JOURNAL OF AGRICULTURAL AND FOOD CHEMISTRY

ARTICLE

pubs.acs.org/JAFC

Taste Modulating *N*-(1-Methyl-4-oxoimidazolidin-2-ylidene) α-Amino Acids Formed from Creatinine and Reducing Carbohydrates

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ABSTRACT: Recent investigations led to the discovery of *N*-(1-methyl-4-oxoimidazolidin-2-ylidene)aminopropionic acid as a taste modulator enhancing the typical thick−sour mouthdryness and mouthfulness imparted by stewed beef juice. In the present study, systematic model reactions were targeted toward the generation of a series of *N*-(1-methyl-4-oxoimidazolidin-2-ylidene)-α-amino acids by Maillard-type reactions between creatinine and ribose, glucose, methylglyoxal, or glyoxal, respectively. By application of a comparative taste dilution analysis on fractions isolated from thermally treated creatinine/carbohydrate mixtures by means of hydrophilic liquid interaction chromatography (HILIC), a total of nine *N*-(1-methyl-4-oxoimidazolidin-2-ylidene)-α-amino acids were identified by means of LC-MS, LC-TOF-MS, and 1D/2D NMR experiments. Six of the nine creatinine glycation products were previously not reported in the literature. Whereas creatinine exhibited a bitter taste, none of the *N*-(1-methyl-4-oxoimidazolidin-2-ylidene)-α-amino acids imparted any intrinsic taste activity up to levels of 10 mmol/L (in water). Depending strongly on their chemical structure, these *N*-(1-methyl-4-oxoimidazolidin-2-ylidene)-α-amino acids induced a thick−sour, mouthdrying orosensation and mouthfulness enhancement when evaluated in model broth with recognition thresholds ranging from 31 to >1000 μmol/L.

KEYWORDS: creatinine, Maillard reaction, taste enhancer, taste, taste dilution analysis

INTRODUCTION

Thermal processing has long been known to enhance umami and savory tastes of food products such as sun-dried tomatoes, air-dried mushrooms, cooked vegetables, and stewed beef, respectively. Strongly depending on reaction conditions such as water activity, pH value, and heating time and temperature, as well as the precursor molecules in the raw materials, Maillard-type glycation of amino acids, organic acids, nucleotides, polyphenols, and creatinine in the presence of reducing carbohydrates was identified in recent years as a leading reaction principle giving rise to the generation of taste modulatory molecules upon thermal food processing. For the targeted localization of such taste modulators within the plethora of molecules formed upon food processing, we introduced the so-called "sensomics" approach combining techniques of modern natural product chemistry, advanced instrumental analysis, and analytical psychophysical tools.[1−5] The success of this discovery strategy and the structure determination of the key taste modulators were catalyzed by carefully planned model experiments. These included the thermal reaction of candidate precursor molecules ex food under food-related model conditions and the LC-MS/MS-based verification of the occurrence of the reaction products in the taste modulating fractions located by means of the sensomics approach, followed by studies on their taste enhancement activity by means of human sensory testings.[1−5]

Application of this discovery strategy revealed that the attractive savory taste of sun-dried tomatoes and air-dried morel mushrooms is due to the enhancement of the umami taste of endogenous L-glutamate by *N*-(1-deoxy-D-fructos-1-yl)-L-glutamic acid and (*S*)-malic acid 1-*O*-β-D-glucopyranoside, respectively.[6−10] Whereas the latter glucoside, coined (*S*)-morelid, is formed by the nonenzymatic glucosylation of L-malic acid, the umami-enhancing Amadori product, present in sun-dried tomatoes in yields of up to 1.5% (on a weight basis), is generated upon the Maillard reaction of L-glutamic acid and glucose.

Moreover, *N*-(1-carboxymethyl)-6-hydroxymethyl-pyridinium-3-ol inner salt was discovered as a taste enhancer in beef bouillon by means of the sensomics approach.[11] Model experiments demonstrated that this so-called alapyridaine is generated as a racemic mixture by the Maillard reaction of L-alanine and glucose.[12] Although being tasteless on its own, the enantiomer (+)-(*S*)-alapyridaine was confirmed to significantly decrease the human recognition threshold of umami as well as sweet stimuli, whereas the (−)-(*R*)-alapyridaine was physiologically not active.[13,14] Additional structure/activity studies revealed that removal of the methyl group in the alanine moiety of alapyridaine switched its sweetness-enhancing into a bitter-inhibitory activity.[15,16]

Besides Maillard-type glycation of amino acids and organic acids, the nonenzymatic *C*-glycosylation of flavan-3-ols was reported to give rise to taste modulators during the manufacturing of roasted, alkalized cocoa powder.[17] By means of the sensomics approach, (epi)catechin-6-*C*-β-D-glucosides, (epi)catechin-8-*C*-β-D-glucosides, and (epi)catechin-6,8-*C*-β-D-glucosides were identified as velvety astringent taste molecules and demonstrated to significantly decrease the perceived bitterness of theobromine.[17]

Very recently, application of the sensomics approach on commercial yeast extracts revealed (*S*)-N^2-(1-carboxyethyl)-guanosine 5′-monophosphate as a previously unreported umami-enhancing nucleotide.[18,19] Supported by systematic model

Received: April 28, 2011
Revised: June 24, 2011
Accepted: June 24, 2011
Published: June 25, 2011

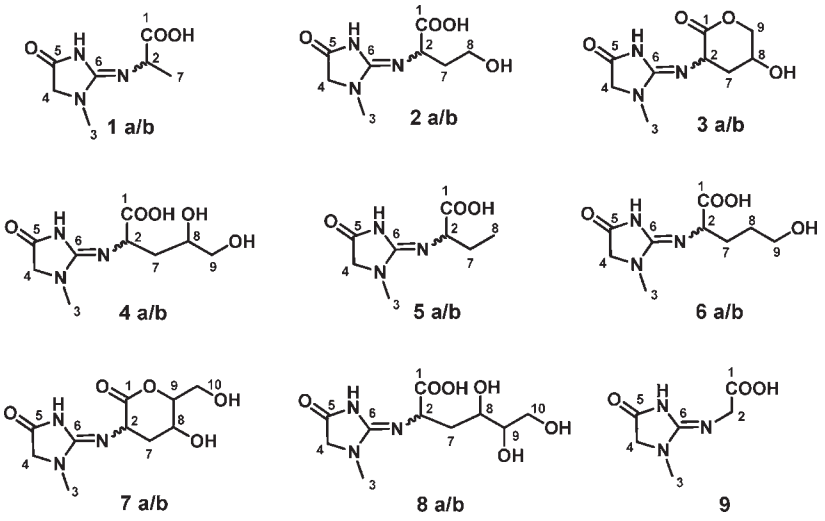

Figure 1. Chemical structures of taste modulating *N*-(1-methyl-4-oxoimidazolidin-2-ylidene)-α-amino acids **1a**/**b**−**9** formed upon Maillard reaction of creatinine.

experiments, this taste modulator, besides a series of structurally related (*S*)-N^2-(1-carboxyalkyl)guanosine 5′-monophosphates and (*S*)-N^2-(1-alkylamino)carbonylalkyl) guanosine 5′-monophosphates, could be confirmed to be generated by Maillard-type reactions between 5′-GMP and reducing monosaccharides.[18,19]

