Characterization of an Intense Bitter-Tasting 1*H*,4*H*-Quinolizinium-7-olate by Application of the Taste Dilution Analysis, a Novel Bioassay for the Screening and Identification of Taste-Active Compounds in Foods

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Thermal treatment of aqueous solutions of xylose and primary amino acids led to rapid development of a bitter taste of the reaction mixture. To characterize the key compound causing this bitter taste, a novel bioassay, which is based on the determination of the taste threshold of reaction products in serial dilutions of HPLC fractions, was developed to select the most intense taste compounds in the complex mixture of Maillard reaction products. By application of this so-called taste dilution analysis (TDA) 21 fractions were obtained, among which 1 fraction was evaluated with by far the highest taste impact. Carefully planned LC-MS as well as 1D and 2D NMR experiments were, therefore, focused on the compound contributing the most to the intense bitter taste of the Maillard mixture and led to its unequivocal identification as the previously unknown 3-(2-furyl)-8-[(2-furyl)methyl]-4-hydroxymethyl-1-oxo-1*H*,4*H*-quinolizinium-7-olate. This novel compound, which we name quinizolate, exhibited an intense bitter taste at an extraordinarily low detection threshold of 0.00025 mmol/kg of water. As this novel taste compound was found to have 2000- and 28-fold lower threshold concentrations than the standard bitter compounds caffeine and quinine hydrochloride, respectively, quinizolate might be one of the most intense bitter compounds reported so far.

Keywords: *Bitter taste; taste dilution analysis; Maillard reaction; quinizolate*

INTRODUCTION

The nonenzymatic reaction between reducing carbohydrates and amino acids, called the Maillard reaction, is known to generate aroma, taste, and browning compounds contributing to the sensory quality of thermally processed foods, such as processed meats, cereals, malt, coffee, or cocoa. Although consumer acceptance of a processed food product is strongly influenced by the aroma-active (detected at the regio olfactoria in the nose) as well as the taste-active compounds (interacting with the receptors on the tongue and in the oral cavity), detailed information on the structures and sensory properties is as yet mainly available only on the volatile odor-active compounds. In the past 10 years, the key odorants in a number of processed foods, such as bread crust (Schieberle and Grosch, 1991), processed beef (Cerny and Grosch, 1992), malt (Fickert and Schieberle, 1998), and chocolate (Schieberle and Pfnuer, 1999) or processed flavorings (Hofmann and Schieberle, 1995, 1997, 1998), could be successfully characterized by application of the aroma extract dilution analysis (AEDA). This technique, using the human nose as a sensitive and selective biosensor to sort out the odor-active compounds from the large number of nonodorous volatiles, is based on the determination of the odor thresholds of volatiles during GC−olfactometry, which is performed with serial dilutions of an aroma extract.

To answer the puzzling question as to which key chromophores are responsible for the attractive browning generated during food processing, one of us applied this "dilution" concept recently to intensely colored fractions obtained by HPLC separation of thermally browned carbohydrate/amino acid mixtures. This so-called color dilution analysis (CDA), which is based on the determination of the visual threshold of chromophores in serial dilutions of colored HPLC fractions, offered the possibility of ranking the browning compounds according to their relative color impact and proved to be a powerful and straightforward technique for the successful identification of various key chromophores of as yet unknown chemical structure (Hofmann, 1998a, 1999; Hofmann and Heuberger, 1999; Frank et al., 2000).

Compared to aroma-active volatiles and browning products, the information available on taste-active compounds generated during thermal food processing is as yet very fragmentary. Recently, Shima et al. (1998) investigated the compounds that impart the "brothy taste" as the characteristic flavor of beef bouillon and succeeded in isolating and identifying *N*-(1-methyl-4-hydroxy-3-imidazolin-2,3-ylidene)alanine with broth-like taste characteristics. This compound was proposed by the authors to be formed from, for example, L-alanine and creatinine, both present in beef broth. Besides the reaction of L-alanine with creatinine, in particular, Maillard reactions with reducing carbohydrates might be involved in the formation of nonvolatile taste compounds during thermal food processing. Whereas some data are reported in the literature (Papst et al., 1985; Tressl et al., 1985; Hofmann, 1998b) on the formation of bitter-tasting compounds from carbohydrates and the secondary amino acid L-proline, almost no information

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is available on the chemical structures and sensory attributes of taste-active compounds formed from Maillard reactions involving primary amino acids.

The purpose of the present investigation was, therefore, to rank the taste compound formed during thermal treatment of pentoses and primary amino acids by their taste impacts by application of a "dilution" concept and, then, to characterize the chemical structure of the most intense-tasting compound.

EXPERIMENTAL PROCEDURES

Chemicals. The following compounds were obtained commercially: xylose, saccharose, *n*-propylamine, L-alanine, 5-(hydroxymethyl)furan-2-aldehyde, lactic acid, NaCl, caffeine, quinine hydrochloride, sodium glutamate, and tannin (Aldrich, Steinheim, Germany). Solvents were of HPLC grade (Aldrich). DMSO-d_6 and CD_3OD were obtained from Isocom (Landshut, Germany). (2*S*,6*S*)-Octahydrodipyrrolo[1,2-*a*;1′,2′-*d*]pyrazine-5,10-dione (Gautschi et al., 1998) and pyrrolidino- and bis-(pyrrolidino)hexose reductone were synthesized following the procedures recently reported (Hofmann, 1998b).

