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Systematic studies of structure and physiological activity of alapyridaine. A novel food-born taste enhancer

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By application of taste dilution analysis (+)-(*S*)-1-(1-carboxyethyl)-5-hydroxy-2-(hydroxymethyl)-pyridinium inner salt was recently successfully identified as a multimodal taste enhancer in beef bouillon. While being taste-less on its own, this so-called alapyridaine was found to intensify the human perception of sweet, salty, and umami taste. To gain information on the molecular requirements of this novel class of taste enhancer, a range of structurally related pyridinium betaines were synthesized, purified, and their physiological activities sensorially evaluated. Removal or modification of the hydroxyl and the hydroxymethyl group, respectively, induced a loss in bioactivity, thus indicating the 2-(hydroxymethyl)-5-hydroxypyridinium moiety as an essential structural element for taste enhancement. Regarding the amino substituent, neither the prolongation or removal of the alkyl chain or the carboxy function in the 1-(1-carboxy-2-ethyl)-moiety, nor the incorporation of an additional carboxy function led to any active derivative, thus demonstrating that also the structure of the nitrogen substituent is rather conserved for taste enhancement. But substitution of the methyl group by a benzyl group yielded a compound showing similar taste enhancing activities as found for alapyridaine. Interestingly, additional insertion of glycine between the 1-(1-carboxy-2-phenylethyl)-moiety and the pyridinium ring resulted in a compound eliciting comparable taste enhancing effects as shown for the compound lacking the glycine spacer. In contrast to these multimodal taste enhancers, substitution of the alanine moiety in alapyridaine by an arginine moiety revealed an one-dimensional taste enhancer exclusively increasing the human sensitivity for salty taste.

Keywords: Alapyridaine / 1-(1-Carboxyethyl)-5-hydroxy-2-(hydroxymethyl)-pyridinium inner salt / Maillard reaction / Taste enhancer / Umami

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1 Introduction

The sensory impression flavor is due to the simultaneous stimulation of the human olfactory and taste systems, and is triggered by chemical compounds in food products [1]. Although the flavor perception is strongly influenced by the interplay of aroma-active volatiles, taste-active nonvolatiles, and flavor modifiers enhancing or suppressing certain taste qualities, the research in the last decades was focused mainly on aroma and taste compounds, rather than on compounds being tasteless on their own, but showing synergistic effects with basic taste compounds. More than 40 years ago [2–4], a first flavor synergism was reported between the umami-like tasting monosodium L-glutamate and purine-5′-ribonucleotides which occur in savory foods such as meat, fish, seafood, and mushrooms, and are widely used as ingredients to enhance the flavor and mouthfeel of culinary products, snacks, soups, and seasonings. Apart from L-glutamic acid, its lower homologue L-aspartic acid as well as the C_4-dicarboxylic acids succinic acid and tartaric acid exhibit some kind of umami taste [5, 6]. In addition, lactic acid was found to contribute to the glutamate-like taste of foods such as beef bouillon and stewed beef juice [7, 8]. Moreover, hydrophilic di-, tri-, and tetrapeptides containing polar side chains, such as Glu-Asp, Glu-Glu, Asp-Glu-Ser, and Asp-Asp-Asp-Asp [9–11], *N*-lactoyl-L-glutamate, the condensation product of lactic acid and glutamic acid [12], as well as the glutamate glycoconjugates *N*-(D-glucos-1-yl)-L-glutamate and *N*-(1-deoxy-D-fructos-1-yl)-L-gluta-

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Abbreviation: DAD, diode-array detector

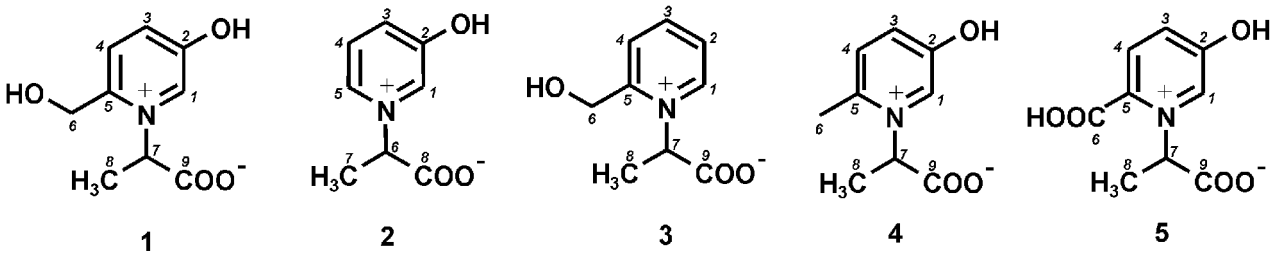


Figure 1. Structures of pyridinium compounds varying in hydroxy and hydroxymethyl groups.

mate [13], were reported to evoke bouillon-like umami taste and to have a long-lasting and mouth-watering sensation in combination with flavor-enhancing characteristics.

In contrast to the taste enhancement induced by umami molecules, the sweetness perception of sugars is reported to be increased by the presence of cyclic enolones such as, *e.g.*, 4-hydroxy-2(or 5)-ethyl-5(or 2)-methyl-3(2*H*)-furanone, 2-hydroxy-3-methyl-2-cyclopenten-1-one, maltol, or ethylmaltol [14, 15]. Furthermore, basic amino acids such as L-lysine and L-arginine [16], the peptide L-ornithyl-β-alanine [17, 18], and the disaccharide trehalose [19] were found to permit reduction of the sodium chloride content in foods without any lack in perceived salt intensity.

Aimed at investigating the structure of potential taste-enhancing compounds which are not present in the foods *per se*, but are generated during food processing from precursors, the taste dilution analysis [20, 21] was recently applied to HPLC fractions isolated from heated solutions of hexoses and L-alanine. This novel approach led to the identification of the previously not reported 1-(1-carboxyethyl)-5-hydroxy-2-(hydroxymethyl)-pyridinium inner salt (**1**; Fig. 1) [22]. This so-called alapyridaine, which was recently found to occur naturally in beef bouillon [23], does not show any taste on its own, but is able to enhance the sweetness of sugars, amino acids, and the artificial sweetener aspartame. While well-known taste enhancers, such as, *e.g.*, monosodium glutamate, guanosine-5′-monophosphate or maltol, intensify single taste modalities only, the (+)-(*S*)-enantiomer of **1** was recently demonstrated to act as a multivalent taste modifier significantly decreasing the human taste thresholds for various sweet, umami, and salty compounds [24, 25]. It is, however, yet not known which structural elements are essential for the physiological activity of that molecule.

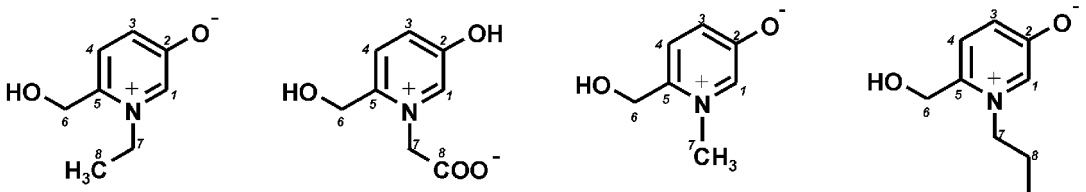
In order to investigate the "gustophore" in various taste molecules, structure/activity investigations based on systematic sensory studies with pure reference compounds have been successfully performed in the past for umami-tasting and/or taste-enhancing carboxylic acids [5], bitter-tasting amino acids and peptides [26, 27], bitter-tasting 1-oxo-2,3-dihydro-1*H*-indolizinium-6-olates [28], sweet-tasting 2-(4-methoxybenzoyl)benzoic acid derivatives [29, 30], pungent and/or tingling shanshool and bungeanool derivatives [31], and, very recently, for 2-(1-pyrrolidinyl)-2-cyclopenten-1-ones and 3-(1-pyrrolidinyl)-2(5*H*)-furanones exhibiting a physiological cooling effect [32].

Because alapyridaine (**1** in Fig. 1) belongs to a novel class of taste enhancer, it is yet not known which molecular features of this pyridinium betaine are required to exhibit the taste modifying activity. The purpose of the present investigation was, therefore, to perform structure/activity studies on a series of synthetic *N*-alkyl-5-hydroxy-2-(hydroxymethyl)-pyridinium inner salts structurally related to the naturally occurring taste enhancer **1**.

