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Synthesis and Sensory Characterization of Novel Umami-Tasting Glutamate Glycoconjugates

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Two glycoconjugates of glutamic acid, namely, the *N*-glycoside dipotassium *N*-(D-glucos-1-yl)-L-glutamate (**1**) and the corresponding Amadori compound *N*-(1-deoxy-D-fructos-1-yl)-L-glutamic acid (**2**), have been synthesized in yields of 35 and 52%, respectively, using new Maillard-mimetic approaches, and their chemical structures have unequivocally been elucidated by 1D- and 2D-NMR and MS experiments. Systematic sensory studies revealed that both glycoconjugates exhibit pronounced umami-like taste with recognition taste thresholds of 1−2 mmol/L, close to that of monosodium glutamate (MSG). Contrary to an aqueous solution of MSG, **1** does not show the sweetish and slightly soapy by-note, but evokes an intense umami taste. Aqueous solutions of **2** were described by the descriptors umami, seasoning, and bouillon-like. Added to a bouillon base, which did not contain any taste enhancers, both glycoconjugates imparted a distinct umami character similar to the control sample containing the same amount of MSG on a molar basis. To the best of our knowledge, these types of glycoconjugates in general and, in particular, *N*-glucosyl glutamate and *N*-deoxyfructosyl glutamate have never been reported as taste active compounds having umami-like properties. Therefore, **1** and **2** represent a new class of umami-type taste compounds showing properties similar to the umami reference compound MSG. Systematic ^{13}C NMR measurements revealed that **1** was fairly stable in aqueous solutions under alkaline conditions (pH 8−10) as well as in dry form. However, it rapidly hydrolyzes in neutral and acidic solutions, giving rise to glucose and glutamate. In contrast, glycoconjugate **2** was observed to be rather stable in aqueous solution as well as in the presence of human saliva.

KEYWORDS: Umami; taste; *N*-glucoside; Maillard reaction; Amadori product; glutamate; MSG; *N*-(D-glucos-1-yl)-L-glutamate; *N*-(1-deoxy-D-fructos-1-yl)-L-glutamic acid; saliva

INTRODUCTION

The Japanese word "umami" means "delicious" and is used as a synonym for the characteristic sensory properties of monosodium glutamate, abbreviated as MSG (*1*). Very recent molecular-biological investigations led to the identification of a truncated form of the metabotropic glutamate receptor mGluR4, which is present in the brain, from rat taste buds and suggested this protein as a taste receptor for L-glutamate (*2*). The identification of this novel G-protein coupled receptor in taste tissue provided evidence for the existence of glutamate-like taste as a basic taste quality. This was further strengthened by the recent finding that two taste receptor proteins, namely, T1R1 and T1R3, are able to form a universal heterodimeric sensor for L-glutamate (*3*, *4*).

Besides glutamic acid, also adenosine-5′-monophosphate (5′-AMP), inosine-5′-monophosphate (5′-IMP), and guanosine-5′-monophosphate (5′-GMP) are well-known to show umami-like sensory characteristics (*5*). These purine-5′-nucleotides occur in many savory foods such as meat, fish, seafood, and mushrooms and are able to enhance the flavor and mouthfeel including the impression of creaminess and viscosity of savory dishes. Therefore, these are widely used as ingredients and taste enhancers in culinary and snack products.

In addition to their distinct sensory quality, another peculiar effect of these compounds is their mutual taste synergism. The synergistic effects between MSG and purine-5′-nucleotides and analogues of both groups were observed more than 35 years ago (*6*, *7*), reporting an exponential increase in the glutamate-like taste intensity of an aqueous solution of MSG when 5′-IMP was added. According to these results the taste activity of

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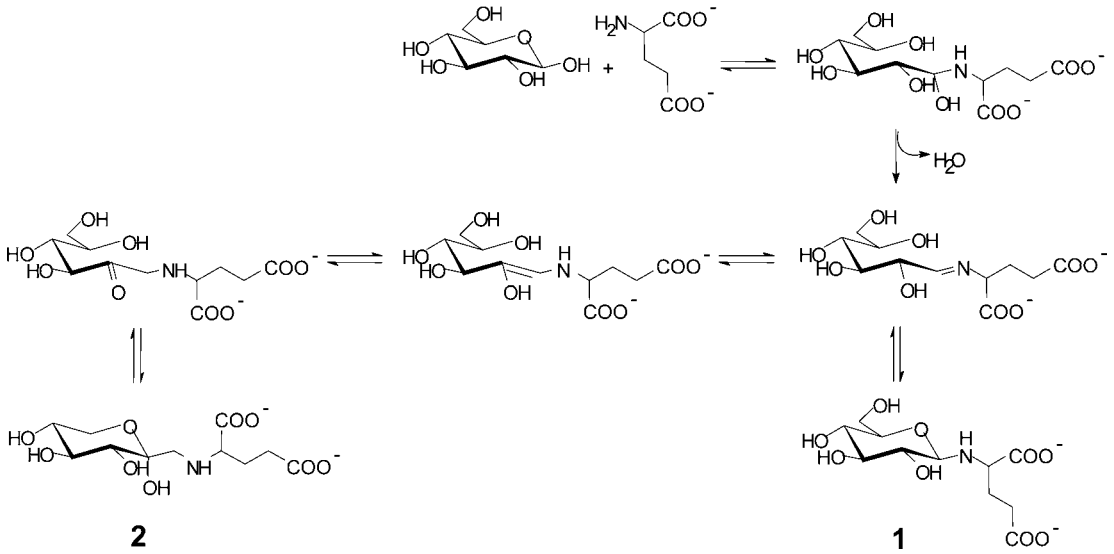


Figure 1. Formation of *N*-(D-glucos-1-yl)-L-glutamate (**1**) and *N*-(1-deoxyfructos-1-yl)-L-glutamate (**2**) from D-glucose and L-glutamic acid.

5′-GMP is 2.3-fold the effect of 5′-IMP, while monosodium aspartate possesses only about 7% of the potency of monosodium glutamate.

Investigations on the structural requirements in compounds exhibiting a taste-enhancing effect led to the suggestion that two negative charges at a distance of 3−9, preferably 4−6, carbon atoms are the essential structural motif required for taste enhancement (*8*). Apart from L-glutamic acid, its lower homologue L-aspartic acid also fulfills this postulate, as well as the C_4-dicarboxylic acids succinic acid and tartaric acid, exhibiting some kind of umami taste and meeting the structural requirement for two negative charges (*8*). Indeed, aqueous solutions of sodium succinate show taste qualities similar to solutions of sodium glutamate (*9*). Furthermore, lactic acid was found to contribute to the glutamate-like taste of foods such as beef bouillon and stewed beef juice (*10*, *11*).

Besides some amino acids, organic acids, and purine-5′-nucleotides, hydrophilic di-, tri-, and tetrapeptides containing polar side chains, such as Glu-Asp, Glu-Glu, Asp-Glu, Thr-Glu, Asp-Glu-Ser, Glu-Gly-Ser, and Asp-Asp-Asp-Asp, have also been described as eliciting a lingering umami-like taste and mouthfeel similar to that of MSG (*12*−*14*). The role of these peptides as umami compounds is, however, controversial. Some researchers described these di- and tripeptides as neutral or even slightly bitter and generally questioned the existence of umami peptides (*15*), whereas others reported that tripeptides such as Glu-Leu-Glu or Glu-Asp-Phe, having a hydrophobic amino acid residue and at least one acidic and another either acidic or hydrophilic amino acid residue, impart mouthfeel and a glutamate-like taste to foods (*16*). Particular emphasis was put on the so-called "delicious" peptide Lys-Gly-Asp-Glu-Glu-Ser-Leu-Ala, which was detected in papain-treated beef and allegedly has taste-enhancing qualities comparable to MSG (*17*). Recent synthetic and sensory experiments, however, could not confirm the glutamate-like taste properties of the octapeptide, but a sour and slightly astringent taste was detected (*18*−*20*).

