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Sugar Fragmentation in the Maillard Reaction Cascade: Isotope Labeling Studies on the Formation of Acetic Acid by a Hydrolytic β-Dicarbonyl Cleavage Mechanism

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The formation of acetic acid was elucidated based on volatile reaction products and related nonvolatile key intermediates. The origin and yield of acetic acid were determined under well-controlled conditions (90−120 °C, pH 6−8). Experiments with various ^{13}C-labeled glucose isotopomers in the presence of glycine revealed all six carbon atoms being incorporated into acetic acid: C-1/C-2 (∼70%), C-3/C-4 (∼10%), and C-5/C-6 (∼20%). Acetic acid is a good marker of the 2,3-enolization pathway since it is almost exclusively formed from 1-deoxy-2,3-diulose intermediates. Depending on the pH, the acetic acid conversion yield reached 85 mol % when using 1-deoxy-2,3-hexodiulose (**1**) as a precursor. Hydrolytic β-dicarbonyl cleavage of 1-deoxy-2,4-hexodiuloses was shown to be the major pathway leading to acetic acid from glucose without the intermediacy of any oxidizing agents. The presence of key intermediates was corroborated for the first time, i.e., tetroses and 2-hydroxy-3-oxobutanal, a tautomer of 1-hydroxy-2,3-butanedione, also referred to as 1-deoxy-2,3-tetrodiulose. The hydrolytic β-dicarbonyl cleavage represents a general pathway to organic acids, which corresponds to an acyloin cleavage or a retro-Claisen type reaction. Although alternative mechanisms must exist, the frequently reported hydrolytic α-dicarbonyl cleavage of **1** can be ruled out as a pathway forming carboxylic acids.

KEYWORDS: Maillard reaction; acetic acid; 1-deoxy-2,3-hexodiulose; hydrolytic β-dicarbonyl cleavage; labeling studies; sugar fragmentation

INTRODUCTION

The reaction of reducing sugars and amino acids or proteins, also referred to as the "nonenzymatic browning reaction" or "Maillard reaction", is known to result in color, flavor, and physiologically active compounds (recent review in *1*). The initial phase of the Maillard cascade starts with the formation of the *N*-glycosyl derivative of the amine component, which is susceptible to enolization due to the presence of the sugar hydroxyl groups. The resulting eneaminol may form 3-deoxyhexos-2-ulose (**2**) via the 1,2-enolization pathway and/or rearrange to the corresponding 1-amino-1-deoxyketose derivative (Amadori compound) (*2*, *3*). Alternatively, further enolization of the Amadori compound may lead to the enediol, which gives rise to 1-deoxy-2,3-hexodiulose (**1**) via 2,3-enolization. Both deoxy-α-dicarbonyls are unstable Maillard intermediates and easily decompose, generating by isomerization, cyclization, and fragmentation a complex mixture of reaction products including volatiles and brown pigments. Characteristic reaction products are 2,3-dihydro-3,5-dihydroxy-6-methyl-4(*H*)-pyran-4-one and 5-hydroxymethyl-2-furaldehyde bearing the intact carbon chain as well as acetic acid and formic acid formed by fragmentation of the α-dicarbonyls **1** and **2**, respectively (*4*).

In our recent study on the degradation of the Amadori compound *N*-(1-deoxy-D-fructos-1-yl)glycine (**3**) under different reaction conditions (*5*), we observed up to 60 mol % conversion yield of acetic acid formed (pH 7−8, 90 °C, 3 h). Thus, under neutral and alkaline conditions, acetic acid belongs to the major degradation products of Amadori compounds. This result is in line with the data reported by van Boekel and co-workers (*4*, *6*) who found significant amounts of acetic acid (about 45 mol %) generated upon thermal treatment of the casein-bound Amadori compound (pH 6.8, 120 °C, 40 min). Lower amounts of acetic acid were obtained when heating glucose with glycine or casein (pH 6.8, 100 °C, 2 h), resulting in up to 8 (*7*) and 1 mol % (*8*), respectively.

Acetic acid was first reported in 1926 as a volatile degradation product (7% yield) formed from hexose sugars under alkaline conditions in air atmosphere (*9*). However, the presence of oxygen is not a prerequisite for its formation: Acetic acid was also generated by thermal alkaline degradation of polysaccharides in inert atmosphere (*10*). Although acetic acid is known for a long time as a sugar degradation product, its formation mechanism is still not yet fully understood. Already about 50

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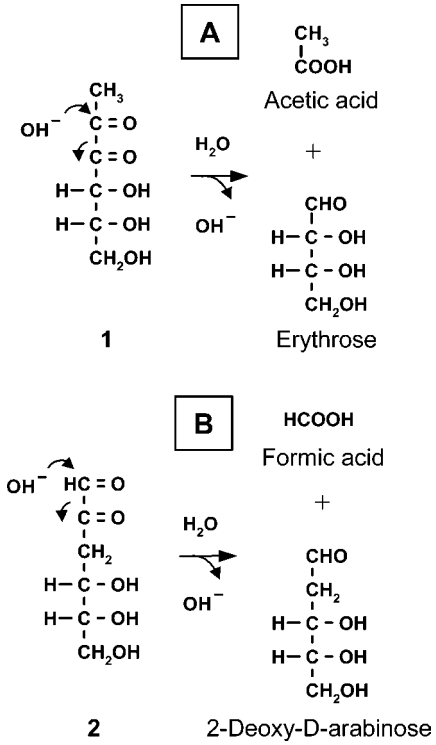


Figure 1. Hypothetical formation mechanism based on hydrolytic α-dicarbonyl cleavage of (**A**) 1-deoxy-2,3-hexodiulose (**1**) and (**B**) 3-deoxy-hexos-2-ulose (**2**) leading to acetic acid and formic acid, respectively (according to ref *12*).

years ago, Hodge postulated **1** as a possible precursor of acetic acid (*11*). The proposed mechanism included retro-aldolization of **1** followed by the metasaccharinic rearrangement of the arising glycolaldehyde. Recent kinetic studies (*4*, *6*) and labeling experiments (*12*, *13*) support the hypothesis of **1** being the precursor of acetic acid. However, these authors suggested the direct degradation of **1** via a hydrolytic α-dicarbonyl cleavage at C-2/C-3 as the reaction mechanism leading to acetic acid and erythrose as the C_4-fragment (**Figure 1A**). In analogy, **2** should result in formic acid and 2-deoxy-D-arabinose as C_5-fragment by cleavage at C-1/C-2 (**Figure 1B**). However, neither of the putative C_4- or C_5-fragments have so far been identified in this context; thus, there is no final proof for this hypothesis. Furthermore, other mechanisms must exist, as acetic acid has also been reported to originate from the C-5/C-6 carbon atoms of glucose (*14*).

This paper deals with the formation of acetic acid by Maillard type reactions under cooking conditions. An in-depth mechanistic study was performed to elucidate the most probable pathway of acetic acid formation and to clarify the validity of the hydrolytic α-dicarbonyl hypothesis.

