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Sensory-Guided Identification of *N*-(1-Methyl-4-oxoimidazolidin-2-ylidene)-α-amino Acids as Contributors to the Thick-Sour and Mouth-Drying Orosensation of Stewed Beef Juice

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Sensory-guided fractionation of stewed beef juice using ultrafiltration, gel permeation chromatography, PFPP-HPLC, and HILIC combined with analytical sensory techniques led to the identification of the dipeptides β-alanyl-*N*-methyl-L-histidine and β-alanyl-L-histidine, as well as the creatinine derivatives *N*-(1-methyl-4-oxoimidazolidin-2-ylidene)aminopropionic acid, *N*-(1-methyl-4-oxoimidazolidin-2-ylidene)aminoacetic acid, and *N*-(1-methyl-4-oxoimidazolidin-2-ylidene)amino-4,5,6-trihydroxyhexanoic acid as taste modulators in stewed beef juice. Model experiments demonstrated for the first time that the latter three *N*-(1-methyl-4-oxoimidazolidin-2-ylidene)-α-amino acids are formed by Maillard-type reactions from creatinine and reducing hexoses. Quantitative analysis, followed by taste recombination and omission experiments, revealed that subthreshold concentrations of these taste modulators enhance the typical thick-sour and mouth-drying orosensation and the mouthfulness imparted by stewed beef juice, although none of these compounds exhibited any significant intrinsic taste when tasted individually in water.

KEYWORDS: Beef juice; taste enhancer; taste modulator; anserine; carnosine; creatinine; Maillard reaction

INTRODUCTION

Due to its desirable umami taste as well as its thick-sour orosensation, stewed beef and its juice are highly appreciated as sapid ingredients in savory dishes, processed food compositions, and convenience products, respectively. Although several hundreds of volatiles have been identified in beef products, combining gas chromatographic separation and human evaluation of the volatile components by means of aroma extract dilution techniques, followed by quantitative analysis and aroma reconstitution experiments, demonstrated that only rather limited numbers of 12 and 16 compounds are enough to blueprint the characteristic aroma signature of beef bouillon and stewed beef juice, respectively (*1*−*4*).

Several studies have been performed in the past to identify the nonvolatile key players evoking the typical taste profile of beef products such as beef bouillon (*3*), stewed beef juice (*4*), and beef broth (*5*, *6*). Whereas most studies addressed primarily the basic taste compounds such as amino acids, organic acids, sugars, nucleotides, and minerals in meat products, the knowledge of taste modulators, which do not show intrinsic taste on their own but do significantly enhance one or the other taste quality, is rather scarce.

For example, *N*-(1-methyl-4-hydroxy-3-imidazolin-2,2-ylidene)alanine, **1** (**Figure 1**), was identified in beef broth (*5*, *6*), but any sensory analyses and profound taste recombination experiments to unequivocally confirm the proposed brothy and thick-sour taste enhancement induced by this molecule are still lacking. By application of the comparative taste dilution analysis (cTDA), the inner salt of (*S*)-*N*-(1-carboxyethyl)-6-(hydroxymethyl)pyridinium-3-ol, coined (*S*)-alapyridaine, **2** (**Figure 1**), was discovered in beef broth as a tasteless, but umami- and sweet taste-enhancing, Maillard reaction product formed from hexoses and L-alanine (*7*, *8*). Although this taste enhancer, when applied in its natural concentration, was found to contribute to the typical taste signature of cooked meat products such as beef bouillon and beef broth, quantitative studies demonstrated that this compound is not formed during stewing of beef (data not published). More recently, sensory-directed fractionation led to the identification of β-alanyl-*N*-methyl-L-histidine (**3**), β-alanyl-L-histidine (**4**), and β-alanylglycine, **5** (**Figure 1**), as important taste modulators in chicken broth (*9*). Whereas the peptides **3** and **4** are long-known to enhance the thickness of foods (*10*), the taste modulatory activity of β-alanylglycine (**5**) was previously not reported. Quantitative analysis, followed by taste recombination and omission experiments, revealed for the first time that, when present together with L-glutamic acid, sodium, and/or potassium ions, subthreshold concentrations of these β-alanyl dipeptides enhance the typical thick-sour orosensation and white-meaty character known for poultry meat, although these taste-modulatory peptides exhibited only a faint sour and slightly astringent intrinsic taste when tasted individually (*9*).

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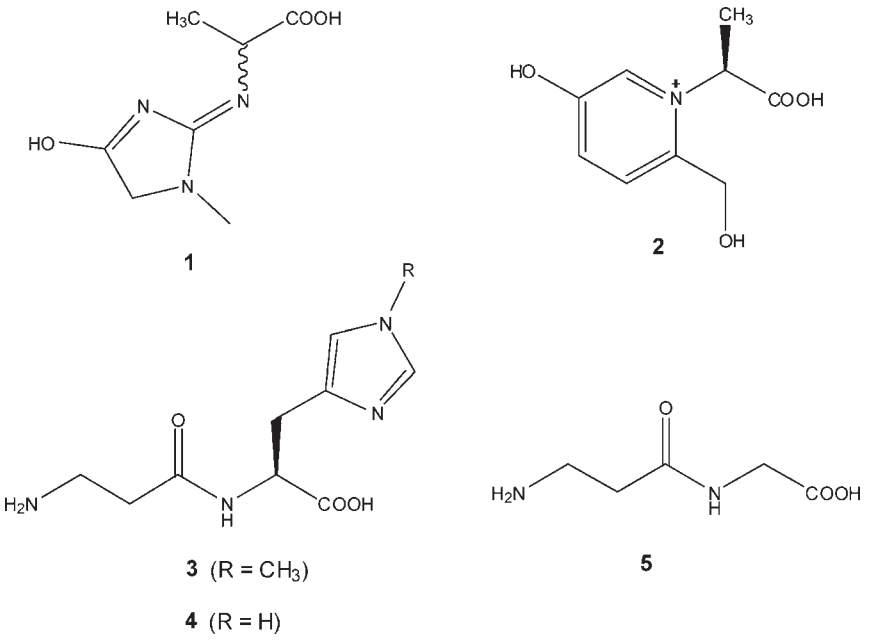

Figure 1. Chemical structures of the taste modulators *N*-(1-methyl-4-hydroxy-3-imidazolin-2,2-ylidene)alanine (**1**), reported in beef broth (*5*, *6*), (*S*)-alapyridaine (**2**), reported in beef bouillon (*7*), and β-alanyl-*N*-methyl-L-histidine (**3**), β-alanyl-L-histidine (**4**), and β-alanylglycine (**5**), reported in chicken broth (*9*).

As preliminary studies on taste recombinants indicate that the knowledge on taste modulators in stewed beef juice is still fragmentary, the objective of the present study was to identify the key molecules enhancing the thick-sour and mouth-drying orosensation induced by stewed beef juice by application of a sensory-directed fractionation approach using a basic taste recombinant as the matrix.

MATERIALS AND METHODS

Chemicals. Creatinine, D-glucose, formic acid, and disodium hydrogen phosphate dihydrate were purchased from Merck KGaA (Darmstadt, Germany), trifluoroacetic acid was obtained from Fluka (Neu-Ulm, Germany), and β-alanyl-*N*-methyl-L-histidine (**3**) and β-alanyl-L-histidine (**4**) were obtained from Bachem (Weil am Rhein, Germany) and were purified prior to use (*9*). The yeast extract (Gistex X-II LS) was obtained from FID (Werne, Germany); maltodextrin 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), maltodextrin (6.375 g), and sodium chloride (2.9 g) in bottled water.

Preparation of Stewed Beef Juice (SBJ). A beef shoulder (15 kg) from a young bull was cut into smaller pieces (400 g each). Following a common household preparation style, these beef meat pieces were roasted for 4 min in coconut oil (Palmin, 480 g) using a frying pan. After quenching with water (2560 g), the meat pieces were placed in stainless steel dishes, soused with the residual aqueous layer, and then heated for 120 min at 185 °C in a convectomate under steaming conditions while the meat pieces were turned every 30 min. After removal of the meat pieces, the aqueous layer obtained was cooled to 6 °C and the fat layer was removed by filtration to afford the SBJ (2.6 L), which was kept at −20 °C until use.

Solvent Extraction of SBJ. An aliquot (300 g) of the SBJ was defatted by extraction with *n*-pentane (3 × 150 mL) as reported recently (*9*). The combined organic layers were freed from solvent under vacuum to yield the solvent extractables (fraction B; < 0.1 g). The residual aqueous layer was lyophilized to give the aqueous fraction A (13.8 g), which was used for sensory evaluation and chemical analysis.

Ultrafiltration. *Tangential-Flow Ultrafiltration.* Following the protocol reported recently (*9*), an aliquot (10.0 g) of the lyophilized fraction A was dissolved in water (500 mL) and separated by means of tangential-flow ultrafiltration using a Vivaflow 200 filtration unit (Sartorius, Goettingen, Germany) and a 5 kDa cutoff membrane to give fraction A1 (MW < 5 kDa; 84.4% in yield) and fraction A2 (MW ≥ 5 kDa; 15.6% in yield) as amorphous powders, which were kept at −20 °C until use.

