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ARTICLE

Discovery of N^2-(1-Carboxyethyl)guanosine 5′-Monophosphate as an Umami-Enhancing Maillard-Modified Nucleotide in Yeast Extracts

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Sensory-guided fractionation of a commercial yeast extract involving medium-pressure RP-18 chromatography and ion-pair chromatography, followed by LC-MS/MS, LC-TOF-MS, 1D/2D-NMR, and CD spectroscopy, led to the discovery of the previously not reported umami-enhancing nucleotide diastereomers (*R*)- and (*S*)-N^2-(1-carboxyethyl)guanosine 5′-monophosphate. Model experiments confirmed the formation of these diastereomers by a Maillard-type glycation of guanosine 5′-monophosphate with dihydroxyacetone and glyceraldehyde, respectively. Sensory studies revealed umami recognition threshold concentrations of 0.19 and 0.85 mmol/L for the (*S*)- and (*R*)-configured diastereomers, respectively, and demonstrated the taste-enhancing activity of these nucleotides on monosodium L-glutamate solutions.

KEYWORDS: Yeast; umami; taste enhancer; taste dilution analysis; N^2-(1-carboxyethyl)guanosine 5′-monophosphate

INTRODUCTION

In the food industry, yeast extracts, made from the soluble fraction of yeasts, are used as a savory ingredient to impart a broth-like, meaty taste impression, referred to as umami, to soups, sauces, and snack products (*1*, *2*). Depending on the starting material used, namely, spent brewer's yeast, baker's yeast, and torula yeast (*2*, *3*), respectively, as well as the manufacturing process, such as autolysis, plasmolysis, or hydrolysis (*2*−*4*), the yeast extracts available on the market strongly differ in cost as well as flavor profile.

Although a series of studies have been performed on the odor-active volatile fraction of yeast extract (*5*−*12*), knowledge on the taste-active and/or taste-modulating nonvolatiles is rather limited. The amino acids L-glutamic acid (**1**) and L-aspartic acid (**2**) formed upon protein hydrolysis and the organic acid succinic acid (**3**), as well as the purine ribonucleotides inosine 5′-monophosphate (**4**) and guanosine 5′-monophosphate (**5**, 5′-GMP), generated upon breakdown of nucleic acids and showing a remarkable synergistic effect on L-glutamate (**Figure 1**), have been well-known for a long time to contribute to the umami taste of yeast extracts (*2*, *3*, *13*−*16*). Very recently, the molecular mechanism of the synergistic effect of purine ribonucelotides on the umami perception of L-glutamate was found to be mediated by a positive allosteric modulation of the umami taste receptor due to the fixation of the closed conformation of the receptor's venus flytrap domain by interaction of the negatively charged phosphate group of the ribonucleotide with the positively charged pincer residues of the receptor protein (*17*). Although many scientists have been focusing on increasing the yields of the umami compounds **1**−**4** in yeast extracts, comparatively little is known about the presence of additional, yet unknown, taste molecules and/or taste-modulating compounds in yeast products.

In recent years, activity-guided fractionation of foods combining liquid chromatographic separation techniques and analytical sensory tools enabled the straightforward discovery of the key taste molecules in red wine (*18*), Gouda cheese (*19*), dried morel mushrooms (*20*), and beef broth (*21*), as well as previously not reported taste modulators in edible beans (*22*), matured Gouda cheese (*23*), chicken broth (*24*), and stewed beef juice (*25*), respectively.

To identify previously unknown taste modulators in yeast extract, the objective of the present study was to apply a sensory-guided fractionation approach to an intensely umami-like-tasting yeast extract.

MATERIALS AND METHODS

Chemicals and Materials. Unless stated otherwise, all chemicals were obtained from Sigma-Aldrich (Steinheim, Germany) and were of puriss grade; formic acid, hydrochloric acid, and sodium hydroxide were from Grüssing GmbH (Filsum, Germany), and disodium hydrogen phosphate dihydrate and potassium dihydrogen phosphate were from Merck KGaA (Darmstadt, Germany). Deuterium oxide and sodium deuteroxide (40% w/w solution in D_2O) were obtained from Euriso-Top (Gif-Sur-Yvette, France), HPLC grade solvents were from Mallinckrodt Baker (Griesheim, Germany), and membrane filter disks (0.45 μm) were purchased from Satorius AG (Goettingen, Germany). Water used for chromatography was purified by means of a Milli-Q Advantage A10 water purification system (Millipore, Molsheim, France), and bottled water (Evian) was used for sensory analysis. A commercially available yeast extract (Springer 2012/20-MG-L) was obtained in powdered form from the food industry in France.

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Fractionation of Yeast Extract by Means of Medium-Pressure Liquid Chromatography (MPLC). A sample of the yeast extract (2 g) was dissolved in water (20 mL) and injected onto a 150 × 40 mm i.d., LiChroprep, 25−40 μm, RP-18 cartridge of a medium-pressure liquid chromatographic system (RP-MPLC). Using 1% formic acid in water (solvent A) and methanol (solvent B), chromatography (flow rate = 50 mL/min) was performed starting with 100% solvent A for 5 min and then increasing the content of solvent B to 20% within 15 min and to 100% within additional 15 min, followed by a 5 min isocratic elution with 100% solvent B. Ten subfractions (I−X; **Figure 2**) were collected, separated from solvent in vacuum, and lyophilized twice to afford the individual fractions in yields given in parentheses (in g/100 g): I (28.2), II (24.0), III (24.5), IV (3.9), V (7.7), VI (2.8), VII (1.8), VIII (2.4), IX (3.3), and X (1.4). The residues obtained were kept at −20 °C until used for sensory analysis (**Figure 3**) and chemical analysis, respectively.

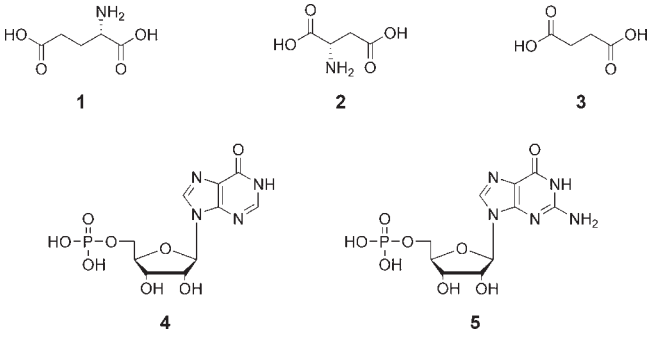

Figure 1. Chemical structures of naturally occurring umami-active compounds L-glutamic acid (**1**), L-aspartic acid (**2**), succinic acid (**3**), inosine 5′-monophosphate (**4**), and guanosine 5′-monophosphate (**5**).

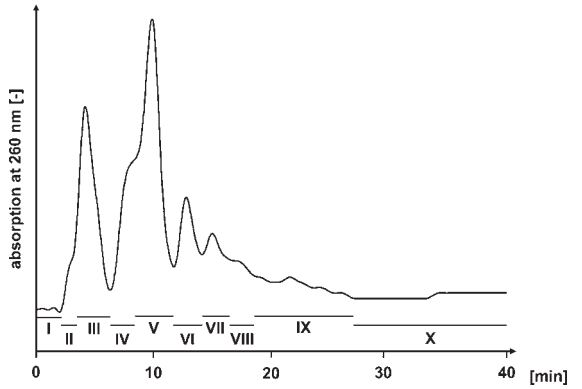

Figure 2. Chromatogram (λ = 260 nm) of the yeast extract separated by means of gradient RP-MPLC.

