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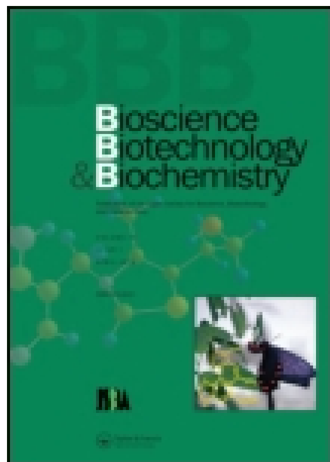

Bioscience, Biotechnology, and Biochemistry
Publication details, including instructions for authors and subscription information:
http://www.tandfonline.com/loi/tbbb20

Identification of 3,4-Dihydroxy-2-oxo-butanal (L-threosone) as an Intermediate Compound in Oxidative Degradation of Dehydro-L-ascorbic Acid and 2,3-Diketo-L-gulonic Acid in a Deuterium Oxide Phosphate Buffer

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Published online: 22 May 2014.

To cite this article: Yoko NISHIKAWA, Yuka TOYOSHIMA & Tadao KURATA (2001) Identification of 3,4-Dihydroxy-2-oxo-butanal (L-threosone) as an Intermediate Compound in Oxidative Degradation of Dehydro-L-ascorbic Acid and 2,3-Diketo-L-gulonic Acid in a Deuterium Oxide Phosphate Buffer, Bioscience, Biotechnology, and Biochemistry, 65:8, 1707-1712, DOI: 10.1271/bbb.65.1707

To link to this article: http://dx.doi.org/10.1271/bbb.65.1707

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Biosci. Biotechnol. Biochem., 65 (8), 1707–1712, 2001

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Identification of 3,4-Dihydroxy-2-oxo-butanal (L-threosone) as an Intermediate Compound in Oxidative Degradation of Dehydro-L-ascorbic Acid and 2,3-Diketo-L-gulonic Acid in a Deuterium Oxide Phosphate Buffer

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Received October 4, 2000; Accepted March 28, 2001

Dehydro-L-ascorbic acid (DAA), an oxidation product of L-ascorbic acid (vitamin C), is unstable in the neutral and basic pH regions. When DAA was incubated in a phosphate buffer with deuterium oxide (pH 7.4), it was degraded to form the main degradation compound, which was identified as 3,4-dihydroxy-2-oxo-butanal (L-threosone). This compound was also formed from diketo-L-gulonic acid (DKG) in a phosphate buffer with deuterium oxide. L-threosone had reducing activity, probably due to its enolization, and is likely to have been involved in the formation of the reducing activity that was observed in aqueous DAA and DKG solutions. As a reactive dicarbonyl compound, L-threosone might also take some role in the cross-linking of tissue proteins that are formed *in vivo* in the Maillard reaction.

Key words: L-ascorbic acid; degradation; L-threosone; diketo-L-gulonic acid

L-Ascorbic acid (AA), also referred to as vitamin C, is a well-known antioxidant in food and biological systems which generally serves as an electron donor. In these antioxidative reactions, dehydro-L-ascorbic acid (DAA) is mostly produced by disproportionation *via* monodehydro-L-ascorbic acid (Fig. 1).[1,2] DAA is either reduced to AA by glutathione-dependent DAA reductases[3] or by glutathione itself,[4] or degraded to 2,3-diketo-L-gulonic acid (DKG)[5] and further degraded to other products due to the instability of DKG in the neutral pH region.[6–8] The hydrolysis reaction of DAA to DKG is essentially irreversible, therefore, DAA is thought to be the key compound in AA catabolism. The reaction of DAA to AA or to DKG is complicated and is influenced by the reaction conditions, pH value, solvent, and presence of oxidants and reductants like glutathione.[9,10]

The degradation reaction of DKG is also complicated and the degradation mechanism after DKG has not been unambiguously clarified, although some degradation products after DKG have been identified. Such degradation products from DKG as the endiol forms of 2,3-diketogulono-δ-lactone[11,12] and erythroascorbic acid[13] have reducing activity and can be expected to contribute in some way as a reducing agent like AA *in vivo*. Some of the other degradation compounds have di- and poly-carbonyl groups[14,15] which are conversely intermediates of the Maillard reaction *in vivo*.[16] The Maillard reaction has been suggested to be closely related to aging and to the development of adult diseases like diabetes[17] that damage the human body. Thus, the oxidative degradation products of AA are considered to have some effects *in vivo*,[18,19] although details of the AA catabolic pathway and the physiological effects of the oxidative degradation products of AA on biological systems have not been clarified.

