Reaction of L-Ascorbic Acid with L-Arginine Derivatives

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When L-ascorbic acid (AA) is heated in the presence of N^{α}-acetyl-L-arginine, a main product can be detected by HPLC/UV. The compound was isolated and identified by spectroscopic data and synthesis as N^{α}-acetyl-N^{δ}-(4-(1,2-dihydroxy-3-propyliden)-3-imidazolin-5-on-2-yl)-L-ornithine (DPI) (**1a**). An analogous derivative (**1b**) was obtained, starting from methylguanidine, a model compound for arginine. **1a** is also formed from L-dehydroascorbic acid (DHA) and xylosone, degradation products of AA. The yield of **1a** was investigated at different reaction temperatures, times, and pH-values.

Keywords: *Maillard reaction; ascorbic acid; L-arginine*

INTRODUCTION

As vitamin C L-ascorbic acid (AA) is an important component of human nutrition. Moreover, it is added to food stuffs as an antioxidative or enzyme inhibiting additive without toxicity. During processing or heating of food, AA is mainly degraded by oxidation reactions, leading in the first step to L-dehydroascorbic acid (DHA). DHA breaks further down to 2,3-diketogulonic acid (DKG), xylosone, threose, oxalic acid, and other low molecular weight compounds (Niemelä, 1987). In the presence of amino acids or proteins, however, AA reacts in a different way. In a Maillard type reaction AA forms brown pigments and melanoidines (Bensch et al., 1985; Ranganna and Setty, 1968). It can be assumed, that amine induced degradation of vitamin C causes the browning of fruit juice and dried food stuffs (Ranganna and Setty, 1968; Nagy et al., 1990; Kirk, 1981; Liao and Seib, 1987). Bensch et al. (1985) could show that L-ascorbic acid plays also a major role in in-vivo and in-vitro protein glycation and cross-link. Incubation of lens crystalline with L-ascorbic acid for example leads to the formation of yellow and brown products, fluorescence, and covalent binding of AA to the protein. There is strong evidence that these adducts are related to senile and diabetes induced cataract (Ortwerth et al., 1988; Ortwerth and Olesen, 1988). Slight et al. (1990) incubated proteins with AA and performed amino acid analysis before and after the reaction. They found that AA mainly binds to lysine residues, but arginine and histidine are also considerably modified.

In spite of the great importance of the reaction of AA with proteins for food chemistry and medicine as well, almost nothing is known about the reaction mechanism and structures of the products. Kurata et al. (1973) found that, in the presence of α-amino acids, DHA undergoes Strecker degradation, resulting in scorbamic acid, which reacts with a second molecule of DHA to a red pigment. This pathway, however, does not explain the reaction of AA with protein bound amino acids forming covalently linked products.

Pischetsrieder et al. (1995) reacted lysine with AA and isolated an amino L-ascorbic acid in which the 3-hydroxy group of AA is substituted by the ε-amino residue of lysine. A similar product can be expected with lysine side chains of proteins.

This paper reports on the reaction of L-arginine derivatives with L-ascorbic acid.

MATERIALS AND METHODS

Apparatus. ^{1}H NMR (400 MHz) and ^{13}C NMR (100 MHz) spectra were recorded with a Jeol 400 GSX spectrometer using $(CH_3)_4Si$ as internal standard. Chemical shifts are reported in parts per million. Mass spectral analyses were obtained with a HP 5989 A MS Engine (CI with CH_4). The IR spectrum was recorded in a KBr disk with a Perkin-Elmer 197 spectrometer. Analytical HPLC was performed with a Merck L-7100 gradient pump and a Merck L-7450 photodiode array detector including Merck-Hitachi Model D-7000 chromatography data station software. For preparative and semipreparative HPLC a Merck L-6250 pump, a Merck L-4000 UV detector, and a Merck D-2500 chromatointegrator were used. UV spectra were recorded with the diode array detector, described above.

