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Discovery and Structure Determination of a Novel Maillard-Derived Sweetness Enhancer by Application of the Comparative Taste Dilution Analysis (cTDA)

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Application of a novel screening procedure, the comparative taste dilution analysis (cTDA), on the non-solvent-extractable reaction products formed in a thermally processed aqueous solution of glucose and L-alanine led to the discovery of the presence of a sweetness- enhancing Maillard reaction product. Isolation, followed by LC-MS and 1D- and 2D-NMR measurements, and synthesis led to its unequivocal identification as *N*-(1-carboxyethyl)-6-(hydroxymethyl)pyridinium-3-ol inner salt. This so-called alapyridaine, although being tasteless itself, is the first nonvolatile, sweetness-enhancing Maillard reaction product reported in the literature. Depending on the pH value, the detection thresholds of sweet sugars, amino acids, and aspartame, respectively, were found to be significantly decreased when alapyridaine was present; for example, the threshold of glucose decreased by a factor of 16 in an equimolar mixture of glucose and alapyridaine. Studies on the influence of the stereochemistry on taste-enhancing activity revealed that the (+)−(*S*)-alapyridaine is the physiologically active enantiomer, whereas the (−)-(*R*)-enantiomer did not affect sweetness perception at all. Thermal processing of aqueous solutions of alapyridaine at 80 °C demonstrated a high thermal and hydrolytic stability of that sweetness enhancer; for example, more than 90 or 80% of alapyridaine was recovered when heated for 5 h at pH 7.0, 5.0, or 3.0, respectively.

KEYWORDS: Alapyridaine; Maillard reaction; sweetness enhancer; comparative taste dilution analysis; taste compound; *N*-(1-carboxyethyl)-6-(hydroxymethyl)pyridinium-3-ol inner salt

INTRODUCTION

The Maillard reaction between reducing carbohydrates and amino acids is undoubtedly one of the most important reactions leading to the development of the unique aroma and taste as well as the typical browning, which contribute to the sensory quality, of thermally processed foods, such as cooked or roasted meat, roasted coffee, or cocoa. Although numerous studies have addressed the structures and sensory attributes of the volatile odor-active compounds (*1*−*4*), the information available on nonvolatile, sensory-active components generated during thermal food processing is as yet extraordinarily scarce.

Whereas some data are reported in the literature on the formation of bitter-tasting compounds from carbohydrates and amino acids (*5*−*8*), hitherto, almost no information is available on the chemical structures and sensory activities of Maillard reaction products exhibiting a salty, sweet, or umami taste or taste-enhancing properties. To detect sapid tastants and to identify the most intense taste compounds in thermally processed foods, we recently developed a novel bioassay-guided screening procedure, the so-called taste dilution analysis (TDA) (*9*). This technique, using the human tongue as a sensitive and selective biosensor to sort out the taste-active compounds from the large number of nontasting compounds, is based on the determination of the relative taste thresholds of compounds in serial dilutions of HPLC fractions. In combination with LC-MS, 1D- and 2D-NMR, and ^{13}C labeling experiments, this technique recently led to the discovery and unequivocal identification of the previously unknown (*E*)-2-[(2-furyl)methylidene]-7-[(2-furyl)methyl]-3-hydroxymethyl- and (*E*)-2-[(2-furyl)methylidene]-7-[(2-furyl)-methyl]-3,8-bis(hydroxymethyl)-1-oxo-1*H*,2*H*,3*H*-indolizinium-6-olate, exhibiting an intense bitter taste at extraordinarily low detection thresholds of 0.00025 and 0.001 mmol/kg of water, respectively (*10*).

By application of this TDA to heated glucose/proline mixtures as well as roasted malt, more recently, 5-methyl-2-(1-pyrrolidinyl)-2-cyclopenten-1-one and 3-methyl-2-(1-pyrrolidinyl)-2-cyclopenten-1-one were identified as the first Maillard reaction products imparting a "cooling" effect to the oral cavity without exhibiting a prominent minty odor as perceived for, for example, (−)-menthol (*11*).

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In addition to taste-active nonvolatiles, knowledge of the chemical structures and sensory activities of compounds showing taste-enhancing activity would open the possibility of evoking desirable taste effects during the consumption of food compositions. Almost 10 years ago, the sweetness perception of sugars was found to be intensified in the presence of the odorants 4-hydroxy-2(or 5)-ethyl-5(or 2)-methyl-3(2*H*)-furanone, 2-hydroxy-3-methyl-2-cyclopenten-1-one, and/or 3-hydroxy-4,5-dimethyl-2(5*H*)-furanone; for example, solutions containing 5% sucrose and 15 parts (based on sugar) 2-hydroxy-3-methyl-2-cyclopenten-1-one tasted as sweet as an aqueous solution containing 20% sucrose (*12*). Although these compounds seem to intensify the sweet taste of sugars, their strong caramel-like and seasoning-like odor might influence the authentic aroma of foods to which they were applied. Therefore, in particular, odorless but sweetness-enhancing compounds would open new avenues to develop low-calorie, sugar-reduced foods without the need of using artificial sweeteners, which, depending on their concentration and type, exhibit slightly bitter and metallic off-notes.

The purpose of the present investigation was, therefore, to develop a TDA-based analytical strategy to identify a Maillard product with sweetness-enhancing activity in thermally processed solutions of glucose and L-alanine.

EXPERIMENTAL PROCEDURES

Chemicals. The following compounds were obtained commercially: D-glucose, L-alanine, lactic acid, NaCl, caffeine, quinine hydrochloride, sucrose, sodium glutamate, tannin (gallustannic acid), 5-(hydroxymethyl)-2-furanaldehyde, potassium dihydrogen phosphate, and ammonium formate (Aldrich, Steinheim, Germany); sodium hydroxide and trifluoroacetic acid (Merck, Darmstadt, Germany). Solvents were of HPLC grade (Merck). DMSO-d_6 and D_2O were obtained from Euroiso-Top (Gif-Sur-Yvette, France).

