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Vitamin C Degradation

Maillard Degradation Pathways of Vitamin C

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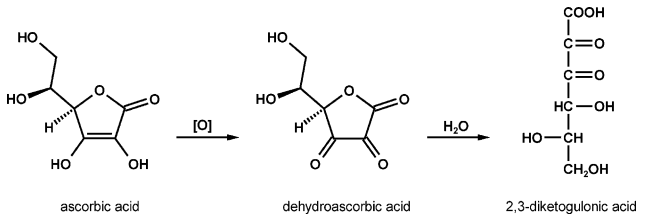
Vitamin C, or L-threo-ascorbic acid (hereafter referred to as ascorbic acid), belongs to the class of essential vitamins in the human diet and is widely found in nature, for example, in herbs, vegetables, and, in particular, in citrus fruits. Because of its antioxidant properties, ascorbic acid has been successfully utilized as an additive in food industry for prolonging the shelf life of foods, and in pharmaceutical applications for the prevention of common cold. Under typical food storage or processing conditions (oxygen atmosphere, pH, and temperature) ascorbic acid is highly unstable and subject to degradation processes that lead to the loss of nutritional value,[1] discoloration,[2] and "off-flavor" formation.[3] Structurally, ascorbic acid is a reducing carbohydrate and therefore an active participant in the non-enzymatic browning reaction (Maillard reaction) with amino acids, peptides, and proteins.[4] Its protein glycation activity has also been recognized in vivo, especially in organs and tissues with negligible protein turnover, such as eye-lens proteins.[5–7] Thus, in view of both food chemistry and biochemistry, there is vital interest in understanding the Maillard degradation of vitamin C.

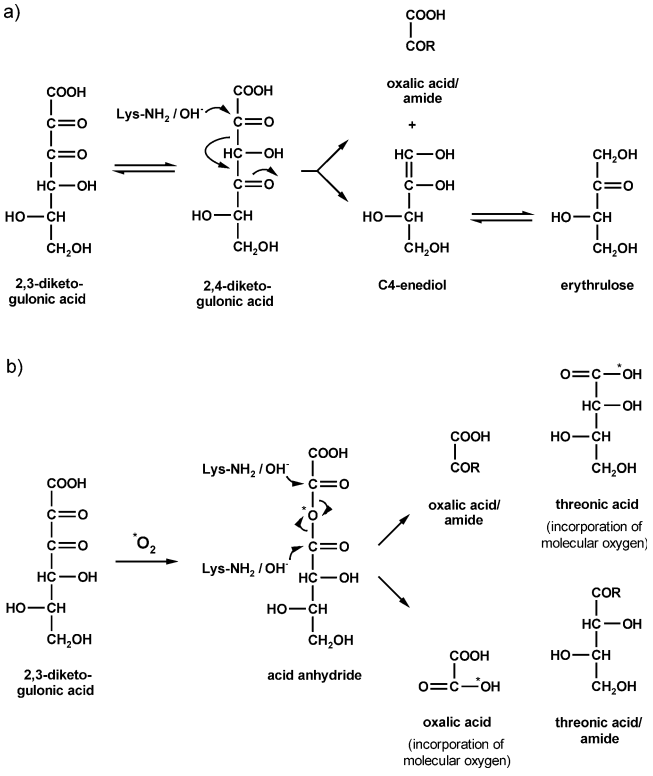
Numerous investigations were undertaken to characterize the decomposition products of ascorbic acid. Dehydroascorbic acid (DHA), 2,3-diketogulonic acid (2,3-DKG), erythrulose, and oxalic and threonic acids were identified as the main degradation products.[8–10] It is well established, that ascorbic acid can be readily oxidized to DHA, which hydrolyzes irreversibly to 2,3-DKG, even at pH 7 (Scheme 1).[11] To gain



Scheme 1. Oxidation of ascorbic acid in neutral solution, leading to DHA and 2,3-DKG.

insight into the degradation pathways of ascorbic acid, isotopic carbon labeling experiments were performed by several investigators. Despite the fact that approximately 30% of decarboxylation was found to take place,[8] the results remained inconclusive because labeling studies were limited to the use of mainly C1-labeled ascorbic acid.[12] Nevertheless, some authors have attempted to propose the main degradation routes of ascorbic acid.[8,13–15] However, in most cases, these pathways seem to be more a cut through the molecule rather than a profound explanation of mechanistic processes.