Stirred-Cell Ultrafiltration. As reported recently (*9*), an aliquot (2.0 g) of fraction A1 was dissolved in water (250 mL) and separated by means of an Amicon 8400-type ultrafiltration cell (Amicon, Witten, Germany) to give fraction A1-1 (MW < 1 kDa; 98.8% in yield) and fraction A1-2 (MW 1−5 kDa; 1.2% in yield), respectively.

Gel Permeation Chromatography (GPC). An aliquot (1.5 g) of fraction A1-1 was dissolved in water (10 mL) and, after adjustment of the pH value to 4.0 with aqueous formic acid (1% in water), was separated on a 100 × 5 cm Sephadex G-15 column (Amersham Bioscience, Uppsala, Sweden) using a flow rate of 2.3 mL/min (*9*). Seven GPC fractions I−VII (**Figure 2**) were collected, freeze-dried, and used for identification of taste-active compounds and taste modulators, respectively.

Subfractionation of GPC-Fractions III and IV. Following the detailed protocol reported recently (*9*), aliquots (0.5 mL) of an aqueous solution (1 g in 5 mL) of the GPC-fractions III and IV, respectively, were separated by means of preparative HPLC on a 250 × 21.2 mm i.d., 5 μm, Monochrom MS column (Varian, Darmstadt, Germany) to give nine or seven HPLC fractions, namely, fractions III-1−III-9 or fractions IV-1−IV-7, respectively. Each collected fraction was diluted with water (20 mL), freeze-dried, and kept at −20 °C until used for sensory analysis.

Identification of Taste-Modulating Compounds. HPLC fraction IV-7 (**Figure 3A**) was dissolved in acetonitrile/water (1:1, v/v; 5 mL) and, after membrane filtration, was fractionated by 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 of the same type (Tosoh Bioscience). Using a flow rate of 6 mL/min, chromatography was performed using aqueous trifluoroacetic acid (0.1% in water) as solvent A and acetonitrile containing 0.1% trifluoroacetic acid as solvent B. Starting with 35% solvent A and 65% solvent B for 5 min and increasing solvent A to 100% within 35 min, the effluent was separated into seven HILIC subfractions, namely, fractions IV-7/1−IV-7/7, which were

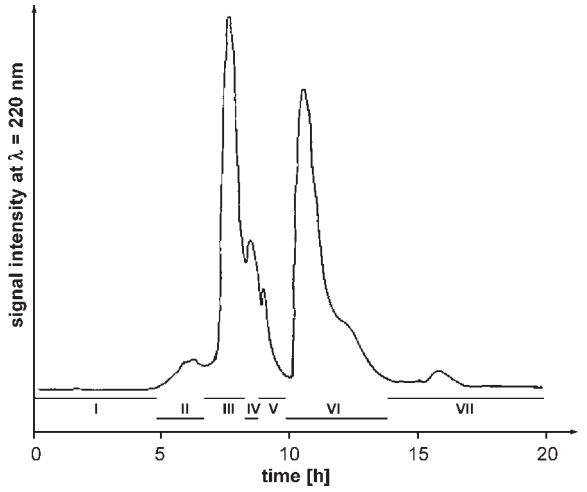

Figure 2. GPC chromatogram (λ = 220 nm) of the low molecular ultrafiltration fraction A1-1 isolated from stewed beef juice.

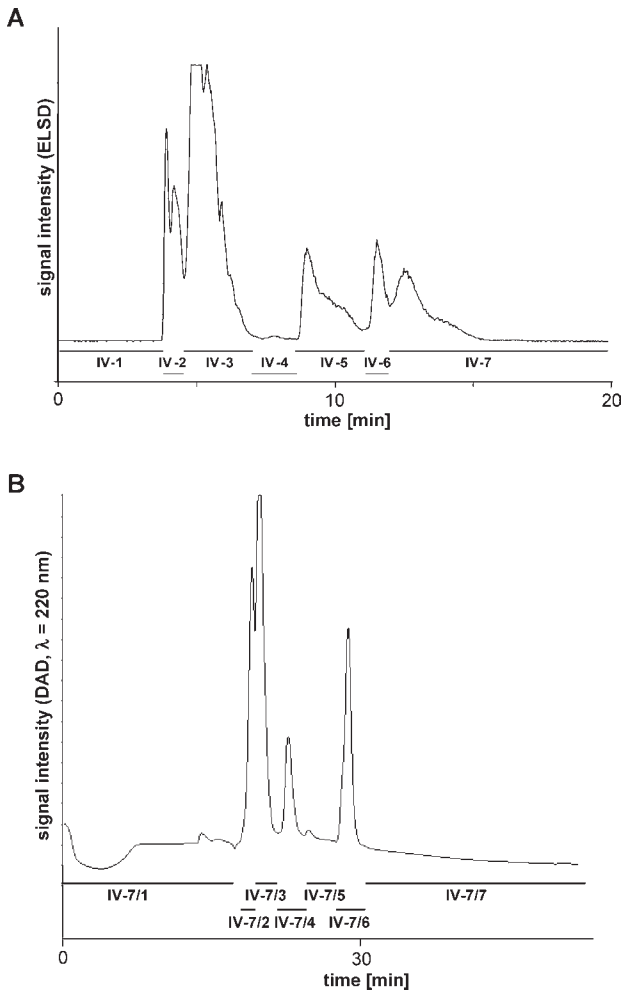

Figure 3. (**A**) PFPP-HPLC-ELSD chromatogram of GPC-fraction IV and (**B**) HILIC HPLC-DAD chromatogram of HPLC-fraction IV-7 isolated from beef juice.

collected separately, freed from solvent under vacuum, and lyophilized. Sensory evaluation of the individual HILIC fractions in model broth solution revealed that fraction IV-7/2 induced a mouth-drying and slightly umami-like taste impression and fraction IV-7/6 imparted a sour, slightly metallic, and salty orosensation. The compounds eluting in HILIC

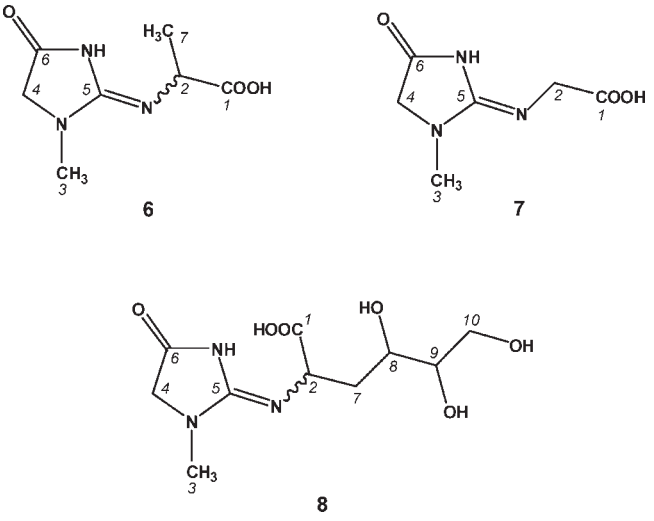

Figure 4. Chemical structures of Maillard-derived taste modulators *N*-(1-methyl-4-oxoimidazolidin-2-ylidene)aminopropionic acid (**6**), *N*-(1-methyl-4-oxoimidazolidin-2-ylidene)aminoacetic acid (**7**), and *N*-(1-methyl-4-oxoimidazolidin-2-ylidene)amino-4,5,6-trihydroxyhexanoic acid (**8**).

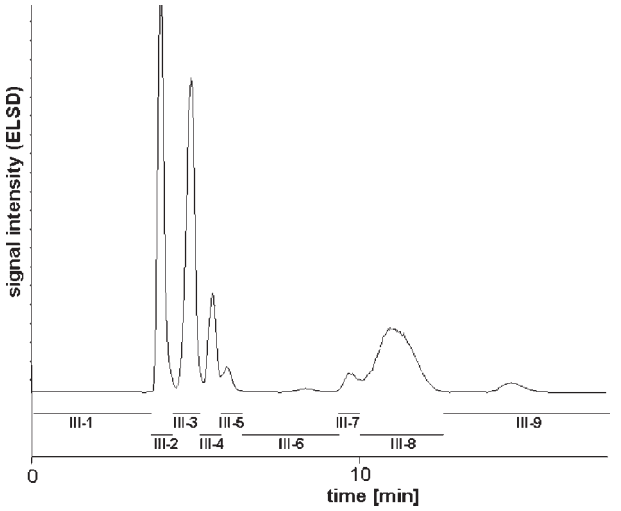

Figure 5. PFPP-HPLC-ELSD chromatogram of GPC-fraction III isolated from beef juice.

fractions IV-7/2 and IV-7/6 (**Figure 3B**) were collected, separated from solvent in vacuum, and freeze-dried. Sensory, LC-MS/MS, and NMR studies led to the identification of the taste modulators in fractions IV-7/2 and IV-7/6 as *N*-(1-methyl-4-oxoimidazolidin-2-ylidene)aminopropionic acid (**6**) and *N*-(1-methyl-4-oxoimidazolidin-2-ylidene)aminoacetic acid (**7**), respectively (**Figure 4**). Comparison of chromatographic (PFPP, HILIC) and spectroscopic data (UV−vis, LC-MS/MS, ^{1}H NMR) with those obtained for the synthetic reference compounds confirmed the identity of these compounds. Compound **6** occurred as a mixture of diastereomers due to the additional chiral center at position C(2).

