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Model Studies on the Influence of High Hydrostatic Pressure on the Formation of Glycated Arginine Modifications at Elevated Temperatures

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Aqueous solutions of N^{α}-acetylarginine and glucose were reacted for 2 h with pressure application from 0 to 600 MPa and varying temperatures between 90 and 120 °C. After enzymatic deacetylation of the reaction products, the glycated amino acids were separated by means of a self-assembled preparative ion exchange chromatography system using ninhydrin detection. On the basis of the use of eight synthesized reference compounds known in the literature as posttranslational arginine modifications, first, the presence of several glycated amino acids could be excluded. On the other hand, N^5-[[(1-carboxyethyl)amino]iminomethyl]ornithine [N^7-(1-carboxyethyl)arginine; N^7-CEA; **12**] was identified as a previously unknown arginine modification based on LC-MS, NMR measurements, and synthesis. In addition, N^5-(5-hydro-5-methyl-4-imidazolon-2-yl)-L-ornithine (**1**) was identified as a further major reaction product. In further experiments, the formation of **1** and **12** was quantitatively followed at different pressures and/or temperatures. The results indicated that high hydrostatic pressure at elevated temperatures significantly increased the amounts of both arginine modifications. 2-Oxopropanal, known to form **1** in a reaction with arginine, was also quantified to explain the different yields observed after pressure application. A new formation mechanism leading to **12** by a reaction of the guanidine group or arginine with 2-oxopropanal is discussed.

KEYWORDS: Maillard reaction; posttranslational modification; arginine; high hydrostatic pressure; N^5-(5-hydro-5-methyl-4-imidazolon-2-yl)-L-ornithine; N^7-(1-carboxyethyl)arginine

INTRODUCTION

Besides the advantageous effects of high hydrostatic pressure (HHP) for food preservation (*1*), this new method of food processing may also enable the development of innovative food products by means of appropriate temperature/pressure regimens, for example, in the production of process flavorings. Very recently, a clear influence of HHP on the yields of certain aroma compounds formed in reaction flavors has been shown for the first time (*1*). In this study, it was shown that the application of HHP on Maillard-type reactions increases, in particular, the yields of α-dicarbonyl compounds such as ethane dialdehyde (glyoxal) and 2-oxopropanal in a pressurized amino acid/carbohydrate mixture, thereby significantly changing the spectrum of aroma-active compounds (*1*).

Besides the formation of flavor or color, the Maillard reaction is also known to generate posttranslational modified amino acids. At normal pressure, the identification of such modifications has been the subject of many studies, because the functional properties of proteins may be altered and the nutritional value is reduced. Lysine and arginine side chains are mainly susceptible to such reactions. Some of the modified amino acids, also known as advanced glycation end products (AGEs), are even reported to show mutagenic or antiproteolytic properties, for example, pyrraline (*2*). However, the physiological relevance of most of the glycated amino acids remains to be clarified.

The guanidino function of arginine may react with α-dicarbonyl compounds, such as 2-oxopropanal, glyoxal, or deoxyosones, which are generated during carbohydrate degradation (*3*, *4*). Thus, a broad spectrum of arginine modifications have been characterized up to now.

Henle et al. (*5*) have characterized the first product from arginine and 2-oxopropanal as N^5-(5-hydro-5-methyl-4-imidazolon-2-yl)-L-ornithine (**1** in **Figure 1**) and have also identified **1** in pretzels. The oxidation product of **1**, N^5-(5-methyl-4-imidazolon-2-yl)-L-ornithine (**4** in **Figure 1**), was at the same time described by Lo et al. (*4*), when N^{α}-acetylarginine was reacted with 2-oxopropanal at lower temperatures and at blood pH. On the basis of mass spectrometry (MS) and nuclear magnetic resonance spectroscopy (NMR) measurements, Ahmed et al. (*6*) recently characterized two further arginine modifications (**5** and **6** in **Figure 1**), which may be formed from 2-oxopropanal by bridging N^5 and N^7 instead of N^7 and N^8 of the guanidino group.

A major fluorescent product, assigned as "argpyrimidine" (**8** in **Figure 1**), was identified from the reaction of 2-oxopropanal

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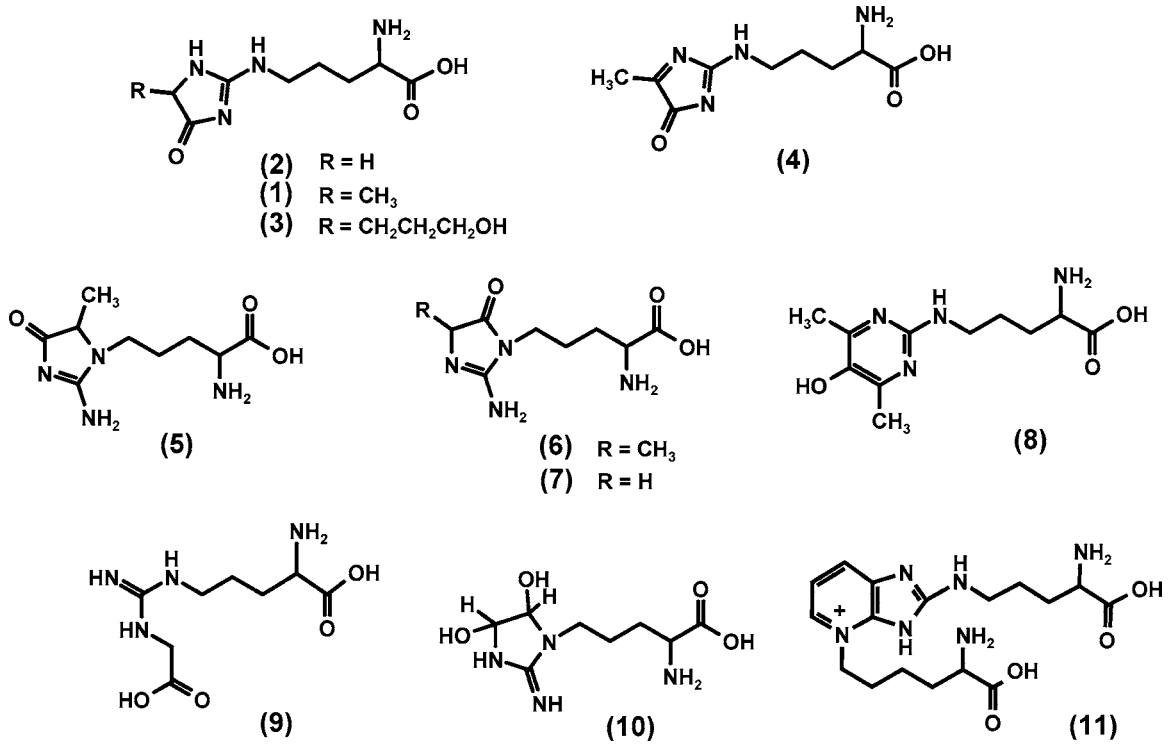


Figure 1. Posttranslational arginine modifications reported in the literature: (**1**) N^5-(5-hydro-5-methyl-4-imidazolon-2-yl)-L-ornithine; (**2**) N^5-(5-dihydro-4-imidazolon-2-yl)-L-ornithine; (**3**) N^5-[5-hydro-5-(3-hydroxypropyl)-4-imidazolon-2-yl]-L-ornithine; (**4**) N^5-(5-methyl-4-imidazolon-2-yl)-L-ornithine; (**5**) 2-amino-5-(2-amino-5-hydro-5-methyl-4-imidazolon-1-yl)pentanoic acid; (**6**) 2-amino-5-(2-amino-4-hydro-4-methyl-5-imidazolon-1-yl)pentanoic acid; (**7**) 2-amino-5-(2-amino-4-dihydro-5-imidazolon-1-yl)-pentanoic acid, "Glarg"; (**8**) N^5-(5-hydroxy-4,6-dimethyl-2-pyrimidinyl)-L-ornithine, "argpyrimidine"; (**9**) N^5-[[(carboxymethyl)amino]iminomethyl]-L-ornithine, "carboxymethylarginine"; (**10**) 2-amino-5-(4,5-dihydro-4,5-dihydroxy-2-iminoimidazol-1-yl)pentanoic acid; (**11**) pentosidind.

with N^α-*t*-BOC-arginine (*7*). **8** was also recently quantified in beer, and concentrations of ~27 nmol/L have been measured (*8*).

