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Sensory Activity, Chemical Structure, and Synthesis of Maillard Generated Bitter-Tasting 1-Oxo-2,3-dihydro-1*H*-indolizinium-6-olates

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Thermal treatment of aqueous solutions of xylose, rhamnose, and L-alanine led to a rapid development of a bitter taste of the reaction mixture. To characterize the key compounds causing this bitter taste, the recently developed taste dilution analysis (TDA), which is based on the determination of the taste threshold of reaction products in serial dilutions of HPLC fractions, was performed to locate the most intense taste compounds in the complex mixture of Maillard reaction products. By application of this TDA, 26 fractions were obtained, among which seven fractions were evaluated with a high taste impact. LC/MS and NMR spectroscopy as well as synthetic experiments revealed the 1-oxo-2,3-dihydro-1*H*-indolizinium-6-olates **1**−**5** as the key compounds contributing the most to the intense bitter taste of the Maillard mixture. Calculation of the taste impact of these compounds based on a dose/activity relationship indicated that these five compounds already accounted for 56.8% of the overall bitterness of the Maillard mixture, thus demonstrating this class of 1-oxo-2,3-dihydro-1*H*-indolizinium-6-olates as the key bitter compounds. First synthetic studies on the relationship between the chemical structure and the human psychobiological activity of 1-oxo-2,3-dihydro-1*H*-indolizinium-6-olates revealed that substitution of the furan rings of **1** by 5-methylfuryl moieties (compounds **3**−**5**) or by 5-(hydroxymethyl)furyl groups (compound **6**) led to a significant increase of the bitter threshold. In contrast, the substitution of the oxygen atoms in the furan rings of **1** by sulfur atoms induced a significant decrease of the detection threshold of the 1-oxo-2,3-dihydro-1*H*-indolizinium-6-olate; for example, the thiophene derivative **7** showed the extraordinarily low bitter detection threshold of 6.3 $\times$ 10^{-5} mmol/kg (water).

KEYWORDS: Flavor compounds; bitter compounds; taste dilution analysis; quinizolate; Maillard reaction; structure/activity

INTRODUCTION

To elucidate the bitter key tastants formed during thermal food processing from Maillard-type reactions, we recently developed the so-called taste dilution analysis (TDA), which is based on the determination of the taste threshold of reaction products in serial dilutions of HPLC fractions (*1*, *2*). Application of this novel bioassay led to the detection of intense bitter-tasting compounds in heated aqueous solutions of xylose and L-alanine. On the basis of LC/MS and NMR experiments, the structure of the most intense bitter tastant, named quinizolate, was identified as (*E*)-2-[(2-furyl)methylidene]-7-[(2-furyl)methyl]-3-(hydroxymethyl)-1-oxo-2,3-dihydro-1*H*-indolizinium-6-olate (*3*). This novel compound, exhibiting an intense bitter taste at an extraordinarily low detection threshold of 0.000 25 mmol/kg water, belongs to one of the most potent bitter compounds reported so far (*1*, *3*). In addition, the structure of a homologous compound, recently isolated from a heated xylose/L-alanine solution, exhibited an additional hydroxymethyl group and was identified as (*E*)-2-[(2-furyl)methylidene]-7-[(2-furyl)-methyl]-3,8-bis(hydroxymethyl)-1-oxo-2,3-dihydro-1*H*-indolizinium-6-olate (*3*). This compound, named homoquinizolate, imparted a strong bitter taste at a detection threshold of 0.001 mmol/kg water, which is 4-fold above the threshold found for quinizolate but below the threshold of caffeine (0.5 mmol/L) by a factor of 500 and indicated that the bitter threshold is strongly influenced already by small changes in the molecular structure of the 1-oxo-2,3-dihydro-1*H*-indolizinium-6-olate.