The leading role of Maillard-type reactions in the generation of taste modulators during food processing was even more highlighted by the recent discovery of the diastereomeric *N*-(1-methyl-4-oxoimidazolidin-2-ylidene)aminopropionic acid, **1a**/**b** (Figure 1), as taste modulators in stewed beef juice.[20] This *N*-(1-methyl-4-oxoimidazolidin-2-ylidene)-α-amino acid, enhancing the typical thick−sour and mouthdrying orosensation and the mouthfulness imparted by stewed beef juice, was demonstrated by preliminary model experiments to be formed upon Maillard-type reactions involving creatinine.[20]

As food-related model experiments have proven to be successful in the discovery of previously unknown taste modulators in processed foods,[5] the objective of the present study was to target the Maillard reaction toward the generation of a series of *N*-(1-methyl-4-oxoimidazolidin-2-ylidene)-α-amino acids by application of appropriate reaction conditions to determine their chemical structure and to evaluate their taste recognition concentrations by means of human sensory experiments.

MATERIALS AND METHODS

Chemicals. Formic acid, potassium dihydrogen phosphate, and dipotassium hydrogen phosphate were purchased from Merck KGaA (Darmstadt, Germany), trifluoroacetic acid and D-(−)-ribose were obtained from Fluka (Neu-Ulm, Germany), and all other chemicals were purchased from Sigma-Aldrich (Steinheim, Germany). Deionized water used for chromatography was prepared by means of a Milli-Q water gradient A 10 system (Millipore, Schwalbach, Germany). For sensory analysis, bottled water (Evian) was adjusted to pH 5.9 with trace amounts of formic acid. Solvents were of high-performance liquid chromatography (HPLC) grade (Fisher Scientific, Schwerte, Germany), and deuterated solvents were supplied by Euriso-Top (Saarbruecken, Germany). A model broth solution (1 L) was prepared by dissolving monosodium glutamate monohydrate (1.9 g), yeast extract (2.1 g; Gistex XII LS, DSM Food Specialities, Düsseldorf, Germany), maltodextrin (6.375 g), and sodium chloride (2.9 g) in bottled water and adjusting the pH value to 5.9 using trace amounts of formic acid (0.1 mmol/L in water). *N*-(1-Methyl-4-oxoimidazolidin-2-ylidene)aminopropionic acid (**1a**/**b**) was prepared as reported recently.[20]

Reaction of Creatinine and Carbohydrates. A binary mixture of creatinine (4 mmol) and D-(−)-ribose (4 mmol) or D-(+)-glucose (4 mmol), respectively, in aqueous Na_2HPO_4 buffer (0.5 mol/L; pH 7.0; 40 mL) was heated for 2 h at 120 °C in a closed vessel. After cooling to room temperature, the reaction mixtures were lyophilized and kept at −18 °C until used for further analysis.

Fractionation of Creatinine/Carbohydrate Reaction Mixtures. The lyophilized reaction mixtures were solubilized in methanol (200 mL) upon ultrasonification for 30 min at 40 °C and, after filtration, the solutions were separated from solvent in vacuum, lyophilized twice, and then dissolved in acetonitrile/water (60:40, v/v; 5 mL). After membrane filtration (0.45 μm), each solution was fractionated by semipreparative hydrophilic interaction liquid chromatography (HILIC) on a 300 × 21.5 mm i.d., 10 μm, TSKgel Amide-80 column (Tosoh Bioscience, Stuttgart, Germany) equipped with a 75 × 21.5 mm i.d., 10 μm, guard column (Tosoh Bioscience). Monitoring the effluent at 200 nm and using a flow rate of 8 mL/min, chromatography was performed with isocratic elution with a mixture (40:60, v/v; for creatinine/ribose products) of aqueous formic acid (0.1% in water) and acetonitrile or a mixture (20:80, v/v; for creatinine/glucose products) of water and acetonitrile containing 0.1% trifluoracetic acid, respectively. To sensorially evaluate the creatinine/ribose reaction products, the effluent was separated into fractions R-I−R-VIII, which were collected separately, freed from solvent under vacuum, lyophilized twice, and then used for the comparative taste dilution analysis. Among the creatinine/glucose reaction products, the effluent of fractions G-I−G-VI was collected, freed from solvent under vacuum, lyophilized twice, and then used for the identification experiments.

Analytical Sensory Experiments. *Panel Training and Pretreatment of Fractions.* Nine assessors (four males, five females, ages 26−40 years), who gave informed consent to participate in the sensory tests of the present investigation and have no history of known taste disorders, participated for at least two years in sensory training sessions with solutions (2.0 mL) of purified reference compounds by using the sip-and-spit method as reported recently.[20−22] For the training of the thick−sour and mouthdrying orosensation and mouthfulness enhancement, the panel was asked to compare the gustatory impact of a model broth solution (pH 5.9) as control with a solution of

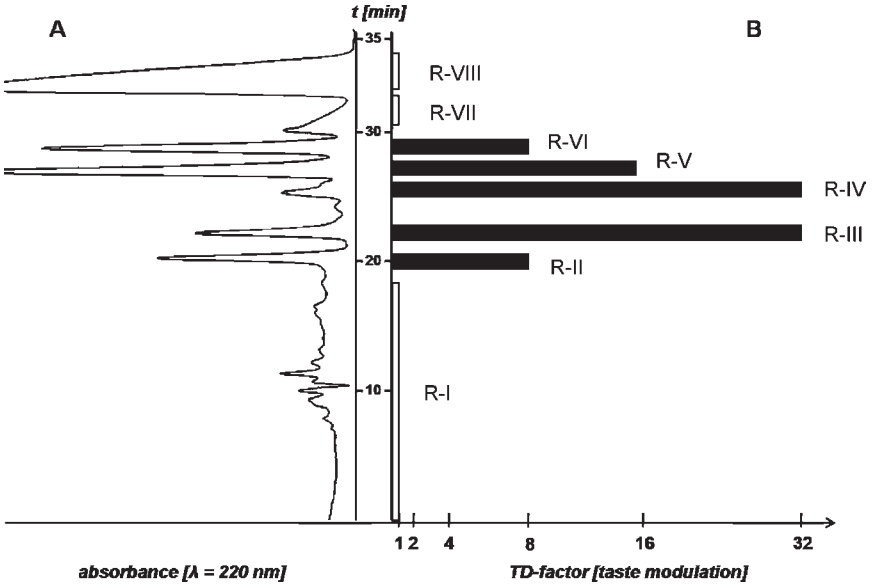

Figure 2. HILIC chromatogram (A) and comparative taste dilution analysis (B) of a thermally treated mixture of creatinine and ribose.

N-(1-methyl-4-oxoimidazolidin-2-ylidene)aminopropionic acid (1.0 mmol/L) in the same tastant solution. The sensory sessions were performed in a sensory panel room at 18−23 °C in three independent sessions. To prevent cross-modal interactions with odorants, the panelists used nose clips. Prior to sensory analysis, the fractions or compounds isolated were analytically confirmed to be essentially free of solvents and buffer compounds. Formate was determined using the anion procedure reported recently,[21] and solvents were analyzed by means of GC-MS after headspace-SPME extraction. To minimize the uptake of any compound to the best of our knowledge, all of the sensory analyses were performed by using the sip-and-spit method, which means that the test materials were not swallowed but terminally expectorated.