2 Materials and methods

2.1 Chemicals

The following compounds were obtained commercially: L-alanine, L-arginine, bromine, caffeine, citric acid, ethylamine solution (2 M in MeOH), formic acid, D-glucose, glycine, hydrochloric acid, L-isoleucine, D-isoleucine, methylamine solution (41% in water), monosodium L-glutamic acid salt, L-phenylalanine, potassium permanganate, quinine hydrochloride, sodium chloride, sodium hydroxide, sucrose, sulfuric acid, gallustannic acid, L-valine from Merck (Darmstadt, Germany). Ammonium formate, 5-(hydroxymethyl)-furaldehyde, 5-methyl-2-furaldehyde, 2-furaldehyde from Raney Nickel (Aldrich, Steinheim, Germany). H-Gly-Phe-OH from Bachem Biochemica (Heidelberg, Germany). Solvents were of HPLC grade (Merck). Deuterated solvents were obtained from Euroiso-Top (Gif-Sur-Yvette, France). EVIAN® mineral water was used for sensory evaluation. Racemic 1-(1-carboxyethyl)-5-hydroxy-2-(hydroxymethyl)-pyridinium inner salt (**1**; Fig. 1) was prepared following the procedure recently reported in the literature and was purified using food-grade solvents [22]. 1-Propyl-5-hydroxy-2-hydroxymethyl-pyridinium inner salt (**9**; Fig. 2) was synthesized and purified as reported recently [20]. Both the enantiomers, (+)-(*S*)-**1** and (–)-(*R*)-**1**, were stereospecifically synthesized and purified using food-grade solvents [25]. All alapyridaine samples were essentially free of sodium and chloride.



6 7 8 9

Figure 2. Structures of pyridinium compounds varying in the amino substituent.

2.2 Syntheses

2.2.1 Compound 2 (Fig. 1)

A solution of 2-furaldehyde (40 mmol) and L-alanine (50 mmol) in water/ethanol (1/1 v/v, 80 mL) was adjusted to pH 9.4 with aqueous sodium hydroxide solution (32% in water). After stirring at room temperature for 0.5 h, the mixture was refluxed for 2 h and cooled to room temperature. The sample was then concentrated under reduced pressure, taken up in water (25 mL), and extracted with ethyl acetate (3 × 50 mL). Aliquots of the aqueous phase were applied onto the top of a water-cooled glass column (4 × 30 cm) filled with a slurry of RP-18 material (LiChroprep 25–40 µm; Merck) in trifluoroacetic acid (0.1% in water). The effluent was monitored at 300 nm and the elution of the target compound was confirmed by RP-HPLC/DAD. The purification step was repeated and the fractions collected were freeze-dried twice affording the target compound as a white solid.

1-(1-Carboxyethyl)-3-hydroxy-pyridinium inner salt, racemate (**2**; yield 42%): LC-MS (ESI^+): *m/z* 168 (100, $[M+1]^+$), 335 (57, $[2M+1]^+$), 357 (21, $[2M+Na]^+$); ^1H NMR (400 MHz; DMSO-d_6): δ 1.88 [d, 3H, *J* = 7.3 Hz, H-C(7)], 5.71 [q, 1H, *J* = 7.3 Hz, H-C(6)], 7.97 [dd, 1H, *J* = 6.0, 8.7 Hz, H-C(4)], 8.06 [dd, 1H, *J* = 1.6, 8.7 Hz, H-C(3)], 8.61 [d, 1H, *J* = 6.0 Hz, H-C(5)], 8.73 [d, 1H, *J* = 1.6 Hz, H-C(1)]; ^{13}C NMR (100 MHz; DMSO-d_6; HMBC, HMQC): δ 18.2 [CH_3, C(7)], 68.2 [CH, C(6)], 127.5 [CH, C(4)], 132.5 [CH, C(3)], 134.2 [CH, C(1)], 136.3 [CH, C(5)], 158.5 [CO, C(2)], 170.5 [COOH, C(8)].

2.2.2 Compound 3 (Fig. 1)

A solution of 2-(hydroxymethyl)-pyridine (80 mmol) and (±)-2-bromopropionic acid (80 mmol) in ethanol (10 mL) was stirred for 24 h at room temperature and then heated for 96 h at 60°C. After cooling to room temperature, the mixture was freed from solvent and recrystallized two times with ethanol. After filtration, the reaction products were dissolved in water (20 mL), an excess of powdered Ag_2O was added, and the AgBr precipitated was filtered off. The aqueous solution was dried *in vacuo*, the residue was dissolved in methanol and applied to the top of a water-cooled glass column (2.5 × 20 cm) filled with a slurry of silica gel (silica gel 60, 0.063–0.200 mm; Merck) in ethyl acetate. After flushing the column with ethyl acetate (250 mL), followed by ethyl acetate/methanol (8/2 v/v; 125 mL), the title compound was eluted with methanol (500 mL). After removing the solvent *in vacuo*, compound **3** was obtained as an amorphous powder in yields of 25%.

N-(1-Carboxyethyl)-2-(hydroxymethyl)pyridinium inner salt, racemate (**3**): LC-MS (ESI^+): *m/z* 182 (100, $[M+1]^+$); ^1H NMR (400 MHz; MeOH-d_4): δ 1.96 [d, 3H, *J* = 7.1 Hz, H-C(8)], 4.93 [d, 1H, *J* = 16 Hz, H_a-C(6)], 5.05 [d, 1H, *J* = 16 Hz, H_b-C(6)], 5.41 [q, 1H, *J* = 7.1 Hz, H-C(7)], 8.00 [dd, 1H, *J* = 6.4, 8.0 Hz, H-C(2)], 8.24 [d, 1H, *J* = 8.0 Hz, H-C(4)], 8.54 [dd, 1H, *J* = 6.4, 8.0 Hz, H-C(3)], 8.98 [d, 1H, *J* = 6.4 Hz, H-C(1)]; ^{13}C NMR (100 MHz; MeOH-d_4; HMQC, HMBC): δ 17.7 [CH_3, C(8)], 59.8 [CH_2, C(6)], 64.8 [CH, C(7)], 126.3 [CH, C(2)], 127.8 [CH, C(4)], 144.1 [CH, C(3)], 146.0 [CH, C(1)], 157.6 [C, C(5)], 171.5 [COOH, C(9)].

2.2.3 Compound 4 (Fig. 1)

A solution of 5-methyl-2-furaldehyde (25 mmol) and L-alanine (35 mmol) in water/ethanol (1/1 v/v, 50 mL) was adjusted to pH 9.4 with aqueous sodium hydroxide solution (32% in water). After stirring at room temperature for 1.5 h, the mixture was refluxed for 14 h and cooled to room temperature. The sample was then concentrated under reduced pressure, taken up in water (25 mL), and extracted with ethyl acetate (3 × 50 mL). Aliquots of the aqueous phase were applied onto the top of a water-cooled glass column (4 × 30 cm) filled with a slurry of RP-18 material (LiChroprep 25–40 µm; Merck) in a mixture (99.0/1.0 v/v) of ammonium formate (10 mmol/L, pH 8.2) and methanol. Fractionation of the reaction product was performed by chromatography using the same solvent mixture. The effluent was monitored at 330 nm and the elution of the target compounds was confirmed by RP-HPLC/DAD. The purification step was repeated and the fractions collected were freeze-dried twice affording the target compound as a white solid.

1-(1-Carboxyethyl)-5-hydroxy-2-methyl-pyridinium inner salt, racemate (**4**; yield 15%): LC-MS (ESI$^+$): *m/z* 182 (36, $[M+1]^+$), 204 (15, $[M+Na]^+$), 363 (34, $[2M+1]^+$), 385 (55, $[2M+Na]^+$), 566 (92, $[3M+Na]^+$), 588 (100, $[3M+2Na]^+$); ^{1}H NMR (250 MHz; DMSO-d_6): δ 1.57 [d, 3H, *J* = 7.0 Hz, H-C(8)], 2.37 [s, 3H, H-C(6)], 4.76 [q, 1H, *J* = 7.0, H-C(7)], 6.90 [dd, 1H, *J* = 2.8, 8.9 Hz, H-C(3)], 7.12 [d, 1H, *J* = 8.9 Hz, H-C(4)], 7.40 [d, 1H, *J* = 2.8 Hz, H-C(1)]; ^{13}C NMR (62.5 MHz; DMSO-d_6): δ 18.4 [CH_3, C(6)], 19.0 [CH_3, C(8)], 65.7 [CH, C(7)], 128.4 [CH, C(4)], 132.1 [C, C(5)], 132.4 [2 × CH, C(1) and C(3)], 166.7 [CO, C(2)], 169.4 [COOH, C(9)].

