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Application of Hydrophilic Interaction Liquid Chromatography/ Comparative Taste Dilution Analysis for Identification of a Bitter Inhibitor by a Combinatorial Approach Based on Maillard Reaction Chemistry

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Activity-directed fractionation of heated carbohydrate/alanine solutions recently led to the discovery of (+)-(*S*)-1-(1-carboxyethyl)-5-hydroxy-2-(hydroxymethyl)pyridinium inner salt (**1**, alapyridaine), and it has been shown that this compound lowers the detection thresholds of sugars, glutamate, and NaCl solutions, whereas no influence on bitter perception was observed. As this class of Maillard-derived pyridinium betaines seemed to be promising targets for further research on their taste modulatory activity, the objective of the present investigation was to screen for bitter taste-suppressing target molecules in combinatorial libraries of pyridinium betaines prepared from 5-(hydroxymethyl)-furan-2-aldehyde and amino acid mixtures by use of Maillard-type reaction chemistry instead of synthesizing and purifying each derivative individually. By application of hydrophilic interaction liquid chromatography in combination with the recently developed comparative taste dilution analysis, followed by structure determination, synthesis, and sensory studies, we have now succeeded in identifying 1-carboxymethyl-5-hydroxy-2-hydroxymethylpyridinium inner salt (**2**) as a potential bitter-suppressing candidate. While tasteless on its own, **2** was found to reduce the bitterness of various bitter tastants such as the amino acid L-phenylalanine, the peptide Gly-Leu, the alkaloid caffeine, and the bitter glycosides salicin and naringin.

KEYWORDS: Bitter inhibitor; bitter blocker; pyridinium betaine; alapyridaine; Maillard reaction; taste dilution analysis; hydrophilic interaction liquid chromatography

INTRODUCTION

Mammalian taste perception consists of the gustatory taste sensation, that is, the sweet, bitter, sour, salty and umami basic taste modalities, as well as lingual somatosensory sensitivity resulting from temperature and tactile stimulation as well as chemical activation of chemosensory receptors on the perigemmal fibers (*1*). In combination, this sensory detection system provides valuable information on the sensory active ingredients of the food we eat.

In consequence, the development of healthy food products reduced in sugar, salt, glutamate, and fat, respectively, or enriched in plant-derived bioactive, health-promoting micronutrients, which are claimed to be beneficial for health and well-being of the consumers, induced nonacceptable flavor defects in the products and unexpected flavor challenges for the food industry. In response to consumer demand for healthy but tasty foods, novel ingredient discovery is essential to overcome such flavor challenges associated with fortified or functional foods and beverages. One important step to make healthy food taste better is to control bitter off-tastes of food products, either by masking but preferably by suppressing the perception of bitter taste by compounds coined "bitter inhibitors".

By means of an in vitro assay using taste receptor-containing membranes isolated from bovine circumvallate papillae, several purine nucleotides such as adenosine monophosphate (AMP) and chemically related compounds were recently shown to inhibit taste receptor activation by several bitter tastants, such as, for example, denatonium, quinine, or strychnine (*2*). On the basis of these experiments, it was claimed that, in general, purine and pyrimidine nucleotides or derivatives thereof bearing an ionizable phosphate group are able to inhibit the human perception of bitter taste in food, pharmaceutical, and cosmetic products (*3*). Since these nucleotides are known to exhibit a strong umami taste on their own, it is suggested that the perceived bitterness modulation is caused by simple masking effects rather than by a specific taste receptor inactivation. In contrast, the tasteless and odorless neodiosmin was reported to act as a specific "bitter inhibitor" in citrus fruit juices exhibiting

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undesirable bitter taste caused by the triterpenoids limonin and naringin (*4*). Moreover, neodiosmin was also found to partly suppress the bitter taste perception induced by the alkaloids caffeine and quinine as well as the artificial sweetener saccharin (*5*).

The literature survey shows that the sensory evaluation of potential bitter inhibitors has been mainly focused on compounds of which the chemical structure was already known. In particular, the structures and sensory activities of taste-modifying compounds that are not present in the foods per se but are generated during food processing from precursors, e.g. by Maillard-type reactions from carbohydrates and amino acids, remain mainly unknown. To bridge the gap between pure structural chemistry and human taste perception, very recently, a screening procedure, the so-called comparative taste dilution analysis, cTDA (*6*), was applied to high-performance liquid chromatographic (HPLC) fractions isolated from heated foods and heated carbohydrate/amino acid solutions of hexoses and L-alanine in order to locate a sweetness-enhancing compound formed upon food processing. This novel approach led to the discovery of the previously not reported (+)-(*S*)-1-(1-carboxyethyl)-5-hydroxy-2-(hydroxymethyl)pyridinium inner salt, named alapyridaine, in heated hexose/L-alanine mixtures as well as in beef bouillon, and it has been shown that this compound lowers the detection thresholds of sugars, glutamate, and NaCl solutions, whereas no influence on bitter perception was observed (*6*−*8*).

As this class of Maillard-derived pyridinium betaines seemed to be promising targets for further research on their taste modulatory activity, the objective of the present investigation was to evaluate alapyridaine-related pyridinium betaines for their bitter-suppressing activity. Instead of choosing the laborious way of synthesizing, purifying, and testing a range of derivatives individually, the screening for target compounds should be performed by using a combinatorial approach based on Maillard reaction chemistry.

