Maillard-Induced Carbohydrate Fragmentations

DISSERTATION

zur Erlangung des akademischen Grades
DOCTOR RERUM NATURALIUM
(Dr. rer. nat.)

vorgelegt der

Naturwissenschaftlichen Fakultät II – Chemie, Physik und Mathematik
der Martin-Luther-Universität Halle-Wittenberg

von Frau Diplom-Lebensmittelchemikerin Mareen Smuda
geb. am 21. August 1983 in Lutherstadt Wittenberg

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Halle (Saale), 19. Juni 2013

DANKSAGUNG

An Herrn Prof. Dr. M. A. Glomb möchte ich an dieser Stelle ein besonderes Wort des Dankes richten, da er durch die Überlassung des außerordentlich interessanten Themas sowie durch wertvolle Anregungen, Hinweise und Diskussionen zum Gelingen der Arbeit beigetragen hat.

Mein herzlicher Dank gilt meinen beiden ehemaligen Diplomanden Frau J. Kahlert-Wohlrab und Herrn Armin Schaub, die im Rahmen ihrer Diplomarbeiten mit viel Sorgfalt Teile des hier vorgestellten Themas bearbeitet haben.

Herrn Dr. D. Ströhl, Frau R. Flächsenhaar sowie Frau Y. Schiller sei für die Anfertigung von NMR-Spektren gedankt.

Für die Aufnahme von zahlreichen ESI-Massenspektren möchte ich mich bei Herrn Dr. J. Schmidt bedanken.

Allen Kollegen und ehemaligen Kollegen des Arbeitskreises Prof. Glombs gebührt Dank für das sehr angenehme Arbeitsklima und die interessanten Diskussionen und Gespräche. Besonderer Dank geht dabei an Martin und Christian: Danke, dass ich rund ums analytische Bauen und Basteln so viel von euch lernen durfte und natürlich für die Milchreis-Flatrate sowie andere kulinarische Köstlichkeiten während der Mittagspause!

Des Weiteren möchte ich mich besonders bei meinen Eltern, meiner Familie und meinen Freunden bedanken, die mir stets Rückhalt und Unterstützung geboten haben.

VORWORT

Die vorliegende Arbeit wurde von Mai 2009 bis März 2013 am Institut für Chemie (Bereich Lebensmittelchemie) der Martin-Luther-Universität Halle-Wittenberg im Arbeitskreis von Prof. Dr. M. A. Glomb angefertigt.

Finanziell gestützt wurde diese Arbeit im ersten Jahr durch eine Graduierten-förderung des Landes Sachsen-Anhalt und für den verbleibenden Zeitraum durch die Anstellung als wissenschaftliche Mitarbeiterin der Universität.

Die erhaltenen Forschungsergebnisse wurden vollständig in international anerkannten Fachzeitschriften publiziert, denen die experimentellen Daten, die Einzelergebnisse sowie deren Diskussion zu entnehmen sind. Teile der kumulativen Arbeit wurden bereits als Review-Artikel im *Journal of Agricultural and Food Chemistry* veröffentlicht. Stellen, an denen der Review-Artikel für die kumulative Dissertation erweitert oder verändert wurde, sind durch Kursivschrift gekennzeichnet. Da der Review-Artikel in englischer Sprache publiziert ist, wurde diese auch für weitere Ausführungen beibehalten.

PUBLIKATIONSLISTE

PUBLIKATIONEN

Voigt, M.; **Smuda, M.**; Pfahler, C.; Glomb, M. A. Oxygen-Dependent Fragmentation Reactions during the Degradation of 1-Deoxy-D-erythro-hexo-2,3-diulose *Journal of Agricultural and Food Chemistry* **2010**, *58*, 5685-6591

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Henning, C.; **Smuda, M.**; Girndt, M.; Ulrich, C.; Glomb, M. A. Molecular Basis of Maillard Amide-AGE Formation in vivo *Journal of Biological Chemistry* **2011**, *286*, 44350-44356

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Smuda, M.; Glomb, M. A. Maillard Degradation Pathways of Vitamin C *Angewandte Chemie – International Edition* **2013**, *52*, 4887-4891 ***(Publication C)***

TAGUNGSVORTRÄGE

Smuda, M.; Glomb, M. A. Chemie von α-Dicarbonylverbindungen beim Abbau von Maltose 22. Arbeitstagung der Regionalverbände Südost und Nordost der Lebensmittelchemischen Gesellschaft der GDCh, 29.-30.03.**2012**, Jena

Smuda, M.; Glomb, M. A. Maillard-induzierte Fragmentierungswege von Vitamin C 23. Arbeitstagung des Regionalverbandes Südost der Lebensmittelchemischen Gesellschaft der GDCh, 14.-15.03.**2013**, Dresden

POSTERBEITRÄGE

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Smuda, M.; Glomb, M. A. α-Dicarbonylstrukturen als Schlüsselintermediate des Maillard-induzierten Abbaus von Maltose 40. Deutscher Lebensmittelchemikertag, 2011, Halle (Saale) (Abstract in: *Kurzreferateband Gesellschaft Deutscher Chemiker e.V.* **2011**)

Smuda, M.; Kahlert-Wohlrab, J.; Glomb, M. A. Neuartige N^{ε}-Lysin-Amide aus Vitamin C 41. Deutscher Lebensmittelchemikertag, 2012, Münster (Abstract in: *Kurzreferateband Gesellschaft Deutscher Chemiker e.V.* **2012**)

Smuda, M.; Glomb, M. A. Maillard Degradation of Vitamin C: β-Dicarbonyl cleavage leads to novel Amide-AGEs 11th International Symposium on the Maillard Reaction, 16.-20.09.**2012**, Nancy/France

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1 Introduction and Objectives

The nonenzymatic browning reaction between reducing carbohydrates and compounds bearing an amino group (amino acids, peptides and proteins) is referred to as the Maillard reaction. In the course of the Maillard reaction carbohydrates are subject to degradation processes resulting in lower molecular weight compounds.[1] Among these products carbonyl structures[2] and carboxylic acids[3] occur in the highest amounts. Furthermore, a class of highly reactive diketones, namely, α-dicarbonyl compounds, is present in Maillard reaction systems.[2,4] Although 1,2-dicarbonyls were detected only in small quantities, they have drawn major attention to food chemistry research regarding their pivotal role as color, flavor, and aroma precursors and also to medical sciences because their participation in AGE (advanced glycation endproduct) formation in vivo was established.[5]

Reactive α-dicarbonyl compounds were identified in various foods, for example, in cookies,[6] soy sauces,[7] milk products,[8] honey,[9] coffee,[10] beer,[11] wine,[12] and sweetened beverages (with high fructose corn syrup as the major source),[13] but also in peritoneal fluids[14] as a medical device. Moreover, α-dicarbonyl compounds were found to be produced in vivo, where they can mediate chronic and age-related diseases such as atherosclerosis,[15] diabetes,[16] uremia,[17] and Alzheimer's disease.[18] Recently in our working group the spectrum of α-dicarbonyl compounds present in the human body was extended by the structures 2-glucosulose (glucosone), 1-deoxy-2,3-glucodiulose (1-deoxy-glucosone) and 3,4-dihydroxy-2-oxo-butanal (threosone) among others, which are of considerably higher relevance than, for instance, the more stable 3-deoxy-2-glucosulose (3-deoxyglucosone) as the most frequently detected dicarbonyl in vivo besides glyoxal and methylglyoxal.[19]

However, as highly reactive and, thus, unstable Maillard intermediates, α-dicarbonyl compounds play a key role in carbohydrate decomposition.[5] Knowledge about the underlying fragmentation pathways is the basic prerequisite to understand changes occurring during storage and processing of food as well as during adverse alterations in vivo. Due to their high reactivity α-dicarbonyl compounds easily degrade, resulting in complex mixtures of reaction products, for example, again in short-chained α-dicarbonyls.[20] In the literature five major

pathways exist, operating as mechanistic explanations for carbohydrate fragmentation reactions: (i) retro-aldol fragmentation,[1] (ii) hydrolytic α-dicarbonyl cleavage,[21] (iii) oxidative α-dicarbonyl cleavage,[22] (iv) hydrolytic β-dicarbonyl cleavage,[23] and (v) amine-induced β-dicarbonyl cleavage.[24]

The aim of the present work was to study the lysine-catalyzed degradation of different carbohydrates in Maillard model incubation systems with focus on mechanistic relationships. As carbohydrate structures maltose, as a representative of disaccharide chemistry, and L-ascorbic acid with wide distribution in nature as vitamin C were chosen for the decomposition studies. Furthermore, the direct degradation of the central α-dicarbonyl intermediate in Maillard hexose chemistry 1-deoxy-2,3-glucodiulose (1-deoxyglucosone) was investigated. Results will be helpful in understanding alterations that occur in Maillard systems related to food chemistry and biochemistry, respectively.

2 Approaches to investigate fragmentation pathways

Published fragmentation pathways from the early Maillard chemistry were more of a hypothetical nature than experimentally proven and also more oriented toward the formation of only one compound without identification of the postulated fragmentation counterpart or the suggested precursor structure. Investigations were limited by analytical techniques and procedures and, therefore, restricted to stable Maillard reaction products like carboxylic acids that are easily detectable.[1]

It was a long time before investigators were able to identify reactive intermediates such as dicarbonyl compounds.[25] The use of trapping reagents such as *O*-alkyl hydroxylamines,[26] cysteamine,[27] aminoguanidine,[28] and diaminobenzene derivatives[29] allowed a reliable detection of the various dicarbonyl compounds in Maillard reaction systems and, thus, displayed a major step forward to understand mechanistic relationships in fragmentation processes. *o*-Phenylenediamine has prevailed as the generally accepted and most often used derivatization reagent in dicarbonyl analyses.[30]

Modern approaches to verify fragmentation pathways in model systems are based mainly on three procedures. (i) Kinetic studies of model incubation systems conducted under aerobic and anaerobic conditions monitor the formation and degradation rates of Maillard reaction products. This method provides information about mechanistically related compounds and also allows to identify precursor structures.[31,32] (ii) In most cases fragmentation pathways are examined by degradation experiments starting from the carbohydrate. Of course, more detailed results regarding the formation of fragmentation products can be achieved by decomposition studies of the direct precursor compound. This implies syntheses of reactive intermediates. For example, 3-deoxyglucosone,[33-35] 4-*O*-α-glucopyranosyl-2-glucosulose (maltosone) and 1,4-dideoxy-2,3-glucodiulose (1,4-dideoxyglucosone)[32] were successfully synthesized for such investigations. The synthesis of the key intermediate in hexose chemistry, namely 1-deoxyglucosone,[36] has to be considered as a milestone in this field.[37] (iii) An elegant tool to elucidate underlying reaction mechanisms in general is the use of isotopically labeled reactants in incubation systems. Thus, atoms in Maillard reaction products can be easily traced back to their original positions in the precursor carbohydrate. The working groups of Tressl[38] and Yaylayan[39] have pioneered this procedure.

However, this method is hampered by the high cost of labeled precursors and by the fact that many of the desirable carbohydrates or carbohydrate intermediates are not commercially available. Therefore, in most studies, carbohydrates labeled only at position C-1 were used.[40,41] As a consequence, many approaches with single-labeled sugars to investigate the origin of fragmentation products smaller than C_6 result in inconclusive data.[42] Nevertheless, the use of ^{13}C-enriched carbohydrates, if carefully planned and performed, delivers comprehensive results regarding fragmentation reactions.[43] A cost-effective method to realize labeling studies was developed by Yaylayan. He conducted the degradation of labeled sugars in a microreactor of a coupled pyrolysis-gas chromatography-mass spectrometry system.[44] Furthermore, ^{13}C-labeling applications, namely CAMOLA experiments (carbon module labeling)[45,46] and the CBL-technique (carbon bond labeling),[47] were established to clarify the mechanistic pathways of Maillard reaction products.

3 Fragmentation pathways in the Maillard reaction

Investigations of Weenen in 1998[20] and of Tressl and Rewicki in 1999[48] basically took three fragmentation pathways of α-dicarbonyl compounds into consideration within the Maillard reaction cascade: retro-aldolization, α-dicarbonyl cleavage and β-dicarbonyl cleavage. From today's perspective this mechanistic framework has to be refined and extended (**figure 1**) in view of two aspects: First, the α-dicarbonyl cleavage phenomenon has to be differentiated into a hydrolytic[21] and into an oxidative α-dicarbonyl cleavage. The latter was verified as a new sugar fragmentation pathway by Davidek et al. to occur under oxidative conditions.[22] Second, besides the hydrolytic β-dicarbonyl cleavage, *with publication A of the present Ph.D. thesis* recently an amine-induced β-dicarbonyl cleavage was established leading to amide-AGEs directly.[24] **Figure 1** presents all five pathways described for carbohydrate decomposition in an overview scheme. Each fragmentation pathway is discussed extensively on the basis of one of several examples selected from the literature summarized in the **tables 1-5**.

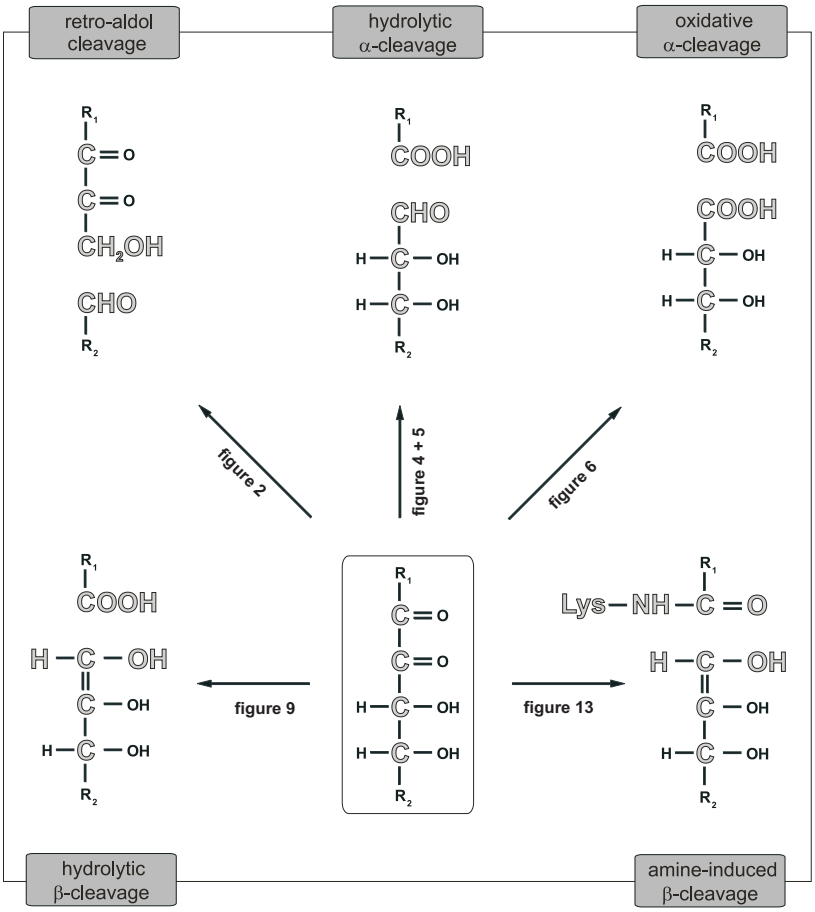


Figure 1: Fragmentation pathways for α-dicarbonyl compounds reported in Maillard literature

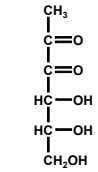
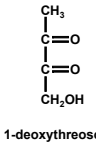
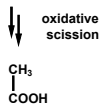
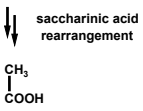
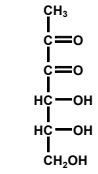
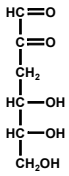
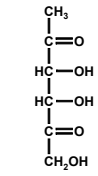
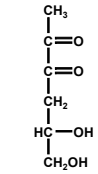
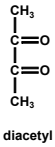
3.1 Retro-aldol cleavage

Compounds being prone to retro-aldol reactions must contain a β-hydroxy carbonyl moiety as a structural feature. Sugars, Amadori and Heyns rearrangement products and α-dicarbonyl compounds are reported in the literature to undergo retro-aldolization.[5,20] The carbon-carbon bond cleavage takes place between C-α and C-β next to the carbonyl functionality, resulting in shorter-chained hydroxy ketones, hydroxy aldehydes and dicarbonyl compounds.[2] *In the following explanations the focus is placed on* the retro-aldol reactions described for α-dicarbonyl fragmentation.

Aldol condensation and retro-aldol reactions can be assigned to topics of the early Maillard literature, for example, acetic acid was suggested to be formed as a follow-up product of glycolaldehyde by retro-aldol fragmentation of 1-deoxy-glucosone.[1] Up to now retro-aldolization is by far the most accepted fragmentation pathway, for example, used to explain the formation of 1-hydroxy-2-propanone (acetol), methylglyoxal and glyceraldehyde from deoxyosones generated during degradation of glucose.[20,42,49] **Table 1** reveals selected examples of different α-dicarbonyl precursors that are postulated to be split into the given fragmentation products via retro-aldolization. Various efforts have been made to substantiate the proposed fragmentation mechanism.

The formation of methylglyoxal and glyceraldehyde via retro-aldol fragmentation of 1-deoxyglucosone was postulated by Weenen based on a degradation experiment of 1-^{13}C glucose resulting in labeled methylglyoxal and unlabeled glycer-aldehyde.[20] In contrast, research of Voigt et al. has shown that degradation incubations of independently synthesized 1-deoxyglucosone led to extremely mismatched amounts of methylglyoxal and glyceraldehyde.[43] Thus, under the tested reaction conditions retro-aldolization seems to be very unlikely to explain the formation of methylglyoxal from 1-deoxyglucosone.

Table 1: Retro-aldol fragmentation of different α-dicarbonyl compounds reported in the literature

α-dicarbonyl precursor	fragmentation product	expected counterpart	experimental basis	comments	ref
 1-deoxyglucosone	 1-deoxythreosone  oxidative scission acetic acid	 glycolaldehyde  saccharinic acid rearrangement acetic acid	identification of acetic acid	hypothetical mechanism to explain acetic acid formation	[1]
 1-deoxyglucosone	 methylglyoxal	 glyceraldehyde	labeling studies with 1-^{13}C glucose	results disproven by Voigt[43]	[20]
 3-deoxyglucosone	 methylglyoxal	 glyceraldehyde	model incubations with synthesized 3-deoxyglucosone	degradation of 3-deoxyglucosone yielded only 0.18 mol-% methylglyoxal	[49]
 2,5-tautomer of 1-deoxyglucosone	 acetol	 3-hydroxy-pyruvaldehyde	labeling studies with 1-^{13}C, 2-^{13}C, 3-^{13}C, 4-^{13}C, 5-^{13}C, 6-^{13}C glucose	results of labeling experiments for both counterparts in support of retro-aldol reaction quantification of counterparts not performed	[42]
 1,4-dideoxyglucosone	 diacetyl	 glycolaldehyde	inverse experiment: aldol condensation of diacetyl and formaldehyde	results disproven by incubation of 1,4-dide-oxyglucosone, diacetyl was not detected[32]	[50]

Thornalley et al. synthesized 3-deoxyglucosone to demonstrate in an incubation experiment that methylglyoxal is generated from this precursor compound via retro-aldol fragmentation. Although methylglyoxal could be detected in that model experiment concentrations reached only 0.09 µM when starting from 50 µM 3-deoxyglucosone. Investigations of Thornalley et al. did not include the detection and quantification of glyceraldehyde as the expected counterpart which had to be generated in similar concentrations to confirm the hypothetical retro-aldol cleavage.[49] In addition, a yield of 0.18 mol-% methylglyoxal rejects the retro-aldolization route as an important fragmentation pathway to form methylglyoxal.

Yaylayan and Keyhani conducted labeling studies with all possible ^{13}C-glucose isotopomers. As a result, acetol was formed by 70 % from former positions C1–C2–C3 of glucose. The expected counterpart, 3-hydroxypyruvaldehyde, was detected as its pyrazine derivative containing carbon atoms C4–C5–C6. Based on this results retro-aldol fragmentation of the proposed 2,5-tautomer of 1-deoxy-glucosone was postulated as the major pathway to yield acetol.[42] Even though the labeling studies were in full support of the retro-aldolization the quantification of acetol and 3-hydroxypyruvaldehyde in incubations of the direct precursor 1-deoxyglucosone is missing to confirm the suggested mechanism.

