DOI:10.1021/jf100334r

JOURNAL OF AGRICULTURAL AND FOOD CHEMISTRY
ARTICLE

Degradation of 1-Deoxy-D-*erythro*-hexo-2,3-diulose in the Presence of Lysine Leads to Formation of Carboxylic Acid Amides

MAREEN SMUDA, MICHAEL VOIGT, AND MARCUS A. GLOMB*

Institute of Chemistry, Food Chemistry, Martin-Luther-University Halle-Wittenberg, Kurt-Mothes-Strasse 2, 06120 Halle/Saale, Germany

A novel species of amides formed from degradation of one of the most important key intermediates in Maillard hexose chemistry−1-deoxyhexo-2,3-diulose−was investigated. In 1-deoxyhexo-2,3-diulose/N^{α}-*t*-BOC-lysine reaction mixtures four amides, N^{ε}-acetyl lysine, N^{ε}-formyl lysine, N^{ε}-lactoyl lysine and N^{ε}-glycerinyl lysine, were identified and their structures verified by authentic reference standards. Amides and corresponding carboxylic acids (acetic acid, formic acid, lactic acid and glyceric acid) accumulated over time. Both N^{ε}-lysine amides and carboxylic acids were thus determined as stable Maillard end products. Results of model incubations suggested the synthesis of amides to be mechanistically closely related to the formation of their corresponding carboxylic acids by β-dicarbonyl cleavage. Due to the different chemical properties of all the compounds monitored, various analytical strategies had to be carried out (LC−MS^2, GC−MS, GC−FID, enzymatic determination).

KEYWORDS: Maillard reaction; β-dicarbonyl cleavage; 1-deoxyhexo-2,3-diulose; 1-DG; N^{ε}-lysine amides; carboxylic acid amides; carboxylic acids

INTRODUCTION

During heating or storing of food, the Maillard reaction leads to degradation of reducing sugars and amino acids, peptides or proteins (*1*). In addition to the formation of color, taste and flavor, the Maillard reaction results in a decline of the biological value of proteins caused by reactions of the ε-amino group of lysine (*2*). In the past decades the outstanding role of α-dicarbonyl compounds as intermediates of high reactivity in the complex Maillard chemistry was manifested. Among these intermediates 1-deoxyhexo-2,3-diulose (1-DG) is of major importance in hexose chemistry (*3*−*5*). Since Glomb and Pfahler succeeded in the synthesis of 1-DG, it is possible to study carbohydrate Maillard degradation mechanistically isolated on this intermediate (*6*). Davidek et al. postulated a formation pathway of acetic acid and glyceric acid that can be explained by a hydrolytic β-dicarbonyl cleavage mechanism starting from 1-DG (*7*, *8*). In our study we now investigated a novel type of Maillard reaction products: carboxylic acid amides formed by the ε-amino group of lysine during degradation of 1-DG. In the literature, formation of amides in Maillard chemistry is only scarcely described. In 1985 Hayase and co-workers investigated the formation of *N*-butylacetamide and *N*-butylformamide in model reaction systems of butylamine and the α-dicarbonyl structures diacetyl and glyoxal (*9*). Several α-hydroxy acid amides such as lactic acid propylamide have been identified in reaction mixtures of glucose and propylamine (*10*), but it is still unknown if lysine side chains can react in a similar way. Nagaraj et al. studied the formation of oxalate monoalkylamide in the human lens in 1999 (*11*). In 2001 Glomb and Pfahler postulated the formation pathway of GOLA (N^{ε}-{2-[(5-amino-5-carboxypentyl)amino]-2-oxoethyl}lysine) and GALA (N^{ε}-glycoloyllysine) in glyoxal/lysine reaction mixtures. With the detection of GOLA and GALA in brunescent lens proteins the importance of these amide protein modifications with respect to pathological processes *in vivo* was demonstrated (*12*). N^{ε}-Formyl lysine was already mentioned in the literature as a glycation product of proteins, but a formation mechanism was not given (*13*).

The present work extends the knowledge of amide formation in Maillard reactions. The four amides N^{ε}-acetyl lysine, N^{ε}-formyl lysine, N^{ε}-lactoyl lysine, and N^{ε}-glycerinyl lysine as well as the corresponding carboxylic acids acetic acid, formic acid, lactic acid, and glyceric acid were found to be formed in 1-DG/N^{α}-*t*-BOC-lysine reaction mixtures. Quantification of the compounds was done by various analytical methods to gain further insights into the degradation mechanisms of 1-DG.

MATERIALS AND METHODS

Materials. The following chemicals of analytical grade were commercially available: *N*,*O*-bis(trimethylsilyl)acetamide with 5% trimethylchlorosilane, acetic acid, heptafluorobutyric acid (Fluka/Sigma-Aldrich, Seelze, Germany), ninhydrin, dipotassium hydrogen phosphate trihydrate, potassium dihydrogen phosphate (Merck, Darmstadt, Germany), decylchloroformate, *N*-ethyl-*N*′-(3-dimethylaminopropyl)carbodiimide (EDC), 2,3-*O*-isopropylidene-D-glyceric acid methyl ester, D-glyceric acid calcium salt, N^{α}-*t*-BOC-lysine, N^{ε}-acetyl-L-lysine, N^{ε}-formyl-L-lysine (Sigma-Aldrich, Steinheim, Germany), 1-hydroxybenzotriazole (HOBt), pyridine, thioanisole,

*To whom correspondence should be addressed. E-mail: marcus.glomb@chemie.uni-halle.de. Fax: ++049-345-5527341.

 Published on Web 04/29/2010 © 2010 American Chemical Society

diethylenetriaminepentaacetic acid (Fluka/Sigma-Aldrich, Taufkirchen, Germany), TFA (Carl Roth, Karlsruhe, Germany), Dowex 50W × 8 (H^+-form, 50−100 mesh) (Serva/Boehringer, Ingelheim-Heidelberg, Germany), L-lactic acid (Alfa Aesar, Karlsruhe, Germany), and formic acid (Grüssing GmbH, Filsum, Germany). 1-Deoxy-4,5-*O*-isopropylidene-D-*erythro*-hexo-2,3-diulose and 1-deoxy-D-*erythro*-hexo-2,3-diulose (*6*), N^{α}-(*t*-butoxycarbonyl)-L-lysine *tert*-butyl ester (**1**) (*14*), and O^{α}-tetrahydropyranyl-L-lactic acid (**2**) (*15*) were synthesized according to the literature.