The compounds eluting in HPLC-fractions III-5, III-7, III-8, and III-9 (**Figure 5**) and showing interesting taste modulatory activity were collected, separated from solvent under vacuum, and freeze-dried. LC-MS/MS and NMR experiments led to the identification of the taste modulators *N*-(1-methyl-4-oxoimidazolidin-2-ylidene)amino-4,5,6-trihydroxyhexanoic acid (**8**) in fraction III-5, β-alanyl-L-histidine (**4**) in fractions III-7 and III-8, and β-alanyl-3-methyl-L-histidine (**3**) accompanied with small amounts of *N*-(1-methyl-4-oxoimidazolidin-2-ylidene)aminopropionic acid (**6**) in fraction III-9. Both compounds **6** and **8** occurred as a mixture of diastereomers.

*N-(1-Methyl-4-oxoimidazolidin-2-ylidene)aminopropionic acid, **6**, **Figure 4**:* UV−vis (MeOH), λ_{max} = 204 nm; LC-TOF-MS, *m*/*z* 186.0878 ([M + H]$^+$,

measured), m/z 186.0873 ($[M + H]^+$, calcd for $C_7H_{12}N_3O_3^+$); LC-MS (ESI^+), m/z 186.1 (100, $[M + H]^+$).

N-(1-Methyl-4-oxoimidazolidin-2-ylidene)aminoacetic acid, 7, ***Figure 4****:* UV−vis (MeOH), λ_{max} = 204 nm; LC-TOF-MS, m/z 172.0728 ($[M + H]^+$, measured), m/z 172.0716 ($[M + H]^+$, calcd for $C_6H_{10}N_3O_3^+$); LC-MS (ESI^+), m/z 172.1 (100, $[M + H]^+$).

N-(1-Methyl-4-oxoimidazolidin-2-ylidene)amino-4,5,6-trihydroxyhexanoic acid, 8, ***Figure 4****:* UV−vis (MeOH), λ_{max} = 204 nm; LC-TOF-MS, m/z 276.1196 ($[M + H]^+$, measured), m/z 276.1190 ($[M + H]^+$, calcd for $C_{10}H_{18}N_3O_6^+$); LC-MS (ESI^+), m/z 276.1 (100, $[M + H]^+$).

The chromatographic (HILIC), spectroscopic (UV−vis, LC-MS/MS), and sensory data of the taste modulators **6**−**8** matched well with those obtained for *N*-(1-methyl-4-oxoimidazolidin-2-ylidene)aminopropionic acid (**6**), *N*-(1-methyl-4-oxoimidazolidin-2-ylidene)aminoacetic acid (**7**), and *N*-(1-methyl-4-oxoimidazolidin-2-ylidene)amino-4,5,6-trihydroxyhexanoic acid (**8**) prepared from creatinine and glucose.

Synthetic Preparation of *N*-(1-Methyl-4-oxoimidazolidin-2-ylidene)-α-amino Acids (6−8) by a Maillard-Type Reaction. A mixture of D-glucose (20 mmol) and creatinine (2 mmol) in aqueous Na_2HPO_4 buffer (2.5 mL; 1.0 mol/L, pH 7.0) was heated for 4 h at 100 °C in a closed vessel. After cooling to room temperature, the reaction mixture was dissolved in water (50 mL) using an ultrasonic bath and, after membrane filtration (0.45 μm), aliquots (0.5 mL) were separated by means of preparative HPLC on a 250 × 21.2 mm i.d., 5 μm, Monochrom MS column (Varian, Darmstadt, Germany) equipped with a 50 × 21.2 mm i.d., 5 μm, guard column (Varian). Monitoring the effluent at λ = 220 nm, chromatography was performed at a flow rate of 18 mL/min using isocratic conditions with aqueous trifluoroacetic acid (0.1% in water) as solvent. Over a run time of 20 min, the effluent of one main HPLC fraction was collected. The main fraction were removed from solvent, freeze-dried, dissolved in a mixture (80:20, v/v; 5 mL) of acetonitrile and water and, then, fractionated by 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). Using a flow rate of 6 mL/min, isocratic chromatography was performed using aqueous trifluoroacetic acid (0.1% in water) as solvent A and acetonitrile with 0.1% trifluoroacetic acid in a ratio of 20:80 for 40 min. The major peaks were collected, separated from solvent in vacuum, freeze-dried, and analyzed by means of UV−vis, LC-MS/MS, LC-TOF-MS, and NMR spectroscopy. The chromatographic (HILIC) and spectroscopic data (UV−vis, LC-MS/MS, ^{1}H NMR) of the synthesized compounds matched with those obtained for *N*-(1-methyl-4-oxoimidazolidin-2-ylidene)aminopropionic acid (**6**), *N*-(1-methyl-4-oxoimidazolidin-2-ylidene)aminoacetic acid (**7**), and *N*-(1-methyl-4-oxoimidazolidin-2-ylidene)amino-4,5,6-trihydroxyhexanoic acid (**8**) isolated from beef juice.

N-(1-Methyl-4-oxoimidazolidin-2-ylidene)aminopropionic acid, 6, ***Figure 4****:* UV−vis (MeOH), λ_{max} = 204.0 nm; LC-TOF-MS, m/z 186.0878 ($[M + H]^+$, measured), m/z 186.0873 ($[M + H]^+$, calcd for $C_7H_{12}N_3O_3^+$); LC-MS (ESI^+), m/z 186.1 (100, $[M + H]^+$); ^{1}H NMR (500 MHz, D_2O, COSY), diastereomer A, δ 1.42 [d, 3H, J = 7.2 Hz, H−C(7)], 3.13 [s, 3H, H−C(3)], 4.18 [s, 2H, H−C(4)], 4.43 [q, 1H, J = 7.2 Hz, H−C(2)]; diastereomer B, δ 1.39 [d, 3H, J = 7.2 H−C(7)], 3.16 [s, 3H, H−C(3)], 4.16 [s, 2H, H−C(4)], 4.40 [q, 1H, J = 7.2 Hz, H−C(2)]; ^{13}C NMR (125 MHz, D_2O, HMQC, HMBC), diastereomer A, δ 15.44 [C(7)], 37.34 [C(3)], 53.64 [C(4)], 56.03 [C(2)], 157.29 [C(5)], 171.47 [C(6)], 177.97 [C(1)]; diastereomer B, δ 15.48 [C(7)], 38.49 [C(3)], 52.53 [C(4)], 56.01 [C(2)], 157.4 [C(5)], 171.02 [C(6)], 177.81 [C(1)].

N-(1-Methyl-4-oxoimidazolidin-2-ylidene)aminoacetic acid, 7, ***Figure 4****:* UV−vis (MeOH), λ_{max} = 204 nm; LC-TOF-MS, m/z 172.0728 ($[M + H]^+$, measured), m/z 172.0716 ($[M + H]^+$, calcd for $C_6H_{10}N_3O_3^+$); LC-MS (ESI^+), m/z 172.1 (100, $[M + H]^+$); ^{1}H NMR (400 MHz, D_2O; COSY), δ 3.11 [s, 3H, H−C(3)], 4.24 [s, 2H, H−C(4)], 4.30 [s, 2H, H−C(2)]; ^{13}C NMR (100 MHz, D_2O, HMQC, HMBC), δ 31.3 [C(3)], 42.4 [C(2)], 53.2 [C(4)], 157.2 [C(5)], 170.9 [C(1)], 171.4 [C(6)].

N-(1-Methyl-4-oxoimidazolidin-2-ylidene)amino-4,5,6-trihydroxyhexanoic acid, 8, ***Figure 4****:*. UV−vis (MeOH), λ_{max} = 204 nm; LC-TOF-MS, m/z 276.1196 ($[M + H]^+$, measured), m/z 276.1190 ($[M + H]^+$, calcd for $C_{10}H_{18}N_3O_6^+$); LC-MS (ESI^+), m/z 276.1 (100, $[M + H]^+$); ^{1}H NMR (500 MHz, D_2O; COSY), diastereomer A, δ 1.79 [m, 2H, H−C(7)], 3.04 [s, 3H, H−C(3)], 3.51 [m, 2H, H−C(9), H−C(10_a)], 3.66 [m, 2H, H−C(8), H−C(10_b)], 3.98 [s, 2H, H−C(4)], 4.26 [dd, 1H, J = 3.2 Hz, 9.7 Hz, H−C(2)]; diastereomer B, δ 1.79 [m, 2H, H−C(7)], 3.03 [s, 3H, H−C(3)], 3.51 [m, 2H, H−C(9), H−C(10_a)], 3.66 [m, 2H, H−C(8), H−C(10_b)], 3.97 [s, 2H, H−C(4)], 4.24 [dd, 1H, J = 3.5 Hz, 9.3 Hz, H−C(2)]; ^{13}C NMR (125 MHz, D_2O, HMQC, HMBC), diastereomer A, δ 34.96 [C(7)], 37.66 [C(3)], 54.37 [C(4)], 59.32 [C(2)], 62.29 [C(10)], 68.77 [C(8)], 74.60 [C(9)], 169.58 [C(5)], 171.01 [C(6)], 175.60 [C(1)]; diastereomer B, δ 34.78 [C(7)], 35.68 [C(3)], 53.03 [C(4)], 59.19 [C(2)], 62.28 [C(10)], 68.71 [C(8)], 74.58 [C(9)], 169.44 [C(5)], 171.01 [C(6)], 174.60 [C(1)].