In the literature, two degradation pathways are described for carbohydrate fragmentation, both of which are unequivocally underpinned on the basis of the conducted experiments: β-dicarbonyl cleavage as the main degradation pathway[16,17] and, to a lesser extent, oxidative α-dicarbonyl fragmentation.[18] Shin and Feather found that Maillard degradation of ascorbic acid, DHA, and 2,3-DKG results in the same follow-up products. Therefore, 2,3-DKG must be considered as the educt to explain the formation of most of the fragmentation products with a carbon backbone smaller than C_6.[8] Based on the established carbohydrate degradation mechanisms Scheme 2 shows 2,3-DKG following both the β-dicarbonyl fragmentation (Scheme 2a) and the oxidative α-dicarbonyl cleavage route (Scheme 2b). The latter includes incorporation of molecular oxygen as a key step and leads to a pair of carboxylic acids. Consequently, oxygen is 50% incorporated into both the resulting oxalic and threonic



Scheme 2. Pathways for β-dicarbonyl fragmentation (a) and oxidative α-dicarbonyl cleavage (b) of 2,3-diketogulonic acid. R = OH or NH-Lys.

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acids.[18] Furthermore, both pathways have in common the scission of the carbon chain induced by the attack of a nucleophile. Recently, we showed that, in the case of β fragmentation in parallel with the hydrolytic form (attack of a hydroxyl anion), an amine-induced β cleavage (attack of the ε-amino moiety of lysine) is existent, thus leading directly to amide-advanced glycation endproducts (AGEs).[19] It can be hypothesized that a nucleophilic attack of lysine is also valid for the intermediate acid anhydride in the oxygen-triggered mechanism.

Herein, we deliver a comprehensive study of the amine-catalyzed degradation of ascorbic acid with a focus on the mechanisms. On the basis of isotopic labeling experiments and by quantification of all important fragmentation products, we are able to explain about 75% of the Maillard induced decomposition of vitamin C.

Figure 1a presents the kinetic data for different carbonyl and dicarbonyl compounds in a Maillard model system of ascorbic acid and *N*1-BOC-lysine (BOC = *tert*-butoxycarbonyl). Highly reactive α-dicarbonyl compounds, threo-2-pentosulose (xylosone), 3,4-dihydroxy-2-oxobutanal (threosone), 1-hydroxybutane-2,3-dione (1-deoxythreosone), 4-hydroxy-2-oxobutanal (3-deoxythreosone), and methylglyoxal were analyzed as their quinoxaline derivatives, and glyceric aldehyde as the corresponding benzimidazole by the established trapping method with *o*-phenylenediamine.[17,20,21] The time-course formation of carboxylic acids is shown in Figure 1b. Incubations of different ^{13}C-labeled ascorbic acid isotopomers (labeled positions 1, 2, 3, 5, 6) allowed atoms in the Maillard reaction products to be traced back to their original positions in the precursor carbohydrate (Table 1). The formation of carboxylic acids through oxidative α-dicarbonyl cleavage was evidenced by incubation experiments conducted under an $^{18}O_2$ atmosphere, in an adaption of the method of Davidek et al.[18] As stated above, a 50% incorporation of oxygen into the respective carboxylic acid indicates that the acid is formed solely by this fragmentation route. Table 2 reveals the results for the ^{18}O experiment. In line with the literature, oxalic acid (59.6 mol%), threonic acid (24.9 mol%), and erythrulose (28.3 mol%; max. conc. after 3 days) were detected as the main degradation products.[8,10] Thus, threonic acid was found to be formed exclusively through oxidative α-dicarbonyl cleavage from the original C3–C6 atoms of ascorbic acid. Starting from 2,3-DKG as the precursor compound, the fitting counterpart would be oxalic acid, as proposed in Scheme 2. Indeed, oxalic acid originated at 90% from the upper part, C1 and C2, but only half of the total amount of oxalic acid was formed by the oxidative route. Assuming that 2,3-DKG undergoes isomerization to the tautomer 2,4-DKG, hydrolytic β cleavage would also release oxalic acid from the former C1 and C2 positions of ascorbic acid. The expected counterpart is a C4-enediol (see Scheme 2), which instantly isomerizes to erythrulose; erythrulose evolved solely from C3–C6 of ascorbic acid. Further follow-up products of the C4-enediol were threosone, 1-deoxythreosone, and 3-deoxythreosone, as established in a previous paper by our group regarding degradation studies on 1-deoxy-2,3-glucodiulose.[17] These findings completely