In this report, an intermediate product in the degradation reaction of DAA and DKG at pH 7.4 was investigated in a preliminary study to clarify the degradation products of AA and its degradation pathway *in vivo*.

Materials and Methods

Materials. The deuterium-labeled solvents and reagents used in the ^{1}H-NMR and ^{13}C-NMR measurements were all guaranteed-grade reagents from Merck. The other reagents used in this research were purchased from Wako Pure Chemical Industries Co.

NMR and FAB-MS analyses. ^{1}H-NMR and ^{13}C-NMR spectra were obtained by JEOL GSX 270 and JEOL GX 400 FT NMR spectrometers. FAB-MS spectra were recorded with a JEOL JMS-700 instrument, using glycerol as a matrix and ionization by FAB with Xe atoms.

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Abbreviations: AA, L-ascorbic acid; DAA, dehydro-L-ascorbic acid; DKG, 2,3-diketo-L-gulonic acid; D_2O-PB; phosphate buffer with D_2O; TLC, thin-layer chromatography; t_R, retention time

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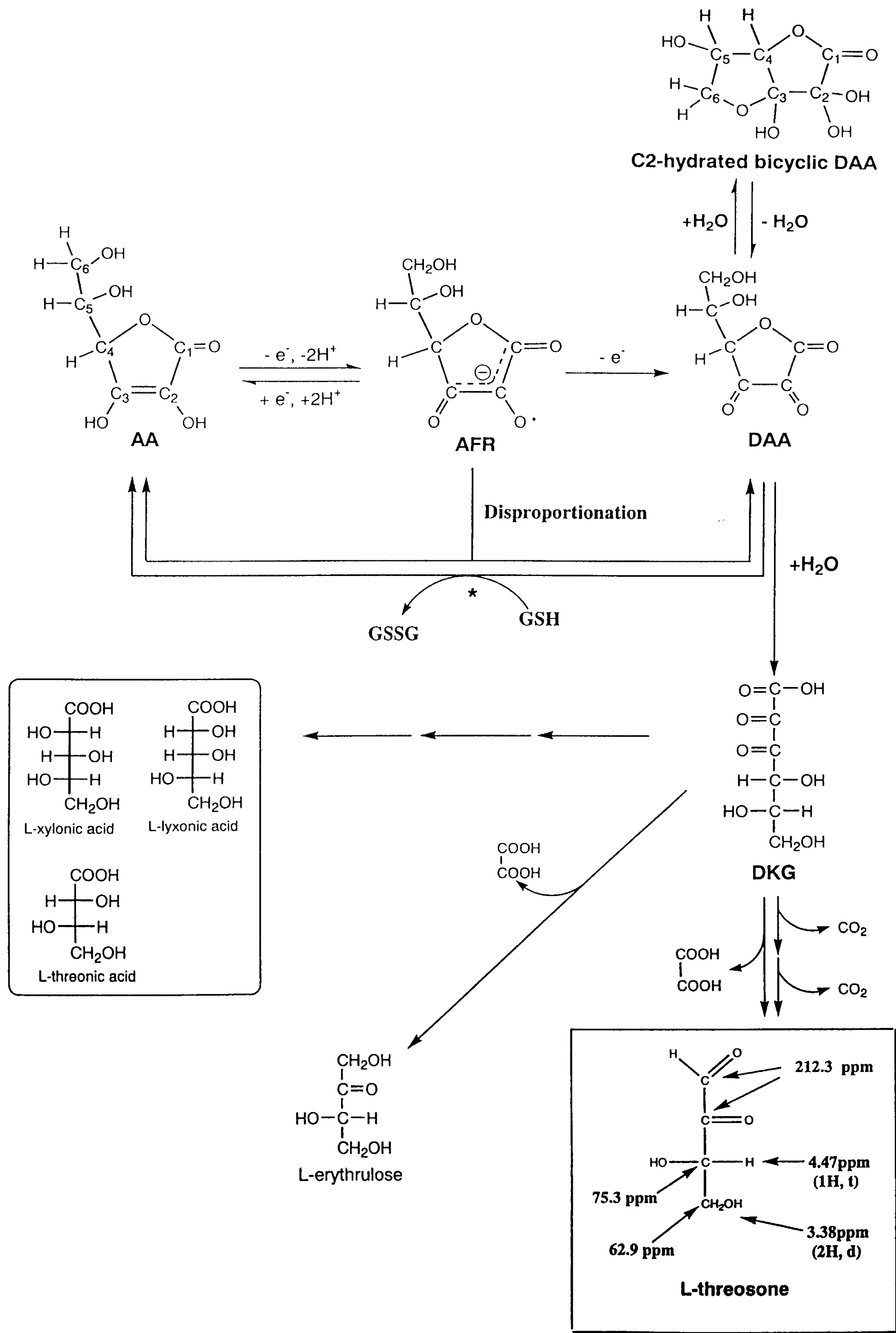