N^{α}-(*t*-Butoxycarbonyl)-N^{ε}-(O^{α}-(tetrahydropyranyl)-L-lactoyl)-L-lysine *tert*-Butyl Ester (3). **2** (70 mg, 0.4 mmol) was dissolved in CH_2Cl_2 (2 mL) under argon atmosphere at 0 °C, and HOBt (54 mg, 0.4 mmol) was added. After 10 min a solution of EDC (80 μL, 0.45 mmol) in CH_2Cl_2 (1 mL) was added dropwise. To the stirred solution, **1** (120 mg, 0.4 mmol) dissolved in CH_2Cl_2 (1 mL) was added dropwise. The reaction mixture was warmed to room temperature and stirred overnight. Then, the mixture was diluted with CH_2Cl_2 (10 mL) and washed with 15 mL each of saturated $NaHCO_3$ solution and brine. The organic layer was dried over $MgSO_4$, and the solvent was removed under reduced pressure. The crude product was purified by column chromatography on silica gel 60 using EtOAc−hexane (1:1). Fractions containing **3** (TLC: R_f 0.83 in EtOAc, ninhydrin detection) were collectively concentrated in vacuo to afford compound **3** as a yellow viscous oil (90 mg, 49%). ^{1}H NMR (400 MHz, $CDCl_3$): δ [ppm] = 1.42−1.82 (m, 12 H), 1.31 (d, ^3J = 6.8 Hz, 3H), 1.38 (s, 9H), 1.39 (s, 9H), 3.13−3.24 (m, 2H), 3.41−3.49 and 3.75−3.86 (m, 2H), 4.02−4.18 (m, 2H), 4.52−4.57 (m, 1H). ^{13}C NMR (100 MHz, $CDCl_3$): δ [ppm] = 17.9, 19.6/19.9, 20.4/22.6, 25.1/25.2, 28.0, 28.4, 29.3/29.4, 31.0, 32.5, 38.6, 53.7/53.8, 62.8/64.0, 73.3/74.3, 79.5, 81.7, 98.4/98.9, 155.4, 171.7/171.8, 173.0/173.3. HR-MS: *m*/*z* 481.2884 (found); *m*/*z* 481.2886 (calculated for $C_{23}H_{42}O_7N_2Na$ $[M + Na]^+$).

N^{ε}-L-Lactoyl-L-lysine Trifluoroacetate (4). To **3** (66 mg, 0.14 mmol) a solution of 25% thioanisole in TFA (8 mL) was added. The reaction mixture was stirred at room temperature for 6 h under argon atmosphere while the color of the solution changed from green to brown. The mixture was concentrated in vacuo, and the residue was partitioned between 20 mL each of diethyl ether and water. The organic layer was further extracted with water (10 mL). The combined aqueous layers were concentrated under reduced pressure to a final volume of 3 mL. The crude product was purified by column chromatography (Lichroprep RP C18 material). The column was pre-equilibrated with methanol (50 mL) followed by 0.1% TFA in water (50 mL). The product was eluted with 0.1% TFA in water. Fractions containing **4** (positive ninhydrin stain) were collectively lyophilized to give a colorless glassy foam in quantitative yield. ^{1}H NMR (400 MHz, D_2O): δ [ppm] = 1.23 (d, ^3J = 6.8 Hz, 3H), 1.25−1.40 (m, 2H), 1.42−1.52 (m, 2H), 1.75−1.95 (m, 2H), 3.09−3.16 (m, 2H), 3.90−3.95 (m, 1H), 4.10 (q, ^3J = 6.6 Hz, 1H). ^{13}C NMR (100 MHz, D_2O): δ = 19.8, 21.6, 28.0, 29.5, 38.4, 53.0, 67.8, 172.2, 177.4. HR-MS: *m*/*z* 219.1339 (found); *m*/*z* 219.1335 (calculated for $C_9H_{19}O_4N_2$ $[M + H]^+$).

2,3-*O*-Isopropylidene-glyceric Acid (6). To a solution of KOH (95 mg, 1.7 mmol) in 250 μL of water and 500 μL of absolute EtOH, 2,3-*O*-isopropylidene-D-glyceric acid methyl ester (**5**) (160 mg, 1 mmol) was added. The reaction mixture was stirred 30 min at room temperature. After adjusting the pH value to 4.3 by using HCl (1 M), solution was extracted with EtOAc (5 × 3 mL), and the combined organic layers were dried ($MgSO_4$) and concentrated to give 62 mg (42%) of a colorless oil. ^{1}H NMR (400 MHz, $CDCl_3$): δ [ppm] = 1.38 (s, 3H), 1.48 (s, 3H), 4.14 (dd, ^3J = 5.0 and ^2J = 8.9 Hz, 1H), 4.25 (dd, ^3J = 7.5 and ^2J = 8.7 Hz, 1H), 4.60 (dd, ^3J = 5.0 and ^3J = 7.5 Hz, 1H), 9.54 (br, 1H). ^{13}C NMR (100 MHz, $CDCl_3$): δ [ppm] = 25.3, 25.8, 67.2, 73.6, 111.8, 175.8.

N^{α}-(*t*-Butoxycarbonyl)-N^{ε}-2,3-*O*-*isopropylidene-glycerinyl*-L-lysine *tert*-Butyl Ester (7). **6** was reacted in an equimolar ratio with HOBt and **1** and with a 1.1-fold excess of EDC as described above for **3**. Purification was done by column chromatography (silica gel 60, EtOAc−hexane 1:1). Fractions containing **7** (TLC: R_f 0.87 in EtOAc, ninhydrin detection) were collectively concentrated in vacuo to afford **7** as a yellow viscous oil (105 mg, 61%). ^{1}H NMR (400 MHz, $CDCl_3$): δ [ppm] = 1.24−1.44 (m, 2H), 1.33 (s, 3H), 1.38 (s, 9H), 1.40 (s, 9H), 1.42 (s, 3H), 1.46−1.60 (m, 3H), 1.70−1.80 (m, 1H), 3.21 (m, 2H), 4.01 (dd, ^3J = 5.4 and ^2J = 8.7 Hz, 1H), 4.03−4.13 (m, 1H) 4.21 (dd, ^3J = 7.7 and ^2J = 8.7 Hz, 1H), 4.40 (dd, ^3J = 5.4 and ^3J = 7.5 Hz, 1H). ^{13}C NMR (100 MHz, $CDCl_3$): δ [ppm] = 22.5, 25.0, 26.1, 28.0, 28.3, 29.2, 32.5, 38.6, 53.7, 67.7, 75.0, 79.5, 81.7, 110.7, 155.2, 170.9, 171.6. HR-MS: *m*/*z* 453.2571 (found); *m*/*z* 453.2570 (calculated for $C_{21}H_{38}O_7N_2Na$ $[M + Na]^+$).

N^{ε}-Glycerinyl-L-lysine Trifluoroacetate (8). Deprotection and purification of **7** was done as described above for compound **4**. **8** was obtained as a colorless glassy foam in quantitative yield after lyophilization. ^{1}H NMR (400 MHz, D_2O): δ [ppm] = 1.25−1.39 (m, 2H), 1.42−1.49 (m, 2H), 1.76−1.95 (m, 2H), 3.15 (t, ^3J = 6.6 Hz, 2H), 3.62−3.71 (m, 2H), 3.93 (t, ^3J = 6.0 Hz, 1H), 4.07−4.12 (m, 1H). ^{13}C NMR (100 MHz, D_2O): δ [ppm] = 21.6, 28.0, 29.5, 38.6, 53.1, 63.4, 72.4, 172.3, 174.2. HR-MS: *m*/*z* 235.1288 (found); *m*/*z* 235.1286 (calculated for $C_9H_{19}O_5N_2$ $[M + H]^+$).