Thermally Treated Glucose/L-Alanine Mixture. A mixture of D-glucose (0.2 mol) and L-alanine (0.4 mol) was refluxed for 30 min in phosphate buffer (400 mL; 1.0 mol/L, pH 5.0). After cooling to room temperature, the aqueous solution was extracted with ethyl acetate (3 × 100 mL), the aqueous layer was freeze-dried, and the residue obtained was dissolved in distilled water (40 mL). An aliquot of this stock solution was used for the comparative taste dilution analysis (4 mL) as well as for the identification experiments (36 mL).

Comparative Taste Dilution Analysis (cTDA). After membrane filtration (Spartan 13/0.45 RC; Schleicher & Schuell, Dassel, Germany), aliquots (100 μL) of the stock solution were analyzed by RP-HPLC. The effluent was separated into 10 fractions, which were separately collected in ice-cooled glass vials. The corresponding fractions obtained were collected, divided into two aliquots (A and B), and both aliquots per fraction were freeze-dried separately. The residues obtained from the aliquot A of the 10 pooled HPLC fractions were dissolved in tap water (1 mL), whereas aliquot B was dissolved in an aqueous solution of sucrose (50 mmol/L, 1 mL). For both aliquots, the 10 pooled HPLC fractions were then diluted stepwise 1 + 1 with pure tap water. The serial dilutions of each of these fractions were presented to the sensory panel in order of increasing concentrations, and, while wearing a nose clamp, each dilution was sensorially evaluated for sweetness in a triangle test. The dilution at which a sweet taste difference between the diluted fraction and two blanks (tap water) could just be detected was defined as the taste dilution (TD) factor. Aliquot A did not show any sweetness in any HPLC fraction, whereas HPLC fractions of aliquot B showed sweetness with various TD factors (**Figure 1**). The TD factors evaluated by three different assessors in three different sessions were averaged. The TD factors between individuals and separate sessions differed not more than one dilution step.

Sensory Analyses. *Training of the Sensory Panel.* Panelists were trained to evaluate the taste of aqueous solutions (1 mL each) of the following standard taste compounds by using a triangle test as described in the literature (*13*): sucrose (50 mmol/L) for sweet taste; lactic acid (20 mmol/L) for sour taste; NaCl (12 mmol/L) for salty taste; caffeine (1 mmol/L) for bitter taste; sodium glutamate (8 mmol/L, pH 5.7) for umami taste; gallustannic acid (0.05%) for astringency.

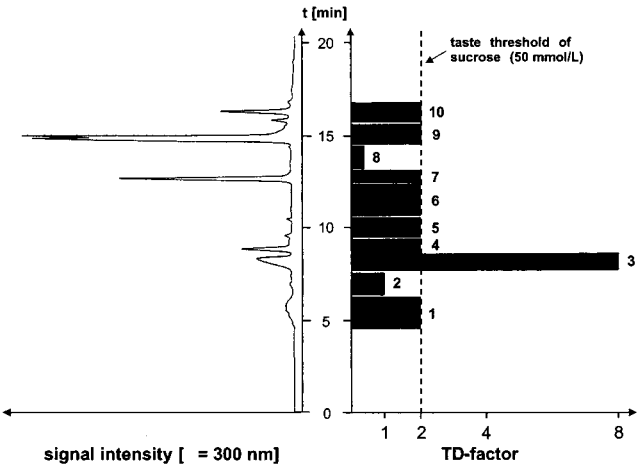


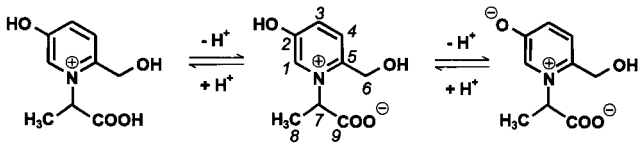
Figure 1. RP-HPLC chromatogram (left) and TD chromatogram (right) of the non-solvent-extractable fraction of a heated D-glucose/L-alanine solution.

Determination of Taste Thresholds. The taste thresholds were determined in a triangle test using tap water as the solvent. The pH of the solvent was adjusted to 5.0, 7.0, or 9.0 by adding trace amounts of hydrochloric acid (0.01 mmol/L) or sodium hydroxide solution (0.01 mmol/L). The samples were presented in order of increasing concentrations (serial 1:1 dilutions), and the threshold values evaluated in three different sessions by eight panelists each were averaged. The values between individuals and separate sessions differed not more than one dilution step; that is, a threshold value of 12.0 mmol/L for L-alanine represents a range from 6.0 to 24.0 mmol/L.

Determination of Isointensity for Sweetness. Aqueous solutions containing constant amounts of glucose (80 or 267 mmol/kg water) but increasing concentrations of alapyridaine (0−125 mmol/kg of water) were stepwise 1 + 1 diluted with water, and the sensory panel was asked to evaluate the sweetness intensity of each dilution and to identify the dilution showing isointensity for sweetness with an aqueous solution containing glucose only (80 or 267 mmol/kg of water). The relative taste strength (taste-enhancing factor) of a mixture containing the glucose and alapyridaine was related to glucose as the standard substance and was determined as the ratio of the concentrations c of isointensely tasting solutions of the basic tastant glucose (glc), and the mixture containing tastant plus pyridinium betain, that is

$$f_{\mathrm{glc,g}}(c_{\mathrm{glc}}) = c_{\mathrm{glc}}/c_{\mathrm{glc+alapyridaine}}$$

