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Electrospray ionization mass spectrometric investigations of α-dicarbonyl compounds—Probing intermediates formed in the course of the nonenzymatic browning reaction of L-ascorbic acid

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Abstract

A new method is presented which allows the simultaneous detection of various α-dicarbonyl compounds generated in the course of the nonenzymatic browning reaction initiated by thermal treatment of L-ascorbic acid, namely: glyoxal, methylglyoxal, diacetyl, 3-deoxy-L-pentosone, and L-threosone. 3-Deoxy-L-threosone was successfully identified as a new C_4-α-dicarbonyl structure for the first time in the degradation of Vitamin C by application of this non-chromatographic mass spectrometric approach. Moreover, a more detailed elucidation of the mechanistic scenario with respect to the oxidative and nonoxidative pathways is presented by using dehydro-L-ascorbic acid and 2,3-diketo-L-gulonic acid instead of L-ascorbic acid as a starting material. Furthermore, the postulated pathways are corroborated with the aid of ^{13}C-isotopic labeling studies. The investigations were extended to baby food, and the successful detection of α-dicarbonyl compounds characteristic for Vitamin C degradation proved the matrix tolerance of the introduced method.

Keywords: L-Ascorbic acid; Nonenzymatic browning; α-Dicarbonyl compounds; 3-Deoxy-L-threosone; Electrospray ionization mass spectrometry

1. Introduction

Chemically, L-ascorbic acid belongs to the family of carbohydrates, functionally it is classified as an organic acid and a reducing reagent, and physiologically the compound is attributed to the class of essential vitamins. The so-called Vitamin C is a natural component in a wide range of esculent goods. Furthermore, artificial addition of L-ascorbic acid is successfully utilized to protect food against oxidation. Hence, it is vital to have knowledge and control over the Vitamin C content in foods. Unfortunately, L-ascorbic acid is relatively unstable under common storage and processing conditions such as heat, oxygen, and exposure to transition metals. Thus, it is of essential importance to understand the nonenzymatic degradation of Vitamin C. However, this process is complex and hence it is not completely understood yet. It is widely accepted to classify the decomposition into two types of reactions, namely the oxidative and a nonoxidative pathway. Hitherto, more than 100 different intermediates and degradation products are known to play a role in the course of the nonenzymatic browning reaction [1]. In this context, fragments with α-dicarbonyl structure represent a category, which has not yet been in the center of attention. Therefore, we focussed on the formation of the highly reactive intermediates, which need to be derivatized prior to detection. The trapping reagent fulfills two important roles: on the one hand it is necessary to stabilize the α-dicarbonyl compounds to avoid fast subsequent reactions and on the other hand it is compulsory to enhance the sensitivity in electrospray ionization (ESI) at the same time. Here, the derivatizing agent of choice is *o*-phenylenediamine (OPD) as it is capable of fulfilling both tasks. In a two-fold condensation reaction the α-dicarbonyl structures are converted to stable quinoxalines. The latter are probed by a high electrospray ionization response owing to the relatively high basicity of nitrogen centers associated with more facile protonation, which has been verified by test calculations.

Furthermore, it would be desirable to develop a method which allows the direct detection of highly reactive α-dicarbonyl intermediates generated in course of the browning reaction of

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L-ascorbic acid in the presence of complex matrices as encountered in food systems. If chromatographic or laboratory work-up could be omitted, this method would be even more attractive for time and cost efficiency reasons. In order to allow a simultaneous analysis of the various α-dicarbonyl compounds generated via the oxidative and nonoxidative Vitamin C degradation pathway, we describe here, is the successful development and application of such an approach.

2. Experimental

2.1. Materials

L-Ascorbic acid was generously provided from Pascoe pharmazeutische Präperate GmbH. 2,3-Diketo-L-gulonic acid was synthesized according to established preparative procedures [2]. In brief, L-ascorbic acid was oxidized to dehydro-L-ascorbic acid by addition of *p*-benzochinone, oxalic acid, and formic acid to a dimethylacetamide solution of L-ascorbic acid. The reaction mixture was heated to 50 °C and the newly generated dehydro-L-ascorbic acid dimer was crystallized. The intermediate dimer was converted to the barium salt of 2,3-diketo-L-gulonic acid by formation of the monomer in an alkaline sodium hydroxide solution, and addition of hydriodic acid induced spontaneous lactone cleavage followed by crystallization with the aid of bariumiodide. In a final step, the 2,3-diketo-L-gulonic acid was liberated by ion exchange.