MATERIALS AND METHODS

Chemicals. The following compounds were obtained commercially: β-alanine, γ-amino-*n*-butyric acid, ammonium formate, 5-(hydroxymethyl)-2-furanaldehyde, and gallustannic acid (Aldrich, Steinheim, Germany); caffeine, citric acid, formic acid, D-glucose, hydrochloric acid, lactic acid, salicin, sodium chloride, sodium hydroxide, and sucrose (Merck, Darmstadt, Germany); amino acids, denatonium benzoate, naringin, and trifluoroacetic acid (Fluka, Buchs, Switzerland); d-fructose (Riedel-de Haën, Seelze, Germany); and H-Gly-Leu-OH (Bachem Biochemica, Heidelberg, Germany). Solvents were of HPLC grade (Merck). Deuterated solvents were obtained from Euriso-top (Gif-sur-Yvette, France). Bottled water (Evian) was used for sensory evaluation. Alapyridaine (**1**) was synthesized following the procedure reported recently (*6*).

Acid-Catalyzed Generation of 5-(Hydroxymethyl)furan-2-aldehyde from Fructose. A solution of D-fructose (130 mmol) in water (60 mL) and concentrated hydrochloric acid (32%, 0.4 g) was heated for 2 h at 130 °C in a laboratory autoclave to generate 5-(hydroxymethyl)-2-furanaldehyde. Quantitative analysis by means of gas chromatography/mass spectrometry (GC/MS) revealed that the fructose was converted into the target compound (35 mmol) in a yield of 26%.

Preparation of Pyridinium Betaine Libraries. Amino acid mixture A containing glycine, l-alanine, L-valine, L-leucine, and L-threonine, mixture B containing L-serine, L-aspartic acid, L-arginine, and L-tryptophan, mixture C containing L-methionine, L-histidine, L-lysine, and L-asparagine, or mixture D containing L-cysteine, L-glutamate, L-glutamine, L-phenylalanine, and L-tyrosine (4.0 mmol of each amino acid) was added to aliquots of the fructose hydrolysate containing 5-(hydroxymethyl)furan-2-aldehyde (17.0 mmol). After addition of ethanol (25 mL) and adjusting the solution to pH 9.4 with sodium hydroxide, the solutions were refluxed for 72 h. After cooling, the reaction mixtures were freed from solvent in a vacuum, and taken up in water (50 mL).

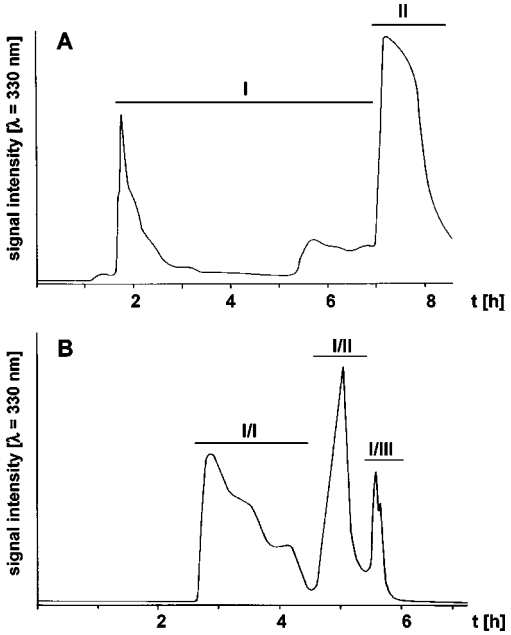

Figure 1. (**A**) RP-18 chromatogram of fructose hydrolysate heated in the presence of amino acid mixture A containing glycine, L-alanine, L-valine, L-leucine, and L-threonine. (**B**) Gel-permeation chromatogram of RP-18 fraction I.

Fractionation of Pyridinium Betaines Libraries. The aqueous solution of each reaction mixture was extracted with ethyl acetate (3 × 100 mL), the organic layer was discarded, and aliquots of the aqueous layer were fractionated by RP18 column chromatography on a water-cooled glass column (300 × 40 mm i.d.) filled with a slurry of LiChroprep 25−40 μm (Merck, Darmstadt, Germany) in aqueous ammonium formate (10 mmol/L, pH 8.2). Chromatography was performed with ammonium formate (10 mmol/L, pH 8.2, 600 mL) and aqueous ammonium formate/methanol (90/10 v/v, pH 8.2, 200 mL), followed by aqueous ammonium formate/methanol (75/25 v/v, pH 8.2, 400 mL) as the mobile phase. The effluent was monitored at λ 330 nm, and fraction I (**Figure 1**), exhibiting the typical UV/vis absorption maxima at λ 251 and 328 nm (pH 8.2) or λ 298 nm (pH 3.5) reported for pyridinium betaines (*6*), was collected, freeze-dried, taken up in water (10 mL), and then applied onto the top of a glass column (750 × 55 mm) (Pharmacia, Uppsala, Sweden) filled with a slurry of Sephadex G15 (Pharmacia) in Millipore water. Gel-permeation chromatography (GPC) was performed with water as the mobile phase (3 mL/min), and the effluent was monitored by means of a UV/vis detector operating at 330 nm. Three GPC fractions (fractions I/I−I/III; **Figure 2**) were collected by a fraction collector. Reverse-phase (RP) HPLC/diode-array detection (DAD) analysis identified pyridinium betaines in GPC fraction I/II, which was freeze-dried and used for hydrophilic interaction liquid chromatography (HILIC) and comparative taste dilution analysis (cTDA).