Isolation of (*S*)-N^2-(1-Carboxyethyl)guanosine 5′-Monophosphate, (*S*)-6. RP-MPLC fraction VIII was dissolved in a mixture (93:7, v/v) of an aqueous triethylammonium acetate solution (250 mM TEAA, pH 6.0) and methanol and then fractionated by means of semipreparative ion pair high-performance liquid chromatography (IP-HPLC) using a Microsorb-MV C18 column, 250 × 10 mm i.d., 5 μm (Varian, Darmstadt, Germany). Monitoring the effluent at 260 nm, chromatography was performed at a flow rate of 3.8 mL/min using an aqueous TEAA buffer (250 mM, pH 6.0, solvent A) and methanol (solvent B) as solvents. After isocratic elution with 7% solvent B for 10 min, the content of solvent B was increased to 25% within 20 min and, finally, to 40% within an additional 2 min. Within 8 min the organic solvent content was reduced to starting conditions. As shown in **Figure 4A**, a total of 16 fractions were collected individually, separated from solvent in vacuum, and lyophilized to remove the buffer. Subfraction VIII-8 was subjected to rechromatography using a preparative Microsorb-MV C18 column, 250 × 21.2 mm i.d., 5 μm (Varian) as stationary phase. Chromatography was performed with a mixture of 1% formic acid in water (solvent A) and acetonitrile (solvent B) at a flow rate of 18 mL/min, monitoring the effluent at 260 nm. After isocratic elution with 0% B for 5 min, the content of B was increased to 5% in 10 min and to 10% B within an additional 5 min, followed by a linear gradient to 30% solvent B within 10 min and to 100% within an additional 10 min. The UV-absorbing fractions (**Figure 4B**) were collected, separated from organic solvent in vacuum, diluted 5-fold with water, and then lyophilized twice. UV−vis, LC-MS, and ^{1}H NMR analysis of subfraction VIII-8/5, obtained as an amorphous white powder after freeze-drying, led to the identification of (*S*)-N^2-(1-carboxyethyl)guanosine 5′-monophosphate, (*S*)-**6**. Comparison of chromatographic (RP-18) and spectroscopic data (UV−vis, LC-MS/MS, ^{1}H NMR) with those obtained for the reference compound generated by a Maillard-type reaction confirmed the identity of (*S*)-**6**.

(*S*)-N^2-(*1-Carboxyethyl*)*guanosine 5′-monophosphate,* (*S*)-***6***, ***Figure 6***: UV−vis (100 mM PO_4^{3-} buffer, pH 7.5), λ_{max} = 254, 276 nm (sh); LC-MS (ESI$^-$), *m*/*z* (%) 434 (100) [M − H]$^-$; LC-TOF-MS, *m*/*z* 434.0705 (found), *m*/*z* 434.0719 (calcd for [$C_{13}H_{17}N_5O_{10}P$]$^-$); ^{1}H NMR (500 MHz, D_2O/NaOD, COSY), δ 1.45 [d, 3H, *J* = 7.2 Hz, H−C(3″)], 3.97−4.10 [m, 2H,

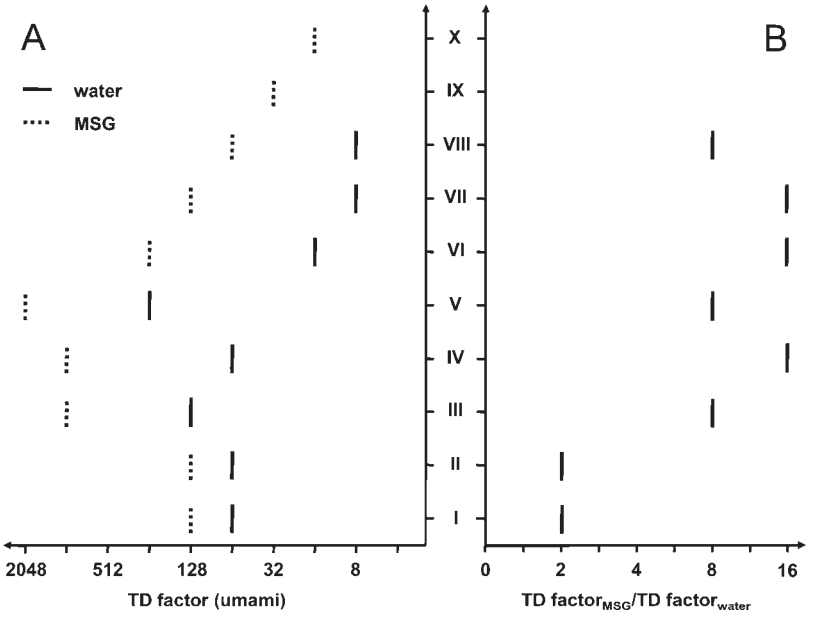

Figure 3. (**A**) Taste dilution (TD) factors determined for the umami taste of the RP-MPLC fractions isolated from yeast extract in water (TDA_{water}) and in an aqueous monosodium L-glutamate solution (3 mmol/L in water; TDA_{MSG}). (**B**) Quotient of the TD factors obtained from TDA_{MSG} and TDA_{water}.

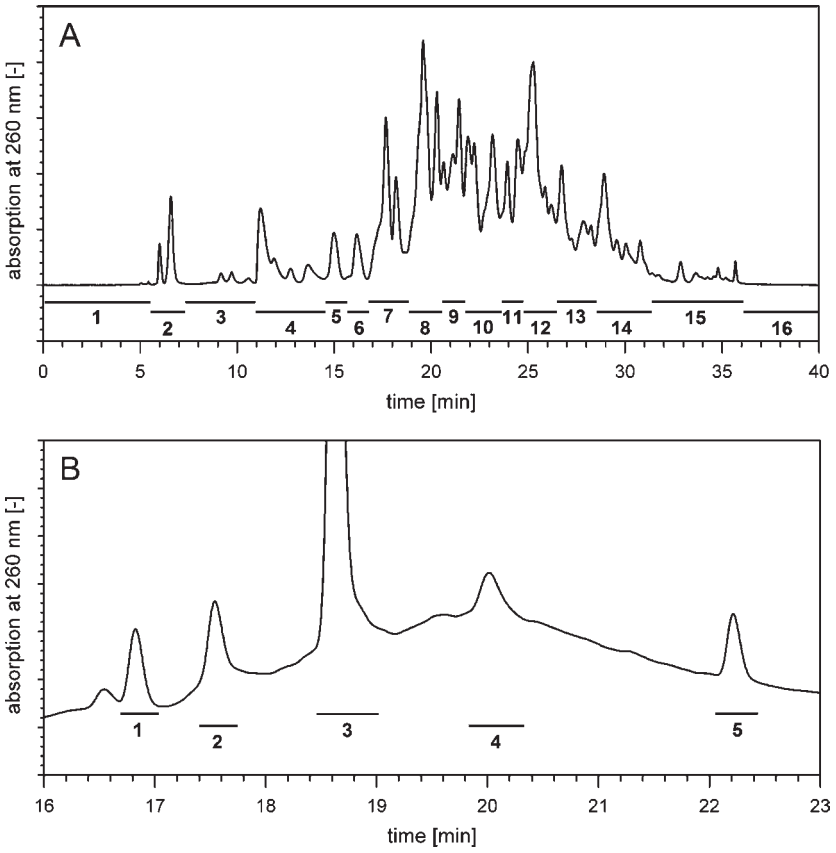

Figure 4. Semipreparative IP-HPLC chromatogram (λ = 260 nm) of fraction VIII (**A**) and excerpt of preparative RP-HPLC chromatogram (λ = 260 nm) of fraction VIII-8 (**B**).

H−C(5′)], 4.25 [q, 1H, J = 7.2 Hz, H−C(2″)], 4.31 [m, 1H, H−C(4′)], 4.44 [pt, 1H, J = 4.8 Hz, 1H, H−C(3′)], 4.72 [pt, 1H, J = 5.2 Hz, H−C(2′)], 6.02 [d, 1H, J = 5.2 Hz, H−C(1′)], 8.17 [s, 1H, H−C(8)]; ^{13}C NMR (125 MHz, D_2O/NaOD, HMQC, HMBC), δ 20.2 [C(3″)], 55.4 [C(2″)], 66.7 [d, $^2J_{C,P}$ = 4.6 Hz, C(5′)], 73.1 [C(3′)], 77.1 [C(2′)], 86.7 [d, $^3J_{C,P}$ = 8.7 Hz, C(4′)], 89.9 [C(1′)], 118.8 [C(5)], 140.4 [C(8)], 154.8 [C(4)], 155.0 [C(2)], 162.0 [C(6)], 184.2 [C(1″)].

