Elucidation of Chemical Pathways in the Maillard Reaction by $^{17}$O-NMR Spectroscopy

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ABSTRACT: $^{17}$O-NMR spectroscopy was employed as an innovative method to help understand mechanistic pathways in sugar fragmentation. Elucidation of reaction mechanisms to final Maillard end products was achieved by starting from specific intermediates obtained by synthesis, such as 1-deoxy-D-erythro-hexo-2,3-diulose. This $\alpha$-dicabonyl was thermally treated in the presence of $^{17}$O-enriched water under alkaline conditions. The reaction products were monitored by $^{17}$O-NMR spectroscopy and their structures corroborated by complementary techniques. For the first time, evidence is shown for the direct formation of acetic acid from 1-deoxy-D-erythro-hexo-2,3-diulose by an oxidative $\alpha$-dicarbonyl cleavage and incorporation of a $^{17}$OH group into the acetic acid released as sugar fragment.

KEYWORDS: Maillard reaction; acetic acid; $^{17}$O-NMR spectroscopy; mechanism; $\alpha$-dicarbonyl cleavage

INTRODUCTION

Despite numerous studies conducted to understand the Maillard reaction cascade, many chemical pathways are still speculative. For example, the role of oxygen and water in oxidation and other reaction phenomena is largely unclear. Even after 90 years of Maillard chemistry, the formation mechanism of acetic acid is still hypothetical. Currently, the 1-deoxyhexo-2,3-diulose 6 is generally accepted as the key Maillard intermediate, which is thought to degrade to acetic acid 8 and erythrose 7a ($X = H$) as shown in FIGURE 1. Similarly, it is assumed that formic acid 5 can be released from 3-deoxyhexos-2-ulose 4. However, no relevant experiments have been performed so far to substantiate this hypothesis, despite the fact that up to 60 mol-% acetic acid can be generated by Maillard reactions. In general, the limited knowledge of mechanisms is partially due to the complexity of the reaction cascade, but also to the limitation of the analytical techniques employed. Application of novel analytical tools combined with labeling experiments may be an attractive approach to answer specific questions related to formation mechanisms.

Up to the 1980s the application of $^{17}$O spectroscopy to organic chemistry has been relatively limited due to the low natural abundance (0.037%) and the quadrupolar properties of the $^{17}$O nucleus. However, $^{17}$O enrichment and the use of high-
FIGURE 1. Reaction of an aldohexose 1 and an amino acid to the corresponding N-glycosylconjugate 2 followed by rearrangement to the Amadori compound 3 shown in the open-chain form, leading to the α-dicarbonyls 3-deoxyhexos-2-ulose 4 and 1-deoxy-2,3-hexodiulose 6, formed via 1,2-enolization and 2,3-enolization, respectively, yielding formic acid 5 and acetic acid 8 as sugar fragmentation products. Erythrose: 7a (X = H); erythronic acid: 7b (X = OH).

field FT-NMR spectrometers has dramatically encountered these difficulties.\(^7\) Thus, \(^{17}\text{O-}\text{NMR}\) has been used as a tool to investigate reaction mechanisms, especially in organic chemistry.\(^8\) Although the reaction between a reducing sugar and an amino acid has been known for long time, \(^{17}\text{O}\) has never been employed to substantiate mechanistic steps.

In this paper we report on a new application of \(^{17}\text{O-}\text{NMR}\) spectroscopy and the use of labeled oxygen as a powerful tool to validate formation mechanisms in the Maillard reaction, which are frequently mentioned but still of a hypothetical nature. The putative key intermediate 1-deoxyhexo-2,3-diulose 6 was synthesized and its degradation studied in the presence of \(^{17}\text{O-enriched water}\). We show for the first time unequivocal evidence, based on \(^{17}\text{O-}\text{NMR}\) and GC-MS data, that the Maillard intermediate 1-deoxyhexo-2,3-diulose 6 is, indeed, a direct precursor of acetic acid formed by oxidative α-dicarbonyl cleavage.
Materials and Methods

Materials

2,3-O-Isopropylidene-D(-)-erythronolactone, tert-butyllithium (1.7 M solution in pentane), ethylvinyl ether, tetrahydrofuran (THF, anhydrous), ammonium chloride (NH₄Cl), magnesium sulfate (MgSO₄), Florisil (100–200 mesh), Dowex 50X8 (100–200 mesh) ion exchange resin (H⁺-form), metallic sodium, methanol (MeOH), diethyl ether (Et₂O), petroleum ether, ethyl acetate (EtOAc), aqueous hydrochloric acid (HCl, 1 M), and deuterochloroform (CDCl₃, >99%) were from Aldrich/Fluka (Buchs, Switzerland). Ten percent ¹⁷O-enriched water was from CIL (Andover, MA).