Fig. 1. AA Degradation Pathway and Possible Formation Pathway for L-Threosone.
AFR, monodehydro-L-ascorbic acid; *DAA reductase.

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Preparation of DAA. DAA was prepared from AA in methanol by the method described in the literature[20] with minor modifications and obtained as a pale syrup which was kept in a freezer at −20°C before its use. The identification and purification of DAA was accomplished by NMR and thin-layer chromatography (TLC), using a solvent system of acetonitrile-acetone-water-acetic acid (80:5:15:1, v/v) and silicagel 60 as the adsorbent, and by high-performance liquid chromatography (HPLC) under

the following conditions: column, ODS-2 (150 × 4.6 mm i.d., GL Sciences); mobile phase, sodium phosphate buffer (50 mM at pH 2.3); flow rate, 0.7 ml/min; column temperature, room temperature of around 20°C; detection, electrochemical detector (BAS LC-4B, 400 mV). DAA was reduced to AA by DTT before the HPLC analysis.

Preparation of the DKG potassium salt. The DKG potassium salt was prepared from AA by the method described in the literature[5] and obtained as a white hygroscopic powder that was stored at −20°C before use. The DKG potassium salt was identified by TLC under the same condition as those just described for the DAA preparation.

LC-MS analysis of DKG-1. The DKG potassium salt (5 g) was dissolved in 100 ml of a phosphate buffer with deuterium oxide (D_2O-PB; 0.5 M, pH 7.4) and incubated at room temperature for 7 h. The formation of DKG-1 in the incubated solution was confirmed by TLC and ^{1}H-NMR under the same conditions as those just described for the preparation of DAA and DKG. DKG-1 was eluted at t_R 2.1 min under the following HPLC operating conditions: column, Inertsil ODS-2 (150 × 4.6 mm i.d.; GL Sciences); mobile phase, methanol; flow rate, 0.8 ml/min; detection, UV spectrometer (Shimadzu SPD-6A at 220 and 280 nm) and electrochemically (BAS LC-4B, 400 mV). The incubated sample was analyzed by LC-MS. HPLC with a TSP p4000 instrument (Thermoquest) was carried out under the following conditions: column, Inertsil ODS-2 (150 × 4.6 mm i.d., GL Sciences); mobile phase, methanol; flow rate, 1.0 ml/min; detection, UV spectrometer at 220 nm. A Finnigan LCQ instrument was used for mass spectrometry under the following conditions: ionization, chemically at atmospheric pressure; sease gas, nitrogen 85 unit; auxiliary gas, nitrogen 30 unit; vaporizer temperature, 450°C; heating capillary temperature, 175°C; corona current, 5 μA.