Recently, *N*-lactoyl-L-glutamate, the condensation product of lactic acid and glutamic acid, was reported to evoke a bouillon-like umami taste and to have a long-lasting and mouth-watering sensation in combination with flavor-enhancing characteristics, although weaker compared to MSG (*16*). In agreement with the sensory qualities of *N*-lactoyl-L-glutamate assessed by a human taste panel, a comprehensive molecular-biological investigation demonstrated that *N*-lactoyl-L-glutamate activated the rat mGlu4a receptor and inhibited the binding of L-AP4, a selective L-glutamate agonist for mGlu4 receptors (*21*).

Besides the N-acylation of glutamic acid either by lactic acid or by hydrophilic amino acids, the Maillard reaction between glutamic acid and reducing sugars yields glutamic acid derivatives that might exhibit umami-like and mouth-watering sensations as well as flavor-enhancing characteristics. It is well accepted that the reaction between amino acids and aldoses first proceedes through the corresponding *N*-glycoside, **1** (**Figure 1**), which is, however, not stable and can be rearranged to produce the so-called Amadori product, **2** (**Figure 1**), as the first relatively stable amino acid glycoconjugate (*22*). Such Amadori products are important aroma precursors, formed as intermediates during drying or heat treatment of foods such as fruits, milk powder, cocoa, coffee, and meat (*23*−*25*). To the best of our knowledge, Amadori compounds, however, have not yet been reported as taste compounds exhibiting glutamate-like organoleptic properties. The objectives of the present investigation were, therefore, (i) to synthesize the glycoconjugates *N*-(glucos-1-yl)-L-glutamate and *N*-(1-deoxy-D-fructos-1-yl)-L-glutamic acid, (ii) to evaluate their organoleptic properties, and (iii) to study their stability in aqueous media.

MATERIALS AND METHODS

Chemicals. The following chemicals were obtained from commercial sources: monopotassium L-glutamate (MPG) monohydrate, anhydrous D-glucose, trifluoromethanesulfonic acid anhydride, trifluoro acetic acid (Aldrich, Buchs, Switzerland); anhydrous MPG (Sigma, Germany); sulfuric acid (98%), acetonitrile, formic acid, acetone, methanol (H_2O < 0.02%), hexane, ethyl acetate, dichloromethane, paraffin, potassium hydroxide (Merck, Darmstadt, Germany), D_2O (99.8%); ammonium acetate, sodium hydroxide, magnesium sulfate, hydrochloric acid, 2,6-di-*tert*-butyl-4-methylpyridine, *N*,*N*-dimethylformamide, glutamic acid dimethyl ester (Fluka, Buchs, Switzerland). HPLC grade water was purified using a Milli-Q system (Millipore, Volketswil, Switzerland). A model bouillon base without taste enhancers was used in sensory experiments (PTC Kemptthal, Switzerland).

Synthesis of Dipotassium *N*-(D-Glucos-1-yl)-L-glutamate (1). A solution of potassium hydroxide (167 mmol) in methanol (100 mL) was added to MPG monohydrate (167 mmol), and the mixture was stirred until the amino acid was completely dissolved. Methanol (300 mL) and anhydrous D-glucose (100 mmol) were then added, and the suspension was refluxed for 1 h at 75 °C while stirring. After cooling the reaction mixture to ambient temperature, the volume was reduced to 100 mL under vacuum, the surplus of the amino acid was removed

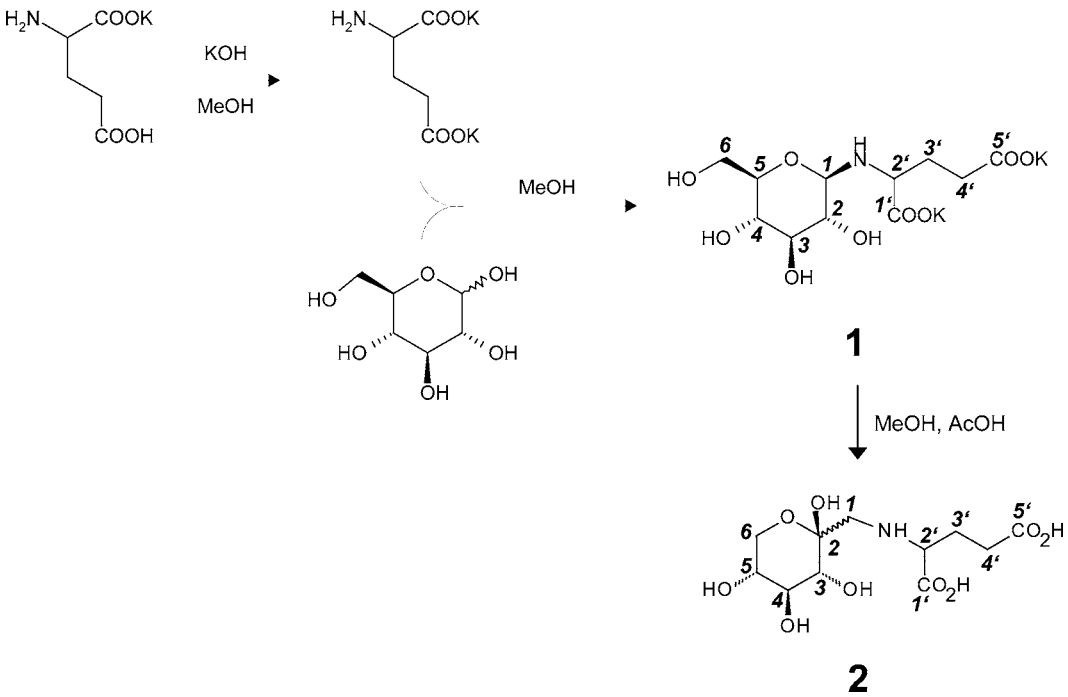


Figure 2. Preparation of dipotassium *N*-(D-glucos-1-yl)-L-glutamate (**1**) and *N*-(1-deoxyfructos-1-yl)-L-glutamic acid (**2**) using Maillard-mimetic chemistry.