Thermally Treated Xylose/L-Alanine Mixture. A solution of D-xylose (0.75 mol) and L-alanine (0.19 mol) in phosphate buffer (500 mL; 0.1 mol/L, pH 5.0) was heated under reflux for 3 h. After cooling to room temperature, the aqueous solution was extracted with ethyl acetate (8 × 200 mL), and the organic layer was dried over Na_2SO_4 and concentrated at 25 °C in vacuo (100 mbar) to 100 mL. An aliquot (2 mL) of this solvent extract was freed from solvent in vacuo (100 mbar), and the residue was dissolved in tap water (4 mL). Sensory analysis with three trained panelists revealed an intense bitter taste of this aqueous solution. The other aliquot of the solvent extract was used for the taste dilution analysis (TDA) (1 mL) as well as for the identification experiments (97 mL).

TDA. An aliquot (1 mL) of the solvent extract was freed from the volatiles by high-vacuum distillation (0.04 mbar, 30 °C), the residue was dissolved in methanol (1 mL), and, after membrane filtration, aliquots (100 μL) were analyzed by RP-HPLC. The effluent was separated into 21 fractions, which were separately collected in glass vials. The corresponding fractions obtained from 10 HPLC runs were collected, combined, and freeze-dried. The residues obtained from these 21 pooled HPLC fractions were taken up in exactly 1.0 mL of water and then stepwise 1+1-diluted with tap water. The serial dilutions of each of the 21 fractions were then presented to a trained sensory panel in order of increasing concentrations, and each dilution was sensorially judged in a triangle test. The dilution at which a taste difference between the diluted fraction and two blanks (tap water) could just be detected was defined as the taste dilution (TD) factor; for example, fraction 19 was evaluated with the highest TD factor. The TD factors evaluated by three different assessors were averaged. The TD factors among individuals and three separate sessions differed not more than one dilution step.

Sensory Analyses. *Training of the Sensory Panel.* Assessors were recruited from the German Research Center for Food Chemistry and 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 (Wieser and Belitz, 1975): saccharose (50 mmol/L) and L-alanine (15 mmol/L), respectively, for sweet taste; lactic acid (20 mmol/L) for sour taste; NaCl (12 mmol/L) for salty taste; caffeine (1 mmol/L) and quinine hydrochloride (0.05 mmol/L), respectively, for bitter taste; sodium glutamate (8 mmol/L, pH 5.7) for umami taste; and tannin (0.05%) for astringency. Sensory analyses were performed in a sensory panel room at 22−25 °C in three different sessions.

Determination of Taste Thresholds. The detection thresholds of taste compounds were determined in a triangle test using tap water (pH 6.5) as the solvent. The samples were presented in order of increasing concentrations (serial 1:1 dilutions), and the threshold values evaluated in three different sessions by six panelists each were averaged. The values among individuals and separate sessions differed not more than one dilution step; that is, a threshold value of 0.5 mmol/L for caffeine represents a range from 0.25 to 1.0 mmol/L.

Isolation of Bitter-Tasting 3-(2-Furyl)-8-[(2-furyl)-methyl]-4-hydroxymethyl-1-oxo-1*H*,4*H*-quinolizinium-7-olate (Quinizolate) from a Heated Aqueous Xylose/L-Alanine Solution. The major aliquot (97 mL) of the solvent extract of the Maillard mixture was distilled in a high vacuum (0.04 mbar) at 35 °C to remove volatile reaction products. The intensely colored residue was dissolved in ethyl acetate (10 mL) and then fractionated by column chromatography (35 × 450 mm) on silica gel (200 g, silica gel 60, Merck, Darmstadt, Germany). After application of an aliquot (5 mL) of the crude material onto the top of the column conditioned with toluene/ethyl acetate (4:6, v/v), chromatography was performed using toluene/ethyl acetate (4:6, v/v; 400 mL; fraction A), followed by toluene/ethyl acetate (2:8, v/v; 400 mL; fraction B), ethyl acetate (400 mL; fraction C), ethyl acetate/methanol (8:2, v/v; 400 mL; fraction D), ethyl acetate/methanol (6:4, v/v; 400 mL; fraction E), ethyl acetate/methanol (4:6, v/v; 400 mL; fraction F), and ethyl acetate/methanol (2:8, v/v; 400 mL; fraction G). Fractions A−G were separately collected, and aliquots (5 mL) were taken, freed from solvent in vacuo, and taken up in tap water (3 mL). To localize the most intense bitter-tasting compound, these solutions were evaluated by the sensory panel. Fraction E was judged to have by far the highest bitter intensity and was therefore analyzed by RP-HPLC/degustation following the TDA procedure reported above. The effluent of fraction 19 was collected in three HPLC runs, freed from solvent, and again analyzed for bitter taste in aqueous solution. An intense bitter taste was detected, thus confirming that the bitter-tasting target compound detected in HPLC fraction 19 is present in fraction E of the column chromatography. For further purification, fraction E was subfractionated by column chromatography (30 × 500 mm) on aluminum oxide (200 g, basic Al_2O_3, activity III−IV, Merck), which was conditioned with ethyl acetate. After application of the material, the column was flushed with ethyl acetate (200 mL). Elution with the same solvent (200 mL) affords a fraction in which the bitter compound could be sensorially detected by using the same procedure as described above. This fraction was freed from solvent in vacuo, dissolved in methanol (1 mL), and then stored at −18 °C, affording crystals of an intensely fluorescent compound (yield = 33 mg, 0.1 mmol). RP-HPLC/degustation of an aqueous solution of this substance confirmed this reaction product to cause the bitter taste of fraction 19, which was evaluated by TDA to contain the most intense tastant in the Maillard mixture: LC-MS, *m*/*z* 338 (100, $[M + 1]^+$). CHN Anal. Calcd for $C_{19}H_{15}O_5N$: C, 67.65%; H, 4.48%; N, 4.15%. Found: C, 67.30%; H, 4.60%; N, 4.05% (CHN Rapid instrument, Heraeus, Germany). Determination of a melting point was not successful because the compound decomposed rapidly on dry heating. The ^1H NMR spectrum is displayed in Figure 3, and ^1H and ^{13}C NMR data are summarized in Tables 2 and 3.