Glyoxal is another α-dicarbonyl compound rapidly reacting with arginine. Schwarzenbolz et al. (*9*) have observed the formation of "Glarg" (**7** in **Figure 1**), when reacting N^α-acetylarginine with glyoxal. In addition, "carboxymethylarginine" (**9** in **Figure 1**) was identified in in vitro glycated collagen and was characterized by matrix-assisted laser desoprtion ionization (MALDI)-MS and NMR studies (*10*).

Glomb et al. (*11*) have recently been able to characterize the dihydroxy derivative **10** (cf. **Figure 1**) from N^α-*t*-BOC-arginine and glyoxal. They proposed that Glarg (**7**) is an artifact of an acid hydrolysis of **10**. In fact, many authors have used strong mineral acids to hydrolyze proteins or acetylated amino acids prior to the identification experiments.

An ornithino-imidazolinone likely formed from arginine and glyoxal (**2** in **Figure 1**) was isolated from incubations of collagen with ribose and characterized by means of mass spectrometry as N^5-(5-dihydro-4-imidazolon-2-yl)-L-ornithine (*12*). Synthesis of the compound and characterization by NMR spectroscopy later on confirmed its structure (*6*).

Several other imidazolone compounds were additionally identified as arginine adducts with 3-deoxy-D-hexos-2-ulose (3-DG) in incubation mixtures with N^α-benzoyl-L-arginine (*13*−*15*).

"Pentosidine" (**11** in **Figure 1**), a fluorescent cross-link amino acid derivative of lysine, arginine, and a pentose, was already identified in 1989 (*16*). Amounts of pentosidine in foods, however, are relatively low (*17*). Further modifications comprising one or more molecules of arginine were recently identified by Lederer and Klaiber (*18*), Biemel et al. (*19*), and Hofmann (*20*). Finally, an ornithino-imidazolinone was observed to be formed after heating of N^α-acetylarginine with lactose (*21*). The compound assigned as "PIO" (**3** in **Figure 1**) is described as a disaccharide-specific arginine modification and is proposed to be formed from the intermediate 3,4-didesoxypentosulose (*21*).

Although the literature results indicate a very complex pattern of arginine modifications, it must be taken into consideration that many authors used strong mineral acids in the workup procedure, thus probably causing a degradation of the carbohydrate into α-dicarbonyls during workup. Furthermore, carbohydrate degradation products, such as 2-oxopropanal or glyoxal, but not the intact carbohydrate, have often been used in the model studies.

Like the temperature, high hydrostatic pressure (HHP) also influences chemical equilibria and reaction rates (*22*). It is, therefore, very likely that the Maillard reaction and, in particular, the formation of glycated amino acids are influenced by HHP as well. Although the Maillard reaction has previously been shown to be influenced by HHP (*1*), currently only limited information is available about the formation of glycated amino acids during the Maillard reaction under HHP. Only Schwarzenbolz et al. (*23*) have recently reported on an enhanced formation of pentosidine (**11** in **Figure 1**) at HHP.

The aim of this study was, therefore, to gain a more detailed insight into the influence of HHP and high termperatures on the extent of the formation of arginine modifications in the presence of glucose. Furthermore, a method should be developed to quantify the modified amino acids without the application of acid hydrolysis.

EXPERIMENTAL PROCEDURES

Chemicals. The following compounds were obtained commercially: L-arginine, N^{α}-acetyl-L-arginine, D-glucose, 2-oxopropanal (methylglyoxal; 40% aqueous solution), ethane dialdehyde (glyoxal; 40% aqueous solution), 3-(*N*-morpholino)propanesulfonic acid (MOPS), cyanamide, sodium methoxide, methyl 2-oxopropropanoate, sodium cyanoborohydride, 1,2-diaminobenzene, tetramethylsilane (TMS), and Acylase I (porcine liver) (Sigma, Deisenhofen, Germany). Deuterated solvents and sodium 3-(trimethylsilyl)propionate-*2,2,3,3-d*$_4$ (TMSP) were obtained from euriso-top (Saarbrücken, Germany). [$^{13}C_4$]Butane-2,3-dione was a laboratory standard synthesized according to the method given in ref *26*. Dowex 50W-X8 (50−100 mesh, H^+ form) was from Aldrich (Sigma-Aldrich Chemie, Taufkirchen, Germany). N^{α}-*t*-BOC-ornithine (Bachem, Weil am Rhein, Germany) and N^{α}-*t*-BOC-arginine were from Merck (Biosciences, Schwalbach/Taunus, Germany). All other chemicals were of analytical grade.

Syntheses. *2-Amino-5-(2-amino-5-hydro-5-methyl-4-imidazolon-1-yl)pentanoic acid (**5**).* Through a modification of a procedure of Ahmed et al. (*6*), **5** was synthesized by dissolving N^{α}-*t*-BOC-ornithine (1.0 mmol), methyl 2-oxopropanoate (2.2 mmol), and sodium cyanoborohydride (2.2 mmol) in methanol (9 mL). Modifications were done by omitting the purification of the intermediate N^{α}-*t*-BOC-N^{δ}-(methylpropion-2-yl)ornithine by reversed phase high-performance liquid chromatography (RP-HPLC) with 0.1% trifluoroacetic acid (TFA), because **5** could be isolated by direct RP-HPLC from the final mixture in good yields and high purity.

After 3 days of stirring at room temperature, the solvent was evaporated in vacuo. The solid residue was dissolved in deionized water (1 mL) and freeze-dried. A solution of sodium methoxide (250 mg) in methanol (8 mL) was added to the solid residue followed by addition of cyanamide (200 mg). The mixture was stirred at room temperature for another 4 days, and after evaporation of the solvent in vacuo, the residue was taken up in deionized water (4 mL). One milliliter of the solution was separated by semipreparative RP-HPLC. The mobile phase used was 0.1% TFA in water for 0−30 min, followed by a linear gradient of 0−50% methanol over 30−50 min. The eluate was monitored at 210 nm, and the peak eluting between 39 and 41 min was collected. After freeze-drying, the solid residue was treated with ice-cold TFA (60 μL) for 15 min. TFA was removed in vacuo to yield a white amorphous solid (8.6 mg). The ^{1}H and ^{13}C NMR data (MeOD-d_3, internal standard TMS) agreed with published data (*6*). In MS/ESI$^+$ measurements a main peak (100%) at *m*/*z* 229 was found.

*2-Amino-5-(2-amino-4-hydro-4-methyl-5-imidazolon-1-yl)-pentanoic acid (**6**).* **6** was synthesized following the procedure of Ahmed et al. (*6*) with some modifications. After freeze-drying of the incubation mixture, the extraction step with methanol was omitted, because this was found to be unnecessary. The residue was taken up in 0.1% aqueous TFA (2 mL) and purified by semipreparative RP-HPLC. The mobile phase was 0.1% aqueous TFA with 20% methanol (0−10 min) followed by a linear gradient of 20−50% methanol between 10 and 25 min. The effluent was monitored at 225 nm. The effluent between 27 and 29 min was collected. After freeze-drying, the solid residue was treated with ice-cold TFA (60 μL) for 15 min, and the TFA evaporated in vacuo to yield a white amorphous solid (21 mg). The ^{1}H and ^{13}C NMR data (MeOD-d_3, internal standard TMS) agreed with published data (*6*). In MS/ESI$^+$ measurements a main peak (100%) at *m*/*z* 229 was observed.