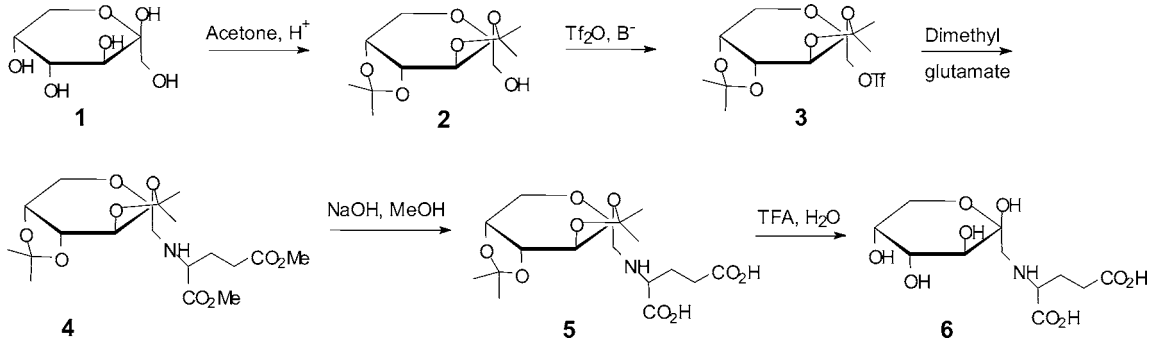


Figure 3. Reaction sequence used in the synthesis of *N*-(1-deoxyfructos-1-yl)-L-glutamic acid (**2**).

by filtration, and the filtrate was cooled to −20 °C. Dropwise addition of acetone precipitated the *N*-glucoside **1** as an amorphous powder. Concentration, cooling, and addition of acetone were repeated and yielded additional target compound. The two batches of crude **1** were combined and, according to the procedure described above, recrystallized twice from methanol/acetone, yielding pure dipotassium *N*-(D-glucos-1-yl)-L-glutamate (**1**; yield: 35%) as a white amorphous powder. LC/MS (ESI^-): *m*/*z* 146 (100), 89 (62), 75 (61), 97 (58), 61 (53), 59 (44) 121 (12), 179 (11), 326 (5), 308 (5). ^1H NMR (360 MHz; D_2O; DQF-COSY; arbitrary numbering of carbon atoms refers to structure **1** in **Figure 2**): δ 1.82 (m, 2H, H−C(3′)), 2.22 (m, 2H, H−C(4′)), 3.20 (dd, 1H, ^3J = 8.8 Hz, ^3J = 8.8 Hz, H−C(2)), 3.33−3.37 (m, 2 × 1H, H−C(4) and H−C(5)), 3.41 (m, 1H, H−C(2′)), 3.45 (m, 1H, H−C(3)), 3.67 (dd, 1H, ^2J = 12.3 Hz, ^3J = 5.4 Hz, H_a−C(6)), 3.85 (dd, 1H, ^2J = 12.3 Hz, ^3J = 0.8 Hz, H_b−C(6)), 3.89 (d, 1H, ^3J = 8.8 Hz, H−C(1)). ^{13}C NMR (360 MHz; D_2O; HMQC, HMBC): δ 33.1 (CH_2, C(3′)), 37.1 (CH_2, C(4′)), 63.0 (CH, C(2′)), 63.9 (CH_2, C(6)), 72.7 (CH, C(5)), 75.8 (CH, C(2)), 79.5 (CH, C(3)), 79.7 (CH, C(4)), 91.7 (CH, C(1)), 185.0 (C, C(1′)), 185.6 (C, C(5′)).

Synthesis of *N*-(1-Deoxy-D-fructos-1-yl)-L-glutamic Acid (2). *Via N-Glucoside 1*. Dipotassium *N*-(D-glucos-1-yl)-L-glutamate (**1**; 7.9 mmol) was refluxed for 30 min in a mixture of methanol (30 mL) and acetic acid (1 mL). After cooling to 0 °C, acetone (20 mL) was added, and the precipitate formed was filtered under reduced pressure and dried under high vacuum to yield a pale yellowish powder. This procedure was repeated twice. After dissolving the powder in water, followed by lyophilization, *N*-(1-deoxy-D-fructos-1-yl)-L-glutamic acid (**2**; 1.23 g; 52% yield) was obtained as a white powder. LC/MS (ESI^+): *m*/*z* 310 (100), 292 (92), 274 (24), 226 (10), 148 (4). ^1H NMR (360 MHz; D_2O; COSY; arbitrary numbering of carbon atoms refers to structure **2** in **Figure 2**): δ 2.04−2.10 (m, 1H, H−C(3′)), 2.39−2.55 (m, 3H, H−C(3′), H−C(4′)), 3.13 (d, 2H, ^2J = 14.5 Hz, H−C(1), 3.42−3.98 (m, 6H, H−C(2′), H−C(3), H−C(4), H−C(5), H−C(6)). ^{13}C NMR (360 MHz; D_2O/H_2O; HMQC, HMBC): δ 28.51 (CH_2, C(3′), 37.0 (CH_2, C(4′)), 55.5 (CH_2, C(1)), 66.2 (CH, C(2′)); 66.7 (CH_2, C(6)); 71.8 (CH, C(5)), 72.2 (CH, C(4)), 72.7 (CH, C(3)), 98.3 (C, C(2)), 176.3 (C, C(1′)), 183.9 (C, C(5′)).

Via D-Fructose. Following the general procedure described in the literature (*26*), *N*-(1-deoxy-D-fructos-1-yl)-L-glutamic acid (**2**) was synthesized via the intermediates 2,3:4,5-di-*O*-isopropylidene-*β*-D-fructopyranose (**3**), 2,3:4,5-di-*O*-isopropylidene-1-*O*-(trifluoromethansulfonyl)-*β*-D-fructopyranose (**4**; 76% yield), dimethyl *N*-(2,3:4,5-di-*O*-isopropylidene-1-deoxy-D-fructos-1-yl)-L-glutamate (**5**), and disodium *N*-(2,3:4,5-di-*O*-isopropylidene-1-deoxy-D-fructos-1-yl)-L-glutamate (**6**). Deprotection of the acetal groups in **6** at mild acidic conditions yielded the target product **2** in 80% yield.

*2,3:4,5-Di-O-isopropylidene-β-D-fructopyranose, 3 (**Figure 3**).* D-Fructose (**1**, 66 mmol) was added to a cooled mixture of concentrated sulfuric acid (11.6 mL) and acetone (233 mL). The suspension was stirred at room temperature until the sugar was dissolved. The solution was kept under these conditions for 90 min and then cooled in ice. An ice-cooled aqueous solution of sodium hydroxide (36.6 g) was gradually added while stirring. The acetone was removed by evaporation under reduced pressure, and the resulting aqueous suspension was extracted with dichloromethane (3 × 100 mL). The extracts were combined, washed with water (2 × 50 mL), and dried over $MgSO_4$. Evaporation

of the solvent yielded **3** as a crystalline solid (14.9 g) in 86% yield. ^{1}H NMR (360 MHz, $CDCl_3$): δ 1.32, 1.37, 1.45, 1.51 (4 s, 3H each, $C(CH_3)_2$), 2.35 (s, 1H, OH), 3.64 (2×d, 2H, ^2J = 7.9 Hz, H−C(1)), 3.74 (dd, 1H, ^2J = 13.2 Hz, ^3J = 1.0 Hz; H_a−C(6)), 3.90 (dd, 1H, ^2J = 13.2 Hz, ^3J = 2.0 Hz, H_b−C(6)), 4.21 (ddd, 1H, ^3J = 7.9, 2.0, 1.0 Hz, H−C(5)), 4.32 (d, 1H, ^3J = 2.6 Hz, H−C(3)), 4.59 (dd, 1H, ^3J = 7.9, 2.6 Hz, H−C(4)). ^{13}C NMR (90 MHz, $CDCl_3$): δ 23.9 (CH_3), 25.3 (CH_3), 25.7 (CH_3), 26.4 (CH_3), 61.2 (CH_2, C(6)), 65.4 (CH_2, C(1)), 70.0−70.9 (3 CH, C(3), C(4), C(5)), 103.1 (C, C(2)); 108.5 (C), 109.0 (C).