From the mechanistic point of view, one of the selected examples is illustrated in **figure 2** and will be discussed in more detail. Pfeifer and Kroh postulated the formation of 1,4-dideoxyglucosone via a “peeling-off” mechanism from oligo-saccharides followed by degradation into 2,3-butanedione (diacetyl) and glycolaldehyde via retro-aldolization. To elucidate this fragmentation pathway, an inverse experiment was conducted. In a model incubation (aqueous solution, pH 8) diacetyl and formaldehyde were reacted for 10 min at ambient temperature to give 1,4-dideoxypentosulose (1,4-dideoxypentosone) detected as its quinoxaline derivative. Obviously as expected, 1,4-dideoxypentosone was generated as the aldol condensation product. This finding led to the assumption that if diacetyl is operating as an educt of aldol condensation, it must be also a product of retro-aldolization.[50]

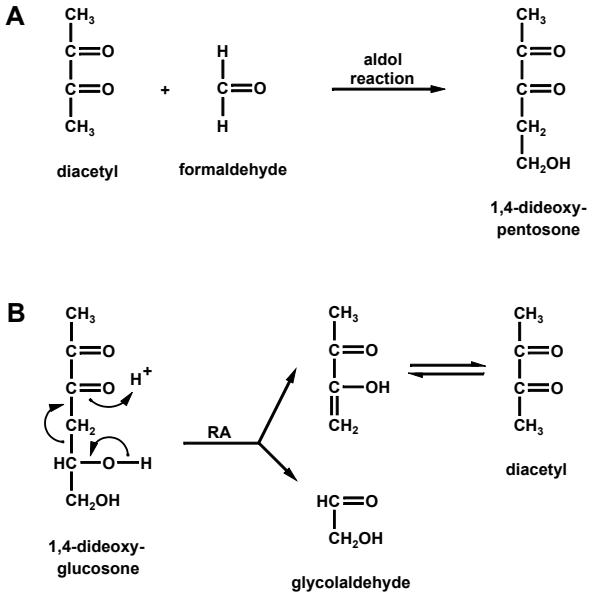


Figure 2: Aldol reaction of diacetyl and formaldehyde (A) and retro-aldolization (RA) of 1,4-dideoxy-2,3-hexodiulose (B), adapted from Pfeifer and Kroh[50]

In order to test this hypothesis, *in publication B the successful synthesis of 1,4-dideoxyglucosone was established* for direct degradation experiments of the suggested C6-precursor structure. Neither diacetyl nor glycolaldehyde as the counterpart were detected in incubations of 1,4-dideoxyglucosone conducted under similar conditions to Pfeifers inverse experiment. Thus, retro-aldolization has to be ruled out as the formation pathway of diacetyl from 1,4-dideoxy-glucosone.[32] *Although the origin of diacetyl in maltose Maillard systems remained unidentified publication B reveals mechanistic relationships of various α-dicarbonyl compounds important for disaccharide degradation. The overview scheme presented in* ***figure 3*** *is based on the thoroughly conducted degradation experiments. Most of the maltose decomposition pathways can be assigned to redox reactions.*

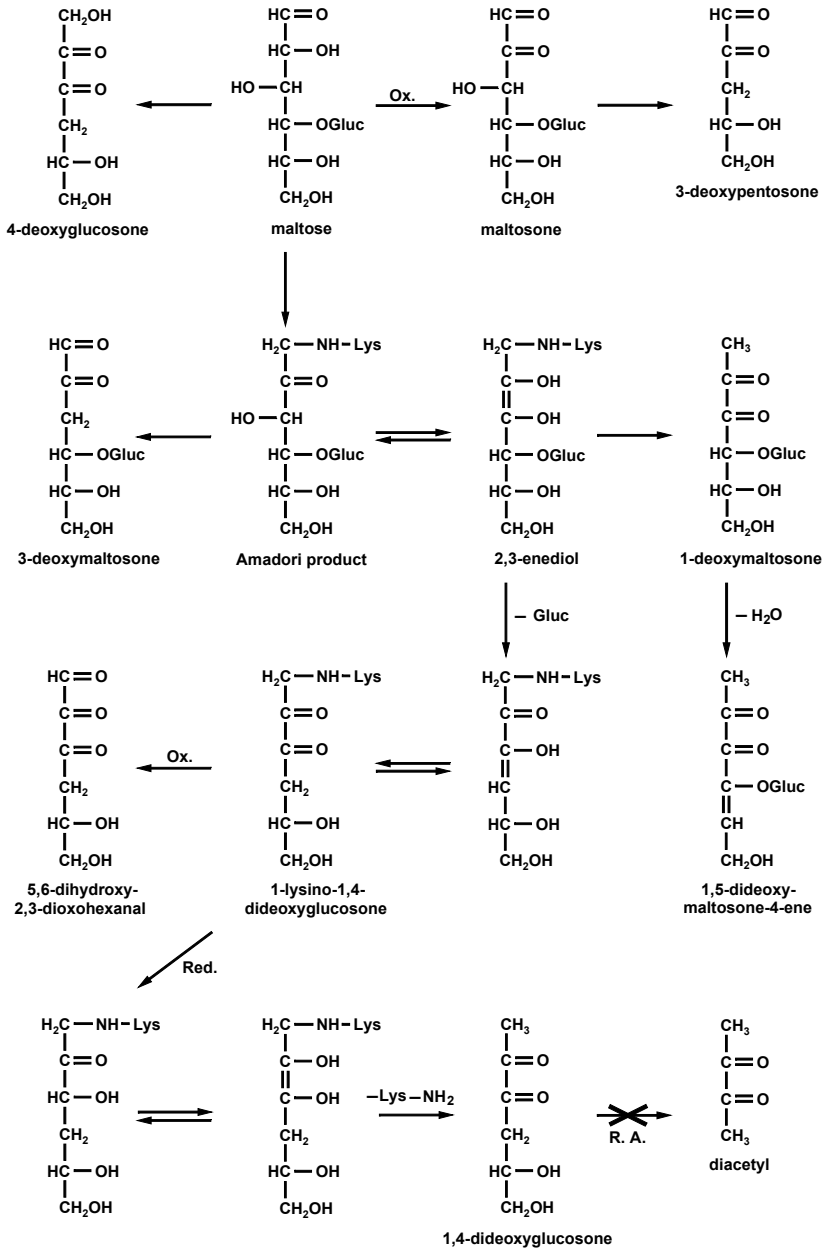


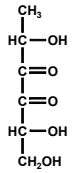
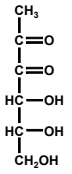
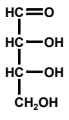
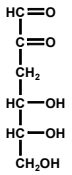
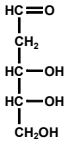
Figure 3: *Formation of α-dicarbonyl compounds in maltose Maillard reaction systems*[32]

In conclusion, literature research and own experiments … toward fragmentation pathways of dicarbonyl compounds in the Maillard reaction cascade revealed a surprising fact regarding retro-aldol reactions. Retro-aldolization seemed to be well understood and is also most widely used in current Maillard literature to explain various fragmentation products without questioning its validity. However, no mechanistic study conclusively underpinned its existence for dicarbonyl degradation. Thus, the concept of retro-aldolization needs to be challenged and should be re-examined on a mechanistic basis and for its importance to Maillard reactions.

3.2 Hydrolytic α-dicarbonyl cleavage

Second to retro-aldol reactions the hydrolytic α-dicarbonyl cleavage is the most frequently reported dicarbonyl fission reaction in the Maillard reaction network.[21,51-53] The hydrolytic α-dicarbonyl fragmentation reaction is understood as the breakage of the carbon-carbon bond between both carbonyl moieties toward a carboxylic acid and an aldehyde. From a mechanistic view this reaction must be based on an intramolecular disproportionation without changing the stereo chemistry given by the precursor. **Table 2** lists selected examples of α-dicarbonyl precursors suggested in the literature to trigger the α-dicarbonyl fragmentation route.

Table 2: Hydrolytic α-dicarbonyl cleavage of different α-dicarbonyl compounds reported in the literature

α-dicarbonyl precursor	fragmentation product	expected counterpart	experimental basis	comments	ref
 3,4-tautomer of 1-deoxyglucosone	 lactic acid	 glyceraldehyde	model incubations with synthesized 1-^{13}C-γ-pyranone	results of labeling experiments for both counterparts in support of a hydrolytic α-dicarbonyl cleavage quantification of counterparts not performed	[21]
 1-deoxyglucosone	 acetic acid	 erythrose	labeling studies with 1-^{13}C, 2-^{13}C, 3-^{13}C glucose	results of labeling experiments (labeled acetic acid) in support of a hydrolytic α-dicarbonyl cleavage detection of erythrose not performed	[51]
 3-deoxyglucosone	HCOOH formic acid	 2-deoxypentose	model incubations of glucose and fructose quantification of formic acid and furfurylalcohol	2-deoxypentose not detected, but furfurylalcohol as a possible follow-up product mismatched amounts of formic acid and furfurylalcohol	[52]

2,3-Dihydro-3,5-dihydroxy-6-methyl-4(*H*)-pyrane-4-one (γ-pyranone) is an established important intermediate in Maillard hexose chemistry resulting from 1-deoxyglucosone.[54,55] Kim and Baltes synthesized 1-^{13}C-γ-pyranone for mechanistic oriented degradation studies. After hydrolytic ring opening of the cyclic γ-pyranone, the 3,4-tautomer of 1-deoxyglucosone was hypothesized as an open-chained intermediate to degrade in lactic acid and glyceraldehyde via a hydrolytic α-dicarbonyl fragmentation reaction. The hydrolytic carbonyl decomposition was based on the detection of lactic acid exclusively labeled at the methyl group and unlabeled glyceraldehyde as a byproduct.[21] A final convincing proof for the suggested mechanism by quantification of the respective counterparts or by experiments with complementary labeled precursors was not performed.

Ginz et al. conducted labeling experiments with different glucose isotopomers (1-^{13}C, 2-^{13}C, 3-^{13}C) to explain the formation of various carboxylic acids. For example, NMR-results for acetic acid led to the assumption that the carboxylic acid is formed from 1-deoxyglucosone by hydrolytic α-dicarbonyl cleavage of the C-2/C-3 bond with erythrose as the expected counterpart. The final concluding link, the detection of erythrose, was not performed.[51] Thus, the proposed fragmentation mechanism has to be regarded as a mere assumption.

In another degradation study of glucose and fructose by Brands and van Boekel, the α-dicarbonyl cleavage route was considered as a self-evident fragmentation pathway to yield formic acid. A C5 compound (2-deoxypentose) should be formed via a C-1/C-2 scission of 3-deoxyglucosone in parallel at similar concentrations (**figure 4**). Unexpectedly, a 2-deoxypentose sugar was not detected in the model experiments. Instead, furfurylalcohol was found as a possible follow-up product known to be generated from 2-deoxypentose after cyclization and dehydration.[56] The authors mentioned that the amounts of furfurylalcohol did not equal the concentration of formic acid (approximately factor 1000 difference) and, therefore, expressed doubts themselves concerning the accuracy of the underlying fragmentation pathway. They speculated the mismatched amounts to be explained by further unknown follow-up products formed from the 2-deoxypentose sugar.[52]

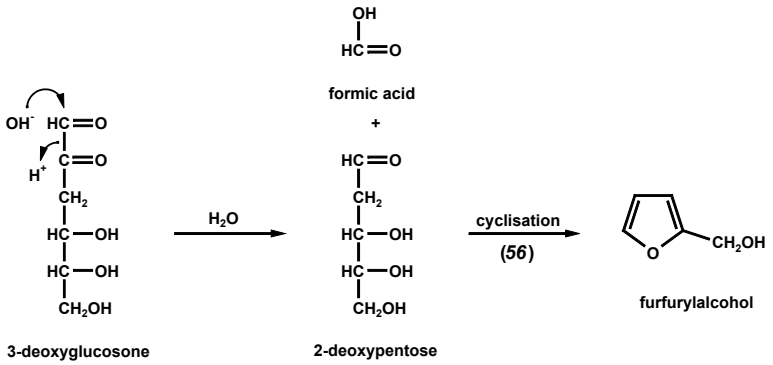


Figure 4: Hydrolytic α-dicarbonyl cleavage reaction of 3-deoxyglucosone, adapted from Brands and van Boekel[52]

The unsatisfactory results from known mechanistic studies regarding the hydrolytic α-dicarbonyl cleavage reaction gave rise to in-depth investigations by Davidek and co-workers with focus on carboxylic acid formation. The validity of the hydrolytic α-dicarbonyl cleavage hypothesis was disproved by the authors in a simple model experiment with 2,3-pentanedione (**figure 5**). The theory of hydrolytic α-dicarbonyl fragmentation calls for two possible sets of fragmentation counterparts: on the one hand acetic acid and propanal and, on the other hand, propanoic acid and acetaldehyde. Neither propanal nor acetaldehyde was identified in Davideks model experiment. Instead, the detection of acetic acid and propanoic acid at similar concentration levels rather pointed to an oxidative α-dicarbonyl cleavage.[57] In view of the published data, … it must be stated explicitly that the frequently suggested hydrolytic α-dicarbonyl cleavage is nonexistent, and has only been brought up based on the adventitious, but nonquantitative coexistence of matching counterparts.

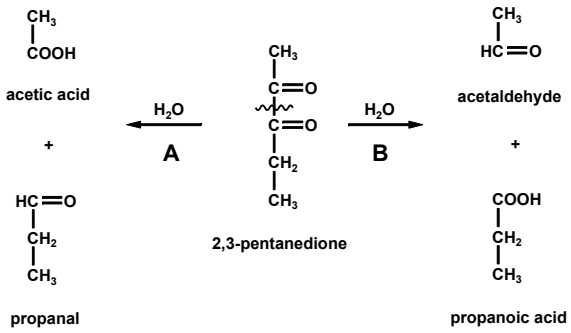
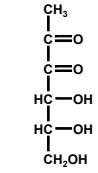
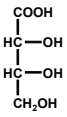
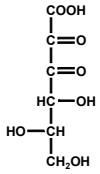
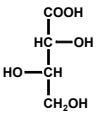


Figure 5: Hypothetical hydrolytic α-dicarbonyl cleavage of 2,3-pentanedione leads to acetic acid and propanal (A) or acetaldehyde and propanoic acid (B)

3.3 Oxidative α-dicarbonyl cleavage

As mentioned above, Davideks experiments of 2,3-pentanedione clearly indicated that an alternative oxidative α-dicarbonyl cleavage reaction indeed occurs as a previously not ascertained sugar fragmentation pathway to result in carboxylic acid formation.[22] *In support with publication C, it was shown that 31 % of ascorbic acid degradation pathways can be assigned to the oxidative α-dicarbonyl fragmentation reaction. For example, threonic acid is formed via this dicarbonyl cleavage reaction.* **Table 3** briefly summarizes the findings of Davidek et al. *for 1-deoxyglucosone and of publication C for 2,3-diketogulonic acid* concerning the oxidative α-dicarbonyl cleavage reaction.

Table 3: Oxidative α-dicarbonyl cleavage of different α-dicarbonyl compounds reported in the literature.

α-dicarbonyl precursor	fragmentation product	expected counterpart	experimental basis	comments	ref
 1-deoxyglucosone	 acetic acid	 erythronic acid	model incubations of synthesized 1-deoxyglucosone labeling studies with 1,2-^{13}C, 3-^{13}C, 6-^{13}C glucose model incubations of glucose performed under $^{18}O_2$-atmosphere	results of ^{13}C labeling and $^{18}O_2$ experiments in complete support of an oxidative α-dicarbonyl cleavage	[22]
 2,3-diketo-gulonic acid	 oxalic acid	 threonic acid	labeling studies with 1-^{13}C, 2-^{13}C, 3-^{13}C, 5-^{13}C, 6-^{13}C ascorbic acid quantification of both counterparts	results of labeling experiments and amounts of both counter-parts in complete support of an oxidative α-dicarbonyl cleavage	[58]

Following the degradation mechanism of 2,3-pentanedione, an oxidative α-dicarbonyl cleavage of 1-deoxyglucosone should give rise to erythronic acid as the counterpart of acetic acid. Indeed, Davidek et al. identified erythronic acid and smaller amounts of threonic acid in 1-deoxyglucosone incubations. Labeling studies with several ^{13}C glucose isotopomers have shown that both C_4-aldonic acids originate from the expected lower part of the original carbon backbone

C3–C4–C5–C6. From in-depth investigations with isotopically labeled ^{18}O-dioxygen Davidek and co-workers were able to establish the oxidative α-dicarbonyl cleavage of 1-deoxyglucosone as the most likely degradation pathway in complete support of their results (**figure 6**). This pathway requires activated molecular oxygen generated by photooxidation or hydroperoxide species. The dioxygen-triggered mechanism starts with the incorporation of molecular oxygen by an attack at the C-2 or C-3 carbonyl moiety resulting in two alkoxyradicals. After a single electron transfer reaction, the corresponding hydroperoxide anions occur, leading via a Baeyer-Villiger type rearrangement reaction to mixed asymmetric acid anhydrides with only one ^{18}O atom remaining in the molecule. The second ^{18}O atom was proposed to leave the hydroperoxide anion as a hydroxyl anion. Hydrolysis of the acid anhydride yields a mixture of monooxygen-labeled and unlabeled acetic acid and erythronic acid in ratio 1:1, respectively.[22]

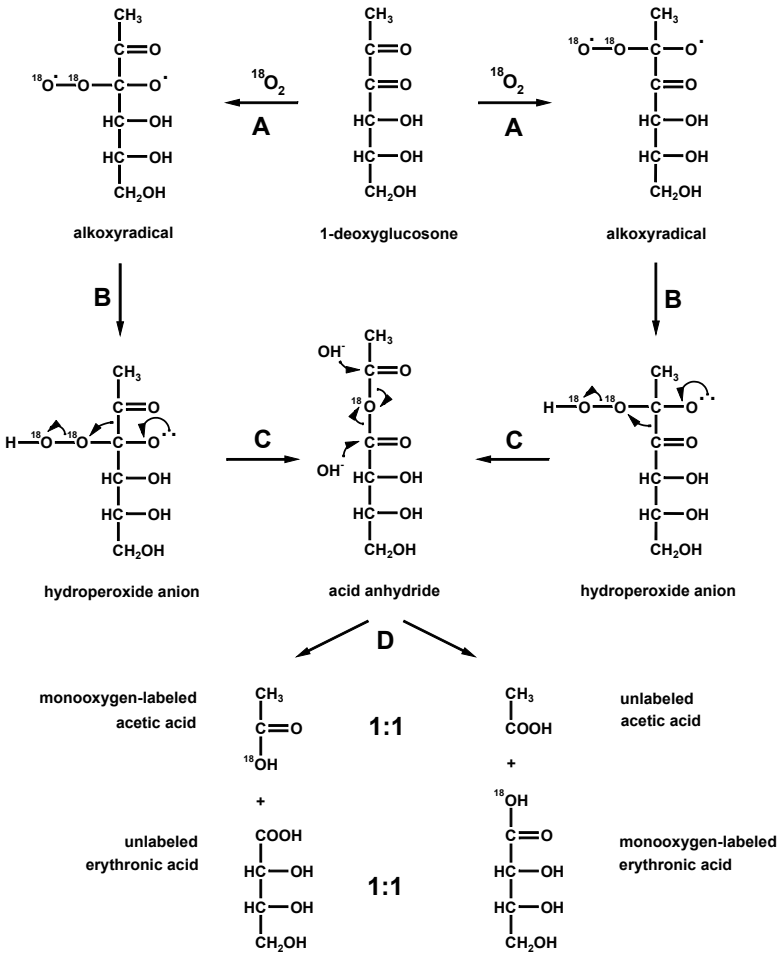


Figure 6: Oxidative α-dicarbonyl cleavage reaction of 1-deoxyglucosone, adapted from Davidek, with the following key steps: incorporation of molecular oxygen (A), single electron transfer reaction (B), Baeyer-Villiger type rearrangement (C), hydrolysis (D)[22]

*In publication C the ^{18}O experiment of Davidek was adapted to the own experiments to determine the amount of oxidative α-cleavage in ascorbic acid degradation. It was found that threonic acid, glyceric acid and glycolic acid were formed exclusively by this fragmentation pathway, whereas with oxalic acid it was half of the amount. The respective counterpart acids were identified by model incubations of different ^{13}C ascorbic acid isotopomers. Furthermore, it was shown that formation of the corresponding carboxylic acid amides from 2,3-diketogulonic acid can be also explained by this cleavage reaction (**figure 7**), assuming that step D in **figure 6** is initiated by the ε-amino function of lysine in parallel to a hydroxyl anion.*

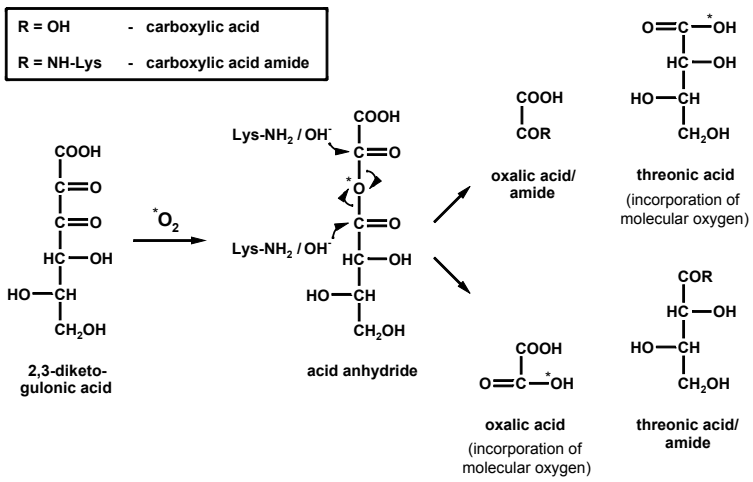


Figure 7: *Oxidative α-dicarbonyl cleavage leads to carboxylic acids and corresponding amides, adapted from [58]*

*Results of both labeling experiments and quantification of counterpart compounds led to fragmentation pathways presented in **figure 8**.*

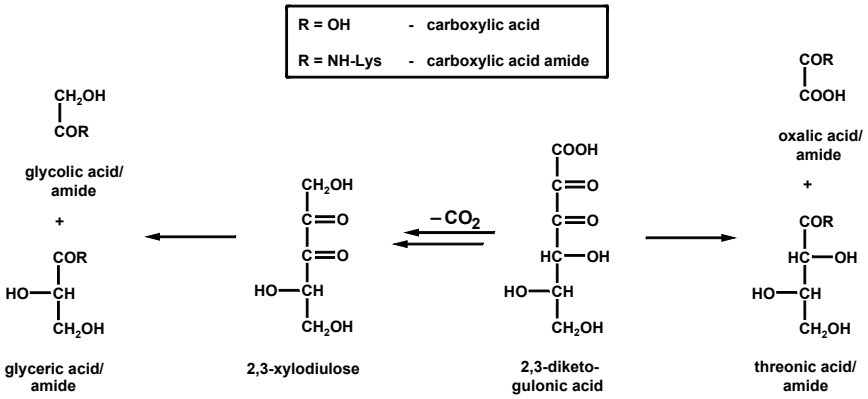


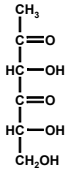
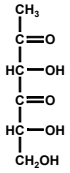
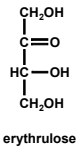
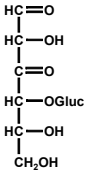
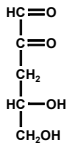
Figure 8: *Oxidative α-dicarbonyl cleavage leads to carboxylic acids and corresponding amides*

3.4 Hydrolytic β-dicarbonyl cleavage

The hydrolytic β-dicarbonyl cleavage is known as a retro-Claisen reaction[59] and was first reported by Hayami as an extreme case of an acyloin cleavage. During the decomposition reaction β-diketones (diacylcarbinols) are cleaved into α-hydroxy carbonyl compounds and carboxylic acids.[23]

Table 4 presents two typical examples of the hydrolytic β-dicarbonyl cleavage[23,57] and another case resulting in the formation of a carboxylic acid and an α-dicarbonyl compound instead of the anticipated α-hydroxy carbonyl structure.[32] Hydrolytic splitting of β-dicarbonyl sugars was mentioned already in 1961 to explain the formation of acetol from glucose and fructose as an alternative fragmentation pathway to retro-aldol reactions.[23] This fragmentation route postulated by Hayami first seemed to be largely hypothetical, but the results were later confirmed by Weenen who identified labeled acetol and unlabeled glyceric acid in 1-^{13}C glucose degradation experiments.[20]

Table 4: Hydrolytic β-dicarbonyl cleavage of different α-dicarbonyl compounds reported in the literature

α-dicarbonyl precursor	fragmentation product	expected counterpart	experimental basis	comments	ref
 2,4-tautomer of 1-deoxyglucosone	 acetol	 glyceric acid	identification of acetol in glucose and fructose incubation system	postulated mechanism later confirmed by Weenen[20]: 1-^{13}C glucose labeling studies yielded labeled acetol and unlabeled glyceric acid	[23]
 2,4-tautomer of 1-deoxyglucosone	 acetic acid	 erythrulose	model incubations with synthesized 1-deoxy-glucosone	identification of both counterparts in 1-deoxy-glucosone incubations, results later confirmed by Voigt[43]	[57]
			degradation studies of 2,4-pentanedione as model molecule	amounts of counterparts in support of a hydrolytic β-dicarbonyl cleavage	
 1,3-tautomer of maltosone	HCOOH formic acid	 3-deoxypentosone	model incubations with synthesized maltosone	degradation of maltosone yielded 16 mol-% 3-deoxypentosone	[32]
			kinetic studies of maltosone and 3-deoxypentosone in maltose incubation systems	kinetic studies in support of a hydrolytic β-dicarbonyl cleavage	

Again, Davidek and co-workers performed detailed studies regarding the hydrolytic β-dicarbonyl cleavage reaction. They unequivocally demonstrated that the β-dicarbonyl cleavage is the major pathway leading to the formation of acetic acid in aqueous hexose[54,60] and pentose-based Maillard reaction systems.[61] Supported by investigations of Voigt et al. the β-dicarbonyl cleavage route must be considered as the major carbohydrate fragmentation pathway in general.[36,43] The mechanism was proved by Davidek et al. in model degradation experiments of 2,4-pentanedione yielding almost equal amounts of acetic acid and acetone. The results were applicable to the degradation of 1-deoxyglucosone that is split into acetic acid and erythrulose. Exemplary for this case, mechanistic details for the β-dicarbonyl fragmentation are shown in **figure 9**. 1-Deoxyglucosone isomerizes into the 2,4-tautomer 1-deoxy-2,4-hexodiulose, which can be nucleophilically attacked by a hydroxyl anion, resulting in acetic acid and a C4-enediol. The

C4-enediol is subject to further isomerization reactions mainly leading to tetruloses. This is in contrast to the void mechanism of hydrolytic α-dicarbonyl cleavage, which does not allow for a change in the stereochemistry of a given precursor.