Preparation of Compounds (*R*)-6 and (*S*)-6 by a Maillard Reaction of Guanosine 5′-Monophosphate. Guanosine 5′-monophosphate disodium salt hydrate (2 mmol, calculated on water-free nucleotide) and DL-glyceraldehyde (6 mmol) were suspended in phosphate buffer (5 mL, 1 mol/L, pH 7.0) and incubated in a closed vessel at 40 °C. After 10 days, the reaction mixture was diluted with water (10 mL) and, after membrane filtration, fractionated by means of preparative RP-HPLC using the conditions described above. UV-absorbing fractions were collected individually and, after separation of the organic solvent in vacuum, water and formic acid residues were removed by repeated lyophilization, yielding the title compounds (*R*)-**6** and (*S*)-**6** as amorphous white powders in a purity of >95% (RP-HPLC/ELSD, ^{1}H NMR).

(*R*)-N^2-(*1-Carboxyethyl*)*guanosine 5′-monophosphate,* (*R*)-**6**, ***Figure 6****:* UV−vis (100 mM PO_4^{3-} buffer, pH 7.5), λ_{max} = 254, 276 nm (sh); LC-MS (ESI$^-$), m/z (%) 434 (100, [M − H]$^-$); LC-TOF-MS, m/z 434.0722 (found), m/z 434.0719 (calcd for [$C_{13}H_{17}N_5O_{10}P$]$^-$); ^{1}H NMR (500 MHz, D_2O/NaOD, COSY), δ 1.45 [d, 3H, J = 7.2 Hz, H−C(3″)], 3.95−4.07 [m, 2H, H−C(5′)], 4.26 [q, 1H, J = 7.2 Hz, H−C(2″)], 4.30 [m, 1H, H−C(4′)], 4.47 [pt, 1H, J = 4.8 Hz, 1H, H−C(3′)], 4.83 [pt, 1H, J = 5.3 Hz, H−C(2′)], 5.97 [d, 1H, J = 5.3 Hz, H−C(1′)], 8.14 [s, 1H, H−C(8)]; ^{13}C NMR (125 MHz, D_2O/NaOD, HMQC, HMBC), δ 20.3 [C(3″)], 55.5 [C(2″)], 66.7 [d, $^2J_{C,P}$ = 4.5 Hz, C(5′)], 73.3 [C(3′)], 76.4 [C(2′)], 87.0 [d, $^3J_{C,P}$ = 8.2 Hz, C(4′)], 90.5 [C(1′)], 119.0 [C(5)], 141.2 [C(8)], 154.6 [C(4)], 154.9 [C(2)], 162.1 [C(6)], 184.1 [C(1″)].

(*S*)-N^2-(*1-Carboxyethyl*)*guanosine 5′-monophosphate,* (*S*)-**6**, ***Figure 6****:* spectroscopic data were identical to those obtained for the sample of (*S*)-**6** isolated from the yeast extracts.

Preparation of (*R*)- and (*S*)-N^2-(1-Carboxyethyl)-2′-deoxyguanosine ((*R*)- 7 and (*S*)-7). Following a literature procedure (*26*) with some minor modifications, a mixture of 2′-deoxyguanosine monohydrate (2 mmol) and 1,3-dihydroxyacetone dimer (3 mmol) was suspended in phosphate buffer (5 mL, 1 mol/L, pH 7.0) and thermally treated for 24 h at 70 °C in a closed vessel. After cooling, the reaction mixture was diluted with water (10 mL), membrane filtered, and fractionated by means of preparative RP-HPLC. Monitoring the effluent at 260 nm, chromatography was performed using aqueous ammonium formate (5 mM, pH 7) as eluent A and methanol as eluent B at a flow rate of 15 mL/min. After starting chromatography with an isocratic step of 5% B for 5 min, the content of B was increased linearly to 100% within 35 min and, then, was kept isocratic for an additional 2 min. The UV-absorbing substances were collected individually and, after separation of the solvent in vacuum, the residue was repeatedly lyophilized to afford the title compounds as amorphous yellowish powders in a purity of >95% (RP-HPLC/ELSD, ^{1}H NMR).

(*R*)-N^2-(*1-Carboxyethyl*)-*2′-deoxyguanosine ammonium salt,* (*R*)-*7*, ***Figure 7****:* UV−vis (100 mM PO_4^{3-} buffer, pH 7.5), λ_{max} = 251, 278 nm (sh); LC-MS (ESI$^-$), m/z (%) 338 (100) [M − H]$^-$; ^{1}H NMR (500 MHz, D_2O, COSY), δ 1.50 [d, 1H, J = 7.3 Hz, H−C(3″)], 2.48 [ddd, 1H, J = 3.9 Hz, 6.8 Hz, 14.1 Hz, H−C(2′$_a$)], 3.13 [pdt, 1H, J = 6.8 Hz, 13.8 Hz, H−C(2′$_b$)], 3.82−3.93 [m, 2H, H−C(5′)], 4.13 [m, 1H, H−C(4′)], 4.27 [q, 1H, J = 7.3 Hz, H−C(2″)], 4.69 [m, 1H, H−C(3′)], 6.35 [t, 1H, J = 6.9 Hz, H−C(1′)], 7.95 [s, 1H, H−C(8)]; ^{13}C NMR (125 MHz, D_2O, HMQC, HMBC), δ 20.5 [C(3″)], 40.4 [C(2′)], 55.6 [C(2″)], 64.7 [C(5′)], 74.1[C(3′)], 87.7 [C(1′)], 89.8 [C(4′)], 119.7 [C(5)], 142.1 [C(8)], 154.4 [C(4)], 154.8 [C(2)], 162.0 [C(6)], 184.0 [C(1″)].

(*S*)-N^2-(*1-Carboxyethyl*)-*2′-deoxyguanosine ammonium salt* (*S*)-*7*, ***Figure 7****:* UV−vis (100 mM PO_4^{3-} buffer, pH 7.5), λ_{max} = 251, 278 nm (sh); LC-MS (ESI$^-$), m/z (%) 338 (100) [M − H]$^-$; ^{1}H NMR (500 MHz, D_2O, COSY), δ 1.46 [d, 1H, J = 7.3 Hz, H−C(3″)], 2.51 [ddd, 1H, J = 4.6 Hz, 6.8 Hz, 14.0 Hz, H−C(2′$_a$)], 2.94 [pdt, 1H, J = 7.0 Hz, 13.8 Hz H−C(2′$_b$)], 3.71 [dd, 1H, J = 6.1 Hz, 12.3 Hz, H−C(5′$_a$)], 3.80 [dd, 1H, J = 4.0 Hz, 12.2 Hz, H−C(5′$_b$)], 4.07 [m, 1H, H−C(4′)], 4.23 [q, 1H, J = 7.2 Hz, H−C(2″)], 4.63 [m, 1H, H−C(3′)], 6.30 [t, 1H, J = 6.6 Hz, H−C(1′)], 7.92 [s, 1H, H−C(8)]; ^{13}C NMR (125 MHz, D_2O, HMQC,

HMBC), δ 20.4 [C(3″)], 40.9 [C(2′)], 55.3 [C(2″)], 64.6 [C(5′)], 73.9 [C(3′)], 87.0 [C(1′)], 89.6 [C(4′)], 119.2 [C(5)], 141.1 [C(8)], 154.2 [C(4)], 154.7 [C(2)], 161.8 [C(6)], 184.0 [C(1″)].