*2,3:4,5-Di-O-isopropylidene-1-O-trifluoromethanesulfonyl)-β-D-fructopyranose, **4** (**Figure 3**).* Trifluoromethanesulfonic acid anhydride (1.35 mL, 11 mmol) was added dropwise at −10 °C under an atmosphere of nitrogen to a solution of 2,6-di-*tert*-butyl-4-methylpyridine (2.26 g, 11 mmol) in dry dichloromethane (30 mL). The mixture was stirred and resulted in the formation of a precipitate. To this mixture was added dropwise a solution of **3** (1.25 g, 5.3 mmol) in dichloromethane (10 mL) while stirring. The reaction mixture was stirred for another 2 h before adding ice-cooled water. The solution was extracted with dichloromethane (8 × 30 mL). The combined organic extracts were dried over $MgSO_4$, and the solvent was evaporated. Column chromatography of the crude product on silica gel (hexane/ethyl acetate, 4:1, v/v) and evaporation of the solvent from the appropriate fractions gave **4** (1.45 g, 76%) as a yellow syrup. ^{1}H NMR (360 MHz, $CDCl_3$): δ 1.34, 1.39, 1.45, 1.55 (4 s, 3H each, $C(CH_3)_2$), 3.77 (dd, 1H, ^2J = 12.9 Hz, ^3J = 0.9 Hz, H_a−C(6)), 3.92 (dd, 1H, ^2J = 12.9 Hz, ^3J = 1.7 Hz, H_b−C(6)), 4.22 (ddd, 1H, ^3J = 7.9, 1.7, 0.9 Hz, H−C(5)), 4.30 (d, 1H, ^3J = 2.6 Hz, H−C(3)), 4.38 (d, 1H, ^2J = 10.5 Hz, H_a−C(1)), 4.50 (d, 1H, ^2J = 10.5 Hz, H_b−C(1)), 4.62 (dd, 1H, ^3J = 7.9, 2.6 Hz, H−C(4)). ^{13}C NMR (90 MHz, $CDCl_3$): δ 23.9 (CH_3), 25.0 (CH_3), 25.7 (CH_3), 26.5 (CH_3), 61.6 (CH_2, C(6)), 69.7−70.5 (3 CH, C(3), C(4), C(5)), 74.1 (CH_2, C(1)), 99.8 (C, C(2)), 109.2 (C), 109.8 (C), 118.0 (CF_3, q, J = 319.7 Hz).

*Dimethyl N-(2,3:4,5-Di-O-isopropylidene-1-deoxy-D-fructos-1-yl)-L-glutamate, **5** (**Figure 3**).* The triflate derivative **4** (620 mg, 1.58 mmol) was dissolved in anhydrous DMF (20 mL), and glutamic acid dimethyl ester (554 mg, 2 equiv) was added to the solution. The mixture was refluxed for 2 h, and the product formation was monitored by TLC (pentane/diethyl ether, 3:2). The reaction was stopped by adding water (25 mL), and the solution was extracted with dichloromethane (8 × 20 mL). The combined extracts were washed with tap water (6 × 20 mL), and the solvent was evaporated under reduced pressure. Column chromatography of the crude product on silica gel (hexane/ethyl acetate, 1:1, v/v) and evaporation of the solvent from the appropriate fractions yielded **5** (140 mg, 23%) as a yellow solid. ^{1}H NMR (360 MHz, $CDCl_3$): δ 1.24, 1.31, 1.37, 1.43 (4 s, 3H each, $C(CH_3)_2$), 1.74 (s, 1H, NH); 1.92−1.95 (m, 2H, H−C(3′)); 2.34 (t, 2H, H−C(4′), ^3J = 7.5 Hz); 2.72 (2×d, 2H, ^2J = 12.7 Hz, H−C(1)); 3.30 (t, 1, NCH), 3.57 (s, 3H, OCH_3), 3.61 (s, 3H, OCH_3), 3.65 (dd, 1H, ^2J = 13.1, 0.9 Hz, H_a−C(6)), 3.78 (dd, 1H, ^2J = 13.1, 2.0 Hz, H_b−C(6)); 4.12 (ddd, 1H, ^3J = 7.9, 2.0, 0.9 Hz, H−C(5)), 4.21 (d, 1H, ^3J = 2.4 Hz, H−C(3)), 4.47 (dd, 1H, ^3J = 7.9, 2.4 Hz, H−C(4)). ^{13}C NMR (90 MHz, $CDCl_3$): δ 24.4 (CH_3), 25.8 (CH_3), 26.2 (CH_3), 26.8 (CH_3), 27.8 (CH_2, C(3′)), 30.5 (CH_2, C(4′)), 52.0 (OCH_3), 52.1 (OCH_3), 53.6 (CH_2, C(1)), 60.8 (NCH), 61.5 (CH_2, C(6)), 70.4−71.9 (3 CH, C(3), C(4), C(5)), 103.7 (C, C(2)), 108.4 (C), 109.2 (C), 173.8 (CO); 175.2 (CO).

*Disodium N-(2,3:4,5-Di-O-isopropylidene-1-deoxy-D-fructos-1-yl)-L-glutamate, **6** (**Figure 3**).* **5** (140 mg, 0.36 mmol) was refluxed for 1 h in aqueous sodium hydroxide (1.0 mL, 2 N) and methanol (3.0 mL). The solvent was evaporated, and the crude salt (**6**, 23% yield) was used without further purification for the next step. ^{13}C NMR (90 MHz, D_2O): δ 28.2 (CH_3), 28.6 (CH_3), 29.4 (CH_3), 30.3 (CH_3), 30.6 (CH_2, C(3′)), 34.9 (CH_2, C(4′)), 53.5 (CH_2, C(1)), 65.9 (NCH), 69.6 (CH_2, C(6)), 74.7−76.6 (3 CH, C(3), C(4), C(5)), 108.6 (C, C(2)), 115.1 (C), 115.2 (C), 184.6 (CO), 184.8 (CO).

*N-(1-Deoxy-D-fructos-1-yl)-L-glutamic Acid (**2**).* The disodium salt (**6**) was dissolved in trifluoroacetic acid/water (9:1, v/v, 1 mL) and stirred for 2 h at room temperature. The reagent and the solvent were evaporated under reduced pressure, and the Amadori compound was crystallized from diethyl ether (92 mg, 82% yield) to give a pale yellow solid.

Sensory Analyses. *Taste Quality.* The samples used for sensory evaluation were passed through a sterile filter to obtain a microbiologically safe product. For preliminary taste testing, monosodium glutamate (MSG), a binary mixture of MSG and sodium chloride (NaCl), the dipotassium salt of *N*-glucosylglutamate (**1**), and *N*-(1-deoxy-D-fructos-1-yl)-L-glutamic acid (**2**) were dissolved in a concentration of 10 mmol/L in a mixture (1:3, v/v) of bottled water (Vitel, low mineralization: 405 mg/L) and deionized filtered water (Millipore) to prevent a bitter taste produced by deionized water and to avoid a taste influence of tap water with a higher mineral content. The solution was adjusted to pH 6.0 by addition of aqueous solutions of sodium hydroxide (1.0 mol/L) and hydrochloric acid (1.0 mol/L), respectively. The samples were presented to the sensory panel in coded beakers in alternating order at room temperature (22 °C), and five panelists were asked to describe the taste of the samples using their own descriptors.

Determination of Recognition Taste Thresholds. Taste thresholds were determined by eight panelists using the triangle test. Seven samples of increasing concentrations were presented, i.e., from 0.16 to 12.5 mmol/L, without adjusting the pH of the solutions.