The remaining compounds were all commercially purchased and employed without further purification unless stated otherwise: L-[1-^{13}C] ascorbic acid (Eur iso-top), dehydro-L-ascorbic acid (Aldrich), methanol (Merck), hydrochloric acid (Merck), *p*-benzochinone (Fluka), oxalic acid (Fluka), formic acid (Merck), dimethylacetamide (Merck), sodium hydroxide solution (Merck), hydriodic acid (Merck), bariumiodide (Riedel-de-Haën), sodium hydrogen sulfite (Merck), activated charcoal (Merck) and *o*-phenylenediamine (Aldrich). The latter compound was dissolved in water, augmented with sodium hydrogen sulfite and activated charcoal, refluxed, hot filtrated and recrystallized prior to use.

The quinoxaline standards glyoxal and methylglyoxal were purchased from Serva, diacetyl from Lancaster and 3-deoxy-L-pentosone was synthesized and kindly provided by Hollnagel [3].

The baby food samples, HIPP Baby-C-juice and HIPP Vitamin C enriched fruit tea, were publicly obtained from a supermarket.

2.2. Sample preparation

For preparation of the reaction mixtures L-ascorbic acid, L-[1-^{13}C] ascorbic acid, dehydro-L-ascorbic acid, or 2,3-diketo-L-gulonic acid are dissolved in distilled water and pH adjusted to 3.5 with hydrochloric acid to yield 0.5, 0.5, 0.025, and 0.025 M solutions, respectively. In each case, 0.5 ml aliquots are pipetted into 10 ml ampules and sealed. The thermolysis is initiated by placing the sample ampules in a thermo block (Behrotherm) at 120 °C for 120 min.

The derivatization is brought about by preparing a 1:1 mixture (v/v) of a 0.5 M *o*-phenylenediamine aqueous solution with the thermally treated samples and allowing them to react for 30 min at room temperature, dilution with methanol, and introduction to the ESI-MS without further chromatographic treatment.

The baby food samples were handled analogously by pipetting 0.5 ml of either the unaltered HIPP Baby-C-juice or preparing the HIPP Vitamin C enriched fruit tea according to instructions of the manufacturer (1 g dissolved in 2.5 ml water) into 10 ml ampules and the treated accordingly (see above).

2.3. Electrospray ionization mass spectrometry

The mass spectrometric experiments were carried out on a commercial VG BIO-Q mass spectrometer, which has been described in detail previously [4]. In brief, the VG BIO-Q consists of an ESI source combined with a tandem mass spectrometer of QHQ configuration (Q: quadrupole; H: hexapole). In the present experiments, methanolic mmolar solutions of OPD-derivatized solutions of thermally treated L-ascorbic acid, dehydro-L-ascorbic acid, and 2,3-diketo-L-gulonic acid or the respective food sample were introduced via a syringe pump (flow rate 10 μl min^{-1}) to the fused-silica capillary of the ESI source. Nitrogen was used as drying and nebulizer gas. The source temperature was kept at 80 °C and the cone voltages applied in the desolvation zone of the differentially pumped ESI source were systematically varied for the ions of interest which were then selected at unit mass resolution by means of Q1.

The isotope patterns of all ions described below agreed with expectation on the basis of natural isotope abundances [5]. The cone voltage U_C determines the amount of collisional activation of the ions evolving from solution in the differential pumping system of the ESI source. Collision-induced dissociation (CID) experiments were performed with argon at various collision energies (E_{lab} = 0–30 eV) and a pressure of ca. 3×10^{-4} mbar, which approximately correspond to single-collision conditions [4]. The collision energies were converted to the center-of-mass frame, $E_{CM} = [m/(M+m)]E_{lab}$, where m and M are the masses of the collision gas and the ionic species, respectively. The product ions formed in the hexapole were then analyzed by scanning Q2.

3. Results and discussion

In general, the nonenzymatic browning reaction of L-ascorbic acid is discussed in terms of two major decomposition pathways, which are referred to as the oxidative and nonoxidative pathway, respectively. In this work, the terms oxidative and nonoxidative are used as follows in order to avoid misunderstanding: The oxidative pathway describes the reaction branch which involves the oxidation of L-ascorbic acid to dehydro-L-ascorbic acid as an initial step. Likewise, the nonoxidative pathway relates to the direct decomposition of L-ascorbic acid with exclusion of dehydro-L-ascorbic acid as an intermediate structure. It is noted that the expression nonoxidative refers solely to the nature of the initial step, subsequent transformations may indeed involve various oxidation steps.