Hydrophilic Interaction Liquid Chromatography and Comparative Taste Dilution Analysis. After membrane filtration, aliquots (100 μL) of GPC fraction I/II were analyzed by hydrophilic interaction liquid chromatography on a semipreparative TSK-Gel Amide-80 column (300 × 7.8 mm i.d.; 10 × 1.4 mm i.d. guard column) (Tosoh BioSep, Stuttgart, Germany). Chromatography (flow rate 1.5 mL/min) was performed with a linear gradient that starting with mixture A (8/2 v/v, pH 7.0) of acetonitrile and aqueous ammonium formate (7.0 mmol/L) and then increased the content of solvent mixture B, which consisted of a mixture (2/8 v/v, pH 7.0) of acetonitrile and aqueous ammonium formate (7.0 mmol/L), to 12.5% within 60 min and then to 75% within 5 min. Monitoring the effluent by diode-array detection revealed five fractions showing the typical absorption maxima of pyridinium betaines. Fractions I/II-1 to I/II-5 from several HPLC runs were separately

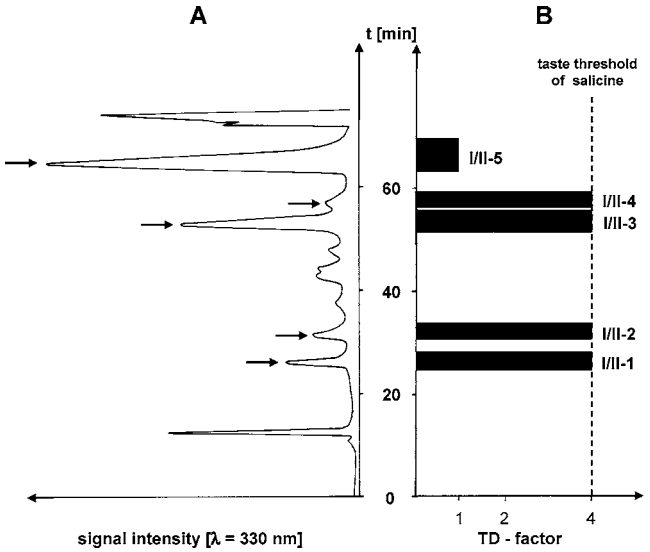

Figure 2. HILIC–HPLC chromatogram (**A**) and comparative taste dilution chromatogram (**B**) of GPC fraction I/II with an aqueous salicin solution (1.0 mmol/L) as the basic taste compound; peaks labeled with arrows showed typical UV/vis absorption spectra of pyridinium betaines.

collected, and traces of solvents and volatile buffers were removed under high vacuum (<5 mPa), followed by a triple freeze-drying procedure. The same amounts of the individual fractions were dissolved in an aqueous solution (2 mL) of the bitter tastant salicin (1.0 mmol/L), and the individual 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 the tester was wearing a nose clamp, each dilution was sensorially evaluated for bitterness in a triangle test with water as the control. The dilution at which a bitter taste difference between the diluted fraction and two controls could just be detected was defined as the taste dilution (TD) factor (*9*). The TD factors evaluated by 10 different assessors on three different sessions were averaged. The TD factors between individuals and separate sessions differed not more than one dilution step.

Synthesis of Bitter-Suppressing Pyridinium Betaines 2−4. A solution of 5-(hydroxymethyl)furan-2-aldehyde (30 mmol) and either glycine, β-alanine, or γ-aminobutyric acid (40 mmol) in water/ethanol (1/1 v/v, 100 mL) was adjusted to pH 9.4 with aqueous sodium hydroxide solution (32%). After stirring at room temperature for 1.5 h, the mixture was refluxed for 24 h and then cooled to room temperature. After the addition of 5-(hydroxymethyl)furaldehyde (5 mmol each time), this procedure was repeated twice. The sample was then concentrated in a vacuum, taken up in water (25 mL), and extracted with ethyl acetate (3 × 50 mL). Aliquots of the aqueous layer were applied onto the top of a water-cooled glass column (300 × 40 mm i.d.) filled with a slurry of LiChroprep 25−40 μm (Merck, Darmstadt, Germany) in aqueous ammonium formate (10 mmol/L, pH 8.2). Chromatography was performed with ammonium formate (10 mmol/L, pH 8.2, 600 mL) and aqueous ammonium formate/methanol (90/10 v/v, pH 8.2, 200 mL), followed by aqueous ammonium formate/methanol (75/25 v/v, pH 8.2, 400 mL) as the mobile phase. The effluent was monitored at λ 330 nm, and the fractions containing the target compounds were collected and freed from solvents by high-vacuum sublimation followed by triple freeze-drying, thus affording 1-carboxymethyl-5-hydroxy-2-(hydroxymethyl)pyridinium inner salt (**2**, 22% yield), 1-(2-carboxyethyl)-5-hydroxy-2-(hydroxymethyl)pyridinium inner salt (**3**, 12% yield), and 1-(3-carboxypropyl)-5-hydroxy-2-(hydroxymethyl)pyridinium inner salt (**4**, 5% yield) as white amorphous powders in a high purity of 99%.

1-Carboxymethyl-5-hydroxy-2-(hydroxymethyl)pyridinium Inner Salt **(2**, **Figure 3).** LC/MS (ESI$^+$) *m/z* 184 (72, $[M + 1]^+$), 206 (57, $[M + Na]^+$), 228 (76, $[M + 2Na]^+$), 411 (79, $[2M + 2Na]^+$), 433 (100, $[2M + 3Na]^+$); ^{1}H NMR (400 MHz; D_2O; TMS-P) δ 4.63 (s, 2H, CH_2), 4.94 (s, 2H, CH_2), 7.43−7.46 (dd, 1H, J = 2.7 and 8.9 Hz, CH), 7.56−7.58 (d, 1H, J = 8.9 Hz, CH), 7.67−7.68 (d, 1H, J = 2.7 Hz, CH); ^{13}C NMR (400 MHz; D_2O; TMS-P; HMBC, HMQC) δ 59.8 (CH_2), 60.3 (CH_2), 129.7 (CH), 131.0 (CH), 132.1 (CH), 138.7 (C), 165.2 (CO), 171.4 (COOH).

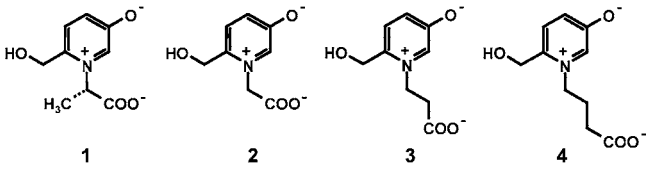

Figure 3. Structures of taste-enhancing alapyridaine (**1**), and bitter-suppressing pyridinium betaines derived from glycine (**2**), β-alanine (**3**), and γ-aminobutyric acid (**4**).