Taste Dilution Analysis (TDA) and Comparative Taste Dilution Analysis (cTDA). The HILIC fractions R-I−R-VIII (Figure 2) were taken up in their "natural" concentrations (i) in water (5.0 mL) to perform the TDA[23] or (ii) in a model broth solution (5.0 mL) to perform the cTDA,[16] and the pH value was adjusted to 5.9 using trace amounts of formic acid (0.1 mmol/L) or potassium hydroxide solution (0.1 mmol/L), respectively. These stock solutions were sequentially diluted 1:2 with water (pH 5.9; for TDA) or the model broth solution (for cTDA), respectively. The fractions were randomly presented to the sensory panel, each as serial dilutions in order of increasing concentration. By means of a duo test with one sample as the blank (water or model broth solution), panelists were asked to determine the dilution step at which a difference between sample and blank water could be detected. These so-called taste dilution (TD) factors[23] determined by the sensory subjects in three separate sessions were averaged.

Determination of Taste Modulation Detection Thresholds. Prior to sensory analysis, the purity of creatinine and the compounds **1a**/**b**−**9** was checked by ^{1}H NMR spectroscopy as well as HPLC-MS. Recognition thresholds were determined in water (pH 5.9; for intrinsic taste) and in model broth (pH 5.9, for taste modulating activity) by nine trained panelists using a triangle test with ascending concentrations of the stimulus, as reported previously.[1,4,20] The threshold value of the sensory group was approximated by averaging the threshold values of the individuals in three independent sessions.

Identification of *N*-(1-Methyl-4-oxoimidazolidin-2-ylidene)-α-amino Acids. The taste modulatory HILIC fractions R-II−R-VI of the creatinine/ribose system and fractions G-II−G-VI of the creatinine/glucose system, respectively, were purified by rechromatography to yield the target compounds as amorphous white powders with a purity of >98% (HPLC-UV−vis, ^{1}H NMR). UV−vis, LC-MS/MS, LC-TOF-MS, and 1D/2D NMR experiments led to the identification of diastereomeric mixtures of the taste modulators *N*-(1-methyl-4-oxoimidazolidin-2-ylidene)aminopropionic acid (**1a**/**b**) in fractions R-III and G-II, *N*-(1-methyl-4-oxoimidazolidin-2-ylidene)amino-4-hydroxybutanoic acid (**2a**/**b**) in fractions R-IV and G-III, *N*-(1-methyl-4-oxoimidazolidin-2-ylidene)amino-4-hydroxypentanoic lactone (**3a**/**b**) in fraction R-V, *N*-(1-methyl-4-oxoimidazolidin-2-ylidene)amino-4,5-hydroxypentanoic acid (**4a**/**b**) in fraction R-VI, *N*-(1-methyl-4-oxoimidazolidin-2-ylidene)aminobutanoic acid (**5a**/**b**) in fractions R-II and G-I, *N*-(1-methyl-4-oxoimidazolidin-2-ylidene)amino-5-hydroxypentanoic acid (**6a**/**b**) in fraction G-IV, *N*-(1-methyl-4-oxoimidazolidin-2-ylidene)amino-4,5,6-trihydroxyhexanoic lactone (**7a**/**b**) in fraction G-V, and *N*-(1-methyl-4-oxoimidazolidin-2-ylidene)amino-4,5,6-trihydroxyhexanoic acid (**8a**/**b**) in fraction G-VI (Figure 1).

N-(1-Methyl-4-oxoimidazolidin-2-ylidene)aminopropionic acid, ***1a/b*** *(Figure 1):* UV−vis (MeOH), λ_{max} 204.0 nm; LC-TOF-MS, *m*/*z* 186.0878 ([M + H]$^+$, measured), 186.0873 ([M + H]$^+$, calcd for $C_7H_{12}N_3O_3^+$); LC-MS and 1D/2D-NMR data were well in agreement with those reported recently.[20]

N-(1-Methyl-4-oxoimidazolidin-2-ylidene)amino-4-hydroxybutanoic acid, ***2a/b*** *(Figure 1):* UV−vis (MeOH), λ_{max} 204.0 nm; LC-MS (ESI$^+$), *m*/*z* 216 (100, [M + H]$^+$); LC-TOF-MS, *m*/*z* 216.0975 ([M + H]$^+$ measured), 216.0978 ([M + H]$^+$ calcd for $C_8H_{14}N_3O_4^+$); ^{1}H NMR (500 MHz, D_2O, COSY) diastereomer **2a**, δ 2.06−2.15 [m, 2H, C(7)], 3.17 [s, 3H, H−C(3)], 3.69 [t, 2H, *J* = 6.1 Hz, H−C(8)], 4.16 [s, 2H, H−C(4)], 4.48−4.55 [m, 1H, H−C(2)]; diastereomer **2b**, δ 2.06−2.15 [m, 2H, C(7)], 3.19 [s, 3H, H−C(3)], 3.71 [m, 2H, H−C(8)], 4,18 [s, 2H, H−C(4)], 4.48−4.55 [m, 1H, H−C(2)]; ^{13}C NMR (125 MHz, D_2O, HMQC, HMBC, ^{13}C) diastereomer **2a**, δ 32.40 [C(7)], 37.37 [C(3)], 54.05 [C(4)], 56.84 [C(8)], 57.52 [C(2)], 156.85 [C(5)], 171.81 [C(6)], 177.49 [C(1)]; diastereomer **2b**, δ 32.41 [C(7)], 38.60 [C(3)], 52.94 [C(4)], 53.68 [C(8)], 57.49 [C(2)], 157.37 [C(5)], 171.42 [C(6)], 177.24 [C(1)].

N-(1-Methyl-4-oxoimidazolidin-2-ylidene)amino-4,5-dihydroxypentanoic lactone, ***3a/b*** *(Figure 1):* UV−vis (MeOH), λ_{max} 204.0 nm; LC-MS (ESI$^+$), *m*/*z* 228 (100, [M + H]$^+$); LC-TOF-MS, *m*/*z* 228.0973 ([M + H]$^+$ measured), 228.0978 ([M + H]$^+$ calcd for $C_9H_{14}N_3O_4^+$); ^{1}H NMR (500 MHz, D_2O; COSY) diastereomer **3a**, δ 1.89−2.10 [m, 2H, H−C(7)], 3.07 [s, 3H, H−C(3)], 3.47

[m, 2H, H−C(9)], 3.87 [m, 1H, H−C(8)], 3.96 [s, 2H, H−C(4)], 4.43 [m, 1H, H−C(2)]; diastereomer **3b**, δ 1.89−2.10 [m, 2H, H−C(7)], 3.09 [s, 3H, H−C(3)], 3.47 [m, 2H, H−C(9)], 3.79 [m, 1H, H−C(8)], 3.98 [s, 2H, H−C(4)], 4.43 [m, 1H, H−C(2)]; ^{13}C NMR (125 MHz, D_2O, HMQC, HMBC) diastereomer **3a**, δ 32.70 [C(7)], 37.40 [C(3)], 52.47 [C(4)], 57.43 [C(2)], 65.28 [C(9)], 67.50 [C(8)], 157.72 [C(5)], 171.19 [C(6)], 177.27 [C(1)]; diastereomer **3b**, δ 32.79 [C(7)], 38.62 [C(3)], 53.52 [C(4)], 57.43 [C(2)], 65.36 [C(9)], 67.36 [C(8)], 157.65 [C(5)], 170.78 [C(6)], 177.27 [C(1)].