Reagents. Unless otherwise noted, phosphate buffer contains 0.02 M phosphate buffer pH 7.4, 0.0054 M KCl, and 0.274 M NaCl. DHA was purchased from Aldrich (Steinheim, Germany); D,L-glyceraldehyde and N^{α}-acetyl-L-arginine, from Sigma (St. Louis, MO). The water used for HPLC was distilled and filtered through a nylon membrane of 0.45 μm. HPLC grade methanol was used without further purification. All solvents were degassed with helium.

High-Performance Liquid Chromatography. *Solvent A, 10 mmol KH_2PO_4, pH 3.0 adjusted with H_3PO_4; solvent B, methanol.* Analytical high-performance liquid chromatography (HPLC) was performed on a column packed with LiChroSorb (RP 18, 280 × 4 mm i.d., 5 μm particle size). The column was protected with a guard cartridge (25 × 4 mm) packed with the same material as the column. For elution a gradient was used of 0-75% solvent B in 0−25 min and then continued with 100% solvent B from 25.1 to 30 min and a flow rate of 0.8 mL/min. The substances were detected by a diode array detector from 200 to 400 nm. Preparative HPLC was performed on a HiBar column packed with LiChroSorb (Merck, Darmstadt, Germany) (RP 18, 250 × 20 mm i.d., 7 μm particle size) with 100% solvent A as eluent and a flow rate of 10 mL/min. For semipreparative HPLC a column packed with LiChrospher RP 18 (Bischoff, Leonberg, Germany) (250 × 10 mm i.d., 5μm particle size) and 100% solvent A as eluent with a flow rate of 2.4 mL/min were used.

Isolation of 4-(1,2-Dihydroxy-3-propyliden)-2-(*N*-methylamino)-3-imidazolin-5-one (1b). Methylguanidine hydrochloride (3.175 g, 29 mmol) was dissolved in 57.5 mL phosphate buffer, and the pH value was adjusted with NaOH to 7.5. DHA (500 mg, 2.9 mmol) was added, and the solution heated for 1 h at 100 °C under reflux while being stirred. After cooling the mixture was freeze-dried, dissolved in 10 mL solvent A, and separated by preparative HPLC (detection at 226 nm, injection of 1 mL). The peak with a retention time of 15 min was collected and the fractions freeze-dried, redissolved in 400 μL solvent A, and purified by semipreparative HPLC

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(detection at 226 nm, 100 μL injected each time). The fractions with a retention time of 13.5 min were collected and freeze-dried. The residue was redissolved in 20 mL methanol and filtered and the filtrate evaporated. The light yellow residue was used for spectral analyses. ^{1}H NMR (CD_3OD, COSY): δ 3.25 (s, 3H, CH_3), 3.65 (d, J = 5.13 Hz, 2H, CH_2OH), 4.70 (dd, J = 5.13, 5.98 Hz, 1H, C*H*OH), 6.17 (d, J = 5.98 Hz, 1H, C*H*=). ^{13}C NMR (CD_3OD, COSY, DEPT): δ 28.2 (*C*H_3), 66.2 (*C*HOH), 70.9 (*C*H_2OH), 121.2 (*C*H=), 128.7 (5-C), 157.2 (2-C), 163.2 (4-C). MS (m/z, CI): 186 (M + 1), 168 (100%), 152. UV: λ_{max} = 238, 283 nm.