Preparation of the hydrazone of DKG-1. Phenylhydrazine derivatives of DKG-1 was prepared by dissolving the DKG potassium salt (5 g) in 100 ml of D_2O-PB (0.5 M, pH 7.4) and leaving at room temperature for 7 h. To the incubated solution was added a phenyl hydrazine hydrochloride solution dissolved in methanol (6 g/30 ml), and the mixture was incubated overnight at room temperature. The reaction mixture was extracted three times with diethyl ether, dried with anhydrous sodium sulfate, and concentrated by evaporation. The obtained phenylhydrazone mixture was further purified by TLC. The phenylhydrazone of DKG-1 compound corresponding to the main spot at R_f 0.4 was separated by using silica gel 60 plates (Merck Art. No. 5745) with toluene-ethyl acetate (1:3, v/v) as the developing solvent, extracted with ethyl acetate, and obtained as a yellow syrup after removing the solvent by evaporation.

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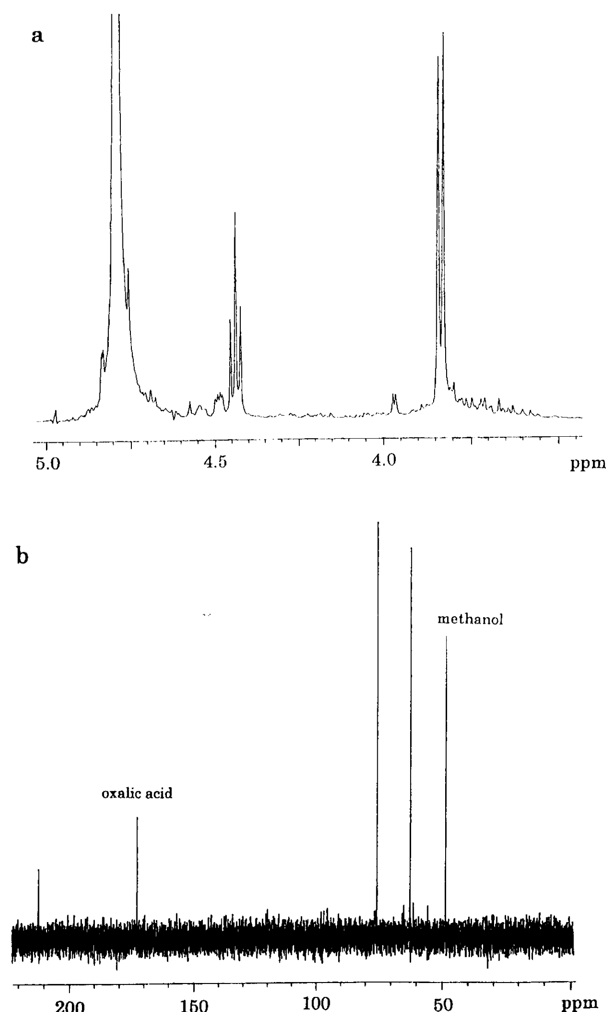

Fig. 2. NMR Spectra of a DKG Solution (250 mM) Incubated for 7 h in a Phosphate Buffer with D_2O (pH 7.4).

a: ^{1}H-NMR spectrum; **b**: ^{13}C-NMR spectrum. Methanol (1% v/v) was added as an internal standard for reference before the NMR analysis.

Results and Discussion

A relatively stable degradation compound (DKG-1) was produced when DAA or the DKG potassium salt was incubated in D_2O-PB (pH 7.4) at room temperature for 7 h, this being identified by an NMR analysis (Fig. 2).

In the ^{1}H-NMR spectrum of the incubated solution of DKG, which included DKG-1 as the main degradation compound, two signals were observed at δ_H = 4.47 ppm (1H, t) and 3.38 ppm (2H, d), the coupling constant being J = 3.96 Hz (Fig. 2(a)). In the ^{13}C-NMR spectrum of the solution, four signals were observed at δ_C = 212.3, 173.0, 73.0 and 63.2 ppm (Fig. 2(b)), the signal at δ_C = 212.3 ppm being split into 212.3 and 212.2 ppm when the pH value of the sample solution was reduced by adding phosphoric