*N-(1-Methyl-4-oxoimidazolidin-2-ylidene)amino-4,5-dihydroxypentanoic acid, **4a/b** (Figure 1):* UV−vis (MeOH), λ_{max} 204.0 nm; LC-MS (ESI$^+$), *m/z* 246 (100, [M + H]$^+$); LC-TOF-MS, *m/z* 246.1078 ([M + H]$^+$ measured), 246.1084 ([M + H]$^+$ calcd for $C_9H_{16}N_3O_5^+$); ^{1}H NMR (500 MHz, D_2O; COSY) diastereomer **4a**, δ 1.80−1.98 [m, 2H, H−C(7)], 3.10 [s, 3H, H−C(3)], 3.49 [m, 2H, H−C(9)], 3.83 [m, 1H, H−C(8)], 4.01 [s, 2H, H−C(4)], 4.51 [m, 1H, H−C(2)]; diastereomer **4b**, δ 1.80−1.98 [m, 2H, H−C(7)], 3.14 [s, 3H, H−C(3)], 3.49 [m, 2H, H−C(9)], 3.83 [m, 1H, H−C(8)], 4.04 [s, 2H, H−C(4)], 4.51 [m, 1H, H−C(2)]; ^{13}C NMR (125 MHz, D_2O, HMQC, HMBC, ^{13}C) diastereomer **4a**, δ 34.16 [C(7)], 37.53 [C(3)], 53.49 [C(4)], 57.43 [C(2)], 65.32 [C(9)], 67.86 [C(8)], 158.08 [C(5)], 171.05 [C(6)], 177.14 [C(1)]; diastereomer **4b**, δ 34.26 [C(7)], 38.65 [C(3)], 52.39 [C(4)], 57.40 [C(2)], 65.26 [C(9)], 67.80 [C(8)], 157.91 [C(5)], 170.74 [C(6)], 176.96 [C(1)].

*N-(1-Methyl-4-oxoimidazolidin-2-ylidene)aminobutanoic acid, **5a/b** (Figure 1):* UV−vis (MeOH), λ_{max} 204.0 nm; LC-MS (ESI$^+$), *m/z* 200 (100, [M + H]$^+$); LC-TOF-MS, *m/z* 200.1025 ([M + H]$^+$ measured), 200.1029 ([M + H]$^+$ calcd for $C_8H_{14}N_3O_3^+$); ^{1}H NMR (500 MHz, D_2O, COSY) diastereomer **5a**, δ 0.91 [t, 3H, *J* = 7.5 Hz, H−C(8)], 1.78−1.94 [dq, 2H, H−C(7)], 3.17 [s, 3H, H−C(3)], 4.17 [s, 2H, H−C(4)], 4.42 [dd, 1H, *J* = 5.5 Hz, 8.6 Hz, H−C(2)]; diastereomer **5a**, δ 0.88 [t, 3H, *J* = 7.5 Hz, C(8)], 1.78−1.94 [dq, 2H, H−C(7)], 3.18 [s, 3H, H−C(3)], 4.18 [s, 2H, H−C(4)], 4.45 [dd, 1H, *J* = 8.3 Hz, 11.4 Hz, H−C(2)]; ^{13}C NMR (125 MHz, D_2O, HMQC, HMBC, ^{13}C) diastereomer **5a**, δ 7.42 [C(8)], 23.60 [C(7)], 37.40 [C(3)], 54.00 [C(4)], 61.20 [C(2)], 157.62 [C(5)], 171.72 [C(6)], 177.52 [C(1)]; diastereomer **5b**, δ 7.30 [C(8)], 23.55 [C(7)], 38.61 [C(3)], 52.87 [C(4)], 61.17 [C(2)], 157.94 [C(5)], 171.28 [C(6)], 177.29 [C(1)].

*N-(1-Methyl-4-oxoimidazolidin-2-ylidene)amino-5-hydroxypentanoic acid, **6a/b** (Figure 1):* UV−vis (MeOH), λ_{max} 204.0 nm; LC-TOF-MS, *m/z* 230.1133 ([M + H]$^+$, measured), 230.1135 ([M + H]$^+$, calcd for $C_9H_{16}N_3O_4^+$); LC-MS (ESI$^+$), *m/z* 230.1 (100, [M + H]$^+$); ^{1}H NMR (500 MHz, D_2O; COSY) diastereomer **6a**, δ 1.45−1.70 [m, 2H, H−C(8)], 1.79−1.98 [m, 2H, H−C(7)], 3.16 [s, 3H, H−C(3)], 3.58 [t, 2H, *J* = 6.6 Hz, H−C(9)], 4.17 [s, 2H, H−C(4)], 4.49 [m, 1H, *J* = 5.5 Hz, H−C(2)]; diastereomer **6b**, δ 1.45−1.70 [m, 2H, H−C(8)], 1.79−1.98 [m, 2H, H−C(7)], 3.18 [s, 2H, H−C(3)], 3.56 [t, 2H, *J* = 6.6 Hz, H−C(9)], 4.18 [s, 2H, H−C(4)], 4.46 [m, 1H, *J* = 5.9 Hz, H−C(2)]; ^{13}C NMR (125 MHz, D_2O, HMQC, HMBC, ^{13}C) diastereomer **6a**, δ 27.27 [C(8)], 28.04 [C(7)], 38.52 [C(3)], 55.07 [C(4)], 61.02 [C(2)], 61.97 [C(4)], 164.23 [C(5)], 172.74 [C(6)], 178.41 [C(1)]; diastereomer **6b**, δ 27.11 [C(8)], 28.04 [C(7)], 39.73 [C(3)], 53.95 [C(4)], 60.99 [C(2)], 61.95 [C(9)], 163.95 [C(5)], 172.32 [C(6)], 175.07 [C(1)].