1-(2-Carboxyethyl)-5-hydroxy-2-(hydroxymethyl)pyridinium Inner Salt **(3, Figure 3).** LC/MS (ESI$^+$) *m/z* 198 (100, $[M + 1]^+$), 220 (18, $[M + Na]^+$), 395 (31, $[2M + 1]^+$); ^{1}H NMR (360 MHz; D_2O; TMS-P; COSY) δ 2.54−2.58 (t, 2H, J = 6.8 Hz, CH_2), 3.17−3.21 (t, 2H, J = 6.8 Hz, CH_2), 4.89 (s, 2H, CH_2), 7.62−7.65 (dd, 1H, J = 2.7 and 8.6 Hz, CH), 7.72−7.75 (d, 1H, J = 8.6 Hz, CH), 8.03−8.04 (d, 1H, J = 2.7 Hz, CH); ^{13}C NMR (360 MHz; D_2O; TMS-P; HMBC, HMQC) δ 36.3 (CH_2), 39.4 (CH_2), 61.8 (CH_2), 131.4 (CH), 136.8 (CH), 137.4 (CH), 139.9 (C), 164.7 (CO), 173.8 (COOH).

1-(3-Carboxypropyl)-5-hydroxy-2-hydroxymethylpyridinium Inner Salt **(4; Figure 3).** LC/MS (ESI$^+$) *m/z* 212 (100, $[M + 1]^+$), 234 (33 $[M + Na]^+$); ^{1}H NMR (400 MHz; D_2O; TMS-P; COSY) δ 2.16−2.24 (m, 2H, CH_2), 2.31−2.38 (m, 2H, CH_2), 4.45−4.49 (m, 2H, CH_2), 4.92 (s, 2H, CH_2), 7.76−7.79 (dd, 1H, J = 2.5 and 8.9 Hz, CH), 7.83−7.85 (d, 1H, J = 8.9 Hz, CH), 8.16−7.17 (d, 1H, J = 2.5 Hz, CH); ^{13}C NMR (360 MHz; D_2O; TMS-P; HMBC, HMQC) δ 29.7 (CH_2), 35.9 (CH_2), 59.5 (CH_2), 61.7 (CH_2), 131.3 (CH), 136.2 (CH), 137.0 (CH), 146.7 (C), 161.9 (CO), 173.6 (COOH).

Sensory Analysis. Ten subjects with no history of known taste disorders had participated earlier at regular intervals for at least 2 years in similar sensory experiments and were, therefore, familiar with the techniques and rating scales applied. Sensory training of the panelists with chemically defined reference stimuli was done as reported recently (*7*, *9*). Sensory analyses were performed in a sensory panel room at 22−25 °C on three different sessions.

Recognition Threshold Concentrations. The taste threshold concentrations were determined in a triangle test with bottled water (Evian) as the solvent. The samples were presented in order of increasing concentrations (serial 1:1 dilutions) to a trained panel of 10 persons who were asked to determine the threshold values in three different sessions by the sip-and-spit method (*7*). At the start of the session and before each trial, the subject rinsed with table water and expectorated. The samples, blanks as well as the taste solutions, were swirled around in the mouth briefly and expectorated. After indicating which glass vial contained 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 individual 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. The geometric mean of the last and the second last concentration was calculated and taken as the individual threshold. The values between individuals and separate sessions differed by not more than one dilution step.

Influence of Pyridinium Betaines on the Perceived Intensity of Bitter tastants. To study the influence of compounds **1**−**4** on the perception of bitter compounds, concentration series of aqueous solutions (pH 7.0) containing L-phenylalanine, glycyl-L-leucine, caffeine, naringin, salicin, or denatonium benzoate, and compounds **1**−**4** either alone or in combinations, were stepwise 1:1 diluted with water, and the sensory panel was asked to evaluate the intensity of the bitterness on a scale from 0 (no taste detectable) to 5 (strong taste) as reported recently (*10*, *11*). The dose/response curves determined on three different sessions were averaged. The intensity values between individuals and separate sessions differed by less than ±0.5 unit.

*Time-Resolved Influence of Compound **2** on Bitter Intensity of* L*-Phenylalanine.* Following a methodology reported recently for taste

adaptation experiments (*10*, *11*), first, an aqueous solution (5 mL) of L-phenylalanine (30 mmol/L) was maintained for 5 s in the oral cavity of eight panelists; the solution was then expectorated while the bitter taste intensity perceived was evaluated after 0, 5, 20, 35, and 65 s as given above. After an intermission of 30 min, the experiment was repeated but, after expectoration of the bitter stimulus, the oral cavity was briefly rinsed with water (3 mL) or an aqueous solution (3 mL) of compound **2** (1.5 or 15.0 mmol/L). After expectoration, the trained sensory panel was then asked to rate the perceived bitter intensity as given above. In a second set of experiments, the corresponding inverse experiment was performed by rinsing the oral cavity first with the solutions of compound **2** (3 mL; 1.5 or 15.0 mmol/L) and then with the l-phenylalanine solution (30 mmol/L; 5 mL). The data of three different sessions were averaged for each panelist. Intensity values between individuals and separate sessions differed by not more than ±0.5 unit.

High-Performance Liquid Chromatography. The HPLC apparatus (Jasco, Gross-Umstadt, Germany) consisted of a HPLC pump system PU 1580 with an in-line degasser (DG-1580-53), a LG-1580-02 type low-pressure gradient unit, and a diode-array detector (DAD) type MD 1515.