2.2.4 Compound 5 (Fig. 1)

To a cooled solution (0°C) of 1-(1-carboxyethyl)-5-hydroxy-2-(hydroxymethyl)-pyridinium inner salt (2.0 mmol) in water (12.5 mL) was added dropwise a solution of potassium permanganate (1.6 mmol) and sulfuric acid (2.5 mmol) in 12.5 mL water. The reaction mixture was stirred for 1 h at 0°C, neutralized with sodiumhydroxide (30% in water), filtrated and evaporated. The residue was taken up in water (5 mL) and applied onto the top of a water-cooled glass column (4 × 30 cm) filled with a slurry of RP-18 material (LiChroprep 25 – 40 µm; Merck) in aqueous ammonium formate (10 mmol/L, pH 8.2). Fractionation of the reaction products was performed by chromatography using ammonium formate (10 mmol/L, pH 8.2) and methanol mixtures as the mobile phase with increasing methanol content. The effluent was monitored at 330 nm and the elution of the target compounds was confirmed by RP-HPLC/DAD. The freeze-dried fractions were then purified by automated HPLC collection on a Jasco-HPLC-System using a semipreparative RP-18 column (ODS-Hypersil, 5 µm, 10 × 250 mm; Phenomenex, Aschaffenburg, Germany). After freeze-drying twice, 1-(1-carboxyethyl)-2-carboxy-5-hydroxy-pyridinium inner salt was obtained with a purity of more than 95%.

1-(1-Carboxyethyl)-2-carboxy-5-hydroxy-pyridinium inner salt, racemate (**5**; yield 15%): LC-MS (ESI$^+$): *m/z* 212 (100, $[M+1]^+$), 423 (40, $[2M+1]^+$); ^{1}H NMR (400 MHz; D_2O; COSY): δ 1.83 [d, 3H, *J* = 6.9 Hz, H-C(8)], 5.81 [q, 1H, *J* = 6.9 Hz, H-C(7)], 7.58 [d, 1H, *J* = 9.0 Hz, H-C(3)], 7.75 [d, 1H, *J* = 9.0 Hz, H-C(4)], 7.86 [s, 1H, H-C(1)]; ^{13}C NMR (100 MHz; D_2O; HMQC, HMBC): δ 19.0 [CH_3, C(8)], 67.1 [CH, C(7)], 128.1 [CH, C(4)], 133.6 [CH, C(1)], 134.0 [CH, C(3)], 139.3 [C, C(5)], 163.8 [CO, C(2)], 172.6 [COO, C(6)], 175.1 [COOH, C(9)].

2.2.5 Compounds 6 and 8 (Fig. 2)

A binary solution of 5-(hydroxymethyl)-2-furaldehyde (20 mmol) and either methylamine, ethylamine, or propylamine (28 mmol each) in water/ethanol (1/1 v/v, 40 mL) was adjusted to pH 9.5 with concentrated hydrochloric acid and then heated to 90°C for 20 h in sealed glass vials. After cooling to room temperature the reaction mixture was freed from solvent *in vacuo* (45 mbar), diluted with water (20 mL), then extracted with ethyl acetate (5 × 20 mL), and evaporated to dryness. The dark residue was taken up in ethanol (10 mL) and applied onto the top of a glass column (30 × 500 mm) filled with a slurry of silica gel (200 g, silica gel 60; Merck), which was conditioned with ethyl acetate/methanol (50/50 v/v). Fractionation of the reaction products was performed by chromatography using ethyl acetate/methanol (50/50 v/v) as the mobile phase with increasing methanol contents. The fractions containing the target compound were freed from solvent and applied to the top of a water-cooled glass column (4 × 30 cm) filled with a slurry of RP-18 material (LiChroprep 25 – 40 µm; Merck) in a mixture (95.0/5.0 v/v) of ammonium formate (10 mmol/L, pH 8.2) and methanol. The same solvent mixture was used as the mobile phase, the effluent was monitored at 330 nm and the elution of the target compound was confirmed by RP-HPLC/DAD. After freeze-drying, the reaction products were recrystallized from 2-propanol/pentane mixtures affording colorless needles.

1-Ethyl-5-hydroxy-2-(hydroxymethyl)-pyridinium inner salt (**6**; yield 16%): LC-MS (ESI$^+$): *m/z* 154 (100, $[M+1]^+$), 307 (56, $[2M+1]^+$); ^{1}H NMR (250 MHz; DMSO-d_6): δ 1.44 [t, 3H, *J* = 7.3 Hz, H-C8)], 4.33 [q, 2H, *J* = 7.3 Hz, H-C(7)], 4.56 [s, 2H, H-C(6)], 7.07 [dd, 1H, *J* = 3.1, 8.9 Hz, H-C(3)], 7.37 [d, 1H, *J* = 8.9 Hz, H-C(4)], 7.65 [d, 1H, *J* = 3.1 Hz, H-C(1)]; ^{13}C NMR (62.5 MHz; DMSO-d_6; HMBC): δ 17.4 [CH_3, C(8)], 52.4 [CH_2, C(7)], 58.3 [CH_2, C(6)], 128.9 [CH, C(4)], 132.8 [CH, C(3)], 134.5 [C, C(5)], 134.7 [CH, C(1)], 166.3 [CO, C(2)].

1-Methyl-5-hydroxy-2-(hydroxymethyl)-pyridinium inner salt (**8**; yield 11%): LC-MS (ESI$^+$): *m/z* 140 (100, $[M+1]^+$); ^{1}H NMR (360 MHz; MeOH-d_4): δ 4.15 [s, 3H, H-C(7)], 4.69 [s, 2H, H-C(6)], 7.32 [dd, 1H, *J* = 2.7, 8.9 Hz, H-C(3)], 7.53 [d, 1H, *J* = 8.9 Hz, H-C(4)], 7.65 [d, 1H, *J* = 2.7 Hz, H-C(1)]; ^{13}C NMR (90 MHz; MeOH-d_4; HMQC, HMBC): δ 44.8 [CH_3, C(7)], 60.5 [CH_2, C(6)], 129.1 [CH, C(4)], 135.1 [CH, C(3)], 137.3 [CH, C(1)], 139.0 [C, C(5)], 168.5 [CO, C(2)].

2.2.6 Compounds 7, 10 – 11, 14 – 15 (Figs. 2, 3)

A binary solution of 5-(hydroxymethyl)-2-furaldehyde (30 mmol) and either glycine, L-phenylalanine, L-valine, L-glutamate, or L-arginine (40 mmol each) in water/ethanol (1/1 v/v, 100 mL) was adjusted to pH 9.4 with aqueous sodium hydroxide solution (32% in water). After stirring at room temperature for 1.5 h, the mixture was refluxed for 24 h and then cooled to room temperature. This procedure was repeated twice after the addition of 5 mmol of 5-(hydroxymethyl)-2-furaldehyde each time. The sample was then

concentrated under reduced pressure, taken up in water (25 mL), and extracted with ethyl acetate (3 × 50 mL). Aliquots of the aqueous phase were applied onto the top of a water-cooled glass column (4 × 30 cm) filled with a slurry of RP-18 material (LiChroprep 25 – 40 µm; (Merck) in aqueous ammonium formate (10 mmol/L, pH 8.2). Fractionation of the reaction products was performed by chromatography using ammonium formate (10 mmol/L, pH 8.2) and methanol mixtures as the mobile phase with increasing methanol contents. The effluent was monitored at 330 nm and the elution of the target compounds was confirmed by RP-HPLC/DAD. The purification step was repeated and the fractions collected were freeze-dried twice affording the target compounds as white solids.