MATERIALS AND METHODS

Materials. The following chemicals of analytical grade were commercially available: glycine, D-(+)-glucose, *o*-phenylenediamine (1,2-benzenediamine), *N*,*N*-dimethylformamide, *N*,*O*-*bis*(trimethylsilyl)-trifluoroacetamide, 1-(trimethylsilyl)-1*H*-imidazole, 1-hydroxy-2-propanone (acetol), methoxamine hydrochloride, ammonium formate, D-erythrose, Dowex 50W × 8 (H^+-form, 16−40 mesh), methanol (MeOH), and diethyl ether (Fluka/Aldrich, Buchs, Switzerland); disodium monohydrogenphosphate, monosodium dihydrogenphosphate, and phosphoric acid (85%) (Merck, Darmstadt, Germany); 2-methylquinoxaline, 2,3-pentanedione, 2,4-pentanedione, and D-threose (Sigma-Aldrich, St. Louis, MO); acetonitrile (Mallinckrodt Baker, Deventer, Holland); D-[1-^{13}C]glucose (99%), D-[2-^{13}C]glucose (99%), D-[3-^{13}C]-glucose (99%), D-[4-^{13}C]glucose (99%), D-[5-^{13}C]glucose (99%), D-[6-^{13}C]glucose (99%), D-[1,2-$^{13}C_2$]glucose (99%), [$^{13}C_3$]acetone (99%), and [$^2H_5$]propanoic acid (98%) (Cambridge Isotope Laboratories, Andover, MA); [2,2,2-$^2H_3$]acetic acid (Isotec, Miamisburg, OH); 3-deoxy-D-*erythro*-hexos-2-ulose (**2**) (Toronto Research Chemicals, Ontario, Canada); and L-tetrulose (L-erythrulose, Kraeber GmbH, Ellerbek, Germany). 1-Deoxy-4,5-*O*-(1-methylethylidene)-D-*erythro*-2,3-hexodiulose (**4**) and 1-deoxy-D-*erythro*-2,3-hexodiulose (**1**) were synthesized as recently described (*15*) following the Glomb procedure (*16*).

Degradation of Glucose. A solution of glycine (0.35 mmol) and D-glucose or various ^{13}C-labeled glucose isotopomers (0.35 mmol) in a phosphate buffer (3.5 mL, 0.2 mol/L) was dispatched (0.4 mL) into screw-cap vials (1.5 mL, Infochroma, Zug, Switzerland) and thermally treated in a silicone bath (90 °C, 15 h or 120 °C, 4 h). Experiments were performed at pH 4, pH 6, and pH 8. After the mixture was cooled to room temperature (RT), an aliquot of the reaction mixture (0.3 mL) was spiked with a defined amount of [$^2H_3$]acetic acid in water (250−600 μg) for the analysis of acetic acid by stable isotope dilution assay (*17*). The samples were acidified to pH 3 with phosphoric acid (85%) and analyzed by solid-phase microextraction combined with gas chromatography−mass spectrometry (SPME GC-MS). Each sample was prepared at least in duplicate and injected twice.

For the identification of (i) tetroses as methoxime trimethylsilyl (TMS) derivatives and (ii) 1-hydroxy-2,3-butandione as quinoxaline derivative by GC-MS, a solution of glycine (0.5 mmol) and D-glucose or various ^{13}C-labeled glucose isotopomers (0.5 mmol) in a phosphate buffer (5 mL, 0.2 mol/L, pH 6 or pH 8) was placed into a Pyrex tube (26 mm × 100 mm) and thermally treated as described above.

Degradation of 2,3-Pentanedione and 2,4-Pentanedione. A solution of glycine (0.5 mmol) and 2,3-pentanedione (0.5 mmol) or 2,4-pentanedione (0.5 mmol) in a phosphate buffer (5 mL, 0.2 mol/L) was dispatched (0.4 mL) into screw-cap vials (1.5 mL, Infochroma) and thermally treated at 120 °C in a silicone bath for a defined period of time (10−240 min). The experiments were performed at pH 6, pH 8, and pH 10. After the reaction cooled down, an aliquot of the reaction mixture (0.35 mL) containing 2,3-pentanedione was spiked with a defined amount of [$^2H_3$]acetic acid (250−600 μg) and [$^2H_5$]propanoic acid (93 μg) in water. An aliquot of the reaction mixture (0.35 mL) containing 2,4-pentanedione was spiked with [$^2H_3$]acetic acid (250−600 μg) and [$^{13}C_3$]acetone (170 μg). Samples were acidified to pH 3 with phosphoric acid (85%) and analyzed by SPME GC-MS. Each sample was prepared at least in duplicate and injected twice.

Degradation of 3-Deoxy-D-*erythro*-hexos-2-ulose (2). A solution of glycine (0.05 mmol) and **2** (0.05 mmol) in a phosphate buffer (0.5 mL, 0.2 mol/L) was placed into screw-cap vials (1.5 mL) and thermally treated in a silicone bath (120 °C, 4 h). The experiments were performed at pH 4, pH 6, and pH 8. After the reaction was cooled down, an aliquot of the reaction mixture (0.3 mL) was spiked with a defined amount of [$^2H_3$]acetic acid in water (250−600 μg). The samples were acidified with phosphoric acid (85%, 80 μL) and analyzed by SPME-GC-MS. Each sample was prepared at least in duplicate and injected twice.

Degradation of 1-Deoxy-D-*erythro*-2,3-hexodiulose (1). *Deprotection.* A solution of 1-deoxy-4,5-*O*-(1-methylethylidene)-D-*erythro*-2,3-hexodiulose (**4**, 0.1 mmol) in water (2 mL) was stirred with Dowex 50W × 8 (H^+-form, 16−40 mesh, 2 mL) at RT. At regular time intervals, an aliquot of the solution (0.1 mL) was withdrawn and diluted with water (0.9 mL). A methanolic solution of *o*-phenylenediamine (1 mL, 1 mol/L) was added, and the mixture was maintained overnight at 25 °C and analyzed by high-performance liquid chromatography (HPLC) to check the progress of the deprotection (*18*). The optimum deprotection time was determined by reversed phase (RP) HPLC measuring both **1** and the protected derivative **4** (**Figure 2A**). Complete deprotection was accomplished after 2.5 h (**Figure 2B**).

Reaction. A solution of **4** (0.1 mmol) in water (2 mL) was stirred with Dowex 50W × 8 (H^+-form, 16−40 mesh, 1.5 mL). After 2.5 h, the resin was filtered off and the solution of **1** was diluted five times with phosphate buffer (0.2 mol/L). The solution was dispatched (0.5 mL) into a screw-cap vial (1.5 mL, Infochroma) and thermally treated in a silicone bath for a defined period of time (120 °C, up to 60 min).

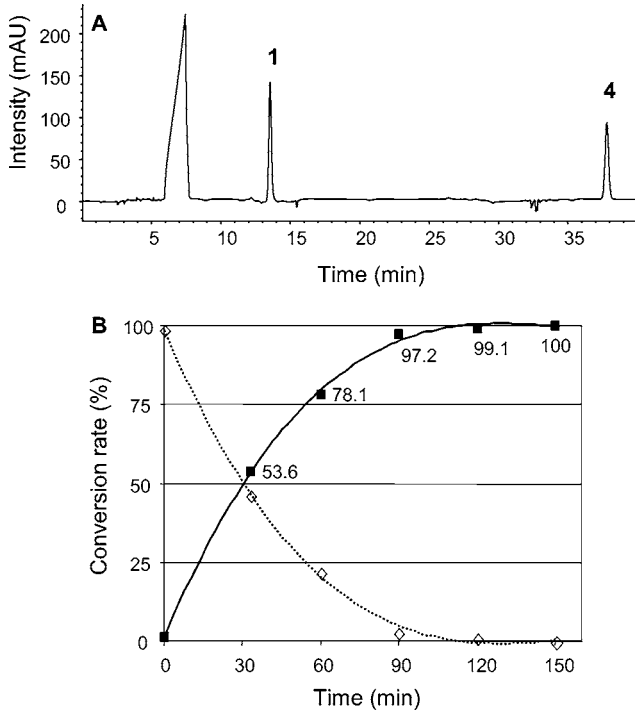


Figure 2. (**A**) RP-HPLC chromatogram of free 1-deoxy-D-*erythro*-2,3-hexodiulose (**1**) and its protected derivative 1-deoxy-4,5-*O*-(1-methylethylidene)-D-*erythro*-2,3-hexodiulose (**4**). (**B**) Effect of reaction time on the deprotection of **4** (◇) liberating the α-dicarbonyl **1** (■). The variation coefficients were less than 3%.