acid. These signals in the ^{1}H- and ^{13}C-NMR spectra are apparently different from those of DKG (δ_H = 3.75 ppm (4H, m); δ_C = 174.2, 94.4, 94.9, 74.4, 68.4 and 62.2 ppm). The signal observed at δ_C = 173.0 is considered to have been due to oxalic acid, which agrees quite well with the NMR data reported in the previous work.[15] The results of ^{13}C-NMR therefore suggest that DKG-1 consisted of four carbons. Furthermore, in the off-resonance decoupling ^{13}C-NMR and C-H COSY analysis made with the same sample solution, mutual coupling was observed between 4.47 ppm (δ_H) and 73.0 ppm (δ_C), and 3.38 ppm (δ_H) and 63.2 ppm (δ_C), suggesting that the glycol structure corresponding to C5-C6 of AA was retained in DKG-1. On the other hand, the formation of DKG-1 was only observed when the DKG potassium salt was incubated in D_2O-PB, and not in the other solvents (Fig. 3).

In order to elucidate the structure and chemical characteristics of DKG-1, DKG-1 was isolated by TLC. DKG-1 gave a round spot (R_f = 0.61) on the TLC plate which was positive to the coloring detection reagents, 2,4-dinitrophenyl hydrazine and α,α'-dipyridyl ferric chloride.

The molecular weight of DKG-1 was deduced to be 118 from the result of an LC-MS analysis carried out in the negative mode (data not shown). Further confirmation of the molecular weight of DKG-1 was provided by preparing its phenylhydrazone (DKG-1-PZ) whose molecular weight was determined to be 298 by an FAB-MS analysis (Fig. 4, **a-1** and **a-2**). The result of the FAB-MS analysis indicates that DKG-1-PZ contained two molecules of a phenylhydrazine residue, this being supported by the results of a ^{1}H-NMR analysis (Fig. 4, **b**). This set of experimental results led to the structure of DKG-1 being concluded to be L-threosone (Fig. 1). Although L-threosone seemed to be comparatively stable in D_2O-PB, it could not be completely purified due to its unexpectedly easy degradation during the separation procedure.

Simpson *et al.* have recently identified L-erythrulose, which had different ^{1}H- and ^{13}C-NMR spectral data from those of L-threosone, as the main degradation product of DAA and DKG under similar experimental conditions to ours, by which DAA or DKG were incubated in a phosphate buffer at a neutral pH value at room temperature.[15] In order to clarify the apparent discrepancy between these two experiments, we used a phosphate buffer solution [D_2O:H_2O = 1:9] as the reaction medium used by Simpson *et al.*[15] instead of D_2O used in our original experiments. L-Erythrulose was confirmed to be the major reaction product by ^{1}H- and ^{13}C-NMR (data not shown). This result strongly suggests that not only the phosphate buffer, but also D_2O strongly influenced the formation of L-threosone. The C-D bond is about 10 times as strong as the C-H bond, the