Isolation of Sweetness-Enhancing *N*-(1-Carboxyethyl)-6-(hydroxymethyl)pyridinium-3-ol Inner Salt (Alapyridaine) from a Heated Aqueous Glucose/L-Alanine Solution. The major aliquot (36 mL) of the aqueous stock solution prepared from the Maillard mixture was fractionated using a water-cooled glass column (40 × 200 mm) filled with a slurry of RP-18 material (LiChroprep 25−40 μm, Merck), which was conditioned in a mixture (97:3, v/v) of aqueous ammonium formate (10 mmol/L, pH 8.2) and methanol. Aliquots (8−10 mL) of the stock solution were applied onto the top of the column, and chromatography was performed using the same solvent mixture as the mobile phase. Monitoring the effluent at $\lambda = 250$ nm, fractions of 20 mL were collected, freeze-dried, and dissolved in water (2 mL). To localize the sweetness-enhancing compound, these residues were taken up in an aqueous solution of sucrose (50 mmol/kg of water), and the sweetness intensities of these solutions were compared to that of the reference saccharose solution by a sensory panel. The fractions eluting between 140 and 180 mL were judged to have an intensely sweet taste quality and were therefore analyzed by RP-HPLC/degustation following the TDA procedure reported above. The effluent of HPLC fraction 3 (**Figure 1**) was collected in four HPLC runs, freed from solvent, and again analyzed for sweetness enhancement in an aqueous sucrose solution. An intense sweet taste was detected, thus confirming that the



1a 1b 1c

Figure 2. Structures of the sweetness-enhancing *N*-(1-carboxyethyl)-6-(hydroxymethyl)pyridinium-3-ol inner salt (alapyridaine).

Table 1. Assignment of ^{1}H NMR Signals (400 MHz, DMSO-d_6) of *N*-(1-Carboxyethyl)-6-(hydroxymethyl)pyridinium-3-ol Inner Salt (Alapyridaine)

H at relevant C atom[a]	δ[b]	I[c]	M[c]	J[c] (Hz)	homonuclear ^{1}H,^{1}H connectivity[d]
H–C(8)	1.81	3	d	7.1	H–C(7)
H_a–C(6)	4.69	1	d	13.3	H_b–C(6)
H_b–C(6)	4.78	1	d	13.3	H_a–C(6)
H–C(7)	5.23	1	q	7.1	H–C(8)
HO–C(6)	6.49	1	bs		
H–C(3)	7.06	1	dd	8.9, 2.7	H–C(4), H–C(1)
H–C(4)	7.45	1	d	8.9	H–C(3)
H–C(1)	7.50	1	d	2.7	H–C(3)

[a] Arbitrary numbering of carbon atoms refers to structure in **Figure 2**. [b] The ^{13}C chemical shifts are given in relation to DMSO-d_6. [c] Determined from 1D spectrum. [d] Homonuclear ^{1}H,^{1}H connectivities observed by a GRASP-DQF-COSY experiment.

Table 2. Assignment of ^{13}C NMR Signals (500 MHz, DMSO-d_6) of *N*-(1-Carboxyethyl)-6-(hydroxymethyl)pyridinium-3-ol Inner Salt (Alapyridaine)

H at relevant C atom[a]	δ[b]	DEPT[c]	heteronuclear ^{1}H,^{13}C connectivity[d] via ^1J(C,H)	via $^{2,3}J$(C,H)
C(8)	19.0	CH_3	H–C(8)	H–C(7)
C(6)	59.1	CH_2	H–C(6)	H–C(4)
C(7)	64.1	CH	H–C(7)	H–C(8)
C(4)	127.6	CH	H–C(4)	H–C(6)
C(3)	131.3	CH	H–C(3)	H–C(1)
C(5)	131.8	C		H–C(6), H–C(7), H–C(4), H–C(3), H–C(1)
C(1)	132.3	CH	H–C(1)	H–C(7)
C(2)	166.0	C		H–C(4)
C(9)	167.7	C		H–C(8), H–C(7)

[a] Arbitrary numbering of carbon atoms refers to structure in **Figure 2**. [b] The ^{13}C chemical shifts are given in relation to DMSO-d_6. [c] DEPT-135 spectroscopy. [d] Assignments based on GRASP–HSQC (^1J) and GRASP–HMBC ($^{2,3}J$) experiments.

sweetness-modifying compound detected in HPLC fraction 3 is present in the effluent between 140 and 180 mL of the column chromatography. For further purification, the target compound **1** (**Figure 2**) was isolated from that fraction by RP-HPLC; HPLC fraction 3 was collected and freeze-dried, affording a white amorphous powder (yield = 0.6%, 1.2 mmol): UV−vis λ_{max} 251, 328 nm (pH 8.2), 298 nm (pH 3.5); LC-MS (ESI$^+$), *m*/*z* 198 (100, [M + 1]$^+$), 220 (57, [M + Na]$^+$), 395 (19, [2M + 1]$^+$), 417 (29, [2M + Na]$^+$); LC/MS (ESI$^-$), *m*/*z* 197 (100, [M]$^-$). CHN-Anal. Calcd for $C_9H_{11}O_4N$: C, 56.25%; H, 5.58%; N, 7.11%. Found: C, 56.11%; H, 5.68%; N, 7.24% (CHN Rapid instrument, Heraeus, Germany); ^{1}H and ^{13}C NMR data are summarized in **Tables 1** and **2**.