Synthesis of 2-Amino-4-(1,2-dihydroxy-3-propylidenyl)-3-methyl-3-imidazolin-5-one (2). D,L-Glyceraldehyde (1.960 g, 21.8 mmol), creatinine (490 mg, 4.4 mmol), and acetamide (20 g) were stirred at 100 °C for 1 h. Acetamide was removed under high vacuum (70 °C, 10^{-1} Torr), and the residue was redissolved in 20 mL of solvent A and separated by preparative HPLC. Each time 1 mL was injected, detection was performed at 280 nm, and the peaks at 20.3 min were collected, freeze-dried and redissolved in 30 mL of methanol and filtered. The solvent was removed under reduced pressure, and the remaining bright yellow solid was used for spectral analyses. ^{1}H NMR (CD_3OD): δ 3.28 (s, 3H, CH_3), 3.6 (d, J = 5.13 Hz, 2H, CH_2OH), 5.4 (dd, J = 5.13, 8.55 Hz, 1H, C*H*OH), 5.9 (d, J = 8.55 Hz, 1H, C*H*=). ^{13}C NMR: δ 28.6 (*C*H_3), 66.7 (*C*HOH), 68.7 (*C*H_2OH), 123.4 (*C*H=), 131.6 (5-C), 158.1 (2-C), 165.2 (4-C). MS (m/z, CI): 186 (M + 1), 168 (100%). UV: λ_{max} = 221, 282 nm. IR: 3305, 1706, 1670 cm^{-1}.

Isolation of N^α-Acetyl-N^δ-(4-(1,2-dihydroxy-3-propyliden)-3-imidazolin-5-on-2-yl)-L-ornithine (1a). N^α-Acetyl-L-arginine (6.3 g, 29 mmol) was dissolved in 57.5 mL phosphate buffer, and the pH value was adjusted to 7.5 with diluted NaOH. DHA (500 mg, 2.9 mmol) was added, and the solution was stirred for 1 h at 100 °C under reflux. After cooling the mixture was freeze-dried, redissolved in 20 mL solvent A, and separated by preparative HPLC using 7% solvent B in solvent A with a flow rate of 12 mL/min and detection at 232 nm. The peaks with a retention time of 18.7 min were collected and the fraction evaporated and dissolved in 20 mL of methanol. The residue was used for spectral analyses after filtration and evaporation. ^{1}H NMR (CD_3OD): δ 1.7−1.9 (m, 4H, 3′- and 4′-CH_2), 2.0 (s, 3H, N^α-CH_3CO), 3.6 (d, 2H, CH_2OH), 3.8 (m, 2H, 5′-CH_2), 4.4 (m, 1H, 2′-C*H*), 4.7 (dd, 1H, C*H*OH), 6.2 (d, 1H, C*H*=). MS (m/z, CI): 328 (M + 1), 277, 253. UV: λ_{max} = 238, 283 nm.

Preparation of the Samples. *Reaction of AA: Sample A.* N^α-Acetyl-L-arginine (756 mg, 3.5 mmol) and L-ascorbic acid (52 mg, 0.3 mmol) was dissolved in 6 mL of phosphate buffer (pH 7.35) and stirred for 2 days at 40 °C. The solution was injected into the analytical HPLC.

Reaction of DHA: Sample B. N^α-Acetyl-L-arginine (126 mg, 0.58 mmol) was dissolved in 1 mL of phosphate buffer, DHA (8.7 mg, 0.05 mmol) was added, and the mixture was heated in a closed vessel at 100 °C. After 1 h a sample was injected into the HPLC.

Reaction of Xylosone: Sample C. Xylosone was prepared according a method of Salomon et al. (1952), and purity was determined by GC/MS after conversion into the alditol acetate derivative (Shin and Feather, 1990). Methylguanidine hydrochloride (55 mg, 0.5 mmol) was dissolved in 1 mL of phosphate buffer, the pH value was adjusted to 7.5 with diluted NaOH, and xylosone was added (10 mg, 0.076 mmol). After heating for 2 h at 100 °C in a closed vessel, the solution was injected into the HPLC.

Reaction of DHA and N^α-Acetyl-L-arginine. N^α-Acetyl-L-arginine (252 mg, 1.17 mmol) was dissolved in 1 mL of phosphate buffer (0.1 M, pH 7.0), and the pH was adjusted with diluted NaOH. DHA (17.4 mg, 0.1 mmol) was added and the mixture was heated at 40 or 70 °C while being stirred. Samples were taken at different time points as indicated. Reaction mixtures, as described above, were dissolved in citrate−phosphate buffer (0.2 M; pH 3.0, 3.6, 4.0, 4.6, 5.0, 5.6, and 6.0), phosphate buffer (0.2 M, pH 5.7, 6.0, 6.5, 7.0, 7.5, 8.0), or tris buffer (0.2 M; pH 8.0, 8.4, 9.0), and if necessary, pH was adjusted with diluted NaOH or HCl. Saccharine (0.075 mg/mL) was used as internal standard. The solutions were heated in closed vessels at 100 °C.