The experiments were performed at pH 5, pH 6, pH 6.5, pH 7, and pH 8. After it was cooled to RT, an aliquot of the reaction mixture (0.4 mL) was spiked with a defined amount of [$^2H_3$]acetic acid (61 μg) dissolved in water. The samples were acidified to pH 3 with phosphoric acid (85%) and extracted with diethyl ether (1 mL, 1 min). The extract was dried over anhydrous sodium sulfate and analyzed by GC-MS. For the analysis of **1**, an aliquot of the reaction mixture (0.1 mL) was diluted with water (0.9 mL). A methanolic solution of *o*-phenylenediamine (1 mL, 1 mol/L) was added, and the mixture was kept overnight at 25 °C and analyzed by RP-HPLC. Each sample was prepared at least in duplicate and injected twice.

Derivatization Reactions. *Methoxime Trimethylsilyl (TMS) Derivatives of Tetrose Sugars.* An aliquot of the reaction mixture containing glucose (2 mL) was mixed with methoxamine hydrochloride (0.2 g), the pH of the solution was adjusted to 7.0 with aqueous NaOH (1 mol/L), and the mixture was heated (40 °C, 2 h). After it was cooled to RT, the mixture was freeze-dried and the residue was dissolved in a mixture of acetonitrile and *N*,*N*-dimethylformamide (1 mL, 1:1, v/v). *N*-(Trimethylsilyl)imidazole (0.2 mL) and *N*,*O*-*bis*(trimethylsilyl)-trifluoroacetamide (0.2 mL) were added, and the mixture was treated in an ultrasonic bath (10 s). Finally, the mixture was heated (70 °C, 10 min) and analyzed by GC-MS (*19*).

Quinoxaline Derivative of 1-Hydroxy-2,3-butanedione. The derivatization was performed as described by Hofmann (*18*) using some modifications. An aliquot of the reaction mixture containing glucose (5 mL) was adjusted to pH 7.0 with aqueous NaOH (1 mol/L), 1.5 mL of a freshly prepared solution of *o*-phenylenediamine (1 M in MeOH) was added, and the mixture was heated (40 °C, 2 h). After it was cooled to RT, the pH of the reaction mixture was adjusted to 6.0 using aqueous HCl (1 M) and extracted with diethyl ether (20 mL, 15 min). The organic layer was dried over anhydrous sodium sulfate and concentrated on a Vigreux column (50 cm × 1 cm) followed by microdistillation to a final volume of 1.5 mL prior to analysis by GC-MS.

SPME GC-MS. After an equilibration phase (20 °C, 60 min), the polydimethylsiloxane-divinylbenzene (PDMS-DVB; film thickness, 65 μm; Supelco, Bellefonte, PA) fiber was exposed to the headspace above the samples in the glass vials without agitation (20 °C, 30 min). After sampling, the SPME device was placed in the GC injector (5 min), operating in the splitless mode and heated at 250 °C. GC-MS analyses were performed on a GC 6890A coupled to an MSD 5973 (both Agilent, Palo Alto, CA) using a DB-Wax capillary column (30 m × 0.25 mm; film thickness, 0.25 μm; J&W Scientific, Folsom, CA). Helium was used as the carrier gas with a constant flow (1 mL/min). After insertion of the SPME device into the injector, the oven temperature program was started and the temperature rose at 6 °C/min from 25 (2 min) to 240 °C (10 min). The temperature of the ion source was 280 °C. Mass spectra in the electron impact (EI) mode were generated at 70 eV and at a scan range from *m*/*z* 28 to *m*/*z* 350. Quantification of acetic acid, acetone, and propanoic acid was performed by stable isotope dilution assay (*17*) in the scan mode by measuring the molecular ions of analytes and labeled internal standards. [$^2H_3$]Acetic acid was used as internal standard for the quantification of formic acid. The naturally occurring percentage of ^{13}C (1.10%) was considered in the calculations.

GC-MS. *Acetic Acid and Quinoxaline Derivatives (Method 1).* The solvent extracts were analyzed on a GC 6890A coupled to an MSD 5973 using a DB-Wax capillary column and the operating conditions for the GC-MS as described above. The oven temperature program was as follows: 25 °C (2 min), 40 °C/min to 50 °C (1 min), and 6 °C/min to 240 °C (10 min). Quantification of acetic acid was performed in the scan mode by measuring the molecular ions of the analyte (*m*/*z* 60) and labeled internal standard (*m*/*z* 63). Quinoxaline derivatives were analyzed using the same conditions; however, they were not quantified. The retention index of the quinoxaline derivative of 1-hydroxy-2,3-butanedione was $RI_{DB-Wax} = 2760$.

Tetrose Methoxime TMS Derivatives (Method 2). The analyses were performed on a GC 6890A coupled to an MSD 5973 (both Agilent) using a HP-PONA capillary column (50 m × 0.20 mm; film thickness, 0.50 μm; Agilent). Helium was used as the carrier gas with a constant flow (1 mL/min). Samples were introduced via split injection at 250 °C (1 μL, split ratio 1:10). The oven temperature program was as follows: 80 °C (1 min), 6 °C/min to 240 °C (20 min). The temperature of the ion source was 280 °C. Mass spectra in the EI mode were generated at 70 eV over a mass range of 28−550 Da. Retention indices of the corresponding tetrose derivatives on the apolar stationary phase PONA were as follows: RI = 1729 and RI = 1744 for erythrose; RI = 1738 and RI = 1748 for threose; and RI = 1756 and RI = 1767 for tetrulose. The two retention indices for each tetrose derivative correspond to the syn and anti forms of the corresponding methoximes.

HPLC. RP-HPLC analyses were performed using an integrated HP-1090 system with a binary pumping system and a diode array detector. Analytical separation was achieved on a Nucleosil 100/5 C18 HPLC column (240 mm × 4 mm i.d., Macherey Nagel, Düren, Germany). The elution mode was a solvent gradient starting with a mixture (10/90, v/v) of acetonitrile and ammonium formate (pH 3.5, 20 mmol/L) and increasing the acetonitrile content to 30% within 50 min. The flow rate was 0.6 mL/min. The column temperature was maintained at 40 °C with a column heater. The injection volume was 20 μL. Compounds **1** and **4** were detected as their corresponding quinoxaline derivatives by monitoring the effluent at the wavelength of 320 nm. The quantification of both compounds was based on calibration curves by comparing the peak areas with those of standard solutions containing known amounts of 2-methylquinoxaline. 2-Methyl-3-(1,2,3-trihydroxypropyl)quinoxaline and 2-methylquinoxaline were shown to have the same extinction coefficients (*20*).

RESULTS

Degradation of Glucose and C_6-Deoxy-α-dicarbonyls. The formation of acetic acid was studied in terms of origin and yield in phosphate-buffered model systems containing equimolar concentrations of glucose and glycine (0.1 mol/L) as affected by pH, temperature, and reaction time. First experiments with unlabeled glucose at 120 °C indicated that the concentration of acetic acid increased with pH and the reaction time (**Table 1**). After 4 h of reaction, about 1, 17, and 38 mol % acetic acid were formed at pH 4, pH 6, and pH 8, respectively. The

Table 1. Formation of Acetic Acid from Glucose/Glycine in Phosphate-Buffered (0.2 mol/L) Model Systems at 120 °C as a Function of Time and pH

	acetic acid concentration (mol %)[a]		
reaction time (min)	pH 4	pH 6	pH 8
15	ND[b]	ND[b]	21.4 ± 0.1
30	ND[b]	9.7 ± 0.1	28.3 ± 0.2
60	0.28 ± 0.02	12.6 ± 0.1	33.0 ± 0.1
120	0.48 ± 0.04	15.1 ± 0.1	37.0 ± 0.2
240	1.00 ± 0.01	16.9 ± 0.2	38.4 ± 0.4
480	1.69 ± 0.17	ND[b]	ND[b]

[a] The data obtained by SPME GC-MS showed variation coefficients of less than 10%. [b] ND, not determined.