1-Carboxymethyl-5-hydroxy-2-(hydroxymethyl)-pyridinium inner salt (**7**; yield 22%): LC-MS (ESI^+): *m/z* 184 (72, $[M+1]^+$), 206 (57, $[M+Na]^+$), 228 (76, $[M+2Na]^+$), 411 (79, $[2M+2Na]^+$), 433 (100, $[2M+3Na]^+$); ^1H NMR (400 MHz; D_2O; TMS-P): δ 4.63 [s, 2H, H-C(6)], 4.94 [s, 2H, H-C(7)], 7.45 [dd, 1H, *J* = 2.7, 8.9 Hz, H-C(3)], 7.57 [d, 1H, *J* = 8.9 Hz, H-C(4)], 7.67 [d, 1H, *J* = 2.7 Hz, H-C(1)]; ^{13}C NMR (100 MHz; D_2O; TMS-P; HMBC, HMQC): δ 59.8 [CH_2, C(6)], 60.3 [CH_2, C(7)], 129.7 [CH, C(4)], 131.0 [CH, C(3)], 132.1 [CH, C(1)], 138.7 [C, C(5)], 165.2 [CO, C(2)], 171.4 [COOH, C(8)].

1-(1-Carboxy-2-phenylethyl)-5-hydroxy-2-(hydroxymethyl)-pyridinium inner salt, racemate (**10**; yield 5%): LC-MS (ESI^+): *m/z* 274 (100, $[M+1]^+$), 547 (10, $[2M+1]^+$); ^1H NMR (400 MHz; MeOH-D_4): δ 3.39 [dd, 1H, *J* = 11.0, 14.7 Hz, H_a-C(8)], 3.83 [dd, 1H, *J* = 4.5, 14.7 Hz, H_b-C(8)], 4.39 [d, 1H, *J* = 14.8 Hz, H_a-C(6)], 4.49 [d, 1H, *J* = 14.8 Hz, H_b-C(6)], 5.57 [dd, 1H, *J* = 4.5, 11.0 Hz, H-C(7)], 7.18 [m, 5 × 1H, H-C(10), H-C(14); 1H, H-C(3)], 7.53 [m, 2 × 1H, H-C(1), H-C(4)]; ^{13}C NMR (100 MHz; MeOH-D_4; HMQC, HMBC): δ 39.4 [CH_2, C(8)], 59.5 [CH_2 C(6)], 71.1 [CH, C(7)], 127.3 [ArH, C(12)], 127.5 [CH, C(4)], 128.9 [ArH, C(10) and C(14)], 129.0 [ArH, C(11) and C(13)], 131.0 [CH, C(3)], 132.7 [CH, C(1)], 136.6 [Ar, C(9)], 143.6 [C, C(5)], 161.2 [CO, C(2)], 170.7 [COOH, C(15)].

1-(1-Carboxy-2-methylpropyl)-5-hydroxy-2-(hydroxymethyl)-pyridinium inner salt, racemate (**11**; yield 4%): LC-MS (ESI^+): *m/z* 226 (35, $[M+1]^+$), 264 (30, $[M+K]^+$), 451 (20, $[2M+1]^+$), 264 (35, $[2M+K]^+$), 676 (20, $[3M+1]^+$), 714 (100, $[3M+K]^+$), 752 (85, $[3M+2K]^+$); ^1H NMR (400 MHz; DMSO-D_6; COSY): δ 0.65 [d, 3H, *J* = 6.7 Hz, H-C(9)], 1.06 [d, 3H, *J* = 6.7 Hz, H-C(10)], 2.38 [m, 1H, H-C(8)], 4.48 [d, 1H, *J* = 10.5 Hz, H-C(7)], 4.59 [d, 1H, J = 14.0, H_a-C(6)], 4.68 [d, 1H, *J* = 14.0, H_b-C(6)], 7.21 [dd, 1H, *J* = 2.2, 8.8 Hz, H-C(3)], 7.46 [d, 1H, *J* = 8.8 Hz, H-C(4)], 7.83 [d, 1H, *J* = 2.2, H-C(1)]; ^{13}C NMR (100 MHz; DMSO-D_6; HMQC, HMBC): δ 18.9 [CH_3, C(9/10)], 20.6 [CH_3, C(9/10)], 31.5 [CH, C(8)], 59.8 [CH_2, C(6)], 76.1 [CH, C(7)], 128.6 [CH, C(4)], 132.7 [CH, C(3)], 132.9 [CH, C(1)], 138.1 [C, C(5)], 165.2 [CO, C(2)], 169.2 [COOH, C(11)].

1-(1,3-Dicarboxypropyl)-5-hydroxy-2-(hydroxymethyl)-pyridinium inner salt, racemate (**14**; yield 5%): LC-MS (ESI^+): *m/z* 256 (65, $[M+1]^+$), 511 [42, $2M+1]^+$), 533 [11, $2M+Na]^+$), 766 [100, $3M+1]^+$), 788 [46, $3M+Na]^+$); ^1H NMR (360 MHz; D_2O; TMS-P): δ 2.17 [t, 2H, H-C(9)], 2.39 [m, H, H_a-C(8)], 2.66 [m, H, H_b-C(8)], 4.80 [s, 2H, H-C(6)], 5.30 [dd, 1H, *J* = 5.2, 10.2 Hz, H-C(7)], 7.70 [dd, 1H, *J* = 2.5, 8.9 Hz, H-C(3)], 7.76 [d, 1H, *J* = 8.9 Hz, H-C(4)], 8.06 [d, 1H, *J* = 2.5 Hz, H-C(1)]; ^{13}C NMR (100 MHz; D_2O; TMS-P; HMBC, HMQC): δ 29.7 [CH_2, C(8)], 33.6 [CH_2, C(9)], 59.8 [CH_2, C(6)], 69.3 [CH, C(7)], 129.0 [CH, C(4)], 133.5 [CH, C(1)], 134.3 [CH, C(3)], 143.4 [C, C(5)], 160.9 [CO, C(2)], 173.4 [COOH, C(11)], 179.5 [COOH, C(10)].

1-[4-(*N*-(Aminoiminomethyl)-amino)-1-carboxy-butyl]-5-hydroxy-2-(hydroxymethyl)-pyridinium inner salt, racemate (**15**; yield 6%): LC-MS (ESI^+): *m/z* 283 (100, $[M+1]^+$), 327 (39, $[M+2Na]^+$); ^1H NMR (400 MHz; D_2O; TMS-P): δ 1.36 [m, 1H, H_a-C(9)], 1.65 [m, 1H, H_b-C(9)], 2.15 [m, 1H, H_a-C(8)], 2.38 [m, 1H, H_b-C(8)], 3.16 [t, 2H, *J* = 6.8 Hz, H-C(10)], 4.77 [s, 2H, H-C(6)], 5.23 [dd, 1H, *J* = 5.7, 9.6 Hz, H-C(7)], 7.48 [dd, 1H, *J* = 2.7, 8.9 Hz, H-C(3)], 7.58 [d, 1H, *J* = 8.9 Hz, H-C(4)], 7.83 [d, 1H, *J* = 2.7 Hz, H-C(1)]; ^{13}C NMR (100 MHz; D_2O; TMS-P; HMBC, HMQC): δ 25.2 [CH_2, C(9)], 29.7 [CH_2, C(8)], 40.7 [CH_2, C(10)], 59.6 [CH_2, C(6)], 69.1 [CH_2, C(7)], 129.6 [CH, C(4)], 133.3 [CH, C(1)], 134.2 [CH, C(3)], 140.2 [C, C(5)], 156.9 [C, C(11)], 164.0 [CO, C(2)], 173.4 [COOH, C(12)].