Degradation of 1-Deoxyhexo-2,3-diulose. A solution of 1-deoxy-4,5-*O*-isopropylidene-D-*erythro*-hexo-2,3-diulose (0.35 mmol) in water (3 mL) was stirred with Dowex 50W × 8 (H^+-form, 50−100 mesh, 4 mL) for 4 h under argon atmosphere. The resin was filtered off and washed with MeOH (3 × 2 mL). After evaporation of the combined solvents, the residue was dissolved in phosphate buffer (0.1 M, pH 7.4, 4.2 mL). Aliquots (150 μL) of this solution and a solution of N^{α}-*t*-BOC-lysine (0.35 mmol) in phosphate buffer (0.1 M, pH 7.4, 4.2 mL) were mixed in screw-cap vials giving an incubation solution of 1-DG and N^{α}-*t*-BOC-lysine (42 mM, respectively). Incubation solutions were shaken at 37 °C, and samples were taken over time.

Amides were analyzed by HPLC−MS^2 after deprotection of the BOC group. Lactic and glyceric acid were analyzed by GC−MS after silylation, acetic acid by GC-FID as its decylchloroformate derivative and formic acid by enzymatic determination. Each sample was prepared at least three times.

Deaerated Incubations. Degradation of 1-deoxy-D-*erythro*-hexo-2,3-diulose under deaerated conditions was carried out using phosphate buffer with 1 mM diethylenetriaminepentaacetic acid. Buffer was degassed with helium before samples were prepared; samples were deaerated with argon before incubation.

Deprotection Reaction of the Amides. To aliquots (100 μL) of the incubation solutions 6 M HCl (100 μL) was added, and samples were kept at room temperature for 30 min. Solutions were diluted on a scale of 1:100 with water prior to injection into the HPLC−MS^2 system.

Control Experiment. Carboxylic acids (acetic acid, lactic acid, glyceric acid and formic acid, 13 mM, respectively) were separately incubated with N^{α}-*t*-BOC-lysine (42 mM) in phosphate buffer (0.1 M, pH 7.4). Incubation solutions were shaken at 37 °C, and samples were taken after 7 days. Further workup was performed analogous to the amide samples prior to injection into the HPLC−MS^2 system.

Derivatization Reactions for the Carboxylic Acids. *Trimethylsilyl Derivatives of Lactic Acid and Glyceric Acid.* Adopting the method described in ref *16*, aliquots of the samples (50 μL) were dried in vacuo, residues were dissolved in pyridine (50 μL), and *N*,*O*-bis(trimethylsilyl)-acetamide with 5% trimethylchlorosilane (50 μL) was added. Samples were kept 3 h at room temperature prior to injection into the GC−MS system. Quantification was carried out by comparison of peak areas obtained in the TIC with those of standard solutions containing known amounts of the pure authentic reference compounds. Signals of target compounds were standardized using the signal of silylated phosphoric acid present in all samples. Data for silylated compounds obtained by GC−MS showed standard deviations of < 5 mmol/mol 1-DG, resulting in coefficients of variation < 5%.

Decylchloroformate Derivative of Acetic Acid. Adopting the method described in ref *17*, samples (300 μL) were spiked with chlorosuccinic acid (50 μg) dissolved in water as internal standard, pyridine (40 μL) and decylchloroformate (50 μL) were added. The mixture was then sonicated for 10 min. Decyl esters were extracted with hexane, and the organic layer was analyzed by GC−FID. Quantitative results were obtained by internal calibration using commercially available acetic acid. Data obtained by GC−FID showed standard deviations of < 10 mmol/mol 1−DG, resulting in coefficients of variation < 3.5%.

Analytical HPLC−MS^2. A Jasco PU−2080 Plus quaternary gradient pump with degasser and Jasco AS-2057 Plus autosampler (Jasco, Gross-Umstadt, Germany) were used. Chromatographic separations were performed on a stainless steel column (VYDAC 218TP54, 250 × 4.6 mm, RP18, 5 μm, Hesperia, CA) using a flow rate of 1 mL/min. The mobile phase used consisted of water (solvent A) and MeOH/water (7:3 (v/v), solvent B). To both solvents (A and B), 1.2 mL/L heptafluorobutyric acid (HFBA) was added. Samples were injected at 2% B (held 25 min), gradient then changed to 100% B in 5 min (held 10 min) and then changed to 2% B in 5 min (held 15 min). Elution of amides (N^{ε}-glycerinyl lysine at 7.0 min,

Table 1. Mass Spectrometer Parameters for Amide Quantification (MRM Mode)

	Q1 mass (amu)	Q3 mass (amu)	dwell time (ms)	DP	CE	CXP
N^{ε}-acetyl lysine	189.20	126.10	75.00	30.00	18.00	10.00
N^{ε}-formyl lysine	175.10	112.10	75.00	25.00	20.00	13.00
N^{ε}-lactoyl lysine	219.20	156.20	75.00	32.00	20.00	8.00
N^{ε}-glycerinyl lysine	235.30	84.20	75.00	48.00	37.00	6.00

N^{ε}-formyl lysine at 9.3 min, N^{ε}-acetyl lysine at 13.6 min, and N^{ε}-lactoyl lysine at 13.8 min) was monitored by mass detection. The mass analyses were performed using an Applied Biosystems API 4000 quadrupole instrument (Applied Biosystems, Foster City, CA) equipped with an API source using an electrospray ionization (ESI) interface. The LC system was connected directly to the probe of the mass spectrometer. Nitrogen was used as sheath and auxiliary gas. To measure the amides in incubation solutions the MRM mode of HPLC−MS^2 was used. The optimized parameters for mass spectrometry are given in **Table 1**. Quantification was based on the standard addition method using solutions containing known amounts of the pure authentic reference compounds. Data for amides obtained by HPLC−MS^2 showed standard deviations of < 0.07 mmol/mol 1-DG, resulting in coefficients of variation < 5%.

GC−MS. Samples were analyzed on a Thermo Finnigan Trace GC Ultra coupled to a Thermo Finnigan Trace DSQ (both Thermo Fisher Scientific GmbH, Bremen, Germany). The GC column was a HP-5 (30 m × 0.32 mm, film thickness 0.25 μm; Agilent Technologies, Palo Alto, CA); injector, 220 °C; split ratio, 1:30; transfer line, 250 °C. The oven temperature program was as follows: 100 °C (0 min), 5 °C/min to 200 °C (0 min), 10 °C/min to 270 °C (10 min). Helium 5.0 was used as carrier gas in constant flow mode (linear velocity 28 cm/s, flow 1 mL/min). Mass spectra were obtained with EI at 70 eV (source: 210 °C) in full scan mode (mass range m/z 50−650).