Synthesis of Racemic *N*-(1-Carboxyethyl)-6-(hydroxymethyl)-pyridinium-3-ol Inner Salt. A solution of 5-(hydroxymethyl)-2-furanaldehyde (25 mmol) and alanine (25 mmol) in water/ethanol (1:1, v/v; 60 mL) was adjusted to pH 9.4 with concentrated sodium hydroxide solution and was then refluxed for 48 h. After cooling to room temperature, the reaction mixture was freed from solvent in vacuo (45 mbar), diluted with water (20 mL), and then extracted with ethyl acetate (2 × 10 mL). Aliquots (5 mL) of the aqueous phase were separated by column chromatography (200 × 40 mm) on RP-18 material (LiChroprep 25−40 μm, Merck) using a mixture (97:3, v/v) of aqueous ammonium formate (10 mmol/L, pH 8.2) and methanol as the mobile phase. The target compound was eluted between 150 and 290 mL as confirmed by LC-MS and RP-HPLC/degustation, which enabled the sensory detection of the target compound in the HPLC effluent. After repetition of that purification step and freeze-drying, (*R*)- and (*S*)-*N*-(1-carboxyethyl)-6-(hydroxymethyl)pyridinium-3-ol inner salt (12.8 mmol, 51% yield) were obtained as a 1:1 racemic mixture with a purity of >99%. The spectroscopic data (LC-MS, NMR), the retention times (RP-HPLC), and the sensory attributes of the synthetic *N*-(1-carboxyethyl)-6-(hydroxymethyl)pyridinium-3-ol inner salt were identical to those obtained for the sweetness-enhancing alapyridaine isolated from the heated glucose/L-alanine mixture: LC-MS (ESI$^-$), *m*/*z* 197 (100, [M]$^-$); ^{1}H NMR (360 MHz; DMSO-d_6) δ 1.81 (d, 3H, J = 7.08 Hz), 4.69 (d, 1H, J = 13.27 Hz), 4.78 (d, 1H, J = 13.27 Hz), 5.23 (q, 1H, J = 7.08 Hz), 7.06 (dd, 1H, J = 2.65, 8.85 Hz), 7.45 (d, 1H, J = 8.85 Hz), 7.50 (d, 1H, J = 2.65 Hz); ^{13}C NMR (360 MHz; DMSO-d_6) δ 19.0 [CH_3], 59.1 [CH_2], 64.1 [CH], 127.6 [CH], 131.3 [CH], 131.8 [C], 132.3 [CH], 166.0 [C], 167.7 [COOH].

Influence of pH on ^{13}C NMR data (400 MHz; D_2O/H_2O, 2/8; 135° DEPT; HMBC): pH 2.0, δ 20.7 [C(8), CH_3], 61.9 [C(6), CH_2], 68.0 [C(7), CH], 130.6 [C(4), CH], 134.6 [C(1), CH], 135.3 [C(3), CH], 148.5 [C(5), C], 158.9 [C(2), CO], 176.2 [C(9), COOH]; pH 7.0, δ 20.8 [C(8), CH_3], 62.0 [C(6), CH_2], 67.3 [C(7), CH], 131.0 [C(4), CH], 136.1 [C(1), CH], 136.9 [C(3), CH], 140.8 [C(5), C], 168.0 [C(2), CO], 177.2 [C(9), COOH].

Quantification of Alapyridaine. Alapyridaine was quantified in solutions of D-glucose (1 mmol) and L-alanine (2 mmol) in phosphate buffer (2 mL; 1 mol/L; pH 5.0) and in solutions of alapyridaine (1.0 mg/mL) in phosphate buffer (0.1 mmol/L; pH 3.0, 5.0, or 7.0), which had been heated in closed vials for 0.5, 1, 2, and 5 h at 80 °C. After membrane filtration, aliquots (10−100 μL) of these reaction mixtures were analyzed by analytical RP-HPLC, and the amounts of alapyridaine present were quantified by comparing the peak areas determined at λ = 328 nm with those of defined standard solutions of the synthetic reference compound in methanol.

High-Performance Liquid Chromatography (HPLC). The HPLC apparatus (Kontron, Eching, Germany) consisted of two pumps (type 522), Rheodyne injectors (100 μL loop), and a diode array detector (DAD type 540), monitoring the effluent in a wavelength range between 220 and 500 nm. Separations were performed on a stainless steel column packed with RP-18 (ODS-Hypersil, 5 μm, 10 nm, Shandon, Frankfurt, Germany) in either an analytical (4.6 × 250 mm, flow rate = 0.8 mL/min) or a semipreparative scale (10 × 250 mm, flow rate = 1.6 mL/min). After injection of the sample (10−100 μL), analysis was performed isocratically using a solvent mixture (99.9:0.1, v/v) of aqueous ammonium formate (10 mmol/L; pH 8.2) and methanol.

Liquid Chromatography—Mass Spectrometry (LC-MS). An analytical HPLC column (Nucleosil 100-5C18, Macherey and Nagel, Dürren, Germany) was coupled to an LCQ-MS (Finnigan MAT GmbH, Bremen, Germany) using positive (ESI$^+$) and negative (ESI$^-$) electronspray ionization. After injection of the sample (2−20 μL), analysis was performed using isocratic solvent mixtures (99:1, v/v) consisting of methanol and either aqueous ammonium formate (10 mmol/L; pH 8.2), or aqueous trifluoroacetic acid (0.1%; pH 2.2), respectively.

Nuclear Magnetic Resonance Spectroscopy (NMR). ^{1}H, ^{13}C, and DEPT-135 NMR experiments were performed on a Bruker-AMX 400-III. COSY, HSQC, and HMBC measurements were performed on a Bruker-Avance 500 spectrometer (Bruker, Rheinstetten, Germany) at 300 K by means of a gradient selected (GRASP) experiment (*14*, *15*). The DEPT-135 experiment (distortionless enhancement by polarization transfer) was performed using a transfer pulse of 135° to obtain positive signals for methine and methyl carbons and negative signals for methylene carbon atoms. Evaluation of the experiments was done with 1D- and 2D-WIN NMR as well as UX-NMR software (Bruker, Rheinstetten, Germany).

RESULTS AND DISCUSSION

RP-HPLC analysis of a thermally processed aqueous solution of glucose and L-alanine demonstrated that a multiplicity of hydrophilic reaction products were produced. To identify potential sweetness-enhancing compounds in this mixture, it was necessary, first, to sort out the taste-modifying compounds from the bulk of sensorially inactive substances. To locate novel, sweetness-enhancing compounds in the glucose/L-alanine reaction mixture, we screened the reaction products by means of a novel HPLC-assisted bioassay, the so-called comparative taste dilution analysis.