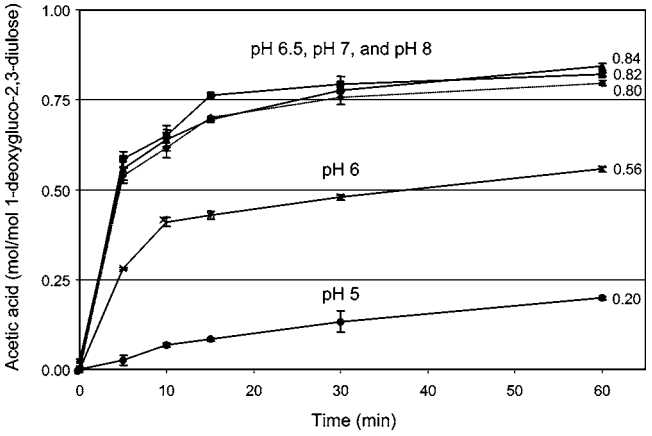


Figure 3. Effect of the reaction time and pH on the yield of acetic acid generated from 1-deoxy-D-*erythro*-2,3-hexodiulose (**1**) at 120 °C: pH 5 (●), pH 6 (×), pH 6.5 (▲), pH 7 (◆), and pH 8 (■). The data obtained by GC-MS showed variation coefficients of less than 10%.

corresponding Amadori compound, *N*-(1-deoxy-D-fructos-1-yl)-glycine (**3**), has been reported to show a similar effect (*21*). However, the conversion yields of acetic acid arising from **3** were higher than those from glucose, i.e., **3** resulted after 4 h of reaction at pH 4, pH 6, and pH 8, in about 4, 28, and 56 mol % acetic acid, respectively. The final conversion yields of acetic acid obtained by the degradation of **3** at 120 °C were comparable to those performed at 90 °C (*5*). These data are well in line with the assumption that acetic acid is formed by the 2,3-enolization pathway, which is favored with increasing pH (*3*), with 1-deoxy-D-*erythro*-2,3-hexodiulose (**1**) as a key intermediate.

Thermal treatment of **1**, obtained by synthesis (*15*), indicated a very rapid decomposition of this labile Maillard intermediate at 120 °C. Already after 5 min of heating, only 8% of **1** was found by RP-HPLC in the pH 5 sample, and no **1** could be detected anymore at pH ≥ 6 (data not shown). On the other side, the acetic acid conversion yields increased with reaction time in the whole pH range studied (**Figure 3**). The yields also increased with the pH of the reaction mixture up to pH 6.5. There were no significant differences in the acetic acid amounts generated at pH 6.5, pH 7, and pH 8. The high conversion yields of acetic acid, especially at pH ≥ 6.5, directly prove **1** as a major precursor of acetic acid at the early stage of the Maillard reaction. To the best of our knowledge, these data provide for the first time direct evidence for the formation of acetic acid from **1**.

To confirm that acetic acid is not formed in significant amounts via the 1,2-enolization pathway, 3-deoxyhexos-2-ulose (**2**) was heated in the presence of glycine for 4 h at 120 °C. Indeed, irrespectively of the pH, only small amounts of acetic acid were detected, i.e., 0.8, 1.5, and 2.4 mol % at pH 4, 6, and 8, respectively. Surprisingly, the quantities of formic acid were also very low, resulting in 0.9, 1.5, and 1.7 mol % at pH 4, 6, and 8, respectively. Hence, contrary to the hypotheses proposed in the literature (*4*, *6*, *12*), the hydrolytic α-dicarbonyl cleavage of **2** (**Figure 1B**) cannot be considered as a major pathway leading to formic acid from glucose or glucose-derived Amadori compounds.

Hydrolytic *β*-Dicarbonyl Cleavage. The results obtained with **2** were unexpected and questioned the up to now well-established hydrolytic α-dicarbonyl cleavage of **2** and **1** as the major mechanism leading to formic acid and acetic acid, respectively (*12*, *13*). On the other hand, the carbonyl mobility in Maillard intermediates by isomerization is well-known (*3*, *22*). Therefore, **1** may readily isomerize to 1-deoxy-2,4-hexodiulose (**5**), which was reported to degrade to 1-hydroxy-2-propanone (acetol) and glyceric acid via a *β*-dicarbonyl cleavage mechanism by nucleophilic attack of the hydroxyl ion at the C-4 carbonyl group of **5** (**Figure 4A**) (*23*). In analogy, nucleophilic attack of the hydroxyl ion at the C-2 carbonyl group of **5** would theoretically result in acetic acid and a tetrose sugar (**Figure 4B**). The validity of this alternative pathway was studied in more detail by using model intermediates.

To clarify the hypothesis discussed above, the α-dicarbonyl 2,3-pentanedione and the *β*-dicarbonyl 2,4-pentanedione were reacted with glycine at 120 °C in a phosphate buffer (0.2 mol/L) at pH 6, pH 8, and pH 10 with the aim of monitoring the acetic acid formed. 2,4-Pentanedione (**Figure 5A**) turned out to be a much better precursor of acetic acid as compared to 2,3-pentanedione (**Figure 5B**). For example, after 4 h of reaction at pH 8, the yield of acetic acid from 2,4-pentanedione and 2,3-pentanedione reached 89 and 11%, respectively. The data also demonstrated that this reaction already takes place at pH 6, which means that it is relevant under food processing conditions. More interestingly, acetic acid was accompanied by acetone in the model system containing 2,4-pentanedione. The concentrations of both compounds were similar, and they constantly increased as the reaction progressed. The rate of the formation of both compounds increased with the pH of the reaction mixture. These data fully conform to the *β*-dicarbonyl cleavage mechanism shown in **Figure 6A** and support the hypothesis of sugar fragmentation via a *β*-cleavage mechanism leading to acetic acid and C_4-fragments as counterparts (**Figure 4B**).

Interestingly, the reaction system containing 2,3-pentanedione resulted not only in acetic acid but also propanoic acid (**Figure 5B**). Following the hypothesis of the hydrolytic α-dicarbonyl cleavage (*12*, *13*), the low amounts of these acids might have been formed by a nucleophilic attack of the hydroxyl ion at the C-2 and C-3 carbonyl group, respectively. However, neither propanal nor acetaldehyde were detected in the reaction mixture (data not shown). On the other hand, our data suggest the C−C scission to occur via an oxidative α-dicarbonyl cleavage (**Figure 6B**), leading to both acetic and propanoic acid, which were detected at similar concentrations levels. This alternative fragmentation pathway will be discussed in the following paper (*24*).