Liquid Chromatography/Mass Spectroscopy. An ODS-Hypersil, 250 × 4.6 mm i.d., 5 μm, analytical HPLC column (Phenomenex Aschaffenburg, Germany) was coupled to a LCQ-MS (Finnigan MAT GmbH, Bremen, Germany) with positive (ESI^+) and negative (ESI^-) electrospray ionization. The samples were separated by varying gradients with aqueous ammonium formate (10 mmol/L, pH 8.2) or trifluoroacetic acid (0.1%, pH 2.5) and methanol as the mobile phase.

Nuclear Magnetic Resonance Spectroscopy. ^1H and ^{13}C experiments were performed at 298 K on a Bruker-AM-360 spectrometer (Bruker, Rheinstetten, Germany). ^1H, GRAD-correlation spectroscopy (COSY), GRAD-heteronuclear multiple quantum correlation (HMQC) as well as GRAD-heteronuclear multiple-bond correlation (HMBC) spectroscopy was performed on a Bruker-AMX 400-III spectrometer (Bruker, Rheinstetten, Germany). The samples were dissolved in D_2O spiked with TMS-P as the internal standard. Data processing was done with one-and two-dimensional (1D- and 2D-) WIN NMR as well as UX-NMR software (Bruker, Rheinstetten, Germany).

RESULTS AND DISCUSSION

To investigate the potential of alapyridaine-related bitter inhibitors, the following experiments aimed at screening for bitter taste-suppressing target molecules in combinatorial libraries of pyridinium betaines prepared from 5-(hydroxymethyl)-furan-2-aldehyde, recently identified as a alapyridaine precursor (*6*, *8*), and amino acid mixtures by using Maillard-type reaction chemistry instead of chosing the laborious method of synthesizing and purifying each derivative individually.

To achieve this, fructose was first converted into 5-(hydroxymethyl)furan-2-aldehyde upon acid catalysis in a yield of 26%. This solution was then reacted under alkaline conditions for 72 h in the presence of amino acid mixture A containing glycine, L-alanine, L-valine, L-leucine, and L-threonine, mixture B containing L-serine, L-aspartic acid, L-arginine, and L-tryptophan, mixture C containing L-methionine, L-histidine, L-lysine, and L-asparagine, or mixture D containing L-cysteine, L-glutamate, L-glutamine, L-phenylalanine, and L-tyrosine. HPLC/DAD and HPLC/MS analysis of the Maillard-type libraries revealed that a multiplicity of hydrophilic byproducts had been produced, among which the target compounds were located upon comparison of their UV/vis absorption spectra with the absorption maxima at λ 251 and 328 nm (pH 8.2) or λ 298 nm (pH 3.5) observed for alapyridaine (*6*). Exemplified with reaction mixture A, the screening of these pyridinium betaines for bitter-suppressing activity will be demonstrated in detail.

Screening for Bitter-Suppressing Pyridinium Betaines. To sort out the pyridinium betaines from the bulk of byproducts, further preseparation was required prior to screening by means of a comparative taste dilution analysis (cTDA). As shown in **Figure 1A**, reaction mixture A was first separated by preparative RP-18 column chromatography into two major fractions I and II. Fraction I containing the pyridinium betaines was collected and further separated by means of gel-permeation chromatography (GPC) with Sephadex G15 material as the stationary phase and water as the mobile phase, thus yielding three subfractions I/I−I/III, which were collected separately (**Figure 1B**).

HPLC/DAD analysis detected the target compounds in GPC fraction I/II, which was then used for bitter suppressor screening by means of comparative taste dilution analysis (cTDA). To achieve a suitable resolution of the highly hydrophilic pyridinium betaines, aliquots of the GPC fraction I/II were analyzed by hydrophilic interaction liquid chromatography (HILIC) on a semipreparative TSK-gel Amide-80 column (**Figure 2A**). Monitoring the effluent at 330 nm revealed the presence of multiple polar compounds, among which those five compounds marked with an arrow in **Figure 2A** showed the typical UV/vis absorption spectra expected for pyridinium betaines (*6*). The effluent of these five target compounds were collected, freeze-dried, and then dissolved at the same concentration levels in 2 mL of water. Sensory analysis demonstrated that these compounds are tasteless on their own. With the aim of investigating their putative bitter-suppressing activity, aliquots of these fractions were dissolved in aqueous solutions of the bitter reference compound salicin, the concentration of which was 4-fold above its bitter detection threshold of 0.25 mmol/L. 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 bitter taste difference between the diluted fraction and two blanks (tap water) could just be detected. Since this so-called taste dilution (TD) factor, obtained for each fraction, is related to the bitter taste activity of the salicin solution, the five HPLC fractions collected were rated in their relative impact on bitter taste as given in **Figure 2B**. Evaluated with a TD factor of 4, the threshold concentration of fractions I/II-1 to I/II-4 was found to be 0.25 mmol/L, matching the bitter threshold of the salicin reference solution. These data clearly indicate that the pyridinium betaines eluting in fractions I/II-1 to I/II-4 did not influence bitter perception of salicin. In contrast, fraction I/II-5, judged with a TD factor of 1, exhibited just a faint bitter taste at the highest concentration level. This 4-fold increase of the bitter threshold of salicin clearly indicated that this fraction contained a compound exhibiting some bitter-suppressing activity. Therefore, the following identification experiments were focused on the taste modifier in fraction I/II-5.