Sensory Experiments with a Model Bouillon Base. A preliminary taste testing with the *N*-glucoside **1** (10 mmol/L) and the Amadori product **2** (10 mmol/L), respectively, was performed using a model bouillon base (19 g/L) that did not contain any taste enhancer, such as MSG, IMP, or GMP. By the addition of an aqueous solution of hydrochloric acid (1.0 mol/L) the pH of the mixture was adjusted to 5.8, corresponding to the pH of a solution of the bouillon base alone (19 g/L). The bouillon containing one of the glycoconjugates was compared to a solution of the bouillon base alone and to a solution of the bouillon base with added MSG (10 mmol/L, pH 5.8). The bouillon samples were tasted at 65 °C in coded beakers. The panelists were asked to describe the taste of the samples using their own descriptors and to compare the taste intensities of the solutions.

Stability of Dipotassium *N*-(D-Glucos-1-yl)-L-glutamate (1) and *N*-(1-Deoxy-D-fructos-1-yl)-L-glutamate (2). Solutions of **1** (50 mg) or **2** (50 mg) in D_2O/H_2O (10:50, v/v; 0.7 mL) were prepared and transferred to an NMR tube after pH adjustment with sulfuric acid. ^{13}C NMR experiments were performed on a regular basis over a defined period of time.

Influence of Human Saliva on the Stability of Dipotassium *N*-(D-Glucos-1-yl)-L-glutamate (1) and *N*-(1-Deoxy-D-fructos-1-yl)-L-glutamate (2). Solutions of glycoconjugates **1** and **2** (10 mg/mL each), respectively, in either phosphate buffer (pH 7.4) or human saliva, which was obtained from 3 women and 3 men, were incubated at 37 °C in closed vials. After 0, 5, and 20 min, samples were withdrawn and subsequently analyzed by an LC 3000 type amino acid analyzer (Biotronic, Maintal, Germany) using ninhydrin detection.

Direct Infusion Mass Spectrometry. Mass spectrometric experiments were carried out on a Micromass Quattro-LC triple quadrupole mass spectrometer (Micromass, Manchester, UK) equipped with a "Z-Spray" electrospray ion source. The electrospray capillary voltage was set to 4.0 kV, and the source block temperature to 80 °C. The cone gas was operated at 70 L/h, and desolvation gas at 550 L/h. The desolvation temperature was set to 150 °C. The samples were introduced at 10 μL/min in a mixture of methanol/water (1:1, v/v). Data were acquired in negative mode from 50 to 800 Da with a dwell time of 1 s. Data acquisition was performed using the software package MassLynx 3.4 (Micromass, Manchester, UK).

Nuclear Magnetic Resonance Spectroscopy (NMR). The samples for NMR spectroscopy were prepared in Wilmad 528-PP 5 mm Pyrex NMR tubes, using D_2O/H_2O (3:7, v/v) as the solvent (0.7 mL). The NMR spectra were acquired on a Bruker AM-360 spectrometer, equipped with a quadrinuclear 5 mm probe head, at 360.13 MHz for ^{1}H and at 75.56 MHz for ^{13}C. Chemical shifts given in ppm are relative to the solvent signal. One-dimensional ^{1}H NMR, ^{13}C NMR, distortionless enhancement by polarization transfer (DEPT 135), and two-dimensional COSY and HETCOR spectra were acquired as described previously using standard conditions (*27*).

RESULTS AND DISCUSSION

Syntheses of Glycoconjugates. It is well-known in the literature that the reaction of amino compounds with reducing

sugars leads to *N*-glycosides, **1** (**Figure 1**) as the first reaction products (*22*), which, however, rapidly rearrange into the so-called Amadori products, **2** (**Figure 1**) (*28*). Our preliminary studies on the formation of these glycoconjugates indicated that it is possible to stop the reaction of glucose and glutamate at the level of *N*-glucosyl glutamate (**1**), when the reaction was performed under nonaqueous, alkaline conditions (*29*). Using a Maillard-mimetic approach, dipotassium *N*-(D-glucos-1-yl)-L-glutamate (**1**) was obtained by a new and simple synthetic route in two steps starting from monopotassium L-glutamate, which was first solubilized in anhydrous methanol in the presence of potassium hydroxide and then coupled with D-glucose under reflux conditions (**Figure 2**). After precipitation, the *N*-glucoside (**1**) was obtained in an overall yield of about 40%. Absolute anhydrous conditions were mandatory to avoid hydrolysis of the *N*-glucoside into the educts or its rearrangement into the Amadori compound. On the other hand, the latter reaction offered an elegant synthetic approach toward the *N*-(1-deoxy-D-fructos-1-yl)-L-glutamic acid, **2** (**Figure 2**). Acidic, but anhydrous conditions allowed dehydration and enolization, affording the Amadori rearrangement product **2** in about 50% yield (**Figure 2**). This is a new synthetic pathway to obtain Amadori compounds in relatively high yields, which also allows the preparation of **2** in a multigram pilot scale as well (data not shown).

As an alternative to the Maillard-mimetic approach, a synthetic route to **2** was developed starting from β-D-fructose, which first was successively protected in the positions C(2)/C(3) and C(4)/C(5) with acetone (*26*). The intermediate 2,3:4,5-di-*O*-isopropylidene-β-D-fructopyranose, **3** (**Figure 3**), was then activated at the C-1 position by a triflate group, giving rise to 2,3:4,5-di-*O*-isopropylidene-1-*O*-(trifluoromethansulfonyl)-β-D-fructopyranose (**4**) in 76% yield. The leaving group was then substituted by glutamic acid dimethyl ester in a 23% yield. The substitution reaction resulted in dimethyl *N*-(2,3:4,5-di-*O*-isopropylidene-1-deoxy-D-fructos-1-yl)-L-glutamate (**5**). However, an intramolecular cyclization of the glutamate moiety was also observed leading to the corresponding pyrrolidone derivative. Alkaline treatment of **5** gave rise to the acetal-protected glutamate derivative disodium *N*-(2,3:4,5-di-*O*-isopropylidene-1-deoxy-D-fructos-1-yl)-L-glutamate (**6**) by saponification and, in the case of the pyrrolidone derivative, opening of the lactam ring. Deprotection of the acetal groups in **6** under mild acidic conditions yielded the target product **2** in 80% yield (**Figure 3**).