2.2.7 Compounds 12 and 13 (Fig. 3)

2.2.7.1 (*S,S*)-*N*-(1-Carboxy-2-methylbutyl)-2-hydroxymethyl-5-(methylamino)furan

A solution of 5-(hydroxymethyl)-2-furanaldehyde (15 mmol) and L-isoleucine (12.5 mmol) in water (35 mL) was adjusted to pH 8.0 with conc. sodium hydroxide solution and stirred in a hydrogenation vessel for 30 min at room temperature. After the addition of Raney nickel (0.75 g) the solution was stirred in an hydrogen atmosphere (5 bar) for 48 h. This procedure was repeated after the addition of 5-(hydroxymethyl)-2-furaldehyde (5 mmol). The catalyst was then filtered off, washed with methanol, and the filtrate was concentrated *in vacuo*. The residue was taken up in ammonium formate buffer (10 mmol/L, pH 8.2) and applied to the top of a water-cooled glass column (4 × 30 cm) filled with a slurry of RP-18 material (LiChroprep 25 – 40 µm; Merck) in a mixture (92.5/7.5 v/v) of ammonium formate (10 mmol/L, pH 8.2) and methanol. Using the same solvent mixture as the mobile phase, the effluent was monitored at 220 nm and the elution

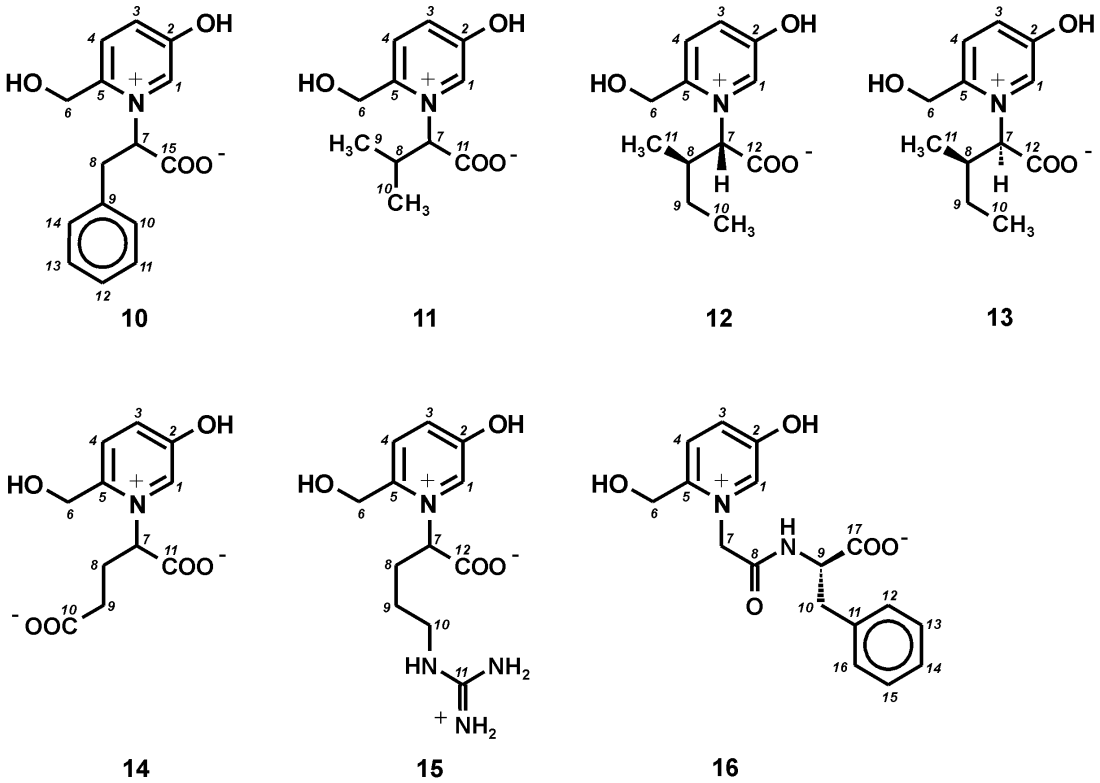


Figure 3. Structures of pyridinium compounds varying in the amino acid moieties.

of the target compound was confirmed by RP-HPLC/DAD. After freeze-drying, (*S*,*S*)-*N*-(1-carboxy-2-methylbutyl)-2-hydroxymethyl-5-(methylamino)furan (3.2 mmol, 26% in yield) was obtained. Following the same procedure, the corresponding (*R*,*S*)-isomer was prepared using D-isoleucine.

(*S*,*S*)-*N*-(1-Carboxy-2-methylbutyl)-2-hydroxymethyl-5-(methylamino)furan: LC-MS (ESI^+): 242 (100, $[M+1]^+$), 264 (42, $[M+Na]^+$); ^1H NMR (400 MHz; TMS-P; D_2O): δ 0.90 [t, 3 H, J = 7.5 Hz, CH_3], 1.01 [d, 3 H, J = 7.2 Hz, CH], 1.27 (m, 1 H, CH_aH), 1.48 [m, 1 H, CH_bH], 1.90 [m, 1 H, CH], 3.64 [d, 1 H, J = 3.9 Hz, CH], 4.23 [d, 1 H, J = 14.7 Hz, CH_aH], 4.34 [d, 1 H, J = 14.7 Hz, CH_bH], 4.58 [s, 2 H, CH_2], 6.42 [d, 1 H, J = 3.3 Hz, CH], 6.58 [d, 1 H, J = 3.3 Hz, CH].

2.2.7.2 Compounds 12 and 13

To a cooled (0°C) solution of (*R*,*S*)- or (*S*,*S*)-*N*-(1-carboxy-2-methylbutyl)-2-hydroxymethyl-5-(methylamino)furan (1.7 mmol) in water (15 mL) was added dropwise a solution of bromine in methanol (1.0 mmol in 5 mL) over a period of 30 min and stirring was continued for 30 min. The resulting mixture was neutralized by the addition of a strongly basic ion exchange resin (Amberlite IRA-400 (OH^-) ion exchanger resin; Aldrich), filtered and evaporated. The residue was taken up in ammonium formate buffer (10 mmol/L, pH 8.2) and applied onto the top of a water-cooled glass column (3 × 30 cm) filled with a slurry of RP-18 material (LiChroprep 25–40 µm; Merck) in a mixture (92.5/7.5 v/v) of ammonium formate (10 mmol/L, pH 8.2) and methanol. Using the same solvent mixture as the mobile phase, the effluent was monitored at 330 nm and the elution of the target compound was confirmed by RP-HPLC/DAD. From the freeze-dried material the target compounds was purified by HPLC using an analytical (4.6 × 250 mm, flow rate 0.8 mL/min) phenyl-hexyl-column (Luna 5µ; Phenomenex) with a mixture (95/5 v/v) of ammonium formate (10 mmol/L, pH 8.2) and methanol as the mobile phase.

(*S*,*S*)-1-(1-Carboxy-2-methylbutyl)-5-hydroxy-2-(hydroxymethyl)-pyridinium inner salt (**12**; yield 1%): LC-MS (ESI^+): *m/z* 240 (100, $[M+1]^+$); ^1H NMR (400 MHz; D_2O; TMS-P): δ 0.87 [t, 3 H, J = 7.5 Hz, H-C(10)], 1.14 [d, 3 H, J = 6.6 Hz, H-C(11)], 1.30 [m, 1 H, H_a-C(9)], 1.56 [m, 1 H, H_b-C(9)], 2.34 [m, 1 H, H-C(8)], 4.73 [s, 2 H, H-C(6)], 4.92 [d, 1 H, J = 10.2 Hz, H-C(7)], 7.44 [dd, 1 H, J = 2.7, 8.9 Hz, H-C(3)], 7.59 [d, 1 H, J = 8.9 Hz, H-C(4)], 8.00 [d, 1 H, J = 2.7 Hz, H-C(1)].

(*R*,*S*)-1-(1-Carboxy-2-methylbutyl)-5-hydroxy-2-(hydroxymethyl)-pyridinium inner salt (**13**; yield 1%): LC-MS (ESI^+): *m/z* 240 (100, $[M+1]^+$); ^1H NMR (400 MHz; D_2O;

TMS-P): δ 0.80 [d, 3H, *J* = 6.6 Hz, H-C(11)], 1.01 [t, 3H, *J* = 7.5 Hz, H-C(10)], 1.30 [m, 1H, H_a-C(9)], 1.69 [m, 1H, H_b-C(9)], 2.39 [m, H, H-C(8)], 4.72 [s, 2H, H-C(6)], 4.93 [d, 1H, *J* = 10.5 Hz, H-C(7)], 7.45 [dd, 1H, *J* = 2.7, 8.9 Hz, H-C(3)], 7.59 [d, 1H, *J* = 8.9 Hz, H-C(4)], 7.99 [d, 1H, *J* = 2.7 Hz, H-C(1)].