Synthesis of 6-Hydroxymethyl-1-propylpyridinium-3-olate. According to a procedure reported by Koch et al. (1998), *n*-propylamine (60 mmol) was added to a solution of 5-(hydroxymethyl)furan-2-aldehyde (50 mmol) in water/ethanol (1:1, v/v; 80 mL), the pH value was adjusted to 9.5 with aqueous hydrochloric acid (1 mol/L), and the mixture was then heated at reflux for 48 h. After cooling, the organic solvent was removed in vacuo and the aqueous layer was freeze-dried. The dark residue was taken up in ethyl acetate/methanol (1:1, v/v; 5 mL) and then fractionated by column chromatography (35 × 450 mm) on silica gel (200 g, silica gel 60, Merck), which was conditioned with ethyl acetate. After application of the crude material, chromatography was performed with ethyl acetate (400 mL; fraction A), ethyl acetate/methanol (6:4, v/v; 200 mL; fraction B), ethyl acetate/methanol (4:6, v/v; 200 mL; fraction C), ethyl acetate/methanol (3:7, v/v; 200 mL; fraction D), ethyl acetate/methanol (2:8, v/v; 200 mL; fraction E), and ethyl acetate/methanol (1:9, v/v; 200 mL; fraction F), followed by methanol (400 mL; fraction G). Fractions E and F were

combined, rechromatographed as described above, and then freed from solvent, affording the target compound as a pale amorphous powder. Recrystallization from mixtures of ethanol and ethyl acetate yielded 6-hydroxymethyl-1-propylpyridinium-3-olate as colorless needles (22.5 mmol, 45% yield): UV_{max} = 252, 322 nm; ^{1}H NMR (360 MHz; CD_3OD; DQF-COSY; the arbitrary numbering of the carbon atoms refers to Figure 4) δ 1.04 [t, 3H, 7.5 Hz, H−C(1)], 1.95 [m, 2H, H−C(2)], 4.36 [t, 2H, 7.5 Hz, H−C(3)], 4.71 [s, 2H, H−C(5)], 7.28 [dd, 1H, 2.66 Hz, 8.82 Hz, H−C(7], 7.50 [d, 1H, 8.82 Hz, H−C(6)], 7.66 [d, 1H, 2.66 Hz, H−C(9)]; ^{13}C NMR (360 MHz; CD_3OD; the arbitrary numbering of the carbon atoms refers to Figure 4) δ 11.1 [CH_3, C(1)], 25.9 [CH_2, C(2)], 59.2 [CH_2, C(3)], 60.4 [CH_2, C(5)], 129.8 [C, C(6)], 135.4 [C, C(7)], 136.3 [C, C(4)], 138.5 [C, C(9)], 168.7 [C, C(8)];^{15}N NMR (600 MHz; CD_3OD) 209.3 ppm. ^{1}H and ^{13}C NMR data are well in line with those reported in the literature (Koch et al., 1998).

High-Performance Liquid Chromatography (HPLC). The HPLC apparatus (Kontron, Eching, Germany) consisted of two pumps (type 422), a gradient mixer (M 800), a Rheodyne injector (100 μL loop), and a diode array detector (DAD type 540) monitoring the effluent in a wavelength range from 220 to 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 (20−100 μL), analysis was performed using a gradient starting with a mixture (8:2, v/v) of aqueous ammonium formate (10 mmol/L; pH 3.5) and methanol and increasing the methanol content to 100% within 40 min.

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 electrospray ionization (ESI). After injection of the sample (2−20 μL), analysis was performed using a gradient starting with a mixture (8:2, v/v) of aqueous ammonium formate (10 mmol/L; pH 3.5) and methanol and increasing the methanol content to 100% within 40 min.

UV−Vis Spectrocopy. UV−vis spectra were obtained in water using a U-2000 spectrometer (Colora Messtechnik GmbH, Lorch, Germany).

Nuclear Magnetic Resonance Spectroscopy (NMR). ^{1}H, ^{13}C, DEPT-135, DQF-COSY, TOCSY, HMQC, and HMBC spectroscopy (Hofmann, 1997) and the ^{15}N HMBC experiment (hmbc.en from Bruker standard pulse sequence) with an evolution time of 55 ms were performed on Bruker-AC-200 and Bruker-DMX-600 spectrometers (Bruker, Rheinstetten, Germany) using the acquisition parameters described recently. Chemical shifts were measured either by using tetramethylsilane (TMS) as the internal standard or from residual DMSO-d_5 (2.49 ppm) in the proton dimension and with the carbon signal of DMSO-d_6 (39.5 ppm) in the carbon dimension.

RESULTS AND DISCUSSION

Sensory analysis of an aqueous solution of the solvent-extractable compounds isolated from a thermally treated mixture of xylose and L-alanine revealed an intense bitter taste of the hydrophobic fraction of reaction products. To characterize the taste-active substances formed, odor-active volatiles were removed by high-vacuum distillation, and the nonvolatile compounds were separated by RP-HPLC and detected using a diode array detector monitoring in the wavelength range from 220 to 500 nm. To identify the key tastant responsible for the bitter taste of the xylose/L-alanine reaction mixture, we first screened the reaction products for the most intense taste compounds by means of a bioassay and then focused on the structure determination on the compound that was evaluated as having the highest taste activity.