Comparative Taste Dilution Analysis. Aliquots of the aqueous glucose/L-alanine mixture were analyzed by RP-HPLC (**Figure 1**, left), and the effluents were separated into 10 fractions, which were freeze-dried and then made up to a volume of 1 mL with an aqueous solution of sucrose, the concentration of which was 2-fold above the sweetness detection threshold. Each fraction was then stepwise diluted 1:1 with water and then presented in order of increasing concentrations to trained sensory panelists, who were asked to evaluate the taste quality and to determine the dilution at which a sweet taste difference between the diluted fraction and two blanks (tap water) could just be detected. Because this so-called taste dilution (TD) factor, obtained for each fraction, is related to its taste activity in water, the 10 HPLC fractions collected were ranked in their relative sweet taste impact as given in **Figure 1** (right). Due to the highest TD factor of 8, fraction 3 exhibiting a sweet taste quality was judged with the highest sweetness intensity. With the exception of fractions 2 and 8, all of the other fractions showed a sweet taste quality with a TD factor of 2, corresponding to the sweet taste threshold of the sucrose.

In comparison, the 10 HPLC fractions obtained from another aliquot of the glucose/alanine mixture were taken up in 1 mL of tap water and were also judged by the sensory panel for sweetness, but no fraction imparted any sweet taste per se. The high TD factor of fraction 3 evaluated in the presence of sucrose, therefore, clearly demonstrated that this fraction contained a reaction product enhancing the sweetness of the sucrose solution by a factor of 4. Because sweetness-enhancing compounds were as yet not reported to be formed by Maillard reactions, the following identification experiments were focused on the sapid taste modifier present in HPLC fraction 3 (**Figure 1**).

Structure Determination of Sweetness-Enhancing Compound. For isolation of the sweetness enhancer in HPLC fraction 3, the non-solvent extractables of the glucose/L-alanine solution were separated by column chromatography using RP-18 material as stationary phase and a mixture of methanol and aqueous ammonium formate as the mobile phase. Monitoring the effluent at $\lambda = 250$ nm, 20 mL fractions were collected, freeze-dried, and dissolved in an aqueous saccharose solution, the concentration of which was 2-fold above the detection threshold. To localize the sweetness-enhancing compound, these solutions were evaluated for sweet taste by a trained sensory panel. The fractions imparting the most intense sweet taste were then analyzed by RP-HPLC/degustation. An intense sweet taste was detected in HPLC fraction 3, thus confirming that the sweetness-enhancing compound isolated matched with the sweetness-enhancing compound detected by means of the TDA. After final purification by semipreparative RP-HPLC and freeze-drying, the compound was obtained as a white amorphous powder with a purity of >99%. The spectroscopic data obtained by 1D- and 2D-NMR experiments, LC-MS, and UV−vis spectroscopy were consistent with the structures displayed in **Figure 2**.

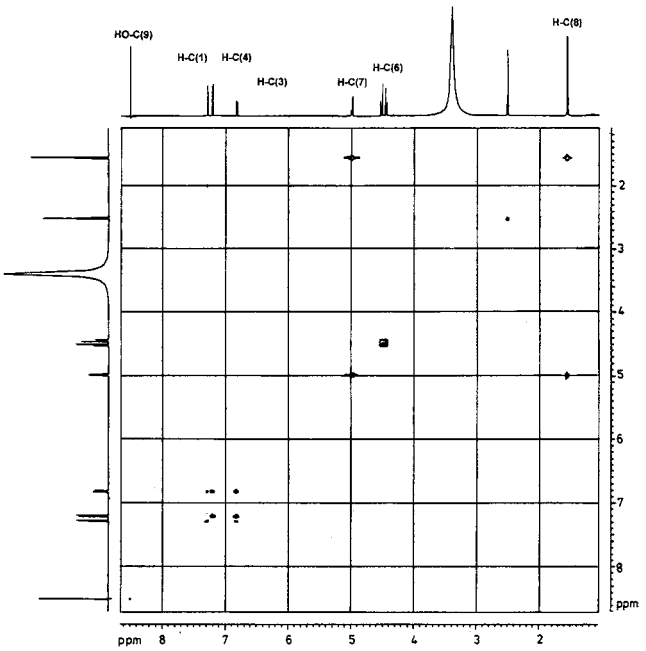


Figure 3. GRASP−COSY spectrum of the unknown sweet principle in HPLC fraction 3.

LC-MS measurements showed an intense $[M + 1]^+$ ion at *m*/*z* 198 indicating that one nitrogen atom should be incorporated in the molecule. LC/MS^2 revealed a loss of 18 amu and 2×18 amu, yielding *m*/*z* 180 and 162, respectively, most likely corresponding to the elimination of one and two molecules of water. These data indicated the presence of two activated hydroxyl groups in the tastant, fitting well with the structure proposed in **Figure 2**.

The ^{1}H NMR spectrum showed nine resonance signals, among which one integrated for the three protons of a methyl group and eight signals integrated for one proton only (**Table 1**). The two geminal protons H_a−C(6) and H_b−C(6) were deduced from the large coupling constant of 13.3 Hz of the signals resonating at 4.69 and 4.78 ppm. This was further confirmed by a gradient-enhanced, double-quantum filtered homonuclear δ,δ-correlation (GRASP−COSY) experiment (**Figure 3**), indicating the expected strongly coupled ^{1}H spin system in the methylene group C(6). Comparison of the ^{13}C NMR spectrum, in which nine signals appeared, with the results of the DEPT-135 experiment showing six signals, revealed three signals corresponding to quaternary carbon atoms (**Table 2**). Unequivocal assignment of these quaternary carbon atoms as well as the hydrogen-substituted carbon atoms, respectively, could be successfully achieved by means of heteronuclear multiple bond correlation spectroscopy (HMBC) optimized for $^2J_{C,H}$ and $^3J_{C,H}$ coupling constants and heteronuclear single-quantum correlation spectroscopy (HSQC) optimized for $^1J_{C,H}$ coupling constants, respectively (**Table 2**); that is, the HMBC experiment revealed a $^3J_{C,H}$ correlation between the aromatic proton H−C(1) resonating at 7.50 ppm and the carbon atoms C(3) and C(5) or between H−C(4) and the quarternary carbon C(2) as well as the methylene carbon atom C(6). Comparison of the ^{1}H and ^{13}C NMR data with those recently reported for 6-(hydroxymethyl)-1-propylpyridinium-3-olate (*9*) further confirmed the pyridinium-3-olate structure proposed in **Figure 2**.