Structural Analysis of Glycoconjugates 1 and 2. The purified dipotassium salt of *N*-(D-glucos-1-yl)-L-glutamic acid (**1**) was characterized by various 1D- and 2D-NMR as well as MS techniques. ^{13}C NMR spectroscopy in combination with a DEPT-135 experiment showed 11 resonance signals, six of which corresponded to methine and methyl carbon atoms, three as methylene carbon atoms, and two as quaternary carbon atoms. The chemical shifts of the two carboxylate groups resonating at 185.6 and 185.0 ppm clearly showed that these are present in their deprotonated form. By means of homonuclear H,H-correlation spectroscopy (COSY) (**Figure 4**) and heteronuclear C,H-correlation spectroscopy (HETCOR), the complete set of ^{1}H and ^{13}C signals could be unequivocally assigned. HETCOR (data not shown) defined the anomeric proton at 3.89 ppm, which coupled with the carbon at 91.7 ppm. This carbon resonance signal was assigned as the anomeric carbon, thus proving the *N*-glucosyl-type structure of compound **1**. From this signal, all other proton shifts of the sugar skeleton could be attributed in the COSY experiment (**Figure 4**). As expected,

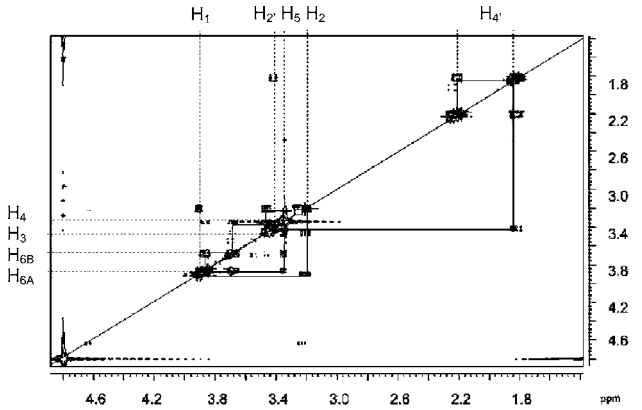


Figure 4. COSY spectrum of dipotassium *N*-(D-glucos-1-yl)-L-glutamate (**1**).

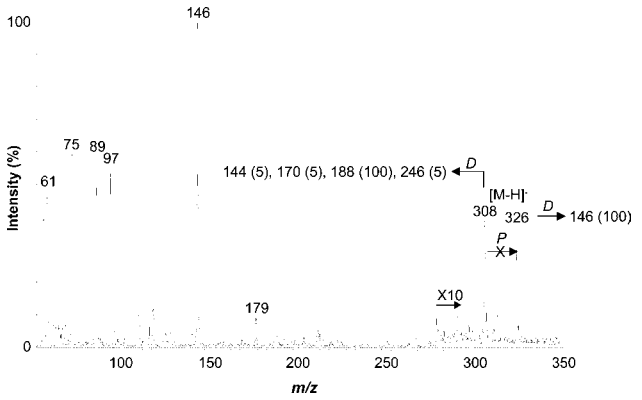
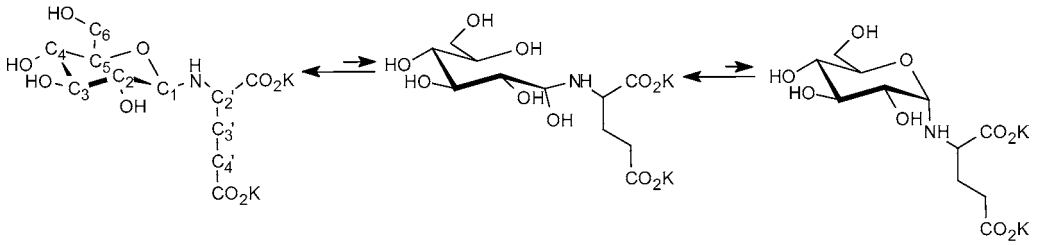


Figure 5. MS-ESI(−) spectrum of the dipotassium salt of *N*-(D-glucos-1-yl)-L-glutamic acid (**1**; 10 ng/μL). D, daughter ions; P, parent ions.

the carbons of the five CHOH groups of the sugar moiety were shifted between 79.7 and 72.7 ppm, whereas the CH_2OH group was detected at 63.9 ppm. The signals at 63.0, 37.1, and 33.1 ppm were assigned as the methine and the two methylene groups of the glutamate backbone, respectively. The presence of only three methylene protons ruled out the Amadori structure in **1** and confirmed the structure of an *N*-glucoside. Moreover, the anomeric proton at 3.89 ppm was observed as a doublet with a coupling constant of $^{3}J = 8.8$ Hz, which is characteristic for a *trans* axial−axial coupling. These data suggest the β-glucopyranose as the main form of *N*-glucoside **1**.

Direct infusion mass spectrometry operating in the negative electrospray ionization mode confirmed the molecular weight and the proposed structure of **1**. In dilute solutions of 10 ng/μL, the ion at *m*/*z* 326, corresponding to the molecular ion of the open-chain form, was found to be associated with an ion at *m*/*z* 308, which was suggested to be the cyclic form of the molecule (**Figure 5**). MS/MS experiments of the ions *m*/*z* 308 and 326 confirmed the presence of both compounds occurring individually in the synthesized sample. Fragmentation of the molecular ion *m*/*z* 326 resulted only in *m*/*z* 146 as the daughter ion, thus confirming that *m*/*z* 308 was not formed by dehydration of *m*/*z* 326. In agreement with these observations, parent ion measurement of *m*/*z* 308 did not yield the ion *m*/*z* 326, thereby confirming the presence of an equilibrium between the cyclic and the open-chain form of **1** (**Figure 6**).

The Amadori-type structure of *N*-(1-deoxy-D-fructos-1-yl)-L-glutamic acid (**2**) was mainly determined by ^{13}C NMR/DEPT 135 data indicating four methylene carbons out of 11 carbon signals, instead of three as detected for the *N*-glucoside **1**. In agreement with these suggestions, C(1) in compound **2** was



1a 1b 1c

Figure 6. Mutarotation of dipotassium *N*-(D-glucos-1-yl)-L-glutamate (**1**).

Table 1. Recognition Threshold Concentrations for the Umami Taste of *N*-Glucoside **1**, the Amadori Product **2**, and Monosodium Glutamate

compound	threshold concentration [mmol/kg][b]
dipotassium *N*-(D-glucos-1-yl)-L-glutamate (**1**)[a]	1.6
N-(1-deoxy-D-fructos-1-yl)-L-glutamate (**2**)[a]	1.8
monosodium glutamate (MSG)	1.5

[a] Structure of compound is given in **Figure 1**. [b] The umami taste threshold was determined in water using the triangle test.

clearly defined as a methylene carbon, instead of the methine carbon in **1**. Furthermore, carbon C(2) was represented by a quaternary carbon in **2**, whereas C(2) was assigned as a methine carbon in **1**.

Sensory Properties of the Glycoconjugates. Prior to sensory analysis, the purity of the glycoconjugates **1** and **2** was checked by LC/MS (*30*) and ^{1}H NMR spectroscopy. Preliminary sensory experiments revealed a pronounced umami-like taste character of aqueous solutions of **1** and **2**, respectively. To study the sensory activity of these compounds, the recognition thresholds of **1** and **2** for the umami taste were determined by means of triangle tests. Both glycoconjugates showed taste thresholds between 1.5 and 2.0 mmol/L, which is close to the threshold concentration of MSG (**Table 1**).

In addition, the sensory panel was asked to describe the taste profile and to rate the perceived taste intensity of aqueous, equimolar solutions of the glycoconjugates as well as MSG, either alone or in mixture with NaCl, on a scale from 0 (not perceived) to 3 (perceived as intense). Dipotassium *N*-(D-glucos-1-yl)-L-glutamate (**1**) on its own revealed a bouillon-like, long-lasting taste sensation and a more pronounced umami character (intensity 2.0) as compared to MSG, which was evaluated with a score of 1−2 (**Table 2**). Contrary to an aqueous solution of MSG, **1** did not show the sweetish and slightly soapy note, but evoked an intense umami taste in aqueous solutions. In addition, **1** elicited a distinct mouthfeel effect comparable to that observed for MSG (**Table 2**). The bouillon-like taste of **1** was further enhanced when sodium chloride was present in equimolar concentrations; that is, the mixture was evaluated with an intensity of 3 and was similar to the binary mixture of MSG and NaCl (**Table 2**). Also the binary mixture of **1** and MSG was rated with an intensity of 3 and was described to impart an umami, bouillon-like, viscous, and lingering taste sensation. The solution containing MSG alone was judged only with an intensity of 1−2. Similar to glycoconjugate **1**, the *N*-(1-deoxy-D-fructos-1-yl)-L-glutamate (**2**) showed the same umami and bouillon-like overall sensation with an intensity of 1−2 as observed for MSG (**Table 2**).