Thermal treatment of L-ascorbic acid, dehydro-L-ascorbic acid, and 2,3-diketo-L-gulonic acid leads to the formation of various types of α-dicarbonyl compounds as important degradation intermediates. Due to their high reactivity it becomes necessary to trap them with OPD as quinoxaline derivatives [6–8]. However, although these intermediates are detected in their derivatized form, we will refer to them as α-dicarbonyl for the sake of simplicity.

In the course of the nonenzymatic browning reaction of L-ascorbic acid, a wide range of degradation products have been reported previously in literature [1,9–23]. With the aid of ESI-MS, we succeeded in the detection of three classes of α-dicarbonyl compounds as highly reactive intermediates differing in their number of carbon atoms in the backbone. Glyoxal and methylglyoxal are representatives of C_3 structures. Diacetyl, L-threosone, and unprecedentedly 3-deoxy-L-threosone (4-hydroxy-2-oxo-butanal) could be identified as C_4 α-dicarbonyl compounds. 3-Deoxy-L-pentosone was discovered as a C_5-intermediate. The α-dicarbonyl derivatives have been characterized by CID of the protonated quinoxalines and comparison of the resulting spectra with fragmentation of the respective external standards, where available. For two of the C_4 α-dicarbonyls, 3-deoxy-L-threosone and L-threosone, the respective standards were inaccessible and in these cases the CID spectra of the experimentally detected α-dicarbonyls were surveyed for their general compatibility with the theoretically expected fragmentation channels of these compounds. Exemplarily, we will discuss the CID spectrum of 3-deoxy-L-threosone as this is the α-dicarbonyl compound, which is reported the first time as a degradation intermediate in the course of the nonenzymatic browning reaction of L-ascorbic acid. In order to verify the identity of the compound corresponding to the signal of $m/z = 175$ observed in the L-[1-^{13}C] ascorbic acid sample, the CID spectrum is characterized (Fig. 1).

Elimination of water is expected to originate from a dehydration of the quinoxaline side chain and consecutive loss of acetylene is in agreement with the expected structure as well. The signal at $m/z = 131$ may also originate from a direct elimination of C_2H_4O from the parent ion. The last characteristic fragment ion detected is at $m/z = 147$; this ion corresponds to an elimination of ethene from the side chain. Additionally, the CID spectra were later cross-checked with signals of the browned L-[1-^{13}C] ascorbic acid solution. An analogous characterization has also been carried out in case of L-threosone, and the analysis is in accordance with the expected structure; for the sake of conciseness, it is refrained from presenting these results here in detail.

In the following section, we will focus on the two possible pathways discussed for the nonenzymatic browning reaction of L-ascorbic acid. In order to distinguish between the oxidative and the nonoxidative type, the oxidative pathway is enforced by choosing dehydro-L-ascorbic acid as a starting point of the reaction, which is the first characteristic intermediate of L-ascorbic acid degradation via the oxidative route. Mass spectrometric investigations of the dehydro-L-ascorbic acid sample yielded the following α-dicarbonyl compounds: glyoxal, methylglyoxal, diacetyl, L-threosone, and 3-deoxy-L-threosone. This finding indicates that these five α-dicarbonyl compounds are presumably formed from L-ascorbic acid on the oxidative pathway. However, at this point of the investigations, it cannot be ruled out that they are also accessible via the nonoxidative route. On the other hand, the absence of 3-deoxy-L-pentosone confirms previous findings [9] that this intermediate is exclusively formed nonoxidatively.

It should be noted that starting from dehydro-L-ascorbic acid does not strictly exclude the nonoxidative reaction branch as the back reaction from dehydro-L-ascorbic acid to ascorbic acid competes with the degradation of dehydro-L-ascorbic acid [24]. This problem can be circumvented by choosing the 2,3-diketo-L-gulonic acid, the product obtained after lactone hydrolysis of dehydro-L-ascorbic acid as this step is considered practically irreversible under the given experimental conditions. Hence,

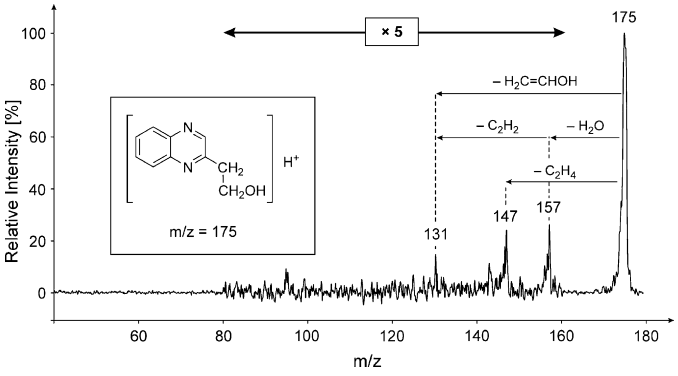

Fig. 1. CID spectrum of *o*-phenylenediamine derivatized and protonated 3-deoxy-L-threosone, derived by thermal treatment of Vitamin C.