In addition, the heteronuclear connectivities between the proton H−C(7) and the carbons C(1) and C(5) permitted the connection of the pyridinium ring with carbon C(7) resonating at 64.1 ppm. The correlations between the protons H−C(7) and

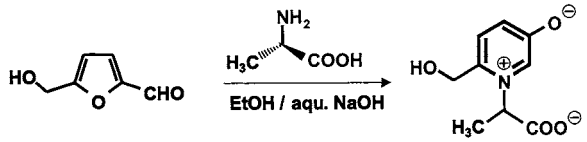


Figure 4. Synthesis of *N*-(1-carboxyethyl)-6-(hydroxymethyl)pyridinium-3-ol inner salt.

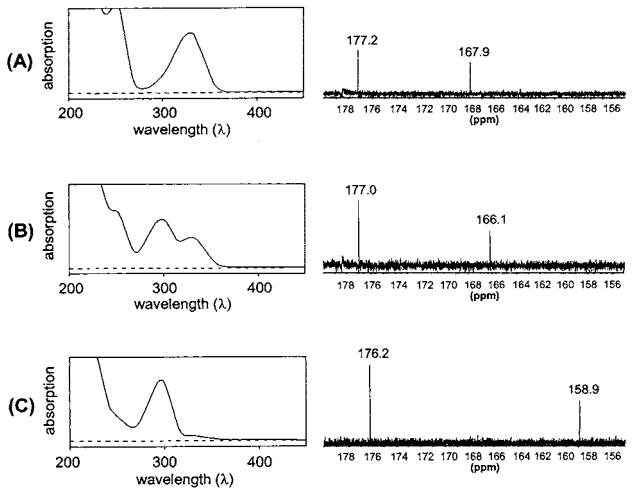


Figure 5. Influence of the pH value on the UV–vis spectrum (left) and the ^{13}C NMR data (excerpt on right side) of alapyridaine: (A) pH 7.0; (B) pH 5.0; (C) pH 2.0.

the carbon C(8) indicated the existence of the L-alanine moiety, thus offering a complete picture of the structure given in **Figure 2**.

For a final confirmation of the proposed structure, *N*-(1-carboxyethyl)-6-(hydroxymethyl)pyridinium-3-ol inner salt was synthesized in yields of >50% by reacting 5-(hydroxymethyl)-2-furanaldehyde with L-alanine under alkaline conditions (**Figure 4**). After purification, the synthetic product showed identical spectroscopic and chromatographic data as well as the sweetness-enhancing characteristics as did the Maillard reaction product isolated from HPLC fraction 3 of the glucose/L-alanine mixture.

To answer the question of how the charge distribution in the sweet tastant is influenced by the pH value, alapyridaine was analyzed by UV−vis as well as ^{13}C NMR spectroscopy in aqueous solutions at pH 2.0, 5.0, and 7.0. The results of the UV−vis measurements revealed that the absorption spectrum of the Maillard compound in aqueous solution is strongly influenced by the pH value, for example, the UV−vis spectrum at pH 7.0 exhibited two absorption maxima, one at $\lambda = 251$ nm and the other at 328 nm (A, left, in **Figure 5**). Lowering the pH to 5.0 revealed a decrease in the absorption at $\lambda = 251$ and 328 nm and led to the detection of an additional maximum at $\lambda = 298$ nm (B, left, in **Figure 5**). In contrast, only a sole maximum at $\lambda = 298$ nm was detectable at pH 2.0 (C, left, in **Figure 5**). Measuring the ^{13}C NMR spectrum of an aqueous solution of the pyridinium betain at pH 7.0 showed a resonance signal at 177.2 and 167.9 ppm corresponding to the carboxy group C(9) and the quarternary oxygen-bound carbon C(2) in the pyridinium moiety (A, right, in **Figure 5**). Decreasing the pH value to 5.0 induced a high-field shift of the resonance signal of C(2) by 1.8 ppm, whereas the chemical shift of C(9) was only slightly affected (B, right, in **Figure 5**), thus indicating that the oxygen atom in the pyridinium-3-olate moiety starts to be protonated as the pH is lowered. At pH 2.0, the chemical shift of the resonance signal of C(2) was shifted by 7.2 ppm from 166.1 to 158.9 ppm, whereas the signal of the carboxy group C(9) was high-field shifted only by 0.8 ppm (C, right, in **Figure 5**). Taking all of these data into account, it can be summarized that, strongly depending on the pH value, alapyridaine exists in an equilibrium of the three structures **1a**−**1c** displayed in **Figure 2** and in both neutral and alkaline media the carboxy group in the alanine moiety as well the oxygen atom of the pyridinium-3-olate moiety are deprotonated. When the pH is lowered to 5.0, first the oxygen group in the pyridinium-3-olate moiety is protonated, being reflected by the hypsochromic shift of the absorption maximum from $\lambda = 328$ to 298 nm and the high-field shift of the resonance signal of C(2) in the ^{13}C NMR spectrum. Thereafter, the carboxy function starts to be protonated at very low pH values, which are, however, not relevant for food products (data not shown).