*N^5-[[(1-Carboxyethyl)amino]iminomethyl]-L-ornithine, N^7-CEA (**12**).* **6** (10 mg), dissolved in deionized water (3 mL) and adjusted to pH 9.0 with a 10% solution of NH_4OH, was incubated at 37 °C for 3 days. The mixture was freeze-dried and the solid residue redissolved in deionized water (1 mL). After the pH had been adjusted to 2.2 with HCl (2 mol/L), the solution was applied to a column (10 × 140 mm), filled with a cation exchange resin Dowex 50W-X8 (H^+-form), equilibrated with a 10% solution of NH_4OH (20 mL) and a solution of 0.3 M NH_4HCO_3, pH 3.3 (40 mL). The column was eluted with aqueous NH_4HCO_3 (20 mL each) by increasing the pH stepwise from 3.3 via pH 4.2 and 6.1 to pH 8.0 at a flow rate of 1 mL/min. Fractions (1 mL) were collected and analyzed for N^7-(1-carboxyethyl)arginine by thin-layer chromatography (TLC) on silica gel plates, 20 × 20 cm (Merck, Darmstadt, Germany) with *n*-butanol/H_2O/acetic acid/pyridine (4:2:3:3, by vol) as the mobile phase. Fractions at R_f 0.33 were combined and twice freeze-dried after the addition of water (5 mL) to yield a white amorphous solid (4.8 mg). N^7-(1-Carboxyethyl)arginine (**12**) was characterized by MS-ESI$^+$ and NMR measurements.

*2-Amino-5-(2-amino-4-dihydro-5-imidazolon-1-yl)pentanoic acid (**7**).* **7** was synthesized following the procedure of Glomb et al. (*11*) with slight modifications. The last purification step was modified by dissolving 20 mg of the yellowish residue in H_2O (1 mL). Aliquots of 50 μL were purified by semipreparative RP-HPLC with 0.1% TFA as mobile phase monitoring the eluate at 210 nm. The use of heptofluorobutanoic acid as done in ref *11* was avoided, because it interfered with the LC-MS measurements. Furthermore, despite the weaker ion pairing of TFA, a separation from byproducts was achieved.

7 eluting at 6.8 min was collected. The sample was freeze-dried to give a white amorphous solid (5.4 mg). The ^{1}H NMR and ^{13}C NMR data agreed with published spectra (*11*) (D_2O, external standard TMS). In MS-ESI$^+$ measurements a main peak (100%) at *m*/*z* 215 was found.

*N^5-[[(Carboxymethyl)amino]iminomethyl]-L-ornithine (**9**).* **9** was synthesized by hydrolytic cleavage of the amide function in **7**. The yellowish residue obtained prior to purification of **7** by RP-HPLC (500 mg) was dissolved in deionized water (40 mL), and the solution was adjusted to pH 8.0 with 0.1 N HCl. After incubation at 37 °C for 2 days with readjustment of the pH between, the mixture was freeze-dried. Forty milligrams of the residue was dissolved in deionized water (0.5 mL), and the pH was adjusted to 2.0 with 1 N HCl. The solution was applied onto a column (10 × 140 mm), filled with cation exchange resin Dowex 50W-X8 (H^+ form), and equilibrated with a 10% solution of NH_4OH (20 mL) and a solution of 0.3 M NH_4HCO_2, pH 3.3 (40 mL). The column was flushed with solutions (20 mL each) of 0.3 M NH_4HCO_3 by increasing the pH stepwise from 3.3 via pH 4.2 and 6.1 to pH 8.0 at a flow rate of 1 mL/min. Fractions (1 mL) were collected and analyzed for **9** by TLC on silica gel, 20 × 20 cm (Merck) with *n*-butanol/H_2O/HOAc/pyridine (4:2:3:3, v/v/v/v) as the mobile phase. Fractions at R_f 0.30 were combined and freeze-dried after the addition of deionized water (5 mL) to yield a white amorphous solid (6.2 mg). N^7-Carboxymethylarginine was characterized by MS-ESI$^+$ measurements showing a main peak (100%) at *m*/*z* 233. The ^{1}H NMR and ^{13}C NMR data were in agreement with data published in ref *11* (D_2O, external standard TMS).

*2-Amino-5-(4,5-dihydro-4,5-dihydroxy-2-iminoimidazol-1-yl)pentanoic Acid (**10**).* By modifying a procedure published by Glomb et al. (*11*), L-arginine (5.9 mmol) and a 40% aqueous solution of glyoxal (8.9 mmol) were dissolved in water (59 mL) and the pH was adjusted to 7.4 with 0.1 N HCl. The solution was incubated at 37 °C for 4 h with adjustment of pH inbetween. After freeze-drying, the resulting yellowish residue was dissolved in H_2O (25 mg/mL), and the solution was purified by RP-HPLC using 0.1% aqueous TFA as the mobile phase. The effluent was monitored at 210 nm, and the peak eluting at 5.4 min was collected. Sample loading was 25 μL. A total of 0.5 mL of the solution was purified and freeze-dried to yield a white amorphous solid (9.0 mg). The ^{1}H and ^{13}C NMR data (D_2O, external standard TMS) agreed with published data (*11*). In MS/ESI$^+$ measurements a molecular peak at *m*/*z* 233 was observed.

Further arginine modifications were prepared without modifications as described in the literature given in parentheses: N^5-(5-hydroxy-4,6-dimethyl-2-pyrimidinyl)-L-ornithine (**8**) (*7*), N^5-(5-hydro-5-methyl-4-imidazolon-2-yl)-L-ornithine (**1**) (*5*), and N^5-(5-dihydro-4-imidazolon-2-yl)-L-ornithine (**2**) (*6*). The molecular weights of the target compounds were checked by MS/ESI$^+$ measurements.

Model Systems. N^{α}-Acetylarginine (2 mmol) and D-glucose (6 mmol) were dissolved in MOPS buffer (0.5 mol/L; 20 mL; pH 7.0). Aliquots of the solution (1.5 mL) were filled in polypropylene vials (Brand, Germany) with screw caps suitable for autoclave treatment (VWR International, Ismaning, Germany), avoiding any headspace volume. For high-pressure application, vials were placed in a larger polypropylene vial (20 mL) filled with water. Through the stopper was placed a temperature sensor. Samples were incubated at the conditions described in the tables. After pressure application, samples were immediately cooled on ice and stored at −20 °C until analyzed.

Table 1. Buffer and Reagent Formulations Used in Ion Exchange Chromatography[a]

	loading buffer	buffer					
		A	B	C	D	E	F
pH	2.20	2.85	3.30	4.25	8.0	10.6	
lithium hydroxide × H_2O (g)					5.0	6.0	12.6
lithium acetate × H_2O (g)	16.3	16.3	18.4	20.4	10.2	12.6	
methanol (mL)		25	10				
formic acid (mL)	7.5	7.5	6.0	3.5	6.0	4.0	
lithium tetraborate (g)					4.0	4.0	
$Li_2EDTA \times H_2O$					0.5		
thiodiglycol 25% in water (mL)	20						
boric acid (g)					4.0		
octanoic acid (μL)	100	100	100	100	100	100	
Millipore water		to 1000 mL					

[a] The ninhydrin reagent was prepared as follows: 100 mL of methanol, 0.6 g of hydrindantin, 20 g of ninhydrin, dissolved in 450 mL of potassium acetate (5 mol/L), and 450 mL of ethylene glycol.

Control samples were incubated in an autoclave (Carl Roth, Karlsruhe, Germany) at the respective temperatures and times.

Enzymatic Hydrolysis. Reaction mixtures (1 mL) were deacetylated by incubation with Acylase I (2.0 mg) for 24 h at 25 °C.