Structure Determination of Bitter-Suppressing Compound in Fraction I/II-5. As already mentioned above, the compound eluting in fraction I/II-5 showed the typical UV/vis absorption maxima expected for pyridinium betaines (*6*). LC/MS analysis of this fraction revealed an intense $[M + 1]^+$ ion with *m*/*z* 184, suggesting the formation of the corresponding pyridinium betaine of glycine, **2** in **Figure 3**. To unequivocally confirm the chemical structure of this compound, the 1-carboxymethyl-5-hydroxy-2-hydroxymethylpyridinium inner salt was prepared in suitable amounts from 5-(hydroxymethyl)furan-2-aldehyde and glycine. After chromatographic purification, the structure was confirmed by means of LC/MS and 1D/2D NMR spectroscopy, and the spectroscopic (LC/MS, UV/vis) and chromatographic data (retention time) were compared with those obtained for the compound in fraction I/II-5 isolated from the

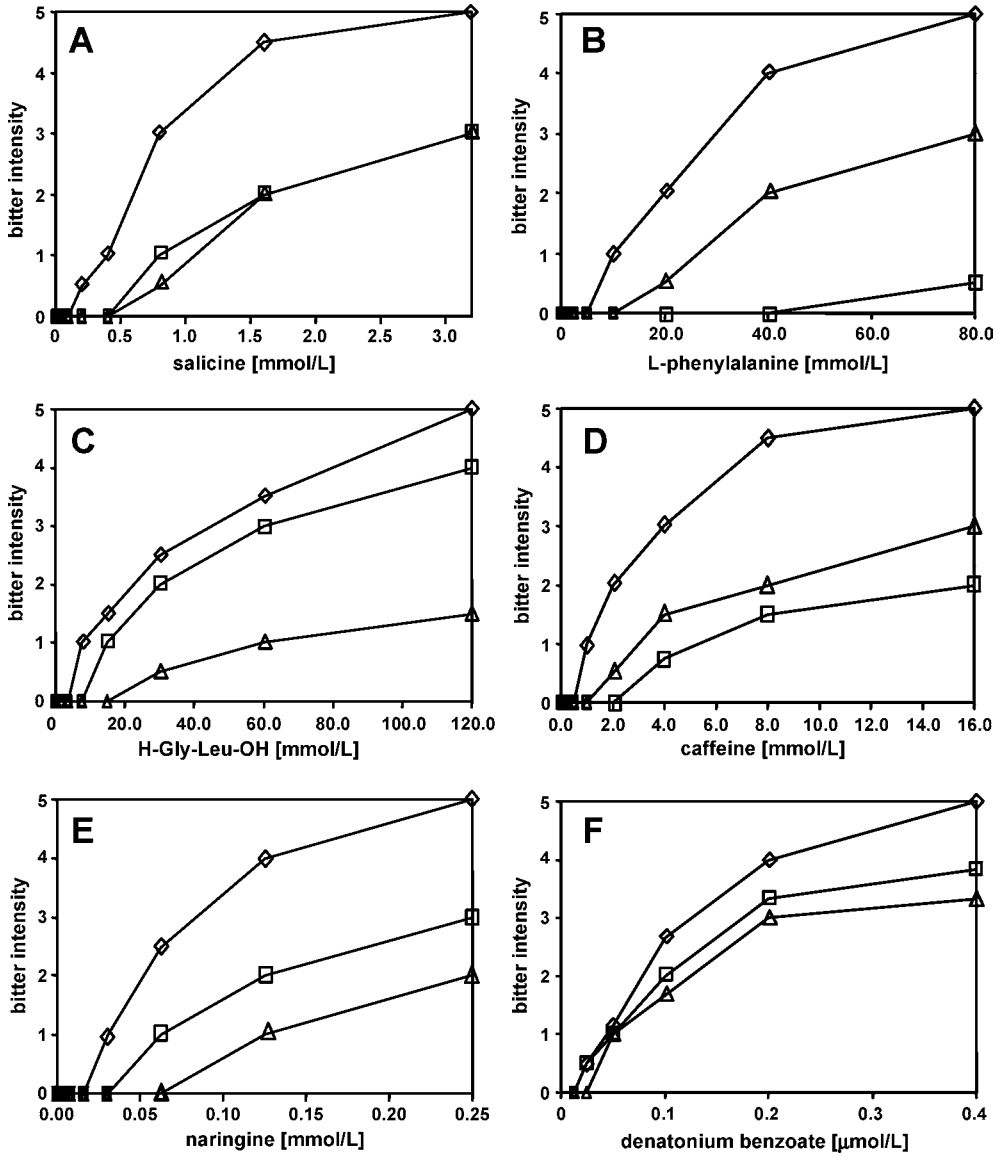

Figure 4. Influence of pyridinium betaine **2** on taste intensity of aqueous solutions of bitter taste compounds (pH 7.0): (**A**) salicin (◇), salicin + **2** (1:0.1, □), and salicin + **2** (1:1, △); (**B**) L-phenylalanine (◇), L-phenylalanine + **2** (1:0.1, △), and L-phenylalanine + **2** (1:1, □); (**C**) Gly-Leu (◇), Gly-Leu + **2** (1:0.1, □), and Gly-Leu + **2** (1:1, △); (**D**) caffeine (◇), caffeine + **2** (1:0.2, △), and caffeine + **2** (1:1, □); (**E**) naringin (◇), naringin + **2** (1:1, □), and naringin + **2** (1:10, △); (**F**) denatonium benzoate (◇), denatonium benzoate + **2** (1:1000, □), and denatonium benzoate + **2** (1:10 000, △).

Table 1. Influence of 1-(Carboxymethyl)-5-hydroxy-2-hydroxymethylpyridinium Inner Salt (**2**) on the Bitter Detection Thresholds of Selected Bitter Compounds (pH 7.0)

compounds	detection threshold (mmol/kg of water)	factor of threshold decrease
L-phenylalanine	16.0	
L-phenylalanine + compound **2** (1+1)	50.0	3
caffeine	1.0	
caffeine + compound **2** (1+1)	3.0	3
salicin	0.2	
salicin + compound **2** (1+1)	0.8	4

Maillard reaction product library A. Finally, cochromatography and sensory analysis confirmed that the bitter-suppressing compound in fraction I/II-5 is the 1-(carboxymethyl)-5-hydroxy-2-hydroxymethylpyridinium inner salt (**2**, **Figure 3**), which does not show any taste on its own. Comparison of LC/MS and UV/vis data with those obtained for the synthetic reference compound (*6*) as well as cochromatography led to the identification of the pyridinium compound eluting in fraction I/II-3 as (+)-(*S*)-1-(1-carboxyethyl)-5-hydroxy-2-(hydroxymethyl)-pyridinium inner salt (alapyridaine; **1** in **Figure 3**). It is interesting to note that only the glycine derivative (**2**) exhibited bitter-suppressing activity whereas the alanine derivative (**1**), just differing in a methyl group, did not influence bitter taste perception at all.