The purpose of the present investigation was, therefore, (i) to react xylose/alanine solutions in the presence of additional carbohydrates such as rhamnose, to increase the potential spectrum of 1-oxo-2,3-dihydro-1*H*-indolizinium-6-olates formed, (ii) to locate and identify these taste compounds by application

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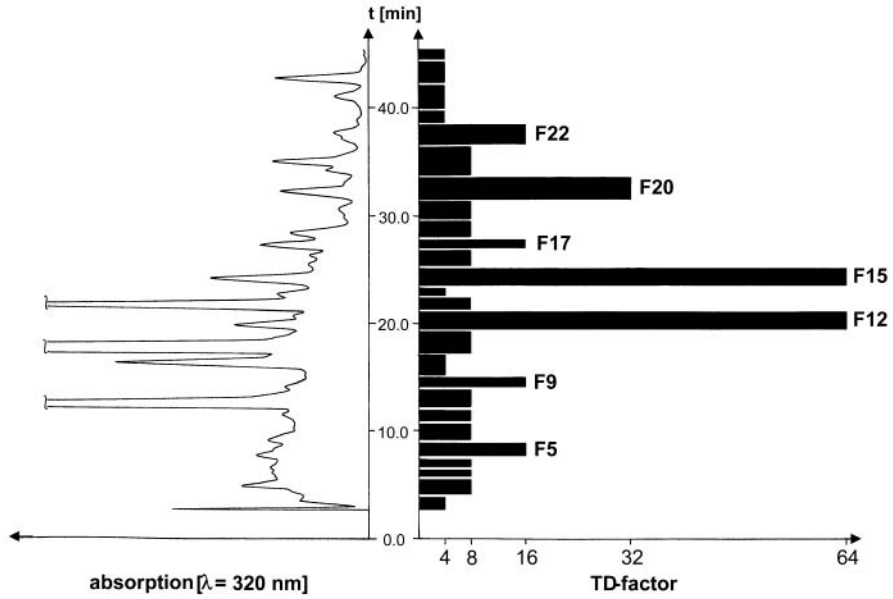


Figure 1. RP-HPLC chromatogram (left side) and taste dilution (TD) chromatogram (right side) of the solvent-extractable, nonvolatile fraction of a heated aqueous solution of xylose, rhamnose, and L-alanine.

of the taste dilution concept, (iii) to estimate their contribution to the overall bitter taste, and (iv) to gain first insights into the relationship between chemical structure and sensory activity of this novel class of potent bitter tastants.

MATERIALS AND METHODS

Chemicals. The following compounds were obtained commercially: D-xylose, L-alanine, furan-2-aldehyde, 5-(hydroxymethyl)furan-2-aldehyde, thiophene-2-aldehyde (Aldrich, Steinheim, Germany). Solvents were HPLC grade (Aldrich, Steinheim, Germany) and distilled twice prior to use. DMSO-d_6 was obtained from Isocom (Landshut, Germany). The 3-deoxypentosulose was synthesized following the procedure reported in the literature (*4*). Human taste thresholds were determined by a trained sensory panel using a triangle test as reported recently (*1*).

Thermally Treated Xylose/Rhamnose/Alanine Mixture. A solution of xylose (100 mmol), rhamnose (150 mmol), and L-alanine (50 mmol) in phosphate buffer (125 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 (10 × 50 mL). 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 five trained panelists revealed an intense bitter taste of this aqueous solution. The major aliquot of the solvent extract was used for the taste dilution analysis (1 mL) as well as for the identification and quantification experiments (97 mL).

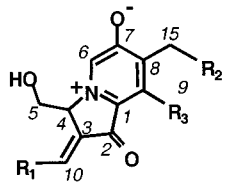
Taste Dilution Analysis (TDA). An aliquot (1 mL) of the solvent extract was freed from the volatiles by high-vacuum distillation (0.04 mbar, 30 °C), the residue was dissolved in methanol (1 mL), and after membrane filtration, aliquots (100 μL) were analyzed by RP-HPLC (**Figure 1**). The effluent was separated into 26 fractions, which were separately collected in glass vials. The corresponding fractions obtained from 12 HPLC runs were collected, combined, and freeze-dried. The residues obtained from these 26 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 26 fractions were then presented to a trained sensory panel while wearing a nose clamp 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 recently defined as the taste dilution (TD) factor (*1*). The TD factors evaluated by five different assessors were averaged. The TD factors between individuals and three separate sessions differed by not more than one dilution step.