*N-(1-Methyl-4-oxoimidazolidin-2-ylidene)amino-4,5,6-trihydroxyhexanoic lactone, **7a/b** (Figure 1):* UV−vis (MeOH), λ_{max} 204.0 nm; LC-MS (ESI$^+$), *m/z* 258 (100, [M + H]$^+$); LC-TOF-MS, *m/z* 258.1084 ([M + H]$^+$ measured), 258.1084 ([M + H]$^+$ calcd for $C_{10}H_{16}N_3O_5^+$); ^{1}H NMR (500 MHz, D_2O; COSY) diastereomer **7a**, δ 2.29 [m, 2H, H−C(7)], 3.17 [s, 3H, H−C(3)], 3.51 [m, 2H, H−C(9), H−C(10_a)], 3.70 [m, 2H, H−C(8), H−C(10_b)], 4.10 [s, 2H, H−C(4)], 4.56 [dd, 1H, *J* = 6.6 Hz, H−C(2)]; diastereomer **7b**, δ 2.09 [m, 2H, H−C(7)], 3.16 [s, 3H, H−C(3)], 3.51 [m, 2H, H−C(9), H−C(10_a)], 3.70 [m, 2H, H−C(8), H−C(10_b)], 4.09 [s, 2H, H−C(4)], 4.20 [dd, 1H, *J* = 6.3 Hz, H−C(2)]; ^{13}C NMR (125 MHz, D_2O, HMQC, HMBC) diastereomer **7a**, δ 32.67 [C(7)], 38.51 [C(3)], 54.57 [C(4)], 57.97 [C(2)], 62.50 [C(10)], 67.19 [C(8)], 74.58 [C(9)], 157.79 [C(5)], 172.30 [C(6)], 172.43 [C(6)], 177.97 [C(1)]; diastereomer **7b**, δ 32.61 [C(7)], 37,23 [C(3)], 54.46 [C(4)], 57.43 [C(2)], 62.37 [C(10)], 67.01 [C(8)], 74.55 [C(9)], 157.44 [C(5)], 172.30 [C(6)], 172.43 [C(6)], 177.80 [C(1)].

*N-(1-Methyl-4-oxoimidazolidin-2-ylidene)amino-4,5,6-trihydroxyhexanoic acid, **8a/b** (Figure 1):* UV−vis (MeOH), λ_{max} 204.0 nm; LC-MS (ESI$^+$), *m/z* 276 (100, [M + H]$^+$); LC-TOF-MS, *m/z* 276.1196 ([M + H]$^+$ measured), 276.1190 ([M + H]$^+$ calcd for $C_{10}H_{18}N_3O_6^+$); LC-MS and 1D/2D NMR data were well in agreement with those reported recently.[20]

Identification of *N*-(1-Methyl-4-oxoimidazolidin-2-ylidene)aminopropionic acid (1a/b) and *N*-(1-Methyl-4-oxoimidazolidin-2-ylidene)aminoacetic acid (9) in Creatinine/Dicarbonyl Reaction Systems. A binary mixture of creatinine (4 mmol) and glyoxal (6 mmol) or methylglyoxal (6 mmol), respectively, was heated in water (40 mL) at pH 7.0 for 24 h at 70 °C in a closed vessel. After cooling to room temperature, the solution was freeze-dried, and the lyophilisate was taken up in acetonitrile/water (80:20, v/v; 5 mL) and fractionated by means of semipreparative HILIC on a 300 × 21.5 mm i.d., 10 μm, TSKgel Amide-80 column (Tosoh Bioscience) equipped with a 75 × 21.5 mm i.d., 10 μm, guard column (Tosoh Bioscience). The major reaction products formed from creatinine/glyoxal and creatinine/methylglyoxal were isolated, lyophilized, and then identified as *N*-(1-methyl-4-oxoimidazolidin-2-ylidene)aminoacetic acid, **9** (Figure 1), and *N*-(1-methyl-4-oxoimidazolidin-2-ylidene)aminopropionic acid (**1a**/**b**), respectively, by means of UV−vis, LC-TOF-MS, LC-MS/MS, and NMR experiments. Spectroscopic data and retention time (HILIC) were well in agreement with those reported recently.[20]

High-Performance Liquid Chromatography (HPLC). Analytical chromatography was performed on an HPLC apparatus (Jasco, Gross-Umstadt, Germany) equipped with a type PU-2087 PLUS HPLC-pump, an AS-2055 PLUS type autoinjector unit, a DAD MD2010 PLUS type detector, and a Sedex 85 type evaporative light scattering detector (LT-ELSD, Sedere S.A., Alfortville Cedex, France), which was operated at 40 °C with air as operating gas (3.5 bar). Data acquisition was done by means of Chrompass software. Analytical separations were performed on a 300 × 7.8 mm i.d., 5 μm, HILIC column (TSKgel Amide-80, Tosoh Bioscience) operated at a flow rate of 1.0 mL/min.

For preparative fractionation, the HPLC system consisted of S 1122 type pumps (Sykam, Eresing, Germany), an Rh 7125i type Rheodyne injection valve (Bensheim, Germany), an ERC-3215α type solvent degasser (ERC, Riemerling, Germany), a gradient mixer (Sunchrom, Friedrichsdorf, Germany), a splitter (Upchurch, Oak Harbor, WA), a spectraflow 600 type DAD detector (Sunchrom), and a PrepELS type ELSD detector (Gilson Int. Germany, Limburg-Offheim, Germany). The split ratio was set to a flow of 1 mL/min for the ELSD detector. Data acquisition was performed by means of ChromStar v. 6.2 software. The chromatographic separation was performed on a 300 × 21.5 mm i.d., 10 μm, TSKgel Amide-80 column (Tosoh Bioscience).

Liquid Chromatography−Mass Spectrometry (LC-MS/MS). Mass spectrometric analysis was performed in electrospray ionization (ESI) mode on an API 4000 Q-Trap LC-MS/MS system (AB Sciex Instruments, Darmstadt, Germany) connected to an Agilent 1100 series HPLC system (Agilent, Waldbronn, Germany). The ion spray voltage was set at −4500 V in the ESI$^-$ mode and at +5500 V in the ESI$^+$ mode. Nitrogen served as the curtain gas (20 psi), and the collision energy as well as the declustering potential was adjusted depending on the single substance. The mass spectrometer was operated in the full scan mode monitoring positive ions

and negative ions, respectively. Fragmentation of $[M + H]^+$ and $[M - H]^-$, respectively, molecular ions into specific product ions was tuned by flow injection (10 μL/min) of the purified reference compounds and induced by collision with nitrogen (4×10^{-5} Torr). The determination was done using the MRM mode. Analysis of mass spectrometry data was performed with Sciex Analyst software v. 1.4.2.

Liquid Chromatography−Time of Flight Mass Spectrometry (LC-TOF-MS). High-resolution mass spectra of the target compounds were measured on a Bruker Micro-TOF (Bruker Daltonics, Bremen, Germany) mass spectrometer with flow injection referenced to sodium formate. The compounds were dissolved in MeOH, and 10 μL of a saturated solution of sodium formate in MeOH was added to measure the exact mass of the sodium adducts. Data processing was performed by using Daltonics Data Analysis software (v. 3.4, Bruker Daltonics)

Nuclear Magnetic Resonance Spectroscopy (NMR). The ^1H, ^{13}C, and 2D NMR data were acquired on a Bruker DRX-400 equipped with a QNP probehead or on an AVANCE-III-500 equipped with a Cryo-CTCI-Probe, spectrometer (Bruker BioSpin, Rheinstetten, Germany). D_2O was used as solvent and 20 μL of MeOD (3.31/49.05 ppm) as internal standard for peak referencing. For structural elucidation and NMR signal assignment, COSY, HSQC, and HMBC as well as ^{13}C spectroscopic experiments were carried out using the pulse sequences taken from the Bruker software library. Data acquisition was performed by using Topspin software (v. 2.1; Bruker BioSpin), data processing was performed by using Mestre-Nova software (v. 5.1.0−2940; Mestrelab Research, Santiago de Compostella, Spain).