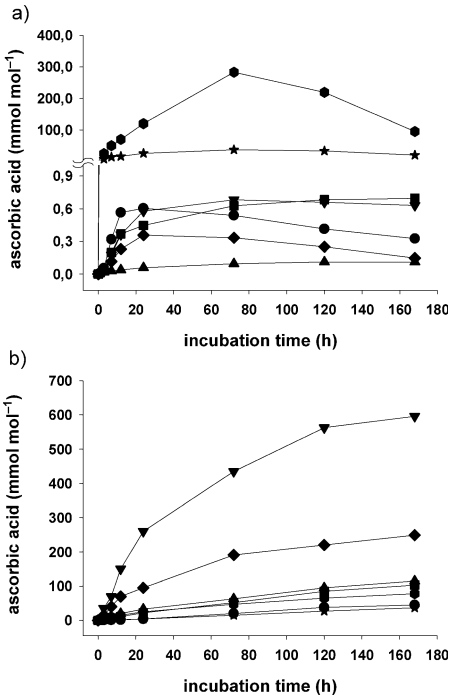


Figure 1. Time course of the formation of carbonyl and dicarbonyl compounds, and carboxylic acids. a) Erythrulose (●), glyceric aldehyde (★), 3-deoxythreosone (■), threosone (▼), 1-deoxythreosone (●), xylosone (◆), methylglyoxal (▲). b) Oxalic acid (▼), threonic acid (◆), glycolic acid (▲), glyceric acid (■), xylonic acid (●), tartronic acid (●), and lyxonic acid (★) in incubations of ascorbic acid and *N*1-BOC-lysine (42 mM each in pH 7.4 phosphate buffer at 37°C).

Table 1: Degradation of different ^{13}C-ascorbic acid isotopomers revealing the formation of carbonyl compounds, carboxylic acids, and amide-AGEs from the indicated parts of the original carbon backbone.