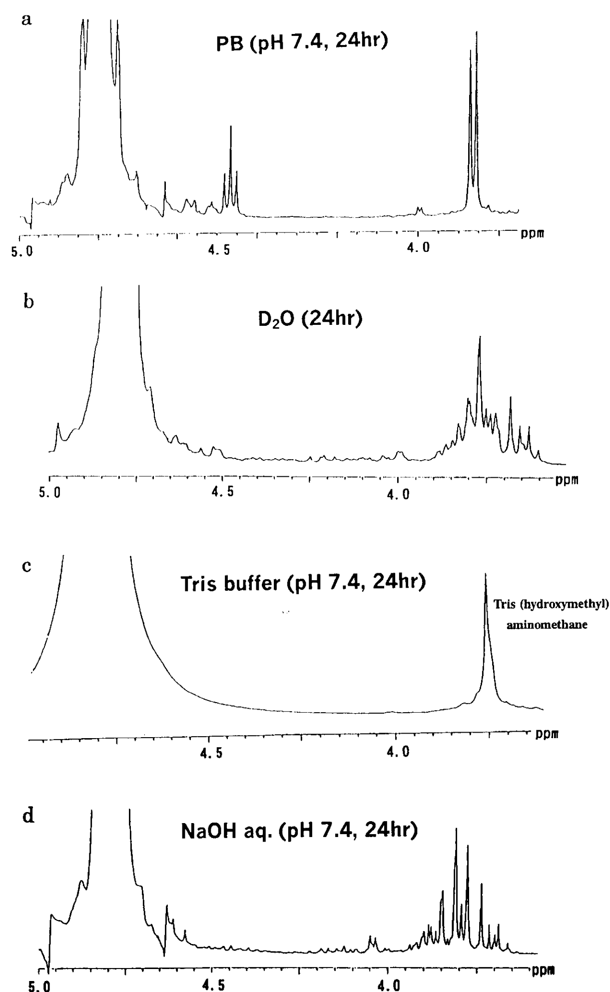

Fig. 3. ^{1}H-NMR Spectra of the Incubated Solutions of DKG.
The DKG concentration in each solution was 250 mM, and all solutions were incubated for 24 h at room temperature. **a**: phosphate buffer with D_2O (0.5 M, pH 7.4); **b**: D_2O; **c**: tris buffer with D_2O (0.5 M, pH 7.4); **d**: NaOH solution with D_2O (controlled to pH 7.4).

O-D bond is also stronger than the O-H bond, and there are some differences in the physical properties between heavy and light water.[21] Furthermore, it has been reported that the oxidation of pentoses and hexoses was more strongly promoted in D_2O than in H_2O.[22] L-Threosone was the compound oxidized in the C1-alcohol group of L-erythrulose, and it is thought that the oxidation reaction was accelerated in D_2O and that L-threosone was mainly produced in this reaction.

On the other hand, the yield of oxalic acid was about 20% of the starting compound (DKG) under our incubation conditions for DKG. Therefore, as shown in Fig. 1, L-threosone is considered to have been produced either by a single fission reaction of the C2-C3 bond of DKG with the equimolar formation of oxalic acid, or by two successive decarboxylation reactions.

It has been reported that there were some di- or poly-carbonyl compounds in the degradation

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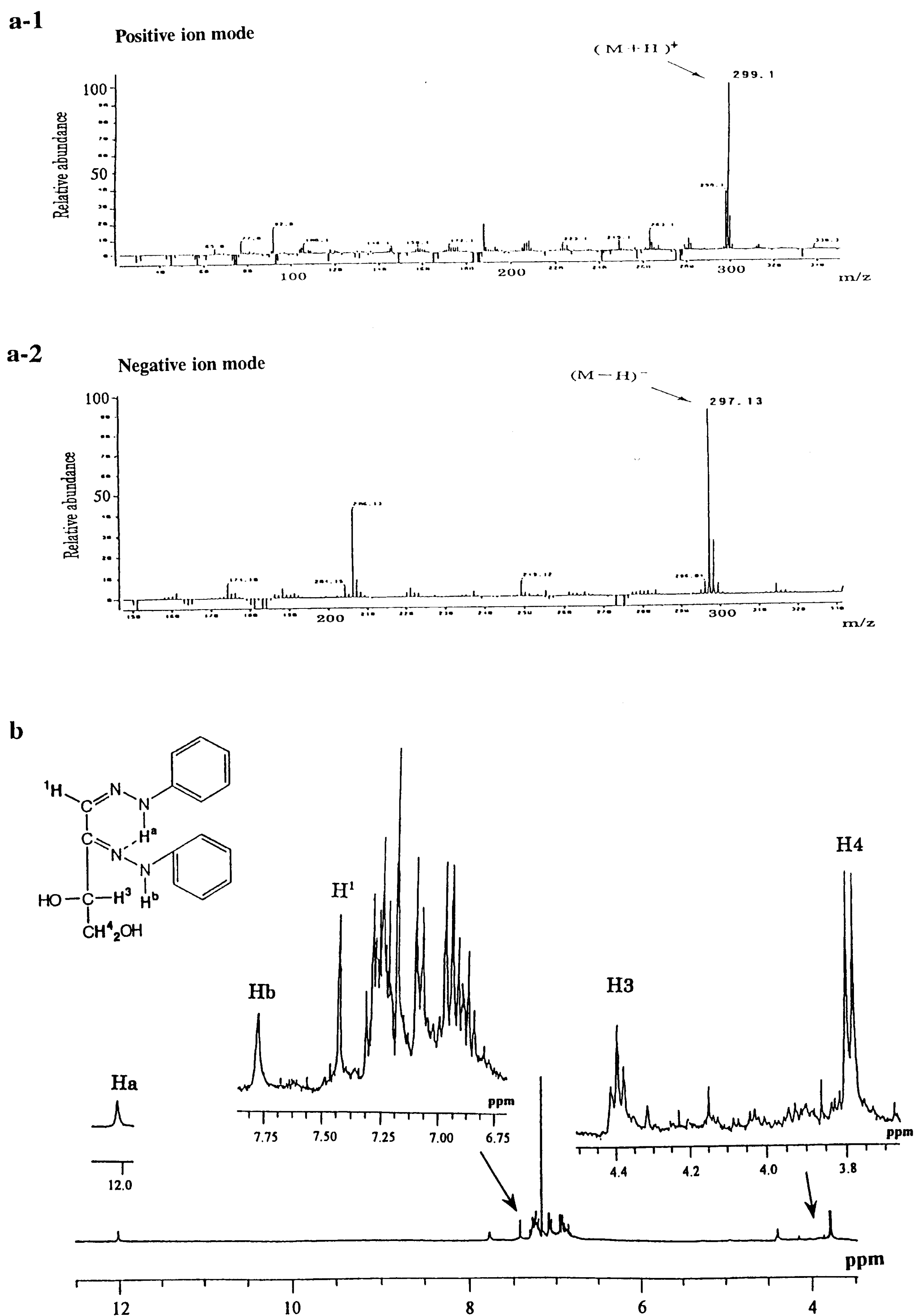