Labeling Experiments with ^{13}C-Glucose Isotopomers. *Formation of Acetic Acid.* On the basis of the results discussed above, the *β*-dicarbonyl cleavage of 1-deoxy-2,4-hexodiulose (**5**) (**Figure 4B**) can be considered as the most probable pathway leading to the formation of acetic acid in hexose-based Maillard reaction systems under food processing conditions. However,

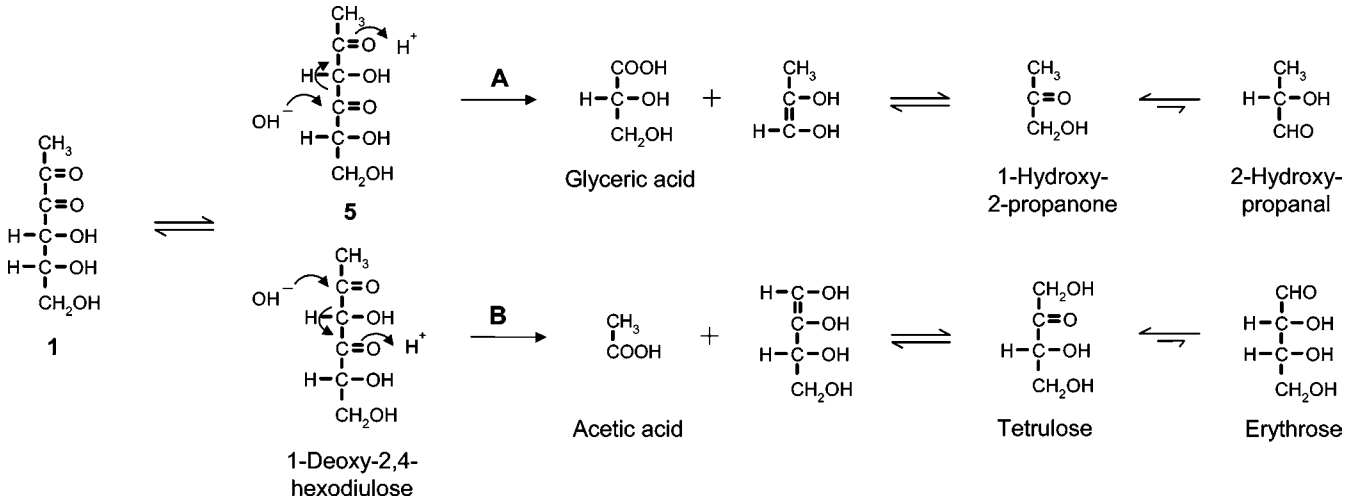


Figure 4. Hydrolytic β-dicarbonyl cleavage of 1-deoxy-2,4-hexodiulose (**5**) leading to (**A**) 1-hydroxy-2-propanone and glyceric acid (according to refs *23* and *33*) as well as to (**B**) acetic acid and tetrulose.

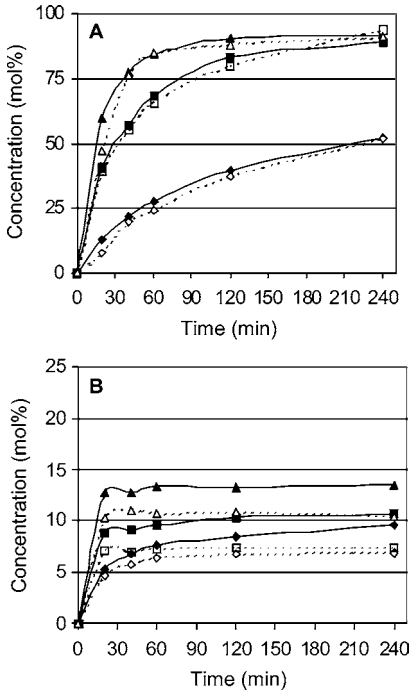


Figure 5. Effect of pH on the formation of acetic acid at 120 °C. (**A**) Formation of acetic acid (—) and acetone (· · ·) from 2,4-pentanedione at pH 6 (◆), pH 8 (■), and pH 10 (▲); (**B**) formation of acetic acid (—) and propanoic acid (· · ·) from 2,3-pentanedione at pH 6 (◆), pH 8 (■), and pH 10 (▲). The data obtained by SPME GC-MS showed variation coefficients of less than 5%.

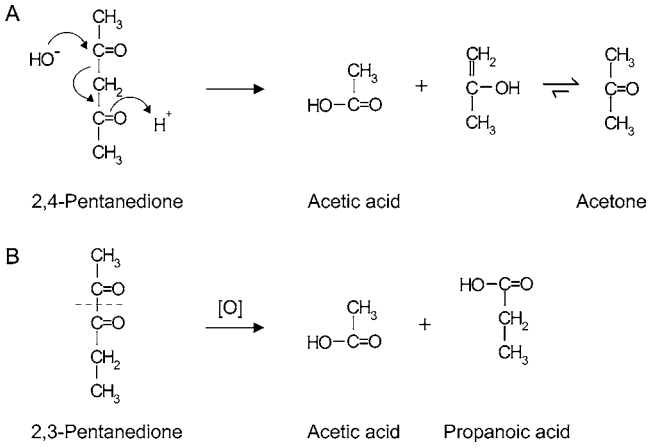


Figure 6. Schematic presentation of the formation of (**A**) acetic acid and acetone by hydrolytic β-dicarbonyl cleavage of 2,4-pentanedione and (**B**) acetic and propanoic acid by an oxidative α-dicarbonyl cleavage.

acetic acid has been reported to originate not only from the C-1 and C-2 carbon atoms of glucose (80%) but also from the C-5 and C-6 carbon atoms (20%) (*14*). Therefore, labeling experiments were performed using various ^{13}C-labeled glucose isotopomers reacted with glycine (90 °C and 120 °C; pH 6 and pH 8) to obtain further insight into the mechanisms of acetic acid formation. Acetic acid was quantified by SPME GC-MS using [$^2H_3$]acetic acid as internal standard. As shown in **Table 2**, singly labeled acetic acid was formed from all ^{13}C-labeled glucose isotopomers. The major pathway reflected the incorporation of the C-1 and C-2 carbon atoms of glucose (about 70%). The minor pathways involved the C-5 and C-6 carbon atoms of glucose (about 22%). We also report here for the first time the incorporation of C-3 and C-4 carbon atoms of glucose (about 8%) into acetic acid, which is in contradiction to the data recently reported by Wnorowski and Yaylayan (*14*). While the reaction conditions applied by these authors were similar (120 °C, 3 h), they used oversaturated aqueous solutions of precursors (0.8 g/mL) without buffer.

The pH had only a negligible effect on the origin of acetic acid from glucose. Although the absolute amounts of acetic acid formed at pH 6 were lower as compared to pH 8 (**Table 1**), the relative amounts of acetic acid arising from different carbon atoms of glucose were similar (**Table 2**). The effect of reaction temperature on the origin of acetic acid from glucose was also relatively small (**Table 2**). At 90 °C as compared to 120 °C, more acetic acid was formed from the C-5 and C-6 carbon atoms and less acetic acid was formed from the C-3 and C-4 carbon atoms of glucose. On the other hand, the temperature had almost no effect on the formation of acetic acid from C-1 and C-2 carbon atoms of glucose, which remained the major source of acetic acid also at 90 °C.