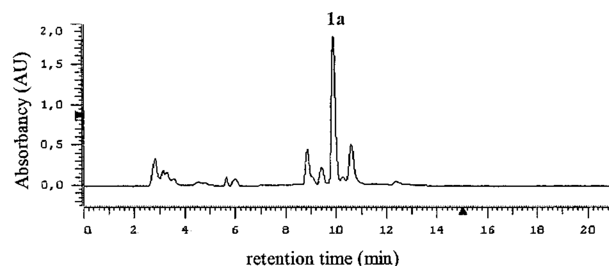

Figure 1. HPLC chromatogram of a reaction mixture of AA and N^α-acetyl-L-arginine (sample A). UV detection at 284 nm.

Table 1. ^{1}H and ^{13}C NMR Data of 1b and 2^a

	δ (ppm)	
	1	**2**
^{1}H NMR		
CH_3	3.3	3.3
CH_2OH	3.7	3.6
C*H*OH	4.7	5.4
C*H*=	6.2	5.9
^{13}C NMR		
CH_3	28.2	28.6
H*C*OH	66.2	66.7
H_2*C*OH	70.9	67.6
H*C*=	121.2	123.4
5-C	128.7	131.6
2-C	157.2	158.1
4-C	163.2	165.2

^{a} Numbers of carbons refer to Figures 2 and 3.

RESULTS

L-Ascorbic acid was heated with L-arginine derivatives in aqueous solution, and the reaction mixtures were analyzed by HPLC using a diode array detector (DAD). In a wavelength range between 200 and 400 nm, one peak predominates the chromatograms (**1a**) (Figure 1). It displays a characteristic UV absorbency with maxima at 238 and 283 nm. The reaction was carried out with N^α-acetyl-L-arginine, N^α-carbobenzoxy-L-arginine, and, as a model compound for arginine, with methylguanidine (MG). Variation of the amino acid component leads to a shift of the retention time of the product.

Isolation and Identification of 1b. To facilitate isolation and identification of the product, the reaction was performed in the first place with MG. MG can be considered as a suitable model compound for arginine, because it can be expected that the guanidine group of arginine reacts in the same way as MG.

For isolation of the product, DHA and MG were heated in phosphate buffer and the reaction mixture was separated by preparative and semipreparative HPLC. The pure substance was used for spectral analyses. The mass spectrum shows a molecule peak at 186 (m/z) which can count for a C_5 degradation product of AA like xylosone which is substituted by MG.

The ^{1}H NMR (Table 1) displays four signals: a singlet at 3.25 ppm for a methyl group, a doublet at 3.65 ppm integrating for two protons, a multiplet at 4.70 ppm for one proton, and an olefin proton at 6.17 ppm. In the ^{13}C NMR seven signals can be distinguished (Table 1). The links between protons and carbons were determined by H,H-COSY, C,H-COSY, and DEPT experiments. Taking all of these data into consideration, an imidazolinone structure can be postulated to which a CH−

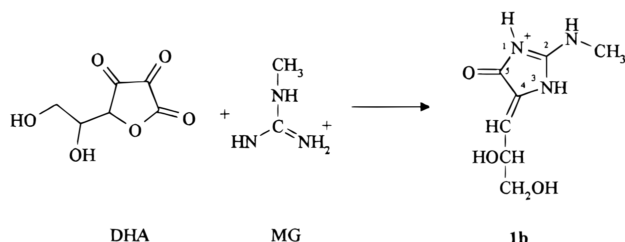

Figure 2. Formation of **1b** from DHA and MG.