To evaluate the potential of these glycoconjugates in food-type applications, the impact of **1** and **2** on the taste profile of a model bouillon base was evaluated. The model bouillon base,

Table 2. Sensory Profiling of Aqueous Solutions of Monosodium Glutamate (MSG), Dipotassium *N*-(D-Glucos-1-yl)-L-glutamate (**1**), and *N*-(1-Deoxy-D-fructos-1-yl)-L-glutamate (**2**)[a]

sample	quality attributes	intensity
MSG	umami, sweet, slightly soapy, mouthfeel, lingering sensation	1–2
MSG+NaCl	salty, umami, bouillon-like, mouthfeel, long-lasting taste sensation	3
1	bouillon-like, sweet, mouthfeel	2
1+NaCl	salty, umami, bouillon-like, glutamate-like, long-lasting taste sensation	3
1+MSG	umami, bouillon-like, sweet, mouthfeel, viscous and lingering taste sensation	3
2	umami, bouillon-like, seasoning-like, mouthfeel	1–2

[a] The samples (10 mmol/L each) were dissolved in water (pH 6.0) and instantaneously evaluated at 22 °C by six sensory panelists on a scale from 0 (not perceived) to 3 (intensely perceived).

Table 3. Influence of Monosodium Glutamate (MSG), *N*-(D-Glucos-1-yl)-L-glutamate (**1**), and *N*-(1-Deoxy-D-fructos-1-yl)-L-glutamate (**2**) on the Taste Profile of a Model Bouillon Base

additive[a]	quality attributes	intensity
(no additive)	salty, bland, fatty, weak umami taste	1
MSG	umami, bouillon-like, salty, slightly sweet	3
1	umami, bouillon-like, salty	2–3
2	umami, bouillon-like, salty, fresh	2–3

[a] A bouillon base (19 g/L; pH 5.8) was evaluated in either the absence or presence of additives (10 mmol/L) at 65 °C by five sensory panelists on a scale from 0 (not perceived) to 3 (intensely perceived).

which was essentially free of any taste enhancer, such as MSG or purine-5′-ribonucleotides, was spiked with either the *N*-glucoside (**1**) or the Amadori product **2**, and the taste qualities were compared either to a bouillon base containing MSG or to an aqueous solution of the bouillon base alone. The pure bouillon base was evaluated with an overall intensity of 1 and was perceived as bland and very salty with only a weak glutamate-like taste (**Table 3**). The bouillon spiked with glycoconjugate **1**, however, elicited a bouillon-like umami taste with an intensity of 2−3, which was close to that with added MSG (intensity 3). Similar to the effect of **1**, the addition of *N*-(1-deoxy-D-fructos-1-yl)-L-glutamate (**2**) imparted a pronounced umami and bouillon-like taste (**Table 3**).

To the best of our knowledge, these types of glycoconjugates in general and *N*-glucosyl glutamate and *N*-deoxyfructosyl glutamate in particular have never been reported as taste active compounds exhibiting umami-like properties. Therefore, glycosylglutamates represent a new class of umami-type tasting compounds showing properties similar to the umami reference compound MSG. While the occurrence of the *N*-glucoside (**1**)

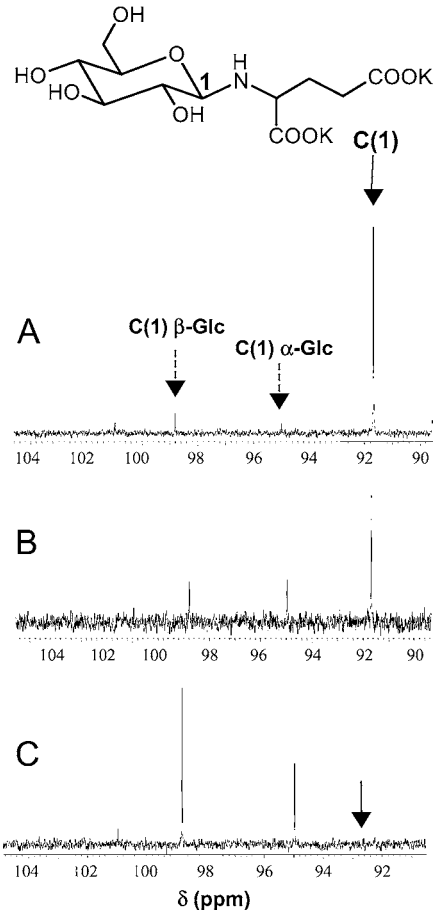


Figure 7. ^{13}C NMR spectra of dipotassium *N*-(D-glucos-1-yl)-L-glutamate in D_2O at (A) pH 9.3, (B) pH 6.5, and (C) pH 2.3 after 15 min of sample preparation. Full arrow indicates the C(1) signal at 91.7 ppm for **1**; the dotted arrows represent the anomeric C atoms of free glucose (95.0, 98.8 ppm).

in foods has never been reported, the Amadori compound **2** is a known constituent of processed foods; for example, high amounts of up to 3.6 g of **2** per 100 g of dry matter have been reported in dried tomatoes (*31*), but it has also been found in dried celery, asparagus, cauliflower, carrots, and red pepper as well as in dark malts (*25*, *31*).

Stability of Glycoconjugates 1 and 2. Preliminary analytical measurements on the stability of **1** by means of RP-HPLC and high-performance anion exchange chromatography, respectively, indicated L-glutamate and glucose as the major hydrolysis products (data not shown), thus suggesting that the *N*-glucoside **1** was relatively unstable in water. To gain a more detailed insight into the stability of the *N*-glucoside, aqueous solutions of **1** were incubated in NMR tubes as function of temperature, reaction time, and pH value, and the hydrolysis of **1** was followed by ^{13}C NMR spectroscopy by monitoring the chemical shift range between 90 and 100 ppm (**Figure 7**). The release of free glucose was indicated by the increase of the resonance signals at 95.0 and 98.8 ppm corresponding to the α- and β-anomeric carbon atom of the hexose, respectively. As displayed in **Figure 7**, the N-glucoside **1** was found to be stable after an incubation time of 15 min at pH 9.3 (sample A) as the signals representing the anomeric carbon atoms of free glucose remained unchanged. In contrast, the lower pH of 6.5 (sample B) induced a partial hydrolysis of **1** as indicated by the two anomeric carbon atoms of glucose. Under more acidic conditions (sample C), hydrolysis occurred nearly instantaneously; for example, no resonance signal of **1** was detectable when the *N*-glucoside was maintained at pH 2.3.