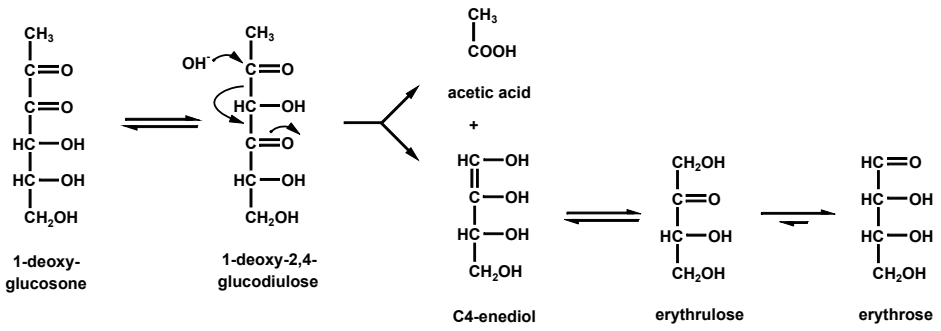


Figure 9: Hydrolytic β-dicarbonyl cleavage reaction of 1-deoxyglucosone, adapted from Davidek[57]

β-Dicarbonyl cleavage can also generate α-dicarbonyl compounds which was demonstrated for a maltose Maillard reaction system *in publication B*. As a result of kinetic experiments inquired in the presence and absence of oxygen the formation of 3-deoxypentosone was assigned to oxidative conditions. The experiments clearly pointed out maltosone as the most likely precursor structure that fulfills the criteria of being formed via oxidation *(**figure 10**)*. For verification, maltosone was synthesized independently and incubated separately. Maltosone was transformed by an amount of 16 mol-% into 3-deoxypentosone which is in good support to the assumed β-dicarbonyl cleavage. *The fragmentation pathway is shown in **figure 11**.* Here, the glucose located at position C-3 of the intermediate C5-enediol immediately triggers β-elimination to result in 3-deoxypentosone.[32]

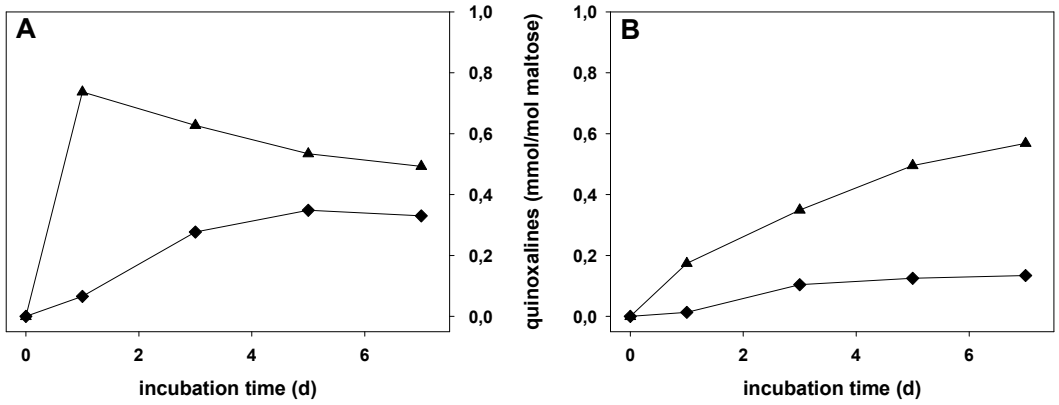


Figure 10: *Formation of maltosone (▲) and 3-deoxypentosone (◆) under aerobic (A) and anaerobic conditions (B)*

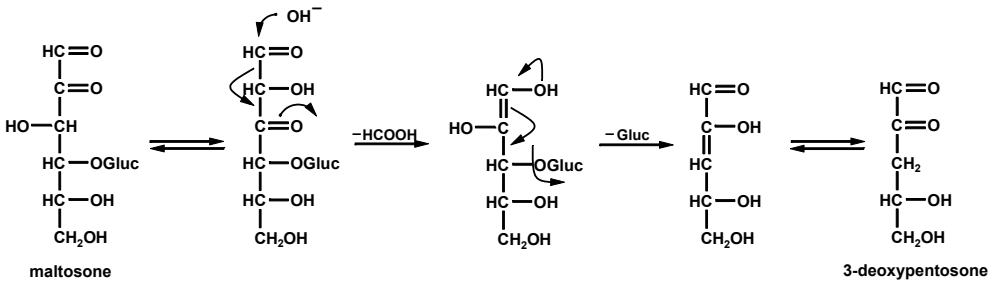


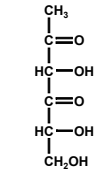
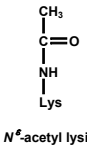
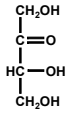
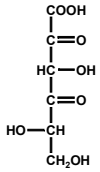
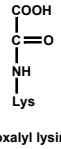
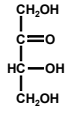
Figure 11: *Hydrolytic β-dicarbonyl cleavage reaction of maltosone leads to 3-deoxypentosone*[32]

3.5 Amine-induced β-dicarbonyl cleavage

With publication A in 2010 a novel class of amides (amide-AGEs) formed during the degradation of the hexose key intermediate 1-deoxyglucosone was established in aqueous Maillard model systems[24] and later also in the human blood plasma.[62] It was found that in analogy to the hydrolytic β-dicarbonyl cleavage an amine-induced β-dicarbonyl fragmentation reaction occurs generating carboxylic acid amides and α-hydroxy carbonyl counterparts.[24]

Table 5 includes one example of amine-induced β-dicarbonyl fragmentation for 1-deoxyglucosone[24] and for 2,3-diketogulonic acid,[58] respectively. Carboxylic acid amides were already mentioned before to be formed in Maillard reaction systems, for example, N^{ε}-oxalyl-lysine was identified in human lens proteins[63] and N^{ε}-formyl-lysine was detected in glycated *β*-lactoglobulin.[64]

Table 5: Amine-induced β-dicarbonyl cleavage of different α-dicarbonyl compounds

α-dicarbonyl precursor	fragmentation product	expected counterpart	experimental basis	comments	ref
 2,4-tautomer of 1-deoxyglucosone	 N^{ε}-acetyl lysine	 erythrulose	model incubations with synthesized 1-deoxyglucosone quantification of acetic acid/amide and erythrulose	formation of N^{ε}-acetyl lysine follows acetic acid formation at lower concentrations	[24]
 2,4-diketo-gulonic acid	 N^{ε}-oxalyl lysine	 erythrulose	labeling studies with 1-^{13}C, 2-^{13}C, 3-^{13}C, 5-^{13}C, 6-^{13}C ascorbic acid quantification of oxalic acid/amide and erythrulose	results of labeling experiments in support of a β-dicarbonyl cleavage formation of N^{ε}-oxalyl-lysine follows oxalic acid formation at lower concentrations	[58]

However, the mechanism of formation was not studied. Amounts of carboxylic acid amides are generally low due to stoichiometric and stereochemical reasons and thus not appropriate as a single elucidation criteria. On the other hand their corresponding carboxylic acids are operating as the quantitatively important counterparts to the α-hydroxy carbonyl compounds formed in the reaction systems. Thus, the identification of both, the amides in parallel to the corresponding acids, provides strong evidence for the underlying amine-induced β-dicarbonyl cleavage. Kinetic experiments indeed showed the same formation progress for both classes of stable Maillard end-products (***figure 12***). For example, N^{ε}-acetyl-lysine was formed in parallel to acetic acid with erythrulose as the counterpart from the 2,4-tautomer of 1-deoxyglucosone *(1-DG)* inline with the amine-induced β-dicarbonyl fragmentation pathway.[24]

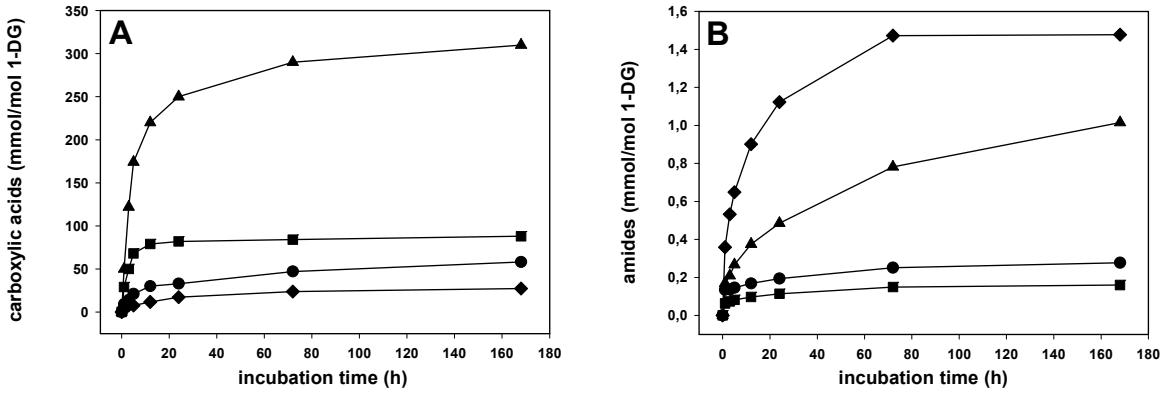


Figure 12: *Formation of carboxylic acids (A) and corresponding amides (B) in 1-DG/N^{α}-t-BOC-lysine incubation mixtures: acetic acid/N^{ε}-acetyl lysine (▲), glyceric acid/N^{ε}-glycerinyl lysine (■), lactic acid/N^{ε}-lactoyl lysine (●) and formic acid/N^{ε}-formyl lysine (◆)*[24]

Mechanistically, the fragmentation reaction is working analogous to the hydrolytic β-dicarbonyl cleavage and is explained in detail for the formation of N^{ε}-oxalyl-lysine from 2,4-diketogulonic acid in **figure 13**. Nucleophilic attack of the ε-amino function of lysine and subsequent β-cleavage releases N^{ε}-oxalyl lysine and a C4-enediol that isomerizes into erythrulose. This fragmentation pathway was proved indirectly by matching quantification of the corresponding oxalic acid and erythrulose. Conducted labeling studies were in complete support of the fragmentation pathway as erythrulose was found to originate at 100 % from C3–C4–C5–C6 and oxalic acid and the amide approximately at 90 % from C1–C2 of former ascorbic acid (*publication C*). As both fragmentation pathways rely on the same mechanism the parallel detection of corresponding carboxylic acids and amides is self-evident.

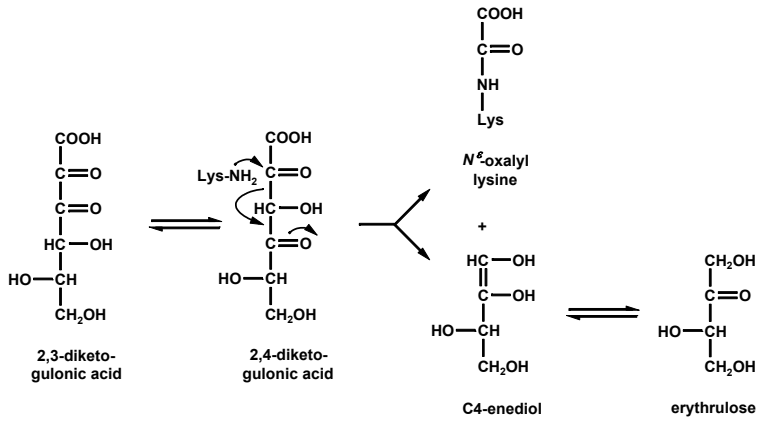


Figure 13: Amine-induced β-dicarbonyl cleavage reaction of 2,3-diketogulonic acid

4 Summary

In the present Ph.D. thesis the lysine-induced breakdown of different carbohydrates (1-deoxyglucosone, maltose, and L-ascorbic acid) was investigated with the aim of gaining insight into the mechanistic scenario. In general, α-dicarbonyl compounds are the most important key players during degradation processes. The β-dicarbonyl cleavage reaction and the oxidative α-dicarbonyl fragmentation are confirmed dicarbonyl decomposition pathways, whereas the retro-aldol fragmentation could not be verified for dicarbonyls. For mechanistic inquiries, in the chosen carbohydrate model systems formation and degradation rates of stable and reactive Maillard reaction products were analyzed. Furthermore, reactive α-dicarbonyl intermediates were independently synthesized to define follow-up products directly. By conducting isotopic labeling experiments the origin of the Maillard reaction products from the precursor carbohydrate was determined.

The β-dicarbonyl fragmentation route is described in the literature as the main degradation pathway in hexose chemistry for 1-deoxyglucosone as its central intermediate. The cleavage is induced by a nucleophilic attack of a hydroxyl anion. Within the model incubation system 1-deoxyglucosone/N^{α}-t-BOC-lysine it was shown that the ε-amino moiety of lysine can also operate as a nucleophile. Thus, the amine-induced β-dicarbonyl cleavage was established as a new α-dicarbonyl fragmentation pathway. Quantitatively, this pathway is of minor importance due to stoichiometric and stereochemical reasons, but can be used as an additional verification of the hydrolytic β-dicarbonyl cleavage. While the hydrolytic form releases carboxylic acids as stable Maillard endproducts the amine-induced alternative leads to the corresponding carboxylic acid amides. As a result of the incubation studies N^{ε}-formyl lysine, N^{ε}-acetyl lysine, N^{ε}-lactoyl lysine, and N^{ε}-glycerinyl lysine were identified as amide-advanced glycation endproducts (AGEs). The three latter compounds can be considered as novel compounds in Maillard systems. The relevance of this class of amide-AGEs was demonstrated by their identification in human blood plasma.

In case of maltose/lysine incubations the focus was placed on mechanistic relationships between arising α-dicarbonyls. It was found that fragmentation of the carbohydrate backbone occurs to a remarkable lesser extend in comparison to glucose degradation. This phenomenon can be explained by the differing reactivity

of 1-deoxymaltosone versus 1-deoxyglucosone. Enolisation along the entire carbon backbone is hampered by the linkage of glucose at position C-4 of 1-deoxymaltosone. As a result, possible fragmentation reactions are extremely limited. In contrast, redox processes were found to be specific for maltose degradation. Herein, 1-lysino-1,4-dideoxyglucosone has emerged as the key intermediate. As a typical fragmentation route β-dicarbonyl cleavage was verified as the formation pathway for 3-deoxypentosone from maltosone. Retro-aldol fragmentations were not identified. In the literature, for the formation of diacetyl from 1,4-dideoxyglucosone a retro-aldol cleavage was postulated. This hypothesis was disproven by the own experiments. As expected, glucose that is split off during the maltose degradation followed the typical hexose chemistry.

For a L-ascorbic acid/N^{α}-t-BOC-lysine model system 75 % of the Maillard-induced degradation pathways could be explained by oxidative α-fragmentations (31 %), β-cleavages (32 %), and decarboxylation from a hydrate/hemiaminal intermediate of the key player 2,3-diketogulonic acid (12 %). Carboxylic acids, that arose as stable Maillard endproducts via all the three mentioned decomposition routes, occured in the highest amounts. Again, all three pathways were verified by their amine-induced form. Thereby, the spectrum of novel amide-AGEs was extended by the compounds N^{ε}-xylonyl-lysine, N^{ε}-lyxonyl-lysine, and N^{ε}-threonyl-lysine. With these carboxylic acid amides AGEs being specific for ascorbic acid degradation were identified for the first time. Thus, Maillard-degradation pathways of ascorbic acid can be distinguished from other carbohydrate precursors. This is of vital interest because in most biological systems glucose and Vitamin C are present at the same time.

Knowledge regarding fragmentation pathways in the model systems are prerequisite for a better understanding of reaction processes in biological systems although reaction conditions can differ. The novel class of amide-AGEs established for Maillard systems can be useful for instance, to indicate thermal food processing or as marker compounds of diseases where dicarbonyls are involved. For the mentioned 1-deoxyglucosone mediated amides it was already shown that concentrations in plasma of patients suffering from uremia are significantly higher than in healthy subjects.

5 Zusammenfassung

In der vorliegenden Dissertation wurde der Lysin-induzierte Abbau verschiedener Kohlenhydratstrukturen (1-Desoxyglucoson, Maltose und L-Ascorbinsäure) mit dem Ziel untersucht, die dabei ablaufenden Fragmentierungswege detailiert zu erfassen. Als Fragmentierungsreaktionen von Dicarbonylverbindungen, die als Schlüsselintermediate des Kohlenhydratabbaus gelten, sind die β-Spaltung und die oxidative α-Spaltung relevant. Für eine Retro-Aldolspaltung gibt es für Dicarbonylverbindungen keine experimentell gesicherte Grundlage. Für die Kohlenhydrat-Maillard-Modellsysteme war es nötig, die Umsatzraten sowohl reaktiver als auch stabiler Maillard-Reaktionsprodukte zu bestimmen. Weiterhin wurden reaktive Intermediate synthetisiert, um deren Einfluss auf die Folgeproduktbildung zu charakterisieren. Mit Hilfe von Isotopenmarkierungs-experimenten konnte lokalisiert werden, aus welchen Positionen der Precursor-struktur Abbauprodukte hervorgehen.

Für 1-Desoxyglucoson als zentrales Intermediat der Hexosenchemie war die hydrolytische β-Dicarbonylspaltung als Hauptfragmentierungsweg bereits literatur-beschrieben. Die β-Spaltung wird dabei durch einen nucleophilen Angriff eines Hydroxylions eingeleitet. Mit Lysin als Aminkomponente im Modellsystem 1-Desoxyglucoson/N^{α}-t-BOC-Lysin konnte gezeigt werden, dass gleichermaßen die ε-Aminofunktion von Lysin als Nucleophil fungiert. Damit wurde die amin-induzierte β-Spaltung als neuer Fragmentierungsweg von Dicarbonylen etabliert, der aufgrund stöchiometrischer und stereochemischer Verhältnisse weniger von quantitativer Bedeutung ist als vielmehr der Verifizierung der hydrolytischen β-Spaltung dient. Im Vergleich zur hydrolytischen Form, bei der Carbonsäuren als stabile Maillard-Endprodukte resultieren, führt die amin-induzierte Form entsprechend zu den korrespondierenden Carbonsäureamiden. Im Zuge der Untersuchungen wurde die Bildung der Amid-advanced glycation endproducts (AGEs) N^{ε}-Formyllysin, N^{ε}-Acetyllysin, N^{ε}-Lactoyllysin und N^{ε}-Glycerinyllysin aufgeklärt. Die drei letztgenannten Amidstrukturen wurden dabei erstmals in Maillard-Systemen nachgewiesen. Die Bedeutung dieser neuen Klasse der Amid-AGEs zeigte sich durch deren Identifizierung in humanem Blutplasma.

In den Maltose/Lysin-Inkubationen lag der Fokus auf der Klärung mechanistischer Zusammenhänge zwischen den aus Maltose hervorgehenden α-Dicarbonyl-

verbindungen. Hinsichtlich der Abbauwege war auffällig, dass die Fragmentierung des Kohlenhydratgrundgerüsts weniger stark ausgeprägt ist als im Vergleich zu Glucose. Grund dafür ist der Unterschied der Reaktivität von 1-Desoxymaltoson versus 1-Desoxyglucoson. Der an Kohlenstoffatom C-4 gebundene Glucoserest des 1-Desoxymaltosons lässt eine Enolisierung über diese Position hinaus nicht zu, so dass im Vergleich zum 1-Desoxyglucoson mögliche Fragmentierungen erheblich limitiert sind. Für die Chemie der Maltose treten vielmehr Redoxprozesse als typische Abbauwege mit 1-Lysino-1,4-dideoxyglucoson als Schlüsselintermediat in Erscheinung. Als eine klassische Fragmentierungsreaktion wurde die Bildung von 3-Desoxypentoson aus Maltoson via β-Dicarbonylspaltung gezeigt. Retro-Aldolspaltungen konnten hingegen nicht nachgewiesen werden. Die in der Literatur beschriebene Bildung von Diacetyl aus 1,4-Didesoxyglucoson durch Retro-Aldolfragmentierung wurde eindeutig widerlegt. Die im Zuge des Maltose-Abbaus freiwerdende Glucose folgte erwartungsgemäß der Hexosenchemie.

Für das L-Ascorbinsäure/N^{α}-t-BOC-Lysin-System konnten 75 % der Maillard-induzierten Abbauwege aufgeklärt werden. 32 % sind der hydrolytischen β-Dicarbonylspaltung zuzuweisen, 31 % entfallen auf die oxidative α-Spaltung. Die verbleibenden 12 % lassen sich durch Hydratisierung und anschließende Decarboxylierung der 2,3-Diketogulonsäure erklären, die das Schlüsselintermediat des Vitamin C Abbaus darstellt. Wieder ist nur die Bildung von Carbonsäuren, die als stabile Maillard-Endprodukte aus allen der drei angeführten Abbauwege hervorgehen, von quantitativer Bedeutung. Auch hier konnten alle drei Fragmentierungsmechanismen durch deren amin-induzierte Form abgesichert werden. Das Spektrum neuartiger Amid-AGEs wurde im Zuge dessen um die Strukturen N^{ε}-Xylonyllysin, N^{ε}-Lyxonyllysin und N^{ε}-Threonyllysin erweitert. Mit diesen drei Carbonsäureamiden ist es erstmals gelungen spezifische Ascorbinsäure-Abbauprodukte zu identifizieren. Sie erlauben daher, Maillard-Abbauwege der Ascorbinsäure von denen anderer Precursor-Kohlenhydrate zu unterscheiden. Dies ist von entscheidendem Interesse, da in den meisten biologischen Sysmtemen beispielsweise Glucose und Vitamin C nebeneinander vorliegen.

Die für Modellsysteme erarbeiteten Fragmentierungsmechanismen tragen maßgeblich dazu bei, Reaktionsabläufe in biologischen Systemen besser zu

verstehen, auch wenn die Reaktionsbedingungen nicht direkt übertragbar sind. Die in Maillard-Systemen neu identifizierten Amid-AGEs können beispielsweise herangezogen werden, um Erhitzungsprozesse während der Lebensmittelverarbeitung nachzuweisen oder als Markerverbindungen verschiedener Krankheitsbilder dienen, bei denen Dicarbonylverbindungen involviert sind. Für die Urämie wurde dieser Nachweis bereits erbracht.