These experimental results can well be explained by the β-cleavage mechanism employing suitable Maillard intermediates as summarized in **Figure 7**. Hydrolytic β-dicarbonyl

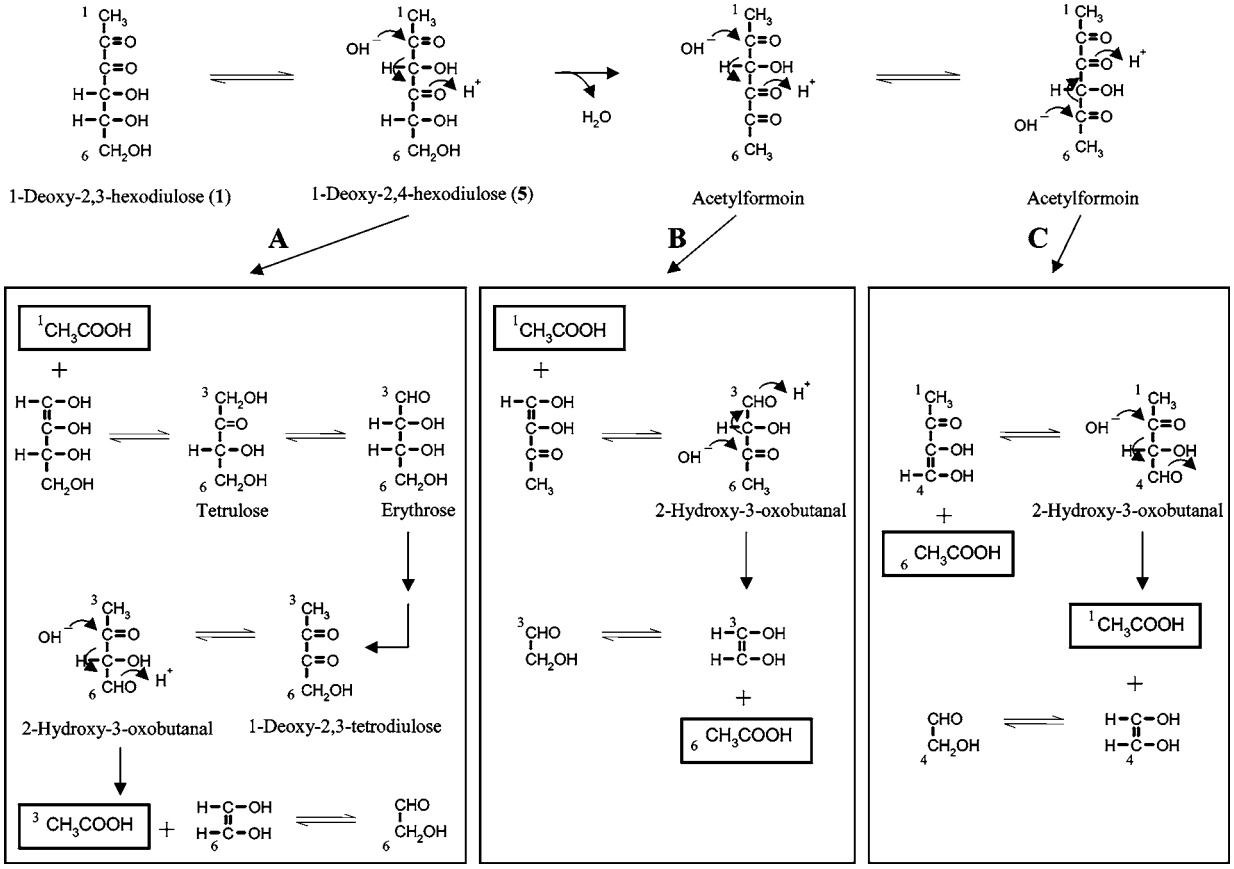


Figure 7. Formation of acetic acid from glucose via 1-deoxy-2,3-hexodiulose as the key intermediate by the hydrolytic β-dicarbonyl cleavage mechanism indicating the various possible degradation pathways. See the text for an explanation.

Table 2. Percentage of Singly Labeled Acetic Acid Arising from ^{13}C-Labeled Glucose Isotopomers in the Presence of Glycine under Different Reaction Conditions

	relative amounts of singly labeled acetic acid (%)[a]			
reaction systems	pH 6, 90 °C, 15 h	pH 6, 120 °C, 4 h	pH 8, 90 °C, 15 h	pH 8, 120 °C, 4 h
[1-^{13}C]glucose/glycine	68.9 ± 0.2	70.3 ± 0.7	69.8 ± 0.2	67.0 ± 0.1
[2-^{13}C]glucose/glycine	70.3 ± 0.7	70.5 ± 0.1	71.4 ± 0.1	68.7 ± 0.7
[3-^{13}C]glucose/glycine	4.3 ± 0.1	7.7 ± 0.3	8.1 ± 0.3	8.6 ± 0.2
[4-^{13}C]glucose/glycine	4.5 ± 0.1	7.4 ± 0.7	7.3 ± 0.1	11.0 ± 0.9
[5-^{13}C]glucose/glycine	25.0 ± 0.4	19.4 ± 0.6	21.9 ± 0.3	22.1 ± 0.1
[6-^{13}C]glucose/glycine	24.2 ± 0.5	18.6 ± 0.3	22.4 ± 0.9	19.6 ± 0.1

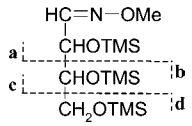
[a] The data obtained by SPME GC-MS showed that variation coefficients were less than 10%.

cleavage of 1-deoxy-2,4-hexodiulose (**5**) (pathway A) leads to acetic acid, containing the C-1/C-2 carbon atoms of glucose, and a C_4-enediol. The latter compound may isomerize to tetrose sugars that are transformed into 1-deoxy-2,3-tetrodiulose via the Maillard reaction cascade, which after isomerization to 2-hydroxy-3-oxobutanal forms by β-dicarbonyl cleavage glycolaldehyde and acetic acid, the latter bearing the C-3/C-4 carbon atoms of glucose. Pathways B and C show the hydrolytic β-dicarbonyl cleavage of 4-hydroxy-2,3,5-hexanetrione (acetylformoin) leading to acetic acid and 2-hydroxy-3-oxobutanal, which can form again via β-cleavage another molecule of acetic acid and glycolaldehyde. These pathways lead to acetic acid containing C-5/C-6 and C-1/C-2 carbons of glucose, respectively. In summary, the scheme proposes three possibilities to form acetic acid from C-1/C-2 carbon atoms, two pathways to form acetic acid from C-5/C-6 carbon atoms, and one option to form acetic acid from C-3/C-4 carbon atoms.

This scheme is in good agreement with the labeling experiments using various ^{13}C-glucose isotopomers (**Table 2**), explaining that (i) all carbon atoms can serve as a source of acetic acid, (ii) the carbon atom pairs C-1/C-2, C-3/C-4, and C-5/C-6 behave similarly, and (iii) acetic acid is preferably formed from C-1/C-2, followed by C-5/C-6, and then C-3/C-4. Furthermore, it explains well why the yield of acetic acid continued to increase (**Figure 3**) whereas no **1** could be detected at pH 6 or higher already after 5 min at 120 °C. Even if **1** is consumed, its reaction products continue to generate acetic acid that can accumulate as a stable end product. Therefore, acetic acid can be seen as a marker of 2,3-enolization in Maillard systems, provided microbial processes can be excluded as an additional source of acetic acid.

Formation of Tetrose Sugars. The following key intermediates are suggested in **Figure 7**: (i) 1-deoxy-2,4-hexodiulose (**5**) formed by isomerization of 1-deoxy-2,3-hexodiulose (**1**), (ii) acetylformoin obtained by dehydration of **5**, (iii) tetroses as degradation products of hexose sugars, and (iv) 2-hydroxy-3-oxobutanal generated from acetylformoin and aldotetroses through the 2,3-enolization pathway. All of these intermediates do not accumulate due to their instability; therefore, it remains difficult to identify them in Maillard reaction samples. While 1-deoxy-2,3-hexodiulose (*25*) and acetylformoin (*26*) have been reported in Maillard reaction systems, C_4-sugars, i.e., erythrose, threose, and tetrulose, have never been unequivocally identified in Maillard samples based on hexose sugars. We have applied