Fractionation and cTDA studies on the other pyridinium betaine libraries prepared from amino acid mixtures B−D did not result in any bitter-suppressing compounds (data not shown).

Sensory Studies. Prior to sensory analysis, the purity of compound **2** was verified by LC/MS and ^{1}H NMR spectroscopy. To get first insight into the bitter-suppressing activity of 1-carboxymethyl-5-hydroxy-2-hydroxymethylpyridinium inner salt (**2**), the bitter detection thresholds were determined for the bitter compounds L-phenylalanine, caffeine, and salicin in the absence or presence of equimolar amounts of compound **2**. As given in **Table 1**, the bitter threshold concentrations of 16.0, 1.0, and 0.25 mmol/L were found for single solutions of L-phenylalanine, caffeine, and salicin, respectively. When compound **2** was added, the detection thresholds of the bitter

compounds were increased by a factor of 3 and 4, thus confirming some bitter-suppressing activity of the pyridinium betaine.

In a second set of experiments, a trained sensory panel was asked to rate the bitter intensity of various bitter compounds compared to solutions containing the same concentration of these compounds plus various amounts of compound **2** on a scale from 0 (no bitterness) to 5 (strong bitterness). As given in **Figure 4A**, a 0.2 mmol/L salicin solution was judged as tasting bitter by the sensory panel, whereas in the presence of compound **2**, either at equimolar or at a tenth the concentration, the solution was not perceived as bitter up to a salicin concentration of 0.8 mmol/L. The bitter-suppressing activity of **2** was also detectable at higher tastant concentrations, for example, at a concentration level of 3.2 mmol/L the salicin solution was rated with a score of 5.0 while both pyridinium betaine-containing mixtures were evaluated with a significantly lower score of 3.0. Similar experiments with the bitter-tasting amino acid L-phenylalanine showed that the bitter intensity score of a 40.0 mmol/L L-phenylalanine solution was reduced from 4.0 to 2.0 by adding 2 mmol/L of compound **2** and was completely suppressed upon addition of 20 mmol/L of the betaine (**Figure 4B**). A complete inhibition of the bitterness of L-phenylalanine by equimolar amounts of compound **2** was observed up to a concentration of 40 mmol/L. Also, the bitter taste intensity of the dipeptide glycyl-L-leucine was considerably reduced in the presence of compound **2**; for example, a 60.0 mmol/L H-Gly-Leu-OH solution was reduced in its bitter intensity from a score of 3.5 to 1.0 by the addition of an equimolar amount of **2** (**Figure 4C**). Similarily, the intensity of the bitter taste impression induced by the alkaloid caffeine (**Figure 4D**) and the flavanoid naringin (**Figure 4E**) was decreased by compound **2**; however, the effects were somewhat less pronounced compared to those found for the bitter-tasting amino acid and the peptide. In contrast, the bitterness of denatonium benzoate, eliciting bitter taste at 0.025 μmol/L (water), was influenced to a far less extent by the presence of compound **2;** for example addition of compound **2** (0.2 mmol/L) induced just a slight reduction in bitterness of denatonium benzoate (0.2 μmol/L) from 4.0 to 3.0 (**Figure 4F**). Although the concentrations of **2** in the experiment exceed the concentration of denatonium benzoate by factors of 1000 and 10 000, only slight bitter-suppressing activities were observed. However it is interesting to note that the sensory panel described the bitter taste of the pure denatonium benzoate solution as rising rapidly but short lasting, whereas the bitter taste of the binary mixture with compound **2** was evaluated as rising much more slowly and lasting longer.

Finally, we investigated the time-resolved influence of compound **2** on the time/intensity functions of the bitter perception induced by L-phenylalanine (**Figure 5**). First, a solution of the bitter amino acid (30 mmol/L) was placed on the tongue, and after 5 s the solution was expectorated and the bitter taste intensity perceived was evaluated after 0, 5, 20, 35, and 65 s on a scale from 0 (no bitterness) to 5 (strong bitterness). The results presented in **Figure 5** demonstrate that the bitter intensity of the pure solution of L-phenylalanine swirled in the mouth was still rated after 60 s with a score of 1.0. Then the experiment was repeated, but the mouth was swirled with a 1.5 and 15.0 mmol/L solution of compound **2**, respectively. The trained sensory panel was then asked to rate the perceived bitter intensity after 0, 5, 20, 35, and 65 s on a scale from 0 (no bitterness) to 5 (strong bitterness). As given in **Figure 5A**, the bitter taste of the L-phenylalanine solution was instantaneously suppressed after oral application of 15 mmol/L compound **2**.