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AGE	advanced glycation endproduct
t-BOC	*tert*-butoxycarbonyl
CAMOLA	carbon module labeling
CBL	carbon bond labeling
1-DG	1-deoxyglucosone
NMR	nuclear magnetic resonance
o	*ortho*

10 Appendix

Publication A:

Smuda, M.; Voigt, M.; Glomb, M. A. Degradation of 1-Deoxy-D-erythro-hexo-2,3-diulose in the Presence of Lysine Leads to Formation of Carboxylic Acid Amides *Journal of Agricultural and Food Chemistry* **2010**, *58*, 6458-6464

Publication B:

Smuda, M.; Glomb, M. A. Novel Insights into the Maillard Catalyzed Degradation of Maltose *Journal of Agricultural and Food Chemistry* **2011**, *59*, 13254-13264

Publication C:

Smuda, M.; Glomb, M. A. Maillard Degradation Pathways of Vitamin C *Angewandte Chemie – International Edition* **2013**, *52*, 4887-4891

6458 J. Agric. Food Chem. 2010, 58, 6458–6464
DOI:10.1021/jf100334r

JOURNAL OF AGRICULTURAL AND FOOD CHEMISTRY
ARTICLE

Degradation of 1-Deoxy-D-*erythro*-hexo-2,3-diulose in the Presence of Lysine Leads to Formation of Carboxylic Acid Amides

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A novel species of amides formed from degradation of one of the most important key intermediates in Maillard hexose chemistry−1-deoxyhexo-2,3-diulose−was investigated. In 1-deoxyhexo-2,3-diulose/N^{α}-*t*-BOC-lysine reaction mixtures four amides, N^{ε}-acetyl lysine, N^{ε}-formyl lysine, N^{ε}-lactoyl lysine and N^{ε}-glycerinyl lysine, were identified and their structures verified by authentic reference standards. Amides and corresponding carboxylic acids (acetic acid, formic acid, lactic acid and glyceric acid) accumulated over time. Both N^{ε}-lysine amides and carboxylic acids were thus determined as stable Maillard end products. Results of model incubations suggested the synthesis of amides to be mechanistically closely related to the formation of their corresponding carboxylic acids by β-dicarbonyl cleavage. Due to the different chemical properties of all the compounds monitored, various analytical strategies had to be carried out (LC−MS^2, GC−MS, GC−FID, enzymatic determination).

KEYWORDS: Maillard reaction; β-dicarbonyl cleavage; 1-deoxyhexo-2,3-diulose; 1-DG; N^{ε}-lysine amides; carboxylic acid amides; carboxylic acids

INTRODUCTION

During heating or storing of food, the Maillard reaction leads to degradation of reducing sugars and amino acids, peptides or proteins (*1*). In addition to the formation of color, taste and flavor, the Maillard reaction results in a decline of the biological value of proteins caused by reactions of the ε-amino group of lysine (*2*). In the past decades the outstanding role of α-dicarbonyl compounds as intermediates of high reactivity in the complex Maillard chemistry was manifested. Among these intermediates 1-deoxyhexo-2,3-diulose (1-DG) is of major importance in hexose chemistry (*3*−*5*). Since Glomb and Pfahler succeeded in the synthesis of 1-DG, it is possible to study carbohydrate Maillard degradation mechanistically isolated on this intermediate (*6*). Davidek et al. postulated a formation pathway of acetic acid and glyceric acid that can be explained by a hydrolytic β-dicarbonyl cleavage mechanism starting from 1-DG (*7*, *8*). In our study we now investigated a novel type of Maillard reaction products: carboxylic acid amides formed by the ε-amino group of lysine during degradation of 1-DG. In the literature, formation of amides in Maillard chemistry is only scarcely described. In 1985 Hayase and co-workers investigated the formation of *N*-butylacetamide and *N*-butylformamide in model reaction systems of butylamine and the α-dicarbonyl structures diacetyl and glyoxal (*9*). Several α-hydroxy acid amides such as lactic acid propylamide have been identified in reaction mixtures of glucose and propylamine (*10*), but it is still unknown if lysine side chains can react in a similar way. Nagaraj et al. studied the formation of oxalate monoalkylamide in the human lens in 1999 (*11*). In 2001 Glomb and Pfahler postulated the formation pathway of GOLA (N^{ε}-{2-[(5-amino-5-carboxypentyl)amino]-2-oxoethyl}lysine) and GALA (N^{ε}-glycoloyllysine) in glyoxal/lysine reaction mixtures. With the detection of GOLA and GALA in brunescent lens proteins the importance of these amide protein modifications with respect to pathological processes *in vivo* was demonstrated (*12*). N^{ε}-Formyl lysine was already mentioned in the literature as a glycation product of proteins, but a formation mechanism was not given (*13*).

The present work extends the knowledge of amide formation in Maillard reactions. The four amides N^{ε}-acetyl lysine, N^{ε}-formyl lysine, N^{ε}-lactoyl lysine, and N^{ε}-glycerinyl lysine as well as the corresponding carboxylic acids acetic acid, formic acid, lactic acid, and glyceric acid were found to be formed in 1-DG/N^{α}-*t*-BOC-lysine reaction mixtures. Quantification of the compounds was done by various analytical methods to gain further insights into the degradation mechanisms of 1-DG.

MATERIALS AND METHODS

Materials. The following chemicals of analytical grade were commercially available: *N*,*O*-bis(trimethylsilyl)acetamide with 5% trimethylchlorosilane, acetic acid, heptafluorobutyric acid (Fluka/Sigma-Aldrich, Seelze, Germany), ninhydrin, dipotassium hydrogen phosphate trihydrate, potassium dihydrogen phosphate (Merck, Darmstadt, Germany), decylchloroformate, *N*-ethyl-*N*′-(3-dimethylaminopropyl)carbodiimide (EDC), 2,3-*O*-isopropylidene-D-glyceric acid methyl ester, D-glyceric acid calcium salt, N^{α}-*t*-BOC-lysine, N^{ε}-acetyl-L-lysine, N^{ε}-formyl-L-lysine (Sigma-Aldrich, Steinheim, Germany), 1-hydroxybenzotriazole (HOBt), pyridine, thioanisole,

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 Published on Web 04/29/2010

diethylenetriaminepentaacetic acid (Fluka/Sigma-Aldrich, Taufkirchen, Germany), TFA (Carl Roth, Karlsruhe, Germany), Dowex 50W × 8 (H^+-form, 50−100 mesh) (Serva/Boehringer, Ingelheim-Heidelberg, Germany), L-lactic acid (Alfa Aesar, Karlsruhe, Germany), and formic acid (Grüssing GmbH, Filsum, Germany). 1-Deoxy-4,5-*O*-isopropylidene-D-*erythro*-hexo-2,3-diulose and 1-deoxy-D-*erythro*-hexo-2,3-diulose (*6*), N^{α}-(*t*-butoxycarbonyl)-L-lysine *tert*-butyl ester (**1**) (*14*), and O^{α}-tetrahydropyranyl-L-lactic acid (**2**) (*15*) were synthesized according to the literature.

N^{α}-(*t*-Butoxycarbonyl)-N^{ε}-(O^{α}-(tetrahydropyranyl)-L-lactoyl)-L-lysine *tert*-Butyl Ester (3). 2 (70 mg, 0.4 mmol) was dissolved in CH_2Cl_2 (2 mL) under argon atmosphere at 0 °C, and HOBt (54 mg, 0.4 mmol) was added. After 10 min a solution of EDC (80 μL, 0.45 mmol) in CH_2Cl_2 (1 mL) was added dropwise. To the stirred solution, **1** (120 mg, 0.4 mmol) dissolved in CH_2Cl_2 (1 mL) was added dropwise. The reaction mixture was warmed to room temperature and stirred overnight. Then, the mixture was diluted with CH_2Cl_2 (10 mL) and washed with 15 mL each of saturated $NaHCO_3$ solution and brine. The organic layer was dried over $MgSO_4$, and the solvent was removed under reduced pressure. The crude product was purified by column chromatography on silica gel 60 using EtOAc−hexane (1:1). Fractions containing **3** (TLC: R_f 0.83 in EtOAc, ninhydrin detection) were collectively concentrated in vacuo to afford compound **3** as a yellow viscous oil (90 mg, 49%). ^{1}H NMR (400 MHz, $CDCl_3$): δ [ppm] = 1.42−1.82 (m, 12 H), 1.31 (d, ^3J = 6.8 Hz, 3H), 1.38 (s, 9H), 1.39 (s, 9H), 3.13−3.24 (m, 2H), 3.41−3.49 and 3.75−3.86 (m, 2H), 4.02−4.18 (m, 2H), 4.52−4.57 (m, 1H). ^{13}C NMR (100 MHz, $CDCl_3$): δ [ppm] = 17.9, 19.6/19.9, 20.4/22.6, 25.1/25.2, 28.0, 28.4, 29.3/29.4, 31.0, 32.5, 38.6, 53.7/53.8, 62.8/64.0, 73.3/74.3, 79.5, 81.7, 98.4/98.9, 155.4, 171.7/171.8, 173.0/173.3. HR-MS: *m*/*z* 481.2884 (found); *m*/*z* 481.2886 (calculated for $C_{23}H_{42}O_7N_2Na$ $[M + Na]^+$).

N^{ε}-L-Lactoyl-L-lysine Trifluoroacetate (4). To **3** (66 mg, 0.14 mmol) a solution of 25% thioanisole in TFA (8 mL) was added. The reaction mixture was stirred at room temperature for 6 h under argon atmosphere while the color of the solution changed from green to brown. The mixture was concentrated in vacuo, and the residue was partitioned between 20 mL each of diethyl ether and water. The organic layer was further extracted with water (10 mL). The combined aqueous layers were concentrated under reduced pressure to a final volume of 3 mL. The crude product was purified by column chromatography (Lichroprep RP C18 material). The column was pre-equilibrated with methanol (50 mL) followed by 0.1% TFA in water (50 mL). The product was eluted with 0.1% TFA in water. Fractions containing **4** (positive ninhydrin stain) were collectively lyophilized to give a colorless glassy foam in quantitative yield. ^{1}H NMR (400 MHz, D_2O): δ [ppm] = 1.23 (d, ^3J = 6.8 Hz, 3H), 1.25−1.40 (m, 2H), 1.42−1.52 (m, 2H), 1.75−1.95 (m, 2H), 3.09−3.16 (m, 2H), 3.90−3.95 (m, 1H), 4.10 (q, ^3J = 6.6 Hz, 1H). ^{13}C NMR (100 MHz, D_2O): δ = 19.8, 21.6, 28.0, 29.5, 38.4, 53.0, 67.8, 172.2, 177.4. HR-MS: *m*/*z* 219.1339 (found); *m*/*z* 219.1335 (calculated for $C_9H_{19}O_4N_2$ $[M + H]^+$).

2,3-*O*-Isopropylidene-glyceric Acid (6). To a solution of KOH (95 mg, 1.7 mmol) in 250 μL of water and 500 μL of absolute EtOH, 2,3-*O*-isopropylidene-D-glyceric acid methyl ester (**5**) (160 mg, 1 mmol) was added. The reaction mixture was stirred 30 min at room temperature. After adjusting the pH value to 4.3 by using HCl (1 M), solution was extracted with EtOAc (5 × 3 mL), and the combined organic layers were dried ($MgSO_4$) and concentrated to give 62 mg (42%) of a colorless oil. ^{1}H NMR (400 MHz, $CDCl_3$): δ [ppm] = 1.38 (s, 3H), 1.48 (s, 3H), 4.14 (dd, ^3J = 5.0 and ^2J = 8.9 Hz, 1H), 4.25 (dd, ^3J = 7.5 and ^2J = 8.7 Hz, 1H), 4.60 (dd, ^3J = 5.0 and ^3J = 7.5 Hz, 1H), 9.54 (br, 1H). ^{13}C NMR (100 MHz, $CDCl_3$): δ [ppm] = 25.3, 25.8, 67.2, 73.6, 111.8, 175.8.

N^{α}-(*t*-Butoxycarbonyl)-N^{ε}-2,3-*O*-*isopropylidene-glycerinyl*-L-lysine *tert*-Butyl Ester (7). 6 was reacted in an equimolar ratio with HOBt and **1** and with a 1.1-fold excess of EDC as described above for **3**. Purification was done by column chromatography (silica gel 60, EtOAc−hexane 1:1). Fractions containing **7** (TLC: R_f 0.87 in EtOAc, ninhydrin detection) were collectively concentrated in vacuo to afford **7** as a yellow viscous oil (105 mg, 61%). ^{1}H NMR (400 MHz, $CDCl_3$): δ [ppm] = 1.24−1.44 (m, 2H), 1.33 (s, 3H), 1.38 (s, 9H), 1.40 (s, 9H), 1.42 (s, 3H), 1.46−1.60 (m, 3H), 1.70−1.80 (m, 1H), 3.21 (m, 2H), 4.01 (dd, ^3J = 5.4 and ^2J = 8.7 Hz, 1H), 4.03−4.13 (m, 1H) 4.21 (dd, ^3J = 7.7 and ^2J = 8.7 Hz, 1H), 4.40 (dd, ^3J = 5.4 and ^3J = 7.5 Hz, 1H). ^{13}C NMR (100 MHz, $CDCl_3$): δ [ppm] = 22.5, 25.0, 26.1, 28.0, 28.3, 29.2, 32.5, 38.6, 53.7, 67.7, 75.0, 79.5, 81.7, 110.7, 155.2, 170.9, 171.6. HR-MS: *m*/*z* 453.2571 (found); *m*/*z* 453.2570 (calculated for $C_{21}H_{38}O_7N_2Na$ $[M + Na]^+$).

N^{ε}-Glycerinyl-L-lysine Trifluoroacetate (8). Deprotection and purification of **7** was done as described above for compound **4**. **8** was obtained as a colorless glassy foam in quantitative yield after lyophilization. ^{1}H NMR (400 MHz, D_2O): δ [ppm] = 1.25−1.39 (m, 2H), 1.42−1.49 (m, 2H), 1.76−1.95 (m, 2H), 3.15 (t, ^3J = 6.6 Hz, 2H), 3.62−3.71 (m, 2H), 3.93 (t, ^3J = 6.0 Hz, 1H), 4.07−4.12 (m, 1H). ^{13}C NMR (100 MHz, D_2O): δ [ppm] = 21.6, 28.0, 29.5, 38.6, 53.1, 63.4, 72.4, 172.3, 174.2. HR-MS: *m*/*z* 235.1288 (found); *m*/*z* 235.1286 (calculated for $C_9H_{19}O_5N_2$ $[M + H]^+$).

Degradation of 1-Deoxyhexo-2,3-diulose. A solution of 1-deoxy-4,5-*O*-isopropylidene-D-*erythro*-hexo-2,3-diulose (0.35 mmol) in water (3 mL) was stirred with Dowex 50W × 8 (H^+-form, 50−100 mesh, 4 mL) for 4 h under argon atmosphere. The resin was filtered off and washed with MeOH (3 × 2 mL). After evaporation of the combined solvents, the residue was dissolved in phosphate buffer (0.1 M, pH 7.4, 4.2 mL). Aliquots (150 μL) of this solution and a solution of N^{α}-*t*-BOC-lysine (0.35 mmol) in phosphate buffer (0.1 M, pH 7.4, 4.2 mL) were mixed in screw-cap vials giving an incubation solution of 1-DG and N^{α}-*t*-BOC-lysine (42 mM, respectively). Incubation solutions were shaken at 37 °C, and samples were taken over time.

Amides were analyzed by HPLC−MS^2 after deprotection of the BOC group. Lactic and glyceric acid were analyzed by GC−MS after silylation, acetic acid by GC-FID as its decylchloroformate derivative and formic acid by enzymatic determination. Each sample was prepared at least three times.

Deaerated Incubations. Degradation of 1-deoxy-D-*erythro*-hexo-2,3-diulose under deaerated conditions was carried out using phosphate buffer with 1 mM diethylenetriaminepentaacetic acid. Buffer was degassed with helium before samples were prepared; samples were deaerated with argon before incubation.

Deprotection Reaction of the Amides. To aliquots (100 μL) of the incubation solutions 6 M HCl (100 μL) was added, and samples were kept at room temperature for 30 min. Solutions were diluted on a scale of 1:100 with water prior to injection into the HPLC−MS^2 system.

Control Experiment. Carboxylic acids (acetic acid, lactic acid, glyceric acid and formic acid, 13 mM, respectively) were separately incubated with N^{α}-*t*-BOC-lysine (42 mM) in phosphate buffer (0.1 M, pH 7.4). Incubation solutions were shaken at 37 °C, and samples were taken after 7 days. Further workup was performed analogous to the amide samples prior to injection into the HPLC−MS^2 system.

Derivatization Reactions for the Carboxylic Acids. *Trimethylsilyl Derivatives of Lactic Acid and Glyceric Acid.* Adopting the method described in ref *16*, aliquots of the samples (50 μL) were dried in vacuo, residues were dissolved in pyridine (50 μL), and *N*,*O*-bis(trimethylsilyl)-acetamide with 5% trimethylchlorosilane (50 μL) was added. Samples were kept 3 h at room temperature prior to injection into the GC−MS system. Quantification was carried out by comparison of peak areas obtained in the TIC with those of standard solutions containing known amounts of the pure authentic reference compounds. Signals of target compounds were standardized using the signal of silylated phosphoric acid present in all samples. Data for silylated compounds obtained by GC−MS showed standard deviations of < 5 mmol/mol 1-DG, resulting in coefficients of variation < 5%.

Decylchloroformate Derivative of Acetic Acid. Adopting the method described in ref *17*, samples (300 μL) were spiked with chlorosuccinic acid (50 μg) dissolved in water as internal standard, pyridine (40 μL) and decylchloroformate (50 μL) were added. The mixture was then sonicated for 10 min. Decyl esters were extracted with hexane, and the organic layer was analyzed by GC−FID. Quantitative results were obtained by internal calibration using commercially available acetic acid. Data obtained by GC−FID showed standard deviations of < 10 mmol/mol 1−DG, resulting in coefficients of variation < 3.5%.

Analytical HPLC−MS^2. A Jasco PU−2080 Plus quaternary gradient pump with degasser and Jasco AS-2057 Plus autosampler (Jasco, Gross-Umstadt, Germany) were used. Chromatographic separations were performed on a stainless steel column (VYDAC 218TP54, 250 × 4.6 mm, RP18, 5 μm, Hesperia, CA) using a flow rate of 1 mL/min. The mobile phase used consisted of water (solvent A) and MeOH/water (7:3 (v/v), solvent B). To both solvents (A and B), 1.2 mL/L heptafluorobutyric acid (HFBA) was added. Samples were injected at 2% B (held 25 min), gradient then changed to 100% B in 5 min (held 10 min) and then changed to 2% B in 5 min (held 15 min). Elution of amides (N^{ε}-glycerinyl lysine at 7.0 min,

Table 1. Mass Spectrometer Parameters for Amide Quantification (MRM Mode)

	Q1 mass (amu)	Q3 mass (amu)	dwell time (ms)	DP	CE	CXP
N^{ε}-acetyl lysine	189.20	126.10	75.00	30.00	18.00	10.00
N^{ε}-formyl lysine	175.10	112.10	75.00	25.00	20.00	13.00
N^{ε}-lactoyl lysine	219.20	156.20	75.00	32.00	20.00	8.00
N^{ε}-glycerinyl lysine	235.30	84.20	75.00	48.00	37.00	6.00

N^{ε}-formyl lysine at 9.3 min, N^{ε}-acetyl lysine at 13.6 min, and N^{ε}-lactoyl lysine at 13.8 min) was monitored by mass detection. The mass analyses were performed using an Applied Biosystems API 4000 quadrupole instrument (Applied Biosystems, Foster City, CA) equipped with an API source using an electrospray ionization (ESI) interface. The LC system was connected directly to the probe of the mass spectrometer. Nitrogen was used as sheath and auxiliary gas. To measure the amides in incubation solutions the MRM mode of HPLC−MS^2 was used. The optimized parameters for mass spectrometry are given in **Table 1**. Quantification was based on the standard addition method using solutions containing known amounts of the pure authentic reference compounds. Data for amides obtained by HPLC−MS^2 showed standard deviations of < 0.07 mmol/mol 1-DG, resulting in coefficients of variation < 5%.

GC−MS. Samples were analyzed on a Thermo Finnigan Trace GC Ultra coupled to a Thermo Finnigan Trace DSQ (both Thermo Fisher Scientific GmbH, Bremen, Germany). The GC column was a HP-5 (30 m × 0.32 mm, film thickness 0.25 μm; Agilent Technologies, Palo Alto, CA); injector, 220 °C; split ratio, 1:30; transfer line, 250 °C. The oven temperature program was as follows: 100 °C (0 min), 5 °C/min to 200 °C (0 min), 10 °C/min to 270 °C (10 min). Helium 5.0 was used as carrier gas in constant flow mode (linear velocity 28 cm/s, flow 1 mL/min). Mass spectra were obtained with EI at 70 eV (source: 210 °C) in full scan mode (mass range m/z 50−650).

GC−FID. Samples were analyzed on a HP 6890N chromatograph (Agilent Technologies, Palo Alto, CA) equipped with a flame ionization detector. The column was a HP-5 (30 m × 0.32 mm, film thickness 0.25 μm; Agilent Technologies, Palo Alto, CA); injector, 250 °C; split ratio, 1:10; detector, 270 °C. Helium 4.6 was used as carrier gas in constant flow mode (linear velocity 28 cm/s, flow 1.6 mL/min). The oven temperature program was as follows: 50 °C (1 min), 4 °C/min to 120 °C (15 min), 40 °C/min to 220 °C (3 min), 30 °C/min to 260 °C (15 min).

Nuclear Magnetic Resonance Spectroscopy (NMR). NMR spectra were recorded on a Varian VXR 400 spectrometer operating at 400 MHz for ^{1}H and 100 MHz for ^{13}C, respectively. Chemical shifts are given relative to external $SiMe_4$.

Accurate Mass Determination (HR−MS). The high-resolution positive ion ESI mass spectra (HR-MS) were obtained from a Bruker Apex III Fourier transform ion cyclotron resonance (FT-ICR) mass spectrometer (Bruker Daltonics, Billerica, MA) equipped with an Infinity cell, a 7.0 T superconducting magnet (Bruker, Karlsruhe, Germany), a radio frequency (RF)-only hexapole ion guide, and an external electrospray ion source (Apollo; Agilent, off-axis spray). Nitrogen was used as drying gas at 150 °C. The samples were dissolved in methanol, and the solutions were introduced continuously via a syringe pump at a flow rate of 120 μL h^{-1}. The data were acquired with 256 k data points and zero filled to 1024 k by averaging 32 scans.

Electrospray Ionization Mass Spectrometry (ESI−MS^2). The mass analyses were performed using the instrument described above for HPLC−MS^2 analyses. The samples were dissolved in water, and the solutions were introduced continuously via a syringe pump at a flow rate of 120 μL h^{-1}. Spectra were obtained by using the following parameters: for N^{ε}-acetyl lysine (DP 50, CE 20, CXP 10), for N^{ε}-formyl lysine (DP 50, CE 20, CXP 10), for N^{ε}-lactoyl lysine (DP 55, CE 22, CXP 10), and for N^{ε}-glycerinyl lysine (DP 50, CE 24, CXP 10). In all cases 42 MCA scans were carried out.

Enzymatic Determination of Formic Acid. Quantification of formic acid in incubation solutions was carried out with an enzyme test kit obtained from R-Biopharm AG (Darmstadt, Germany). Depending on the amount of formic acid after different incubation times, varying volumes of incubation solutions were directly used for the enzyme test (100 to 500 μL). Coefficients of variation (< 2.5%) were in line with the test kit description.

RESULTS

Syntheses of Authentic Amide References. Lactic acid amide (**4**, N^{ε}-lactoyl lysine) was synthesized in a typical amide formation reaction (**Figure 1 a**), starting from lactic acid as the tetrahydropyranyloxy derivative (**2**). **2** was activated with EDC and HOBt and reacted with N^{α}-(*t*-butoxycarbonyl)-L-lysine *tert*-butyl ester (**1**) to give the fully protected amide **3**. In the last step all three protective groups were completely removed by treatment with thioanisole in TFA to result in the lactid acid amide **4**.

In a similar procedure glyceric acid amide (**8**, N^{ε}-glycerinyl lysine) was synthesized (**Figure 1 b**). After activation of isopropylidene protected glyceric acid (**6**) and reaction with **1** the protected amide **7** was isolated. Finally **7** was deprotected under acidic conditions to obtain **8**.

Both target compounds **4** and **8** as well as the intermediates **3**, **6**, and **7** were verified by nuclear magnetic resonance experiments. Furthermore the elemental composition of **3**, **4**, **7**, and **8** was confirmed by accurate mass determination.