GC−FID. Samples were analyzed on a HP 6890N chromatograph (Agilent Technologies, Palo Alto, CA) equipped with a flame ionization detector. The column was a HP-5 (30 m × 0.32 mm, film thickness 0.25 μm; Agilent Technologies, Palo Alto, CA); injector, 250 °C; split ratio, 1:10; detector, 270 °C. Helium 4.6 was used as carrier gas in constant flow mode (linear velocity 28 cm/s, flow 1.6 mL/min). The oven temperature program was as follows: 50 °C (1 min), 4 °C/min to 120 °C (15 min), 40 °C/min to 220 °C (3 min), 30 °C/min to 260 °C (15 min).

Nuclear Magnetic Resonance Spectroscopy (NMR). NMR spectra were recorded on a Varian VXR 400 spectrometer operating at 400 MHz for ^{1}H and 100 MHz for ^{13}C, respectively. Chemical shifts are given relative to external $SiMe_4$.

Accurate Mass Determination (HR−MS). The high-resolution positive ion ESI mass spectra (HR-MS) were obtained from a Bruker Apex III Fourier transform ion cyclotron resonance (FT-ICR) mass spectrometer (Bruker Daltonics, Billerica, MA) equipped with an Infinity cell, a 7.0 T superconducting magnet (Bruker, Karlsruhe, Germany), a radio frequency (RF)-only hexapole ion guide, and an external electrospray ion source (Apollo; Agilent, off-axis spray). Nitrogen was used as drying gas at 150 °C. The samples were dissolved in methanol, and the solutions were introduced continuously via a syringe pump at a flow rate of 120 μL h^{-1}. The data were acquired with 256 k data points and zero filled to 1024 k by averaging 32 scans.

Electrospray Ionization Mass Spectrometry (ESI−MS^2). The mass analyses were performed using the instrument described above for HPLC−MS^2 analyses. The samples were dissolved in water, and the solutions were introduced continuously via a syringe pump at a flow rate of 120 μL h^{-1}. Spectra were obtained by using the following parameters: for N^{ε}-acetyl lysine (DP 50, CE 20, CXP 10), for N^{ε}-formyl lysine (DP 50, CE 20, CXP 10), for N^{ε}-lactoyl lysine (DP 55, CE 22, CXP 10), and for N^{ε}-glycerinyl lysine (DP 50, CE 24, CXP 10). In all cases 42 MCA scans were carried out.

Enzymatic Determination of Formic Acid. Quantification of formic acid in incubation solutions was carried out with an enzyme test kit obtained from R-Biopharm AG (Darmstadt, Germany). Depending on the amount of formic acid after different incubation times, varying volumes of incubation solutions were directly used for the enzyme test (100 to 500 μL). Coefficients of variation (< 2.5%) were in line with the test kit description.

RESULTS

Syntheses of Authentic Amide References. Lactic acid amide (**4**, N^{ε}-lactoyl lysine) was synthesized in a typical amide formation reaction (**Figure 1 a**), starting from lactic acid as the tetrahydropyranyloxy derivative (**2**). **2** was activated with EDC and HOBt and reacted with N^{α}-(*t*-butoxycarbonyl)-L-lysine *tert*-butyl ester (**1**) to give the fully protected amide **3**. In the last step all three protective groups were completely removed by treatment with thioanisole in TFA to result in the lactid acid amide **4**.

In a similar procedure glyceric acid amide (**8**, N^{ε}-glycerinyl lysine) was synthesized (**Figure 1 b**). After activation of isopropylidene protected glyceric acid (**6**) and reaction with **1** the protected amide **7** was isolated. Finally **7** was deprotected under acidic conditions to obtain **8**.

Both target compounds **4** and **8** as well as the intermediates **3**, **6**, and **7** were verified by nuclear magnetic resonance experiments. Furthermore the elemental composition of **3**, **4**, **7**, and **8** was confirmed by accurate mass determination.

Formation of Carboxylic Acids in 1-DG/N^{α}-*t*-BOC-lysine Incubations (pH 7.4, 37 °C). Degradation of 1-deoxyhexo-2,3-diulose (1-DG) in the presence of amine led to the formation of short chain carboxylic acids. Formation of formic, acetic, lactic and glyceric acid was monitored over a period of seven days. Results are shown in **Figure 2 A**. Within the first twelve hours a rapid accumulation of all carboxylic acids was observed. The further progress of the reaction was characterized by a marginal increase of the acids until concentrations remained unchanged. Formation of acetic acid led to the highest amounts after seven days (310 mmol/mol 1-DG), followed by glyceric acid (88 mmol/mol 1-DG), lactic acid (58 mmol/mol 1-DG) and formic acid (27 mmol/mol 1-DG).

Formation of Carboxylic Acid Amides in 1-DG/N^{α}-*t*-BOC-lysine Incubations (pH 7.4, 37 °C). Before incubation solutions could be analyzed for the corresponding carboxylic acid amides, samples were treated with acid to remove the BOC protection group. Amides were found to be stable under these workup conditions. A HPLC−MS^2 method (MRM mode) was developed to perform quantification of the amides in model incubation systems. Collision induced dissociation (CID) experiments were carried out to observe the fragmentation pattern of the different amides. **Table 2** demonstrates that all four amides underlie analogous fragmentation steps. Fragmentation of N^{ε}-lactoyl lysine as an exemplary representative of analyzed amides can be explained as follows: Based on QM^+ ion of N^{ε}-lactoyl lysine (m/z = 219) loss of HCOOH gives the ion at m/z 173. Loss of both HCOOH and ammonia at the α-amino group gives the ion at m/z 156 with the highest abundance. Ions at m/z 130 and m/z 84 are fragments that are prominent in the mass spectra of all four amides. m/z 84 presents the pyrrolinium ion. The ion at m/z 130 occurs after a cyclization step of N^{ε}-lactoyl lysine to a six-membered ring and loss of N^{ε}-functionality. All these fragmentations are similar to the fragmentation of ordinary lysine reported in refs *18* and *19*. Accurate mass determination of synthesized N^{ε}-lactoyl lysine gave m/z 173.1284 for $C_8H_{17}O_2N_2$ (calculated m/z 173.1281) and m/z 156.1019 for $C_{12}H_{14}O_2N$ (calculated m/z 156.1016) and confirmed the results obtained by CID experiments.