RESULTS AND DISCUSSION

Very recently, *N*-(1-methyl-4-oxoimidazolidin-2-ylidene)aminopropionic acid (**1a**/**b**) was discovered as a key taste modulator in stewed beef and demonstrated to be generated upon Maillard-type reaction of creatinine and glucose.[20] As the generation of a series of structural homologues of **1a**/**b** was to be expected from the Maillard reaction of creatinine, the following experiments were targeted toward the discovery of the entire series of putatively taste modulating *N*-(1-methyl-4-oxoimidazolidin-2-ylidene)-α-amino acids from pentoses and hexoses, respectively.

Identification of *N*-(1-Methyl-4-oxoimidazolidin-2-ylidene)-α-amino Acids from Creatinine and Pentoses. To extend the portfolio of putative taste modulating Maillard reaction products of creatinine, an equimolar mixture of creatinine and D-(−)-ribose was thermally treated in aqueous phosphate buffer (pH 7.0) for 2 h at 120 °C. HILIC-UV−vis analysis (220 nm) of the Maillard reaction mixture demonstrated that a remarkable number of different reaction products were formed from creatinine (Figure 2A). To rank these compounds in their taste modulating activity, the effluent was collected to give eight individual fractions (R-I−R-VIII), which were separated from solvent and taken up in their "natural" concentration ratios in water (pH 5.9) to perform a TDA and in a savory tasting model broth solution (pH 5.9) to perform a cTDA. The TDA did not show any significant taste impact for any of the HILIC fractions (data not shown), thus demonstrating that these compounds do not exhibit any intrinsic taste activity. In comparison, the cTDA revealed taste modulating activity for HILIC fractions R-II−R-VI with TD factors between 8 and 32 (Figure 2B). The panelists observed an increased thick−sour sensation and enhanced mouthfulness of these fractions, whereas the solutions containing fractions R-I, R-VII, and R-VIII did not differ from the model broth solution (control).

To identify the Maillard reaction products imparting the taste modulating activity, HILIC fractions R-II−R-VI were separately collected in a preparative scale and, after final purification by rechromatography, the target compounds were identified by means of LC-MS/MS, LC-TOF-MS, and 1D/2D NMR spectroscopic experiments.

LC-MS and LC-TOF-MS analysis of fraction R-III, judged with a high TD factor of 32 (Figure 2B), revealed the pseudomolecular ion m/z 186 ($[M + H]^+$) with an elemental composition of $[C_7H_{12}N_3O_3]^+$. Comparing the LC-MS and NMR data with those reported recently,[20] followed by cochromatography with the corresponding reference compound, led to the identification of the diastereomeric *N*-(1-methyl-4-oxoimidazolidin-2-ylidene)aminopropionic acids **1a** and **1b** (Figure 1), exhibiting a chiral carbon atom C(2) in the alanine moiety as well as a chiral tertiary nitrogen atom N−C(3)/C(4)/C(6) with its free electron pair as part of the creatinine moiety.

Rechromatography of fraction R-IV (Figure 2B) revealed the diastereomeric reaction products **2a**/**b**, which were found by LC-MS and LC-TOF-MS to exhibit the pseudomolecular ion m/z 216 with the sum formula of $[C_8H_{14}N_3O_4]^+$. Comparison of the ^1H NMR data of **2a**/**b** to those found for **1a**/**b** revealed strong similarities with the characteristic singlets of the methyl protons H−C(3) and the methylene protons H−C(4) of the creatinine moiety. Heteronuclear multiple bond correlation (HMBC) spectroscopy showed a heteronuclear J_3 connectivity between C(6) and H−C(2), thus indicating the linkage between the creatinine fragment and the α-amino acid moiety in **2a**/**b**. Moreover, a DQF-COSY experiment revealed homonuclear J_3 couplings between H−C(2) and H−C(7) and between H−C(7) and H−C(8), thus leading to the identification of **2a**/**b** as a diastereomeric mixture of *N*-(1-methyl-4-oxoimidazolidin-2-ylidene)amino-4-hydroxybutanoic acid (Figure 1). To the very best of our knowledge, compounds **2a**/**b** have not been previously reported in the literature.

LC-MS and LC-TOF-MS analysis of compounds **5a**/**b**, isolated from HILIC fraction R-II (Figure 2B), indicated an additional methyl group when compared to **1a**/**b** and the lack of a hydroxyl group when compared to **2a**/**b**. This was further confirmed by the NMR data, showing an additional triplet at 0.88/0.91 ppm integrating for three protons and exhibiting J_2 couplings to H−C(7). Careful assignment of all spectroscopic data led to the identification of compound **5a**/**b** as the previously unreported *N*-(1-methyl-4-oxoimidazolidin-2-ylidene)aminobutanoic acid (Figure 1).

LC-MS and NMR analysis of the compound isolated from fraction R-V (Figure 2B) revealed a molecular weight of 227 Da and the presence of a diastereomeric mixture of two molecules, both exhibiting 11 carbon-bound protons and 9 carbon atoms. Careful assignment of all homo- and heteronuclear connectivities led to the discovery of a 3-amino-5-hydroxypyran-2-one substructure with a characteristic hetereonuclear HMBC coupling between C(1) and H−C(9). With all spectroscopic data taken into consideration, the structure of the diastereomers could be unequivocally identified as the previously unreported *N*-(1-methyl-4-oxoimidazolidin-2-ylidene)amino-4-dihydroxypentanoic lactone, **3a**/**b** (Figure 1).

LC-MS and LC-TOF-MS analysis of the compound isolated from HILIC fraction R-VI (Figure 2B) indicated a molecular weight of 245 Da and an elemental composition of $C_9H_{15}N_3O_5$. The mass deficiency of 18 amu as well as the similarity of the ^1H NMR data compared to the lactone **3a**/**b** (Figure 3) suggested