Quantitative Analysis of Basic Taste Compounds by Means of High-Performance Ion Chromatography. A defined volume (1 mL) of the SBJ-fraction A was membrane filtered (0.45 μm) and used in a dilution of 1:50 for the analysis of carbohydrates and polyols or in a dilution of 1:250 for the analysis of anions, cations, and organic acids, respectively. Aliquots (5−25 μL) were analyzed by means of an ICS 2500 ion chromatography system (Dionex, Idstein, Germany) for anions, cations, carbohydrates, polyols, and organic acids following the protocol reported recently (*9*).

Quantitative Analysis of Carbohydrate-6-phosphates. Sugar-6-phosphates were quantitatively determined in SBJ-fraction A by using an enzyme kit (R-Biopharm, Darmstadt, Germany) and glucose-6-phosphate dehydrogenase (Roche, Penzberg, Germany) following the protocol reported in the literature (*11*).

Quantitative Analysis of Nucleotides. A portion (1.0 mL) of SBJ-fraction A was membrane filtered (0.45 μm) and diluted 1:250 with water, and aliquots (10 μL) were analyzed by HPLC-MS/MS using a 300 × 7.8 mm i.d., 5 μm, TSKgel Amide-80 column (Tosoh Bioscience) as reported recently (*9*).

Quantitative Analysis of Amino Acids. An aliquot (1 mL) of SBJ-fraction A was membrane filtered (0.45 μm) and diluted 1:250 with water prior to analysis. Then an aliquot (10 μL) was injected into the HPLC-MS/MS system equipped with a 300 × 7.8 mm i.d., 5 μm, TSKgel Amide-80 column (Tosoh Bioscience). Using a 90% acetonitrile solution containing 5 mM ammonium acetate, adjusted to pH 3.5 with acetic acid as solvent A, and 40% acetonitrile solution containing 5 mM ammonium acetate, adjusted to pH 3.5 with acetic acid as solvent B, chromatography was performed at a flow rate of 1 mL/min with an initial mixture of 100% solvent A and 0% solvent B for 5 min. Thereafter, the content of solvent B was increased within 47 min from 0 to 100%. After chromatographic separation, the effluent was split in a ratio of 1:5 to reduce the effluent entering the mass spectrometer. The following amino acids were analyzed using the mass transitions given in parentheses: L-aspartic acid (m/z 134.2→74.0), L-glutamic acid (m/z 148.1→84.2), L-asparagine (m/z 133.1→74.0), L-glutamine (m/z 147.2→84.2), L-serine (m/z 105.9→60.0), L-histidine (m/z 156.1→110.0), L-arginine (m/z 175.1→70.1), L-alanine (m/z 89.9→62.0), L-phenylalanine (m/z 166.1→120.1), L-threonine (m/z 120.2→74.0), glycine (m/z 76.0→48.1), L-tyrosine (m/z 182.2→91.0), L-valine (m/z 118.0→72.0), L-tryptophan (m/z 205.1→118.0), L-leucine (m/z 132.2→86.1), L-isoleucine (m/z 132.2→86.1), L-methionine (m/z 150.0→56.0), L-lysine (m/z 147.2→84.2), L-proline (m/z 116.0→70.0), L-4-hydroxyproline (m/z 131.7→86.1), L-ornithine (m/z 133.2→70.3), taurine (m/z 126.1→108.0), pyroglutamic acid (m/z 130.0→84.1), creatine (m/z 132.1→90.0), and creatinine (m/z 114.1→86.1), respectively. Quantitative data are given as the mean of triplicates by comparing the peak areas obtained for the corresponding mass traces with those of defined standard solutions of each reference compound [relative standard deviation (RSD) for each data point < ±10.0%].

Quantitative Analysis of β-Alanyl Dipeptides 3 and 4. A portion (1 mL) of SBJ-fraction A was diluted with water to 1000 mL and membrane-filtered (0.45 μm), and aliquots (10 μL) were analyzed by HPLC-MS/MS using a 300 × 7.8 mm i.d., 5 μm, TSKgel Amide-80 column following the protocol reported recently (*9*).

Quantitative Determination of the Gelatin Content. The gelatin content in SBJ-fraction A was analyzed after acidic hydrolysis and derivatization by means of photometric determination of the released 4-hydroxyproline (*12*).

Quantitative Analysis of *N*-(1-Methyl-4-oxoimidazolidin-2-ylidene)amino Acids (6−8). An aliquot (1 mL) of SBJ-fraction A was made up with water to 100 mL and membrane-filtered (0.45 μm), and, then, aliquots (10 μL) were injected into the HPLC-MS/MS system equipped with a 300 × 7.8 mm i.d., 5 μm, TSKgel Amide-80 column (Tosoh Bioscience). Chromatography was performed at a flow rate of 1 mL/min

using acetonitrile containing 1% formic acid as solvent A and aqueous formic acid (1% in water) as solvent B. Starting with a mixture of 70% A and 30% B for 5 min, the amount of solvent B was increased to 100% within 15 min. After chromatographic separation, the effluent was split in a ratio of 1:5 to reduce the effluent entering the mass spectrometer. The quantification was performed by means of external standard calibration with the reference compounds **6**−**8**. *N*-(1-Methyl-4-oxoimidazolidin-2-ylidene)aminopropionic acid (**6**; *m*/*z* 186.1→86.9), *N*-(1-methyl-4-oxoimidazolidin-2-ylidene)aminoacetic acid (**7**, *m*/*z* 172.1→125.9), and *N*-(1-methyl-4-oxoimidazolidin-2-ylidene)amino-4,5,6-trihydroxyhexanoic acid (**8**; *m*/*z* 276.1→170.0) were analyzed using the mass transitions given in parentheses. Quantitative data are given as the mean of triplicates by comparing the peak areas obtained for the corresponding mass traces with those of defined standard solutions of each reference compound (RSD for each data point < ±12.0%).

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 purified reference compounds by using the sip-and-spit method as reported recently (*9*, *13*), but performing the experiments at pH 5.9 instead of pH 6.5. For intensity scaling, test solutions, containing a tastant in defined concentrations, were used to calibrate the panel for judging the intensities 0, 2.5, and 5.0. Prior to sensory analysis, the fractions or compounds isolated were analytically confirmed to be essentially free of solvents and buffer compounds . Trifluoroacetate was determined using the anion procedure reported recently (*9*), and solvents were analyzed by means of GC-MS after headspace-SPME extraction.

Taste Dilution Analysis (*TDA*). Aliquots of the lyophilized GPC fractions were taken up in "natural" ratios in water (5.0 mL), adjusted to pH 5.9 with trace amounts of aqueous formic acid (0.1 mmol/L) or aqueous sodium hydroxide solution (0.1 mmol/L), diluted stepwise 1:2 with water (pH 5.9), and, then, used for the determination of the taste dilution (TD) factor (*14*−*16*) as detailed recently (*9*).

Comparative Taste Profile Analysis. Lyophilized ultrafiltration fractions (A1, A2, A1-1, or A1-2), HPLC fractions (III-1−III-9 and IV-1−IV-7), as well as HILIC fractions (IV-7/1−IV-7/7), were taken up in their "natural" concentrations either in water (5.0 mL) or in a model broth solution (5.0 mL), 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 solutions were then presented to the trained sensory panel, which was asked to rate the intensity of the descriptors sweetness, saltiness, bitterness, umami taste, acidic taste, thick-sourness, mouthfulness, mouth-dryness, and viscosity on a scale from 0 (no taste impression detectable) to 5 (strong taste impression) in a duo test in comparison to the unspiked model broth (control).

Determination of Taste Modulation Detection Thresholds. Prior to sensory analysis, the purity of compounds **6**−**8** and β-alanyl dipeptides (**3**, **4**) was checked by ^{1}H NMR spectroscopy as well as HPLC-MS. Detection thresholds for the taste modulating activity were determined by nine trained panelists in model broth solution (pH 5.9) as reported recently (*9*). Values between individuals and separate sessions differed by not more than plus or minus one dilution step; as a result, a threshold value of 31.0 mmol/L for compound **7** represents a range of 15.5−62.0 mmol/L.

Preparation of Taste Recombinants. According to the literature (*3*, *4*, *9*, *10*, *17*, *18*), a basic taste recombinant (bRec) was prepared by dissolving the tastants summarized in groups I−V in their "natural" concentrations in bottled water (**Table 2**) and, after solubilizing gelatin (5.04 g/L), the pH value of this solution was adjusted to 5.9 by the addition of trace amounts of an aqueous formic acid solution (0.1 mol/L). In addition, a total taste recombinant (tRec) was prepared with "natural" concentrations of all the taste compounds given in groups I−VI (**Table 2**). Furthermore, a partial taste recombinant (tRec+VIa) was prepared by omitting the creatinine derivatives **6**−**8** summarized in group VIb. A second partial recombinant (tRec-**7**/**8**) was prepared by omitting only compounds **7** and **8** from tRec. The taste profiles of the recombinant solutions bRec, tRec, tRec-VIb, and tRec-**7**/**8** were evaluated by means of taste profile analysis using nose-clips.

High-Performance Liquid Chromatography (HPLC). For analytical chromatography, the HPLC apparatus (Gilson International, Limburg-Offheim, Germany) was equipped with a type 321 HPLC pump, a 506C type system interface module, a 234 type autoinjector unit, an UV−vis-156 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). Analytical separations were performed with a 300 × 7.8 mm i.d., 5 μm, HILIC column containing carbamoyl-derivatized silica gel (TSKgel Amide-80, Tosoh Bioscience) operated at a flow rate of 1.0 mL/min.