Fig. 4. FAB-MS Data and ^{1}H-NMR Spectrum of the Phenylhydrazine Derivative of DKG-1.

a-1: spectrum in the positive ion mode; **a-2**: spectrum in the negative ion mode; **b**: ^{1}H-NMR spectrum in $CDCl_3$. The signals observed between $\delta_H = 6.8$ ppm and $\delta_H = 7.3$ ppm were due to two phenyl groups (5H × 2).

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products of AA.[14,15] Those compounds may promote the formation of protein crosslinks *in vivo* which is considered to be responsible for adult diseases such as diabetes. The results of this experiment indicate L-threosone to be a candidate for the AA oxidation products that would promote the Maillard reaction *in vivo*. It has also been reported that some compounds with reducing activity were produced in DAA and DKG solutions,[23] although details of the reduction mechanism have not yet been clarified. Since L-threosone showed some reducing activity, probably through its enolization, it might have contributed to the reducing activity observed in the DAA and DKG solutions.

In summary, L-threosone was found to be the major degradation compound produced during the incubation of DAA and DKG under conditions close to physiological, although the formation was restricted to the D_2O-PB solution. Moreover, L-erythrulose is thought to have been one of the main products in the oxidative degradation of AA *in vivo* as stated earlier.[15] Further studies on the detailed formation mechanism for these degradation products from DKG are certainly needed.