2.2.8 Compound 16 (Fig. 3)

2.2.8.1 (*S*)-1-[*N*-(1-Carboxy-2-phenylethyl)-carboxamido-methyl]-2-(hydroxymethyl)-5-(methylamino)furan

A solution of 5-(hydroxymethyl)-2-furaldehyde (5 mmol) and H-Gly-Phe-OH (10 mmol) in water (15 mL) was adjusted to pH 9.4 with conc. sodium hydroxide solution and stirred in a hydrogenation vessel for 30 min at room temperature. After the addition of Raney nickel (0.5 g) the solution was stirred in a hydrogen atmosphere (5 bar) for 40 h. The catalyst was filtered off, washed with methanol, and the filtrate was concentrated *in vacuo*. The residue was taken up in ammonium formate buffer (10 mmol/L, pH 8.2) and applied onto the top of a water-cooled glass column (4 × 30 cm) filled with a slurry of RP-18 material (LiChroprep 25–40 μm; Merck) in a mixture (75/25 v/v) of ammonium formate (10 mmol/L, pH 8.2) and methanol. The same solvent mixture was used as the mobile phase, the effluent was monitored at 220 nm and the elution of the target compound was confirmed by RP-HPLC/DAD. After freeze-drying, (*S*)-1-[*N*-(1-carboxy-2-phenylethyl)-carboxamido-methyl]-2-(hydroxylmethyl)-5-(methylamino)furan (2.6 mmol, 53% in yield) was obtained.

(*S*)-*N*-[*N*-(1-Carboxy-2-phenylethyl)-carboxamido-methyl]-2-(hydroxymethyl)-5-(methylamino)furan: LC-MS (ESI^+): *m/z* 333 (37, $[M+1]^+$), 355 (68, $[M+Na]^+$), 655 (82, $[2M+1]^+$), 709 (92, $[2M+2Na]^+$), 731 (100, $[2M+3Na]^+$); ^{1}H NMR (400 MHz; DMSO-d_6): δ 2.94 [dd, 1H, *J* = 6.7, 13.5 Hz, CH_aH], 3.03 [d, 2H, *J* = 4.1 Hz, CH_2], 3.10 [dd, 1H, *J* = 12.8, 13.5 Hz, CH_bH], 3.49 [s, 2H, CH_2], 4.27 [dd, 1H, *J* = 6.7, 12.8 Hz, CH], 4.34 [s, 2H, CH_2], 6.08 [d, 1H, *J* = 3.1 Hz, CH], 6.16 [d, 1H, *J* = 3.1 Hz, CH], 7.18 [m, 5H, 5 × CH]; ^{13}C NMR (100 MHz; DMSO-d_6; HMBC, HMQC): δ 37.9 [CH_2], 45.8 [CH_2], 51.6 [CH_2], 55.0 [CH], 57.3 [CH_2], 107.8 [CH], 108.8 [CH], 127.1 [CH], 128.5 [2 × CH], 129.8 [2 × CH], 136.7 [C], 153.3 [C], 154.8 [C], 170.4 [CO], 172.0 [COOH].

2.2.8.2 Compound 16

To a cooled (0°C) solution of (*S*)-1-[*N*-(1-carboxy-2-phenylethyl)-carboxamido-methyl]-2-(hydroxymethyl)-5-(methylamino)-furan (2.5 mmol) in 15 mL of water was added dropwise a solution of bromine in methanol (2.0 mmol in 3.5 mL) over a period of 30 min and stirring was continued for 1 h. The resulting mixture was neutralized by the addition of a strongly basic ion exchange resin (Amberlite IRA-400 (OH^-) ion exchanger resin, filtered, and evaporated. The residue was taken up in a mixture (90/10 v/v) of ammonium formate (10 mmol/L, pH 8.2) and methanol and applied onto the top of a water-cooled glass column (4 × 30 cm) filled with a slurry of RP-18 material (LiChroprep 25–40 μm; Merck) in a mixture (90/10 v/v) of ammonium formate (10 mmol/L, pH 8.2) and methanol. The same solvent mixture was used as the mobile phase, the effluent was monitored at 330 nm and the elution of the target compound was confirmed by RP-HPLC/DAD. After freeze-drying twice, (*S*)-1-[*N*-(1-carboxy-2-phenylethyl)-carboxamido-methyl]-5-hydroxy-2-(hydroxymethyl)-pyridinium inner salt was obtained.

(*S*)-1-[*N*-(1-Carboxy-2-phenylethyl)-carboxamido-methyl]-5-hydroxy-2-(hydroxymethyl)-pyridinium inner salt (**16**; yield 19%): LC-MS (ESI^+): *m/z* 331 (100, $[M+1]^+$), 661 (25, $[2M+1]^+$), 683 (33, $[2M+Na]^+$), 705 (35, $[2M+2Na]^+$), 727 (42, $[2M+3Na]^+$); ^{1}H NMR (400 MHz; DMSO-d_6): δ 2.84 [dd, 1H, *J* = 8.4, 13.5, H_a-C(10)], 3.11 [dd, 1H, *J* = 3.9, 13.5 Hz, H_b-C(10)], 4.20 [dd, 1H, *J* = 3.9, 8.4 Hz, H-C(9)], 4.35 [s, 2H, H-C(6)], 5.13 [s, 2H, H-C(7)], 7.20 [m, 5 × 1H, H-C(12)–H-C(16); 1H, H-C(3)], 7.44 [d, 1H, *J* = 9.3 Hz, H-C(4)], 7.68 [d, 1H, *J* = 1.8 Hz, H-C(1)]; ^{13}C NMR (100 MHz; DMSO-d_6; HMQC, HMBC): δ 37.4 [CH_2, C(10)], 56.5 [CH_2, C(6)], 58.0 [CH_2, C(7)], 58.6 [CH, C(9)], 126.2 [CH, C(14)], 127.5 [CH, C(4)], 128.3 [2 × CH, C(12), C(16)], 129.6 [2 × CH, C(13), C(15)], 133.4 [CH, C(3)], 136.6 [CH, C(1)], 137.5 [CH, C(11)], 139.3 [C, C(5)], 165.1 [CO, C(8)], 166.2 [CO, C(2)], 173.8 [COOH, C(17)].

2.3 Sensory analyses

2.3.1 Training of the sensory panel

Twelve subjects (five women and seven men, age 25–38 years) with no history of known taste disorders were trained to evaluate the taste of aqueous solutions (2 mL each) of the following standard taste compounds by using a triangle test as described in the literature [24]: 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, sodium glutamate (3 mmol/L) for umami taste, and gallustannic acid (0.05%) for astringency. The assessors had participated earlier at regular intervals for at least two years in sensory experiments and were, therefore, familiar with the techniques applied.

2.3.2 Determination of taste thresholds

Taste recognition thresholds, meaning the concentrations at which the typical taste qualities of the compounds were just detectable, were determined in triangle test using bottled

water (Evian®) as the solvent. The pH value of the solutions was adjusted to 7.0 by adding either aqueous hydrochloric acid (0.01 mmol/L) or aqueous sodium hydroxide solution (0.01 mmol/L). Serial 1 : 1 dilutions of the samples were presented in order of increasing concentrations to a trained panel of twelve persons in three different sessions, using the sip-and-spit method. At the start of the session and before each trial, the subject rinsed with water and expectorated. The samples, both blanks as well as the taste solutions were swirled around in the mouth briefly and expectorated. After indicating which glass vial showed the typical quality (umami, salty, sweet, sour, bitter) of the tastant, the participant received another set of two blanks and one taste sample. To prevent excessive fatigue, tasting began at a concentration level two steps below the threshold concentration that had been determined in a preliminary taste experiment. Whenever the panelist selected incorrectly, the next trial took place at the next higher concentration step. When the panelist selected correctly, the same concentration was presented again besides two blanks as a proof for the correctness of the data. The geometric mean of the last and the second last concentration was calculated and taken as the individual recognition threshold. The values between individuals, and between separate sessions, differed by not more than one dilution step.