Acidic Hydrolysis of (*R*)-6, (*S*)-6, (*R*)-7, and (*S*)-7. A portion (10 mg) of compound of (*R*)-**6**, (*S*)-**6**, (*R*)-**7**, and (*S*)-**7**, respectively, was mixed with hydrochloric acid (1 mol/L; 1 mL) and heated in closed glass vials (1.5 mL) for 2.5 h at 100 °C. The individual hydrolysates were allowed to cool to room temperature, diluted with water (2 mL), and adjusted to pH 3 by the addition of sodium hydroxide solution (1 mol/L), and, then, the effluent of the peak showing UV adsorption at 260 nm was isolated by means of preparative RP-HPLC, using aqueous formic acid and acetonitrile as described. After solvent removal in vacuum and lyophilization, (*R*)-**8** and (*S*)-**8** were obtained as amorphous white powders.

(*R*)-N^2-(*1-Carboxyethyl*)*guanine,* (*R*)-***8***, ***Figure 7****:* UV−vis (100 mM PO_4^{3-} buffer, pH 7.5), λ_{max} = 247, 279 nm (sh); LC-MS (ESI$^-$), *m*/*z* (%) 222 (100) [M − H]$^-$; ^{1}H NMR (400 MHz, D_2O, COSY), δ 1.44 [d, 3H, *J* = 7.2 Hz, H−C(3″)], 4.24 [q, 1H, *J* = 7.2 Hz, H−C(2″)], 7.98 [s, 1H, H−C(8)]; ^{13}C NMR (100 MHz, D_2O, HMQC, HMBC), δ 20.4 [C(3″)], 55.0 [C(2″)], 116.0 [C(5)], 141.6 [C(8)], 155.1 [C(4)], 155.8 [C(2)], 160.9 [C(6)], 183.4 [C(1″)].

(*S*)-N^2-(*1-Carboxyethyl*)*guanine,* (*S*)-***8***, ***Figure 7****:* spectroscopic data of (*S*)-**8** were identical to those of the enantiomer (*R*)-**8**.

Sensory Analyses. *Precautions Taken for Sensory Analysis of Food Fractions and Taste Compounds*. Prior to sensory analysis, the fractions or compounds isolated were, after removal of the volatiles in high vacuum (< 5 mPa), diluted with water and freeze-dried twice. GC-MS and ion chromatographic analysis revealed that food fractions treated by that procedure are essentially free of the solvents and buffer compounds used. For pH adjustment of all samples applied in human sensory experiments to 6.0, trace amounts of formic acid (1 g/100 g) or sodium hydroxide (1 mmol/L or 0.1 mmol/L) were used. Formic acid, which is GRAS listed as a flavoring agent for food and feed applications, was used, because trace amounts of this acid do not influence the sensory profiles of the test solution. To minimize the uptake of any toxic compound to the best of our knowledge, all of the sensory analyses were performed by using the sip-and-spit method, which means the test materials were not swallowed but expectorated.

Training of the Sensory Panel. Thirteen subjects (11 women and 2 men, ages 22−30 years), who gave consent to participate in the sensory tests of the present investigation and have no history of known taste disorders, were trained to evaluate the taste of aqueous solutions of the following standard taste compounds in bottled water (pH 6.0): sucrose (50 mmol/L) for sweet taste, lactic acid (20 mmol/L) for sour taste, NaCl (20 mmol/L) for salty taste, caffeine (1 mmol/L) for bitter taste, monosodium L-glutamate (3 mmol/L) for umami taste. For the puckering astringency and the velvety astringent mouth-drying oral sensation, the panel was trained by using tannic acid (0.05%) and quercetin-3-*O*-β-D-glucopyranoside (0.01 mmol/L), respectively, using the half-tongue test. Additionally the panel was familiarized to differentiate several umami taste qualities by tasting solutions of monosodium L-glutamate (3 mmol/L), a binary mixture of monosodium L-glutamate (3 mmol/L) and guanosine 5′-monophosphate (0.1 mmol/L), and a disodium succinate solution (5 mmol/L). The assessors had participated earlier at regular intervals for at least 18 months in sensory experiments (triangle tests, iso-intensity testings, scale training) and were, therefore, familiar with the techniques applied. To prevent cross-modal interactions with olfactory inputs, the panelists wore nose clips.

Taste Profile Analysis. To evaluate the taste profile of an aqueous solution of the yeast extract, the yeast powder was dissolved in bottled water to give a final concentration of 1%. After adjustment of the pH value to 6.0, the trained sensory panel was asked to rate the taste qualities umami, salty, sweet, bitter, sour, and astringent on an intensity scale ranging from 0 (not detectable) to 5 (strongly detectable).

Taste Dilution Analysis (*TDA*). The lyophilized fractions obtained by RP-MPLC were dissolved in water (10 mL; TDA_{water}) to perform the TDA (*27*) or in an aqueous monosodium L-glutamate solution (3 mmol/L in water; 10 mL) to perform a modified taste dilution analysis (TDA_{MSG}) as an alternative to the previously reported cTDA (*28*). After adjustment of the pH value to 6.0 by adding trace amounts of either sodium hydroxide (1 mol/L) or aqueous formic acid (1% in water), the parent solutions were stepwise diluted 1 + 1 with water (pH 6.0), and the dilutions of each fraction were presented to the sensory panel in order of increasing concentrations by means of a duo test using water (pH 6.0; for TDA_{water}) or a serial dilution of monosodium L-glutamate (3 mmol/L, pH 6.0; for TDA_{MSG}) as blank solutions. The participants were instructed to determine the dilution step at which a taste difference between sample and blank solution could be detected. This so-called taste dilution (TD) factor determined by the sensory subjects in three separate sessions was averaged.

Taste Recognition Threshold Concentrations. Determination of the taste threshold concentrations of the nucleotides was performed in bottled water (pH 6.0) using a triangle test with ascending concentrations of the stimulus, as reported in previous papers (*27*, *29*). The threshold value of the sensory group was approximated by averaging the threshold values of the individuals in three independent sessions. Values between individuals and separate sessions differed by not more than plus or minus one dilution step; as a result, a threshold value of 0.15 mmol/L for **5** represents a range of 0.075−0.30 mmol/L.

Determination of Taste-Enhancing Activity. To determine potential taste-enhancing properties of the nucleotide derivatives, the purified target compounds were subjected to an iso-umami test. To achieve this, an aqueous binary solution (pH 6.0) containing the test nucleotide (0.1 mmol/L) and monosodium L-glutamate (10 mmol/L) was compared to aqueous solutions containing ascending concentrations (in 5 mmol/L steps) of monosodium L-glutamate alone (10−100 mmol/L; pH 6.0) in a duo test. The sensory panel was asked to determine the concentration of monosodium L-glutamate required to match the umami taste intensity of the binary nucleotide/monosodium L-glutamate mixture. This so-called iso-umami concentration was approximated by averaging the values obtained for each individual in three independent sessions.

RP-MPLC. Medium-pressure chromatography was performed on a preparative Sepacore chromatography system (Büchi, Flawil, Switzerland) consisting of two C-605-type pumps, a C-615 pump manager, a C-660 fraction collector, a manual injection port equipped with a 20 mL loop, and a C-635-type UV detector monitoring the effluent at 260 nm. Chromatography (flow rate = 50 mL/min) was performed on LiChroprep, 25−40 μm, RP-18 bulk material (Merck KGaA), filled in a 150 × 40 mm i.d. polypropylene cartridge (Büchi) using a C-670 cartridge (Büchi).

HPLC. The analytical HPLC system (Jasco, Gross-Umstadt, Germany) consisted of a PU-2080 Plus pump, a DG-2080-53 degasser, an LG-2080-02 gradient unit, an AS-2055 Plus autosampler with a 100 μL loop, and an MD-2010 Plus detector. The HPLC apparatus (Jasco) for semipreparative and preparative liquid chromatography consisted of two PU-2087 pumps, a Degasys DG-1310 online degasser (Uniflows Co., Tokyo, Japan), a 1000 μL gradient mixer, a 7725 i injection valve (Rheodyne, Bensheim, Germany), and an MD-2010 Plus detector. Semipreparative separations (flow rate = 3.8 mL/min for IP-HPLC) were performed using a Microsorb-MV C18 column, 250 × 10 mm i.d., 5 μm (Varian, Darmstadt, Germany) column and preparative chromatography (flow rate = 15 or 18 mL for RP-HPLC) using a Microsorb-MV C18 column, 250 × 21.2 mm i.d., 5 μm (Varian). For chromatographic analysis of the isolated fractions an aliquot was dissolved in water and analyzed on an Outstanding B C18 column, 250 × 4.6 mm i.d., 5 μm (Trentec, Rutesheim, Germany).