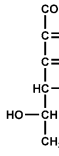
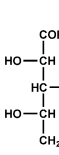
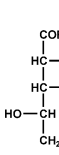
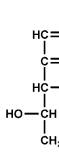
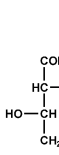
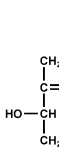
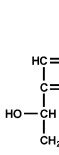
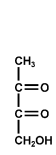
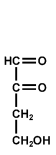
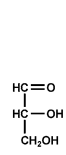
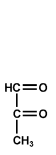
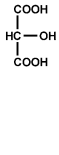
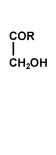
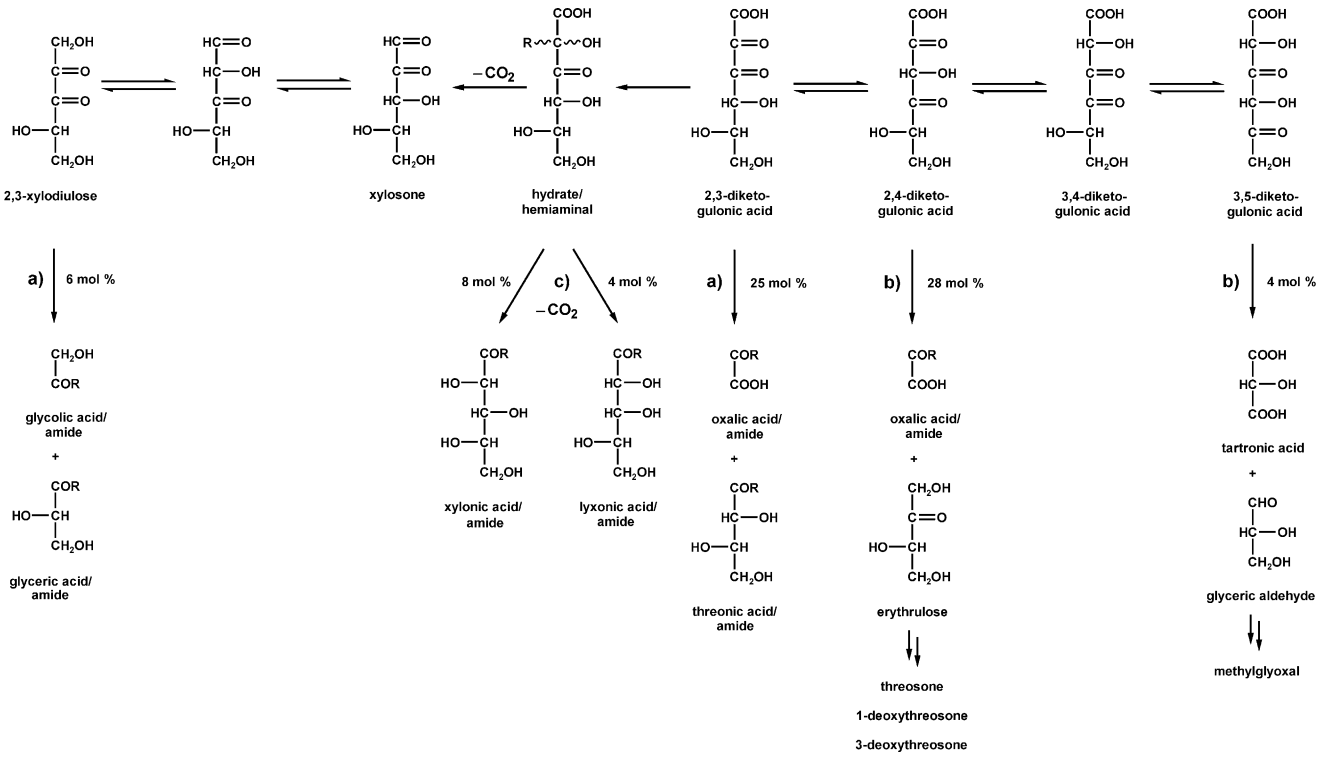
															
	2,3-diketo-gulonic acid	xylonic acid/ amide	lyxonic acid/ amide	xylosone	threonic acid/ amide	erythrulose	threosone	1-deoxy-threosone	3-deoxy-threosone	glyceric acid/ amide	glyceric aldehyde	methyl-glyoxal	tartronic acid	oxalic acid/ amide	glycolic acid/ amide
distribution		(C2–C6) 100%	(C2–C6) 100%	(C2–C6) 100%	(C3–C6) 100%	(C3–C6) 100%	(C3–C6) 100%	(C3–C6) 100%	(C3–C6) 100%	(C4–C6) 100%	(C4–C6) 100%	(C4–C6) 70%	(C1 and C2) 100%	(C1 and C2) 90%	(C2 and C3) 50%

Table 2: Percentages of oxygen incorporation and amounts of carboxylic acids formed through oxidative α cleavage.