Synthesis

1-Deoxy-D-erythro-hexo-2,3-diulose 6. This was synthesized from commercially available 2,3-O-isopropylidene-D(-)-erythronolactone 9 as recently described by Glomb and Pfahler⁹ (Fig. 2) using some modifications detailed below.

1-Deoxy-2-O-ethyl-4,5-O-isopropylidene-D-erythro-hex-1-en-3-ulose 10. tert-Butyllithium in pentane (30 mL, 1.1 eq., 51.4 mmol, 1.7 M) was slowly added to a solution of ethylvinyl ether (7.5 mL, 1.6 eq., 78.4 mmol) in 45 mL THF held at −65°C. The colorless solution was spiked at −65°C with a solution of the starting material 9 (7.7 g, 1.0 eq., 49.0 mmol) in THF (30 mL). After stirring the solution at 0°C for 30 min the reaction mixture was quenched with a saturated NH₄Cl solution (50 mL). The target compound was extracted with Et₂O (3 x 150 mL), dried over MgSO₄, and concentrated under reduced pressure. After purification with medium pressure liquid chromatography on Florisil (eluent: petroleum ether/EtOAc, 9:1, v/v) compound 10 was obtained as colorless oil (4.4 g, 11.3 mmol, 40% yield). ¹H NMR (360 MHz, CDCl₃) δppm: 1.03 (t, J = 7.0 Hz, 3H, H₈); 1.17, 1.36 (s, 6H, H₉); 3.55 (q, J = 7.0 Hz, 2H, H₆); 3.89 (m, 2H, H₇); 4.43 (d, J = 6.0 Hz, 1H, H₃); 4.59 (ddd, J = 6.0 Hz; J = 4.1 Hz, J = 1.1 Hz, 1H, H₄). ¹³C NMR (90 MHz, CDCl₃) δppm: 14.7, 14.8 (C₁, C₁₁); 25.2, 25.7, 26.6, 26.7 (C₁, C₈, C₉); 63.8, 63.9 (C II, C₆); 70.6, 71.7 (C II, C₁₀); 80.7, 81.2 (C III, C₄); 83.4, 84.7 (C II, C₁); 85.9 (C III, C₅); 102.5, 105.1 (C IV, C₃); 113.1, 113.7 (C IV, C₇); 158.3, 159.1 (C IV, C₂).
1-Deoxy-4,5-O-isopropylidene-δ-erythro-hexo-2,3-diulose 11. Aqueous HCl (0.8 mL, 1 M) was added to a solution of compound 10 (1.6 g, 7.0 mmol) in MeOH (40 mL). The solution was stirred at room temperature for 90 min. Reaction mixture was concentrated under reduced pressure, taken up in 10 mL water, and extracted with EtOAc (4 × 20 mL). The organic layer was dried over MgSO₄ leading after recrystallization in Et₂O to yellow crystals of compound 11 (1.1 g, 5.5 mmol, 78% yield). Melting point (Büchi 510): 77°C. 1H NMR (360 MHz, CDCl₃) δ ppm: 1.22, 1.46 (s, 6H, Hₑ); 2.34 (s, 3H, Hₐ); 4.04 (m, 2H, Hᵇ); 4.49 (d, J = 5.7 Hz, 1H, Hᵈ); 4.89 (dd, J = 5.7 Hz, J = 3.9 Hz, 1H, Hᶜ).

13C NMR (90 MHz, CDCl₃) δ ppm: 25.5 (C₁, Cl); 26.2, 27.0 (C₁, C₈, C₉); 73.4 (C₆, C₅); 80.7 (C₃, C₅); 87.5 (C₃, C₄); 105.1 (CⅣ, C₂); 113.7 (CⅣ, C₇); 206.5 (CⅣ, C₃).

