). Even the presence of excess amounts of amine led only to a loss of ∼20%. In contrast, analogous incubations of 1-DG revealed complete degradation as evidenced by the complete absence of detectable trapped material. Detailed time course studies on the degradation of 1-DG with OPD showed that the molecule was completely degraded within 5 h to structures no longer accessible for the trapping reagent; 1-DG has a half-life of ∼0.5 h (Figure 4B). This means that relatively stable compounds such as 3-DG accumulate in reaction mixtures even during amine-catalyzed degradation and, thus, can be detected even by slow-reacting trapping reagents. On the other hand, reactive carbonyl structures such as glucosone and especially 1-DG can be accessed only by very fast reagents. The amounts measured represent only the levels present at the respective time points and will be

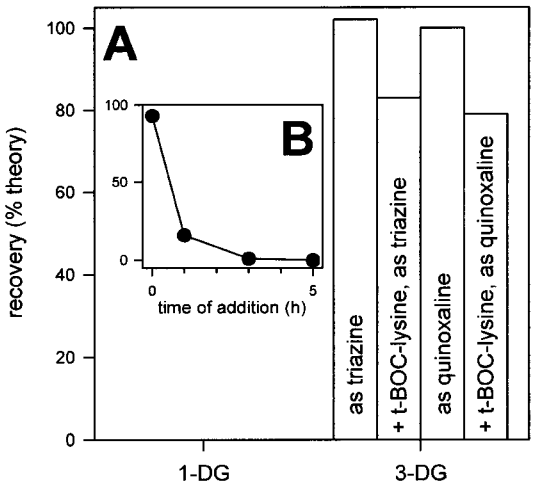

Figure 4. (A) Stability of 1-DG versus 3-DG in incubation mixtures with/without N^{α}-*t*-BOC-lysine (1 mM). Dicarbonyl structures (both 300 μM) were recovered as the corresponding triazines/quinoxalines by addition of AG/OPD (both 5 mM) after 24 h. (B) Incubation of 1-DG without amine, when OPD was added at 0, 1, 3, and 5 h. In each case, after addition of trapping reagent, the reaction mixtures were worked up after 5 h of additional incubation.

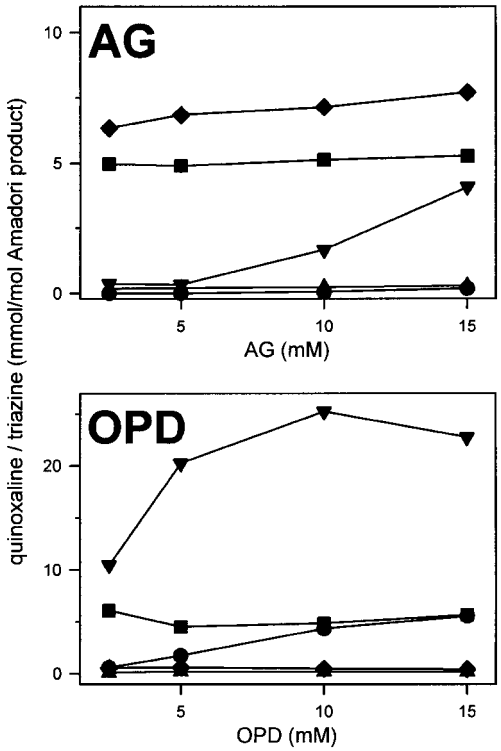

Figure 5. Formation of triazines/quinoxalines [from glyoxal (▲), methylglyoxal (◆), 1-DG (●), 3-DG (■), and glucosone (▼)] in incubations of the Amadori product (42 mM) of glucose and N^{α}-*t*-BOC-lysine in the presence of different concentrations of AG and OPD, respectively. Samples were worked up after 168 h.

significantly influenced by the presence of degradation-catalyzing molecules such as amines.