Screening for Taste-Active Compounds (TDA). The nonvolatile fraction of the heated pentose/alanine mixture consisted of a tremendous variety of different reaction products, of which only a limited number of compounds were expected to contribute significantly to the overall taste of the Maillard mixture. To focus the identification experiments on these key tastants, it was therefore necessary to sort out the strongly taste-active compounds from the less taste-active or tasteless substances.

To achieve this and to rank the reaction products by their relative taste impact, we therefore developed an HPLC-assisted bioassay, which we call the taste dilution analysis (TDA). An aliquot of the reaction mixture was chromatographed by RP-HPLC (Figure 1, left side), and the effluent was separated into 21 fractions, which were freeze-dried and then made up with water to the same volume. Each fraction was then stepwise diluted one-to-one 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 detection threshold in a triangle test. The dilution at which a taste difference between the diluted fraction and two blanks could just be detected was defined as the taste dilution (TD) factor. Because the TD factor, by definition, corresponds to the detection threshold of the taste-active fraction in water, a taste-active solution has a TD factor of ≥1. As the TD factor obtained for each compound is proportional to its taste activity in water, the TD factor therefore ranks the 21 HPLC fractions in their relative taste intensity, which is outlined in Figure 1 (right side).

Due to the high TD factor of 512, fraction 19 was evaluated with by far the highest taste impact, therefore mainly contributing to the taste of the Maillard mixture. As listed in Table 1, this fraction exhibited a strong bitter taste. Fraction 15, which was also judged as bitter tasting (Table 1), was found to have an 8-fold lower taste impact, followed by the burnt-tasting fraction 11 and the bitter-tasting fractions 13 and 16, all of which show somewhat lower taste impacts (Figure 1 and Table 1). Besides these bitter-tasting fractions, Maillard products also exhibiting sweet taste (fractions 1 and 3) or astringency (fractions 4, 5, 18, and 21) were detected; however, due to their low TD factors, these compounds should not contribute significantly to the overall taste of the Maillard mixture.

The striking advantage of this HPLC bioassay is that the most taste-active compounds can be located in complex product mixtures without knowledge of their structures. The identification experiments were then focused on the compound causing the strong bitter taste of fraction 19 and, as a consequence, contributing mainly to the taste of the heated carbohydrate/amino acid mixture.

Structure Determination of Bitter-Tasting Principle in Fraction 19. For isolation of the bitter taste compound in fraction 19, the nonvolatile fraction of the reaction mixture was separated in several chromatographic steps using silica gel and aluminum oxide as stationary phases. After each fractionation procedure, the bitter-tasting compound was detected by RP-HPLC/degustation analysis. Finally, the target compound was successfully crystallized and its chemical structure determined by several one- and two-dimensional NMR techniques, and, in addition, by LC-MS, and UV−vis