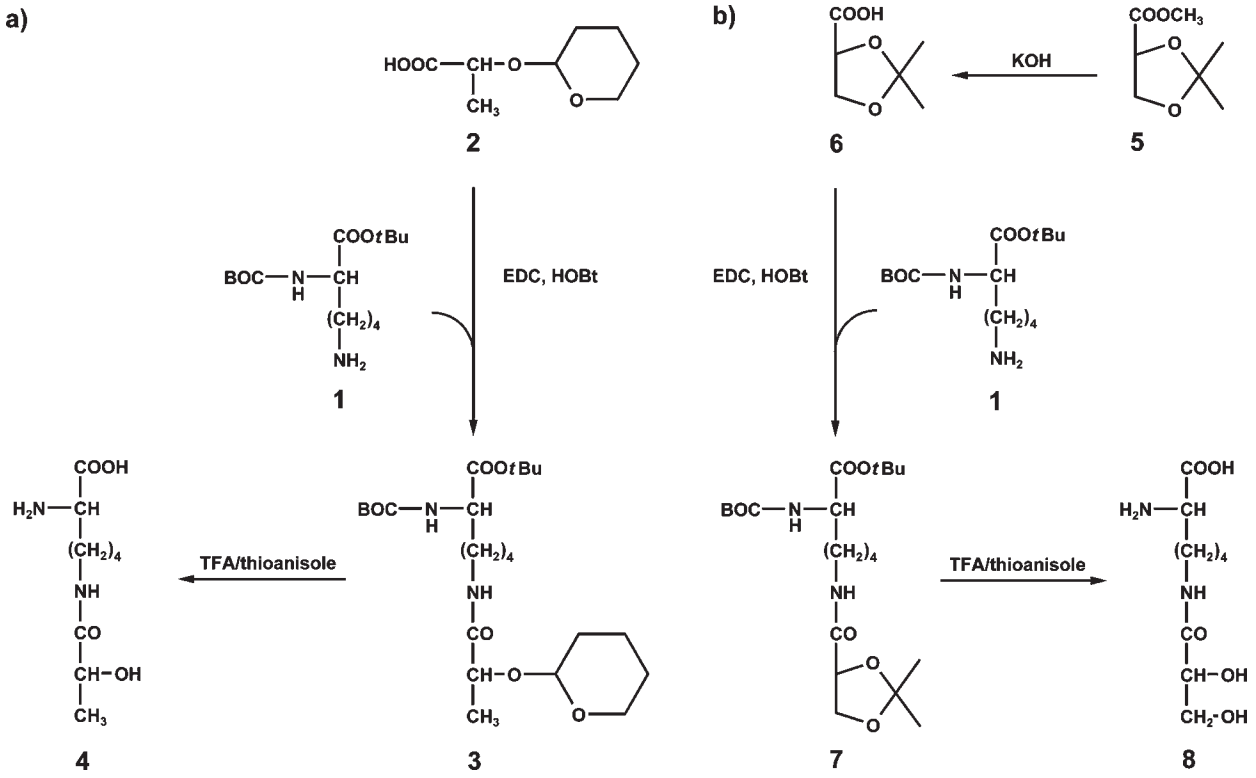

Figure 1. Synthesis of N^{ε}-lactoyl lysine and N^{ε}-glycerinyl lysine.

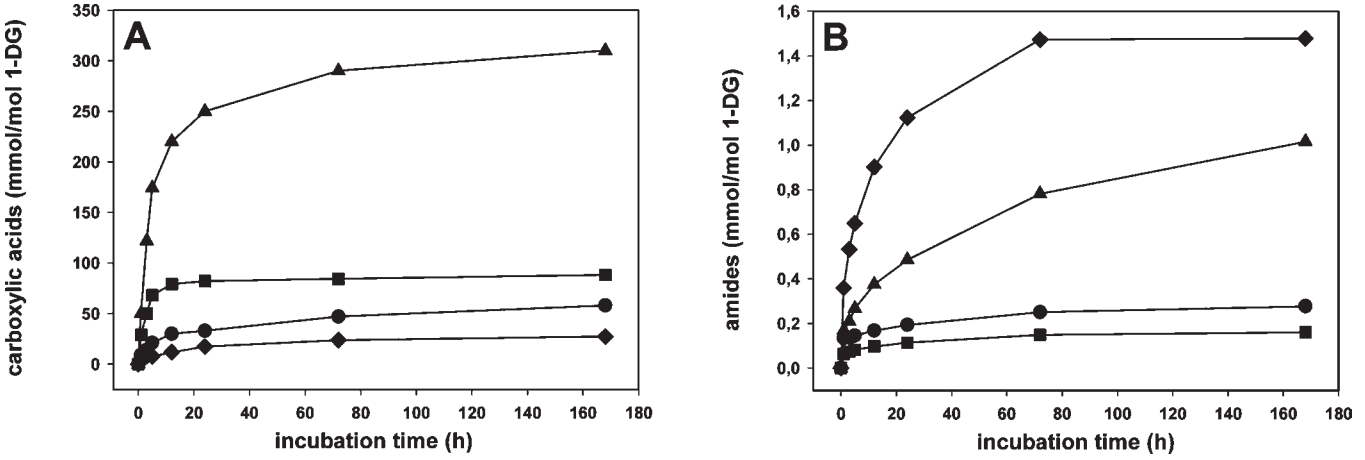

Figure 2. Formation of carboxylic acids (**A**) and corresponding amides (**B**) in 1-DG/N^{α}-*t*-BOC-lysine incubation mixtures (42 mM in phosphate buffer 0.1 M, pH 7.4, at 37 °C under aerated conditions): acetic acid/N^{ε}-acetyl lysine (▲), glyceric acid/N^{ε}-glycerinyl lysine (■), lactic acid/N^{ε}-lactoyl lysine (●) and formic acid/N^{ε}-formyl lysine (◆).

Table 2. Comparison of Fragmentation of the Different Amides in Positive Ion ESI-MS^2−CID Experiments

	fragmentation pattern, *m/z* (amu)				
	(M + H)	(M + H −HCOOH)	(M + H − HCOOH − NH_3)	(M + H − H_2N−CO−R)	pyrrolinium ion
N^{ε}-acetyl lysine	189	143	126	130[a]	84
N^{ε}-formyl lysine	175	129	112	130[b]	84
N^{ε}-lactoyl lysine	219	173	156	130[c]	84
N^{ε}-glycerinyl lysine	235	189	172	130[d]	84

[a] R = CH_3. [b] R = H. [c] R = CH(OH)−CH_3. [d] R = CH(OH)−CH_2OH.

Formation of N^{ε}-acetyl lysine, N^{ε}-formyl lysine, N^{ε}-lactoyl lysine, and N^{ε}-glycerinyl lysine was also monitored over a period of seven days. The time course formation plot of the carboxylic acid amides is shown in **Figure 2 B**. In parallel with the corresponding carboxylic acids there was a rapid increase of all amides within the first twelve hours. Also at later incubation times concentrations reached a steady level. Unexpectedly, formation of N^{ε}-formyl lysine led to the highest amounts after seven days

(1.48 mmol/mol 1-DG), followed by N^{ε}-acetyl lysine (1.01 mmol/mol 1-DG), N^{ε}-lactoyl lysine (0.28 mmol/mol 1-DG), and N^{ε}-glycerinyl lysine (0.16 mmol/mol 1-DG).