Isolation of Arginine Modifications for Structural Assignment. Glycated amino acids were isolated from model incubations (10 mL), which had been reacted at 110 °C and 600 MPa for 2 h. Samples were deacetylated by the addition of Acylase I (20 mg), followed by centrifugation at 5000*g* (centrifuge Z 320, Hermle Labortechnik, Wehingen, Germany) using the centrifugal filter Centricon YM-10 (Millipore GmbH, Schwalbach, Germany) to remove the Acylase. After dilution with loading buffer (20 mL; cf. **Table 1**), glucose was removed by applying the solution onto a column (10 × 120 mm) filled with cation exchange resin Dowex 50W-X8 (H^+-form). Glucose was eluted with deionized water (100 mL), and the amino acids were isolated with 0.3 M NH_4HCO_3. After freeze-drying, samples were diluted with a suitable volume of the loading buffer (cf. **Table 1**).

Preparative isolation of the modified amino acids was performed using a self-constructed ion exchange chromatography system with ninhydrin detection consisting of a Besta low-pressure rotary valve (Besta-Technik GmbH, Wilhelmsfeld, Germany), a Jasco PU-1580 pump, and a Jasco UV-1575 detector (JASCO Labor- und Datentechnik, Gross-Umstadt, Germany). Chromatographic separations were performed on a stainless steel column (125 × 8.0 mm) filled with the same cation exchange resin as used for the analytical amino acid analysis described above. Column temperature was maintained at 50 °C by a water bath. Flow rate was 0.6 mL/min.

For the detection of the arginine modifications, ninhydrin was added to the effluent prior to the detector, using a 140B solvent delivery system (Applied Biosystems, Darmstadt, Germany) at a flow rate of 0.2 mL/min. The composition of the buffers and the ninhydrin reagent are summarized in **Table 1**. For preparative runs, the pH values of the buffers and the time program were modified as follows: buffer B, pH 3.30, 0−10 min; buffer C, pH 3.60, 10−25 min; buffer C, pH 4.20, 25−50 min; buffer D, pH 6.20, 50−75 min; buffer E, pH 8.70, 75−95 min, regeneration with buffer F for 20 min, and equilibration with buffer B, pH 3.30, for 20 min.

For preparation of the analytes, the effluent was continuously monitored at 210 nm without ninhydrin derivatization, and fractions were collected at the respective elution volumes. Aliquots of the fractions were tested for the presence of amino acids using ninhydrin. Desalting of the fraction containing **1** was achieved by preparative RP-HPLC using 0.1% TFA in water as the solvent. The effluent was monitored at 210 nm, and the fraction corresponding to the elution range of **1** (10−13 min) was collected and freeze-dried.

The fraction containing N^7-(1-carboxyethyl)arginine (**12**) was desalted by TLC on cellulose MN 300, 20 × 20 cm, 0.5 mm (Macherey-Nagel, Düren, Germany) using methanol/H_2O/pyridine (20:5:1) as the mobile phase. A single ninhydrin positive band between R_f 0.25 and 0.35 was scraped off and suspended in water (~5 mL per plate) for 30 min. After centrifugation at 3000*g*, the solution was filtered through 0.45 μm centrifuge filters and freeze-dried.

Table 2. Standard Separation Program Used in the Ion Exchange Chromatography

total run time (min)	buffer	temp (°C)	total run time (min)	buffer	temp (°C)
0	A	33	93.5	E	60
15	B	34	109.5	E	70
41.5	C	36	124.5	F	80
48	C	40	134.5	A	80
58	C	46	142.5	A	55
64.5	D	50	148.5	A	33
81.5	D	54			

Ornithine was purified by column chromatography using a glass column (16 × 120 mm) filled with a cation exchange resin (Dowex 50W-X8, 50−100 mesh, H^+ form). The column was flushed with deionized water (100 mL) followed by 1 N HCl (50 mL) at a flow rate of 0.3 mL/min. Finally, ornithine was eluted with HCl (4 mol/L) at a flow rate of 0.6 mL/min. Fractions (1 min) were tested for ornithine using ninhydrin. Fractions containing ornithine were pooled, and the hydrochloric acid was removed in vacuo.

Quantitation of 1, 12, and Ornithine. Aliquots of the deacetylated samples (50 μL) were diluted with loading buffer (100 μL; cf. **Table 1**) and analyzed using an amino acid analyzer LC 3000 (Eppendorf; Laborservice Onken GmbH, Gründau-Breitenborn, Germany) equipped with a stainless steel column (125 × 4.0 mm) filled with a strong acidic cation exchange resin, 4 μm, BTC 2410. Buffer and reagent flow was set at 0.2 mL/min. The composition of the buffers used is shown in **Table 1** and the separation program in **Table 2**. Amino acids were detected after reaction with ninhydrin at a coil temperature of 130 °C. For data acquisition at 570 and 440 nm and for peak integration, the ChromStar 4.10 software (SCPA; Software für Chromatographie und Prozess-Analytik GmbH, Germany) was used. Quantitation of arginine modifications was achieved by external standard curves prepared from solutions containing defined amounts of the target compounds. The limit of quantitation (LoQ) was determined to be 20 nmol/mL for both compounds. The reproducibility of the data determined by analyzing defined solutions of the synthesized reference compounds was ±4%.

Determination of α-Dicarbonyls. Glyoxal and 2-oxopropanal were analyzed after derivatization with 1,2-diaminobenzene (*24*). Briefly, 2 mL of the incubation mixture was diluted with water, and labeled butanedione ([$^{13}C_4$]-2,3-butandione), which was synthesized as described previously (*25*), and 1,2-diaminobenzene (0.1 mmol) were added. After 24 h at 20 °C in the dark, the quinoxalines formed were extracted with diethyl ether (total volume = 90 mL). After concentration to 2 mL, the solution was put on the top of a silica column (20 × 1 cm) filled with *n*-pentane/diethyl ether (9+1 by vol). The quinoxalines were isolated by flushing the column with *n*-pentane/diethyl ether (60 mL; 3+7 by vol).

High-Pressure Plant. A self-assembled high-pressure plant comprising a 250 mL high-pressure vessel with interior heating was used (Klaus Dunze GmbH, Rosengarten/Ehestorf, Germany). Pressure was raised by a type 760.0117 pressure amplifier system (Sitec Sieber Enigneering AG, Zürich, Switzerland). For pressure transmission a mixture of glycol/water was used. The temperature inside the sample and the pressure were recorded throughout the experiments.

High-Performance Liquid Chromatography. The HPLC apparatus (BIO-TEK Instruments, Eching, Germany) consisted of two pumps (type 522), a gradient mixer (M 800), a Rheodyne injector, and a UV detector (type 535). Chromatographic separations were performed on a stainless steel column, Hypersil ODS, 250 × 10 mm, 5 μm (Thermo Hypersil-Keystone, Kleinostheim, Germany) using a flow rate of 3.0 mL/min. Because HPLC was used only in the synthetic experiments, the detailed information is given under Syntheses.

Mass Spectral Analysis. Analysis was performed by means of an LCQ Classic mass spectrometer (ThermoQuest GmbH, Egelsbach, Germany) equipped with an ESI interface (ESI^+) and coupled to an

ion trap mass detector. The scan range was from *m*/*z* 100 to 600. Samples were dissolved in 0.1% aqueous formic acid and introduced by direct infusion via the syringe pump flow technique at a flow rate of 5 μL/min. The capillary temperature was set at 200 °C, spray voltage at 5 kV, capillary voltage at 17 V, and tube lens offset at 10 V.

Nuclear Magnetic Resonance Spectroscopy. ^{1}H NMR spectra, DQF-COSY, HMQC, and HMBC experiments were recorded using a 400 AMX (400 MHz) instrument (Bruker, Rheinstetten, Germany). ^{13}C NMR and DEPT-135 spectra were measured by means of an AM 360 (360 MHz) spectrometer (Bruker).

RESULTS AND DISCUSSION

Setup of the Analytical Method. Several glycated arginine modifications have already been reported in the literature, and Ahmed et al. (*6*) were the first to develop a method for the separation of several arginine modifications in one run. However, they used derivatization of the amino group with 6-aminoquinolyl-*N*-hydroxysuccinimidyl-carbamate in combination with fluorescence detection. Another method, using LC-MS/MS, without derivatization and isotopically labeled internal standards was recently developed (*26*), but this method is not appropriate for preparative purposes.