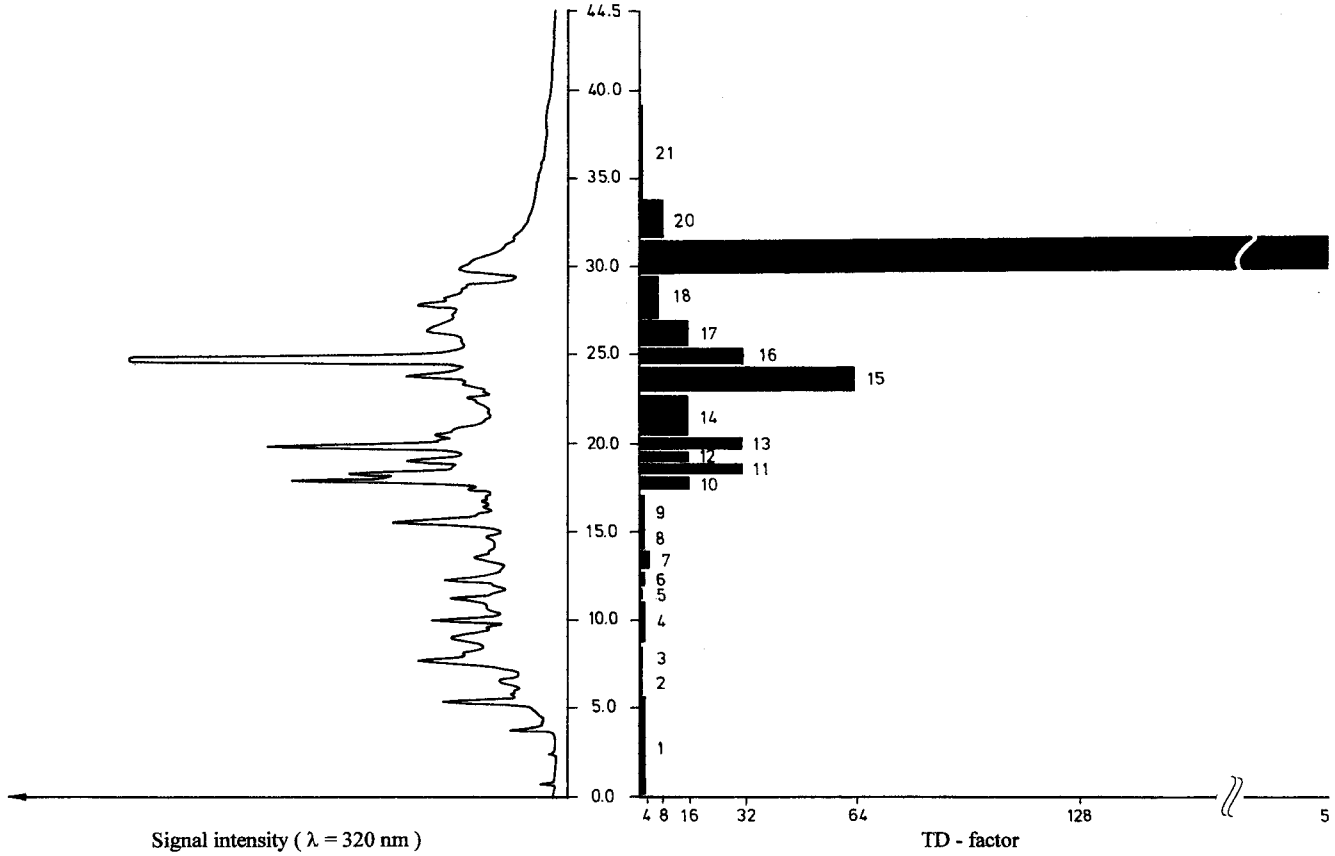

Figure 1. RP-HPLC chromatogram (left) and TD chromatogram (right) of the solvent-extractable, nonvolatile fraction of a heated aqueous xylose/L-alanine solution.

Table 1. Taste Qualities and TD Factors of HPLC Fractions Obtained by TDA of a Heated Xylose/L-Alanine Mixture[a]

fraction[b]	taste quality[c]	TD factor[c]
1	sweet	2
2	bitter	1
3	sweet	1
4	astringent	2
5	astringent	1
6	bitter, coffee-like	2
7	bitter	4
8	bitter, coffee-like	2
9	bitter	2
10	bitter-sweet	16
11	burnt	32
12	bitter	16
13	bitter	32
14	bitter	16
15	bitter	64
16	bitter	32
17	bitter	16
18	astringent	8
19	bitter	512
20	bitter	8
21	astringent	2

[a] A solution of xylose (0.75 mol) and L-alanine (0.19 mol) in phosphate buffer (500 mL; 0.1 mol/L; pH 5.0) was refluxed for 3 h. [b] Number of HPLC fraction referring to Figure 1. [c] The taste quality and the TD factor were determined by using a triangle test.

spectroscopy. The spectroscopic data were consistent with the structure displayed in Figure 2.

LC-MS measurements showed an intense $[M + 1]^+$ ion at *m/z* 338 indicating that one nitrogen atom should be incorporated in the molecule. LC/MS^2 revealed a loss of 30 yielding *m/z* 308, most likely corresponding to the

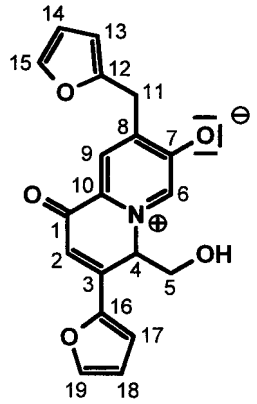

Figure 2. Structure of 3-(2-furyl)-8-[(2-furyl)methyl]-4-hydroxymethyl-1-oxo-1*H*,4*H*-quinolizinium-7-olate (quinizolate).

elimination of one molecule of formaldehyde. These data indicated a hydroxymethyl group and one nitrogen atom in the tastant fitting well with the structure proposed in Figure 2.

The ^1H NMR spectrum displayed in Figure 3 showed 14 resonance signals, among which 1 integrated for 2 protons and 13 signals integrated only for 1 proton. A total of two furan rings, each substituted at the 2-position, was deduced from the characteristic coupling pattern of the hydrogens H−C(13)/H−C(14)/H−C(15) and H−C(17)/H−C(18)/H−C(19). This was further confirmed by a double-quantum filtered homonuclear δ,δ correlation experiment (DQF-COSY) indicating the expected strongly coupled ^1H spin system in the furan rings (Table 2). In addition, the DQF-COSY experiment revealed a coupling of 11.75 Hz between the geminal hydrogen atoms H_a−C(5) and H_b−C(5) resonating at