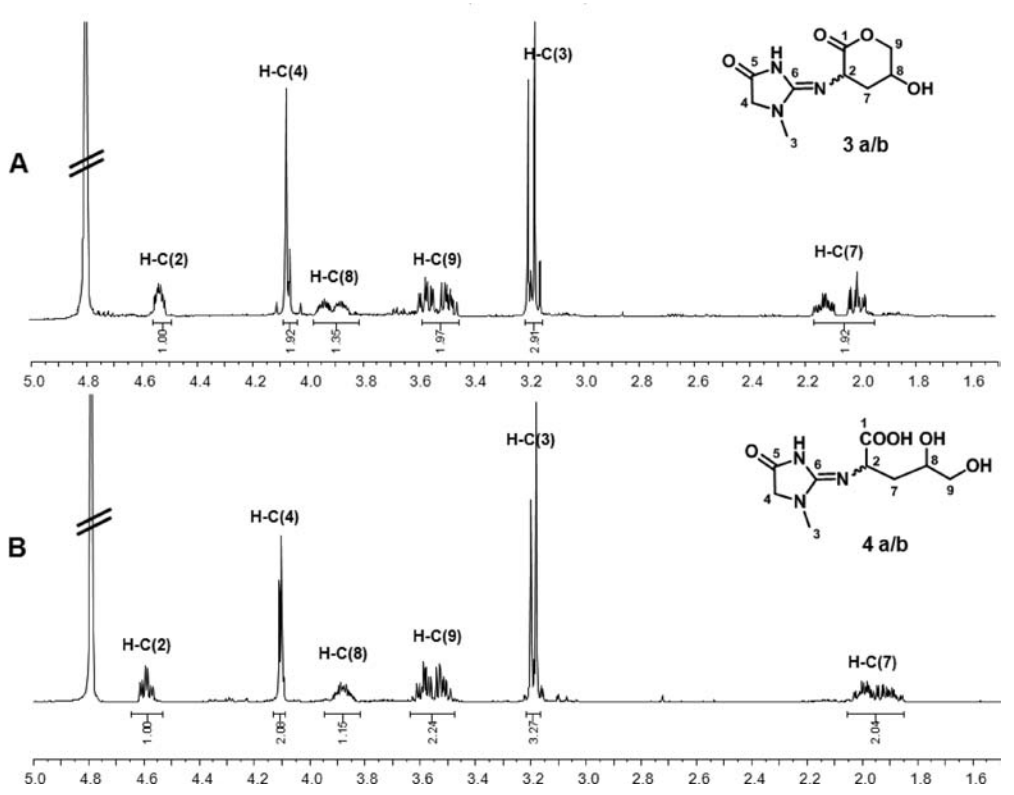

Figure 3. [1]H NMR spectra of (A) *N*-(1-methyl-4-oxoimidazolidin-2-ylidene)amino-4-dihydroxypentanoic lactone (**3a**/**b**) and (B) *N*-(1-methyl-4-oxoimidazolidin-2-ylidene)amino-4,5-dihydroxypentanoic acid (**4a**/**b**).

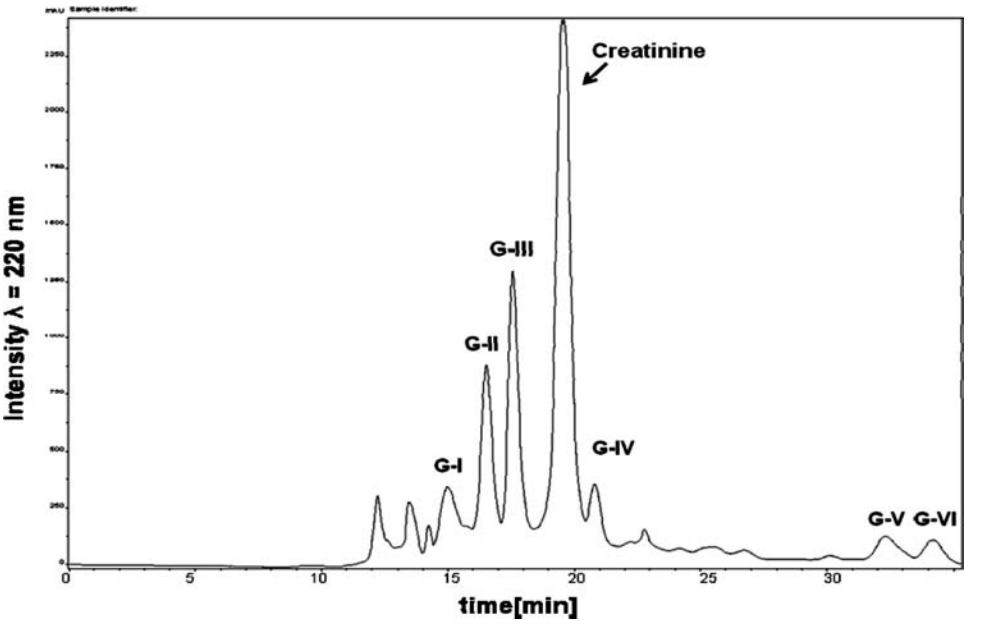

Figure 4. HILIC chromatogram of a thermally treated mixture of creatinine and glucose.

the presence of the open-chain hydroxy acid form of **3a**/**b**. This was confirmed by the observed generation of the target compounds **4a**/**b** upon alkaline treatment (0.05 mmol/L NaOH, 30 min at room temperature) of **3a**/**b**, thus demonstrating *N*-(1-methyl-4-oxoimidazolidin-2-ylidene)amino-4,5-dihydroxypentanoic acid, **4a**/**b** (Figure 1), as the previously unreported structure of the creatinine glycation products isolated form fraction R-VI.

Identification of *N*-(1-Methyl-4-oxoimidazolidin-2-ylidene)-α-amino Acids from Creatinine and Hexoses. To screen for additional hexose-specific glycation products of creatinine, an equimolar mixture of creatinine and glucose was thermally

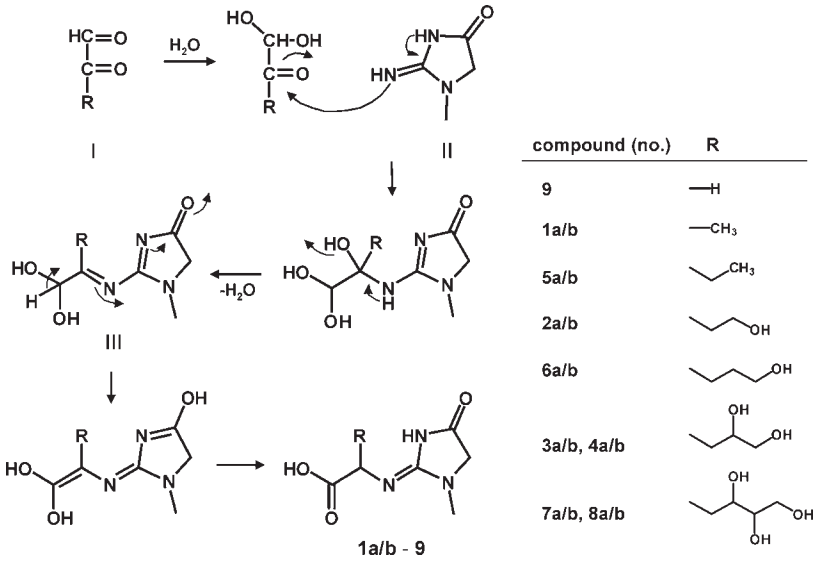

Figure 5. Hypothesized reaction mechanism leading to the formation of *N*-(1-methyl-4-oxoimidazolidin-2-ylidene)-α-amino acids **1a**/**b**−**9** from creatinine and 2-oxoaldehydes.

treated in aqueous phosphate buffer (pH 7.0) for 2 h at 120 °C and, after sample workup, the reaction mixture was screened for creatinine glycation products by means of HILIC-UV−vis (λ = 220 nm), LC-MS, and LC-TOF-MS, respectively (Figure 4). Comparison of spectroscopic data, followed by cochromatography with reference compounds isolated from the creatinine/ribose reaction system, led to the straightforward identification of the reaction products **1a**/**b**, **2a**/**b**, and **5a**/**b** in HILIC fractions G-II, G-III, and G-I, besides three additional creatinine glycation products detected in HILIC fractions G-IV, G-V, and G-VI, respectively (Figure 4). The effluents of the latter three fractions were individually collected, and the target compounds were purified by rechromatography and then analyzed by means of LC-MS, LC-TOF-MS, and 1D/2D NMR experiments.