Formation of Carboxylic Acids in 1-DG/N^{α}-*t*-BOC-lysine Incubations (pH 7.4, 37 °C). Degradation of 1-deoxyhexo-2,3-diulose (1-DG) in the presence of amine led to the formation of short chain carboxylic acids. Formation of formic, acetic, lactic and glyceric acid was monitored over a period of seven days. Results are shown in **Figure 2 A**. Within the first twelve hours a rapid accumulation of all carboxylic acids was observed. The further progress of the reaction was characterized by a marginal increase of the acids until concentrations remained unchanged. Formation of acetic acid led to the highest amounts after seven days (310 mmol/mol 1-DG), followed by glyceric acid (88 mmol/mol 1-DG), lactic acid (58 mmol/mol 1-DG) and formic acid (27 mmol/mol 1-DG).

Formation of Carboxylic Acid Amides in 1-DG/N^{α}-*t*-BOC-lysine Incubations (pH 7.4, 37 °C). Before incubation solutions could be analyzed for the corresponding carboxylic acid amides, samples were treated with acid to remove the BOC protection group. Amides were found to be stable under these workup conditions. A HPLC−MS^2 method (MRM mode) was developed to perform quantification of the amides in model incubation systems. Collision induced dissociation (CID) experiments were carried out to observe the fragmentation pattern of the different amides. **Table 2** demonstrates that all four amides underlie analogous fragmentation steps. Fragmentation of N^{ε}-lactoyl lysine as an exemplary representative of analyzed amides can be explained as follows: Based on QM^+ ion of N^{ε}-lactoyl lysine (m/z = 219) loss of HCOOH gives the ion at m/z 173. Loss of both HCOOH and ammonia at the α-amino group gives the ion at m/z 156 with the highest abundance. Ions at m/z 130 and m/z 84 are fragments that are prominent in the mass spectra of all four amides. m/z 84 presents the pyrrolinium ion. The ion at m/z 130 occurs after a cyclization step of N^{ε}-lactoyl lysine to a six-membered ring and loss of N^{ε}-functionality. All these fragmentations are similar to the fragmentation of ordinary lysine reported in refs *18* and *19*. Accurate mass determination of synthesized N^{ε}-lactoyl lysine gave m/z 173.1284 for $C_8H_{17}O_2N_2$ (calculated m/z 173.1281) and m/z 156.1019 for $C_{12}H_{14}O_2N$ (calculated m/z 156.1016) and confirmed the results obtained by CID experiments.

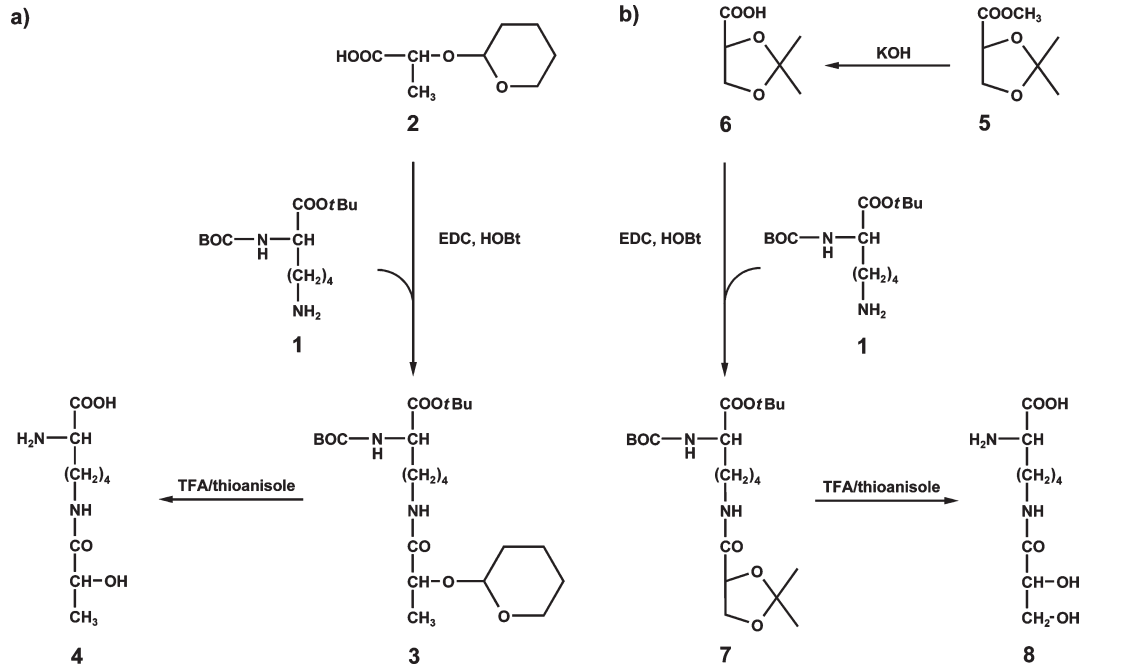

Figure 1. Synthesis of N^{ε}-lactoyl lysine and N^{ε}-glycerinyl lysine.

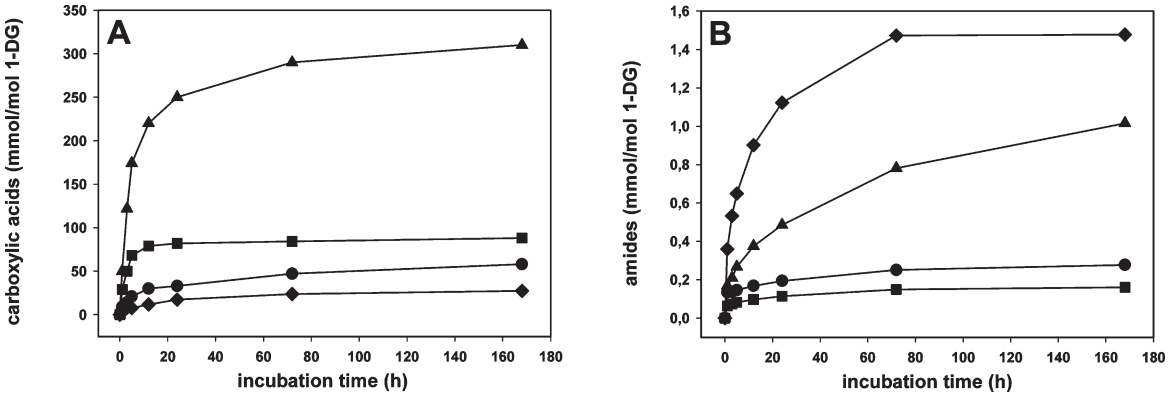

Figure 2. Formation of carboxylic acids (**A**) and corresponding amides (**B**) in 1-DG/N^{α}-*t*-BOC-lysine incubation mixtures (42 mM in phosphate buffer 0.1 M, pH 7.4, at 37 °C under aerated conditions): acetic acid/N^{ε}-acetyl lysine (▲), glyceric acid/N^{ε}-glycerinyl lysine (■), lactic acid/N^{ε}-lactoyl lysine (●) and formic acid/N^{ε}-formyl lysine (◆).

Table 2. Comparison of Fragmentation of the Different Amides in Positive Ion ESI-MS^2−CID Experiments

	fragmentation pattern, *m/z* (amu)				
	(M + H)	(M + H −HCOOH)	(M + H − HCOOH − NH_3)	(M + H − H_2N−CO−R)	pyrrolinium ion
N^{ε}-acetyl lysine	189	143	126	130[a]	84
N^{ε}-formyl lysine	175	129	112	130[b]	84
N^{ε}-lactoyl lysine	219	173	156	130[c]	84
N^{ε}-glycerinyl lysine	235	189	172	130[d]	84

[a] R = CH_3. [b] R = H. [c] R = CH(OH)−CH_3. [d] R = CH(OH)−CH_2OH.

Formation of N^{ε}-acetyl lysine, N^{ε}-formyl lysine, N^{ε}-lactoyl lysine, and N^{ε}-glycerinyl lysine was also monitored over a period of seven days. The time course formation plot of the carboxylic acid amides is shown in **Figure 2 B**. In parallel with the corresponding carboxylic acids there was a rapid increase of all amides within the first twelve hours. Also at later incubation times concentrations reached a steady level. Unexpectedly, formation of N^{ε}-formyl lysine led to the highest amounts after seven days

(1.48 mmol/mol 1-DG), followed by N^{ε}-acetyl lysine (1.01 mmol/mol 1-DG), N^{ε}-lactoyl lysine (0.28 mmol/mol 1-DG), and N^{ε}-glycerinyl lysine (0.16 mmol/mol 1-DG).

Table 3 provides information about concentrations of carboxylic acid amides formed under aerated and under deaerated incubation conditions at 37 °C after 24 and 72 h. Under aerated conditions we measured significant higher amounts of N^{ε}-glycerinyl lysine and of N^{ε}-formyl lysine. 0.15 mmol/mol 1-DG of N^{ε}-glycerinyl lysine and 1.47 mmol/mol 1-DG of N^{ε}-formyl lysine were formed after 72 h at 37 °C under aerated conditions in contrast to 0.09 mmol/mol 1-DG and 1.08 mmol/mol 1-DG under deaerated conditions, respectively. Concentrations of N^{ε}-acetyl lysine and of N^{ε}-lactoyl lysine under aerated versus under deaerated conditions varied only slightly. 0.78 mmol/mol 1-DG of N^{ε}-acetyl lysine and 0.25 mmol/mol 1-DG of N^{ε}-lactoyl lysine were obtained after 72 h at 37 °C under aerated conditions, in comparison to 0.75 mmol/mol 1-DG and 0.26 mmol/mol 1-DG under deaerated conditions, respectively.

Table 3. Comparison of Amide Concentrations Generated from 1-DG and N^{α}-*t*-BOC-lysine under Aerated and Deaerated Conditions

	concentration (mmol/mol 1-DG)			
	24 h		72 h	
	aerated	deaerated	aerated	deaerated
N^{ε}-acetyl lysine	0.48	0.45	0.78	0.75
N^{ε}-formyl lysine	1.12	0.71	1.47	1.08
N^{ε}-lactoyl lysine	0.19	0.16	0.25	0.26
N^{ε}-glycerinyl lysine	0.11	0.05	0.15	0.09

DISCUSSION

Carboxylic acids formed in Maillard reaction systems were already established to be stable degradation products (*7*, *20*). Data obtained in the present work for the carboxylic acids acetic acid, formic acid, lactic acid and glyceric acid confirmed this finding. The corresponding carboxylic acid amides revealed the same behavior. Over a period of seven days all amides monitored accumulated to reach a plateau. Thus, these amides can be regarded as stable degradation end products in Maillard reactions too.

Davidek et al. described a hydrolytic β-dicarbonyl cleavage mechanism of 1-deoxyhexo-2,4-diulose as the major formation pathway for acetic acid as well as for glyceric acid (*7*), based on studies of Hayami to investigate formation of acetol by decomposition of hexoses via a hydrolytic cleavage of 1-deoxyhexo-2,4-diulose (*21*). Also Weenen confirmed a β-cleavage mechanism to give acetol and glyceric acid (*22*). Davidek postulated that first an isomerization reaction of 1-DG to 1-deoxyhexo-2,4-diulose takes place, followed—in the case of acetic acid—by a nucleophilic attack of a hydroxyl ion at the C-2 carbonyl function (likewise glyceric acid is formed by a nucleophilic attack on C-4 position). The cleavage reaction results in acetic acid and a C4-enediole which can undergo further isomerization reactions to yield erythrulose and corresponding deoxyosones (*7*, *8*, *20*). These findings give reason to believe that the cleavage mechanism will

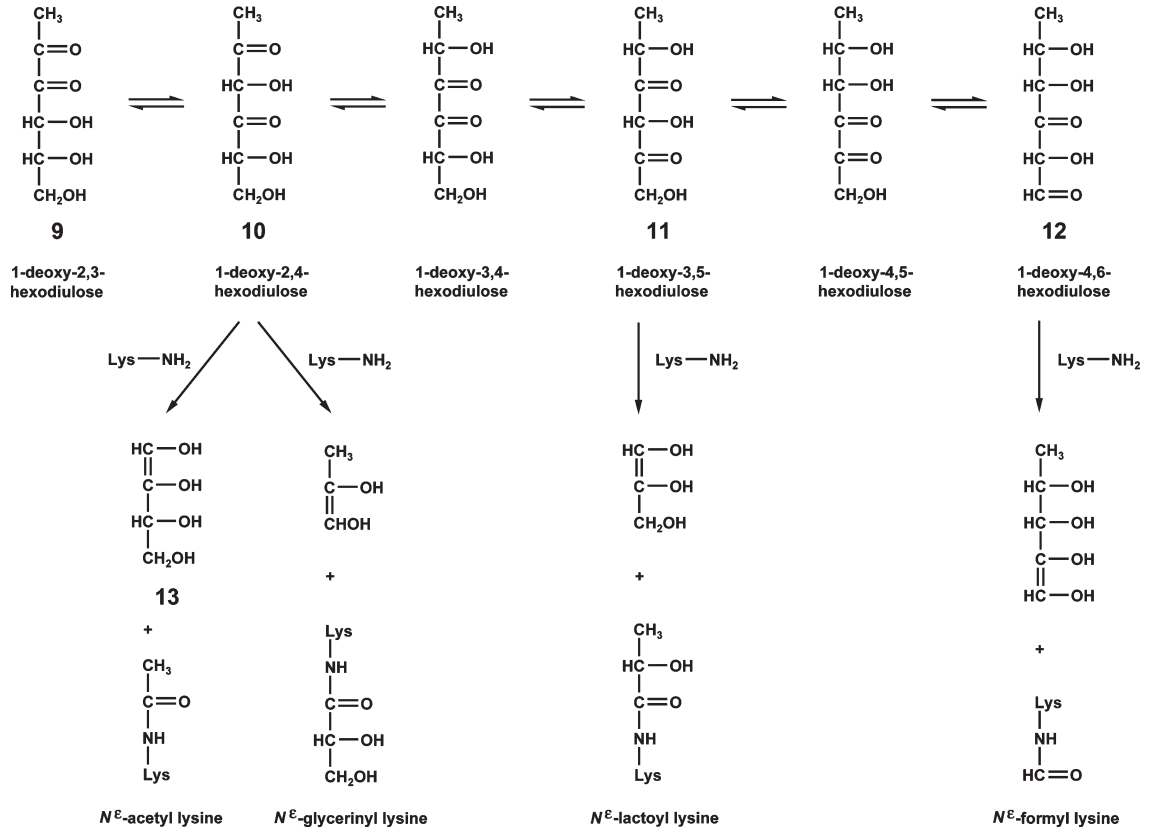

Figure 3. Mechanism of nonoxidative 1-deoxyhexo-2,3-diulose degradation: β-dicarbonyl cleavage leads to N^{ε}-acetyl lysine, N^{ε}-glycerinyl lysine, N^{ε}-lactoyl lysine and N^{ε}-formyl lysine.

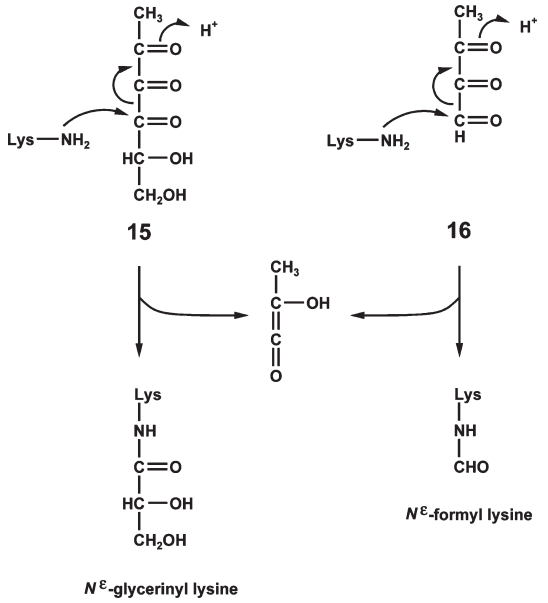

Figure 4. Nucleophilic attack on tricarbonyls formed by oxidation from 1-DG or 1-DT leads to N^{ε}-glycerinyl lysine and N^{ε}-formyl lysine.

also lead to formation of carboxylic acid amides in the presence of amines. In **Figure 3** a mechanism is postulated that is capable of elucidating the formation pathway of N^{ε}-acetyl lysine, N^{ε}-formyl lysine, N^{ε}-lactoyl lysine and N^{ε}-glycerinyl lysine under deaerated conditions. After isomerization of 1-DG (**9**) to 1-deoxyhexo-2, 4-diulose (**10**) a nucleophilic attack by the ε-amino function of lysine at C-2 position of **10** followed by the β-cleavage reaction results in the formation of N^{ε}-acetyl lysine and C4-enediole (**13**). In a similar manner the formation of N^{ε}-glycerinyl lysine can be explained by a nucleophilic attack of lysine at the C-4 position of **10**, analogous to the formation of glyceric acid. Formation of N^{ε}-lactoyl lysine as well as formation of N^{ε}-formyl lysine can equally be explained by a β-cleavage mechanism assuming that isomerization of 1-DG will also lead to 1-deoxyhexo-3,5-diulose (**11**) and 1-deoxyhexo-4,6-diulose (**12**) (**Figure 3**). Such long-range shifts of the carbonyl moiety in Maillard chemistry have been already studied, leading to major intermediates, i.e. Lederer's glucosone (*23*, *24*). Thus, isomerization of α-diketones into β-diketones along the entire carbon backbone must be deemed to be a general transformation.

Strikingly, in comparison to the remainder carboxylic acid amides, N^{ε}-formyl lysine occurred with the highest amounts, whereas formic acid gave the lowest values for all carboxylic acids. With respect to formic acid formation this obviously indicates that the nucleophilic attack of the ε-amino function of lysine emerges as a competitive reaction to the hydrophilic attack of the hydroxyl ion more strongly than in case of the other carboxylic acid/amide pairs. This is based on the assumption that β-dicarbonyl cleavage is the major formation pathway and an additional oxidative α-dicarbonyl cleavage plays only a minor part or is nonexistent (*20*).

Our working group has found a favored formation of glyceric acid and lactic acid under aerated conditions and postulated a formation mechanism coexistent to regular hydrolytic β-cleavage to explain carboxylic acid formation (data will be published elsewhere). Starting with 1-DG or 1-deoxy-2,3-tetrodiulose (1-DT, a major degradation product of 1-DG (*20*)) the tricarbonyl structures **15** and **16** are formed after oxidation, respectively. This step is followed by nucleophilic attack of a hydroxyl ion at the tricarbonyls to give glyceric acid, lactid acid and formic acid as the cleavage products. In our present study we measured significantly higher amounts of N^{ε}-formyl lysine and N^{ε}-glycerinyl lysine under aerated conditions. This is in line with the postulated mechanism, because a nucleophilic attack of the ε-amino function of lysine will lead to N^{ε}-formyl lysine and N^{ε}-glycerinyl lysine as shown in **Figure 4**.

For verification that amides indeed originated from the postulated β-dicarbonyl cleavage reaction and not by a simple amide bond formation between lysine side chains and the formed free carboxylic acids, all these acids were separately incubated with N^{α}-*t*-BOC-lysine. Concentrations of carboxylic acids were chosen according to the acetic acid concentration formed after seven days in 1-DG/N^{α}-*t*-BOC-lysine reaction mixtures. In all carboxylic acid/N^{α}-*t*-BOC-lysine reaction mixtures the corresponding amides were not detected. This control experiment thus clearly depicts that the direct reaction of lysine with carboxylic acids is definitively not an alternative pathway to produce the amides under the given conditions.

While studies by Büttner and Hayase only provide an indication of a possible reaction between lysine side chains and sugars/sugar degradation products (*9*, *10*), we are now capable of verifying Maillard induced amine acylation with the experiments conducted herein. The detection of N^{ε}-acetyl lysine, N^{ε}-formyl lysine, N^{ε}-lactoyl lysine and N^{ε}-glycerinyl lysine in Maillard model reaction systems, resulting from reaction of the ε-lysine side chain and 1-DG, opens a new field with respect to their formation and significance in foods and *in vivo*. Compounds like CML (N^{ε}-(carboxymethyl)lysine) (*25*), CEL (N^{ε}-(carboxyethyl)-lysine) (*26*), GOLA (N^{ε}-{2-[(5-amino-5-carboxypentyl)amino]-2-oxoethyl}lysine), GALA (N^{ε}-glycoloyllysine) and GOLD (1,3-bis-(5-amino-5-carboxypentyl) imidazolium salt) (*12*) that occur via rearrangement reactions have already confirmed that α-dicarbonyl structures are highly potent to modify lysine side chains. Based on our data it must be assumed that acylation induced by α-dicarbonyl compounds is a second major pathway leading to a novel class of amide advanced glycation end products (amide-AGEs) present in food and *in vivo*.

In conclusion, we succeeded in the synthesis of N^{ε}-lactoyl lysine and N^{ε}-glycerinyl lysine−lysine amide modifications that occur in 1-DG/N^{α}-*t*-BOC-lysine reaction mixtures. Furthermore we established N^{ε}-acetyl lysine, N^{ε}-formyl lysine, N^{ε}-lactoyl lysine and N^{ε}-glycerinyl lysine as novel stable end products in Maillard reactions. The data reported in the present study strongly supports a formation mechanism of the amides based on the established β-dicarbonyl cleavage reaction.

ACKNOWLEDGMENT

We thank Dr. D. Ströhl from the Institute of Organic Chemistry, Halle, Germany, for recording NMR spectra and Dr. J. Schmidt from the Leibnitz Institute of Plant Biochemistry, Halle, Germany, for performing accurate mass determination.

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Received for review January 26, 2010. Revised manuscript received April 14, 2010. Accepted April 17, 2010.

JOURNAL OF AGRICULTURAL AND FOOD CHEMISTRY

ARTICLE

pubs.acs.org/JAFC

Novel Insights into the Maillard Catalyzed Degradation of Maltose

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ABSTRACT: Numerous investigations concerning Maillard degradation of carbohydrates clearly depict the important impact of α-dicarbonyl compounds on changes occurring during preparation of food or physiological processes in vivo. To study the formation of these reactive intermediates during degradation of maltose in the presence of lysine, α-dicarbonyl compounds were isolated, identified and quantified after reaction with *o*-phenylenediamine to form their stable quinoxaline derivatives. Maltosone and 1,4-dideoxyglucosone were synthesized and incubated independently with lysine to investigate follow-up products and to gain further insights into the complex degradation mechanisms. Glyoxylic acid as a dicarbonyl structure and 5,6-dihydroxy-2,3-dioxohexanal as a 1,2,3-tricarbonyl compound were established as novel Maillard degradation products of maltose. Conducted experiments unequivocally demonstrated that inter- and intramolecular redox reactions are of major importance during degradation of disaccharides. 1,4-Dideoxyglucosone, 1-lysino-1,4-dideoxyglucosone, 5,6-dihydroxy-2,3-dioxohexanal, 3,4-dideoxypentosone and glyoxylic acid were found to be the central intermediates involved in the redox chemistry. With the present study we deliver a comprehensive overview on the mechanisms behind α-dicarbonyl compounds evolving from Maillard degradation of maltose.