COMPOUND 11

1-Deoxy-δ-erythro-hexo-2,3-diulose 6. A solution of 11 (0.2 g, 1.0 mmol) in water (14 mL) was stirred with Dowex 50 Wx8 (14 mL) at room temperature under argon atmosphere. After 90 min, the resin was filtered off and washed with MeOH. Evaporation of the combined solvents led to the target compound 6 as a colorless oil (0.4 g, 0.2 mmol, 26% yield). 13C NMR (90 MHz, CDCl₃) δ ppm: 22.6 (C₁, C₁); 63.6 (C₁, C₆); 72.9, 75.4 (CⅢ, C₄, C₅); 97.7 (CⅣ, C₂); 204.5 (CⅣ, C₃).

COMPOUND 6

Model Reactions

The α-dicarbonyl 6 (0.05 mmol) and 10%-¹⁷O-enriched water (0.5 mL) under basic conditions (addition of metallic sodium up to pH 10) were placed into a screw-cap vial (1.5 mL, Infochroma, Zug, Switzerland). The solution was then thermally treated at 90°C in a silicone bath for 30 min and diluted 20 times with nonlabeled water prior to ¹⁷O-NMR spectroscopy.
Nuclear Magnetic Resonance (NMR) Spectroscopy

Samples obtained by synthesis were prepared in Wilmad 528-PP Pyrex NMR tubes (5-mm i.d.) using CDCl₃ as solvent (0.7 mL). The NMR spectra were acquired on a Bruker AM-360 spectrometer equipped with a quadrinuclear 5-mm probe head at 360.13 MHz for ¹H and at 90.03 MHz for ¹³C. One-dimensional ¹H NMR, ¹³C NMR, and distortionless enhancement by polarization transfer (DEPT 135) spectra were acquired as described earlier using standard conditions. Chemical shifts are cited in ppm relative to the solvent signal. Abbreviations describing multiplicities are as follows: s, singlet; d, doublet; t, triplet; q, quartet; m, multiplet; br, broad; Cᵢ, primary (sp³) carbon; Cᵢᵢ, secondary (sp²) carbon; Cᵢᵢᵢ, tertiary (sp) carbon; Cᵢᵣ⁴, quaternary carbon.

Samples obtained from the model reaction were diluted 20 times with non-labeled water prior to ¹⁷O-NMR spectroscopy. ¹⁷O-NMR spectra were recorded on a Bruker DPX-360 spectrometer, equipped with a 5-mm BBO grad probe head operating at 48.82 MHz. The instrumental setting was as follows: Spectral width 100 kHz, 8 K data points, 90° pulse angle 9.90 msec, 200 μs acquisition delay, 41 ms acquisition time; 70,000–120,000 scans were required. The spectra were recorded with sample spinning, without lock at room temperature. The pulse sequence was P1(90°) P2(90°) P3(90°). The signal-to-noise ratio was improved by applying a 20-Hz exponential broadening factor to the FID prior to Fourier transformation.

Gas Chromatography–Mass Spectrometry (GC-MS)

The fiber polydimethylsiloxane-divinylbenzene (PDMS-DVB, Supelco) was exposed for 30 min at 25°C to the headspace above the samples in the glass vials. The volatile compounds adsorbed on the SPME fiber were desorbed for 5 min in an injector port 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). After insertion of the SPME device into the injector, the oven temperature program was started, and the temperature was raised at 6°C/min from 20 to 240°C and held for 10 min isothermally. Mass spectra in the electron impact mode (EI) were generated at 70 eV and at a scan range from m/z 28 to 350.