For reversed phase separations, chromatography was performed using 1% formic acid in water (solvent A) and acetonitrile (solvent B) as effluents at a flow rate of 1 mL/min, starting with 100% solvent A for 5 min and increasing solvent B to 5% within 10 min, to 10% within an additional 5 min, to 30% within the following 10 min, and, finally, to 100% B within 10 min.

For ion pair chromatography, an aqueous triethylammonium acetate buffer (40 mM TEAA, pH 6) was used as solvent A and methanol as solvent B (flow rate = 0.8 mL/min). After starting with 3% B for 5 min, the organic solvent content was increased linearly to 40% B within 25 min and finally to 100% B in 7 min.

LC−Time-of-Flight Mass Spectrometry (LC-TOF-MS). Mass spectra of the target compounds were measured on a Bruker Micro-TOF-Q (Bruker Daltonics, Bremen, Germany) mass spectrometer with flow injection referenced on sodium formate. Data processing was performed by using Daltonics DataAnalysis software (version 3.4, Bruker Daltonics).

Liquid Chromatography−Mass Spectrometry (LC-MS). Electrospray ionization (ESI) spectra were acquired on an API 3200 type LC-MS/MS system (AB Sciex Instruments, Darmstadt, Germany) coupled to an

Agilent 1100 pump, an Agilent 1100 degasser, and an Agilent 1200 autosampler (Agilent, Waldbronn, Germany). The spray voltage was set at −4500 V in ESI^- mode. Zero grade air served as nebulizer gas (35 psi) and as turbo gas (350 °C) for solvent drying (45 psi). Nitrogen served as curtain (20 psi) and collision gas (4.5×10^{-5} Torr). Both quadrupoles were set at unit resolution. The declustering potential was set at −10 to −85 V in ESI^- mode. The mass spectrometer was operated in the full-scan mode monitoring negative ions. ESI^- mass and product ion spectra were acquired with direct flow infusion. Fragmentation of $[M - H]^-$ molecular ions into specific product ions was induced by collision with nitrogen and a collision energy of −15 to −70 V. For instrumentation control and data aquisition, the Analyst software version 1.5.1 was used.

HPLC-MS/MS analysis separation was performed on an analytical Luna PFP column, 150 × 2 mm i.d., 3 μm (Phenomenex, Aschaffenburg, Germany), prior to introduction of the eluent into the mass spectrometer operating in the multiple reaction monitoring mode (MRM) detecting negative ions. Three mass transitions, namely, m/z 434→339, m/z 434→204, and m/z 434→79, were selected for the identification of (*R*)- and (*S*)-**6**, respectively. After sample injection (5 μL), chromatography was performed with a flow rate of 200 μL/min starting with a mixture (97:3, v/v) of aqueous formic acid (0.1% in water) and acetonitrile containing 0.1% formic acid. After isocratic elution for 3 min, the acetonitrile content was increased to 100% within 22 min and, finally, kept isocratic for 2 min.

Circular Dichroism Spectroscopy (CD). Measurements of the optical rotation were performed using a J-810 spectropolarimeter (Jasco) equipped with a 0.1 cm path length quartz cuvette 106-QS (Hellma, Müllheim, Germany). Spectra were recorded from 220 to 360 nm at 20 °C by accumulating 16 runs (bandwidth = 1 nm, scan speed = 100 nm/min, DIT = 4 s). For measurement, the samples were dissolved in phosphate buffer (100 mmol/L, pH 7.5), which was ultrasonicated for at least 30 min and filtered through a 0.45 μm membrane filter disk prior to use. Final concentration of the sample solutions, which were freshly prepared prior to analysis, was ∼500 μmol/L. After subtraction of the background noise induced by the buffer, the spectra were analyzed using the instrument software. The molar circular dichroic absorption $\Delta\varepsilon$ (cm^2/mmol) was calculated according to the quotient $\theta_{obs}/32980cl$, where θ_{obs} (mdeg) denotes the measured ellipticity, c (mol/L) the sample concentration, and l (cm) the path length of the quartz cuvette.

Nuclear Magnetic Resonance Spectroscopy (NMR). The ^1H, ^{13}C, COSY, DEPT, HMQC, and HMBC spectroscopic experiments were performed on either a 400 MHz DRX 400 or a 500 MHz Avance III NMR spectrometer from Bruker (Rheinstetten, Germany). Samples were dissolved in D_2O using 3-(trimethylsilyl)propionic-2,2,3,3-d_4 acid sodium salt (TMSP) as reference. In the case of (*R*)- and (*S*)-N^2-(1-carboxyethyl)guanosine 5′-monophosphate, the solvent was prepared by mixing 10 μL of a 40% w/w solution of sodium deuteroxide in D_2O with 750 μL of D_2O. Data processing was performed using Topspin version 1.3 (Bruker), and the individual data interpretation was done with MestReNova 5.1.0-2940 (Mestrelab Research S.L., Santiago de Compostela, Spain).

RESULTS AND DISCUSSION

To evaluate the taste modalities of a yeast extract, a 1% aqueous solution of the extract was subjected to a taste profile analysis. To achieve this, a trained sensory panel was asked to rate the taste descriptors umami, salty, sweet, sour, bitter, and astringent on a linear scale from 0 (no taste impression) to 5 (strong taste impression). Whereas the umami taste impression was rated with the highest intensity of 5.0, sweet and salty tastes were evaluated with intensities of 1.6 and 1.5, respectively. Bitter and sour taste impressions were judged with comparably low intensities of 0.6 and 0.5, whereas an astringent orosensation was not detectable at all.

Sensory-Guided Fractionation of a Yeast Extract. To gain first insight into the polarity of the taste-active and/or taste-modulating compounds, the yeast extract was separated by means of MPLC on RP-18 material to give 10 fractions, I−X (**Figure 2**), with yields ranging from 1.4 to 28.2%. Polar fractions I−V accounted for a total yield of 88.3%, whereas the total yield of the less polar fractions VI−X accounted for 11.7% only. To localize the fractions, contributing to the intense umami taste impression induced by the yeast extract, aliquots of the individual fractions I−X were used for taste dilution analysis using water (TDA_{water}) and an aqueous monosodium L-glutamate solution (3 mmol/L; TDA_{MSG}) as the matrix, respectively. Application of the TDA_{water} revealed an intrinsic umami taste of fractions I−VIII (**Figure 3A**). The highest TD factors of 256 and 128 were observed for fractions V and III, followed by fractions I, II, and IV, all of which were evaluated with a TD factor of 64. The less polar fractions VI−VIII were judged with comparatively low TD factors of 8 and 16, respectively, and the nonpolar fractions IX and X did not show any umami taste at all. In comparison, the results of the TDA_{MSG} demonstrated each of the 10 MPLC fractions to have a taste-enhancing activity on the umami taste of the L-glutamate matrix solution (**Figure 3A**). By far the highest TD factors of 2048 and 1024 were found for fractions V, III, and IV. Calculation of the ratio of the TD factors obtained from TDA_{MSG} and TDA_{water} revealed values of 8 and 16 for fractions III−VIII, whereas for fractions I and II the TD factor of TDA_{MSG} was only 2-fold above that of the TDA_{water} (**Figure 3B**). Most interesting, the less polar fractions VI−VIII showed an increase of the TD factors of 8−16 times when evaluated in the L-glutamate matrix (TDA_{MSG}; **Figure 3B**), although the TDA_{water} revealed these fractions to exhibit only a marginal intrinsic umami taste judged with TD factors of < 32 (**Figure 3A**). As LC-MS/MS screening and cochromatography with the corresponding reference substances revealed the well-known umami stimuli L-glutamic acid (**1**), L-aspartic acid (**2**), and succinic acid (**3**) in fractions I and II, the umami-enhancing purine ribonucleotide inosine 5′-monophosphate (**4**) in fractions III−V, and guanosine 5′-monophosphate (**5**) in fractions V−VII, MPLC fraction VIII, lacking all of these compounds, was expected to contain unknown umami taste modulators.