Table 3. Effect of Alapyridaine on the Sweet Detection Thresholds of Sugars and Artificial Sweeteners

compound	pH	taste quality	detection threshold[a] (mmol/kg of water)	threshold decrease[b]
glucose	5/7	sweet	48.0	
glucose/alapyridaine (1/1)	5	sweet/sour	6.0	8
glucose/alapyridaine (1/1)	7	sweet	3.0	16
glucose/alapyridaine (1/1)	9	sweet	3.0	16
sucrose	7	sweet	12.5	
sucrose/alapyridaine (1/1)	5	sweet	6.0	2
sucrose/alapyridaine (1/1)	7	sweet	3.0	4
sucrose/alapyridaine (1/1)	9	sweet	1.5	8
L-alanine	7	sweet	12.0	
L-alanine/alapyridaine (1/1)	5	sweet	6.0	2
L-alanine/alapyridaine (1/1)	7	sweet	3.0	4
L-alanine/alapyridaine (1/1)	9	sweet	1.5	8
aspartame	5/7	sweet	0.16	
aspartame/alapyridaine (1/1)	7.0	sweet	0.04	4

[a] Taste threshold concentrations were determined by a triangle test using tap water (pH 5.0, 7.0, 9.0) as the solvent. [b] Factor by which the threshold of the sweet tastant was decreased in the presence of alapyridaine.

In summary, LC-MS measurements, 1D- and 2D-NMR experiments, and UV−vis spectroscopy as well as synthesis of the reference compound led to the unequivocal identification of the sweetness-enhancing compound in HPLC fraction 3 as *N*-(1-carboxyethyl)-6-(hydroxymethyl)pyridinium-3-ol inner salt (**Figure 2**). To the best of our knowledge, this tasteless compound, which we name alapyridaine, has not previously been reported in the literature and is the first Maillard reaction product identified that exhibits a pronounced sweetness-enhancing activity.

Additional experiments focusing on the stereochemistry of the alanine moiety in alapyridaine showed that the Maillard product is formed as a 1:1 mixture of the (*R*)- and (*S*)-enantiomers. Studies on the unequivocal determination of the stereochemistry as well as the stereospecific chemical synthesis of the (*R*)- and (*S*)-enantiomers will be published elsewhere (manuscript in preparation).

Sensory Experiments. Prior to sensory analysis, the purity of racemic (*R*)- and (*S*)-alapyridaine was checked by LC-MS and ^{1}H NMR spectroscopy. To study the sweetness-enhancing effect of the novel Maillard reaction product, binary mixtures of the tasteless, racemic alapyridaine and the sweet-tasting compounds glucose, sucrose, L-alanine, or aspartame, respectively, were sensorially evaluated in a triangle test using water with pH 5.0, 7.0, and 9.0, and the sweet detection thresholds determined were compared to the threshold concentrations of aqueous solutions containing the sweet tastants alone (**Table 3**). Depending on the pH value, the detection threshold of

Table 4. Influence of the Stereochemistry of Alapyridaine on Its Sweetness-Enhancing Activity

compound[a]	taste quality	detection threshold[b] (mmol/kg of water)	threshold decrease[c]
sucrose	sweet	12.5	
sucrose/(*R*/*S*)-alapyridaine	sweet	3.0	4
sucrose/(−)-(*R*)-alapyridaine	sweet	12.5	0
sucrose/(+)-(*S*)-alapyridaine	sweet	1.5	8

[a] Aqueous solutions of saccharose alone, or equimolar mixtures of sucrose and (*R*/*S*)-, (*R*)-, or (*S*)-alapyridaine in water (pH 7.0) were presented for determination of the sweet taste threshold. [b] Taste threshold concentrations were determined by a triangle test using tap water as the solvent. [c] Factor by which the threshold of the sweet tastant was decreased in the presence of alapyridaine.

glucose, determined to be 48.0 mmol/L, was significantly decreased when alapyridaine was present; for example, the threshold dropped by a factor of 16 to 3.0 mmol/L. Lowering the pH value to 5.0 led to a less pronounced effect; for example, only a threshold decrease by a factor of 8 was detectable. Furthermore, the sensory panel was able to detect the sweet taste of the disaccharide sucrose as well as the amino acid L-alanine in 4- and 8-fold lower concentrations at pH 7.0 and 9.0, respectively, when alapyridaine was present (**Table 3**). Collaborating well with the data found for glucose, the sweetness-enhancing effect was more pronounced at pH 7.0 and 9.0 than at pH 5.0. Because at pH >5, the alapyridaine was shown by UV−vis and ^{13}C NMR to exist as an anion (**Figure 5**), this strong pH dependency of the sensory activity of the alapyridaine indicates that the deprotonated pyridinium-3-olate, **1C** in **Figure 2**, can be considered to be the physiologically active form of alapyridaine. Besides sugars and the amino acid, also the sweet detection threshold of the artificial sweetner aspartame was significantly affected by alapyridaine; for example, the threshold decreased by a factor of 4 in the presence of the Maillard-type taste modifier (**Table 3**).

In a second set of experiments, we focused on the influence of the stereochemistry at the alanine moiety in alapyridaine on the taste-enhancing activity of the pyridinium betain. The results, given in **Table 4**, revealed that a 1+1 racemic mixture of both synthetic enantiomers decreased the taste threshold of an aqueous sucrose solution by a factor of 4, thus being well in line with the data found for the racemic alapyridaine isolated from the Maillard mixture (**Table 3**). In contrast, the (+)-(*S*)-alapyridaine was found to be twice as efficient as the racemic mixture, whereas the (−)-(*R*)-enantiomer did not affect the sweet threshold concentration of the sucrose solution. These data clearly demonstrate that the stereochemistry of the alapyridaine is of major importance for the sensory activity of that compound.