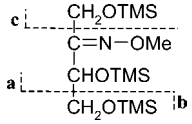
Table 3. Characteristic Mass Spectra (MS) Fragments of Methoxime Trimethylsilyl (TMS) Derivatives of Erythrose (E) and Threose (T) Formed from Glucose and Selected ^{13}C-Labeled Glucose Isotopomers[a]



reaction systems[a]		characteristic fragments, *m/z* (%)					
		[M − Me]$^+$	[a + H]$^+$	[b]$^+$	[c]$^+$	[d]$^+$	[b + H − OTMS]$^+$
D-glucose/glycine	E:	350 (2)	161 (25)	205 (82)	262 (3)	103 (12)	117 (41)
	T:	350 (1)	161 (35)	205 (100)	262 (3)	103 (14)	117 (52)
[1,2-$^{13}C_2$]glucose/glycine	E:	350 (2)	161 (27)	205 (100)	262 (2)	103 (12)	117 (53)
	T:	350 (3)	161 (21)	205 (88)	262 (1)	103 (8)	117 (50)
[4-^{13}C]glucose/glycine	E:	**351** (2)	**162** (15)	205 (76)	**263** (2)	103 (13)	117 (42)
	T:	**351** (1)	**162** (29)	205 (77)	**263** (2)	103 (15)	117 (35)
[6-^{13}C]glucose/glycine	E:	**351** (2)	161 (20)	**206** (77)	262 (3)	**104** (9)	**118** (38)
	T:	**351** (1)	161 (23)	**206** (93)	262 (2)	**104** (8)	**118** (31)

[a] Glycine (0.5 mmol) and D-glucose or various ^{13}C-labeled glucose isotopomers (0.5 mmol) were reacted in a phosphate buffer (5 mL, 0.2 mol/L, pH 6; 120 °C, 2 h). The C-1 of the aldotetrose-TMS derivatives corresponds to C-3 of glucose. Key fragments discussed in the text are marked in bold.

Table 4. Characteristics Mass Spectra (MS) Fragments of Methoxime Trimethylsilyl (TMS) Derivative of Tetrulose Formed from Glucose and Selected ^{13}C-Labeled Glucose Isotopomers



reaction systems[a]	characteristic fragments, *m/z* (%)				
	[M]$^+$	[a]$^+$	[b]$^+$	[c]$^+$	[a − OTMS]$^+$
D-glucose/glycine	365 (4, 4)	262 (9, 32)	103 (36, 35)		173 (24, 26)
[1,2-$^{13}C_2$]glucose/glycine	365 (5, 5)	262 (9, 41)	103 (35, 36)		173 (23, 30)
[4-^{13}C]glucose/glycine	**366** (5, 8)	**263** (8, 55)	103 (36, 52)		**174** (23, 36)
[6-^{13}C]glucose/glycine	**366** (4, 5)	262 (6, 31)	**104** (15, 15)	103 (23, 25)	173 (26, 32)

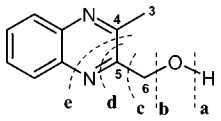
[a] Glycine (0.5 mmol) and D-glucose or various ^{13}C-labeled glucose isotopomers (0.5 mmol) were reacted in a phosphate buffer (5 mL, 0.2 mol/L, pH 6; 120 °C, 2 h). [b] Relative abundance of the corresponding syn and anti forms is shown in brackets. The C-1 of the tetrulose-TMS derivative corresponds to the C-3 of glucose. Key fragments discussed in the text are marked in bold.

various derivatization methods, such as silylation and oximation, to substantiate the presence of tetrose sugars in our model systems. Reference mass spectra and retention indices were obtained by derivatization of commercially available tetrose sugars (Supporting Information), which facilitated their identification. Indeed, we found for the first time unequivocal evidence for the presence of erythrose and threose (**Table 3**) as well as tetrulose (**Table 4**) in Maillard systems based on hexose sugars. The peaks were small indicating only trace amounts of these C_4-sugars, which however is not surprising due to the known high reactivity of these compounds. On the basis of the peak height, more tetrulose (5−10 times) than aldotetroses was obtained under the reaction conditions studied (pH 6 and pH 8).

The labeling pattern of the glucose used was perfectly in agreement with the MS fragments of the C_4-sugar methoxime-TMS derivatives (*27*). The mass spectra obtained with glucose and [1,2-$^{13}C_2$]-labeled glucose were identical, as the C-1 and C-2 atoms are split off leading to labeled acetic acid. On the other hand, the use of [4-^{13}C]- or [6-^{13}C]-labeled glucose resulted in a mass shift of one unit for the molecular mass (**Table 4**) or the [M − Me]$^+$ fragment (**Table 3**). Furthermore, the characteristic fragments of the aldotetrose derivatives at *m/z* 162 ([a + H]$^+$) and *m/z* 263 ([c]$^+$) indicated the labeling position at C-4, and those at *m/z* 206 ([b]$^+$), *m/z* 104 ([d]$^+$), and *m/z* 118 ([b + H − OTMS]$^+$) indicated the labeling position at C-6 (**Table 3**). Similarly, the fragments at *m/z* 263 ([a]$^+$) and *m/z* 174 ([a − OTMS]$^+$) as well as at *m/z* 104 ([b]$^+$) and *m/z* 173 ([a − OTMS]$^+$) were characteristic for the [4-^{13}C]- and [6-^{13}C]-labeling positions in the tetrulose derivatives (**Table 4**).

Formation of 2-Hydroxy-3-oxobutanal. This compound is another key intermediate (**Figure 7**) that has not been reported in mechanistic Maillard reaction studies. It has tentatively been identified by pyrolysis GC-MS as one of the major volatile products of thermally treated caramel syrups (*28*) and amylose (*29*). We could not identify it in our samples using conventional GC-MS. However, it is known that compounds having such chemical functionalities can undergo tautomerization resulting in various $C_4H_6O_3$ entities with methylreductone and dicarbonyl structures (**Figure 8**) (*30*). Only 1-hydroxy-2,3-butanedione (also referred to as 1-deoxy-2,3-tetrodiulose) has been obtained to date as pure material by synthesis, although its preparation is complicated by high instability toward oxidation and aldol type reactions (*30*). The presence of this α-dicarbonyl compound in our Maillard reaction samples was substantiated by derivatization with *o*-phenylendiamine. The corresponding quinoxaline derivative, first reported by Nedvidek et al. (*25*), could indeed be unequivocally attributed to 1-hydroxy-2,3-butanedione by

Table 5. Characteristic Mass Spectra (MS) Fragments of Quinoxaline Derivatives of 1-Hydroxy-2,3-butandione[a] Formed from D-Glucose and Selected ^{13}C-Labeled Glucose Isotopomers



	characteristic MS fragments, *m/z* (%)					
reaction systems[b]	$[M]^+$	$[a]^+$	$[b]^+$	$[c + H]^+$	$[d]^+$	$[e]^+$
D-glucose/glycine	174 (19)	173 (20)	157 (60)	144 (96)	131 (100)	117 (24)
[1,2-$^{13}C_2$]glucose/glycine	174 (19)	173 (19)	157 (60)	144 (98)	131 (100)	117 (23)
[4-^{13}C]glucose/glycine	**175** (20)	**174** (21)	**158** (62)	**145** (100)	**132** (96)	**118** (39)
[6-^{13}C]glucose/glycine	**175** (20)	**174** (21)	**158** (62)	144 (95)	131 (100)	117 (24)

[a] The numbering of carbon atoms in the quinoxaline structure corresponds to that of glucose. [b] Glycine (0.5 mmol) and D-glucose or various ^{13}C-labeled glucose isotopomers (0.5 mmol) were reacted in a phosphate buffer (5 mL, 0.2 mol/L, pH 6, 120 °C, 20 min). Key fragments discussed in the text are marked in bold.