The Amadori product of N^{α}-*t*-BOC-lysine and glucose was incubated in the presence of increasing amounts of AG or OPD to gain further insight to the specific impact of the trapping chemical on the detection of dicarbonyl structures (Figure 5). In general, for incubations of the Amadori product, triazine/quinoxaline concentrations were expressed as millimoles per mole of

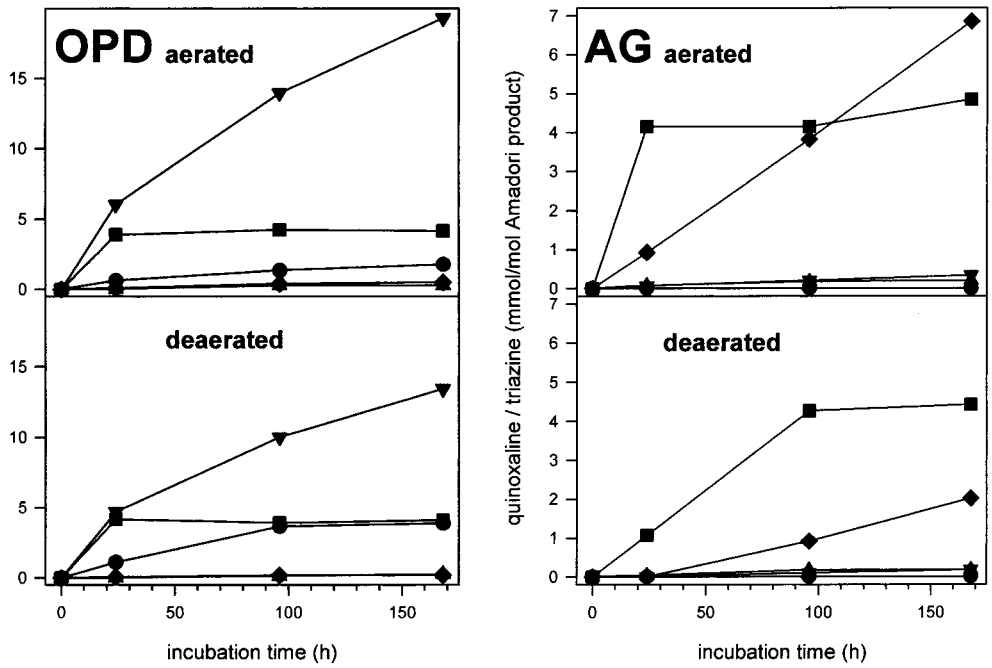

Figure 6. Formation of triazines/quinoxalines [from glyoxal (▲), methylglyoxal (◆), 1-DG (●), 3-DG (■), and glucosone (▼)] in incubations of the Amadori product (42 mM) of glucose and N^{α}-*t*-BOC-lysine under aerated versus deaerated conditions in the presence of AG and OPD (both 5 mM), respectively.

the starting Amadori product concentration. 3-DG derivative levels were not influenced by changes in trapping reagent concentrations at all, leading to the same results for AG and OPD. This was expected, as 3-DG is generated nonoxidatively from the Amadori product by enolization, dehydration, and loss of the amine. Second, the slow-reacting AG was not limiting condensation at low concentrations due to kinetic effects. 3-DG was still trapped quickly enough to prevent degradation of this relatively stable α-dicarbonyl structure. The opposite of the latter consideration indeed counts for the highly unstable 1-DG, for which minor concentrations of the corresponding triazine could be detected only at very high AG levels. This became more obvious in the presence of OPD, when increasing concentrations were clearly positively correlated to quinoxaline levels. Increasing concentrations for the glucosone triazine in AG incubations also imply a similar competition between degradation and trapping action. However, these results were not in line with the OPD incubations, for which significantly higher levels of the respective quinoxalines were present at 1 mM, which then dramatically increased at 5 mM and leveled off at 10 mM. As this cannot be explained by trapping rate limitations, other reasons influencing/limiting the direct formation of glucosone were indicated. Glucosone synthesis from the Amadori product is explained by oxidation catalyzed by, for example, copper metal ions (*1*). OPD represents an electron-rich aromatic structure, the *o*-diamino configuration of which can easily be oxidized to quinonimine derivatives in the presence of oxygen. These represent ideal redox partners for the oxidative degradation of the Amadori compound to glucosone. Thus, the accelerated synthesis of glucosone might be a response to enhanced oxidative stress imposed on the reaction system by the trapping reagent, especially OPD. Another striking difference between the two trapping methods was the ~15-fold higher levels found for the methylglyoxal derivatives in AG reaction mixtures. Several suggestions have been reported for the synthesis of methylglyoxal within the Maillard reaction (*25*−*28*), which are all based on nonoxidative fragmentation of 3-DG, 1-DG, or the Amadori product. Obviously, AG in contrast to OPD significantly enhances the proposed retro-aldol fragmentation. Glyoxal was detected by both trapping systems at similar levels not influenced by increasing concentrations of AG or OPD. The small amounts detected are explained by previous work localizing oxidation and fragmentation of the Schiff base adduct from glucose with N^{α}-*t*-BOC-lysine as the main synthetic route for glyoxal (*18*).