Table 3 provides information about concentrations of carboxylic acid amides formed under aerated and under deaerated incubation conditions at 37 °C after 24 and 72 h. Under aerated conditions we measured significant higher amounts of N^{ε}-glycerinyl lysine and of N^{ε}-formyl lysine. 0.15 mmol/mol 1-DG of N^{ε}-glycerinyl lysine and 1.47 mmol/mol 1-DG of N^{ε}-formyl lysine were formed after 72 h at 37 °C under aerated conditions in contrast to 0.09 mmol/mol 1-DG and 1.08 mmol/mol 1-DG under deaerated conditions, respectively. Concentrations of N^{ε}-acetyl lysine and of N^{ε}-lactoyl lysine under aerated versus under deaerated conditions varied only slightly. 0.78 mmol/mol 1-DG of N^{ε}-acetyl lysine and 0.25 mmol/mol 1-DG of N^{ε}-lactoyl lysine were obtained after 72 h at 37 °C under aerated conditions, in comparison to 0.75 mmol/mol 1-DG and 0.26 mmol/mol 1-DG under deaerated conditions, respectively.

Table 3. Comparison of Amide Concentrations Generated from 1-DG and N^{α}-*t*-BOC-lysine under Aerated and Deaerated Conditions

	concentration (mmol/mol 1-DG)			
	24 h		72 h	
	aerated	deaerated	aerated	deaerated
N^{ε}-acetyl lysine	0.48	0.45	0.78	0.75
N^{ε}-formyl lysine	1.12	0.71	1.47	1.08
N^{ε}-lactoyl lysine	0.19	0.16	0.25	0.26
N^{ε}-glycerinyl lysine	0.11	0.05	0.15	0.09

DISCUSSION

Carboxylic acids formed in Maillard reaction systems were already established to be stable degradation products (*7*, *20*). Data obtained in the present work for the carboxylic acids acetic acid, formic acid, lactic acid and glyceric acid confirmed this finding. The corresponding carboxylic acid amides revealed the same behavior. Over a period of seven days all amides monitored accumulated to reach a plateau. Thus, these amides can be regarded as stable degradation end products in Maillard reactions too.

Davidek et al. described a hydrolytic β-dicarbonyl cleavage mechanism of 1-deoxyhexo-2,4-diulose as the major formation pathway for acetic acid as well as for glyceric acid (*7*), based on studies of Hayami to investigate formation of acetol by decomposition of hexoses via a hydrolytic cleavage of 1-deoxyhexo-2,4-diulose (*21*). Also Weenen confirmed a β-cleavage mechanism to give acetol and glyceric acid (*22*). Davidek postulated that first an isomerization reaction of 1-DG to 1-deoxyhexo-2,4-diulose takes place, followed—in the case of acetic acid—by a nucleophilic attack of a hydroxyl ion at the C-2 carbonyl function (likewise glyceric acid is formed by a nucleophilic attack on C-4 position). The cleavage reaction results in acetic acid and a C4-enediole which can undergo further isomerization reactions to yield erythrulose and corresponding deoxyosones (*7*, *8*, *20*). These findings give reason to believe that the cleavage mechanism will

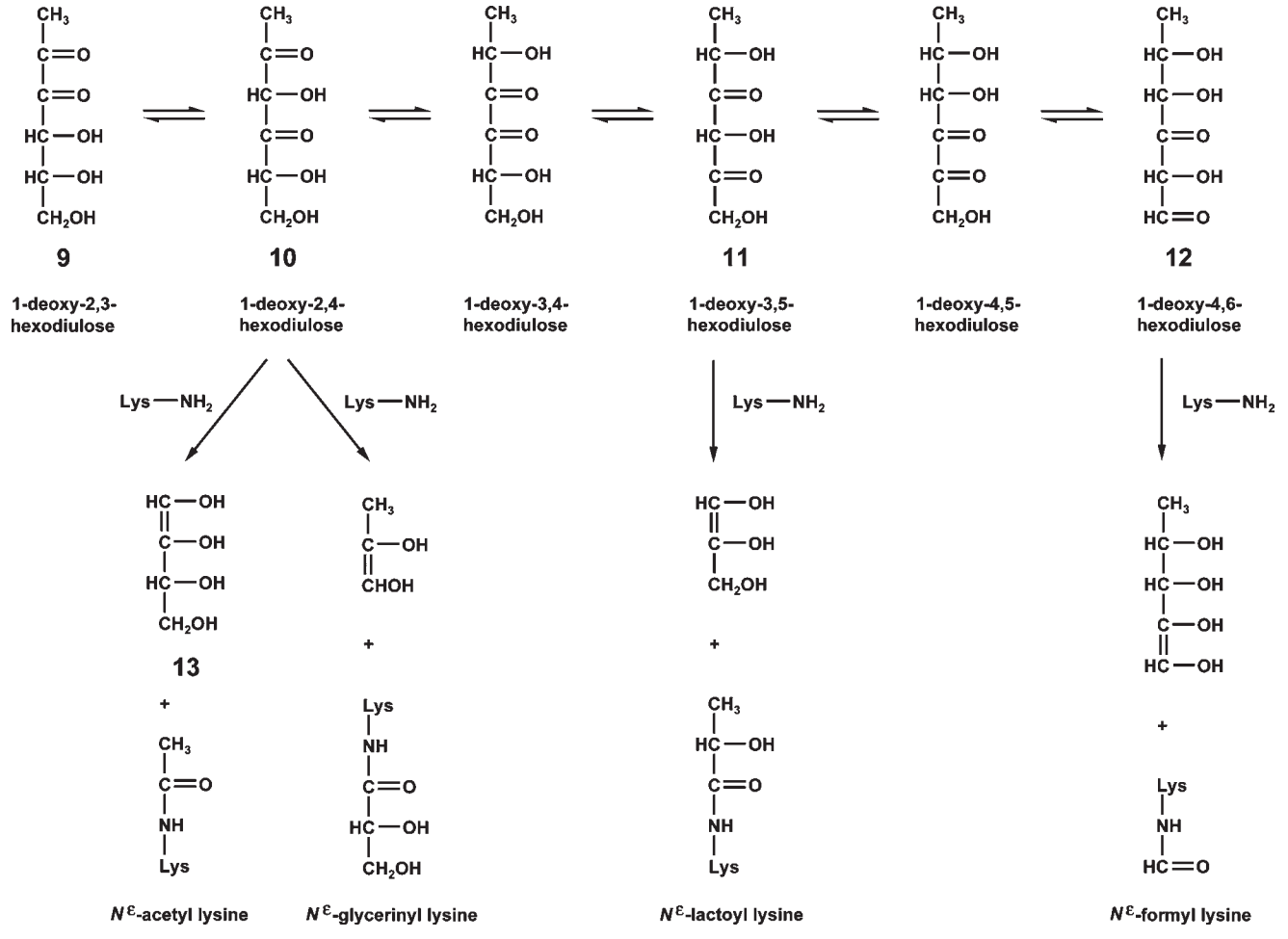

Figure 3. Mechanism of nonoxidative 1-deoxyhexo-2,3-diulose degradation: β-dicarbonyl cleavage leads to N^{ε}-acetyl lysine, N^{ε}-glycerinyl lysine, N^{ε}-lactoyl lysine and N^{ε}-formyl lysine.