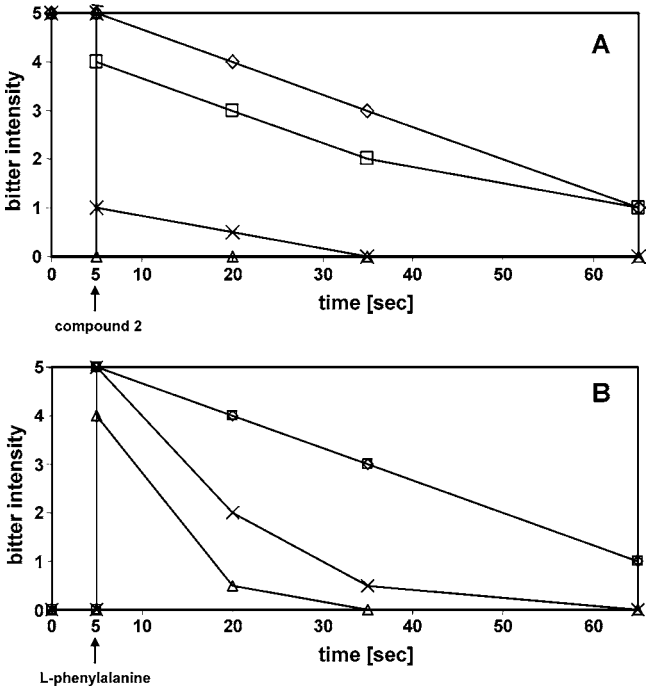

Figure 5. Time-resolved influence of pyridinium betaine **2** on the bitter perception of L-phenylalanine (30 mmol/L). (**A**) L-Phenylalanine solution was taken into the mouth, and expectorated after 5 s, and bitter taste was evaluated without any further additions (◇) or after rinsing with water (□) or 1.5 mmol/L (×) or 15 mmol/L (△) compound **2**. (**B**) Solutions of 1.5 mmol/L (×) or 15 mmol/L (△) compound **2** or water (control; □) were taken into the mouth and, after expectoration, the mouth was rinsed with the L-phenylalanine solution; after 5 s, the solution was spat out while the bitter taste was evaluated.

In comparison, application of only 1.5 mmol/L betaine led to a less pronounced effect and did not induce a complete depletion of the bitter taste perception. A control experiment, which was done by swirling the mouth with pure water instead of a solution of compound **2**, revealed that the bitter taste intensity of phenylalanine was just slightly influenced by the aqueous medium and demonstrated the specific bitter-suppressing effect of compound **2**. In an inverse experiment, the oral cavity was first swirled with a solution of 1.5 or 15.0 mmol/L compound **2**, after 5 s the solution was expectorated, and a solution of L-phenylalanine (30 mmol/L) was applied. As shown in **Figure 5B**, the bitter-suppressing effect of compound **2** could also be observed, but the effect was somewhat delayed and not as drastic as found in the above experiment.

Structure/Activity Considerations. The finding that the achiral glycine derivative **2** reduces the bitterness of various bitter-tasting molecules, whereas the L-alanine derivative **1** and the other pyridinium betaine derivatives from chiral primary amino acids (**Figure 2**) did not influence bitter taste perception, prompted us to study whether the elongation of the side chain in **2** led to achiral compounds of similar bitter taste-suppressing activities. Therefore, 1-(2-carboxyethyl)-5-hydroxy-2-hydroxymethylpyridinium inner salt (**3**) and 1-(3-carboxypropyl)-5-hydroxy-2-hydroxymethylpyridinium inner salt (**4**) were synthesized from 5-(hydroxymethyl)-2-furanaldehyde and the achiral amino acids β-alanine and γ-aminobutyric acid, respectively (**Figure 3**). These compounds were purified by column chromatography, characterized by LC/MS and 1D-/2D-NMR experiments, and then used for sensory experiments demonstrating that compounds **3** and **4** do not exhibit any taste on their own. The trained sensory panel was then asked to rate the bitter

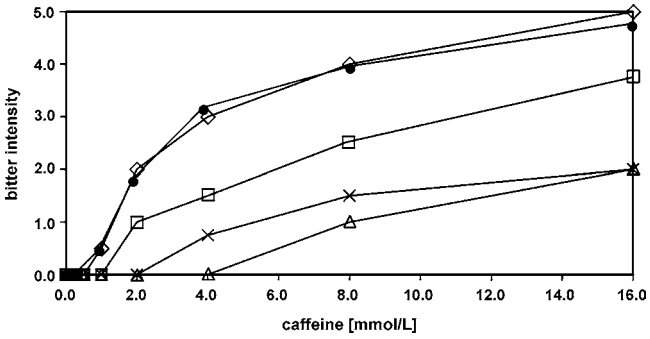

Figure 6. Influence of the pyridinium betaine structure on suppression of the bitter taste intensity of caffeine solutions in the absence of additives (◇) and in the presence of alapyridaine (**1**, ●), pyridinium betaine **2** (△), pyridinium betaine **3** (×), and pyridinium betaine **4** (□).

intensity of caffeine solutions compared to solutions containing the same caffeine concentration plus various amounts of compound **1**−**4** on a scale from 0 (no bitterness) to 5 (strong bitterness). As given in **Figure 6**, the presence of compound **3** induced a distinct reduction of the bitter taste intensity of caffeine solutions; for example, a 2.0 mmol/L equimolar mixture of caffeine and **3** was not described as bitter, while the solution containing caffeine only was evaluated with a score of 2.0 at the same concentration. A similar bitter-suppressing effect was observed for compound **4**, but the activity of this γ-aminobutyric acid derivative was significantly reduced when compared to compounds **2** and **3**, respectively. Again control experiments with (+)-*S*-alapyridaine (**1**) as well as its enantiomeric (−)-*R*-alapyridaine (data not shown) synthesized as reported previously (*12*) demonstrated that pyridinium betaines with chiral amino acid side chains do not exhibit any bitter taste-suppressing activities, thus demonstrating the crucial role of the achiral side chains in compounds **2**−**4** for bitter taste modification.

In summary, it could be shown that the application of a combinatorial approach in Maillard reaction chemistry is a suitable tool to screen for functional compounds. Tasteless but bitter-suppressing compounds such as the pyridinium betaines **2**−**4** might offer new possibilities in reducing unpleasant bitter tastes in food and pharmaceutical products.

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