On the basis of the above experiments, a 5 mM trapping reagent concentration was chosen as a good compromise between impact on oxidation (enhancing glucosone formation) and rate of condensation (enhancing the trapping of 1-DG). The degradation of the Amadori product was first investigated in the presence of the trapping reagents and in comparison of aerated and deaerated conditions (Figure 6). All results were in support of the above considerations. Almost identical 3-DG derivative levels were obtained in all systems, independent from the type of trapping chemical and oxidation. In OPD incubations, glucosone quinoxaline formation was significantly reduced under nonoxidative conditions. Although the glucosone triazines levels found with AG were extremely small, they still corresponded positively to oxidative conditions (0.18 vs 0.34 mmol/mol; at 168 h). As expected, no 1-DG triazine was detected in AG reaction mixtures in contrast to OPD incubations. 1-DG quinoxaline quantities doubled under deaeration, which must be explained by reduced oxidative degradation of this sensible reductone structure. In both systems, the small amounts of glyoxal derivatives observed did not correspond to oxidative conditions (OPD, 0.27 vs 0.30 mmol/mol; AG, 0.18 vs 0.21 mmol/mol; at 168 h). Methylglyoxal triazine levels in AG incubations were more than doubled under aerated conditions, matching exactly the same ratio found in OPD incubations at much lower concentrations (0.21 vs 0.51 mmol/mol; at 168 h). In contrast to the literature,

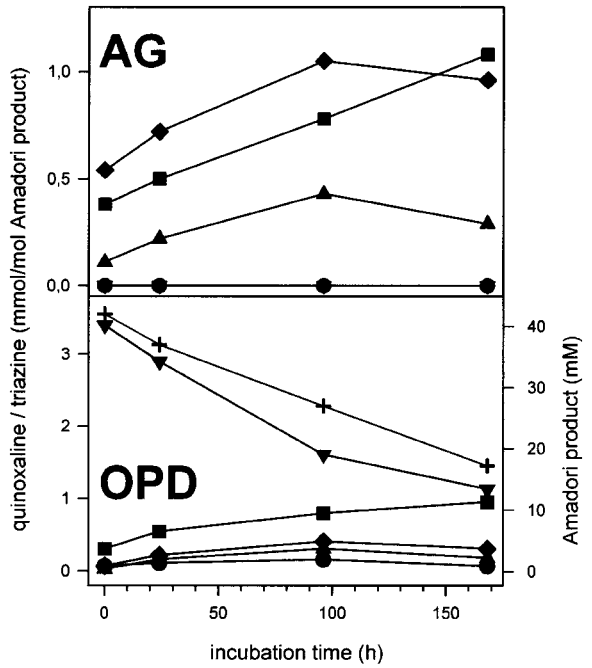

Figure 7. Formation of α-dicarbonyl structures in incubations of the Amadori product (42 mM) of glucose and N^{α}-*t*-BOC-lysine under aerated conditions. Target structures [from glyoxal (▲), methylglyoxal (◆), 1-DG (●), 3-DG (■), and glucosone (▼)] were monitored as the corresponding triazines/quinoxalines by addition of AG and OPD (both 5 mM) at different time points. Samples were worked up after 5 h of additional incubation. Degradation of the Amadori product was followed after reduction as the hexitol derivative (+).

this clearly indicates the involvement of oxidative pathways in the formation of methylglyoxal during Maillard reactions.