Discovery of N^2-(1-Carboxyethyl)guanosine 5′-Monophosphate (6) as an Unkown Umami Enhancer in Fraction VIII. Aimed at the discovery of the umami taste-enhancing principle in fraction VIII, this fraction was further separated on RP-18 material by means of IPC using triethylammonium acetate. To answer the question as to whether the unknown umami taste modulator might be based on the purine backbone as found for **4** and **5**, the effluent of the RP18-IPC separation was monitored at 260 nm and heart-cut into 16 subfractions (**Figure 4A**). Each fraction was lyophilized twice and analyzed for the presence of purine nucleotide derivatives by means of RP-HPLC/DAD analysis. Rechromatography of fraction VIII-8 revealed the subfraction 5, which eluted after 22 min (**Figure 4B**) and exhibited an intense umami taste as well as a guanine-like UV−vis absorption spectrum with a maximum at 255 nm and a shoulder at 280 nm. Preparative isolation of this compound by means of RP-HPLC, followed by lyophilization, afforded an amorphous white powder, which was subjected to LC-TOF-MS, LC-MS, and 1D/2D-NMR spectroscopic experiments.

LC-TOF-MS analysis revealed m/z 434.0705 as the pseudomolecular ion $[M - H]^-$, fitting well with a molecular formula of $[C_{13}H_{17}N_5O_{10}P]^-$. In addition, LC-MS/MS studies, performed in the ESI^- mode, revealed a fragmentation of the pseudomolecular ion m/z 434 to give m/z 336 as the predominant daughter ion, thus implying the cleavage of phosphoric acid. This was further strengthened by the detection of the fragment ion m/z 97 found for $[H_2PO_4]^-$. In addition, the ^{13}C NMR spectrum revealed split signals for the carbon atom C(4′) and C(5′) with coupling constants of $^3J_{C,P}$ = 8.7 Hz and $^2J_{C,P}$ = 4.6 Hz, respectively, thus verifying the presence of the phosphate moiety at C(5′) of the 5′-GMP moiety.

The ^1H NMR spectrum of the unknown compound showed eight resonance signals with integrals between one and three

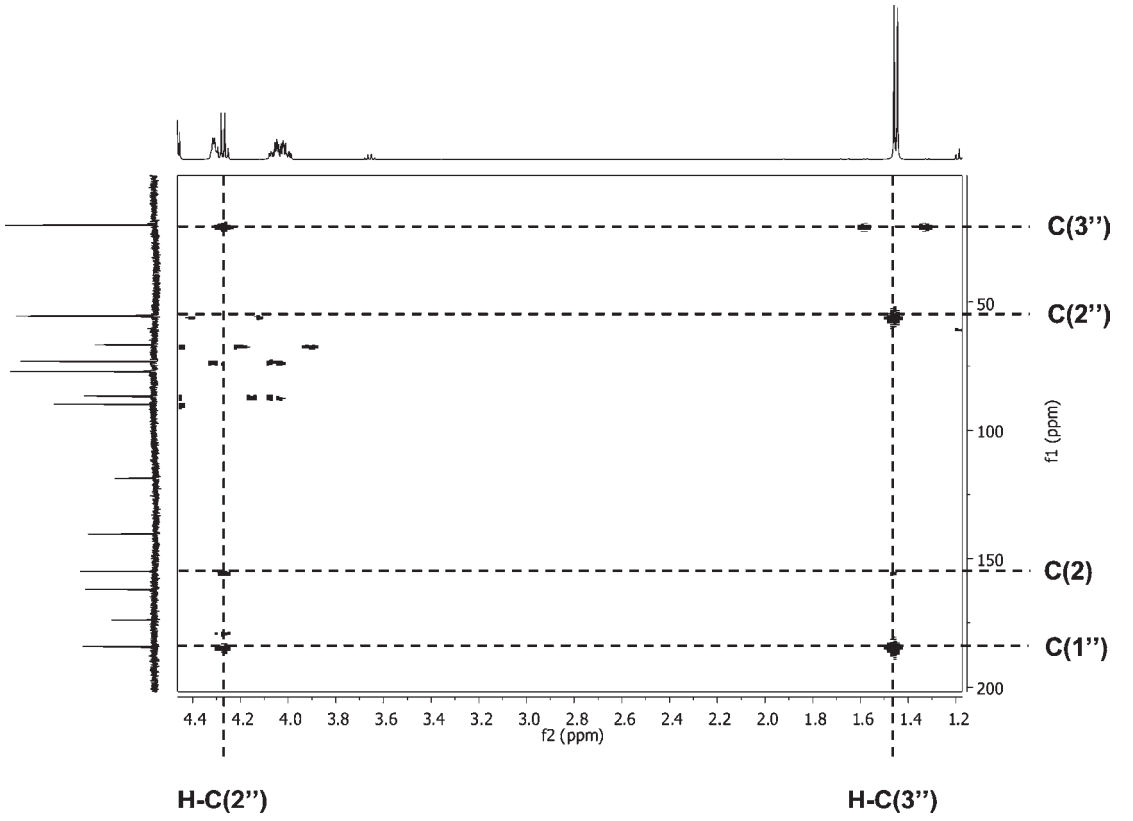

Figure 5. Excerpt from the HMBC spectrum (500 MHz, D_2O/NaOD) of fraction VIII-8/5. Arbitrary numbering of the carbon atoms refers to **Figure 6**.

protons. The signals of the 5′-GMP moiety were easily assigned by comparing the spectral data of the isolated compound with the ^{1}H NMR spectrum recorded for the disodium salt of 5′-GMP (**5**). As expected for the purine nucleotide, the signal of the aromatic proton H−C(8) resonated at 8.17 ppm and the protons of the ribose moiety were observed between 6.02 and 3.97 ppm. The anomeric proton H−C(1′) of the ribose moiety was assigned to the doublet found at 6.02 ppm and showing a coupling constant of 5.2 Hz. In addition to the 5′-GMP related signals, two additional proton signals were observed at 1.45 and 4.25 ppm, respectively. The resonance signal at 4.25 ppm, integrating for one proton and showing the multiplicity of a quartet, was assigned to H−C(2″) and showed homonuclear ^3J coupling (7.2 Hz) to the methyl group H−C(3″) resonating at 1.45 ppm. Furthermore, an HMBC experiment revealed that the proton H−C(2″) showed heteronuclear coupling to the guanine carbon atom C(2), observed at 155.0 ppm, as well as an additional C,H coupling to the carboxy carbon C(1″) resonating at 184.2 ppm (**Figure 5**). In addition, the ^{13}C NMR spectrum revealed C(2″) with a chemical shift of 55.4 ppm as expected for the α-carbon atom of an α-amino acid. These data verified the 5′-GMP derivative to be decorated with an aminocarboxylic acid structure in contrast to the recently reported 5′-GMP lactoylamide showing a resonance signal of 70.7 ppm for the α-carbon atom of the lactic acid amide moiety (*30*). With all of these spectroscopic data taken into consideration, the target compound was identified as the previously not reported (*R*)- or (*S*)-configured N^2-(1-carboxyethyl)guanosine 5′-monophosphate, (*R*)- or (*S*)-**6** (**Figure 6**), but its stereochemistry at C(2″) could not be clarified.