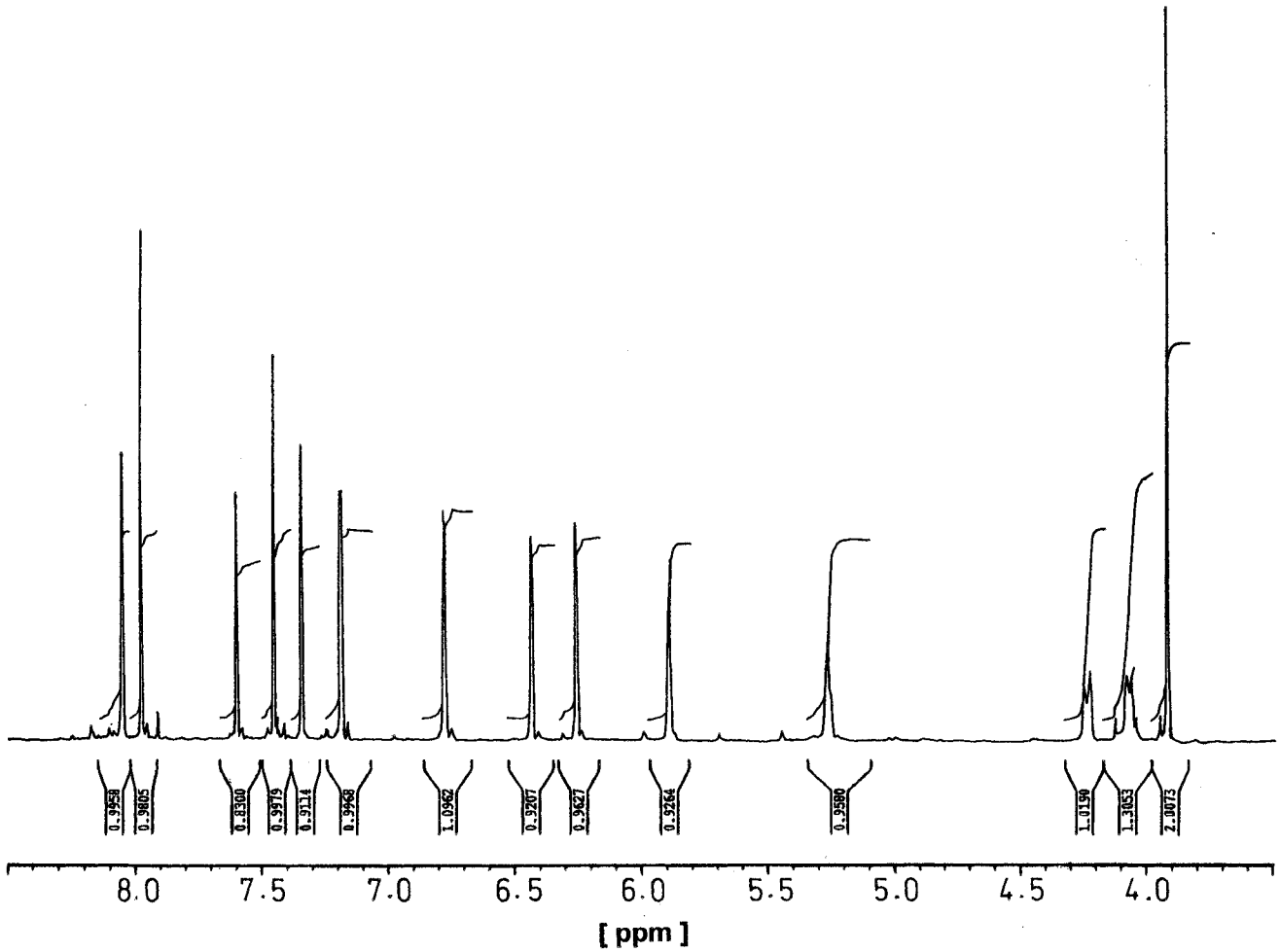

Figure 3. ^{1}H NMR spectrum of the unknown bitter principle in fraction 19.

Table 2. Assignment of ^{1}H NMR Signals (600 MHz, DMSO-d_6) of 3-(2-Furyl)-8-[(2-furyl)methyl]-4-hydroxymethyl-1-oxo-1*H*,4*H*-quinolizinium-7-olate (Quinizolate)

H at relevant C atom[a]	δ^b	I^c	M^c	J^c (Hz)	homonuclear 1H,1H connectivity[d]
H−C (11)	3.92	2	s		
Ha−C(5)	4.06	1	m	11.75	Hb-C(5), HO−C(5), H−C(4)
Hb−C(5)	4.23	1	m	11.75	Ha-C(5), HO−C(5), H−C(4)
HO−C(5)	5.26	1	t	5.44	Ha-C(5), Hb-C(5)
H−C(4)	5.89	1	dd	1.52	H−C(2), Ha-C(5), Hb-C(5)
H−C(13)	6.26	1	d	3.5	H−C(14), H−C(15)
H−C(14)	6.43	1	dd	3.5, 1.8	H−C(13), H−C(15)
H−C(18)	6.78	1	dd	3.5, 1.8	H−C(17), H−C(19)
H−C(17)	7.19	1	d	3.5	H−C(18), H−C(19)
H−C(2)	7.34	1	d	1.52	H−C(4)
H−C(9)	7.45	1	s		
H−C(15)	7.60	1	d	1.8	H−C(13), H−C(15)
H−C(6)	7.96	1	s		
H−C(19)	8.05	1	d	1.8	H−C(17), H−C(18)

[a] Arbitrary numbering of carbon atoms refers to structure in Figure 2. [b] The ^{1}H 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 DQF-COSY experiment.

4.06 and 4.23 ppm and, in addition, a coupling between these protons and the triplet observed at 5.26 ppm and the double doublet detected at 5.89 ppm, respectively. These signals were assigned as the hydroxy proton HO−C(5) and the proton H−C(4). The disappearance of the signal at 5.26 ppm upon H/D exchange by adding trace amounts of D_2O confirmed the proposed hydroxymethyl group in the compound. Considering the 15 protons and

Table 3. Assignment of ^{13}C NMR Signals (600 MHz, DMSO-d_6) of 3-(2-Furyl)-8-[(2-furyl)methyl]-4-hydroxymethyl-1-oxo-1*H*,4*H*-quinolizinium-7-olate (Quinizolate)

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(11)	28.4	CH_2	H−C(11)	H−C(9)
C(5)	62.8	CH_2	H−C(5)	HO−C(5), H−C(4)
C(4)	68.6	CH	H−C(4)	H−C(5), H−C(2), H−C(6)
C(13)	108.1	CH	H−C(13)	H−C(14), H−C(15)
C(14)	111.3	CH	H−C(14)	H−C(13), H−C(15)
C(18)	114.2	CH	H−C(18)	H−C(17), H−C(19)
C(2)	118.7	CH	H−C(2)	
C(17)	120.5	CH	H−C(17)	H−C(18), H−C(19)
C(9)	121.3	CH	H−C(9)	
C(10)	126.5	C		H−C(6), H−C(9)
C(3)	127.6	C		H−C(2), H−C(4)
C(6)	130.7	CH	H−C(6)	
C(15)	142.8	CH	H−C(15)	H−C(13), H−C(14)
C(8)	143.1	C		H−C(6), H−C(9)
C(19)	148.2	CH	H−C(19)	H−C(17), H−C(18)
C(16)	151.0	C		H−C(2), H−C(17), H−C(18), H−C(19)
C(12)	152.9	C		H−C(13), H−C(14), H−C(15)
C(7)	171.7	C		H−C(6), H−C(9)
C(1)	180.8	C		H−C(2), H−C(9)

[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 HSQC (^1J) and HMBC ($^{2,3}J$) experiments.