2.4 HPLC

The HPLC apparatus (Jasco, Groß-Umstadt, Germany) consisted of a HPLC-pump system PU 1580 with an in-line degasser (DG-1580-53), a low-pressure Gradient unit (LG-1580-02) and a DAD type MD 1515. Separations were performed on stainless-steel columns packed with RP-18 material (ODS-Hypersil, 5 µm; Phenomenex) in either analytical (4.6 × 250 mm, flow rate 0.8 mL/min) or semipreparative scale (10 × 250 mm, flow rate 3.2 mL/min). The samples were analyzed using varying gradients with aqueous ammonium formate (10 mmol/L, pH 8.2) or formic acid (0.1%, pH 2.5) and methanol as the mobile phase.

2.5 LC-MS

An analytical HPLC column (ODS-Hypersil, 5 µm; Phenomenex) was coupled to an LCQ-MS (Finnigan MAT, Bremen, Germany) using positive (ESI^+) and negative (ESI^-) electronspray ionization. The samples were separated using varying gradients with aqueous trifluoroacetic acid (0.1%, pH 2.5) and methanol as the mobile phase.

2.6 NMR

^1H, ^{13}C, and DEPT-135 NMR experiments were performed on Bruker AC-250 and AV-360 spectrometers. ^1H, COSY, HMQC, and HMBC measurements were performed on a Bruker AMX 400-III spectrometer (Bruker, Rheinstetten, Germany). Evaluation of the experiments was carried out using 1D- and 2D-WIN-NMR as well as XWin-NMR software (version 3.5; Bruker, Rheinstetten, Germany).

3 Results and discussion

In order to confirm the recently observed effect of 1-(1-carboxyethyl)-5-hydroxy-2-(hydroxymethyl)-pyridinium inner salt (**1**; Fig. 1) on sweetness perception, we reinvestigated its influence on the threshold concentrations of the disaccharide sucrose. Depending on the stereochemistry, the human sensitivity for sweetness was significantly increased when racemic alapyridaine and, even more pronounced, the (+)-(*S*)-enantiomer were present; for example, racemic or (*S*)-configured alapyridaine decreased the oral threshold of sucrose (12.5 mmol/L) by a factor of four or eight to 3.0 or 1.5 mmol/L, respectively (Table 1). In contrast, the (−)-(*R*)-enantiomer did not affect the sweetness threshold of the sugar.

To gain more detailed information on the molecular requirements of 1-(1-carboxyethyl)-5-hydroxy-2-(hydroxymethyl)-pyridinium inner salt (**1**; Fig. 1) for taste enhancement, a range of structurally related pyridinium betaines were synthesized, and their psychophysical activities sensorially evaluated. Suggesting that the oral activities are a reasonable close approximation to the intrinsic activities of the taste enhancer, the determination of the oral threshold values for the taste-modifying effect appeared to give the best measure of intrinsic psychophysical activity.

3.1 Influence of hydroxyl and hydroxymethyl groups on taste-enhancing activity

In a first set of experiments, four pyridinium betaines were synthesized either by removing, or modifying the hydroxymethyl group and the hydroxyl function, respectively, at position 2 and 5 of the pyridinium ring of **1**. The compounds were purified by column chromatography and characterized by LC-MS as well as 1-D and 2-D NMR spectroscopy.

To study whether the presence of the hydroxymethyl function at position 2 is essential for taste-enhancing activity, racemic 1-(1-carboxyethyl)-3-hydroxy-pyridinium inner salt (**2**; Fig. 1) was prepared by using Maillard-type chemistry from L-alanine and furan-2-aldehyde. In order to check the role of the hydroxyl group at position 5, in addition,

Table 1. Influence of equimolar amounts of pyridinium betaines on the detection thresholds for sweet taste of sucrose, umami taste of monosodium glutamate (MSG), and salty taste of sodium chloride (NaCl)

Added compound	Sweet taste of sucrose		Umami taste of MSG		Salty taste of NaCl	
	TC[a)]	fTD[b)]	TC[a)]	fTC[b)]	TD[a)]	fTC[b)]
–	12.5		1.5		10.0	
(+)-(*S*)-1[c)]	1.5	8	0.2	8	2.0	5
(–)-(*R*)-1[c)]	12.5	–	1.5	–	10.0	–
1[c)]	3.0	4	0.4	4	2.0	5
2[c)]	12.5	–	1.5	–	10.0	–
3[d)]	–	–	1.5	–	10.0	–
4[c)]	12.5	–	1.5	–	10.0	–
5[c)]	12.5	–	1.5	–	10.0	–
6[e)]	–	–	1.5	–	10.0	–
7[c)]	12.5	–	1.5	–	10.0	–
8[c)]	12.5	–	1.5	–	10.0	–
9[f)]	–	–	1.5	–	10.0	–
10[c)]	3.0	4	0.2	8	2.0	5
11[c)]	12.5	–	1.5	–	10.0	–
12[c)]	12.5	–	1.5	–	10.0	–
13[c)]	12.5	–	1.5	–	10.0	–
14[c)]	12.5	–	1.5	–	10.0	–
15[g)]	10.0	–	1.5	–	2.0	5
16[c)]	3.0	4	0.4	4	n.d.	n.d.

a) TC, threshold concentration (mmol/L water, pH 7.0)
b) fTD, factor of threshold decrease induced by the test compound
c) Compound did not show any taste on its own up to the concentration of 50 mmol/L.
d)–g) Compound showed bitter taste at the threshold concentration of (d) 1.5 mmol/L, (e) 5.8 mmol/L, (f) 0.8 mmol/L, and (g) 10.0 mmol/L.
n.d., not determined

1-(1-carboxyethyl)-2-hydroxymethyl-pyridinium inner salt (**3**; Fig. 1) was synthesized from 2-(hydroxymethyl)-pyridine and 2-bromopropionic acid. After proving the purity of the compounds by HPLC-DAD (DAD, diode-array detector) and ^{1}H-NMR spectroscopy, sensory analysis of aqueous solutions of the pyridinium betaines **1**–**3** demonstrated that these compounds are entirely tasteless on their own. In order to investigate the taste enhancing potential of these compounds, the influence of the pyridinium betaines **1**–**3** on the oral recognition thresholds for the umami-tasting monosodium glutamate, the sweet-tasting sucrose, and the salty-tasting sodium chloride was evaluated by a trained sensory panel using triangle tests. Confirming the results of previous studies [24], the human recognition threshold of aqueous solutions of sucrose (12.5 mmol/L), monosodium glutamate (1.5 mmol/L), and sodium chloride (20 mmol/L) were found to be significantly decreased in the presence of equimolar amounts of racemic alapyridaine (**1**); for example, the threshold for monosodium glutamate dropped by a factor of 4 from 1.5 to 0.4 mmol/L when compound **1** was present in equimolar amounts (Table 1). In contrast, the sensory threshold for the umami taste of aqueous solutions containing either the pyridinium betaine **2**, or compound **3** in mixture with monosodium glutamate was determined to be 1.5 mmol/L, which is the recognition threshold of monosodium glutamate (Table 1). In addition, human thresholds of 12.5 and 10 mmol/L were measured for solutions containing sucrose and NaCl, respectively, in equimolar mixtures with one of the pyridinium betaines **2** and **3** (Table 1), which exactly match the detection thresholds for sweetness and saltiness of the basic taste compounds. These data clearly indicate that both, the hydroxyl as well as the hydroxymethyl substituent at the pyridinium moiety, act as important structural features required for the taste enhancing activity of compound **1**.

To further investigate whether modifications of the hydroxymethyl group in compound **1** by reduction or oxidation is altering its sensory activity, 1-(1-carboxyethyl)-5-hydroxy-2-methyl-pyridinium inner salt (**4**; Fig. 1) was prepared by using Maillard-type chemistry from 5-methyl-2-furanaldehyde and L-alanine, and 1-(1-carboxyethyl)-2-carboxy-5-hydroxy-pyridinium inner salt (**5**; Fig. 1) was synthesized upon permanganate oxidation of the hydroxymethyl group in compound **1**. After proving that these compounds were essentially tasteless on their own, the taste modifying activity of the pyridinium betaines **4** and **5** were investigated by a trained sensory panel using triangle tests. But neither in aqueous solutions containing monosodium glutamate, sucrose, nor sodium chloride, the addition of equimolar amounts of compounds **4** or **5** induced any significant decrease in human recognition threshold when compared to aqueous solutions containing only the basic tastants (Table 1). On the basis of these data it might be concluded that both the hydroxyl as well as the hydroxymethyl substituent at the pyridinium moiety are essential structural elements required for taste enhancing activity of compound **1**.