Acid	Oxygen incorporation [%]	Amount formed [mol%]	
		Total	Through α cleavage
xylonic acid	0	7.8	0
lyxonic acid	0	3.7	0
threonic acid	50	24.9	24.9
glyceric acid	50	10.3	10.3
tartronic acid	0	4.5	0
oxalic acid	25	59.6	29.8
glycolic acid	50	11.5	11.5

support the present results (all three compounds arose exclusively from C3–C6 of ascorbic acid) and therefore support the β-dicarbonyl cleavage mechanism as well. For clarity, it can be summarized that the formation of 28.3 mol% oxalic acid from C1 and C2 of ascorbic acid must be explained by hydrolytic β fragmentation with erythrulose as the respective counterpart and 24.9 mol% by oxidative α cleavage, which releases the counterpart threonic acid. Approximately 6 mol% oxalic acid resulted from original C2 and C3 positions of an unknown intermediate by oxidative pathways.

Theoretically, 2,4-DKG offers a second possibility (scission between C3 and C4) for cleavage by the β-dicarbonyl route, leading to glyceric acid and a C3-enediol, which is expected to tautomerize to hydroxypyruvic acid. Although glyceric acid stemmed exclusively from the anticipated C4–C6 part of the original carbon backbone, the ^{18}O experiments indicated that the total amount of glyceric acid was formed through oxidative α fragmentation. To further investigate these disparate findings, the quinoxaline of hydroxypyruvic acid was synthesized independently as an authentic reference. In incubation solutions, only traces of hydroxypyruvic acid were detected and ^{13}C labeling studies did not show label incorporation for the expected C1 and C2 positions (data not shown). Alternatively, tartronic acid with a C1–C3 origin was considered as a fitting counterpart for glyceric acid, but had to be excluded because no oxygen incorporation was measured to support oxidative dicarbonyl fragmentation. The only plausible remaining carboxylic acid with a matching concentration in the range of glyceric acid (10.3 mol%) was glycolic acid (11.5 mol%). Indeed, based on ^{18}O incorporation, glycolic acid was also formed solely through oxidative α cleavage, but the findings were not so clear in view of ^{13}C labeling results. Only 50% of the total glycolic acid concentration related to original C2 and C3 positions. This means that about 6 mol% of glycolic acid was available as the counterpart for glyceric acid. By definition, the precursor structure of glycolic acid and glyceric acid must be a C_5 compound. Decarboxylation is an established mechanism for ascorbic acid[8] and C_5 compounds, for example, xylosone,[14,15] xylonic acid, and lyxonic acid[22] have already been identified in ascorbic acid systems. In the overview in Scheme 3, 2,3-xylodiulose is suggested as a feasible C_5 precursor formed by tautomerization of xylosone to release glyceric acid and glycolic acid, which is in line with the



Scheme 3. Degradation of 2,3-diketogulonic acid by oxidative α fragmentation (a), β cleavage (b), and decarboxylation from a hydrate/hemiaminal intermediate (c), leading to carboxylic acids, carboxylic acid amides, and carbonyl and dicarbonyl compounds. R = OH or NH-Lys.

oxidative α cleavage. The remaining glyceric acid (4 mol%) and glycolic acid (6 mol%) must thus be formed by additional unknown pathways. The intriguing extent of oxidative α fragmentation in comparison to 1-deoxy-2,3-glucodiulose, as a typical sugar-derived dicarbonyl compound, might be explained by the differing redox status of the ascorbic acid system.[16–18] Based on quantification results and ^{13}C labeling experiments, we further propose the synthesis of tartronic acid (4.5 mol%) through the β-cleavage route starting from 3,5-DKG with a C3-enediol as the counterpart to yield glyceric aldehyde (formed in up to 3.7 mol% after 3 days solely from C4–C6) after tautomerization. Furthermore, methylglyoxal can be considered as a follow-up product after the elimination of water (70% originated from positions C4–C6; Scheme 3).