19 carbon atoms observed by ^{1}H and ^{13}C NMR spectroscopy (Tables 2 and 3) and the data obtained by CHN analysis, and taking into account that one hydroxy

group and two oxygen atoms in the furan rings are present in the molecule, an elemental composition of $C_{19}H_{15}O_5N$ can be calculated from the molecular weight of 337 Da, fitting well with the proposed structure of the bitter-tasting compound (Figure 2).

A comparison of the ^{13}C NMR spectrum, in which 19 signals appeared, with the results of the DEPT-135 experiment showing 12 signals, revealed 7 signals corresponding to quarternary carbon atoms (Table 3). Unequivocal assignment of these quarternary carbon atoms and 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 3). The HMBC experiment revealed a correlation between the aromatic protons H−C(9) and H−C(6) resonating at 7.45 and 7.96 ppm, respectively, and neighboring carbon atoms, for example, heteronuclear connectivities between the proton H−C(9) and the carbons C(8) and C(10) or between the proton H−C(6) and the quarternary carbons C(7), C(8), and C(10), confirming the pyridinium ring structure proposed in Figure 2. In addition, the long-range correlations between the aromatic proton H−C(6) and the ^{13}C signal at 68.6 ppm as well as between H−C(9) and the ^{13}C signal at 180.8 ppm permitted the connection of the pyridinium ring with carbon C(4) and the carbonyl carbon C(1) of the attached second ring system. The olefinic proton H−C(2) showed heteronuclear correlations via two bonds to the carbonyl group C(1) as well as to the quarternary carbon atom C(3). In addition, heteronuclear long-range couplings were observed between H−C(2) and carbon C(16), confirming the proposed link between C(3) and the furan ring C(16)··· C(19), and carbon C(4), respectively. These heteronuclear couplings, as well as the allylic coupling of 1.52 Hz between the protons H−C(2) and H−C(4) confirmed the finding that the carbonyl group C(1) and carbon C(4) are linked together via a 1,2-substituted (2-furyl)-ethylene bridge, thus revealing a complete picture of the second ring system in the proposed structure.

For an unequivocal confirmation of the proposed 1-oxo-1*H*,4*H*-quinolizinium-7-olate ring system, we also measured heteronuclear δ,δ correlations between the nitrogen atom and neighboring protons by means of $^{15}N/^{1}H$ heteronuclear multiple-bond correlation spectrocopy (^{15}N HMBC). Because the low natural abundance of ^{15}N atoms is reflected in a low sensitivity of this NMR experiment and, depending on their magnetic enviroment, nitrogen atoms in organic compounds resonate in a wide range of ~1000 ppm, the localization of the ^{15}N chemical shift in the weakly concentrated solution of the tastant proved difficult. To locate the ^{15}N chemical shift area of the pyridinium betaine more easily, we aimed at characterizing the ^{15}N chemical shift of a structurally related and synthetically available reference compound. To achieve this, we synthesized 6-hydroxymethyl-1-propylpyridinium-3-olate by reacting 5-(hydroxymethyl)furan-2-aldehyde with propylamine as shown in Figure 4. After isolation and purification, a ^{15}N NMR experiment was performed with a highly concentrated solution of this reference compound, revealing the chemical shift of the nitrogen atom in 6-hydroxymethyl-1-propylpyridinium-3-olate at 209.3 ppm.

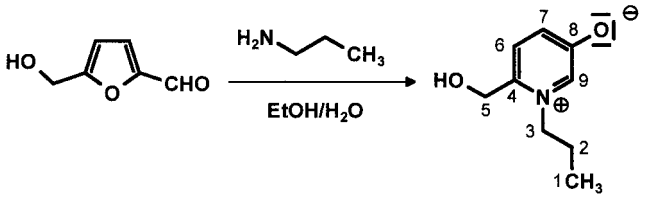

Figure 4. Synthesis and structure of 6-hydroxymethyl-1-propylpyrrolidinium-3-olate.

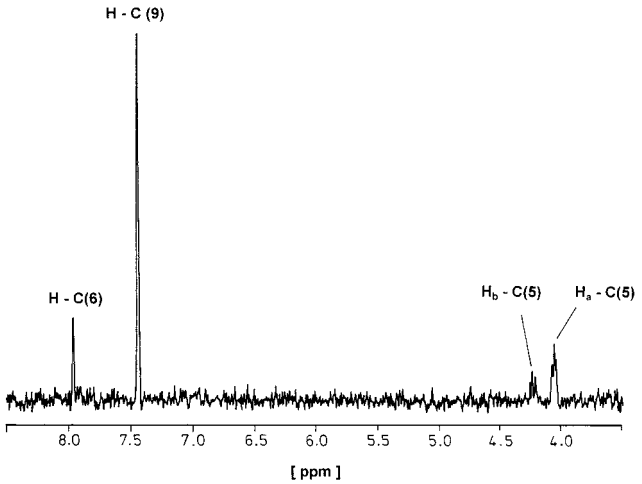

Figure 5. ^{15}N HMBC of the bitter-tasting compound in HPLC fraction 19.

Monitoring the chemical shift range from 190 to 230 ppm by ^{15}N NMR, the nitrogen atom in the bitter compound under investigation was found to resonate at 220.8 ppm (relative to neat NH_3). Using this information, a ^{15}N HMBC experiment was performed showing strong heteronuclear coupling between the nitrogen atom and the protons H−C(9) and H−C(6) detected at 7.45 and 7.96 ppm and weak coupling between the nitrogen atom and the methylene protons H_a−C(5) and H_b−C(5) resonating at 4.06 and 4.23 ppm (Figure 5), thereby, confirming the proposed 1*H*,4*H*-quinolizinium structure.