To eliminate the various influences of the trapping reagents, they were added to incubations only at the various time points of sampling (Figure 7). The reaction mixtures were then reincubated for an additional 5 h to allow sufficient time for condensation prior to workup. Trapping reagents were also added at 0 h to obtain information of how the specific chemical influences the system during the 5 h reincubation time. If the respective "0 h level" is taken as a virtual baseline, the actual dicarbonyl concentration can be estimated by subtracting the "0 h level" from the values determined at the different incubation periods. The "0 h level" time point again visualized the main differences between the trapping reagents. 1-DG was not detected in incubations followed by AG derivatization, whereas with OPD a concentration maximum was observed at 96 h (0.09 mmol/mol). Also at 96 h the maxima for glyoxal and methylglyoxal were obtained with similar concentrations found in both systems (glyoxal, 0.32 vs 0.28 mmol/mol; methylglyoxal, 0.51 vs 0.34 mmol/mol; AG to OPD). Glyoxal, methylglyoxal, and 1-DG represent highly reactive intermediates, and the levels detected will be the result of two competing reactions: synthesis, which is dependent on the Amadori product concentration, and amine-catalyzed degradation. In contrast, 3-DG represents a fairly stable intermediate and thus accumulated in the reaction system as already shown above (cf. Figure 4). Although the raw data values for glyoxal and 3-DG were almost identical for both trapping methods, the presence of AG significantly enhanced the formation of methylglyoxal triazines from the Amadori product at all time points (e.g., 0.07 vs 0.54 mmol/mol at 0 h). This means that the high levels of methylglyoxal triazines were generated mainly by direct interaction of AG with the sugar and not by Maillard-induced degradation. However, as in both trapping systems similar concentration maxima were reached within identical time frames, methylglyoxal was still verified as a Maillard degradation product of the Amadori product. With AG, absolutely no glucosone formation was detected within 168 h of incubation. This might be explained in part, but not totally, by the limited condensation rate. In contrast, values were dramatically high in incubations monitored by OPD (e.g., 3.4 mmol/mol at 0 h) and dropped by 67% within 168 h. Almost in parallel, the concentration of the Amadori product declined by 59%. Taken together, these data unequivocally proved that for this specific incubation system most, if not all, glucosone formed is an artifact based on direct interaction of the Amadori product with the trapping reagent. Kawakishi et al. (*1*) have published glucosone as a main degradation product of the Amadori product in the presence of high amounts of copper metal ions. However, in the reaction system used in this study, only trace amounts of transition metal ions might result from the phosphate buffer used and thereby explain the lack of glucosone formation during the Maillard-catalyzed Amadori product degradation. This is also supported by investigations of Zyzak et al. (*2*), who alternatively used reduction to the corresponding sugar alcohols for the detection of dicarbonyl and other sugar degradation structures in comparable Amadori compound reaction systems.

In conclusion, this study clearly elucidated the problems associated with trapping reagents as the main tools for the detection of α-dicarbonyl compounds in Maillard reaction systems and in vivo. Although the results were obtained for AG in comparison to OPD, they should be applicable to all trapping reagents based on their common feature of electron-rich substituents such as amino functions to allow condensation with the carbonyl moiety. The detection of highly reactive dicarbonyl compounds such as 1-DG is limited by the rate of condensation. In the case of AG this can lead to false negative results. The problem can be eliminated by the use of aromatic *o*-diamines, which boast very fast condensation rates due to the favorable formation of conjugated aromatic ring structures. However, especially these trapping reagents impose high oxidative stress on the system investigated. This enhances dramatically the formation of α-dicarbonyl compounds, which are generated by oxidative pathways as shown by false positive detection of high amounts of glucosone during the degradation of Amadori products under physiological conditions. Trapping reagents can also facilitate retro-aldol fragmentation of higher sugars, leading, for example, to significantly higher amounts of methylglyoxal detectable with AG. And, finally, the wide range in polarity of the trapped derivatives calls for extremely optimized workup procedures. It became clear that for exact qualitative and quantitative statements on α-dicarbonyl structures formed in Maillard reaction systems, detailed studies on the respective trapping reaction are needed. This absolutely requires the availability of both the native α-dicarbonyl and its corresponding derivative. As this is not the case for most of the structures published so far, those results need to be reconsidered very carefully. The same considerations are also warranted for the detection of α-dicarbonyl