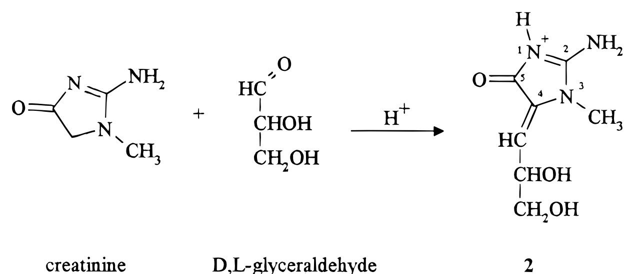

Figure 3. Reaction of creatinine with D,L-glyceraldehyde to give reference compound **2**.

CHOH−CH_2OH group is linked by a double bond. The ^{13}C-signal at 163.0 ppm is in accordance with the carbonyl group in imidazolinones (literature 175 ppm (Duerksen-Hughes et al., 1989; Henle et al., 1994)) which is shifted by ca. 12 ppm to higher field due to the double bond in the α-position. The carbon signals of the guanidinium group and the methyl group appear in accordance with literature values: 157.2 and 28.2 ppm compared to 156−162 and 28.6 in the literature (Henle et al., 1994; Sopio and Lederer, 1995).

The UV spectra are dependent on the pH value. In aqueous solution the compound shows two maxima at 311 and 240 nm which are shifted in phosphate buffer pH 3.0 to 283 and 238 nm, respectively. This is characteristic for conjugated tautomers of imidazolinones (Edward and Lantos, 1972). For the spectral analyses the product was isolated in the protonated form. Summarizing the results, it can be suggested that **1b** is 4-(1,2-dihydroxy-3-propyliden)-2-(*N*-methyl)amino-3-imidazolin-5-one (Figure 2).

Synthesis of 2-Amino-4-(1,2-dihydroxy-3-propyliden)-3-methyl-3-imidazolin-5-one (2). To support the proposed structure of **1b**, synthesis of a compound with similar structure was performed. When creatinine is reacted with glyceraldehyde, a product (**2**) is obtained which differs only in the position of the methyl group from **1b** (Figure 3). The product was isolated, and the spectral data were compared to those of **1b** (Table 1). ^{1}H NMR, ^{13}C NMR- and UV data of both products are in good accordance. Only the proton of **2**, which is linked to C_6 is shifted by 0.7 ppm to lower field, which can be explained because of the steric influence of the methyl group. Therefore the synthesis of **2** lends support to the proposed structure of **1b**.

Isolation of N^{α}-Acetyl-N^{δ}-(4-(1,2-dihydroxy-3-propyliden)-3-imidazolin-5-on-2-yl)-L-ornithine (1a). So far it was assumed that MG reacts in a way similar to arginine. In the reaction mixtures of MG or different L-arginine derivatives, respectively, there is always a main product formed displaying the characteristic UV spectrum of **1b**. The retention time, however, changes dependent on the guanidine derivative. To show that the products possess indeed the same structure, the N^{α}-acetyl-L-arginine derivative of **1** was isolated. UV and ^{1}H NMR spectra show the identity of the product.

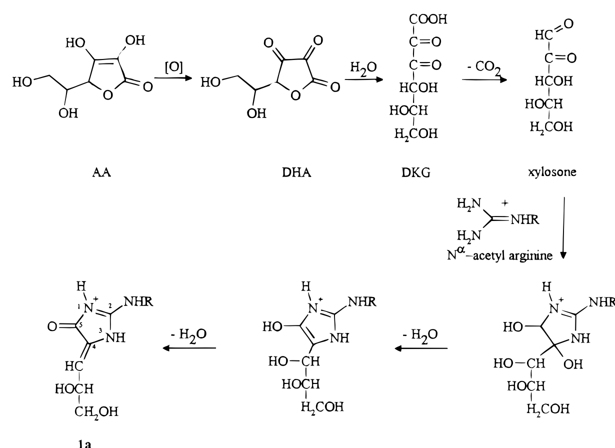

Figure 4. Mechanism of the formation of **1a** (DPI).