To set up a chromatographic system, which can be used for analytical and, also, preparative purposes, eight compounds, namely, **1**, **2**, and **5**−**10** (cf. structures in **Figure 1**) were synthesized, because these were commercially not available. All compounds were found to be quite stable during chromatography, except **10**, which was readily converted into **7** (data not shown).

In **Figure 2**, two chromatographic runs containing the seven resting compounds under investigation are shown. Although **1** and **5** could not be separated in one run (**Figure 2A**), changing the pH of buffer D (cf. **Table 1**) to 6.2 allowed the separation of both compounds (**Figure 2B**). The retention times of all seven arginine modifications determined in single runs are summarized in **Table 3**. For quantitative measurements, external calibration curves were set up using the synthesized reference compounds. The LoQ was found to be 20 nmol/mL for all compounds.

Identification of Arginine Modifications. The use of arginine protected in the α-amino group allows one to study the formation of glycated modifications exclusively at the guanidino group. Furthermore, this procedure is closer to the situation in a protein. Although the same concept has been proposed earlier by other groups, often strong mineral acids have been used to deacetylate the reaction products. However, this approach may lead to carbohydrate degradation as well as structural changes in the reaction products during workup. To avoid such artifact formation, which is likely to occur at low pH, the incubation mixtures were enzymatically deacetylated using Acylase I. Deacetylation was controlled by monitoring the increase of arginine modifications in the ion exchange chromatograms. Releasing curves for most compounds showed a plateau after 24 h of incubation (data not shown).

Analysis of a mixture that had been treated at 600 MPa and 110 °C for 2 h showed six ninhydrin-sensitive peaks, besides ammonia and free arginine (**Figure 3**). The corresponding sample heated at 110 °C without pressure application showed much smaller peaks, but at the same elution times (data not shown).

By comparison of the retention times of the six peaks obtained (I−VI in **Figure 3**) with the synthesized reference compounds, first, the presence of three glyoxal-derived compounds, namely, **2** and **5** as well as **9** (cf. **Figure 1**), could be ruled out. In addition, only very small peaks at the retentions times of compounds **6**/**7** and **8** were observed. Because, thus, the presence of **7** could be more or less excluded, also the presence of **10** is not probable, because **10** was shown to easily degrade into **7** during chromatography (data not shown).

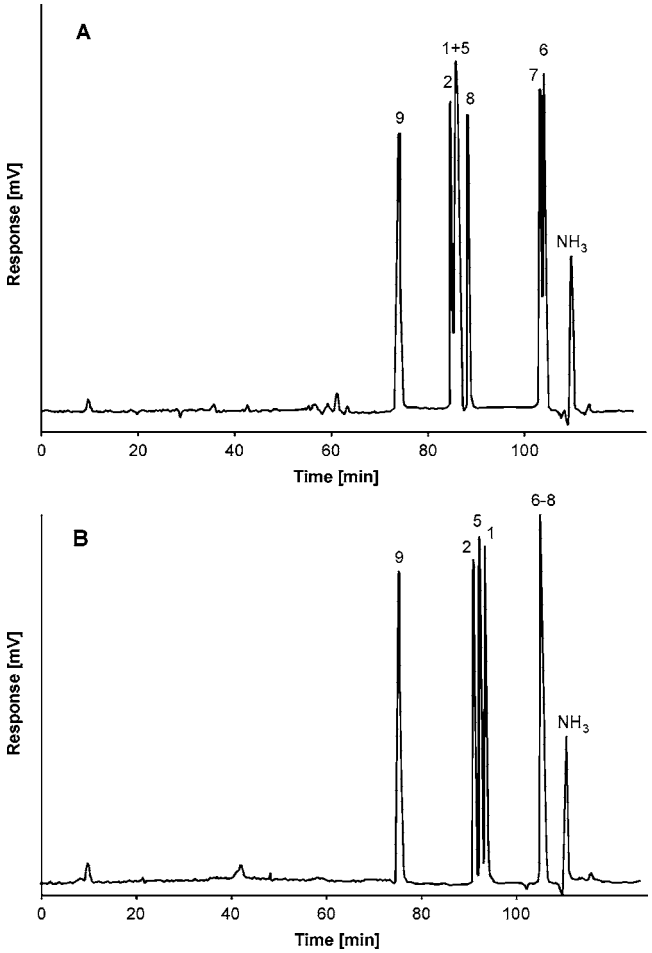


Figure 2. Separation of synthesized arginine derivatives by ion exchange chromatography with ninhydrin detection: (**A**) standard separation program (cf. **Tables 1** and **2**); (**B**) modified separation program with buffer D, pH 6.2. Compounds are numbered as in **Figure 1**.

Spiking of the sample with **1** resulted in an increase in peak IV (**Figure 3**), suggesting the ornithino-imidazolinone to be present.

For unequivocal identification, peak IV was isolated from the incubation mixture. On the basis of one- and two-dimensional NMR experiments as well as the determination of the mass charge ratio of *m*/*z* 229 obtained by MS/ESI, peak IV could unequivocally be identified as **1**.

Using the same method, peak VI was confirmed to be ornithine, which was already reported in the literature to be directly formed from arginine under basic conditions and at elevated temperatures (*27*). Very recently, it was also suggested that ornithine may also result from a breakdown of arginine-derived AGEs (*28*).

Peak III was eluted shortly after the reference compound **9** (N^7-carboxymethylarginine). Because all arginine modifications formed from methylglyoxal were eluted right after the corresponding glyoxal derivatives (cf. **Table 3**), this observation suggested peak III to be the yet unknown N^5-[[(1-carboxyethyl)-amino]iminomethyl]-L-ornithine or N^7-(1-carboxyethyl)arginine (N^7-CEA; **12** in **Table 4**).

To confirm this hypothesis, a synthetic route was developed for **12** by reacting N^{α}-*t*-Boc-arginine with methylglyoxal in a first step as shown in **Figure 4** to yield **6**. After isolation of the

Table 3. Retention Times of Synthesized Reference Compounds during Ion Exchange Chromatography Using the Standard Separation Program and a Modified Program (Buffer D, pH 6.2)

reference compound	compd no.[a]	retention time (min) standard program[b]	retention time (min) buffer D, pH 6.2[c]
N^5-[[(carboxymethyl)amino]iminomethyl]-L-ornithine	**9**	74.6	75.4
N^5-(5-dihydro-4-imidazolon-2-yl)-L-ornithine	**2**	83.9	90.8
2-amino-5-(2-amino-5-hydro-5-methyl-4-imidazolon-1-yl)pentanoic acid	**5**	85.5	91.6
N^5-(5-hydro-5-methyl-4-imidazolon-2-yl)-L-ornithine	**1**	85.6	92.5
N^5-(5-hydroxy-4,6-dimethyl-2-pyrimidinyl)-L-ornithine	**8**	88.4	105.1
2-amino-5-(2-amino-4-dihydro-5-imidazolon-1-yl)pentanoic acid	**7**	103.8	105.2
2-amino-5-(2-amino-4-hydro-4-methyl-5-imidazolon-1-yl)pentanoic acid	**6**	104.2	105.2

[a] See structures in **Figure 1**. [b] See **Table 2**. [c] See **Table 1**.