In summary, LC-MS measurements and carefully planned 1D and 2D NMR experiments, revealing a complete picture of chemical shifts, homo- and heteronuclear single- and multiple-bond correlations, led us to the unequivocal identification of the bitter-tasting Maillard compound as 3-(2-furyl)-8-[(2-furyl)methyl]-4-hydroxymethyl-1-oxo-1*H*,4*H*-quinolizinium-7-olate (Figure 2). To the best of our knowledge, this compound, which we name quinizolate, has not previously been reported in the literature.

Additional experiments, in which L-alanine was substituted with other primary amino acids such as L-valine, L-leucine, or L-isoleucine showed that quinizolate is formed by Maillard reactions from pentoses independent of the amino acid moiety. Studies on the clarification of the carbohydrate-derived precursors and the formation pathways of this interesting bitter-tasting compound gave strong evidence that quinizolate is formed as a racemate and will be published subsequently.

Bitter-Taste Threshold Values. The taste threshold concentration of quinizolate in water was determined by using a triangle test as described by Wieser and Belitz (1975). This novel compound exhibited an intense bitter taste at an extraordinarily low threshold

Table 4. Taste Threshold Concentrations of Selected Bitter Compounds

taste compound	taste threshold[b] (mmol/kg of water)
(2*S*,6*S*)-octahydrodipyrrolo[1,2-*a*;1′,2′-*d*]-pyrazine-5,10-dione (**1**)[a]	1.0
pyrrolidinohexose reductone (**2**)[a]	0.5
7-methyl-2,3,6,7-tetrahydrocyclopenta[*b*]-azepin-8(1*H*)-one (**3**)[a]	0.2[c]
bis(pyrrolidino)hexose reductone (**4**)[a]	0.06
3-(2-furyl)-8-[(2-furyl)methyl]-4-hydroxy-methyl-1-oxo-1*H*,4*H*-quinolizinium-7-olate (quinizolate)	0.00025
caffeine	0.5
quinine hydrochloride	0.007
denatonium chloride	0.00002[d]

[a] The structure is displayed in Figure 6. [b] The taste thresholds were determined by using a triangle test using tap water (pH 6.5) as the solvent. [c] The taste threshold was taken from Papst et al. (1988). [d] The taste threshold was taken from Saroli (1985).

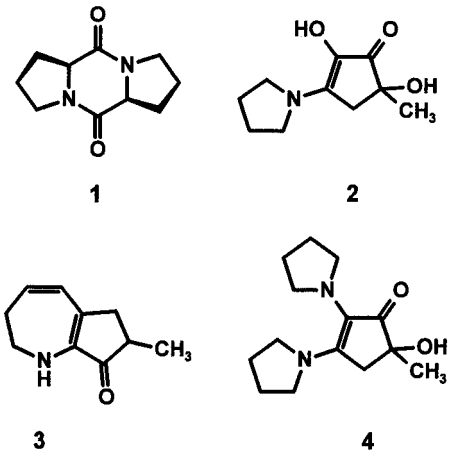

Figure 6. Structures of bitter-tasting Maillard compounds: (2*S*,6*S*)-octahydrodipyrrolo[1,2-*a*;1′,2′-*d*]pyrazine-5,10-dione (**1**), pyrrolidinohexose reductone (**2**), 7-methyl-2,3,6,7-tetrahydrocyclopenta[*b*]azepin-8(1*H*)-one (**3**), and bis(pyrrolidino)hexose reductone (**4**).

of 0.00025 mmol/kg of water (Table 4). Compared to other Maillard reaction products formed from carbohydrates and amino acids, quinizolate is by far the most intense bitter compound reported to date. In contrast, the (2*S*,6*S*)-octahydro-dipyrrolo[1,2-*a*;1′,2′-*d*]pyrazine-5,10-dione (**1** in Figure 6) formed from thermal reactions of L-proline (Gautschi et al., 1997), the pyrrolidinohexose reductone (**2** in Figure 6), or 7-methyl-2,3,6,7-tetrahydrocyclopenta[*b*]azepin-8(1*H*)-one (**3** in Figure 6), both formed by Maillard reactions from glucose and proline (Papst et al., 1985; Tressl et al., 1985; Hofmann, 1998b), showed higher taste thresholds than quinizolate by factors of 4000, 2000, or 800, respectively (Table 4). Also, the bis(pyrrolidino)hexose reductone (**4** in Figure 6), which is the most intensely bitter Maillard reaction product reported so far (Papst et al., 1984, 1988; Hofmann, 1998b), showed a 240-fold higher detection threshold (Table 4). In comparison to bitter-tasting standard compounds, the detection threshold concentration of quinizolate is lower than that reported for caffeine and quinine hydrochloride, respectively, by factors of 2000 and 28 and only 10 times higher than that of denatonium chloride, one of the most intense bitter compounds known so far (Saroli, 1985).

A novel bioassay, TDA, has thus been demonstrated as a straightforward screening technique to localize taste-active compounds in complex mixtures of compounds present in Maillard reaction mixtures or foods and to rank them in their taste impacts. This offers the possibility to evaluate the most intense taste compounds, on which the identification experiments can then be focused. Using this analytical strategy, useful information can be obtained that might be helpful to extend current knowledge on taste-active compounds generated during food processing and to control the taste development more efficiently by either increasing, or decreasing, the concentrations of desired or undesired tastants in foods.

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