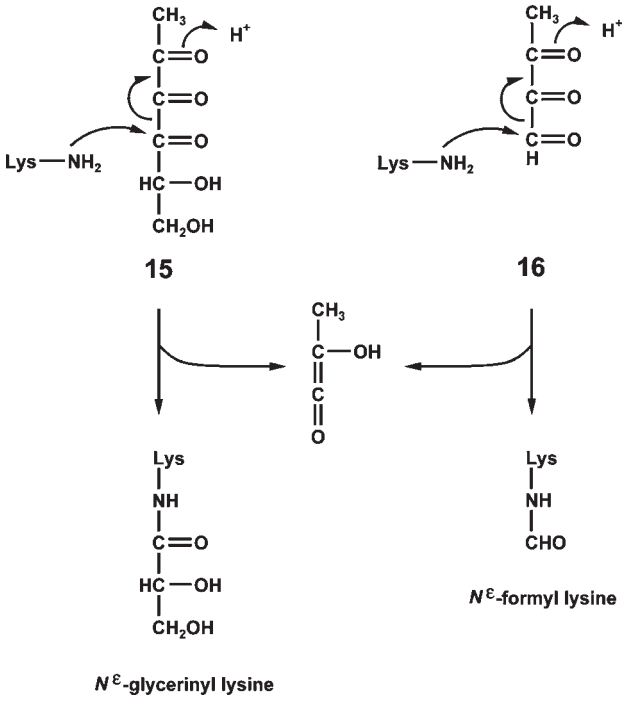

Figure 4. Nucleophilic attack on tricarbonyls formed by oxidation from 1-DG or 1-DT leads to N^{ε}-glycerinyl lysine and N^{ε}-formyl lysine.

also lead to formation of carboxylic acid amides in the presence of amines. In **Figure 3** a mechanism is postulated that is capable of elucidating the formation pathway of N^{ε}-acetyl lysine, N^{ε}-formyl lysine, N^{ε}-lactoyl lysine and N^{ε}-glycerinyl lysine under deaerated conditions. After isomerization of 1-DG (**9**) to 1-deoxyhexo-2, 4-diulose (**10**) a nucleophilic attack by the ε-amino function of lysine at C-2 position of **10** followed by the β-cleavage reaction results in the formation of N^{ε}-acetyl lysine and C4-enediole (**13**). In a similar manner the formation of N^{ε}-glycerinyl lysine can be explained by a nucleophilic attack of lysine at the C-4 position of **10**, analogous to the formation of glyceric acid. Formation of N^{ε}-lactoyl lysine as well as formation of N^{ε}-formyl lysine can equally be explained by a β-cleavage mechanism assuming that isomerization of 1-DG will also lead to 1-deoxyhexo-3,5-diulose (**11**) and 1-deoxyhexo-4,6-diulose (**12**) (**Figure 3**). Such long-range shifts of the carbonyl moiety in Maillard chemistry have been already studied, leading to major intermediates, i.e. Lederer's glucosone (*23*, *24*). Thus, isomerization of α-diketones into β-diketones along the entire carbon backbone must be deemed to be a general transformation.

Strikingly, in comparison to the remainder carboxylic acid amides, N^{ε}-formyl lysine occurred with the highest amounts, whereas formic acid gave the lowest values for all carboxylic acids. With respect to formic acid formation this obviously indicates that the nucleophilic attack of the ε-amino function of lysine emerges as a competitive reaction to the hydrophilic attack of the hydroxyl ion more strongly than in case of the other carboxylic acid/amide pairs. This is based on the assumption that β-dicarbonyl cleavage is the major formation pathway and an additional oxidative α-dicarbonyl cleavage plays only a minor part or is nonexistent (*20*).

Our working group has found a favored formation of glyceric acid and lactic acid under aerated conditions and postulated a formation mechanism coexistent to regular hydrolytic β-cleavage to explain carboxylic acid formation (data will be published elsewhere). Starting with 1-DG or 1-deoxy-2,3-tetrodiulose (1-DT, a major degradation product of 1-DG (*20*)) the tricarbonyl structures **15** and **16** are formed after oxidation, respectively. This step is followed by nucleophilic attack of a hydroxyl ion at the tricarbonyls to give glyceric acid, lactid acid and formic acid as the cleavage products. In our present study we measured significantly higher amounts of N^{ε}-formyl lysine and N^{ε}-glycerinyl lysine under aerated conditions. This is in line with the postulated mechanism, because a nucleophilic attack of the ε-amino function of lysine will lead to N^{ε}-formyl lysine and N^{ε}-glycerinyl lysine as shown in **Figure 4**.

For verification that amides indeed originated from the postulated β-dicarbonyl cleavage reaction and not by a simple amide bond formation between lysine side chains and the formed free carboxylic acids, all these acids were separately incubated with N^{α}-*t*-BOC-lysine. Concentrations of carboxylic acids were chosen according to the acetic acid concentration formed after seven days in 1-DG/N^{α}-*t*-BOC-lysine reaction mixtures. In all carboxylic acid/N^{α}-*t*-BOC-lysine reaction mixtures the corresponding amides were not detected. This control experiment thus clearly depicts that the direct reaction of lysine with carboxylic acids is definitively not an alternative pathway to produce the amides under the given conditions.

While studies by Büttner and Hayase only provide an indication of a possible reaction between lysine side chains and sugars/sugar degradation products (*9*, *10*), we are now capable of verifying Maillard induced amine acylation with the experiments conducted herein. The detection of N^{ε}-acetyl lysine, N^{ε}-formyl lysine, N^{ε}-lactoyl lysine and N^{ε}-glycerinyl lysine in Maillard model reaction systems, resulting from reaction of the ε-lysine side chain and 1-DG, opens a new field with respect to their formation and significance in foods and *in vivo*. Compounds like CML (N^{ε}-(carboxymethyl)lysine) (*25*), CEL (N^{ε}-(carboxyethyl)-lysine) (*26*), GOLA (N^{ε}-{2-[(5-amino-5-carboxypentyl)amino]-2-oxoethyl}lysine), GALA (N^{ε}-glycoloyllysine) and GOLD (1,3-bis-(5-amino-5-carboxypentyl) imidazolium salt) (*12*) that occur via rearrangement reactions have already confirmed that α-dicarbonyl structures are highly potent to modify lysine side chains. Based on our data it must be assumed that acylation induced by α-dicarbonyl compounds is a second major pathway leading to a novel class of amide advanced glycation end products (amide-AGEs) present in food and *in vivo*.