For preparative HPLC, the HPLC system consisted of two S 1122 type pumps (Sykam, Eresing, Germany), a Rh 7125i type Rheodyne injection valve (Bensheim, Germany), an ERC-3215α type solvent degasser (ERC, Riemerling, Germany), a mixing chamber (Sunchrom, Friedrichsdorf, Germany), a P-451 microsplitter (Upchurch, Oak Harbor, WA), a Spectraflow 600 type DAD detector (Sunchrom), and a PrepELS type ELSD detector. Data acquisition was performed by means of ChromStar V. 6.2 software. The split ratio was set to a flow of 1 mL/min for the ELSD detector. The chromatographic separation was performed on a 300 × 21.5 mm i.d., 10 μm, TSKgel Amide-80 column (Tosoh Bioscience) and on a 250 × 21.2 mm i.d., 5 μm, Monochrom MS column (Varian), respectively.

Liquid Chromatography−Time-of-Flight Mass Spectrometry (LC/TOF-MS). High-resolution mass spectra were measured on a Bruker Micro-TOF (Bruker Daltronics, Bremen, Germany) and referenced to sodium formate.

Liquid Chromatography−Mass Spectrometry (LC-MS/MS). Mass spectrometric analyses were 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 1200 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); the collision energy and declustering potential were optimized for the individual target analytes. The mass spectrometer was operated in the full scan mode monitoring positive and negative ions, respectively. Quantitative analysis was done using the multiple monitoring (MRM) mode and the mass transitions described above.

Nuclear Magnetic Resonance Spectroscopy (NMR). NMR data were acquired on a Bruker DRX-400 or an AVANCE-III-500 spectrometer, the latter of which was equipped with a Cryo-CTCI probe (Bruker BioSpin, Rheinstetten, Germany). D_2O was used as solvent and sodium-3-trimethylsilylpropionate (TMSP) as the internal standard. Data processing was performed by using Topspin software (version 2.1; Bruker) as well as Mestre-C software (version 4.8.6; Mestrelab Research, Santiago de Compostella, Spain).

RESULTS AND DISCUSSION

To identify the compounds inducing the typical thick-sour and mouth-drying orosensation induced by stewed beef juice, 2.6 L of SBJ was freshly prepared and, after cooling to 6 °C, the fat layer was removed by filtration. To gain a first insight into the taste profile of SBJ, a trained sensory panel was asked to judge the intensity of the taste descriptors sweetness, saltiness, bitterness, umami taste, acidic taste, thick-sourness, mouthfulness, mouth-dryness, and viscosity on a linear scale from 0 (no taste impression) to 5 (strong taste impression). Umami taste and mouthfulness were rated with the highest intensity of 4.0, followed by the acidic taste as well as the thick-sour orosensation evaluated with intensities of 3.5 and 3.0, respectively (**Table 1**). In addition, a mouth-drying sensation and an increased viscosity were perceived with intensities of 2.5 and 1.5, respectively, whereas sweetness, bitterness, and saltiness were judged with comparatively low intensities of ≤1.0. The mean value obtained for each sensory descriptor was used to calibrate the sensory panel for the precise evaluation of the SBJ and its corresponding fractions in the following experiments.

Solvent Extraction and Molecular Weight Fractionation. To gain first insight into the polarity of the taste-active compounds, the filtered SBJ was repeatedly extracted with *n*-pentane and, after removal of the organic solvent in vacuum, the aqueous layer

Table 1. Taste Profile Analysis of Stewed Beef Juice (SBJ) and the Ultrafiltration Fractions A1-1, A1-2, and A2 Obtained from SBJ

	intensities for individual taste qualities[a]			
taste descriptor	SBJ	A2 (>5 kDa)	A1-2 (1−5 kDa)	A1-1 (<1 kDa)
sweetness	0.5 (±0.10)	0	0	0.5 (±0.15)
saltiness	1.0 (±0.22)	0	0	1.0 (±0.20)
bitterness	1.0 (±0.20)	0	0	1.0 (±0.15)
umami taste	4.0 (±0.25)	0	0	4.0 (±0.22)
acidic taste	3.5 (±0.20)	0	0	3.3 (±0.15)
thick-sourness	3.0 (±0.25)	0	0	2.7 (±0.20)
mouthfulness	4.0 (±0.25)	0	0	3.5 (±0.20)
mouth-dryness	2.5 (±0.20)	0.5 (±0.23)	0.5 (±0.22)	2.5 (±0.18)
viscosity	1.5 (±0.15)	1.0 (±0.20)	0	0

[a] Intensities were judged on a linear scale from 0 (no taste impression) to 5 (strong taste impression) by nine trained panelists. The 95% confidence intervals are given in parentheses.

and the *n*-pentane solubles were freeze-dried to yield the water-soluble fraction A (99.9 g/100 g d.m.) as well as the lipid fraction B (0.1 g/100 g d.m.). Both fractions were taken up in water in their "natural" concentrations, which means 5.89 g of fraction A and 0.006 g of fraction B were solubilized in 100 mL of water and evaluated by the sensory panelists. Although the emulsified fraction B did not induce any taste sensation, the taste profile of the aqueous solution of fraction A matched well that of the authentic beef juice (data not shown), which means the differences in the individual taste descriptors between the samples were not significant on the basis of a 95% confidence interval.

To gain first insight into the molecular weight of the taste-active molecules, fraction A was separated by means of tangential-flow ultrafiltration to obtain fraction A1 (< 5 kDa) and fraction A2 (≥ 5 kDa) after freeze-drying. As the entire taste profile was found to be represented by the molecules present in fraction A1, this fraction was further subfractionated by means of stirred-cell ultrafiltration to give fractions A1-1 (< 1 kDa) and A1-2 (1−5 kDa) after freeze-drying. Fractions A1-1, A1-2, and A2 were dissolved in bottled water, each in its "natural" concentration, which means 4.80 g of fraction A1-1, 0.06 g of fraction A1-2, and 0.90 g of fraction A2 were solubilized in 100 mL of water, and these solutions were then again evaluated by means of a taste profile analysis (**Table 1**). The high molecular weight fractions A1-2 and A2 were evaluated only with a less intense mouth-drying orosensation and, in addition, fraction A2 was the only fraction judged with an increased viscosity, most likely induced by gelatin (**Table 1**). In comparison, the taste profiles of SBJ and A1-1 were not significantly different, with the exception of some lower intensity of mouthfulness in A1-1 (**Table 1**). As these data clearly demonstrated the most taste-active compounds to be present in fraction A1-1, this fraction was used for further analysis.

Quantitative Analysis of Basic Tastants and Taste Re-engineering Experiments. To evaluate the sensory impact of basic taste compounds on the taste profile of beef juice, mono- and disaccharides, alditols, organic acids, cations, and inorganic anions were quantitatively analyzed by means of HPIC. In addition, the amounts of hexose phosphates were determined by means of an enzymatic assay, and purine nucleotides, amino acids, creatinine, and creatine were quantified by means of HILIC-MS/MS. Among these compounds, 25 amino acids, 8 polyols, 6 carbohydrates and hexose-6-phosphates, 6 nucleotides and nucleosides, 6 organic acids, 4 cations, and 2 inorganic anions were quantified in fraction A1-1 and summarized in the basic tastant groups I−V (**Table 2**).

Calculation of dose-over-threshold (DoT) factor, defined as the quotient of the concentration and the threshold concentration of a taste compound (*19*), revealed the DoT factors for the bitter-tasting creatinine, the umami-like-tasting L-glutamic acid, the salty-tasting chlorides and phosphates of potassium, sodium, and magnesium, the sweet-tasting L-alanine, and the sour-tasting organic acids, lactic acid, L-pyroglutamic acid, and acetic acid (**Table 2**). To confirm the results of the instrumental analysis and to check as to whether the compounds already identified can create the typical taste of the beef juice, an aqueous taste reconstitute, containing the 61 basic taste compounds as well as gelatin, each in its "natural" concentration given in **Table 2**, was prepared, and the taste profile of that basic taste recombinant (bRec) was compared with that of the filtered SBJ (**Table 3**). Comparative taste profile analysis revealed that the intensities of sweetness, saltiness, acidic, and umami taste, as well as the viscosity perceived for the bRec solution matched rather well those found for the filtered beef juice. As the characteristic mouthfulness (4.0 → 1.5), thick-sourness (3.0 → 1.3), and mouth-drying impression (2.5 → 1.0) as well as the bitter taste (1.0 → 0.4) were judged significantly less intense in the bRec solution, it was concluded that the basic taste recombinant is lacking molecules modulating the thick-sour and mouth-drying orosensation as well as mouthfulness, respectively.

Sensory-Directed Subfractionation of Fraction A1-1 and Identification of Taste Modulators. To further resolve fraction A1-1 into distinct taste compounds and taste modulating molecules, this fraction was separated by means of GPC on Sephadex G-15. Monitoring the effluent by means of UV−vis detection, fraction A1-1 was separated into the seven GPC-fractions I−VII (**Figure 2**), which were individually freeze-dried.