To explain formation of the two orientations of the C3 hydroxy moiety detected in xylonic and lyxonic acid, we suggest the hydrate form of 2,3-DKG as an intermediate. The nucleophilic attack of a hydroxyl anion at former carbon atom C2 followed by decarboxylation leads to the pentonic acids, which arise solely from C2–C5, respectively, which is in line with the ^{13}C labeling experiments. Again, nucleophilic attack of the ε-amino group of lysine should give rise to a hemiaminal intermediate. After a decarboxylation step, the corresponding amides are formed. In confirmation of this hypothesis, *N*6-xylonyl lysine and *N*6-lyxonyl lysine were independently synthesized as authentic references. Indeed, both amide-AGEs were detected in the incubation solutions. Similarly, an amine-induced α cleavage was proved by the synthesis of authentic *N*6-threonyl lysine. The C4-amide was found to stem 100% from the former C3–C6 positions, which is in line with the proposed mechanism. Except for tartronic acid (owing to the lack of the amide reference) the mechanistic pathways of oxalic, glycolic, and glyceric acid were evidenced by the detection of their corresponding lysine amides.

In conclusion, as summarized in Scheme 3, 75% of the Maillard degradation pathways of ascorbic acid can be explained by the present work. Knowledge of the mechanisms of the ascorbic acid Maillard model system helps in understanding changes occurring during storage and processing of vitamin C containing food, as well as during adverse alterations in vivo, although the pathways will become more complex in biological systems owing to the influence of the oxygen atmosphere, temperature, pH, or metal ions. However, for the first time, we have identified *N*6-xylonyl lysine, *N*6-lyxonyl lysine, and *N*6-threonyl lysine as unique characteristic amide-AGEs of ascorbic acid Maillard systems. Thus, these compounds will now allow researchers to distinguish between ascorbic-acid-mediated Maillard reaction products and those derived from other reducing sugars, especially glucose. In most foods, as well as in vivo, these two carbohydrates are present at the same time.

Experimental Section

Model reactions: In general, incubations (42 mM ascorbic acid and *N*1-BOC-lysine, respectively) were conducted in phosphate buffer (0.1M, pH 7.4) at 37°C. ^{18}O experiments were performed by gassing with $^{18}O_2$. At various time points, aliquots of the reaction mixtures were subjected to a different work-up (AGE assay, chinoxaline/benzimidazole assay, silylation). Amide-AGEs and chinoxalines/benzimidazoles were analyzed by LC-MS/MS measurements, erythrulose and carboxylic acids were analyzed by GC-MS measurements.

LC-MS/MS analysis: Mass spectrometric detection was conducted on an API 4000 QTrap LC-MS/MS system (Applied Biosystems/MDS Sciex, Concord, ON, Canada) equipped with a turbo-ionspray source using electrospray ionization in the positive mode. For analysis, scheduled multiple reaction monitoring (sMRM) mode was used, utilizing collision-induced dissociation (CID) of the protonated molecules with compound-specific orifice potentials and fragment specific collision energies (for detailed parameters, see the Supporting Information).

GC-MS analysis: Samples were analyzed on a Thermo Finnigan Trace GC Ultra coupled to a Thermo Finnigan Trace DSQ (both Thermo Fisher Scientific GmbH, Bremen, Germany). GC-MS was performed with liquid chemical ionization using Methanol as a reactant gas. Mass spectra were obtained at 70 eV (source, 190°C; emission current, 80 μA) in full scan mode (mass range m/z 50–650).

^{13}C labeling experiments: Incubation solutions were analyzed for label distribution in quinoxaline/benzimidazole and amide-AGEs by using the LC-MS/MS system. Mass transitions for sMRM mode were set as indicated in the Supporting Information. Label distribution in carboxylic acids and erythrulose was determined by GC-MS. Extracted target ion masses were $[M+1]$ in comparison to unlabeled molecules. The natural isotope distribution of ^{13}C (1.10%) was considered in the calculations.

$^{18}O_2$ experiments: Incubation solutions were analyzed for label distribution in carboxylic acids using the GC-MS system. Again, extracted target ion masses were $[M+1]$ in comparison to unlabeled molecules.

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