In conclusion, we succeeded in the synthesis of N^{ε}-lactoyl lysine and N^{ε}-glycerinyl lysine−lysine amide modifications that occur in 1-DG/N^{α}-*t*-BOC-lysine reaction mixtures. Furthermore we established N^{ε}-acetyl lysine, N^{ε}-formyl lysine, N^{ε}-lactoyl lysine and N^{ε}-glycerinyl lysine as novel stable end products in Maillard reactions. The data reported in the present study strongly supports a formation mechanism of the amides based on the established β-dicarbonyl cleavage reaction.

ACKNOWLEDGMENT

We thank Dr. D. Ströhl from the Institute of Organic Chemistry, Halle, Germany, for recording NMR spectra and Dr. J. Schmidt from the Leibnitz Institute of Plant Biochemistry, Halle, Germany, for performing accurate mass determination.

LITERATURE CITED

(1) Hodge, J. E. Browning reactions in model systems. *J. Agric. Food Chem.* **1953**, *1*, 928–943.

(2) Ledl, F.; Schleicher, E. The Maillard reaction in food and in the human body - new results in chemistry, biochemistry and medicine. *Angew. Chem.* **1990**, *102*, 597–626.

(3) 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.

(4) Hofmann, T. Quantitative studies on the role of browning precursors in the Maillard reaction of pentoses and hexoses with L-alanine. *Z. Lebensm.-Unters. Forsch. A* **1999**, *209*, 113–121.
(5) Oliver, C. M.; Melton, L. D.; Stanley, R. A. Creating proteins with novel functionality via the Maillard reaction: A review. *Crit. Rev. Food Sci. Nutr.* **2006**, *46*, 337–350.
(6) Glomb, M. A.; Pfahler, C. Synthesis of 1-deoxy-d-erythro-hexo-2,3-diulose, a major hexose Maillard intermediate. *Carbohydr. Res.* **2000**, *329*, 515–523.
(7) Davidek, T.; Devaud, S.; Robert, F.; Blank, I. Sugar Fragmentation in the Maillard Reaction Cascade: Isotope Labeling Studies on the Formation of Acetic Acid by a Hydrolytic beta -Dicarbonyl Cleavage Mechanism. *J. Agric. Food Chem.* **2006**, *54*, 6667–6676.
(8) Davidek, T.; Robert, F.; Devaud, S.; Vera, F. A.; Blank, I. Sugar Fragmentation in the Maillard Reaction Cascade: Formation of Short-Chain Carboxylic Acids by a New Oxidative alpha -Dicarbonyl Cleavage Pathway. *J. Agric. Food Chem.* **2006**, *54*, 6677–6684.
(9) Hayase, F.; Kim, S. B.; Kato, H. Maillard reaction products formed from D-glucose and glycine and the formation mechanisms of amides as major components. *Agric. Biol. Chem.* **1985**, *49*, 2337–2341.
(10) Buttner, U.; Gerum, F.; Severin, T. Formation of alpha -amino-acid amides and alpha -hydroxy-acid amides by degradation of sugars with primary amines. *Carbohydr. Res.* **1997**, *300*, 265–269.
(11) Nagaraj, R. H.; Shamsi, F. A.; Huber, B.; Pischetsrieder, M. Immunochemical detection of oxalate monoalkylamide, an ascorbate-derived Maillard reaction product in the human lens. *FEBS Lett.* **1999**, *453*, 327–330.
(12) Glomb, M. A.; Pfahler, C. Amides are novel protein modifications formed by physiological sugars. *J. Biol. Chem.* **2001**, *276*, 41638–41647.
(13) Hasenkopf, K.; Rönner, B.; Hiller, H.; Pischetsrieder, M. Analysis of glycated and ascorbylated proteins by gas chromathography-mass spectrometry. *J. Agric. Food Chem.* **2002**, *50*, 5697–5703.
(14) Gellett, A. M.; Huber, P. W.; Higgins, P. J. Synthesis of the unnatural amino acid N^{α}-N^{ε}-(ferrocene-1-acetyl)-L-lysine: A novel organometallic nuclease. *J. Organomet. Chem.* **2008**, *693*, 2959–2962.
(15) Kuisle, O.; Quinoa, E.; Riguera, R. A. General Methodology for Automated Solid-Phase Synthesis of Depsides and Depsipeptides. Preparation of a Valinomycin Analog. *J. Org. Chem.* **1999**, *64*, 8063–8075.
(16) Glomb, M. A.; Tschirnich, R. Detection of alpha-dicarbonyl compounds in Maillard reaction systems and in vivo. *J. Agric. Food Chem.* **2001**, *49*, 5543–5550.
(17) Husek, P. Amino acid derivatization and analysis in five minutes. *FEBS Lett.* **1991**, *280*, 354–356.
(18) Milne, G. W. A.; Axenrod, T.; Fales, H. M. Chemical ionization mass spectrometry of complex molecules. IV. Amino acids. *J. Am. Chem. Soc.* **1970**, *92*, 5170–5175.
(19) Weinkam, R. J. Reactions of protonated diamino acids in the gas phase. *J. Org. Chem.* **1978**, *43*, 2581–2586.
(20) Voigt, M.; Glomb, M. A. Reactivity of 1-Deoxy-D-erythro-hexo-2,3-diulose: A Key Intermediate in the Maillard Chemistry of Hexoses. *J. Agric. Food Chem.* **2009**, *57*, 4765–4770.
(21) Hayami, J. Studies on the chemical decomposition of simple sugars. XII. Mechanism of acetol formation. *Bull. Chem. Soc. Jpn.* **1961**, *34*, 927–932.
(22) Weenen, H. Reactive intermediates and carbohydrate fragmentation in Maillard chemistry. *Food Chem.* **1998**, *62*, 393–401.
(23) Biemel, K. M.; Conrad, J.; Lederer, M. O. Unexpected carbonyl mobility in aminoketoses: the key to major Maillard crosslinks. *Angew. Chem., Int. Ed.* **2002**, *41*, 801–804.
(24) Reihl, O.; Rothenbacher, T. M.; Lederer, M. O.; Schwack, W. Carbohydrate carbonyl mobility—the key process in the formation of alpha-dicarbonyl intermediates. *Carbohydr. Res.* **2004**, *339*, 1609–1618.
(25) Reddy, S.; Bichler, J.; Wells-Knecht, K. J.; Thorpe, S. R.; Baynes, J. W. N^{ε}-(Carboxymethyl)lysine Is a Dominant Advanced Glycation End Product (AGE) Antigen in Tissue Proteins. *Biochemistry* **1995**, *34*, 10872–10878.
(26) Ahmed, M.; Brinkmann Frye, E.; Degenhardt, T. P.; Thorpe, S. R.; Baynes, J. W. N^{ε}-(Carboxyethyl)lysine, a product of the chemical modification of proteins by methylglyoxal, increases with age in human lens proteins. *Biochem. J.* **1997**, *324*, 565–570.

Received for review January 26, 2010. Revised manuscript received April 14, 2010. Accepted April 17, 2010.