compounds formed in vivo. Thus, glyoxal, methylglyoxal, and 3-DG might not be the only major structures present in living systems.

LITERATURE CITED

(1) Kawakishi, S.; Tsunehiro, J.; Uchida, K. Autoxidative degradation of Amadori compounds in the presence of copper ion. *Carbohydr. Res.* **1991**, *211*, 167−171.

(2) Zyzak, D. V.; Richardson, J. M.; Thorpe, S. R.; Baynes, J. W. Formation of reactive intermediates from Amadori compounds under physiological conditions. *Arch. Biochem. Biophys.* **1995**, *316*, 547−554.

(3) De Revel, G.; Bertrand, A. A method for the detection of carbonyl compounds in wine: glyoxal and methylglyoxal. *Sci. Food Agric.* **1993**, *61*, 267−272.

(4) Severin, T.; Hiebl, J.; Popp-Ginsbach, H. Untersuchungen zur Maillard-Reaktion. *Z. Lebensm. Unters. Forsch.* **1984**, *178*, 284−287.

(5) Wells-Knecht, K. J.; Zyzak, V. D.; Litchfield, J. E.; Thorpe, S. R.; Baynes, J. W. Mechanism of autoxidative glycosylation: identification of glyoxal and arabinose as intermediates in the autoxidative modification of proteins by glucose. *Biochemistry* **1995**, *34*, 3702−3709.

(6) Hayashi, T.; Shibamoto, T. Analysis of methylglyoxal in foods and beverages. *J. Agric. Food Chem.* **1985**, *33*, 1090−1093.

(7) McLellan, A. C.; Phillips, S. A.; Thornalley, P. J. The assay of methylglyoxal in biological systems by derivatization with 1,2-diamino-4,5-dimethoxybenzene. *Anal. Biochem.* **1992**, *206*, 17−23.

(8) Hirsch, J.; Petrakova, E.; Feather, M. S. The reaction of some dicarbonyl sugars with aminoguanidine. *Carbohydr. Res.* **1992**, *232*, 125−130.

(9) Niyati-Shirkhodaee, F.; Shibamoto, T. Gas chromatographic analysis of glyoxal and methylglyoxal formed from lipids and related compounds upon ultraviolet irradiation. *J. Agric. Food Chem.* **1993**, *41*, 227−230.

(10) Yamaguchi, M.; Ishida, J.; Xuan-Xuan, Z.; Nakamura, M.; Yoshitake, T. Determination of glyoxal, methylglyoxal, diacetyl, and 2,3-pentandione in fermented foods by high-performance liquid chromatography with fluorescence detection. *J. Liq. Chromatogr.* **1994**, *17*, 203−211.

(11) Nedvidek, W.; Ledl, F.; Fischer, P. Detection of 5-hydroxymethyl-2-methyl-3(2*H*)-furanone and α-dicarbonyl compounds in reaction mixtures of hexoses and pentoses with different amines. *Z. Lebensm. Unters. Forsch.* **1992**, *194*, 222−228.

(12) Hirsch, J.; Mossine, V. V.; Feather, M. S. The detection of some dicarbonyl intermediates arising from the degradation of Amadori compounds (the Maillard reaction). *Carbohydr. Res.* **1995**, *273*, 171−177.

(13) Odani, H.; Shinzato, T.; Matsumoto, Y.; Usami, J.; Maeda, K. Increase in three α,*β*-dicarbonyl compound levels in human uremic plasma: specific in vivo determination of intermediates in advanced Maillard reaction. *Biochem. Biophys. Res. Commun.* **1999**, *256*, 89−93.