KEYWORDS: maltose, carbohydrate degradation, α-dicarbonyl compounds, quinoxalines, Maillard reaction

INTRODUCTION

Reactive α-dicarbonyl compounds are well established to play key roles in carbohydrate degradation.[1−3] The formation of reaction products with an α-dicarbonyl moiety has been extensively researched for monosaccharides and central structures, e.g., 1-deoxyhexo-2,3-diulose were synthesized to explore the underlying chemistry.[4] In contrast, for disaccharides and higher polymers, only a few studies have been published, although carbohydrate dimers and polymers with 1,4-glycosidic bonds are much more relevant to food chemistry than monosaccharides. Interestingly, the most studied dicarbonyl compounds in foodstuff are methylglyoxal and glyoxal, found, e.g., in bread crust, soy sauce, baked cereals, cookies and roasted coffee.[5−7] However, these two substances are not exclusive for the degradation of disaccharides and higher polymers.

In studies of Hollnagel and Kramhöller significant differences in the spectrum of follow-up compounds were observed for mono- and disaccharide degradation. Further, the reactivity of disaccharide compounds in the Maillard reaction was reported to depend on the nature of the glycosidic linkage.[8−10] Some α-dicarbonyl compounds are considered to be significant for Maillard degradation of disaccharides, e.g., 1,4-dideoxyglucosone,[8] 3,4-dideoxypentosone[11] and 1-lysino-1,4-dideoxyglucosone.[12]

In most studies with regard to disaccharide degradation the interest is focused on one or very few structures, and the diversity of chosen reaction conditions hinders comparison between results of different investigators with respect to mechanistic relationships. To overcome these deficiencies we now present a thorough study on the complex relations in the formation of 19 α-dicarbonyl compounds (maltosone, 1-deoxymaltosone, 3-deoxymaltosone, 1,5-dideoxymaltoson-4-ene, glucosone, 1-deoxyglucosone, 3-deoxyglucosone, 4-deoxyglucosone, 1,4-dideoxyglucosone, 1-lysino-1,4-dideoxyglucosone, 5,6-dihydroxy-2,3-dioxohexanal, 3-deoxypentosone, 3,4-dideoxypentosone, 3-deoxythreosone, 1-deoxythreosone, glyoxylic acid, glyoxal, methylglyoxal and diacetyl) during degradation of maltose using a defined aqueous setup at 50 °C. Insights to reaction pathways of all relevant dicarbonyls were gained by depletion of oxygen as well as incubations of independent synthesized maltosone and 1,4-dideoxyglucosone.

MATERIALS AND METHODS

Materials. The following chemicals of analytical grade were commercially available: heptafluorobutyric acid (ACROS Organics, Geel, Belgium), dipotassium hydrogen phosphate trihydrate, potassium dihydrogen phosphate (Merck, Darmstadt, Germany), *N*-ethyl-*N*′-(3-dimethylaminopropyl)carbodiimide (EDC), pyridine, diethylenetriaminepentaacetic acid (Fluka/Sigma-Aldrich, Taufkirchen, Germany), maltose monohydrate, *o*-phenylenediamine, ethyl vinyl ether, methyl (4*S*)-(+)-2,2-dimethyl-1,3-dioxolane-4-acetate, 2-quinoxalinol, quinoxaline, *tert*-butyllithium 1.7 M solution in pentane (Sigma-Aldrich, Steinheim, Germany), lysine monohydrochloride, 2,3,5-triphenyltetrazolium chloride (TTC), 2-methyl quinoxaline, *N*,*O*-dimethylhydroxylamine hydrochloride (Fluka/Sigma-Aldrich, Steinheim, Germany), 2,3-dimethyl quinoxaline (SAFC/Sigma-Aldrich, Steinheim, Germany), Dowex 50W × 8 (H^+-form, 50−100 mesh) (Serva/Boehringer, Ingelheim-Heidelberg, Germany), formic acid (Grüssing GmbH, Filsum, Germany), NMR solvents (ARMAR Chemicals, Leipzig/Döttingen, Germany).

4-*O*-(α-D-Glucopyranosyl)-α-(1→4)-D-*arabino*-hexose phenylosazone (1, Maltose Phenylosazone). **1** was synthesized according to ref 13. Isomaltulose monohydrate was replaced by maltose monohydrate. ^{1}H NMR (400 MHz, Me_2SO-d_6): δ [ppm] = 3.11 (m, 1H), 3.24 (m, 1H), 3.33−3.67 (m, 7H), 3.82 (dd, ^3J = 2.9 and ^3J = 8.2 Hz, 1H), 4.29−4.37 (m, 3 OH), 4.40 (dd, ^3J = 3.1 and ^3J = 8.2 Hz, 1H), 4.85 (d, 2 OH), 4.92 (d, ^3J = 3.9 Hz, 1H), 5.28 (d, 1 OH), 5.33

Received: August 19, 2011
Accepted: November 12, 2011
Revised: November 10, 2011
Published: November 28, 2011

(d, 1 OH), 6.83 (t, ^3J = 7.9 Hz, 2H, C_6H_5), 6.98 (d, ^3J = 7.9 Hz, 2H, C_6H_5), 7.13 (d, ^3J = 7.8 Hz, 2H, C_6H_5), 7.32 (m, 4H, C_6H_5), 7.73 ppm (s, 1H), 10.64 (s, 1 NH), 12.12 (s, 1 NH). ^{13}C NMR (100 MHz, Me_2SO-d_6): δ [ppm] = 60.6, 62.2, 69.8, 71.8, 72.4, 73.1, 73.5, 73.7, 85.1, 101.2, 112.0, 112.9, 119.9, 120.4, 129.4, 129.5, 133.6, 135.1, 143.8, 144.2. HR-MS: m/z 521.2247 (found); m/z 521.2242 (calculated for $C_{24}H_{33}O_9N_4$ $[M + H]^+$).

(1′*R*,3′*R*)-2-[2′-*O*-(α-D-Glucopyranosyl)-1′,3′,4′-trihydroxybutyl]quinoxaline (2, Maltosone-Q). **1** (325 mg, 0.63 mmol) was suspended in ethanol/water (2/1, v/v, 2.75 mL), and concentrated HCl (150 μL) was added dropwise at room temperature, giving a dark orange solution. Temperature was raised to 30 °C, and a solution of $NaNO_2$ (87 mg, 1.26 mmol in 625 μL of water) was added over a period of 20 min. Reaction process was checked by TLC ($CHCl_3$−methanol 5:2, UV detection). To the red-colored solution was added NaOAc (103 mg, 1.26 mmol), and ethanol was removed in vacuo. The residual solution was extracted with $CHCl_3$ (5 × 15 mL). To the aqueous phase was added *o*-phenylenediamine (67.5 mg, 0.63 mmol), and the reaction mixture was stirred for 1 h at 70 °C. Then the mixture was cooled to room temperature and stirred overnight. The reaction mixture was filtered and extracted with $CHCl_3$ (10 mL). The aqueous phase was stirred for 20 min with activated charcoal and filtered again. Purification was done by column chromatography (silica gel 60, $CHCl_3$−methanol 5:2). Fractions including the compound with R_f 0.24 (TLC: $CHCl_3$−methanol 5:2, UV detection) were collectively concentrated in vacuo. The crude product was purified again using flash chromatography (Lobar column, Lichroprep RP C18 material, Merck, Darmstadt, Germany; water−methanol 8:2). Fractions containing **2** (TLC: R_f 0.24 in $CHCl_3$−methanol 5:2, UV detection) were combined and concentrated to give 42 mg (16%) as a light yellow powder. ^{1}H NMR (400 MHz, CD_3OD): δ [ppm] = 3.26 (m, 1H), 3.32 (m, 1H), 3.58−3.68 (m, 3H), 3.69−3.83 (m, 4H), 4.39 (t, ^3J = 4.9 Hz, 1H), 4.92 (d, ^3J = 4.0 Hz, 1H), 5.29 (d, ^3J = 4.9 Hz, 1H), 7.82 (m, 2H), 8.12 (m, 2H), 9.17 (s, 1H). ^{13}C NMR (100 MHz, CD_3OD): δ [ppm] = 61.3, 62.9, 70.2, 72.4, 72.6, 73.3, 73.4, 73.9, 82.2, 100.5, 128.3, 128.5, 129.8, 130.2, 141.0, 141.3, 144.6, 156.5. HR-MS: m/z 413.1562 (found); m/z 413.1554 (calculated for $C_{18}H_{25}O_9N_2$ $[M + H]^+$).

4-*O*-(α-D-Glucopyranosyl)-α-(1→4)-D-*arabino*-hexos-2-ulose (3, Maltosone). Synthesis of **3** was carried out by using the method of Henseke.[14] The product was isolated from the reaction mixture by column chromatography (silica gel 60, CH_3CN−water 4:1). Fractions containing **3** (TLC: R_f 0.18 in CH_3CN−water 4:1, TTC detection) were collectively concentrated in vacuo to afford **3** as a colorless foam. HR-MS: m/z 363.0898 (found); m/z 363.0903 (calculated for $C_{12}H_{20}O_{11}Na$ $[M + Na]^+$). The product was unequivocally identified as its quinoxaline derivative. **3** (20 mg, 0.06 mmol) was dissolved in phosphate buffer (2 mL, 0.1 M, pH 7.4), and *o*-phenylenediamine (6.48 mg, 0.06 mmol) was added. The reaction mixture was stirred at 60 °C for 2 h. Compound **2** was formed at a yield of 98% that was determined by comparison of the peak area with that of authentic reference compound **2** by HPLC−UV measurement.

(4*S*)-(2,2-Dimethyl-1,3-dioxolan-4-yl)acetic Acid (4). Methyl (4*S*)-(+)-2,2-dimethyl-1,3-dioxolane-4-acetate (865 mg, 4.92 mmol) was dissolved in 240 μL of methanol. To the stirred solution was added KOH (2.4 mL, 2.5 M) at 0 °C. Then the mixture was stirred at room temperature for 2.5 h. After adjusting the pH value to 4.3 with 1 M HCl, water (8 mL) was added. The mixture was extracted with EtOAc (4 × 6 mL). The combined organic layers were dried over Na_2SO_4 and concentrated to give 466 mg (59%) of a colorless oil. NMR data were in line with the literature.[15]

(4*S*)-2-(2′,2′-Dimethyl-[1′,3′]-dioxolan-4′-yl)-*N*-methoxy-*N*-methyl Acetamide (5). To a stirred solution of **4** (466 mg, 2.9 mmol) in dry CH_2Cl_2 (3 mL) was added dropwise EDC (497 mg, 3.2 mmol) dissolved in dry CH_2Cl_2 (3 mL). After addition of *N*,*O*-dimethylhydroxylamine hydrochloride (311 mg, 3.2 mmol) and pyridine (525 μL, 6.4 mmol) the reaction mixture was stirred at room temperature overnight. To the solution was added water (3 mL), and the mixture was extracted with EtOAc (4 × 3 mL). The organic layer was dried over Na_2SO_4 and concentrated in vacuo. The residue was purified by column chromatography (silica gel 60, hexane−EtOAc 7:3). Fractions containing **5** (TLC: R_f 0.13 in hexane−EtOAc 7:3, UV detection) were collectively concentrated in vacuo to afford 390 mg (66%) of a light yellow oil. NMR data were in line with the literature.[16]

(4*S*)-1-(2′,2′-Dimethyl-[1′,3′]-dioxolan-4′-yl)-3-ethoxy-but-3-en-2-one (6, 5,6-Isopropylidene-1,4-Dideoxyglucosone). To a stirred solution of ethyl vinyl ether (551 μL, 5.8 mmol) in dry THF (3 mL) was added *tert*-butyllithium solution (1.8 mL, 1.7 M in pentane) under argon atmosphere at −65 °C, and the color of the solution changed to bright yellow. The reaction mixture was allowed to warm to 0 °C until the solution was turning colorless again. Temperature was reduced to −65 °C, and **5** (189 mg, 0.94 mmol) dissolved in dry THF (2 mL) was added. The reaction mixture was stirred for 2 h at 0 °C before cold saturated NH_4Cl solution (20 mL) was added. After adjusting the pH value to 5 with 1 M HCl, the mixture was extracted with EtOAc (4 × 30 mL). The combined organic layers were dried over Na_2SO_4, and the solvent was evaporated to afford compound **6** as yellow syrupy liquid in quantitative yield. ^{1}H NMR (400 MHz, $CDCl_3$): δ [ppm] = 1.32 (s, 3H), 1.34 (t, ^3J = 7.0 Hz, 3H), 1.38 (s, 3H), 2.78 (dd, ^3J = 8.1 and ^2J = 18.0 Hz, 1H), 3.22 (dd, ^3J = 5.2 and ^2J = 18.0 Hz, 1H), 3.53 (dd, ^3J = 6.7 and ^2J = 8.4 Hz, 1H), 3.77 (q, ^3J = 7.0 Hz, 2H), 4.19 (dd, ^3J = 6.0 and ^2J = 8.4 Hz, 1H), 4.38 (d, ^2J = 2.4 Hz, 1H), 4.47 (m, 1H), 5.15 (d, ^2J = 2.4 Hz, 1H). ^{13}C NMR (100 MHz, $CDCl_3$): δ [ppm] = 14.3, 25.6, 27.1, 42.7, 64.0, 69.7, 71.8, 91.0, 108.6, 157.3, 195.5. HR-MS: m/z 237.1095 (found); m/z 237.1097 (calculated for $C_{11}H_{18}O_4Na$ $[M + Na]^+$).

3-Deoxy-4-*O*-(α-D-glucopyranosyl)-α-(1→4)-D-*arabino*-hexose bis(benzoylhydrazone) (7, 3-Deoxymaltose Bis(benzoylhydrazone)). **7** was synthesized according to ref 17. D-Glucose was replaced by maltose monohydrate. HR-MS: m/z 559.2059 (found); m/z 559.2046 (calculated for $C_{26}H_{31}O_{10}N_4$ $[M - H]^-$).

(2′*S*,3′*R*)-2-[2′-*O*-(α-D-Glucopyranosyl)-3′,4′-dihydroxybutyl] quinoxaline (8, 3-Deoxymaltosone-Q). Synthesis of **8** was carried out as described above for maltosone-Q while replacing **1** by **7** starting with 141 mg (0.25 mmol) of the bis(benzoylhydrazone). Purification of the quinoxaline solution was done by preparative HPLC−UV (gradient A, t_R 38 min) to yield 43 mg (43%) as a light yellow powder. ^{1}H NMR (500 MHz, D_2O): δ [ppm] = 3.13 (dd, ^3J = 4.2 and ^2J = 14.6 Hz, 1H), 3.20−3.26 (m, 3H), 3.50−3.56 (m, 2H), 3.60−3.72 (m, 4H), 3.80 (m, 1H), 4.19 (m, 1H) 4.71 (d, ^3J = 3.5 Hz, 1H), 7.70 (m, 2H), 7.85 (m, 2H) 8.67 (s, 1H). ^{13}C NMR (125 MHz, D_2O): δ [ppm] = 35.3, 60.4, 62.4, 69.4, 71.3, 72.3, 72.8, 73.3, 78.6, 98.4, 127.3, 127.8, 130.2, 131.0, 139.8, 140.7, 146.5, 154.2. HR-MS: m/z 397.1611 (found); m/z 397.1605 (calculated for $C_{18}H_{25}O_8N_2$ $[M + H]^+$).

Isolation of Quinoxaline Compounds as Authentic References. α-Dicarbonyl quinoxalines were isolated from maltose/lysine incubation mixtures in the presence of OPD after 7 days at 50 °C or after 5 h at cooking conditions under argon atmosphere as well as in reaction mixtures adding OPD at selected incubation times. Isolation of standard material was carried out by means of MLCCC and preparative HPLC techniques.

Incubation of Maltose and Lysine in the Presence of OPD. Maltose monohydrate (42 mM), L-lysine monohydrochloride (42 mM) and OPD (5 mM) were dissolved in phosphate buffered solution (0.1 M, pH 7.4) and incubated at 50 °C for 7 days under aerated and deaerated conditions. Deaerated conditions were achieved by using phosphate buffer with 1 mM diethylenetriaminepentaacetic acid. Buffer was degassed with helium before incubation solutions were prepared; solutions were deaerated with argon before incubation. This incubation setup was used for the quinoxaline compounds which were isolated by MLCCC.

Incubation of Maltose and Lysine with OPD Added after Sampling. To investigate the formation of α-dicarbonyl compounds,

maltose monohydrate (42 mM) and L-lysine monohydrochloride (42 mM) were dissolved in phosphate buffered solution (0.1 M, pH 7.4) and incubated at 50 °C for 7 days under aerated and deaerated conditions. Each sample was prepared at least three times. OPD (5 mM) was added at the time of sampling. Samples were then reincubated for 5 h at 25 °C and subsequently analyzed. Solutions were diluted on a scale of 1:10 with water prior to injection into the HPLC−MS^2 system.

Degradation of 1,4-Dideoxyglucosone and Maltosone. *Deprotection of 5,6-Isopropylidene-1,4-Dideoxyglucosone (**6**).* A solution of **6** (0.2 mmol) in MeOH (2 mL) was stirred with Dowex 50W × 8 (H^+-form, 50−100 mesh, 2 mL) under argon atmosphere. Completeness of deprotection was checked after 2 h with TLC (TTC detection). The resin was filtered off and washed with MeOH. Evaporation of the combined solvents yielded 1,4-dideoxyglucosone as a white powder, which was immediately incubated with L-lysine monohydrochloride.

Incubation Setup. 1,4-Dideoxyglucosone (42 mM) and maltosone (42 mM) were incubated with L-lysine monohydrochloride (42 mM) at 50 °C in phosphate buffered solution (0.1 M, pH 7.4) for 0 h, 1 h, 3 h, 5 h, 7 h, 12 h, 24 h, 72 h, 120 h and 168 h, respectively. Each sample was prepared at least three times. Incubations were conducted to investigate the formation of related α-dicarbonyl compounds. OPD was added at the various time points to form quinoxalines, and samples were reincubated 5 h at 25 °C.

Reincubation of 1-Deoxymaltosone-Q with OPD. 1-Deoxymaltosone-Q (0.01 mM) was incubated with OPD (0.01 mM) at 50 °C in phosphate buffered solution (0.1 M, pH 7.4) for 5 h.

Multilayer Countercurrent Chromatography (MLCCC). The MLCCC system (Ito, Multilayer Separator-Extractor model, P. C. Inc., Potomac, MD) was equipped with a chromatography pump (model 6000 A) by Waters (Eschborn, Germany), a Kontron UV-detector (Munich, Germany) operating at 320 nm, and a sample injection valve with a 10 mL sample loop. Eluted liquids were collected in fractions of 9 mL with a fraction collector (LKB Ultrorac 2070). Chromatograms were recorded on a plotter (Servogor 210). The multilayer coil was prepared from 1.6 mm i.d. poly(tetrafluoroethylene) (PTFE) tubing. The total capacity was 270 mL. The MLCCC was run at a revolution speed of 800 rpm and a flow rate of 2 mL min^{-1} in head to tail mode. Ethyl acetate was used as stationary phase and water (saturated with ethyl acetate) as mobile phase. The coil was first filled with stationary phase. The mobile phase was applied during rotation. Samples were dissolved in 10 mL of water and ethyl acetate 1:1 (v/v) and injected after breakthrough of mobile phase.

Preparative HPLC−UV. A Besta HD 2-200 pump (Wilhelmsfeld, Germany) was used at a flow rate of 10 mL min^{-1}. Elution of material was monitored by a UV detector (Jasco UV- 2075). Detection wavelength was always 320 nm. Chromatographic separations were performed on a stainless steel column (KNAUER, Eurospher-100 C18, 20 mm i.d., 10 μm, Berlin, Germany). The mobile phase used consisted of water (solvent A) and MeOH/water (7:3 (v/v), solvent B). To both solvents (A and B) was added 0.8 mL/L formic acid. Samples were injected at 30% B (gradient A) or at 35% B (gradient B), respectively.

3,4-Dideoxypentosone-Q. This compound was isolated by MLCCC (t_R 920 min, ethyl acetate extract, deaerated incubation setup). NMR results were in line with ref 11. HR-MS: m/z 189.1022 (found); m/z 189.1024 (calculated for $C_{11}H_{12}ON_2$ $[M + H]^+$).

4-Deoxyglucosone-Q. This compound was isolated by MLCCC (t_R 120 min, ethyl acetate extract, deaerated incubation setup). NMR results were in line with ref 18. HR-MS: m/z 257.0897 (found); m/z 257.0898 (calculated for $C_{12}H_{14}O_3N_2Na$ $[M + Na]^+$).

1,4-Dideoxyglucosone-Q. This compound was isolated by MLCCC (t_R 270 min, ethyl acetate extract, deaerated incubation setup). NMR results were in line with ref 19, HR-MS: m/z 241.0947 (found); m/z 241.0950 (calculated for $C_{12}H_{14}O_2N_2Na$ $[M + Na]^+$).

5,6-Dihydroxy-2,3-dioxohexanal-Q (1). This compound was isolated by MLCCC (t_R 610 min, ethyl acetate extract, deaerated incubation setup). ^1H NMR (500 MHz, CD_3OD): δ [ppm] = 3.17 (m, 2H), 3.80 (dd, ^3J = 5.1 and ^2J = 11.6 Hz, 1H), 3.83 (dd, ^3J = 4.4 and ^2J = 11.6 Hz, 1H), 4.64 (m, 1H), 6.09 (s, 1H), 7.81 (m, 2H), 8.01 (m, 1H), 8.06 (m, 1H). ^{13}C NMR: (125 MHz, CD_3OD) δ [ppm] = 33.2, 64.0, 67.2, 92.0, 127.7, 128.3, 129.6, 130.3, 140.9, 141.9, 150.2, 150.8. HR-MS: m/z 255.0735 (found); m/z 255.0740 (calculated for $C_{12}H_{12}O_3N_2Na$ $[M + Na]^+$).

5,6-Dihydroxy-2,3-dioxohexanal-Q (2). This compound was isolated by preparative HPLC−UV (gradient B, t_R 92 min) from maltose/lysine incubation mixtures at 7 days under anaerobic conditions after reincubation with OPD. ^1H NMR (500 MHz, CD_3OD): δ [ppm] = 3.47 (dd, ^3J = 4.7 and ^2J = 16.4 Hz, 1H), 3.56 (dd, ^3J = 8.1 and ^2J = 16.4 Hz, 1H), 3.63 (pd, ^3J = 5.6 Hz, 2H), 4.38 (m, 1H), 7.96 (m, 2H), 8.15 (m, 1H), 8.23 (m, 1H), 9.40 (s, 1H). ^{13}C NMR (125 MHz, CD_3OD): δ [ppm] = 41.2, 65.6, 68.3, 128.5, 130.1, 130.8, 132.2, 140.9, 142.6, 143.3, 146.9, 199.5. HR-MS: m/z 255.0741 (found); m/z 255.0740 (calculated for $C_{12}H_{12}O_3N_2Na$ $[M + Na]^+$).

1-Deoxymaltosone-Q. This compound was isolated by preparative HPLC−UV (gradient B, t_R 19 min) from maltose/lysine/OPD incubation mixtures after 5 h under anaerobic reflux cooking conditions. ^1H NMR (500 MHz, D_2O): δ [ppm] = 2.12 (dd, ^3J = 1.8 and ^2J = 12.5 Hz, 1H), 2.65 (m, 1H), 2.79 (s, 3H), 2.90 (dd, ^3J = 3.9 and ^2J = 12.5 Hz, 1H), 3.16 (t, ^3J = 9.6 Hz, 1H), 3.29 (dd, ^3J = 5.3 and ^2J = 12.1 Hz, 1H), 3.45 (m, 1H), 3.47 (dd, ^3J = 3.9 and ^2J = 12.1 Hz, 1H), 3.66 (t, ^3J = 9.6 Hz, 1H), 4.22 (m, 1H), 5.05 (d, ^3J = 7.6 Hz, 1H), 5.18 (d, ^3J = 4.0 Hz, 1H), 7.76 (m, 2H), 7.90 (m, 1H), 7.79 (m, 1H). ^{13}C NMR: (125 MHz, D_2O) δ [ppm] = 21.5, 58.7, 61.5, 68.8, 71.7, 72.1, 72.7, 74.1, 80.3, 101.8, 127.0, 127.4, 130.4, 131.3, 139.8, 140.3, 153.1, 153.7. HR-MS: m/z 397.1609 (found); m/z 397.1605 (calculated for $C_{18}H_{25}O_8N_2$ $[M + H]^+$).