3.2 Influence of the *N*-(1-carboxyethyl) moiety on taste-enhancing activity

To investigate the essential elements in the *N*-(1-carboxyethyl) group of compound **1**, 1-ethyl-5-hydroxy-2-hydroxymethyl-pyridinium inner salt (**6**; Fig. 2), lacking the carboxy function, and 1-carboxymethyl-5-hydroxy-2-(hydroxymethyl)-pyridinium inner salt (**7**; Fig. 2), lacking the methyl group, were synthesized from 5-(hydroxymethyl)-2-furaldehyde and the corresponding amino compounds. After purification and spectroscopic structure confirmation, the taste thresholds of these pyridinium betaines were determined by means of a trained sensory panel. Whereas compound **7** was evaluated as being essentially tasteless, compound **6** was found to exhibit an intense bitter taste with a threshold concentration of 5.8 mmol/L (Table 1). The oral thresholds of solutions of one of the basic tastants sucrose,

monosodium glutamate or sodium chloride, respectively, in the presence of either compound **6** or **7**, matched those found for the basic tastants (Table 1), thus demonstrating that not only the carboxy group, but also the methyl group, is crucial for the taste enhancing activity of compound **1**.

To further investigate the influence of the chain length of the *N*-alkyl group in compound **6**, 1-methyl-5-hydroxy-2-hydroxymethyl-pyridinium inner salt (**8**; Fig. 2) and 1-propyl-5-hydroxy-2-hydroxymethyl-pyridinium inner salt (**9**; Fig. 2) were prepared by utilizing the Maillard-type reaction of 5-(hydroxymethyl)-2-furaldehyde and methylamine or *n*-propylamine, respectively. After purification and approval of the chemical structure by LC-MS and NMR spectroscopy, the sensory panel described the taste of compound **9** as being intensely bitter, whereas compound **8** did not show any taste on its own. It is interesting to notice that the increase of the chain length of the *N*-alkyl group in compounds **6**, **8**, and **9** lead to a drastic decease of the taste threshold for bitterness; *e.g.*, the bitter intensity increased from the non-bitter compound **8** over the bitter ethyl derivative **6** to the intensely bitter-tasting propyl derivative **9** evaluated with a threshold concentration of 0.8 mmol/L. In addition, none of the pyridinium betaines with an aliphatic chain showed any taste-enhancing activity for umami, sweet, or salty taste modalities (Table 1). As these data demonstrate that the lack of the carboxy function as well as the methyl group in compound **1** is diminishing the taste-enhancing activity, the following experiments were focused on the question as to how the substituents of naturally occurring amino acids are influencing the human bioresponse for pyridinium betaines.

3.3 Influence on amino acid moieties on taste-enhancing activity

To study the role of the amino acid moiety on taste modifying activity, we synthesized six additional pyridinium betaines varying either in the polarity, or the net charge of the side chain. Starting from 5-(hydroxymethyl)-2-furaldehyde and L-phenylalanine, 1-(1-carboxy-2-phenylethyl)-5-hydroxy-2-(hydroxymethyl)-pyridinium inner salt (**10**; Fig. 3), bearing a benzyl group instead of the methyl group in alapyridaine, was prepared and purified. Substitution of phenylalanine with the neutral amino acids L-valine and L-isoleucine revealed 1-(1-carboxy-2-methylpropyl)-5-hydroxy-2-(hydroxymethyl)-pyridinium inner salt (**11**; Fig. 3) as well as the diastereomers (*S*,*S*)- and (*R*,*S*)-1-(1-carboxy-2-methylbutyl)-5-hydroxy-2-(hydroxymethyl)-pyridinium inner salt (**12** and **13**; Fig. 3), all of which bearing an aliphatic side chain as well as the negatively charged carboxy function as the amino substituent (Table 1). For confirmation of the stereochemistry in compounds **12** and **13**, these compounds have been enantioselectively synthesized following the procedure reported recently for (+)-(*S*)-**1** and (−)-(*R*)-**1** [25]. In order to investigate the influence of more polar substituents, 1-(1,3-dicarboxypropyl)-5-hydroxy-2-(hydroxymethyl)-pyridinium inner salt (**14**; Fig. 3) containing an additional carboxy function at the amino substituent, and 1-[4-(*N*-(aminoiminomethyl)-amino)-1-carboxy-butyl]-5-hydroxy-2-(hydroxymethyl)-pyridinium inner salt (**15**; Fig. 3), bearing a basic guanidino group at the amino substituent, were synthesized starting from the amino acids glutamate and arginine, respectively.

After final purification and structure confirmation, sensory analysis revealed all compounds to be tasteless on their own with the exception of pyridinium betaine **15** exhibiting bitter taste at a threshold concentration of 10 mmol/L (Table 1). In equimolar mixtures with the basic taste compounds sucrose, monosodium glutamate, and sodium chloride, respectively, compound **10** induced a significant decrease of the detection threshold of sweetness, umami taste, and saltiness by factors of four and five, respectively (Table 1). In contrast to this compound bearing the benzyl substituent, neither the substitution of the methyl group in the alanine moiety of compound **1** by the 1-methylethyl group of valine, nor by the 1-methylpropyl moiety of isoleucine showed any effect of taste enhancement. Also the polar compounds **14** and **15** did not influence the sweet and umami detection threshold of sucrose and monosodium glutamate, but the guanidino compound **15** was found to affect the salt perception of sodium chloride, *e.g.*, the addition of **15** to an equimolar solution of sodium chloride decreased the sensory threshold for saltiness by a factor of 5 (Table 1).

To evaluate how the taste-enhancing properties of the pyridinium betaine **10** are affected upon insertion of another amino acid into the amino substituent, (*S*)-1-[*N*-(1-carboxy-2-phenylethyl)-carboxamido-methyl]-5-hydroxy-2-(hydroxymethyl)-pyridinium inner salt (**16**) differing from **10** by insertion of a glycine spacer was synthesized by bromine oxidation of (*S*)-1-[*N*(1-carboxy-2-phenylethyl)-carboxamido-methyl]-2-(hydroxymethyl)-5-(methylamino)furan, prepared by reductive amination of 5-(hydroxymethyl)-2-furaldehyde with the dipeptide Gly-Phe. Interestingly, the tasteless compound **16** showed similar taste-enhancing activities as found for compound **10** (Table 2), *e.g.*, in the presence of **16** the threshold for sucrose and monosodium glutamate dropped by factors of four and eight, respectively. None of the compounds **1**–**16** decreased the taste thresholds for the bitter compound caffeine and the sour tasting citric acid (data not shown).

4 Concluding remarks

Taking all these data into consideration, it might be concluded that the 2-(hydroxymethyl)-5-hydroxypyridinium moiety is an essential structural element for taste-enhan-

cing activity. Regarding the amino substituent, the 1-(1-carboxy-2-ethyl)- as well as the 1-(1-carboxy-2-phenylethyl)-moiety from alanine and phenylalanine, respectively, were found to be necessary prerequisites for physiological activity. Neither the prolongation of the alkyl chain (compounds **11** – **13**), the removal of the methyl group (compound **7**), nor the presence of an additional carboxy function (compound **14**), or a guanidine function (compound **15**), respectively, induced any taste enhancing effect. Interestingly, insertion of the amino acid glycine between the phenylalanine moiety and the nitrogen atom of the pyridinium ring of compound **10** resulted in compound **16** showing similar taste enhancing effects as demonstrated for compound **10** lacking the glycine spacer. On the basis of these data, it might be suggested that the benzyl ring in compounds **10** and **16** might favor the activity of these compounds as taste enhancers at the receptor level. In contrast to the multimodal taste enhancers **1**, **10** and **16**, the arginine-derived pyridinium betaine **15** exclusively increases the human sensitivity for salty taste, thus suggesting another biochemical mechanism in salt taste enhancement involving the guanidino function of the L-arginine side-chain.

5 References

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