To investigate the kinetics of the degradation of the dipotassium salt of *N*-(D-glucos-1-yl)-L-glutamate by hydrolysis, **1** was maintained at room temperature under different pH conditions, and the hydrolysis was monitored by ^{13}C NMR spectroscopy over time. As shown in **Figure 8A**, **1** was hydrolyzed relatively slowly under alkaline conditions. After 24 h at pH 10, the *N*-glucoside was still the major constituent with some glucose liberated. With time, however, the degree of hydrolysis increased. After about 1 week, glucose prevailed. The stability of **1** at pH 10 is well reflected in the long half-life, $\tau_{1/2}$, of about 4 days (**Figure 8A**). In contrast, decomposition of **1** was found to be faster under neutral and slightly acidic conditions (**Figure 8B**). The low pH of 2.5 induced a spontaneous decomposition of **1**, which did not allow the measurement of the half-life (data not shown). Between these extreme pH values, the rate of decomposition increased with acidity; for example, the $\tau_{1/2}$ of **1** was about 10 h at pH 7.5 and 2.5 h at pH 5.0 (**Figure 8B**). The hydrolytic instability of the *N*-glycosidic bond in *N*-(D-glucos-1-yl)-L-glutamate (**1**) under acidic and neutral pH conditions is well in line with earlier observations on the stability of *N*-glycosides of amino acids in aqueous solutions (*32*, *33*).

As the *N*-glucoside **1** was shown to be rapidly degraded by acid-catalyzed hydrolysis, the question arose as to whether the umami taste of aqueous solutions of **1** is imparted by the *N*-glucoside itself or by the free L-glutamate liberated upon

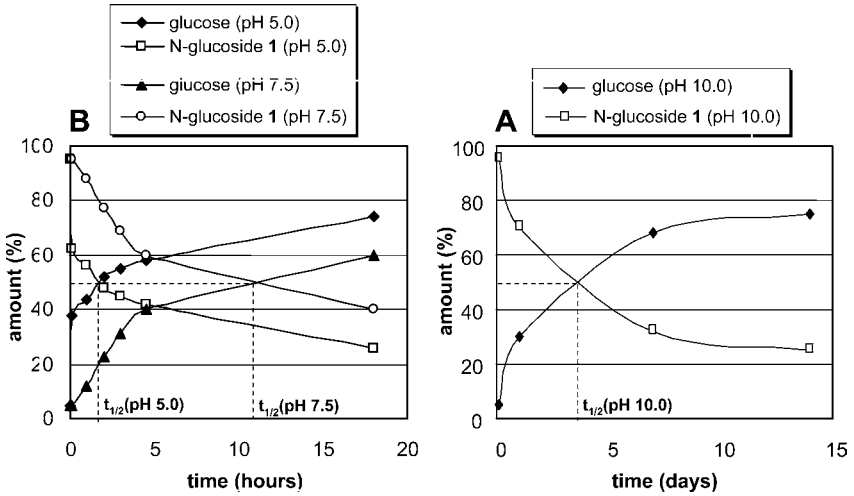


Figure 8. Influence of the pH on the time course of dipotassium *N*-(D-glucos-1-yl)-L-glutamate (**1**) decomposition.

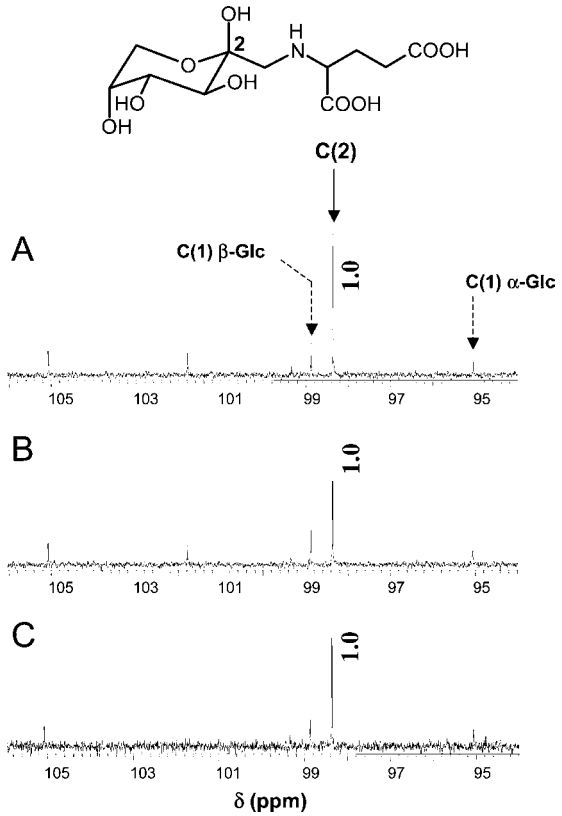


Figure 9. ^{13}C NMR spectra of *N*-(1-deoxy-D-fructos-1-yl)-L-glutamic acid (**2**) in aqueous solution (pH 5.0) after 3 h (A), 24 h (B), and 96 h (C). The full arrow indicates the C(2) signal at 98.3 ppm; the dotted arrows represent the anomeric C atoms of free glucose (95.0, 98.8 ppm). Trace amounts of glucose were present in the purified target compound.

hydrolysis. To answer that question, the pH of an aqueous solution of **1** was measured depending on its concentration. Because the dipotassium *N*-(D-glucos-1-yl)-L-glutamate (**1**) is a base, as expected, the pH of an aqueous solution was significantly changed with its concentration. The pH was found to be stable at concentrations above 1.3 g/L (3.3 mmol/L), slowly approximating a value of 9.1. Under these conditions, the half-life of **1** was $\tau_{1/2} \geq 3$ days (data not shown). The same concentration of **1** (>3.3 mmol/L) in a buffered solution at pH 7.0 resulted in a half-life of $\tau_{1/2} \approx 10$ h. Consequently, the umami taste threshold determined to be 1.6 mmol/L is without any doubt evoked by compound **1** and not by free L-glutamate possibly released upon hydrolysis.

In contrast to *N*-glucosyl-L-glutamate (**1**), ^{13}C NMR measurements of aqueous solutions containing the Amadori compound *N*-(1-deoxy-D-fructos-1-yl)-L-glutamic acid (**2**) revealed this glycoconjugate to be rather stable (**Figure 9**). No increase of the intensity of the resonance signal of the α- (95.0 ppm) and *β*-anomeric carbon atom (98.8 ppm) of the free hexose could be observed at pH 5.0, even after 3 days of storage at room temperature.

Although compounds **1** and **2** were evaluated as rather stable at moderate pH values for at least some hours, it was further investigated whether the umami taste is caused by these glycoconjugates theirselves or by hydrolysis products formed in the oral cavity, e.g., by an enzymatic cleavage of Amadoriase-type enzymes. To achieve this, glycoconjugates **1** and **2**, respectively, were dissolved in human saliva and were incubated in vitro at 37 °C. As the control sample, these compounds were incubated separately in aqueous solution. After 0, 5, and 20 min of incubation at 37 °C, samples were analyzed for free L-glutamic acid. These quantification experiments revealed that after 5 min no significant glutamate release could be measured from the *N*-glucoside in the presence of either saliva or water (control). Increasing the incubation time to 20 min led, however, to the liberation of 12 and 9% glutamate, respectively, from glycoconjugate **1** when maintained in the presence of saliva or water (data not shown). These data show a stability of the *N*-glucoside in human saliva similar to that found in pure aqueous solution. In contrary, neither in the presence of human saliva nor in the aqueous control system were significant amounts of free L-glutamic acid formed from compound **2** (data not shown), thereby clearly demonstrating that the Amadori product is also stable in the presence of human saliva. In summary, these data clearly show that the umami taste of **1** and **2**, which is perceived immediately after taking these compounds into the oral cavity, is caused by the glycoconjugates thereselves, and not by free glutamic acid liberated therefrom.

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