1,5-Dideoxymaltoson-4-ene-Q. This compound was isolated by preparative HPLC−UV (gradient B, t_R 32 min) from maltose/lysine incubation mixtures at 7 days under anaerobic conditions after reincubation with OPD. NMR results were in line with ref 19. HR-MS: m/z 379.1505 (found); m/z 379.1500 (calculated for $C_{18}H_{23}O_7N_2$ $[M + H]^+$).

Glucosone-Q, 1-Deoxyglucosone-Q, 3-Deoxyglucosone-Q, 3-Deoxypentosone-Q, 1-Deoxytheosone-Q and 3-Deoxythreosone-Q. These compounds were verified by authentic quinoxalines isolated from glucose reaction mixtures according to our previous work.[20]

1-Lysino-1,4-dideoxyglucosone-Q. This compound was independently synthesized (data will be published elsewhere). NMR data were in line with the literature.[12]

Analytical HPLC−UV. A Jasco PU-2080 quaternary gradient unit pump with degasser LG-2080-02 (Gross-Umstadt, Germany) and a 715 plus Waters autosampler (Eschborn, Germany) were used. Elution of materials was monitored by a Kontron UV-detector (Munich, Germany) operating at 320 nm. Chromatographic separations were performed on a stainless steel column (KNAUER, Eurospher 100-5 C18, 250 × 4.0 mm, Berlin, Germany) using a flow rate of 1 mL min^{-1}. The mobile phase used consisted of water (solvent A) and MeOH/water (7:3 (v/v), solvent B). To both solvents (A and B) was added 0.6 mL/L heptafluorobutyric acid (HFBA). Samples were injected at 25% B (held 20 min), and the gradient was then changed to 40% B in 20 min and to 70% B in 20 min. Then gradient was changed to 100% B in 5 min (held for 10 min) and to 25% B in 5 min (held 15 min).

Analytical HPLC−MS^2. A Jasco PU-2080 Plus quaternary gradient pump with degasser and a Jasco AS-2057 Plus autosampler (Jasco, Gross-Umstadt, Germany) were used. Chromatographic separations were performed on a stainless steel column (KNAUER, Eurospher 100-5 C18, 250 × 4.0 mm, Berlin, Germany) using a flow rate of 1 mL min^{-1}. The HPLC gradient program used was the same as for HPLC−UV measurements. Elution of quinoxalines (1-deoxymaltosone-Q at 10.7 min, maltosone-Q at 11.5 min, glucosone-Q at 12.4 min, 1-deoxyglucosone-Q at 17.5 min, 3-deoxymaltosone-Q at 19.6 min, 1,5-dideoxymaltosone-Q at 20.7 min, 4-deoxyglucosone-Q at 24.4 min, 3-deoxyglucosone-Q at 25.9 min,

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Table 1. Mass Spectrometer Parameters for Quinoxaline Quantification (Scheduled MRM Mode) with Limits of Detection (LOD) and Quantification (LOQ) for All Quinoxalines Monitored

	mass (amu)					limits (μM)	
	Q1	Q3	DP (V)	CE (eV)	CXP (V)	LOD	LOQ
1-deoxymaltosone-Q	397.3	235.2	65.00	22.00	13.00	0.004	0.011
maltosone-Q	413.3	251.2	50.00	22.00	20.00	0.002	0.006
glucosone-Q	251.1	173.1	60.00	24.00	16.00	0.008	0.025
1-deoxyglucosone-Q	235.2	175.1	50.00	28.00	12.00	0.016	0.049
3-deoxymaltosone-Q	397.3	235.1	60.00	24.00	19.00	0.003	0.008
1,5-dideoxymaltoson-4-ene-Q	379.3	199.2	50.00	23.00	11.00	0.004	0.011
4-deoxyglucosone-Q	235.2	217.1	50.00	18.00	15.00	0.065	0.196
3-deoxyglucosone-Q	235.2	199.1	55.00	24.00	17.00	0.011	0.033
glyoxylic acid-Q	147.1	129.0	27.00	26.50	9.00	0.032	0.096
3-deoxypentosone-Q	205.1	187.1	50.00	21.00	14.00	0.014	0.041
5,6-dihydroxy-2,3-dioxohexanal-Q (1)	233.2	215.1	45.00	15.00	17.00	0.048	0.142
1-lysino-1,4-dideoxyglucosone-Q	363.3	84.2	60.00	55.00	14.00	0.009	0.027
5,6-dihydroxy-2,3-dioxohexanal-Q (2)	233.2	215.2	45.00	12.00	17.00	0.104	0.313
1,4-dideoxyglucosone-Q	219.2	159.1	50.00	27.00	12.00	0.005	0.014
3-deoxythreosone-Q	175.1	157.2	55.00	24.00	10.00	0.007	0.020
1-deoxythreosone-Q	175.1	157.2	43.00	22.00	7.00	0.006	0.018
glyoxal-Q	131.1	77.0	32.00	40.00	6.00	0.051	0.152
3,4-dideoxypentosone-Q	189.1	171.2	40.00	25.00	15.00	0.009	0.025
methyl glyoxyl-Q	145.0	77.0	50.00	41.00	5.00	0.012	0.036
diacetyl-Q	159.2	118.1	75.00	33.00	10.00	0.004	0.012

glyoxylic acid-Q at 30.3 min, 3-deoxypentosone-Q at 33.8 min, 5,6-dihydroxy-2,3-dioxohexanal-Q (1) at 38.0 min, 1-lysino-1,4-dideoxyglucosone-Q at 39.6 min, 5,6-dihydroxy-2,3-dioxohexanal-Q (2) at 43.2 min, 1,4-dideoxyglucosone-Q at 44.1 min, 3-deoxythreosone-Q at 45.1 min, 1-deoxythreosone-Q at 46.9 min, glyoxal-Q at 53.2 min, 3,4-dideoxypentosone-Q at 54.4 min, methyl glyoxal-Q at 61.0 min, and diacetyl-Q at 65.1 min) was monitored by mass detection. The mass analyses were performed using an Applied Biosystems API 4000 quadrupole instrument (Applied Biosystems, Foster City, CA, USA) equipped with an API source using an electrospray ionization (ESI) interface. The LC system was connected directly to the probe of the mass spectrometer. Nitrogen was used as sheath and auxiliary gas. To measure the quinoxalines in incubation solutions, the scheduled MRM mode of HPLC−MS^2 was used. The optimized parameters for mass spectrometry as well as limits of detection (LOD) and quantification (LOQ) for all compounds monitored are given in Table 1. Quantification was based on the standard addition method. More precisely, increasing concentrations of authentic reference compounds at factors of 0.5, 1, 2, and 3 times the concentration of the analyte in the sample were added to separate aliquots of the sample. The aliquots were analyzed, and a regression of response versus concentration is used to determine the concentration of the analyte in the sample. Calibration with this method resolves potential matrix interferences. Data for quinoxalines obtained by HPLC−MS^2 showed coefficients of variation <5%.

Nuclear Magnetic Resonance Spectroscopy (NMR). NMR spectra were recorded on a Varian VXR 400 spectrometer operating at 400 MHz for ^{1}H and 100 MHz for ^{13}C or on a Varian Unity Inova 500 instrument operating at 500 MHz for ^{1}H and 125 MHz for ^{13}C, respectively. Chemical shifts are given relative to external $SiMe_4$.

Accurate Mass Determination (HR-MS). The high-resolution positive ion ESI mass spectra (HR-MS) were obtained from a Bruker Apex III Fourier transform ion cyclotron resonance (FT-ICR) mass spectrometer (Bruker Daltonics, Billerica, MA) equipped with an Infinity cell, a 7.0 T superconducting magnet (Bruker, Karlsruhe, Germany), a radio frequency (RF)-only hexapole ion guide, and an external electrospray ion source (Apollo; Agilent, off-axis spray). Nitrogen was used as drying gas at 150 °C. The samples were dissolved in methanol, and the solutions were introduced continuously via a syringe pump at a flow rate of 120 μL h^{-1}. The data were acquired with 256 k data points and zero filled to 1024 k by averaging 32 scans.

RESULTS

Preparation of Authentic Quinoxaline References. The formation of α-dicarbonyl compounds derived from maltose in the presence of lysine was investigated in a defined aqueous model system under aerated and deaerated incubation conditions. Due to their high reactivity, α-dicarbonyl compounds were converted by o-phenylenediamine (OPD) into their stable quinoxaline derivatives (Q) before analyses. For mechanistic inquiries OPD was added at the time of sampling to avoid artifacts. With the aim to identify and quantify the arising α-dicarbonyls, authentic quinoxaline references were isolated from incubation solutions with MLCCC [3,4-dideoxypentosone-Q, 5,6-dihydroxy-2,3-dioxohexanal-Q (1), 4-deoxyglucosone-Q, 1,4-dideoxyglucosone-Q, glucosone-Q, 1-deoxyglucosone-Q, 3-deoxyglucosone-Q, 3-deoxypentosone-Q, 1-deoxythreosone-Q and 3-deoxythreosone-Q] or preparative HPLC [1-deoxymaltosone-Q, 1,5-dideoxymaltoson-4-ene-Q and 5,6-dihydroxy-2,3-dioxohexanal-Q (2)]. In addition, 3-deoxymaltosone-Q, maltosone-Q and 1-lysino-1,4-dideoxyglucosone-Q were synthesized independently.

Formation of α-Dicarbonyl Quinoxalines in Maltose/Lysine Incubations (pH 7.4, 50 °C). Degradation of maltose in the presence of lysine led to the formation of 20 quinoxalines arising from 19 α-dicarbonyl compounds. 5,6-Dihydroxy-2,3-dioxohexanal offers the possibility to form two different quinoxaline derivatives due to the fact of bearing a 1,2,3-tricarbonyl

moiety: 5,6-dihydroxy-2,3-dioxohexanal-Q (1) (quinoxaline condensation at positions C2 and C3 of the carbon chain) and 5,6-dihydroxy-2,3-dioxohexanal-Q (2) (condensation at C1 and C2). Figure 1 lists all identified quinoxalines.

To perform quantification a HPLC−MS^2 method (scheduled MRM mode) was developed (Table 1). Formation of all arising α-dicarbonyl compounds was monitored via identified quinoxalines

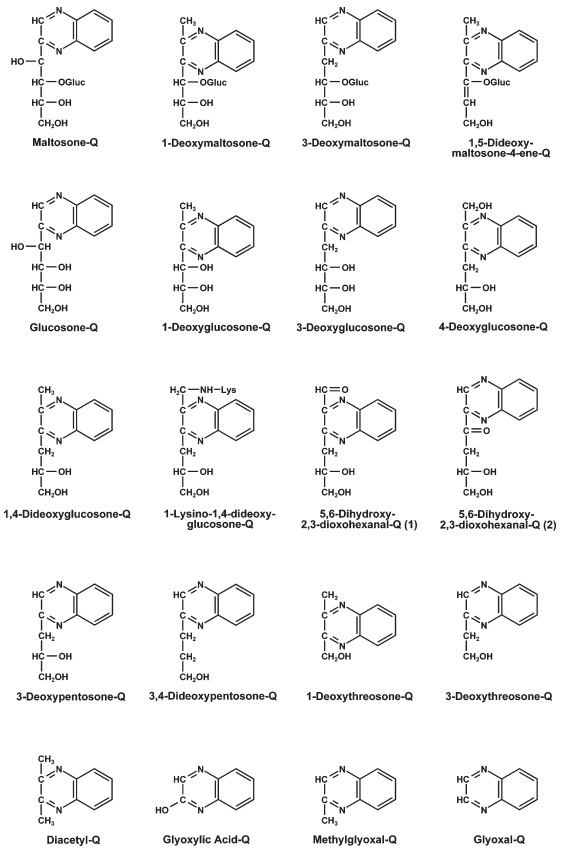

Figure 1. Identified α-dicarbonyl quinoxalines in maltose/lysine incubations.

over a period of seven days. Results for aerated and deaerated incubations are given in Figures 2−5.

The formation of quinoxalines with intact carbon chain of maltose is shown in Figure 2. Formation of 1-deoxymaltosone-Q led to the highest amounts of all quinoxalines. Under deaerated conditions twice the yield of 1-deoxymaltosone-Q was measured after seven days (2.58 vs 4.98 mmol/mol of maltose). The same behavior was observed for 1,5-dideoxymaltoson-4-ene-Q (1.44 vs 3.05 mmol/mol of maltose) and 3-deoxymaltosone-Q (0.02 vs 0.05 mmol/mol of maltose), respectively. Maltosone-Q reached the highest concentrations after 24 h (0.76 mmol/mol of maltose) under aerated incubation conditions and was degraded afterward. Under deaeration maltosone-Q levels increased along the whole time monitored to reach an amount of 0.55 mmol/mol of maltose after seven days.

Kinetic inquiries of quinoxalines with a backbone consisting of six carbon atoms are illustrated in Figure 3. All C6-quinoxalines were formed in very low concentrations except both tricarbonyl quinoxalines. 1,4-Dideoxyglucosone-Q was found under deaerated conditions with an amount of 0.014 mmol/mol of maltose; under aerated conditions only traces were detected. 5,6-Dihydroxy-2,3-dioxohexanal-Q (1) and 5,6-dihydroxy-2,3-dioxohexanal-Q (2) levels were found to be low under aerated conditions (at the limit of quantification); they significantly responded to nonoxidative conditions (2.22 and 3.00 mmol/mol of maltose, respectively). Under deaeration 3-fold higher amounts of 1-lysino-1,4-dideoxyglucosone-Q were observed (0.01 vs 0.03 mmol/mol of maltose). Glucosone-Q doubled under aerated conditions (0.06 vs 0.03 mmol/mol of maltose). For levels of 4-deoxyglucosone-Q (0.09 mmol/mol of maltose) no difference between aerated and deaerated conditions was observed. The formation of 3-deoxyglucosone-Q (0.07 vs 0.09 mmol/mol of maltose) was slightly preferred under deaerated conditions. Under both aerated and deaerated conditions only traces of 1-deoxyglucosone-Q were detected.

Formation of α-dicarbonyl compounds underlying further fragmentation (C_4−C_5 fragments) is given in Figure 4. 3,4-Dideoxypentosone-Q levels were found to be low under aerated conditions; they significantly responded to nonoxidative conditions (0.01 vs 0.39 mmol/mol of maltose). Under aeration 3-fold higher amounts of 3-deoxypentosone-Q were measured than under deaeration (0.33 vs 0.13 mmol/mol of maltose). For 1-deoxythreosone-Q only traces were measured under aerated and deaerated conditions, respectively. 3-Deoxythreosone-Q reached the highest levels after 24 h (0.29 mmol/mol of maltose)

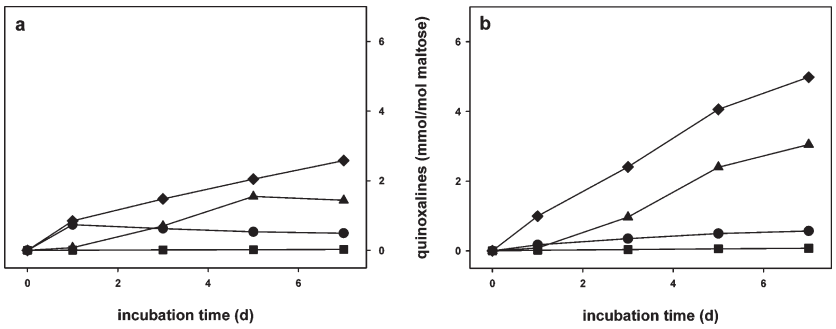

Figure 2. Formation of quinoxalines (Q) with intact carbon chain under aerated (a) and deaerated (b) conditions: 1-deoxymaltosone-Q (◆), 1,5-dideoxymaltoson-4-ene-Q (▲), maltosone-Q (●), 3-deoxymaltosone-Q (■).

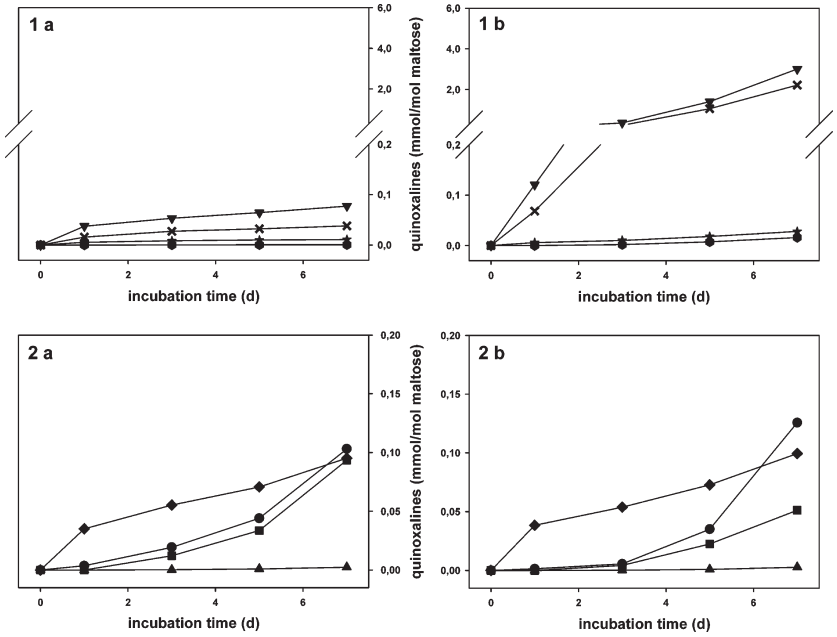

Figure 3. Formation of quinoxalines with a C6-carbon backbone under aerated (a) and deaerated (b) conditions. **1:** 1-lysino-1,4-dideoxyglucosone-Q (★), 5,6-dihydroxy-2,3-dioxohexanal-Q (1) (×), 5,6-dihydroxy-2,3-dioxohexanal-Q (2) (▼), 1,4-dideoxyglucosone-Q (⬢); **2:** glucosone-Q (■), 1-deoxyglucosone-Q (▲), 3-deoxyglucosone-Q (●), 4-deoxyglucosone-Q (◆).

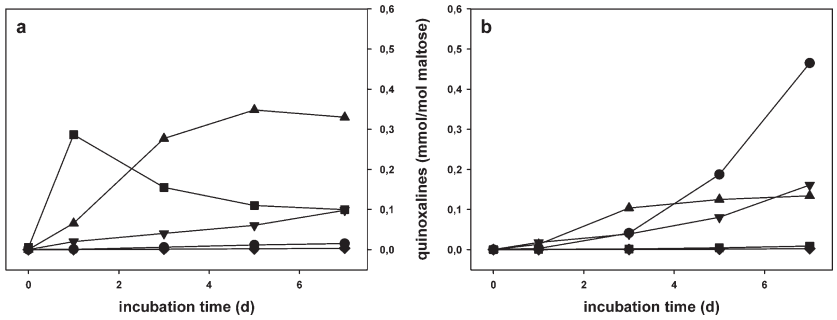

Figure 4. Formation of C_4-C_5 α-dicarbonyl quinoxaline fragments under aerated (a) and deaerated (b) conditions: 3-deoxypentosone-Q (▲), 3,4-dideoxypentosone-Q (●), 1-deoxythreosone-Q (◆), 3-deoxythreosone-Q (■), diacetyl-Q (▼).

under aeration and was degraded afterward. Under deaerated conditions 3-deoxythreosone-Q levels were significantly lower (at the limit of quantification). Thus, they significantly responded to oxidative conditions. Diacetyl-Q increased approximately 2-fold under deaerated conditions (0.08 vs 0.14 mmol/mol of maltose).

Formation of α-dicarbonyl compounds subjected to degradation to C_2-C_3 fragments is shown in Figure 5. Methylglyoxal-Q levels varied only slightly under aerated and deaerated conditions (0.05 vs 0.06 mmol/mol of maltose). Therefore, the formation of methylglyoxal did not depend on the presence of oxygen. Glyoxal-Q reached the highest amounts after 3 days (0.96 mmol/mol of maltose) under aeration and was degraded afterward. A 4-fold lower concentration was obtained under deaeration (0.27 mmol/mol of maltose at seven days). Glyoxylic acid-Q was found in significant higher amounts under deaeration (0.14 mmol/mol of maltose). Levels under aeration were located at the limit of quantification.

Degradation of 1,4-Dideoxyglucosone and Maltosone in the Presence of Lysine. In this experiment 1,4-dideoxyglucosone had a half-life of 11 h. However, after addition of OPD no additional quinoxalines were formed. From maltosone, with a half-life of 9 h, the formation of 3-deoxypentosone up to 19 mmol/mol of maltosone was observed. 3,4-Dideoxypentosone-Q was detected only in traces.

■ DISCUSSION

Some structures investigated herein have been studied previously in Maillard disaccharide systems. Nevertheless, this is the first time that all relevant α-dicarbonyl compounds covering the

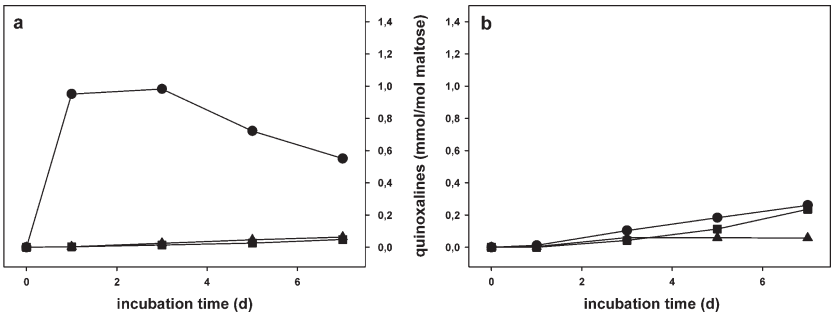

Figure 5. Formation of C_2-C_3 α-dicarbonyl quinoxaline fragments with intact carbon chain under aerated (a) and deaerated (b) conditions: methylglyoxal-Q (▲), glyoxal-Q (●), glyoxylic acid-Q (■).

range of the complete carbon backbone down to C_3 and C_2 fragments are studied thoroughly for Maillard catalyzed maltose degradation. The mechanistic relationships are summarized in Figure 6.

Formation of α-dicarbonyl compounds arising from maltose with intact carbon backbone (maltosone, 1-deoxymaltosone, 3-deoxymaltosone and 1,5-dideoxymaltoson-4-ene) is well established in the literature. Morita et al. detected the quinoxalines of maltosone, 1-deoxymaltosone and 3-deoxymaltosone derived from maltose under weak acidic reflux conditions.[21] Beck et al. described the formation of 1-deoxymaltosone and 3-deoxymaltosone from the Amadori compound of maltose. 1-Deoxymaltosone was formed as the main reaction product and 3-deoxymaltosone as a minor product in model incubation systems of maltose and amines (e.g., propyl amine).[22] These data obtained by Beck et al. are in line with our findings. In the present study degradation of maltosone was observed after 24 h under aerated conditions similar to glucosone derived from glucose (degradation after 72 h) as outlined in our previous work. Also under deaerated conditions maltosone followed the same pattern as glucosone: for both α-dicarbonyl compounds increasing levels of quinoxalines were obtained up to seven days.[20] As expected, maltosone originates during oxidative maltose degradation comparable to glucosone formation from glucose.