RESULTS AND DISCUSSION

Synthesis

1-Deoxy-D-erythro-hexo-2,3-diulose 6 was prepared following the synthesis procedure shown in Figure 2. Chain elongation of 2,3-O-isopropylidene-D(-)-erythronolactone 9 was achieved with ethoxyvinyllithium used as acyl equivalent (40% yield). The intermediary vinylether 1-deoxy-2-O-ethyl-4,5-O-isopropylidene-D-erythro-hex-1-en-3-ulose 10 was transformed under mild acidic conditions into the isopropylidene-protected acyl derivative 11 (78% yield) shown in the cyclic form. Peak splitting of ¹H- and ¹³C-NMR data of 10 and 11 revealed the coexistence of two diastereoisomers. They may result from both the nondiastereoselectivity of
FIGURE 2. Synthesis of 1-deoxy-D-erythro-hexo-2,3-diulose 6 from 2,3-O-isopropylidene-D-erythronolactone 9 via 1-deoxy-2-O-ethyl-4,5-O-isopropylidene-D-erythro-hex-1-en-3-ulose 10, and 1-deoxy-4,5-O-isopropylidene-D-erythro-hexo-2,3-diulose 11. Reagents: a, ethoxyvinylithium, THF, -65°C; b, aqueous HCl (1 M), MeOH, RT; c, Dowex 50 W x 8 (H⁺-form); RT, room temperature.

FIGURE 3. Isomerization of 1-deoxy-2-O-ethyl-4,5-O-isopropylidene-D-erythro-hex-1-en-3-ulose 10, as indicated by NMR data.

the nucleophilic addition and isomerization of the target molecules as illustrated in Figure 3.

The protection group was readily cleaved off under controlled conditions using a protonated ion-exchange resin to afford the target compound 6 (26% yield) shown in the open-chain form. The moderate yield for the last step is most likely due to the high instability of compound 6. So far, it had never been isolated from food products or model reactions. However, it has successfully been trapped as a quinoxazoline derivative, thus verifying its presence in the Maillard reaction. 12, 13

Model Reactions and 17O NMR

The degradation of the α-dicarbonyl 6 was studied in model reactions using 10% 17O-enriched water under alkaline conditions (pH 10). Figure 4 shows the 17O-NMR spectrum of the reaction mixtures using H₂O as reference for the chemical shift (δ 0.00 ppm). Due to 17O enrichment, the spectrum is relatively clear despite the high concentration of the experimental well-colored sample. The observed signals correspond only to oxygen originating from 17O-enriched water, which provides a direct insight into the degradation mechanism. Signals were neither detected in the carbonyl range (+545 to +625 ppm) nor in the alcohol range (−50 to +70 ppm). These data indicate that 17O did not exchange with the initial functional groups of the starting material 6 and that the oxygen from water was not incorporated into the alcohol or carbonyl groups via chemical reactions.
FIGURE 4. $^{17}$O NMR spectra of reaction samples obtained by (A) heating 1-deoxy-d-erythro-hexo-2,3-diulose 6 in $^{17}$O-enriched water (pH 10) at 90°C for 30 min and (B) spiking the heated sample with acetic acid 8 in the ratio 1:1 (v/v).

FIGURE 4A shows in the carboxylic acid range a broad signal (260–270 ppm) and a narrow peak at 282 ppm. According to literature data, the chemical shift of acetic acid as pure liquid is 250.5 ppm at room temperature. However, a spiking experiment, by mixing acetic acid and the reaction mixture in a 1:1 ratio, clearly indicated a matrix effect on the chemical shift of acetic acid: the narrow peak at 282 ppm disappeared, and a new signal was observed at 258 ppm (Fig. 4B). This is the first unequivocal evidence for the reaction $6 \rightarrow 8$ (Fig. 1)—that is, the intermediacy of the sugar-derived Maillard compound 6 in the formation of acetic acid 8.

The broad signal (260–270 ppm) in FIGURE 4A might be attributed to erythronic acid 7b ($X = OH$), which represents the remaining moiety of compound 6 as shown in FIGURE 1. This is supported by data obtained by derivatization of the reaction mixture and identifying the corresponding silylated derivative of 7b by GC-MS (data not shown). These data will be reported elsewhere in a more extended paper dealing with oxidative sugar degradation.