An aliquot of each GPC-fraction was taken up in bottled water in its "natural" concentration, which means in the amounts obtained from the GPC column, and evaluated by means of a TDA. The highest TD factor of 64 was found for the sour taste impression in fraction III, followed by the umami taste as well as the bitter taste perceived in fractions IV and V, respectively, even in 1:32 dilutions (**Table 4**). In addition, the latter two fractions induced an increased mouthfulness judged with a TD factor of 8. The other GPC-fractions showed lower TD factors or were entirely tasteless (**Table 4**).

In addition, aliquots of the GPC-fractions were added to an aqueous model broth, which was used as a tasty matrix solution to enable the localization of tasteless, but taste modulating, molecules in the following sensory-guided fractionation experiments. To achieve this, aliquots of the individual GPC-fractions were dissolved in the model broth in their "natural" concentration ratios and were then evaluated by means of a comparative taste profile analysis using the blank model broth solution as control. The data, given in **Table 4**, show that GPC-fractions III and IV increased the mouthfulness as well as the mouth-drying and thick-sour orosensation when added to the model broth solution. This orosensation was perceivable in neither the blank model broth solution (control) nor the aqueous solution of fraction III or VI alone.

To locate the molecules responsible for the thick-sour and mouth-drying orosensation, GPC-fractions III and IV were separated by means of HPLC using a pentafluorophenylpropyl (PFPP) stationary phase to give the seven subfractions IV-1−IV-7 (**Figure 3A**) and the nine subfractions III-1−III-9 (**Figure 5**), respectively. After lyophilization, the intrinsic taste of each individual fraction was evaluated sensorially in water and, in addition, the influence of each HPLC-fraction on the taste quality of a model broth was judged in comparison to the blank model broth as control. As given in **Table 5**, all HPLC-subfractions of GPC-fraction IV showed taste activity with the exception of fractions IV-1 and IV-4. Interestingly, fraction IV-7 was found to

Table 2. Taste Qualities, Taste Thresholds, Concentrations, and Dose-over-Threshold (DoT) Factors of Nonvolatile Taste Active Compounds in Beef juice

taste compound	TC[a] (μmol/L)	concn (μmol/L) (RSD, %)	DoT[b]
Group I: Bitter-Tasting Compounds			
creatinine[c]	18000	32440 (±8.2)	1.8
creatine[c]	85000	16570 (±7.9)	0.2
L-histidine[d]	48000	11990 (±7.5)	0.2
L-leucine[d]	12000	370 (±8.2)	<0.1
L-tyrosine[d]	5000	260 (±9.4)	<0.1
L-isoleucine[d]	11000	300 (±8.2)	<0.1
L-tryptophan[d]	5000	120 (±8.6)	<0.1
L-lysine[d]	85000	740 (±8.4)	<0.1
L-valine[d]	21000	1080 (±8.4)	<0.1
L-phenylalanine[d]	58000	650 (±9.5)	<0.1
L-arginine[d]	75000	860 (±7.8)	<0.1
taurine	150000	4670 (±8.8)	<0.1
xanthine	60000	320 (±7.3)	<0.1
hypoxanthine	44000	2280 (±8.1)	<0.1
inosine	20000	1200 (±7.8)	<0.1
adenosine	77000	90 (±8.4)	<0.1
Group II: Umami-like Compounds			
L-glutamic acid[c]	1500	2080 (±8.9)	1.4
succinate	900	390 (±5.2)	0.4
5′-IMP[c]	2500	1620 (±8.7)	0.6
5′-AMP[c]	4000	580 (±9.3)	0.1
L-aspartic acid	4000	170 (±8.7)	<0.1
L-glutamine	50000	280 (±9.1)	<0.1
L-asparagine	50000	540 (±9.4)	<0.1
Group III: Salty-Tasting Compounds			
phosphate[c,e]	7500	72050 (±5.2)	9.6
potassium[c,f]	15000	132260 (±2.7)	8.8
sodium[c,f]	7500	42670 (±3.1)	5.7
magnesium[c,f]	4000	12630 (±2.6)	3.2
chloride[c,g]	7500	20880 (±4.9)	2.8
calcium[c,f]	7500	2250 (±2.5)	0.3
Group IV: Sweet-Tasting Compounds			
L-alanine	8000	10360 (±8.4)	1.3
glucose-6-phosphate	1400	720 (±8.4)	0.5
fructose-6-phosphate	2200	430 (±9.3)	0.2
glycine[h]	25000	3940 (±7.2)	0.2
inositol[h]	17700	3440 (±4.2)	0.2
glycerol	81200	9940 (±3.4)	0.1
glucose[i]	90000	1280 (±4.5)	<0.1
fructose[i]	52000	280 (±4.7)	<0.1
sucrose[i]	24000	360 (±5.2)	<0.1
xylose[h]	12500	160 (±4.2)	<0.1
xylitol	20200	30 (±3.7)	<0.1
1,2-propanediol	44200	3720 (±4.2)	<0.1
erythritol	36300	200 (±4.5)	<0.1
sorbitol	33800	90 (±4.9)	<0.1
ribitol[h]	45300	10 (±5.1)	<0.1
L-methionine	5000	200 (±8.4)	<0.1
L-proline	26000	840 (±7.3)	<0.1
L-serine	30000	2060 (±8.9)	<0.1
L-threonine	40000	770 (±9.2)	<0.1
L-ornithine[j,k]	3500	310 (±8.6)	<0.1
L-4-hydroxyproline	6000	180 (±7.9)	<0.1
Group V: Sour-Tasting Compounds			
L-lactate	14000	99620 (±4.8)	7.1
L-pyroglutamic acid[j]	9800	19020 (±9.8)	1.9
acetate	2000	2290 (±7.2)	1.1
glycolic acid	5500	1750 (±6.7)	0.3
L-malate	3700	270 (±6.1)	<0.1
formiate	4300	310 (±6.8)	<0.1
Group VI: Compounds Inducing a Thick-Sour and Mouth-Drying Orosensation			
Group VIa			
3[l]	8560	2430 (±7.9)	0.3
4[l]	22700	15390 (±7.2)	0.7
Group VIb			
6[l]	209	411 (±11.8)	2.0
7[l]	31	32 (±9.2)	1.0
8[l]	278	28 (±9.9)	0.1

[a] Taste threshold concentrations were determined in bottled water by means of a triangle test or taken from the literature. [b] DoT factor is calculated as the ratio of concentration and taste threshold. [c] Value taken from the literature (*3*). [d] Value taken

induce a thick-sour orosensation and mouthfulness when tasted in the model broth matrix, thus suggesting the presence of taste modulator molecules.

Further subfractionation of fraction IV-7 by means of semi-preparative HILIC afforded again seven subfractions, namely, IV-7/1−IV-7/7 (**Figure 3B**), which were then evaluated in water (for intrinsic taste) and in model broth (for taste modulation). As given in **Table 5**, aqueous solutions of fractions IV-7/3, IV-7/4, and IV-7/6 were found to taste slightly bitter, umami-like, and acidic/salty, respectively, but only the latter fraction as well as the tasteless fraction IV-7/2 induced an enhanced thick-sour and mouth-drying sensation when evaluated in the presence of the model broth solution. To identify the molecules inducing this typical orosensation, fractions IV-7/2 and IV-7/6 were analyzed by means of LC-TOF-MS, LC-MS/MS, and NMR experiments.

LC-MS as well as LC-TOF-MS analysis of compound **6** isolated from fraction IV-7/2 showed a pseudomolecular ion ($[M + H]^+$) of m/z 186 and an elemental composition of $C_7H_{12}N_3O_3$ as found earlier for the structure of *N*-(1-methyl-4-hydroxyimidazolin-2-ylidene)aminopropionic acid, **1** (**Figure 1**) (*5*).

LC-MS analysis of compound **7** isolated from fraction IV-7/6 showed a pseudomolecular ion ($[M + H]^+$) of m/z 172 differing from **1** by 14 amu. In addition, LC-TOF-MS analysis revealed an elemental composition of $C_6H_{10}N_3O_3$, thus suggesting that compound **7** is an isomer of **1** lacking a methylene moiety. As both taste modulators isolated from fractions IV-7/2 and IV-7/6 could not be detected by LC-MS/MS in a noncooked beef juice (data not shown), we suggested that they might be formed upon thermal treatment of nontasting precursors, for example, by Maillard-type reactions of reducing carbohydrates and creatine or creatinine, which are present in high concentrations of 85 and 18 mmol/L in the beef juice (**Table 2**).