Maillard-Type Generation of N^2-(1-Carboxyethyl)guanosine 5′-Monophosphate (6) and Stereochemical Investigations. To unequivocally confirm the proposed structure of **6** and to clarify its stereochemistry at C(2″), authentic reference compounds needed to be synthesized for (*R*)- or (*S*)-**6** (**Figure 6**). Although the

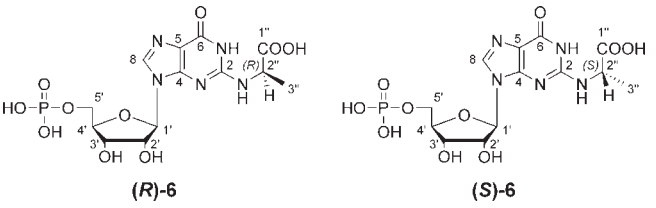

Figure 6. Structures of (*R*)-N^2-(1-carboxyethyl)guanosine 5′-monophosphate (**6a**) and (*S*)-N^2-(1-carboxyethyl)guanosine 5′-monophosphate (**6b**).

structure of compound **6** was not yet reported in literature, the structurally related compounds N^2-(1-carboxyethyl)-2′-deoxyguanosine (**7**) and N^2-(1-carboxyethyl)guanine (**8**; **Figure 7**) are known as advanced glycation end products, derived from the reaction of 2′-deoxyguanosine and guanine with highly reactive C_3 sugar breakdown products such as glyceraldehyde and dihydroxyacetone, respectively (*26*). To investigate whether (*R*)- or (*S*)-**6** is generated by a Maillard-type model reaction of 5′-GMP with C_3 sugar breakdown products, a binary aqueous solution of 5′-GMP (**5**) and DL-glyceraldehyde or 1,3-dihydroxyacetone, respectively, was incubated at pH 7.0 for 10 days at 40 °C. RP-HPLC analysis revealed that 5′-GMP was completely converted into two reaction products (**Figure 7**), which were isolated and purified by means of preparative RP-HPLC and, then, used for MS and NMR spectroscopic experiments. Comparison of chromatographic (RP-HPLC) and spectroscopic data (LC-MS, ^{1}H and ^{13}C NMR) as well as cochromatography demonstrated the later eluting compound to be identical with the stereoisomer of **6** isolated above from the yeast extract.

Next, the absolute stereochemistry of the chiral center C(2″) of the isomers of **6** isolated from the yeast extract and the Maillard reaction system, respectively, should be unequivocally identified.

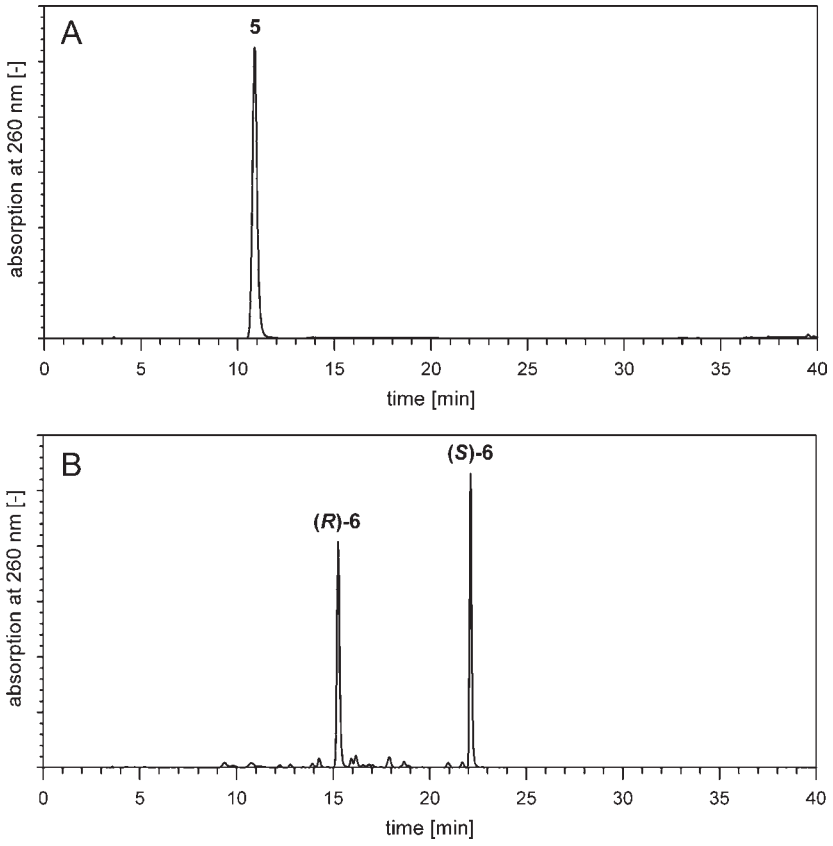

Figure 7. RP-HPLC chromatogram (λ = 260 nm) of an aqueous solution of 5′-GMP (**5**) and DL-glyceraldehyde before (**A**) and after incubation for 10 days at 40 °C (**B**).

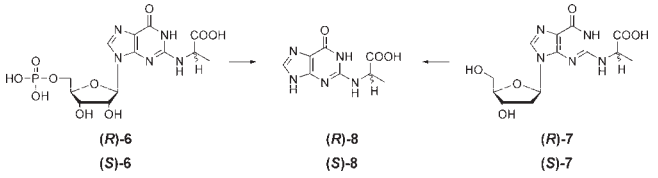

Figure 8. Acidic depurination of the stereoisomers of N^2-(1-carboxyethyl)guanosine 5′-monophosphate (**6**) and N^2-(1-carboxyethyl)-2′-deoxyguanosine (**7**) to give N^2-(1-carboxyethyl)guanine (**8**) as their common hydrolysis product.

To achieve this, the (*R*)- and (*S*)-isomers of N^2-(1-carboxyethyl)-2′-deoxyguanosine (**7**; **Figure 8**), both of which have been synthesized by independent chemical synthesis and assigned in their absolute configuration (*31*), were generated by the Maillard-type reaction of 2′-deoxyguanosine and 1,3-dihydroxyacetone and, after purification, the CD spectra of both stereoisomers were recorded. Comparison of the CD spectra obtained for the two diastereomers of the Maillard-type generated N^2-(1-carboxyethyl)-2′-deoxyguanosine (**Figure 9A**) with the CD data published in literature for the synthetic analogues (*31*) clearly revealed (*R*)-**7** as the earlier and (*S*)-**7** as the later eluting stereoisomer. Although (*R*)- and (*S*)-**7** were lacking the phosphate moiety as well as the chiral center at C(2′) of the sugar backbone when compared to the 5′-GMP derivative **6**, the earlier eluting stereoisomer of **6** showed the same orientations of the CD curve as (*R*)-**7** with a positive absorption maximum at 249 nm and a negative absorption maximum at 282 nm, and the CD spectrum of the later eluting diastereomer of **6** was the corresponding mirror image with a positive absorption maximum at 282 nm and a negative absorption maximum at 249 nm (**Figure 9B**).

To confirm that the CD spectra of the earlier as well as the later eluting stereoisomers of **6**, showing the same orientations of the CD curve as (*R*)- and (*S*)-**7**, respectively, are not influenced by the presence of the phosphate moiety as well as the additional chiral center at C(2′) of the ribose backbone in **6**, both stereoisomers of **6** and **7** structures were truncated to release the common feature of the N^2-(1-carboxyethyl)guanine, **8** (**Figure 8**). To achieve this depurination, both the (*R*)- and (*S*)-**7** and the earlier as well as the later eluting stereoisomers of **6** were individually hydrolyzed by treatment with hydrochloric acid, and the enantiomeric hydrolysis products (*R*)- and (*S*)-**8** were isolated individually and purified by means of preparative RP-HPLC. LC-MS and NMR spectroscopic analyses revealed the identical spectroscopic data for (*R*)- and (*S*)-**8**, being well in line with previous papers (*26*). The CD spectra of the hydrolysis products (*R*)- and (*S*)-**8** (**Figure 9C**) formed upon hydrolysis of (*R*)- and (*S*)-**7** were found to be identical to those of the hydrolysis products released from the earlier and later eluting stereoisomers of **6** (**Figure 9D**). These data clearly confirmed (*R*)-N^2-(1-carboxyethyl)guanosine 5′-monophosphate, (*R*)-**6**, as the earlier eluting isomer and (*S*)-N^2-(1-carboxyethyl)guanosine 5′-monophosphate, (*S*)-**8**, as the later eluting isomer of the umami enhancer isolated from yeast.