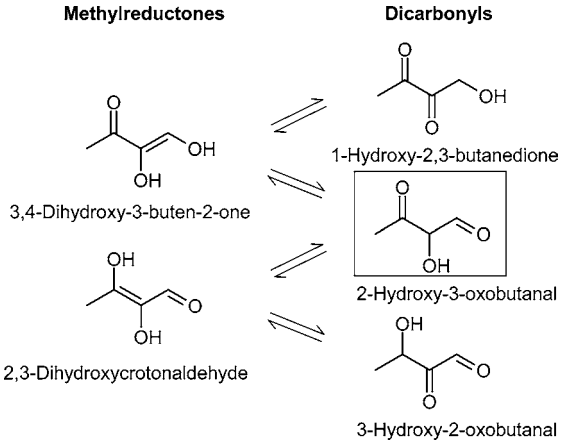


Figure 8. Isomerization of 2-hydroxy-3-oxobutanal into various $C_4H_6O_3$ tautomers with methylreductone and dicarbonyl structures (according to ref *30*).

GC-MS analysis. The MS data of the quinoxaline derivative were in good agreement with those reported by Novotny (*31*). Moreover, the labeling pattern obtained with various ^{13}C-labeled glucose isotopomers was in line with the MS fragments obtained (**Table 5**). Only [4-^{13}C]- and [6-^{13}C]-labeled glucose led to a mass shift of one unit at *m/z* 175 ($[M]^+$), *m/z* 174 ($[M - H]^+$), and *m/z* 158 ($[M - OH]^+$). The fragment at *m/z* 118 ($[e]^+$) was characteristic for the labeling position at C-4, whereas the fragments at *m/z* 144 ($[c + H]^+$), *m/z* 131 ($[d]^+$), and *m/z* 117 ($[e]^+$) were in agreement with the C-6 labeling position. Thus, the experimental results shown in this study, in particular the presence of all key intermediates and the good agreement with the labeling patterns, corroborate our hypothesis on the sugar fragmentation mechanism shown in **Figure 7**. It should be noted that the formation of acetic acid by the hydrolytic β-dicarbonyl cleavage pathway does not require the intermediacy of any oxidizing species, such as radicals or glyoxals.

DISCUSSION

The degradation of sugars to low molecular weight compounds has widely been studied (reviews in *3*, *23*, *32*). In contrast to the literature (*4*, *6*, *12*, *13*) suggesting the direct degradation of **1** via a hydrolytic α-dicarbonyl cleavage at C-2/C-3 as a reaction mechanism (**Figure 1A**), the data reported in this paper corroborated our hypothesis of the hydrolytic β-dicarbonyl cleavage as the major pathway generating acetic acid (**Figure 4B**). Specially designed experiments were performed using model compounds (e.g., 2,3- and 2,4-pentandedione, C_6-deoxy-α-dicarbonyl intermediates) to provide the final evidence. In the Maillard reaction context, the β-cleavage mechanism was first suggested by Hayami (*33*) to explain the formation of acetol from 1-deoxy-2,4-hexodiulose (**5**) by a β-cleavage leading to the scission of the C-3/C-4 bond with glyceric acid as the counterpart. In analogy, acetic acid can be released by scission of the C-2/C-3 bond. This is because the nucleophile HO^- may attack at C-4 and C-2, respectively. Hayami (*33*) suggested α-dicarbonyl sugars to readily isomerize to β-dicarbonyl analogues, also referred to as diacylcarbinols. The isomerization of α-diketones into β-diketones is a well-known transformation of α-ketols. Hesse and Stahl (*34*) have described the isomerization of 4-hydroxy-2,3-pentanedione to diacetylcarbinol via the intermediary eneol compound 3,4-dihydroxy-3-penten-2-one (dimethylreductone). The authors also mentioned the instability of diacetylcarbinol, in particular in alkaline solutions. They also expected a keto cleavage to occur, however, without providing experimental data on the degradation products.

The hydrolytic cleavage of diacylcarbinols can be taken as an extreme case of the acyloin cleavage (*35*): R-CO-CHOH-R → R-COOH + R-CH_2OH. Because β-dicarbonyl sugars belong to the class of diacylcarbinols, they are easily cleaved into an acid and a carbonyl compound, e.g., a β-diketone sugar would result in an α-ketol and a carboxylic acid. In analogy to the formation of acetic acid from β-dicarbonyl sugars presented in this paper, alkaline hydrolysis of diacetylmethylcarbinol (3-methyl-3-hydroxy-2,4-pentanedione) gives rise to acetic acid and acetylmethylcarbinol (3-hydroxy-2-butanone, acyloin) (*36*).

On the other hand, also, β-diketones are known to readily cleave in aqueous alkali to give salts of carboxylic acids (*37*). The mechanism of hydroxyl attack is analogous to that of the saponification of esters. Because β-dicarbonyl sugars also belong to 1,3-dicarbonyl compounds, their base-catalyzed hydrolytic cleavage amounts to a reverse Claisen condensation leading to carboxylic acids and ketones (*38*). The yields of the two possible acids obtained from an asymmetric β-diketone depend on the relative rates of reaction of the two carbonyl groups with the hydroxyl ion. A direct relationship has been found between these yields and the relative rates of alkaline hydrolysis of the corresponding ethyl esters (*38*). In agreement with that, the yields of acetic acids obtained in this study were significantly higher than those of glyceric acid (data not shown, see ref *24*), the two possible carboxylic acids released by alkaline hydrolysis of 1-deoxy-2,4-hexodiulose.

Recently, such a retro-Claisen type reaction has also been employed to explain the formation of formic acid/formamide and 3-deoxypentosulose by a C-1/C-2 splitting of 1-amino-1,4-dideoxy-2,3-hexodiulose, after isomerization to the β-dicarbonyl structure (*39*). Furthermore, Tressl and Rewicki (*40*) have suggested that derivatives of *N*-carboxymethyl amino acids can be formed by a nucleophilic attack (HO^-) at the C-2 carbonyl group of the Maillard intermediate 1,3-dideoxy-1-amino-2,4-hexodiulose leading to a C-2/C-3 cleavage to release the corresponding acetic acid derivative via the so-called "β-dicarbonyl pathway" (*41*). Similarly, Kasper and Schieberle (*42*) have recently suggested a new pathway to N^{ϵ}-carboxymethyllysine without involving free glyoxal or autoxidation reactions. These reactions are in analogy to the mechanism shown in **Figure 4B** and support our hypothesis of the hydrolytic β-dicarbonyl cleavage as a major sugar fragmentation pathway leading to acids or their derivatives without the intermediacy of oxidizing species.

In conclusion, the formation of the simple molecule acetic acid has become clearer. The data reported in this study provide strong evidence for the formation of acetic acid by a hydrolytic β-dicarbonyl cleavage mechanism with 1-deoxy-2,4-hexodiulose as the direct precursor. The corresponding C_4-carbonyl counterparts, obtained by the nucleophilic attack of the hydroxyl ion at the C-2 carbonyl group of 1-deoxy-2,4-hexodiulose via a β-cleavage, are unstable and degrade, thus further releasing additional acetic acid and glycolaldehyde as final reaction products. In general, the formation of acetic acid is a major degradation pathway of sugars and is thought to contribute much to the mass balance in Maillard reaction samples. Therefore, acetic acid can be considered in Maillard samples as a stable marker of the 2,3-enolization pathway, while glycolaldehyde is further reacting. The elucidation of the new pathway was possible thanks to the application of independent and complementary analytical methods to identify both volatile and nonvolatile species. Also, the use of specific intermediates and labeled precursors helped to get a more precise insight into the reaction mechanisms, performed under conditions close to food processing. However, alternative pathways must exist that will be discussed in the following paper (*24*).

ACKNOWLEDGMENT

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Supporting Information Available: Mass spectra and retention indices of sugar derivatives. This material is available free of charge via the Internet at http://pubs.acs.org.

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