To answer the question as to whether *N*-(1-methyl-4-hydroxy-imidazolin-2-ylidene)amino acids such as **1** are thermally generated from these precursors, creatinine was refluxed in the presence of a 10-fold amount of glucose in aqueous phosphate buffer (pH 7.0) for 4 h and the reaction products formed were monitored by HPLC-UV−vis and HPLC-MS/MS, respectively. On the basis of identical spectroscopic data (UV−vis, LC-MS/MS) and chromatographic retention times, two of the reaction products formed were identical to molecules **6** and **7** isolated from the SBJ. In addition, a third major reaction product (**8**), exhibiting a pseudo molecular weight of m/z 276.1 in the ESI^+ mode, was detected in the creatinine/glucose reaction mixture. To obtain suitable

Table 3. Taste Profile Analysis of Stewed Beef Juice (SBJ), Basic Taste Recombinant (bRec), Total Taste Recombinant (tRec), Partial Taste Recominant Lacking Tastant Group VIb (tRec-VIb), and Partial Taste Recombinant Lacking Compounds **7** and **8** (tRec-**7**/**8**), Respectively

	intensities for individual taste qualities[a]				
taste descriptor	SBJ	bRec[b]	tRec[c]	bRec+VIa[d]	tRec-**7**/**8**[e]
sweetness	0.5 (±0.10)	0.5 (±0.10)	0.5 (±0.15)	0.5 (±0.15)	0.5 (±0.12)
saltiness	1.0 (±0.22)	1.0 (±0.20)	1.0 (±0.15)	1.0 (±0.20)	1.0 (±0.18)
bitterness	1.0 (±0.20)	0.4 (±0.12)	0.5 (±0.15)	0.5 (±0.15)	0.5 (±0.18)
umami taste	4.0 (±0.25)	3.8 (±0.22)	3.8 (±0.25)	3.8 (±0.20)	3.8 (±0.25)
acidic taste	3.5 (±0.20)	3.4 (±0.18)	3.6 (±0.20)	3.4 (±0.25)	3.4 (±0.25)
thick sourness	3.0 (±0.25)	1.3 (±0.15)	2.7 (±0.22)	1.7 (±0.15)	2.4 (±0.22)
mouthfulness	4.0 (±0.25)	1.5 (±0.16)	3.6 (±0.20)	2.9 (±0.20)	3.4 (±0.20)
mouth-dryness	2.5 (±0.20)	1.0 (±0.15)	2.4 (±0.22)	2.2 (±0.20)	2.3 (±0.22)
viscosity	1.5 (±0.15)	1.5 (±0.15)	1.5 (±0.18)	1.5 (±0.20)	1.5 (±0.15)

[a] The intensity of the individual taste qualities was evaluated on a linear scale from 0 to 5 by nine trained subjects. The 95% confidence intervals are given in parentheses. [b] The basic taste recombinant solution (bRec) contained the tastant groups I−V in the concentrations given in **Table 2**. [c] The total taste recombinant solution (tRec) contained the tastant groups I−VIII in the concentrations given in **Table 2**. [d] This partial taste recombinant contained the basic taste compounds (group I−V) and the β-alanyl dipeptides (group VIa). [e] This partial taste recombinant was prepared by omitting compounds **7** and **8** from tRec.

Table 4. Taste Dilution Analysis (TDA) of GPC-Fractions I−VII Dissolved in Bottled Water and Comparative Taste Profile Analysis of GPC-Fractions I−VII Dissolved in Model Broth Solution

	TDA in water[a]		in model broth[b]
fraction[c]	TD factor	taste quality	taste modifying effect
I	<1	nd[d]	nd
II	4	bitter	slightly enhanced saltiness
III	64	acidic taste	enhanced mouthfulness, thick-sour orosensation,
	8	bitter	long-lasting, bitter at the side of the tongue
IV	32	umami	long-lasting mouthfulness, thick-sour, and
	16	astringent	mouth-drying orosensation
	8	mouthfulness	
V	32	bitter	salty, slightly bitter
	8	mouthfulness	
VI	16	bitter	long-lasting bitter and astringent
	4	astringent	
VII	1	bitter	slightly bitter and astringent
	1	astringent	

[a] TDA was carried out after dissolving the individual GPC-fractions in bottled water (pH 5.9) in their "natural" concentration ratios. [b] The individual GPC-fractions were dissolved in a model broth solution containing monosodium glutamate monohydrate (1.9 g/L), yeast extract (2.1 g/L), maltodextrin (6.375 g/L), and sodium chloride (2.9 g/L) in bottled water (pH 5.9). The descriptors given by each panelist were collected, and those given by at least seven of the nine panelists are given. The blank model broth solution was used as control. [c] Numbering of GPC-fractions corresponds to **Figure 2**. [d] Not detectable.

Table 5. Intrinsic Taste (in Water) of HPLC-Fractions IV-1−IV-7 and HPLC-Subfractions IV-7/1−IV-7/7 and Their Influence on the Taste Quality of a Model Broth Solution

fraction[a]	intrinsic taste quality in water[b]	influence on the taste quality of a model broth[c]
IV-1	tasteless	nd[d]
IV-2	salty, slightly umami	nd
IV-3	slightly bitter	slightly bitter
IV-4	tasteless	nd
IV-5	umami (glutamate-like)	enhanced umami taste
IV-6	umami (nucleotide-like)	enhanced umami taste
IV-7	slight umami and acidic taste	enhanced thick-sour taste
IV-7/1	tasteless	nd
IV-7/2	tasteless	increased mouth-drying, thick-sour taste
IV-7/3	slightly bitter	slightly bitter
IV-7/4	umami-like	slightly enhanced umami taste
IV-7/5	tasteless	nd
IV-7/6	acidic and salty taste	enhanced mouth-drying, thick-sour taste
IV-7/7	tasteless	nd

[a] HPLC-fraction numbering corresponds to **Figure 3**. [b] The individual HPLC-fractions were dissolved in bottled water (pH 5.9) and sensorially evaluated against bottled water (control). [c] The individual HPLC-fractions were dissolved in model broth solution in a model broth solution containing monosodium glutamate monohydrate (1.9 g/L), yeast extract (2.1 g/L), maltodextrin (6.375 g/L), and sodium chloride (2.9 g/L) in bottled water (pH 5.9). The descriptors given by each panelist were collected, and those given by at least seven of the nine panellists are given. The blank model broth solution was used as control. [d] No difference detectable.

amounts for an unequivocal structure determination, compounds **6**−**8** were isolated from the model reaction mixture in a preparative scale and, after cleanup by rechromatography (purity > 99%), were then analyzed by means of LC-MS/MS and NMR.

The ^{1}H and ^{13}C NMR spectra of compound **6** revealed a double signal set of four proton signals integrating for nine protons and a double signal set of seven carbon signals each in a ratio of 1:1.3, thus implying the presence of two diastereomers. In addition to the two proton singlets of the creatinine moiety, the two proton signals HC(7) and H−C(2) resonating at 1.42 and 4.43 ppm for diastereomer A and at 1.39 and 4.40 ppm for diastereomer B showed homonuclear connectivity in a DQF-COSY experiment. Moreover, a HMBC experiment revealed heteronuclear coupling between the quaternary carbon C(5) of the creatinine moiety and the proton H−C(2) as part of the alanine moiety (**Figure 4**). These NMR data fitt well to the structure of the previously reported *N*-(1-methyl-4-hydroxy-3-imidazolin-2,2-ylidene)alanine, **1** (**Figure 1**), and to its annular exotautomer (**6**) proposed in **Figure 4**. As recent NMR and computational studies on a series of *N*-substituted creatinine derivatives demonstrated the preferred existence of the more stable exotautomers such as **6** when compared to the less stable endotauomers (**1**) (*20*), the structure of the taste modulator isolated was proposed as the *N*-(1-methyl-4-oxoimidazolidin-2-ylidene)aminopropionic acid, **6** (**Figure 4**). A closer look at the stereochemistry of compound **6** revealed two chiral positions; one is the chiral carbon atom C(2) in the alanine moiety, and the other is the chiral tertiary nitrogen atom N−C(3)/C(4)/C(5) with its free electron pair as part of the creatinine moiety, **6** (**Figure 4**). For the first time, these data confirm the formation of the diastereomers of **6** as a Maillard reaction product of creatinine and glucose.

The ^{1}H NMR spectrum of compound **7** showed three proton signals as singlets, two of which integrated for two and the other for three protons. The methyl proton signal resonating at 3.11 ppm and the methylene protons observed at 4.24 ppm did correspond to H−C(3) and H−C(4) of the creatinine moiety. The additional proton signal detected at 4.30 ppm was assigned as the methylene protons H−C(2) of the glycine imine moiety in

N-(1-methyl-4-oxoimidazolidin-2-ylidene)aminoacetic acid, **7** (**Figure 4**). To the best of our knowledge, this compound has not been previously reported in the literature.

The ^{1}H NMR spectrum of compound **8** showed a double signal set, each exhibiting six proton signals in a ratio of 1:1.5, and featured the protons of a creatinine moiety, thus indicating the presence of *N*-(1-methyl-4-oxoimidazolidin-2-ylidene)amino acid diastereomers as already found for compound **6**. The HMBC experiment revealed a heteronuclear C,H correlation between the α-amino acid proton H−C(2) resonating at 4.24/4.26 ppm and the carbon atoms C(1), C(7), and C(8) as part of the 2-amino-2,3-dideoxygluconic acid moiety as well as the quarternary guanidino carbon C(5) being part of the creatinine moiety (**Figure 4**). As the proton and carbon resonances of the 2-amino-2,3-dideoxygluconic acid moiety were well in line with those reported for the same structural element in the Maillard reaction products DOGDIC and DOPDIC (*21*), the reaction product **8** isolated from the thermally treated mixture of creatinine and glucose was unequivocally identified as the previously not reported diastereomeric pair of *N*-(1-methyl-4-oxoimidazolidin-2-ylidene)amino-4,5,6-trihydroxyhexanoic acid, **8** (**Figure 4**).