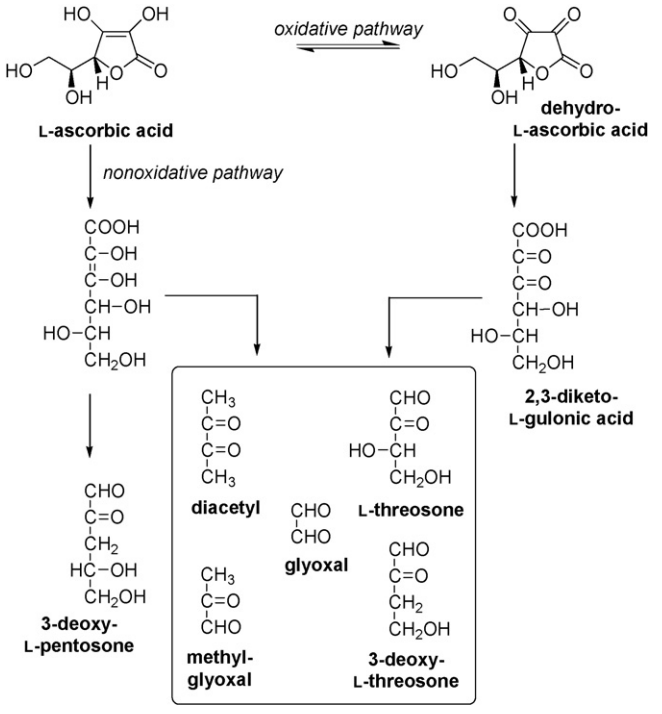

Fig. 2. Degradation of L-ascorbic acid to various α-dicarbonyl compounds via oxidative and nonoxidative pathway.

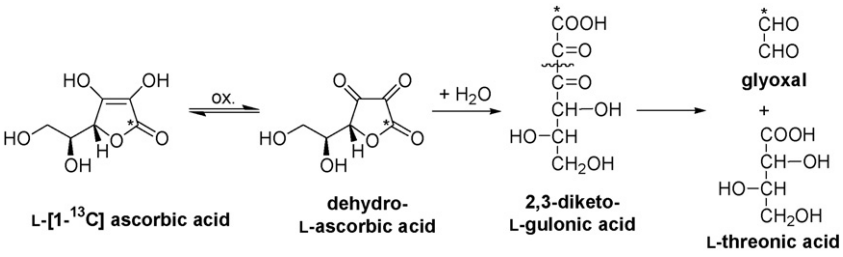

Fig. 3. Postulated formation pathway of isotopically labeled glyoxal from L-[1-^{13}C] ascorbic acid.

the α-dicarbonyls glyoxal, methylglyoxal, diacetyl, L-threosone, and 3-deoxy-L-threosone observed during the browning reaction of the 2,3-diketo-L-gulonic acid sample are definitively formed via the oxidative route. Although this experimental setup still does not allow excluding the possibility that they might additionally be formed nonoxidatively. Moreover, the absence of 3-deoxy-L-pentosone as a possible degradation product from 2,3-diketo-L-gulonic acid proves that the formation of this α-dicarbonyl compound is restricted to the nonoxidative pathway.

Based on the results obtained so far, Fig. 2 describes a possible degradation scenario of L-ascorbic acid considering the formation of the α-dicarbonyl compounds in dependence of the two major reaction branches.

In order to allow a more detailed mechanistic description of the degradation processes, a ^{13}C label is introduced into L-ascorbic acid to trace the position of an isotopically marked carbon atom in the course of the degradation reaction. Investigations of a L-[1-^{13}C] ascorbic acid sample indicate that methylglyoxal, diacetyl, 3-deoxy-L-pentosone, L-threosone, and 3-deoxy-L-threosone do not contain the isotopic label and are hence formed via a decarboxylation step in which the ^{13}C-label is lost in the form of $^{13}CO_2$, irrespective of the oxidative or nonoxidative pathway (see Fig. 2). In contrast, glyoxal contains the ^{13}C-satellite up to 40% and is therefore at least partially formed by a different route, possibly a cleavage of the C_2–C_3 bond which preserves the C_1 position of L-ascorbic acid in glyoxal (Fig. 3).