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References

1) Yamazaki, I. and Piette, L. H., Mechanism of free radical formation and disappearance during the ascorbic acid oxidase and peroxidase reactions. *Biochim. Biophys. Acta*, **50**, 62–69 (1961).
2) Weis, W., Zubrzycki, Z., and Staudinger, H., Redox behavior of L(+)-semidehydro-ascorbic acid. *Hoppe Seyler's Z. Physiol. Chem.*, **349**, 279–282 (1968).
3) Wells, W. W., Xu, D. P., Yang, Y., and Rocque, P.A., Mammalian thioltransferase (glutaredoxin) and protein disulfide isomerase. *J. Biol. Chem.*, **265**, 15361–15364 (1990).
4) Borsook, H., Davenport, H. W., Jeffreys, C. E. P., and Warner, R. C., The oxidation of ascorbic acid and its reduction *in vitro* and *in vivo*. *J. Biol. Chem.*, **117**, 237–279 (1937).
5) Kagawa, Y., Enzymatic studies on ascorbic acid catabolism in animals: I. Catabolism of 2,3-diketo-L-gulonic acid. *J. Biochem.*, **51**, 134–144 (1962).
6) Nishikawa, Y. and Kurata, T., Chemical characteristics of dehydro-L-ascorbic acid. *Biosci. Biotechnol. Biochem.*, **64**, 1651–1655 (2000).
7) Bode, A. M., Cunningham, L., and Rose, R. C., Spontaneous decay of oxidized ascorbic acid (dehydro-L-ascorbic acid) evaluated by high-pressure liquid chromatography. *Clin. Chem.*, **36**, 1807–1809 (1990).
8) Nagaraj, R. H. and Monnier, V. M., Protein modification by the degradation products of ascorbate: formation of a novel pyrrole from the Maillard reaction of L-threose with proteins. *Biochim. Biophys. Acta*, **1253**, 75–84 (1995).
9) Nishikawa, Y. and Kurata, T., Structure and chemical characteristics of dehydro-L-ascorbic acid in solutions. *Food Sci. Technol. Int. Tokyo*, **4**, 155–158 (1998).
10) Nishikawa, Y. and Kurata, T., Interconversion between dehydro-L-ascorbic acid and L-ascorbic acid. *Biosci. Biotechnol. Biochem.*, **64**, 476–483 (2000).
11) Otsuka, M., Kurata, T., and Arakawa, N., Isolation and characterization of an intermediate product in the degradation of 2,3-diketo-L-gulonic acid. *Agric. Biol. Chem.*, **50**, 531–533 (1986).
12) Kimoto, E., Tanaka, H., Ohmori, T., and Choami, M., Analysis of the transformation products of dehydro-L-ascorbic acid by ion-pairing high-performance liquid chromatography. *Anal. Biochem.*, **214**, 38–44 (1993).
13) Jung, C. and Wells, W. W., Spontaneous conversion of L-dehydroascorbic acid to L-ascorbic acid and L-erythroascorbic acid. *Arch. Biochem. Biophys.*, **355**, 9–14 (1998).
14) Kurata, T. and Fujimaki, M., Formation of 3-keto-4-deoxypentosone and 3-hydroxy-2-pyrone by the degradation of dehydro-L-ascorbic acid. *Agr. Biol. Chem.*, **40**, 1287–1291 (1976).
15) Simpson, G. and Ortwerth, B. J., The non-oxidative degradation of ascorbic acid at physiological conditions. *Biochim. Biophys. Acta*, **1501**, 12–24 (2000).
16) Ortwerth, B. J., Slight, S. H., Prabhakaram, M., Sun, Y., and Smith, J. B., Site-specific glycation of lens crystallins by ascorbic acid. *Biochem. Biophys. Acta*, **1117**, 207–215 (1992).
17) Kornig, R. J., Blobstein, S. H., and Cerami, A., Structure of carbohydrate of hemoglobin AIC. *J. Biol. Chem.*, **253**, 2992–2997 (1977).
18) Grandhee, S. K. and Monnier, V. M., Mechanism of formation of the Maillard protein cross-link. *J. Biol. Chem.*, **266**, 11649–11653 (1991).
19) Nwaka, S., Kopp, M., and Holzer, H., Expression and function of the trehalase genes NHT1 and YBR0106 in saccharomyces cerevisiae. *J. Biol. Chem.*, **270**, 10193–10198 (1995).
20) Ohmori, M. and Takagi, M., A facile preparation of dehydro-L-ascorbic acid-methanol solution and its stability. *Agric. Biol. Chem.*, **42**, 173–174 (1978).
21) Kushner, D. J., Baker, A., and Dunstall, T. G., Pharmacological uses and perspectives of heavy water and deuterated compounds. *Can. J. Physiol. Pharmacol.*, **77**, 79–88 (1999).
22) Rangappa, K. S., Raghavendra, M. P., Mahadevappa, D. S., and Gowda, D. C., Kinetics and mechanism of oxidation of erythro-series pentoses and hexoses by *N*-chloro-*p*-toluenesulfonamide. *Carbohydrate Research*, **306**, 57–67 (1998).
23) Retsky, K., Freeman, M., and Frei, B., Ascorbic acid oxidation product(s) protect human low density lipoprotein against atherogenic modification. *J. Biol. Chem.*, **268**, 1304–1309 (1993).