Detected as its β-pyranone form 1,5-dideoxymaltoson-4-ene was found as a major degradation product of maltose Amadori compound,[9] which is comparable to our results. 1,5-Dideoxymaltoson-4-ene-Q was one of the quinoxalines that occurred in higher concentrations compared to the other quinoxalines monitored. In foodstuff 1,5-dideoxymaltoson-4-ene was identified, e.g., in steamed ginseng roots (β-pyranone form)[23] and in beer (quinoxaline derivative).[19] As the most likely precursor structure of 1,5-dideoxymaltoson-4-ene, 1-deoxymaltosone was considered.[9,23] The formation can be explained by β-elimination of water in position C5. In our study this relation is further supported by a paralleled increase of both structures under deaerated conditions. The notion that 1,5-dideoxymaltoson-4-ene-Q might be a direct artifact of 1-deoxymaltosone-Q was excluded by independent incubations of the latter with OPD. No further quinoxalines were monitored.

As α-dicarbonyl compounds with six carbon atoms the formation of glucosone, 1-deoxyglucosone, 3-deoxyglucosone, 4-deoxyglucosone, 1-lysino-1,4-dideoxyglucosone, 1,4-dideoxyglucosone and 5,6-dihydroxy-2,3-dioxohexanal was monitored. From the mechanistic view it is prerequisite to distinguish between compounds that are specific for disaccharide degradation (bearing carbon atoms C1 to C6 of maltose) and those compounds that follow hexose Maillard chemistry arising from glucose after cleavage of the 1,4-glycosidic bond (bearing carbon atoms C1′ to C6′ of maltose). In our previous work[20] glucosone, 1-deoxyglucosone and 3-deoxyglucosone were identified as degradation products of glucose. Measured concentrations of these glucose degradation products followed the same pattern as in glucose incubations, but at much lower levels (factor 5 to 30). Thus, results obtained for maltose degradation concerning glucosone, 1-deoxyglucosone and 3-deoxyglucosone clearly depict that these α-dicarbonyl compounds refer to the Maillard chemistry of glucose. For clarity these structures were excluded from the reaction scheme (Figure 6).

4-Deoxyglucosone,[18,24] 1-lysino-1,4-dideoxyglucosone [12] and 1,4-dideoxyglucosone [8,19] are already mentioned in Maillard literature to be specific for disaccharide degradation. The formation of 4-deoxyglucosone was studied as a degradation product of disaccharides under alkaline conditions.[18] In our study 4-deoxyglucosone was formed in equal yields under aeration and deaeration. This suggests that 4-deoxyglucosone is generated nonoxidatively via enolization and elimination of glucose without incorporation of lysine, but amine-catalyzed.

Beck et al.[22] as well as Huber and Ledl[25] confirmed the existence of 1-lysino-1,4-dideoxyglucosone in incubations with disaccharides. Reihl et al.[12] proposed a formation pathway for this α-dicarbonyl compound via 2,3-enolization and elimination of glucose from maltose. In this work this is underlined as 1-lysino-1,4-dideoxyglucosone was generated preferentially under deaeration.

The formation of 1,4-dideoxyglucosone was described by Hollnagel and Kroh[8] and Bravo et al.[19] Hollnagel and Kroh suggested a mechanism for 1,4-dideoxyglucosone via "peeling off" for the quasi-water-free thermolysis of oligosaccharides.[8] Bravo et al. explained the formation by degradation of a putative precursor 1-deoxyglucosone and other unknown chemical pathways in beer.[19] As a result of our investigations 1,4-dideoxyglucosone has to be assigned to nonoxidative mechanisms.

In this work 5,6-dihydroxy-2,3-dioxohexanal was identified as a specific degradation product of maltose for the first time. 5,6-Dihydroxy-2,3-dioxohexanal was mentioned by Lindström as a degradation product of *O*-β-D-glucopyranosyl-1,4-D-*arabino*-2-hexosulose under alkaline conditions, but only as a nonestablished postulated structure. The author assumed that the 2,3-enediol of *O*-β-D-glucopyranosyl-1,4-D-*arabino*-2-hexosulose

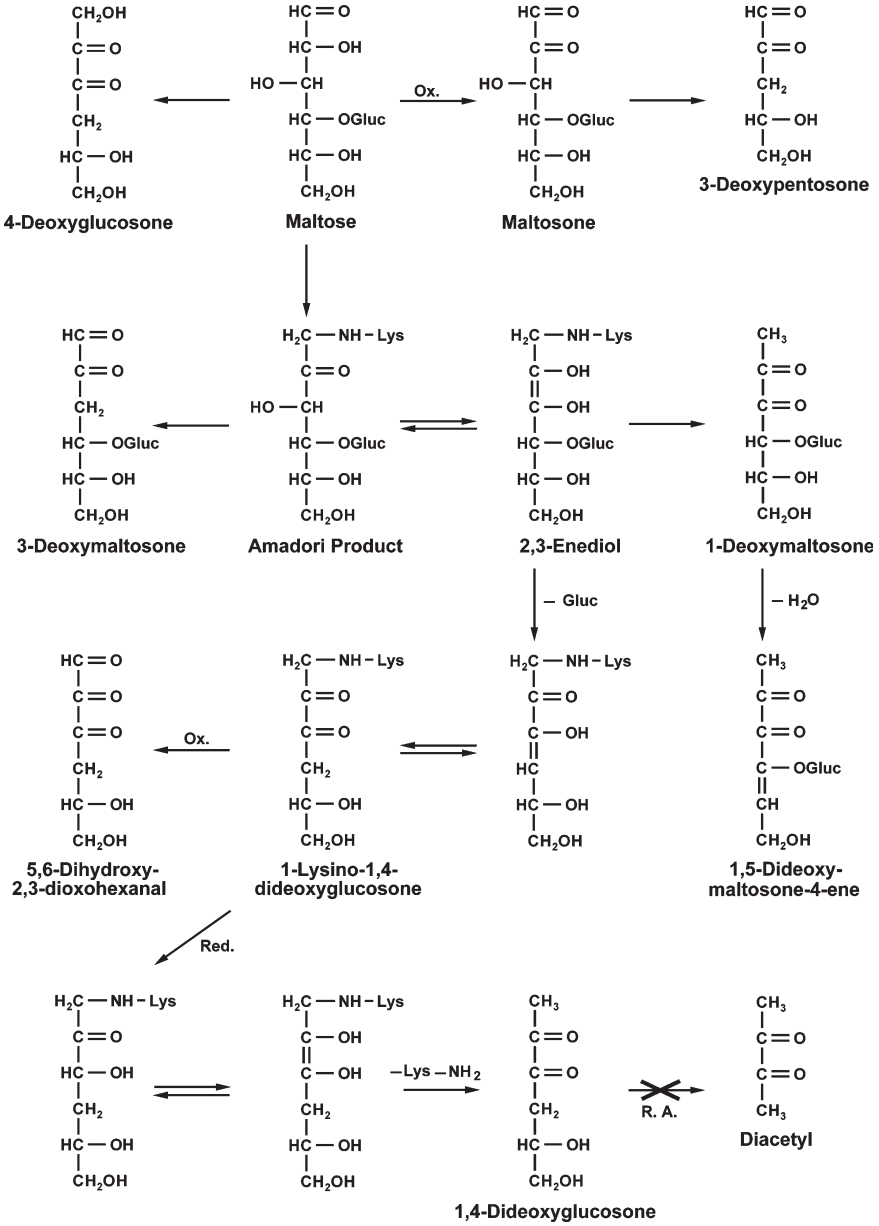

Figure 6. Formation of α-dicarbonyl compounds in maltose Maillard reaction systems.

gives rise to a rapid β-elimination producing glucose and 5,6-dihydroxy-2,3-dioxohexanal as an unstable intermediate subjective to further fragmentation.[26] Curiously enough, 5,6-dihydroxy-2,3-dioxohexanal was detected preferentially under deaeration in this study, where it was generated in high quantities. This clearly excludes Lindström's theory at least for neutral pH values as maltosone continuously accumulated under deaeration and is obviously rather stable under these conditions in contrast to aeration where it was degraded after a maximum. We therefore propose a formation pathway from 1-lysino-1,4-dideoxyglucosone shown in Figure 6. From the structure 5,6-dihydroxy-2,3-dioxohexanal has to result from oxidation in contrast to 1,4-dideoxyglucosone from reduction. Disproportionation is the only type of reaction that enables simultaneous oxidation and reduction in the absence of oxygen. 1-Lysino-1,4-dideoxyglucosone as a structure of high redox activity (nitrogen-analogue reductone) undergoes an intermolecular reaction to 5,6-dihydroxy-2,3-dioxo-hexanal (oxidation) and an aminohexulose intermediate (reduction). To yield 1,4-dideoxyglucosone, elimination of the amine has to take place. As shown in Table 2, an inadequacy of quinoxaline levels between

5,6-dihydroxy-2,3-dioxohexanal and 1,4-dideoxyglucosone was observed. This finding might be explained by unfavorable leaving properties of lysine from the amino-hexulose intermediate.

As α-dicarbonyl fragments with a four and five membered carbon backbone 3-deoxypentosone, 3,4-dideoxypentosone, 1-deoxythreosone, 3-deoxythreosone and diacetyl were identified. Hollnagel and Kroh characterized 3-deoxypentosone as the predominating α-dicarbonyl compound formed from maltose.[27] Mavric and Henle isolated 3-deoxypentosone from lactose. The authors proposed a pathway linked to the formation of 4-deoxyglucosone.[11] In this study 3-deoxypentosone was generated preferentially under aeration. Contrary to the hypothesis of Mavric and Henle this excludes 4-deoxyglucosone as the educt as this structure was formed at same concentration levels independent from oxygen. Clearly 3-deoxypentosone formation suggests a precursor formed via oxidative pathways. The major α-dicarbonyl formed during oxidative maltose degradation was maltosone. Indeed, when maltosone was incubated separately high concentrations of 3-deoxypentosone were found. Therefore, in parallel to our established route for formation of 3-deoxypentosone and 1-deoxypentosone from glucosone via hydrolytic β-dicarbonyl cleavage,[28,29] the following mechanism is strongly suggested (Figure 7). After hydration the 1,3-tautomer of maltosone is split into formic acid and a C11-1,2-enediol. The 1,2-enediol will immediately cleave off glucose. This mechanism is further underlined by the lack of 1-deoxypentosone derivatives in maltose or maltosone incubations in contrast to glucose or glucosone reaction mixtures. The excellent leaving properties of glucose obviously exclusively direct the elimination to position 3 to give solely 3-deoxypentosone.

3,4-Dideoxypentosone is known as another specific degradation product of oligosaccharides with 1,4-glycosidic linkages. As the corresponding quinoxaline, 3,4-dideoxypentosone was isolated after heating lactose followed by trapping with *o*-phenylenediamine by Mavric and Henle. They deduced a possible pathway for the formation of 3,4-dideoxypentosone with 4-deoxyglucosone as the precursor.[11] In the present study 3,4-dideoxypentosone was formed almost exclusively under deaeration. This undermines the hypothesis of Mavric and Henle, as the formation of 4-deoxyglucosone displayed no dependency on oxygen. Together with 5,6-dihydroxy-2,3-dioxohexanal, glyoxylic acid, 1-lysino-1,4-dideoxyglucosone and 1,4-dideoxyglucosone, 3,4-dideoxypentosone has thus to be assigned to a class of α-dicarbonyls calling for intra- and intermolecular redox processes (Table 2). From the mechanistic view the most likely precursor of 3,4-dideoxypentosone is 3-deoxypentosone. The corresponding 2,3-enol form should eliminate water, and reduction results in 3,4-dideoxypentosone. However, 3-deoxypentosone was preferentially generated under oxidative conditions and independent maltosone incubations gave only negligible concentrations of 3,4-dideoxypentosone although 3-deoxypentosone was formed in high levels. This result might be explained by the lack of the corresponding oxidizable redox partner under aeration.

Pfeifer and Kroh postulated a formation mechanism for diacetyl via retro-aldol cleavage from 1,4-dideoxyglucosone within oligosaccharide degradation. In support of their proposed pathway an inverse experiment (aldol condensation of diacetyl and formaldehyde gave 1,4-dideoxypentosone) was conducted.[30] Although the formation of diacetyl was slightly preferred under deaerated conditions in our incubation setup, noticeable amounts were detected under aeration. On the other side 1,4-dideoxyglucosone was detected only under deaeration whereas aerated amounts were negligible. To test the hypothesis of Pfeifer and Kroh a successful synthesis of 1,4-dideoxyglucosone was established. However, no further α-dicarbonyl structures were detected in separate incubations at 50 °C. This unequivocally proves that diacetyl is not formed via retro-aldol reactions from 1,4-dideoxyglucosone. This result is in line with previous experiments conducted in our lab for glucose where retro-aldolization was of minor importance or even negligible concerning α-dicarbonyl fragmentation reactions.[31] However, retro-aldol reactions might have an impact at higher temperatures.

Compared to 3-deoxythreosone, yields of 1-deoxythreosone were found to be nonrelevant for maltose degradation in contrast to glucose degradation where both 1-deoxythreosone and 3-deoxythreosone were established as immediate products of 1-deoxyglucosone under deaerated conditions via hydrolytic β-dicarbonyl cleavage. This means (I) that 3-deoxythreosone is a specific product of maltose degradation and (II) that there must be alternative pathways to explain the significant amounts of 3-deoxythreosone under oxidative conditions.

Table 2. Formation of Selected α-Dicarbonyls in Maltose Incubation Quantified as Quinoxalines after 7 Days

	mmol/mol of maltose	
α-dicarbonyl	aerated	deaerated
Structures Formed by Oxidation		
5,6-dihydroxy-2,3-dioxohexanal (1)	at LOQ	2.22
5,6-dihydroxy-2,3-dioxohexanal (2)	at LOQ	3.00
glyoxylic acid	0.03	0.14
Structures Formed by Reduction		
1,4-dideoxyglucosone	<LOQ	0.01
3,4-dideoxypentosone	0.01	0.39

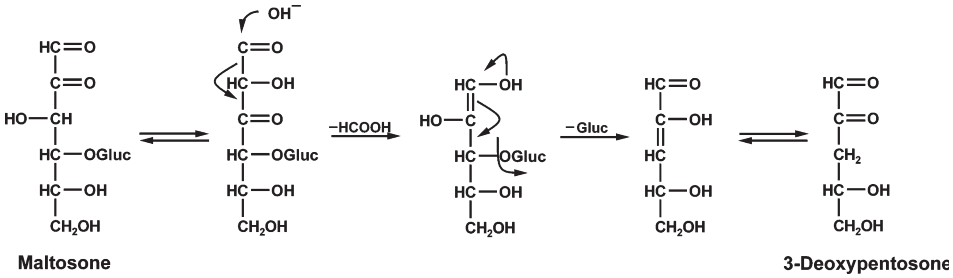

Figure 7. Mechanism of maltosone degradation: β-dicarbonyl cleavage leads to 3-deoxypentosone.

Glyoxylic acid, glyoxal and methyl glyoxal belong to the α-dicarbonyl C_2 and C_3 fragments identified during maltose degradation. Unlike glyoxal and methylglyoxal, glyoxylic acid was a product specific for maltose.[20] Formation of glyoxylic acid regarding Maillard reactions is scarcely described in the literature. Velisek et al. described glyoxylic acid as an oxidation reaction product of glyoxal with glycine.[32] Unexpected from its structure, glyoxylic acid levels were significantly higher under dearation than under aeration (Table 2) and thus revealed the same paradoxical behavior as 5,6-dihydroxy-2,3-dioxohexanal in our experiments. Again, we must assume inter- and intramolecular redox processes. Alternative mechanisms via oxidation of glyoxal can be ruled out, as this α-dicarbonyl structure was detected at significantly higher levels under aeration.

In conclusion, our in-depth study concerning all α-dicarbonyl structures relevant to degradation of maltose in the presence of lysine displays an important step forward to understand the formation of reactive intermediates in disaccharide degradation. The complete reaction mechanisms are summarized in Figure 6. To enhance clarity the formation of degradation products of glucose was excluded from the scheme and is discussed in detail in our previous paper.[20] With the successful syntheses of maltosone and 1,4-dideoxygluosone as free α-dicarbonyl precursors selected mechanistically relationships were unequivocally established. The α-dicarbonyl structures 5,6-dihydroxy-2,3-dioxohexanal and glyoxylic acid were identified as specific maltose degradation products for the first time. Our study obviously depicts the importance of redox processes during Maillard degradation of maltose. We suggest 1-lysino-1,4-dideoxyglucosone as a highly potent player to undergo intermolecular redox reactions.

Further studies have to be accomplished for a complete understanding of disaccharide Maillard-redox chemistry. This must include the independent synthesis of 1-lysino-1,4-dideoxyglucosone as the free α-dicarbonyl structure to study follow-up products and also experiments with complementary ^{13}C stable isotope labeled maltose.

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ACKNOWLEDGMENT

We thank Dr. D. Ströhl from the Institute of Organic Chemistry, Halle, Germany, for recording NMR spectra and Dr. J. Schmidt from the Leibniz Institute of Plant Biochemistry, Halle, Germany, for performing accurate mass determination.

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Angewandte International Edition Chemie

DOI: 10.1002/anie.201300399

Vitamin C Degradation

Maillard Degradation Pathways of Vitamin C

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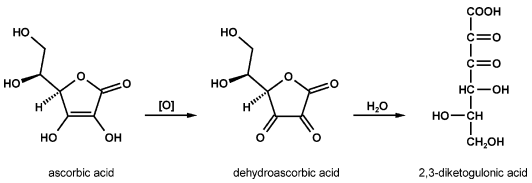
Vitamin C, or L-threo-ascorbic acid (hereafter referred to as ascorbic acid), belongs to the class of essential vitamins in the human diet and is widely found in nature, for example, in herbs, vegetables, and, in particular, in citrus fruits. Because of its antioxidant properties, ascorbic acid has been 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.

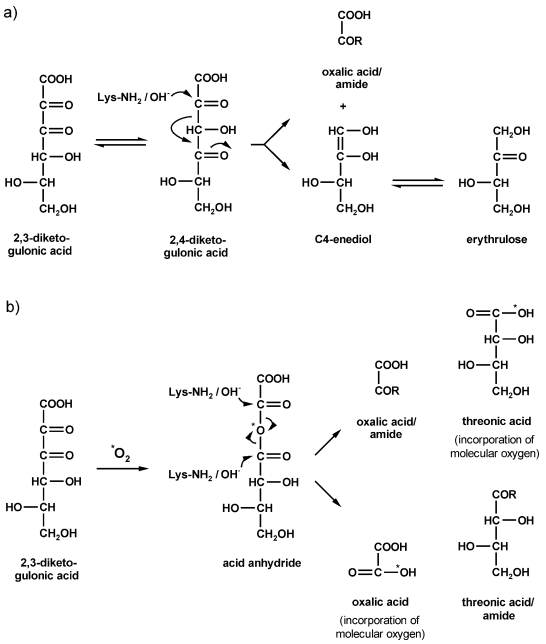
Numerous investigations were undertaken to characterize the decomposition products of ascorbic acid. Dehydroascorbic acid (DHA), 2,3-diketogulonic acid (2,3-DKG), erythrulose, and oxalic and threonic acids were identified as the main degradation products.[8–10] It is well established, that ascorbic acid 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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Supporting information for this article is available on the WWW under http://dx.doi.org/10.1002/anie.201300399.

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

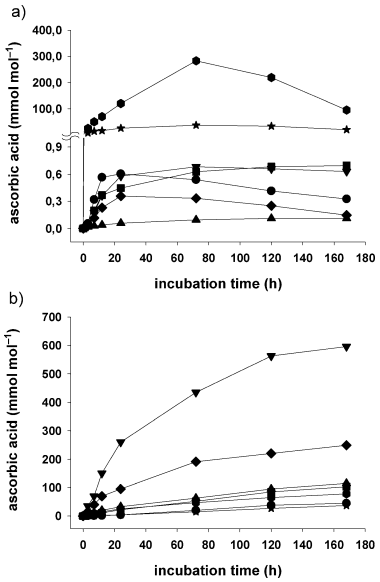


Figure 1. Time course of the formation of carbonyl and dicarbonyl compounds, and carboxylic acids. a) Erythrulose (●), glyceric aldehyde (★), 3-deoxythreosone (■), threosone (▼), 1-deoxythreosone (●), xylosone (◆), methylglyoxal (▲). b) Oxalic acid (▼), threonic acid (◆), glycolic acid (▲), glyceric 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).

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

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.

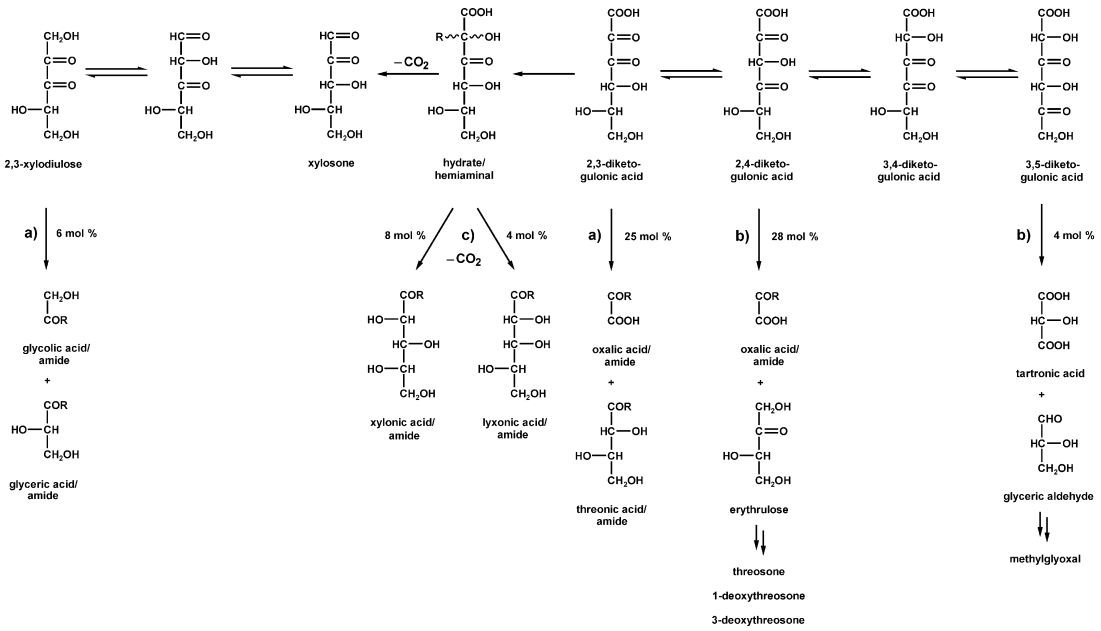
	2,3-diketo-gulonic acid	xylonic acid/ amide	lyxonic acid/ amide	xylosone	threonic acid/ amide	erythrulose	threosone	1-deoxy-threosone	3-deoxy-threosone	glyceric acid/ amide	glyceric aldehyde	methyl-glyoxal	tartronic acid	oxalic acid/ amide	glycolic acid/ amide
distribution		(C2–C6) 100 %	(C2–C6) 100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

Received: January 16, 2013
Published online: March 28, 2013

Keywords: amides · cleavage reactions · Maillard reaction · oxidation · vitamin C

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12 Declaration of originality

Eigenständigkeitserklärung

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