Elucidation of the Reaction Mechanism. Compounds **1a,b** contain five carbons which are derived from AA. It is also obvious that AA must have been oxidized during the reaction. It is known that in the absence of amino acids AA is easily oxidized to give DHA. In a next step the lactone ring opens, resulting in DKG, which, due to its labile β-dicarbonyl structure, is decarboxylated to L-xylosone. It is of interest to know if AA reacts first with the guanidine group and is then oxidized and cleaved or if the guanidine group condenses with an intermediate of the AA degradation. Therefore, the possible intermediates DHA and xylosone were used as starting material. Xylosone was synthesized according to a method of Salomon et al. (1952). DKG was not available. The experiments have shown that **1a,b** are formed from AA, DHA, and xylosone to a similar extent. Therefore the reaction mechanism shown in Figure 4 can be proposed for the formation of **1a,b**.

Correlation of Product Formation with Reaction Time, Temperature, and pH Value. DHA was reacted with N^{α}-acetyl-L-arginine, and the relation of the relative amount of **1a** to the pH value, reaction time, and temperature was determined by HPLC. The time course at 40 °C is shown in Figure 5, panel a. Compound **1a** can be detected after a short incubation time, like 1 h. Prolonged heating increases the amount at the beginning linearly and later with decreasing reaction rate. Raising the temperature to 70 °C (Figure 5, panel b) results in a higher yield of **1a**, but a similar time course can be observed.

The reaction was investigated at a pH range between 3.0 and 9.0. The correlation to the pH value is not significant, but product is formed to a similar extent over the whole pH range.

DISCUSSION

In several experiments with radioactively labeled L-ascorbic acid it was shown, that AA binds covalently to a number of proteins (Ortwerth and Olesen, 1988; Sawamura and Takemoto, 1991; Slight et al., 1990). This reaction leads beside others to browning, flavor formation, and protein cross-link. Amino acid analysis before and after incubation revealed that mainly lysine, arginine, and histidine residues are modified (Ortwerth and Olesen, 1988; Slight et al., 1990). So far only products were identified, which can be responsible for lysine modification (Dunn et al., 1990; Pischetsrieder et al., 1995).