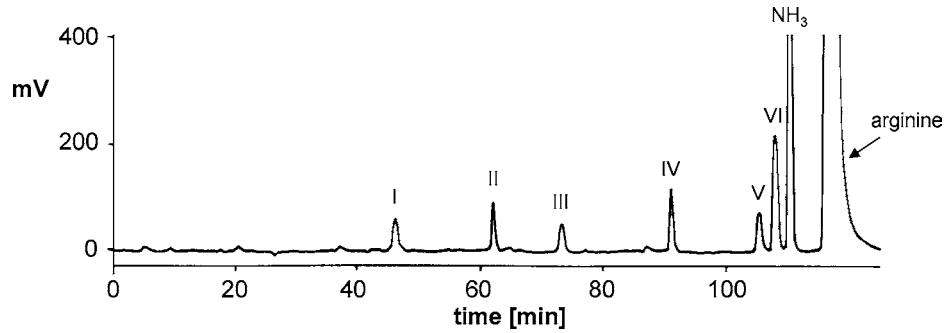
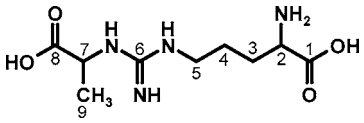


Figure 3. Ion exchange chromatogram of a deacylated mixture of reaction products generated from N^{α}-acetylarginine and glucose after pressurization at 600 MPa and 110 °C for 2 h.

Table 4. Assignment of ^{1}H NMR Signals (400 MHz) Obtained for **12** in D_2O



H at relevant C atom	δ[a]	I[b]	M[b]	J (Hz)[b]	COSY[c]
H–C(9)	1.41	3	d	7.0	H–C(7)
H–C(4)	1.73	2	m		H–C(3), H–C(5)
H–C(3)	1.89	2	m		H–C(2), H–C(4)
H–C(5)	3.28	2	T	6.7	H–C(4)
H–C(2)	3.76	1	t	6.0	H–C(3)
H–C(7)	4.00	1	q	7.1	H–C(9)

[a] ^{1}H chemical shifts are given in relation to TMSP. [b] Determined from 1D spectrum. [c] Observed homonuclear ^{1}H,^{1}H connectivities by DQF-COSY.

imidazolinone **6** by semipreparative HPLC, its incubation with aqueous NH_4OH at pH 9.0 resulted in the formation of **12**.

12 was isolated by ion exchange chromatography, and its structure was elucidated by mass spectrometry (**Figure 6**), as well as by ^{1}H and ^{13}C NMR experiments. In agreement with the suggested structure, six ^{1}H signals were recorded (**Table 4**). The signals of a methyl group at δ 1.41 and a methine H atom at C-7 (δ 4.00) showing coupling constants of J_{9-7} and J_{7-9} of 7.0 and 7.1 Hz, respectively, strongly suggested the methyl group at C-7. This connectivity was confirmed by COSY experiments (**Table 4**). The ^{13}C DEPT experiments (**Table 5**) confirmed the presence of a methyl group (C-9) at δ 20.7 and a second methine group (C-7) at δ 55.2. Also, the HMQC experiments clearly showed a connectivity between the respective carbon and hydrogen atoms at C-7 and C-9, respectively. As reported for the homologous compound **9** in the literature (*11*), also in the HMBC experiments of **12**, no ^3J[C-8,H−C(5)] correlation was observed. The signals of H−C(5), H−C(7), and C(7) in the ^{1}H and ^{13}C NMR spectra (**Tables 4** and **5**) showed a shift toward higher fields as compared to the imidazolinone **6**, thereby corroborating the open-chain structure.

Spiking of a pressure-treated model incubation with synthesized **12** resulted in an increase in peak III (**Figure 3**). For further confirmation, peak III was isolated by ion exchange chromatography. In MS-ESI$^+$, peak III gave a molecular ion at *m/z* 247. Because the retention time of the dihydroxy derivative **10** in the ion exchange chromatogram, having the same molecular weight, was ~86 min (data not shown), other theoretical dihydroxy derivatives of arginine, which would also give an *m/z* of 247, could be excluded. Thus, the formation of N^7-CEA (**12** in **Table 4**) in the model systems was confirmed. N^7-CEA could also reliably be analyzed by ion exchange chromatography with ninhydrin detection, because it was found to be stable under the conditions used.

The remaining peaks, I, II, and V, in the ion exchange chromatogram of the incubated mixture (**Figure 3**) could not be identified as they could not be isolated as pure compounds by means of ion exchange chromatography.

Influence of Temperature and High Hydrostatic Pressure on Amounts of Arginine Derivatives. The influence of HHP and reaction temperature on the amounts of **1**, **12**, and ornithine was measured in model incubations of N^{α}-acetylarginine (0.1 mol/L) with glucose (0.3 mol/L) at pH 7.0. Samples were pressure treated at 0.1, 200, 400, and 600 MPa and at temperatures between 90 and 120 °C for 2 h. No change in the pH was observed after pressure treatment.

Levels of **12** at normal pressure were ~1 mmol/mol of arginine (**Table 6**), but were increased 4-fold with pressure treatment. Levels of **1** were similar to those of **12** in samples without pressure application (0.1 MPa), but increased up to nearly 5-fold at 600 MPa (**Table 6**). Besides **1** and **12**, ornithine was also significantly generated with increasing pressure.

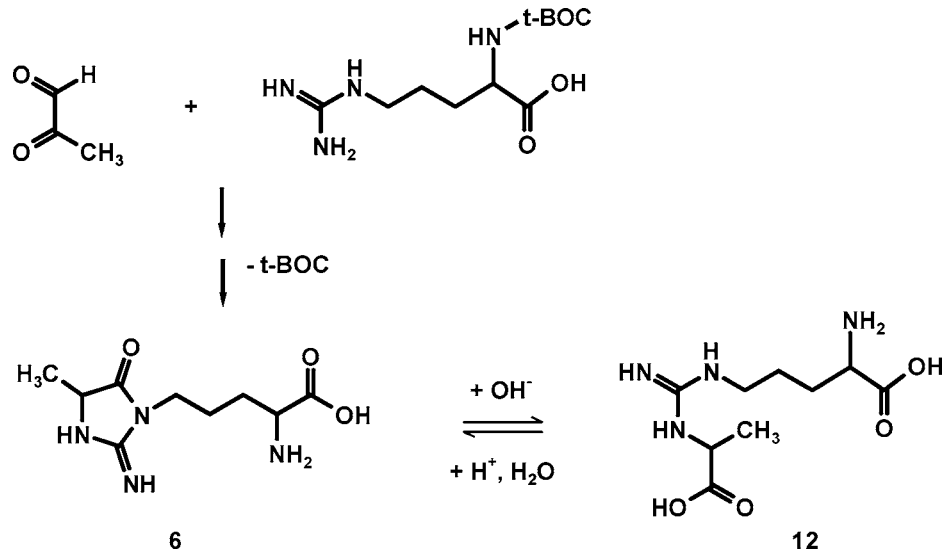


Figure 4. Synthetic route used in the preparation of **12**.

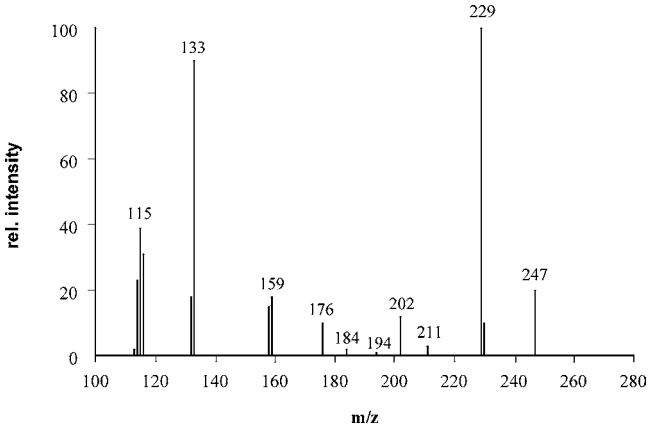
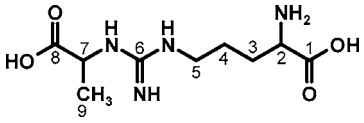


Figure 5. ESI⁺-mass spectrum of **12**.