Model Reactions and GC-MS

GC-MS analysis of the reaction mixture of 6 exhibited both nonlabeled and labeled acetic acid (Fig. 5), as indicated by the mass spectrum with the molecular ions at $m/z$ of 60 and 61, respectively. The ion at $m/z$ 62 corresponds to $^{18}$O-labeled acetic acid originating from $\text{H}_2^{18}$O present in the commercial $^{17}$O-enriched water. The fragments at $m/z$ 43 ([M-OH]$^+$) and 45 ([M-CH$_3$]$^+$) for the nonlabeled acetic acid as well as at $m/z$ 43 ([M-$^{17}$OH]$^+$) and 46 ([M-CH$_3$]$^+$) for the $^{17}$O-labeled analogue and at $m/z$ 43 ([M-$^{18}$OH]$^+$) and 47 ([M-CH$_3$]$^+$) for the $^{18}$O-labeled isotopomer were also observed, suggesting the formation of CH$_3$CO$^{17}$OH and CH$_3$CO$^{18}$OH as reaction products. Furthermore, the similar pattern of the ions at $m/z$ 45, $m/z$ 46, $m/z$ 47 for [M-CH$_3$]$^+$ and $m/z$ 60, $m/z$ 61, $m/z$ 62 for [M]$^+$ indicate the same fragmentation pattern, whereas all acetic acid isotopomers result in the fragment at $m/z$ 43 ([M-*OH]$^+$), which consequently is the base peak in the mass spectrum (Fig. 5).

To verify if the oxygen atom of the acetic acid exchanged with oxygen from water, unlabeled acetic acid was heated under the same experimental conditions (90°C, 30 min, pH 10) in 10% $^{17}$O-enriched water. We did not observe by GC-MS any ox-
ygen exchange between acetic acid and the $^{17}$O-enriched water under these conditions, confirming the validity of our results.

**Reaction Mechanism**

Based on these experimental data obtained by $^{17}$O NMR and GC-MS, we can confirm the formation of acetic acid 8 from the $\alpha$-dicarbonyl 1-deoxy-$d$-erythro-hexo-2,3-diulose 6. Moreover, we found evidence for the presence of erythronic acid 7b ($X = OH$). This is in contrast to literature data$^{3,4}$ assuming erythrose 7a ($X = H$) as a decomposition product of 6, however without providing any experimental proof. We suggest that acetic and erythronic acid are formed from 1-deoxy-$d$-erythro-hexo-2,3-diulose 6 via an oxidative $\alpha$-dicarbonyl cleavage (Fig. 6). The $\alpha$-dicarbonyl 6 may undergo oxidation with molecular oxygen followed by single electron transfer giving rise to the intermediary hydroperoxide anion 12, which leads to anhydride 13 by a Baeyer-Villiger-type rearrangement. The anhydride is susceptible to hydrolysis, finally releasing the organic acids 7b and 8 by incorporating oxygen from water. This hypothesis combines the photooxidation mechanism of $\alpha$-dicarbonyls leading to triplet diketone-oxygen adducts$^{15}$ with the based-catalyzed Baeyer-Villiger reaction of hydroperoxide anions.$^{16}$
FIGURE 6. Hypothetical formation mechanism explaining the formation of erythronic acid 7b and acetic acid 8 by thermally induced decomposition of 1-deoxy-D-erythro-hexo-2,3-diulose 6. Reaction steps: a, oxidation with molecular oxygen followed by a single electron transfer; b, Baeyer-Villiger rearrangement of the hydroperoxide anion 12; c, hydrolysis of the anhydride 13 with 17O-enriched water yielding the organic acids 7b and 8.

CONCLUSIONS

In this paper we show for the first time unequivocal evidence, based on labeling experiments and 17O-NMR and GC-MS data, that the Maillard intermediate 1-deoxy-D-erythro-hexo-2,3-diulose 6 is a direct precursor of acetic acid formed by oxidative α-dicarbonyl cleavage. The mechanism proposed for the formation of acetic and erythronic acid proceeds via oxidation of 6 with molecular oxygen followed by Baeyer-Villiger rearrangement and hydrolysis of the anhydride with 17O-enriched water, incorporating the 17OH group into the acids. However, we found in the course of this study an alternative pathway of acetic acid formation by a β-elimination reaction of a β-dicarbonyl intermediate obtained by isomerization of the α-dicarbonyl 6. We have also demonstrated 17O-NMR spectroscopy and the use of labeled oxygen (17O-enriched water) as a powerful tool in elucidating formation mechanisms in the Maillard reaction, many of which are frequently reported but still of a hypothetical nature. Such work may require the synthesis of specific Maillard intermediates.

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REFERENCES