On the basis of identical spectroscopic (UV−vis, LC-MS/MS), chromatographic (PFPP, HILIC), and sensory data, the taste modulators imparting the thick-sour and mouth-drying orosensation to the beef juice fractions IV-7/2 and IV-7/6 were unequivocally identified as *N*-(1-methyl-4-oxoimidazolidin-2-ylidene)-aminopropionic acid (**6**) and *N*-(1-methyl-4-oxoimidazolidin-2-ylidene)aminoacetic acid (**7**) and, finally, confirmed by cochromatography of an aliquot of the individual SBJ fractions and the corresponding synthetic reference compound.

To investigate putative taste modulators in subfractions III-1−III-9 (**Figure 5**), the intrinsic taste of each individual fraction was evaluated sensorially in bottled water and, in addition, the influence of each HPLC fraction on the taste quality of a model broth was judged in comparison to the blank model broth as control. As given in **Table 6**, all of the HPLC subfractions showed some taste activity with the exception of fractions III-1 and III-6. Interestingly, fractions III-5 and III-7−III-9 were found to induce a thick-sour orosensation and mouthfulness when tasted in the model broth matrix, thus suggesting the presence of additional taste modulators in these fractions. UV−vis, LC-TOF-MS, and LC-MS/MS studies, followed by cochromatography of an aliquot of each individual fraction (III-5, III-7−III-9) and the corresponding synthetic reference compound led to the unequivocal identification of *N*-(1-methyl-4-oxoimidazolidin-2-ylidene)amino-4,5,6-trihydroxyhexanoic acid, **8** (**Figure 4**), in fraction III-5, β-alanyl-L-histidine, **4** (**Figure 1**), in fractions III-7 and III-8, and β-alanyl-3-methyl-L-histidine, **3** (**Figure 1**), in fraction III-9. The impact of these dipeptides on the thick-sour taste of beef juice is well in line with their key role in chicken broth (*9*) as well as beef products (*3*, *4*, *10*). The identification of compound **8** verified for the first time the natural occurrence of taste-modulating Maillard-modified creatinine derivatives in thermally processed foods.

Sensory Activity of *N*-(1-Methyl-4-oxoimidazolidin-2-ylidene)-α-amino Acids. Preliminary studies on the taste modulators **3**, **4** and **6**−**8** revealed that, independent of their chemical structure, none of these compounds imparted any intrinsic taste activity up to levels of 10 mmol/L in water (data not shown). In comparison, triangle tests performed in a model broth solution (pH 5.9) revealed detection thresholds ranging from 31 (**7**) to 278 μmol/L (**8**) for the induction of the thick-sour and mouth-drying orosensation. To correlate the threshold values and the concentration of these compounds in beef juice and to estimate their contribution to the thick-sour and mouth-drying orosensation of beef juice, compounds **3**, **4**, and **6**−**8** were quantitatively analyzed in the stewed beef broth.

Table 6. Intrinsic Taste (in Water) of HPLC-Fractions III-1−III-9 and Their Influence on the Taste Quality of a Model Broth Solution

fraction[a]	intrinsic taste quality in water[b]	influence on the taste quality of a model broth[c]
III-1	tasteless	nd[d]
III-2	acidic taste, slightly umami (lactic acid-like)	increased acidic and umami taste
III-3	acidic taste, metallic-bitter aftertaste, and sweet	increased acidic taste, metallic, bitter
III-4	umami, mouthfulness	mouthfulness, slightly umami
III-5	slightly umami	enhanced thick-sour and mouth-drying orosensation, slightly umami
III-6	tasteless	nd
III-7	slightly acidic taste	more long-lasting, increased thick-sour taste and mouthfulness
III-8	slightly acidic taste	increased thick-sour orosensation and mouthfulness
III-9	slightly umami	increased thick-sour and mouth-drying orosensation and mouthfulness

[a] HPLC fraction numbering corresponds to **Figure 5**. [b] The individual HPLC fractions were dissolved in bottled water (pH 5.9) and sensorially evaluated against bottled water (control). [c] The individual HPLC fractions were dissolved in a model broth solution containing monosodium glutamate monohydrate (1.9 g/L), yeast extract (2.1 g/L), maltodextrin (6.375 g/L), and sodium chloride (2.9 g/L) in bottled water (pH 5.9). The descriptors given by each panellist were collected and those given by at least seven out of the nine panellists are given. The blank model broth solution was used as control. [d] No difference detectable.

Quantitative Analysis of Taste Modulators in Beef Juice and Taste Recombination Experiments. To quantify the taste modulators in beef juice, a HILIC-MS/MS method was developed for the analysis of **3**, **4**, and **6**−**8** using the MRM mode. Compound **6** was found in concentrations of 411 μmol/L as the major *N*-(1-methyl-4-oxoimidazolidin-2-ylidene)-α-amino acid, followed by **7** and **8**, which were present in about 14 times lower amounts of 32 and 28 μmol/L, respectively (**Table 2**). In comparison, the dipeptides **3** and **4** were detected at higher levels of 15390 and 2430 μmol/L, respectively.

On the basis of the threshold concentrations and their "natural" concentration in beef juice, DoT factors were calculated for the taste modulatory activity of the *N*-(1-methyl-4-oxoimidazolidin-2-ylidene)-α-amino acids **6**−**8** as well as the β-alanyl dipeptides **3** and **4**. The thermally generated compounds **6** and **7** were found to reach or exceed their detection threshold levels in stewed beef juice and were evaluated with DoT factors of 2 and 1, respectively, thus giving first evidence for the sensory impact of these compounds (**Table 2**). In addition, the concentrations of β-alanyl dipeptides **3** and **4** were close to their detection thresholds for taste modulation and were evaluated with DoT factors of 0.3 and 0.6, respectively.

To check whether β-alanyl dipeptides and *N*-(1-methyl-4-oxoimidazolidin-2-ylidene)-α-amino acids can explain the difference in the taste profile of the basic taste recombinant (bRec) and the authentic beef juice, a total taste recombinant (tRec) was prepared by spiking bRec with the "natural" amounts of these taste modulators. Taste profile analysis revealed a significant intensity increase in thick-sourness (1.3 → 2.7), mouthfulness (1.5 → 3.6), and mouth-dryness (1.0 → 2.4) when the tastant group VI containing the dipeptides (**3** and **4**) and *N*-(1-methyl-4-oxoimidazolidin-2-ylidene)-α-amino acids (**6**−**8**) were added to bRec (**Table 3**), thus confirming these molecules as key players for the thick-sour and mouth-drying orosensation.

To investigate the contribution of the β-alanyl dipeptides (group VIa) to the thick-sour orosensation of beef broth,

compounds **3** and **4** were added in their "natural" concentration to the solution of the basic taste recombinant bRec. The sensory panel was asked to evaluate the taste profile of this partial taste recombinant (bRec+VIa) and to rate it in comparison to the bRec solution as well as the authentic beef juice by scoring the given taste descriptors on a five-point linear scale. The presence of the β-alanyl dipeptides **3** and **4** in bRec+VIa was detected by all sensory panelists, and the intensity of the thick-sourness (1.3 → 1.7), mouth-dryness (1.0 → 2.2), and mouthfulness (1.5 → 2.9) was judged to be significantly increased (**Table 3**). Although these peptides were slightly below their thresholds, these data clearly demonstrate the important role of **3** and **4** as taste modulators in beef juice. This is well in agreement with the previous findings reporting on the taste modulatory activity of these peptides in chicken broth (*9*), beef broth (*3*, *10*), and stewed beef (*4*).

In a final experiment, another partial taste recombinant was prepared by omitting the *N*-(1-methyl-4-oxoimidazolidin-2-ylidene)-α-amino acids **7** and **8** from the total recombinant. Sensory analysis of this partial recombinant (tRec-**7**/**8**), containing the basic taste compounds (groups I−V) and the β-alanyl dipeptides (group VIa), as well as the quantitatively predominating *N*-(1-methyl-4-oxoimidazolidin-2-ylidene)-α-amino acid **6**, revealed a taste profile differing from that of the partial recombinant tRec+VIa by an increased thick-sour orosensation (1.7 → 2.4) and mouthfulness (2.9 → 3.4), thus indicating the sensory impact of compound **6** in its natural concentrations on the typical taste profile of beef juice (**Table 3**). Comparison of the solutions bRec+VIa and tRec-**7**/**8**, just differing in the presence of compound **6** in the latter recombinant, showed higher mean intensities for the thick-sourness (+ 0.7) and mouthfulness (+ 0.5) in tRec-**7**/**8**, thus verifying the key role of **6** as an important taste modulator in beef juice. Comparison of the taste profile of tRec-**7**/**8** and tRec, differing only in the presence of compounds **7** and **8** in the latter recombinant, showed slightly lower mean intensities for the thick-sourness (−0.3) and mouthfulness (−0.2) in tRec-**7**/**8**, which, however, were not significant. In contrast to compound **6**, no significant taste contribution could be found for **7** and **8** (**Table 3**).

On the basis of the data obtained, it might be concluded that besides the β-alanyl dipeptides (**3**, **4**), previously reported as taste modulators in chicken broth (*9*), beef broth (*3*, *10*), and beef juice (*4*), Maillard-generated *N*-(1-methyl-4-oxoimidazolidin-2-ylidene)-α-amino acids such as **6** are the most important contributors to the thick-sour and mouth-drying orosensation and the mouthfulness imparted by stewed beef juice. Quantitative studies and ^{13}C-labeling experiments are currently in progress to elucidate the formation pathways of *N*-(1-methyl-4-oxoimidazolidin-2-ylidene)-α-amino acids upon thermal processing of meat.

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