Sensory Activity of (*R*)- and (*S*)-6. To gain a first insight into the taste properties of (*R*)- and (*S*)-**6**, the recognition threshold concentrations for umami taste were determined in water (pH 6.0). Whereas the (*R*)-configured isomer showed an umami threshold of 0.85 mmol/L, the corresponding (*S*)-configured molecule exhibited an umami taste at a comparatively lower level of 0.19 mmol/L, which is identical to the threshold concentration of the parent nucleotide 5′-GMP (**Table 1**).

Although several purine 5′-ribonucleotides exhibit an intrinsic taste, the hallmark of umami taste is the pronounced synergism

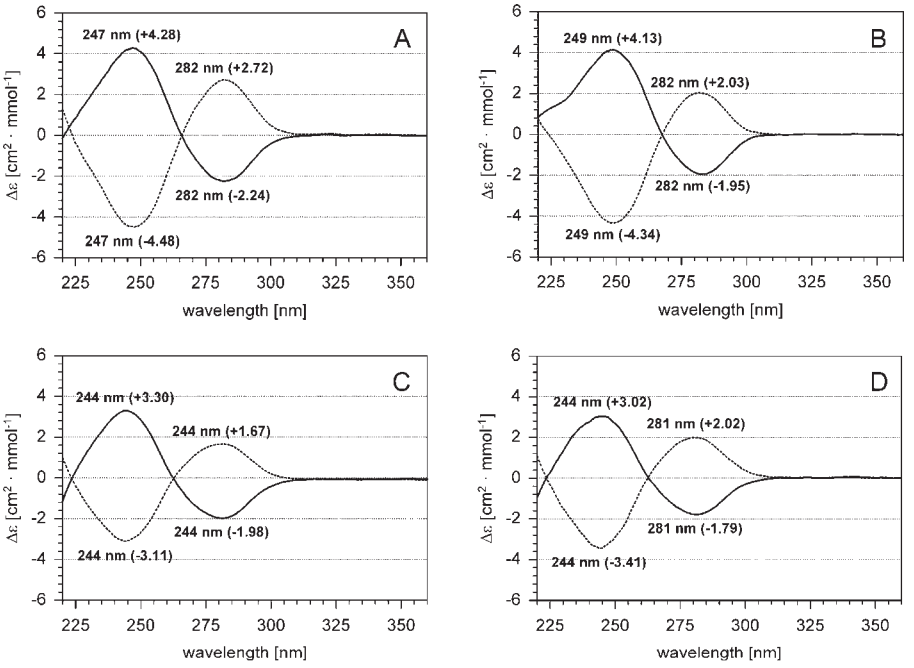

Figure 9. CD spectra of (**A**) N^2-(1-carboxyethyl)-2′-deoxyguanosine, (*R*)-**7** (solid line) and (*S*)-**7** (dotted line), (**B**) N^2-(1-carboxyethyl)guanosine 5′-monophosphate, (*R*)-**6** (solid line), and (*S*)-**6** (dotted line), as well as N^2-(1-carboxyethyl)guanine, (*R*)-**8** (solid line), and (*S*)-**8** (dotted line), obtained by acidic hydroysis of (**C**) (*R*)-**7** (solid line) and (*S*)-**7** (dotted line) and (**D**) (*R*)-**6** (solid line) and (*S*)-**6** (dotted line).

Table 1. Umami Recognition Threshold Concentration and Iso-umami Concentrations of 5′-GMP (**5**) and the Maillard Reaction Products (*R*)-**6** and (*S*)-**6**

	5[a]	(*R*)-**6**	(*S*)-**6**
taste threshold[b] (mmol/L)	0.15	0.85	0.19
iso-umami concn[c] (mmol/L)	45	25	45

[a] Applied as the disodium salt. [b] Taste recognition threshold concentration determined in bottled water by means of a triangle test. [c] Iso-umami concentration determined by comparison of the taste intensity of an aqueous binary solution of the nucleotide (0.1 mmol/L) and monosodium L-glutamate (10 mmol/L) with that of solutions of ascending monosodium L-glutamate concentrations (10−100 mmol/L, in 5 mmol/L steps; pH 6.0) using a duo test.

between those compounds and L-glutamate (*17*). To identify possible taste-enhancing properties of the 5′-GMP derivatives, an iso-umami test was performed. To achieve this, an aqueous binary solution of the test nucleotide (0.1 mmol/L) and monosodium L-glutamate (10 mmol/L) was compared to aqueous solutions containing ascending concentrations of monosodium L-glutamate alone in a duo test, and the sensory panelists were asked to determine the concentration of monosodium L-glutamate required to match the umami taste intensity of the binary nucleotide/monosodium L-glutamate mixture. As shown in **Table 1**, the sensory panel rated the mixture containing monosodium L-glutamate and 5′-GMP (**5**) with an iso-umami value of 45 mmol/L. In the case of the glycated compounds, once again a difference between both diastereomers was observed. Whereas (*R*)-**6** exhibited a significantly reduced taste-enhancing activity with an iso-umami value of 25 mmol/L, the enhancement activity of (*S*)-**6** was equivalent to that of **5**, but the umami taste sensation of the monosodium L-glutamate solution was described to be slightly sweetish and more full-bodied in the presence of (*S*)-**6** when compared to the parent nucleotide **5**. This needs to be more precisely investigated in future studies.

Verification of the Occurrence of (*R*)- and (*S*)-6 in Yeast Extract by Means of LC-MS/MS. To unequivocally confirm the presence of both diastereomers of **6** in the yeast extract, an aqueous solution of a sample of the yeast extract was membrane filtered

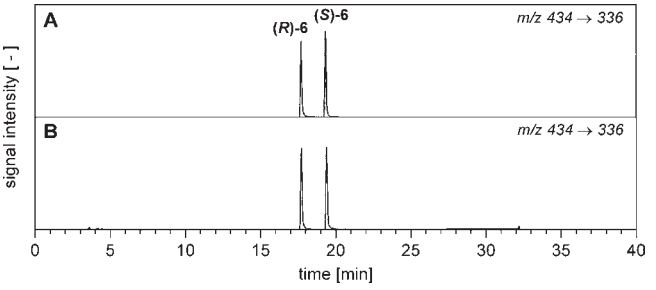

Figure 10. HPLC-MS/MS analysis of (*R*)-**6** and (*S*)-**6** in a reference solution (**A**) and a sample of the authentic yeast extract (**B**). Signal intensity of each mass transition is normalized. Peak numbering refers to the chemical structure given in **Figure 6**.

and separated by means of HPLC using a pentafluorophenylpropyl (PFP) column, and the effluent was screened for three selected mass transitions, namely, *m*/*z* 434→339, *m*/*z* 434→204, and *m*/*z* 434→79, for the identification of (*R*)- and (*S*)-**6** by means of tandem mass spectrometry. Comparison of the mass transitions and retention times of the synthesized reference compounds (**Figure 10A**) with those of the authentic yeast sample (**Figure 10B**) revealed two baseline-separated peaks for (*R*)- and (*S*)-**6**, thus demonstrating the presence of both N^2-(1-carboxyethyl)guanosine 5′-monophosphate diastereomers in the yeast extract. To the best of our knowledge, these umami-active sensometabolites were detected for the first time in a food product.

In conclusion, the present study shows for the first time the isolation and identification of umami-active N^2-(1-carboxyethyl)guanosine 5′-monophosphate diastereomers in a commercial yeast extract and its formation by a Maillard-type glycation of 5′-GMP with a C_3 sugar breakdown product. Studies on structure−activity relationships of a series of umami-tasting glycated 5′-GMP derivatives are ongoing and will be published elsewhere.

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