(14) Araki, A.; Glomb, M. A.; Takahashi, M.; Monnier, V. M. Determination of dicarbonyl compounds as aminotriazines during the Maillard reaction and in vivo detection in aminoguanidine-treated rats. In *The Maillard Reaction in Foods and Medicine*; O'Brien, J., Nursten, H. E., Crabbe, M. J., Ames, J. M., Eds.; RSC Publishing: Cambridge, U.K., 1998; p 400.

(15) Madson, M. A.; Feather, M. S. An improved preparation of 3-deoxy-D-*erythro*-hexos-2-ulose via the bis(benzoylhydrazone) and some related constitutional studies. *Carbohydr. Res.* **1981**, *94*, 183−191.

(16) Glomb, M. A.; Pfahler, C. Synthesis of 1-deoxy-(D-*erythro*)-2,3-hexodiulose, a major hexose Maillard intermediate. *Carbohydr. Res.* **2000**, *329*, 515−523.

(17) Bayne, S. Aldosuloses (osones): from phenylosazones. *Methods Carbohydr. Chem.* **1963**, *2*, 421−423.

(18) Glomb, M. A.; Monnier, V. M. Mechanism of protein modification by glyoxal and glycolaldehyde, reactive intermediates of the Maillard reaction. *J. Biol. Chem.* **1995**, *270*, 10017−10026.

(19) Erikson, J. G. 3-Amino-as-triazines. *J. Am. Chem. Soc.* **1952**, *74*, 4706.

(20) Lo, T. W. C.; Selwood, T.; Thornalley, P. J. The reaction of methylglyoxal with aminoguanidine under physiological conditions and prevention of methylglyoxal binding to plasma proteins. *Biochem. Pharmacol.* **1994**, *48*, 1865−1870.

(21) Hirsch, J.; Petrakova, E.; Feather, M. S. Reaction of some 1-deoxy-2,3-dicarbonyl hexose derivatives with aminoguanidine (guanylhydrazine). *J. Carbohydr. Chem.* **1995**, *14*, 1179−1186.

(22) Morita, N.; Mitzutani, M.; Hayashi, K.; Kirihata, M.; Ichimoto, I.; Ueda, H.; Takagi, M. Quinoxalines derived from d-glucose and maltose with *o*-phenylenediamine under refluxed conditions in alkaline media. *Bull. Univ. Osaka Pref.* **1983**, *35*, 59−70.

(23) Morita, N.; Inoue, K.; Takagi, M. Quinoxalines derived from D-glucose and *o*-phenylenediamine in a weakly acidic medium. *Agric. Biol. Chem.* **1981**, *45*, 2665−2668.

(24) Thornalley, P. J.; Yurek-George, A.; Argirov, O. K. Kinetics and mechanism of the reaction of aminoguanidine with the α-oxoaldehydes glyoxal, methylglyoxal, and 3-deoxyglucosone under physiological conditions. *Biochem. Pharmacol* **2000**, *60*, 55−65.

(25) Hayashi, T.; Mase, S.; Namiki, M. Formation of three-carbon sugar fragment at an early stage of the browning reaction of sugar with amines or amino acids. *Agric. Biol. Chem.* **1986**, *50*, 1959−1964.

(26) Velisek, J.; Davidek, T.; Davidek, J. 1,3-Disubstituted imidazoles in the glucose-glycine Maillard reaction. *Lebensm. Wiss. Technol.* **1992**, *25*, 74−76.

(27) Örsi, F.; Homoki, P.; Phong, V. N. Bestimmung von Methylglyoxal in Karamel-Proben. *Nahrung* **1995**, *39*, 90−97.

(28) Hughues-Despointes, A.; Yaylayan, K. A. Retro-aldol and redox reactions of Amadori compounds: mechanistic studies with variously labeled D-(^{13}C)glucose. *J. Agric. Food Chem.* **1996**, *44*, 672−681.

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