To gain first insights as to how much the glucose concentration can be reduced in the presence of alapyridaine to meet the same sweetness intensity as an aqueous solution containing glucose alone, aqueous solutions containing constant amounts of glucose, but increasing concentrations of alapyridaine, were stepwise 1+1 diluted with water, and the sweetness intensity of each dilution was compared to the standard glucose solution. The sensory panel was asked to evaluate the dilution showing isointensity of sweetness with the standard glucose solution. As shown in **Figure 6**, the relative sweetness of the mixtures containing glucose and alapyridaine increases with increasing amount of the pyridinium betain. This shows the taste-enhancing potential of alapyridaine on the sweet taste of glucose, which is more pronounced at lower glucose concentrations; for example, a solution containing 80 mmol/L glucose and 125 mmol/L alapyridaine has to be diluted by a factor of 8 to match

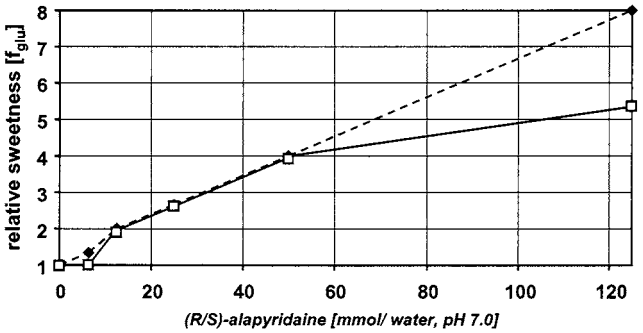


Figure 6. Relative sweetness f_{glc} (*Y* axis) of isointense solutions of glucose (80 mmol/kg of water, ◆; 267 mmol/kg of water, □) and mixtures containing glucose and alapyridaine in various concentrations.

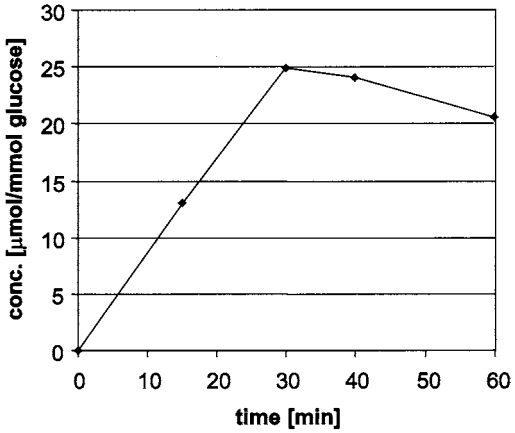


Figure 7. Time course of the formation of alapyridaine (◆) from glucose and L-alanine.

the sweetness of a solution containing 80 mmol/L glucose only. This means that a solution containing 10 mmol/L glucose and 15 mol/L alapyridaine is as sweet as the standard glucose solution (80 mmol of glucose). Lowering the alapyridaine concentration to 50 mmol/L still induced a sweetness enhancement by a factor of 4. Also at higher concentrations of the glucose, for example, 267 mmol/L, the glucose concentration could be decreased by a factor of ∼5 upon alapyridaine addition when compared to the glucose solution without the Maillard compound.

Yields of Alapyridaine. To gain insights into the amounts of the sweetness enhancer formed by Maillard reactions, the time course of alapyridaine formation was determined in the heated glucose/L-alanine solution. As displayed in **Figure 7**, thermal processing of the Maillard mixture led to a rapid formation of the taste modifier; for example, 13.0 μmol of alapyridaine was formed per millimole of glucose already after 15 min. With increasing reaction time, the amounts of alapyridaine increased further, running through a maximum concentration of 24.9 μmol/mmol glucose at 30 min, and decreased only slightly upon further heating; for example, 20.3 μmol/mmol of alapyridaine was detectable after 60 min (**Figure 7**). These data suggest that the novel Maillard reaction product might be a relatively stable compound contributing significantly to the taste of thermally processed foods and that alapyridaine might be suitable as an active food ingredient in order to decrease the concentrations of sugars without losing any sweet taste.

Stability of Alapyridaine. To check the suitability of the novel alapyridaine as a potential sweeteness enhancer and to further investigate its stability during heating, aqueous solutions of racemic alapyridaine were thermally processed at pH 3.0, 5.0, and pH 7.0, and after heating for 0.5, 1, 2, and 5 h at 80

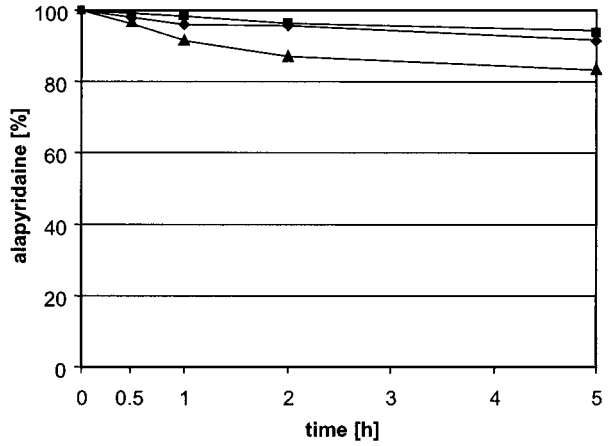


Figure 8. Influence of pH and time on the stability of alapyridaine during thermal processing at 80 °C in aqueous solutions (pH 7.0, ◆; pH 5.0, ■; pH 3.0, ▲).

°C, the residual amounts of the target compound were quantified. The results, given in **Figure 8**, revealed that, independent from the pH value, the alapyridaine is a rather stable Maillard compound; for example, >90% of alapyridaine was recovered after heating for 5 h at pH 5.0 or 7.0, respectively. Also, lowering the pH value to 3.0 did not result in major degradation of alapyridaine; for example, 83% of the sweetness enhancer was recovered after heating for the long period of 5 h (**Figure 8**). These data clearly demonstrate that the sweetness-enhancing alapyridaine shows rather high stability against hydrolytic and thermal degradation during food-related cooking conditions.

Conclusions. The data indicate that the comparative taste dilution analysis, combining instrumental analysis and human taste perception, is a powerful screening procedure to localize and identify tasteless but taste-modifying compounds in complex mixtures of compounds formed upon thermal food processing. Using this analytical strategy, novel food-related sensory active compounds can be characterized, which might be used as culinary ingredients in foods; for example, the tasteless alapyridaine shows sweetness-enhancing properties that might open new avenues for the development of low-calorie, sugar-reduced foods without the need of using artificial sweeteners that may exhibit slightly bitter and metallic off-flavors. Studies aimed at identifying alapyridaine in foods and proving the natural occurrence of this sweetness-enhancing Maillard compound are ongoing and will be reported elsewhere.

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