For the formation of 3-deoxy-L-threosone, we suggest the following mechanism (Fig. 4).

After an initial oxidation of L-ascorbic acid to dehydro-L-ascorbic acid and lactone hydrolysis to 2,3-diketo-L-gulonic acid a cleavage of either the C_2–C_3 or the C_4–C_5 bond leads to the intermediate formation of oxalic acid and L-erythrulose. The ^{13}C experiments show that the isotopic label is found in oxalic acid exclusively thereby corroborating the formation of the fragments by cleavage of the C_2–C_3 bond. Subsequent keto-enol tautomerization (KET) and dehydration of L-erythrulose leads to the formation of 3-deoxy-L-threosone.

Hitherto, we have presented a method which allows the direct analysis of α-dicarbonyl intermediates generated in course of the nonenzymatic browning reaction of L-ascorbic acid which does not involve any chromatographic purification steps. It would now be highly desirable to probe whether this method can be expanded to the detection of degradation products in Vitamin C containing esculents which requires a high matrix tolerance. Quite fortunately, this approach proved to be successful in case

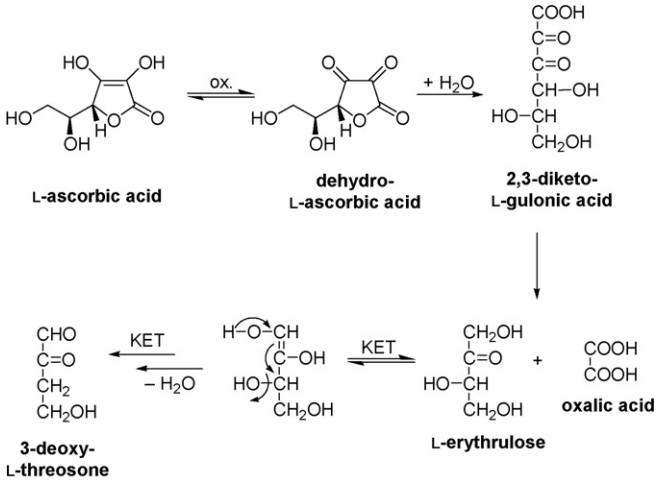

Fig. 4. Suggested formation pathway of 3-deoxy-L-threosone from L-ascorbic acid via the oxidative route.

of HIPP Baby-C-juice and HIPP Vitamin C enriched fruit tea. In these baby food samples glyoxal, methylglyoxal, diacetyl, 3-deoxy-L-pentosone, L-threosone, and 3-deoxy-L-threosone could be identified. The successful detection of 3-deoxy-L-threosone and L-threosone is of particular importance, as it has not been reported so far in the literature according to our knowledge, and their formation is typical for the degradation of L-ascorbic acid and they hence represent characteristic key intermediates. In contrast, the remaining α-dicarbonyl compounds may also originate from the caramelization and the Maillard reaction of mono-, oligo-, and polysaccharides, which are almost ubiquitous in food goods and their detection is hence less specific.

4. Conclusion

In the course of the nonenzymatic browning reaction of L-ascorbic acid, the following α-dicarbonyl compounds glyoxal, methylglyoxal, diacetyl, 3-deoxy-L-pentosone, L-threosone, and 3-deoxy-L-threosone could successfully be identified as the corresponding quinoxaline derivatives with electrospray ionization mass spectrometry omitting any prior chromatographic separation. 3-Deoxy-L-pentosone has been shown to be generated by the nonoxidative pathway which excludes dehydro-L-ascorbic acid as an intermediate. On the other hand, it could be demonstrated that the remaining compounds with α-dicarbonyl

structure are formed via the oxidative route. However, it should be noted that the nonoxidative degradation is not excluded rigorously as an additional source of origin for these intermediates. Nevertheless, it is for the first time that the C_4 structure of 3-deoxy-L-threosone has been reported in this context. Usage of ^{13}C labeling studies allows the corroboration of certain mechanistic details of the decomposition reaction.

Furthermore, the method presented in this work allows the detection of the same characteristic α-dicarbonyl intermediates as expected from analysis of the model systems directly in two exemplary baby food goods following their thermal treatment. Here, L-threosone and 3-deoxy-L-threosone could be identified as key intermediates, which are characteristic for the degradation of Vitamin C.

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