Table 5. Assignment of ^{13}C Signals (360 MHz) Obtained for **12** in D_2O



H at relevant C atom	δ^a	DEPT[b]	heteronuclear $^1H,^{13}C$ multiple-quantum coherence via $^1J(C,H)$[c]	via $^{2,3}J(C,H)$[c]
C(9)	20.7	CH_3	H–C(9)	H–C(7)
C(4)	26.9	CH_2	H–C(4)	H–C(2), H–C(3), H–C(5?)
C(3)	30.4	CH_2	H–C(3)	H–C(2), H–C(4), H–C(5)
C(5)	43.5	CH_2	H–C(5)	H–C(3), H–C(4)
C(7)	55.2	CH	H–C(7)	H–C(9)
C(2)	57.1	CH	H–C(2)	H–C(3), H–C(4)
C(6)	158.2	C		H–C(5), H–C(7)
C(1)	177.4	C		H–C(2), H–C(3)
C(8)	181.8	C		H–C(7), H–C(9)

[a] ^{13}C chemical shifts are given in relation to TMSP. [b] DEPT-135 spectroscopy. [c] Assignments based on HMQC (^1J) and HMBC ($^{2,3}J$) experiments.

Although also at normal pressure (0.1 MPa) the amounts were comparatively high, at 600 MPa the increase was >3-fold.

The promoting effect of the temperature on the amounts of compounds **1** and **12** was shown in models reacted at temperatures between 90 and 120 °C (**Table 7**). Treatment at 90 °C and HHP resulted in low concentrations of only **1** and **12** (0.05 and 0.24 mmol/mol, respectively). However, at 100 °C, a clear influence of HHP on the amounts of both compounds was observed, which became more prevalent at 110 °C (**Table 7**). The levels of ornithine were quite high already at 90 °C, but, in contrast to **1** or **12**, showed a maximum at 100 or 110 °C, respectively. This may be due to the fact that higher amounts of 2-oxopropanal could be present at higher temperatures, which in turn might react with ornithine.

Table 6. Influence of High Hydrostatic Pressure on the Amounts of N^7-(1-Carboxyethyl)-L-arginine (**12**), N^5-(5-Hydro-5-methyl-4-imidazolon-2-yl)-L-ornithine (**1**), and Ornithine Generated from N^α-Acetylarginine and Glucose at 110 °C

	concn[a] (mmol/mol of N^α-acetylarginine)		
pressure (MPa)	**12**	**1**	ornithine
0.1	0.94 ± 0.06	1.01 ± 0.10	9.95 ± 1.06
200	1.29 ± 0.05	2.02 ± 0.29	14.78 ± 1.32
400	2.47 ± 0.11	4.01 ± 0.10	18.78 ± 1.97
600	3.96 ± 0.10	4.76 ± 0.11	34.15 ± 2.51

[a] Values are given as mean value ± standard deviation of triplicates.

These results clearly indicated that at higher temperature, HHP application leads to an increase in the yields of arginine modifications **1** and **12**. The main reason for the higher yields of the arginine modifications at HHP is probably the fact that the formation of α-dicarbonyl is favored under HHP (*1*).

Formation Pathway Leading to N^7-(1-Carboxyethyl)-arginine. To follow this idea, the reaction pathway possibly leading to N^7-CEA must be considered.

It can be proposed that N^7-CEA (**12**) is formed by a reaction of the guanidine group of arginine with 2-oxopropanal. A hypothetical formation mechanism is shown in **Figure 6**. In aqueous solutions, α-dicarbonyls are usually hydrated, and it is likely that in 2-oxopropanal water adds preferentially to the aldehyde function. By forming a Schiff base with the guanidine function of arginine (**Figure 7**) followed by an imine−enamine tautomerism, the unstable structure of an ene 1,1′-dihydroxy compound is formed, which should rapidly isomerize into the more stable acid. This rearrangement is similar to the known formation of 2-hydroxypropanoic acid from hydrated 2-oxopropanal.

On the basis of this reaction scheme, it might be speculated that the increase in **12** observed during pressure treatment of the model solution (**Table 6**) is due to a favored formation of 2-oxopropanal as recently observed by us (*1*).

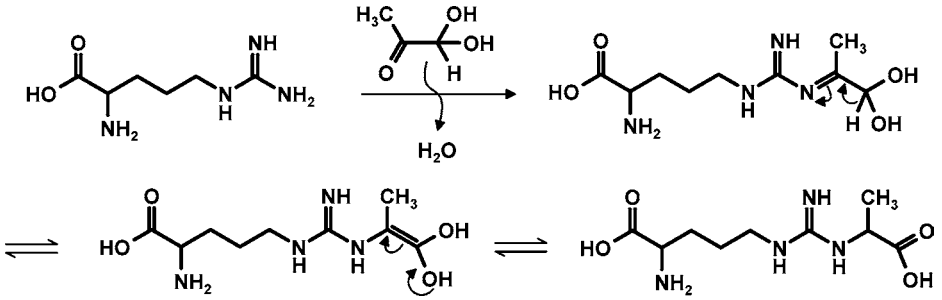


Figure 6. Hypothetical formation pathway leading from arginine and 2-oxopropanal to N^7-carboxyethylarginine.

Table 7. Levels of N^7-(1-Carboxyethyl)-L-arginine (**12**), N^5-(5-Hydro-5-methyl-4-imidazolon-2-yl)-L-ornithine (**1**), and Ornithine Formed after Reaction of N^α-Acetylarginine and Glucose with Variation of the Temperature at 0.1 and 600 MPa, Respectively

	concn[a] (mmol/mol of N^α-acetylarginine)					
temp	**12**		**1**		ornithine	
(°C)	0.1 MPa	600 MPa	0.1 MPa	600 MPa	0.1 MPa	600 MPa
90	0.035 ± 0.003	0.24 ± 0.01	nd	0.05 ± 0.01	7.53 ± 0.65	17.46 ± 1.00
100	0.18 ± 0.02	1.11 ± 0.08	0.11 ± 0.005	0.87 ± 0.07	13.32 ± 0.71	22.90 ± 1.38
110	0.73 ± 0.20	3.96 ± 0.20	0.75 ± 0.28	4.75 ± 0.21	10.80 ± 1.22	34.00 ± 1.84
120	2.48 ± 0.19	5.24 ± 0.30	4.74 ± 0.52	6.88 ± 0.51	9.16 ± 0.76	30.42 ± 2.01

[a] Values are given as mean value ± standard deviation of triplicates. nd, not determined.

Table 8. Influence of High Hhydrostatic Pressure on the Formation of Glyoxal and 2-Oxopropanal from N^α-Acetylarginine and Glucose Reacted for 2 h at 110 °C

	concn[a] (mmol/mol of glucose)	
pressure (MPa)	glyoxal	2-oxopropanal
0.1	0.036 ± 0.011	0.067 ± 0.020
200	0.063 ± 0.014	0.126 ± 0.008
400	0.120 ± 0.020	0.293 ± 0.039
600	0.138 ± 0.021	0.641 ± 0.036

[a] Values are given as mean value ± standard deviation of duplicates.

Quantitation of 2-oxopropanal and glyoxal in the reaction mixtures (**Table 8**) clearly demonstrated that the formation of 2-oxopropanal and, also, glyoxal was favored at HHP, but the levels of 2-oxopropanal were always higher as compared to those of glyoxal. Levels of glyoxal were ~0.035 mmol/mol of glucose and increased ~4-fold at 600 MPa. However, the levels of 2-oxopropanal increased >9-fold from 0.067 to 0.64 mmol/mol of glucose when HHP was applied. The increase of 2-oxopropanal closely followed an exponential curve, whereas the increase of glyoxal became less at pressures >500 MPa. Thus, the difference in levels between glyoxal and 2-oxopropanal increased from ~2-fold at 0.1 MPa to nearly 5-fold at 600 MPa.

This result is in agreement with the observation that glyoxal-derived arginine modifications, such as **2**, **7**, or **9** (cf. **Figure 1**), were not detected as major compounds in the model reactions.