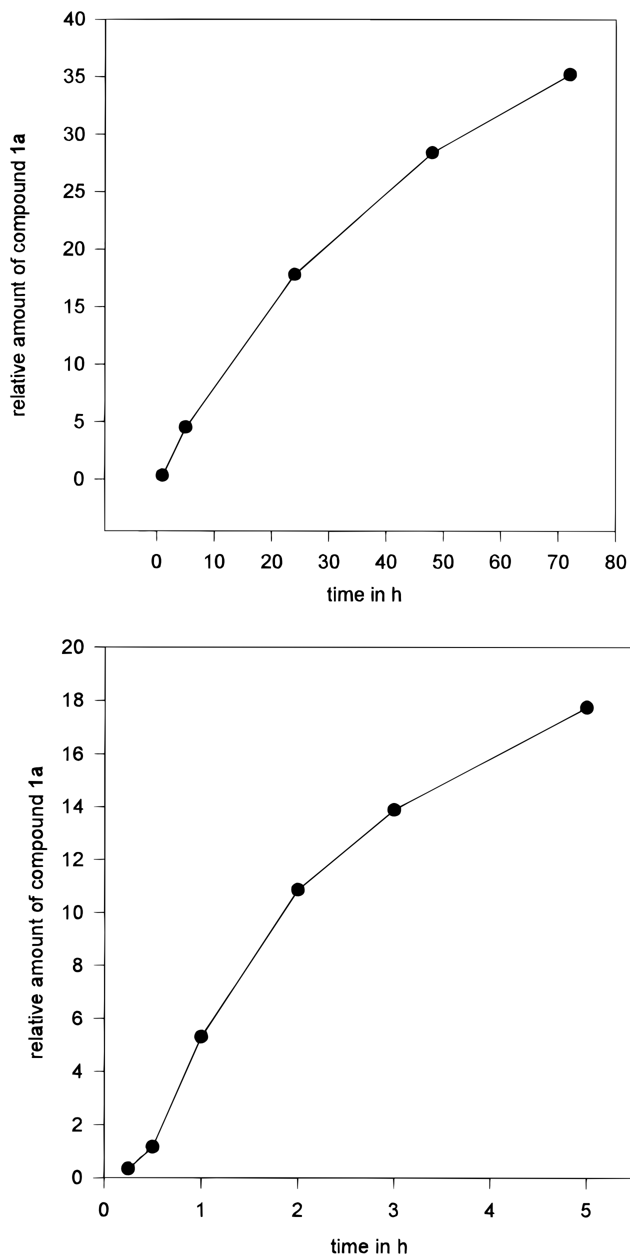

Figure 5. Time dependant formation of **1a** from DHA: (a, top) 40 °C; (b, bottom) 70 °C.

The data presented here report that AA reacts with L-arginine derivatives to a main product, **1a** (DPI). It can be assumed that this product is responsible for the arginine loss during the incubation of proteins with AA. To form DPI, AA must first be oxidized and then decarboxylated to give xylosone (Figure 4). The α-dicarbonyl group of xylosone reacts then with the guanidine residue of arginine to give an imidazoline derivative. The assumption that this intermediate is formed is very likely, since similar reactions are known for a number of α-dicarbonyl compounds. Glyoxal and methylglyoxal react in aqueous solution with guanidine to give the imidazolone derivatives (Lempert, 1959; Bengelsdorf, 1953). The reaction of disubstituted α-dicarbonyl compounds with guanidine results in the formation of dihydroxyimidazoline derivatives (Nishimura and Kitajima, 1976), which can dehydrate to give substituted monohydroxy-4*H*-imidazoles (Nishimura et al., 1975). Phenylglyoxal (Takahashi, 1968) and *p*-hydroxyphenylglyoxal (Yamasaki et al., 1980) were used to modify reversibly the arginine residues of proteins and a bis(phenylglyoxal)−arginine structure for the product was proposed by Takahashi. For the same purpose, proteins were treated with 1,2-cyclohexanedione and a dihydroxyimidazoline derivative was suggested (Patthy and Smith, 1975). For immobilization, proteins were reacted with 4-(oxoacetyl)phenoxyacetic acid and formation of a methylimino-5-oxoimidazolidine was described (Duerksen-Hughes et al., 1989).

Henle et al. (1994) found that methylglyoxal reacts with L-arginine to give two isomeric imidazolidones and detected the compounds in alkali-treated bakery products. The reaction was also carried out under physiological conditions, and for the main product a imidazolone structure was postulated which would be an oxidation product of the above mentioned imidazolinone derivative (Lo et al., 1994). Finally Sopio and Lederer (1995) heated 3-desoxypentosulose with alkylguanidines and isolated the analogous imidazolinone and the enole tautomer hydroxy-5*H*-imidazole.

Therefore, it is reasonable to assume that the α-dicarbonyl compound xylosone, which is an oxidation product of AA, condenses with the guanidine residue of arginine similarly, resulting in the dihydroxylimidazoline derivative (Figure 4). Elimination of water leads then to an imidazolinone derivative which was described for several other α-dicarbonyl compounds. Since the derivative which is obtained from xylosone is the only one which possesses another hydroxyl group in *β*-position to the carbonyl group, further dehydratization can give **1a,b**.

Thus, it can be assumed that **1a,b** represent a general structure which can also be found as a derivative of L-arginine side chains of proteins and AA. Investigations to confirm this assumption are in progress.

ABBREVIATIONS USED

AA, L-ascorbic acid; DHA, L-dehydroascorbic acid; DKG, 2,3-diketogulonic acid; DPI, N^{α}-acetyl-N^{δ}-(4-(1,2-dihydroxy-3-propyliden)-3-imidazolin-5-on-2-yl)-L-ornithine.

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