LC-MS and LC-TOF-MS analysis of the compound **6a**/**b** isolated from HILIC fraction G-IV (Figure 4) revealed a molecular weight of 229 Da and an elemental composition of $C_9H_{15}N_3O_4$. The NMR data were rather similar to those of **2a**/**b**, but showed an additional methylene group, which is well in line with the mass difference of 14 amu when compared to **2a**/**b**. Assignment of all spectroscopic data led to the identification of *N*-(1-methyl-4-oxoimidazolidin-2-ylidene)amino-5-hydroxypentanoic acid, **6a**/**b** (Figure 1), which to the best of our knowledge has not been reported earlier in the literature.

After isolation of compound **7a**/**b** from HILIC fraction G-V (Figure 4), LC-MS analysis indicated *m*/*z* 259 as the pseudomolecular ion. The ^{1}H NMR spectrum showed strong similarity to that of the lactone **3a**/**b** isolated from the creatinine/ribose reaction, but the mass difference of 30 amu indicated an additional hydroxymethylene group in **7a**/**b**, thus indicating the corresponding hexose-derived lactone. This was further strengthened by an HMBC experiment demonstrating a hetereonuclear coupling between C(1) and H−C(9) within the pyran-2-one ring of **7a**/**b**. On the basis of these considerations, compound **7a**/**b** was identified as *N*-(1-methyl-4-oxoimidazolidin-2-ylidene)amino-4,5-dihydroxyhexanoic lactone (Figure 1), which to the best of our knowledge has not been previously identified.

Comparison of the LC-MS, LC-TOF-MS, and NMR data of the compound isolated from HILIC fraction G-VI (Figure 4) with those of the reference compound, followed by cochromatography, revealed the previously reported *N*-(1-methyl-4-oxoimidazolidin-2-ylidene)amino-4,5,6-trihydroxyhexanoic acid, **8a**/**b** (Figure 1), as another reaction product.

Generation of *N*-(1-Methyl-4-oxoimidazolidin-2-ylidene)-α-amino Acids from Creatinine and 2-Oxoaldehydes. To check whether the *N*-(1-methyl-4-oxoimidazolidin-2-ylidene) amino acid **1a**/**b** is also formed from creatinine and methylglyoxal, known as a transient degradation product of reducing carbohydrates,[24−26] a creatinine/methylglyoxal mixture was thermally treated and, then, analyzed by HILIC-UV−vis for the presence of **1a**/**b**. By comparison of the spectroscopic data and retention time with those of the reference compound, **1a**/**b** was identified as the major reaction product.

To answer the question as to whether the corresponding glycine derivative is formed instead of **1a**/**b**, exhibiting an alanine moiety, when methylglyoxal is substituted by glyoxal, a creatinine/glyoxal mixture was thermally reacted. HILIC-UV−vis analysis revealed a quantitatively predominant reaction product, which was isolated and purified by rechromatography. LC-MS, LC-TOF-MS, and 1D/2D NMR experiments led to the identification of that compound as *N*-(1-methyl-4-oxoimidazolidin-2-ylidene)aminoacetic acid, **9** (Figure 1).

On the basis of these findings, the reaction pathway leading to the formation of the *N*-(1-methyl-4-oxoimidazolidin-2-ylidene)-α-amino acids **1a**/**b** and **9** by the Maillard reaction of methylglyoxal and glyoxal, respectively, is proposed in Figure 5. The hydrate of the dicarbonyl (**I**) is attacked by the exocyclic nitrogen atom of creatinine (**II**) to give an imine intermediate (**III**), which upon enolization gives rise to the *N*-(1-methyl-4-oxoimidazolidin-2-ylidene)-α-amino acids. As demonstrated for the generation of **1a**/**b** and **9** from methylglyoxal and glyoxal, respectively, the same reaction route is expected to lead to the other *N*-(1-methyl-4-oxoimidazolidin-2-ylidene)-α-amino acids upon reaction of creatinine with the various 2-oxoaldehydes reported as carbohydrate breakdown products,[24−26] for example, **5a**/**b** from 2-oxobutanal, **2a**/**b** from 3-deoxytetrulose, **6a**/**b** from 5-hydroxy-2-oxopentanal, **3a**/**b** and **4a**/**b** from 3-deoxypentosulose, and **7a**/**b** and **8a**/**b** from 3-deoxyhexosulose, respectively.

Table 1. Taste Recognition Threshold Concentrations of *N*-(1-Methyl-4-oxoimidazolidin-2-ylidene)-α-amino Acids

	threshold concentration[a] (μmol/L) in	
compound no.[b]	water (pH 5.9)	model broth solution (pH 5.9)
1a/b	>1000	209 (±83)
2a/b	>1000	76 (±29)
3a/b	>1000	489 (±105)
4a/b	>1000	159 (±83)
5a/b	>1000	129 (±55)
6a/b	>1000	105 (±43)
7a/b	>1000	>1000
8a/b	>1000	278 (±80)
9	>1000	31 (±11)
creatinine	17.5 ± 7.5 (bitter)	nd[c]

[a] Taste threshold concentrations were determined as the mean of triplicates. [b] Compound numbering refers to Figure 1. [c] nd, not detectable.

Recognition Threshold Concentrations of *N*-(1-Methyl-4-oxoimidazolidin-2-ylidene)-α-amino Acids. Prior to sensory analysis, the purity of each compound was checked by ^{1}H NMR spectroscopy as well as HPLC-MS. Recognition thresholds of creatinine and its glycated derivatives **1a/b**−**9** were determined in water (for intrinsic taste) as well as in model broth solution (for taste modulation) using a triangle test with ascending concentrations of the stimulus, as reported in previous papers.[1,4,20] Whereas creatinine exhibited a bitter taste with a recognition threshold of 17.5 mmol/L (in water), none of the compounds **1a/b**−**9** imparted any intrinsic taste activity up to levels of 10 mmol/L in water (Table 1). In comparison, the sensory experiments performed in model broth solution (pH 5.9) demonstrated that the glycated creatinine derivatives **1a/b**−**9** induced a thick−sour and mouthdrying orosensation with recognition thresholds ranging from 31 (**9**) to >1000 μmol/L (**7**), whereas creatinine did not show any similar taste modulatory activity (Table 1). Interestingly, the recognition thresholds found for the lactones **3a/b** (489 μmol/L) and **7a/b** (>1000 μmol/L) were significantly above those found for the corresponding free hydroxy acid derivatives **4a/b** (159 μmol/L) and **8a/b** (278 μmol/L), respectively.

Besides **1a/b**, **8a/b**, and **9**, six additional previously unreported *N*-(1-methyl-4-oxoimidazolidin-2-ylidene)-α-amino acids (**2a/b**−**7a/b**) were found to be generated by Maillard-type glycation of creatinine, for example, **9** from the reaction with glyoxal, **1a/b** from the reaction with methylglyoxal, **1a/b**−**5a/b**, and **9** from the reaction with ribose, and **1a/b**, **2a/b**, and **5a/b**−**9** from the reaction with glucose, respectively. Although tasteless in water, all of these compounds were found to induce a thick−sour mouthdryness and mouthfulness to a model broth solution with recognition thresholds strongly depending on the chemical structure. The development of a stable isotope dilution analysis for the accurate quantitative analysis of this novel group of taste modulators in meat products is currently in progress and will be reported elsewhere.

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