The experiments showed a significant influence of high hydrostatic pressure on the formation of α-dicarbonyl compounds as well as the respective arginine modifications at pH 7.0, with temperatures >above 90 °C and pressure application promoting their formation. Interestingly, only **1** was identified among the known arginine modifications. Although a few reaction products remained unidentified, it can be concluded from these data that modifications of arginine formed by a reaction with glyoxal are quantitatively much less important as compared to 2-oxopropanal modifications.

LITERATURE CITED

(1) Deters, F.; Hofmann, T.; Schieberle, P. Influence of high hydrostatic pressure on flavor compound formation in Maillard-type reactions. In *Process and Reactions Flavors*; Weerashinge, D., Ed.; ACS Symposium Series; American Chemical Society: Washington, DC, 2005; in press.

(2) Omura, H.; Jahan, N.; Shinohara, K.; Murakami, H. Formation of mutagens by the Maillard reaction. In *The Maillard Reaction in Foods and Nutrition*; Waller, G. R., Feather, M. S., Eds.; ACS Symposium Series 5; American Chemical Society: Washington, DC, 1983; pp 537−563.

(3) Schwarzenbolz, U. Studies on non-enzymatic glycosylation and cross-linking reactions of milk proteins (in German). Dissertation, Technical University of Munich, 1999.

(4) Lo, T. W. C.; Westwood, M. E.; McLellan, A. C.; Selwood, T.; Thornalley, P. J. Binding and modification of proteins by methylglyoxal under physiological conditions. *J. Biol. Chem.* **1994**, *269*, 32299−32305.

(5) Henle, T.; Walter, A. W.; Haessner, R.; Klostermeyer, H. Detection and identification of a protein-bound imidazolone resulting from the reaction of arginine residues and methylglyoxal. *Z. Lebensm. Unters. Forsch.* **1994**, *199*, 55−58.

(6) Ahmed, N.; Argirov, O. K.; Minhas, H. S.; Cordeiro, C. A. A.; Thornalley, P. J. Assay of advanced glycation endproducts (AGEs): surveying AGEs by chromatographic assay with derivatization by 6-aminoquinolyl-*N*-hydroxysuccinimidyl-carbamate and application to N^ϵ-carboxymethyl-lysine- and N^ϵ-(1-carboxyethyl)lysine-modified albumin. *Biochem. J.* **2002**, *364* (1), 15−24.

(7) Shipanova, I. N.; Glomb, M. A.; Nagaraj, R. H. Protein modification by methylglyoxal: chemical nature and synthetic mechanism of a major fluorescent adduct. *Arch. Biochem. Biophys.* **1997**, *344* (1), 29−35.

(8) Glomb, M. A.; Rösch, D.; Nagaraj, R. H. N^5-(5-Hydroxy-4,6-dimethylpyrimidine-2-yl)-L-ornithine, a novel methylglyoxal-arginine modification in beer. *J. Agric. Food Chem.* **2001**, *49*, 336−372.

(9) Schwarzenbolz, U.; Henle, T.; Haessner, R.; Klostermeyer H. On the reaction of glyoxal with proteins. *Z. Lebensm. Unters. Forsch. A* **1997**, *205*, 121−124.

(10) Iijima, K.; Murata, M.; Takahara, H.; Irie, S.; Fujimoto, D. Identification of N^7-carboxymethylarginine as a novel acid-labile advanced glycation end product in collagen. *Biochem. J*. **2000**, *347*, 23−27.

(11) Glomb, M. A.; Lang, G. Isolation and characterization of glyoxal-arginine modifications. *J. Agric. Food Chem*. **2001**, *49*, 1493−1501.

(12) Paul, R. G.; Avery, N. C.; Slatter, D. A.; Sims, T. J.; Bailey, A. J. Isolation and characterization of advanced glycation endproducts derived from the in vitro reaction of ribose and collagen. *Biochem. J*. **1998**, *330*, 1241−1248.

(13) Konishi, Y.; Hayase, F.; Kato, H. Novel Imidazolone compound formed by the advanced Maillard reaction of 3-deoxyglucosone and arginine residues in proteins. *Biosci., Biotechnol., Biochem*. **1994**, *58* (11), 1953−1955.

(14) Hayase, F.; Konishi, Y.; Kato, H. Identification of the modified structure of arginine residues in proteins with 3-deoxyglucosone, a Maillard reaction intermediate. *Biosci., Biotechnol., Biochem*. **1995**, *59* (8), 1407−1411.

(15) Hayase, F.; Koyama, T.; Konishi, Y. Novel dehydrofuroimidazole compounds formed by the advanced Maillard reaction of 3-deoxy-D-hexos-2-ulose and arginine residues in proteins. *J. Agric. Food Chem*. **1997**, *45*, 1137−1143.

(16) Sell, D. R.; Monnier, V. M. Structure elucidation of a senescence cross-link from human extracellular matrix. *J. Biol. Chem*. **1989**, *264* (36), 21597−21602.

(17) Henle, T.; Schwarzenbolz, W.; Klostermeyer, H. Detection and quantification of pentosidine in foods. *Z. Lebensm. Unters. Forsch.* **1997**, *204* (2), 95−98.

(18) Lederer, M. O.; Klaiber, R. G. Cross-linking of proteins by Maillard processes: characterization and detection of lysine-arginine cross-links derived from glyoxal and methylglyoxal. *Bioorg. Med. Chem.* **1999**, *7*, 2499−2507.

(19) Biemel, K. M.; Reihl, O.; Conrad, J.; Lederer, M. O. Formation pathways for lysine-arginine cross-links derived from hexoses and pentoses by Maillard processes. unraveling the structure of a pentosidine precursor. *J. Biol. Chem*. **2001**, *276* (26), 23405−23412.

(20) Hofmann, T. 4-Alkylidene-2-imino-5-[4-alkylidene-5-oxo-1,3-imidazol-2-inyl]aza-methylidene-1,3-imidazolidine−A novel colored substructure in melanoidins formed by Maillard reactions of bound arginine with glyoxal and furan-2-carboxaldehyde. *J. Agric. Food Chem*. **1998**, *46*, 3896−3901.

(21) Schuster, K.; Scheller, D.; Henle, T. A new type of arginine modification formed during food processing. *Czech. J. Food Sci*. **2000**, *18* (Special Issue), 48−49.

(22) Tauscher, B. Pasteurization of food by hydrostatic high pressure: chemical aspects. *Z. Lebensm. Unters. Forsch*. **1995**, *200*, 3−13.

(23) Schwarzenbolz, U.; Klostermeyer, H.; Henle, T. Maillard reaction under high hydrostatic pressure: studies on the formation of protein-bound amino acid derivatives. *Int. Congr. Ser*. **2002**, *No. 1245*, 223−227.

(24) Hofmann, T. Quantitative studies on the role of browning precursors in the Maillard reaction of pentoses and hexoses with L-alanine. *Eur. Food Res. Technol*. **1999**, *209*, 113−121.

(25) Schieberle, P.; Hofmann, T. Evaluation of the character impact odorants in fresh strawberry juice by quantitative measurements and sensory studies on model mixtures. *J. Agric. Food Chem*. **1997**, *45*, 227−232.

(26) Thornalley, P. J.; Battah, S.; Ahmed, N.; Karachalias, N.; Agalou, S.; Babaei-Jadidi, R.; Dawnay, A. Quantitaitve screening of advanced glycation endproducts in cellular and extracellular proteins by tandem mass spectrometry. *Biochem. J*. **2003**, *375*, 581−592.

(27) Hamilton, P. B.; Anderson, R. A. L- and DL-ornithine monohydrochlorides. A. DL-ornithine monohydrochloride. *Biochem. Prep*. **1953**, *3*, 96−97.

(28) Sell, D.; Monnier, V. M: Conversion of arginine into ornithine by advanced glycation in senescent human collagen and lens crystallins. *J. Biol. Chem.* **2004**, *279*, 54173−54183.

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