Isolation of 1-Oxo-2,3-dihydro-1*H*-indolizinium-6-olates 1−5 from a Thermally Treated Mixture of Xylose, L-Alanine, Furan-2-aldehyde, and 5-Methyl-furan-2-aldehyde. A solution of xylose (0.8 mol) and L-alanine (0.2 mol) in phosphate buffer (500 mL; 0.1 mol/L, pH 5.0) was heated under reflux for 20 min. Then furan-2-aldehyde (0.06 mol) and 5-methylfuran-2-aldehyde (0.1 mmol) was added, and heating was continued for an additional 150 min. After the mixture was cooled to room temperature, the pH of the aqueous solution was adjusted to 3.5 with aqueous hydrochloric acid (1 mmol/L), and the mixture was then extracted with ethyl acetate (10 × 200 mL). The combined organic layer was dried over Na_2SO_4 and then freed from solvent at 25 °C in vacuo (100 mbar). The residue was taken up in a mixture (20 mL, 1/5/5, v/v/v) of *n*-hexane, ethyl acetate, and phosphate buffer (0.1 mol/L, pH 3.5), and aliquots (5 mL) of this solvent extract were fractionated by means of high-speed countercurrent chromatography (HSCCC) as detailed below. Fractions of 12 mL were collected, freed from solvent, taken up in methanol, and analyzed for the bitter compounds **1**−**5** by RP-HPLC. Compound **1** (t_R = 19.9 min) was detected in HSCCC fraction III, compound **2** (t_R = 14.2 min) could be isolated from HSCCC fraction V, and compounds **3** (t_R = 24.2 min), **4** (t_R = 27.3 min), and **5** (t_R = 32.3 min) were present in the HSCCC fraction II. These bitter tastants were then isolated from the HSCCC fractions by semipreparative RP-HPLC. Compounds **1** and **2** (**Figure 2**) were identified as (*E*)-2-[(2-furyl)methylidene]-7-[(2-furyl)methyl]-3-hydroxymethyl-1-oxo-2,3-dihydro-1*H*-indolizinium-6-olate and (*E*)-2-[(2-furyl)methylidene]-7-[(2-furyl)methyl]-3,8-bis(hydroxymethyl)-1-oxo-2,3-dihydro-1*H*-indolizinium-6-olate, showing the identical spectroscopic properties as recently reported for the bitter key compounds quinizolate and homoquinizolate in a heated xylose/L-alanine mixture. On the basis of the spectroscopic data, the tastants **3**, **4**, and **5** were identified as (*E*)-2-[2-(5-methylfuryl)methylidene]-7-[(2-furyl)methyl]-3-hydroxymethyl-1-oxo-2,3-dihydro-1*H*-indolizinium-6-olate, (*E*)-2-[(2-furyl)methylidene]-7-[2-(5-methylfuryl)methyl]-3-hydroxymethyl-1-oxo-2,3-dihydro-1*H*-indolizinium-6-olate, and (*E*)-2-[2-(5-methylfuryl)methylidene]-7-[2-(5-methylfuryl)methyl]-3-hydroxymethyl-1-oxo-2,3-dihydro-1*H*-indolizinium-6-olate, respectively (**Figure 2**). Determination of melting points was not successful because compounds **3**−**5** decomposed rapidly on dry-heating. Compound **1**: UV/vis (water) λ_{max} = 412, 335 nm; LC/MS (APCI$^+$) *m*/*z* 338 (100, [M + 1]$^+$), 308 (39); ^{1}H NMR data are given in **Table 1**. Compound **2**: UV/vis (water) λ_{max} = 412, 335 nm; LC/MS (APCI$^+$) *m*/*z* 368 (100, [M + 1]$^+$), 338 (44); ^{1}H NMR data are given in **Table 1**. Compound

Table 1. Assignment of ^{1}H NMR Signals (400 MHz, DMSO-d_6) of 1-Oxo-2,3-dihydro-1*H*-indolizinium-6-olates **1**–**7**

	δ [b] [ppm] (multiplicity[c]) of compound							
H at C atom[a]	**1**	**2**	**3**	**4**	**5**	**6**	**7**	*I*[c]
H–C(19′)				2.23 (s)	2.23 (s)			3
H–C(14′)			2.43 (s)		2.43 (s)			3
H–C(15)	3.92 (s)	4.10 (s)	3.84 (s)	3.84 (s)	3.84 (s)	3.88 (s)	4.04 (s)	2
H_a-C(5)	4.06 (m)	4.06 (m)	4.09 (m)	4.09 (m)	4.09 (m)	4.18 (m)	4.18 (m)	1
H–C(9′)		4.12 (d)						2
H_b-C(5)	4.23 (m)	4.23 (m)	4.22 (m)	4.22 (m)	4.22 (m)	4.23 (m)	4.23(m)	1
HO–C(9′)		4.99 (t)						1
HO–C(5)	5.26 (t)	5.33 (t)	5.23 (t)	5.23 (t)	5.23 (t)	5.22 (t)	5.44 (t)	1
H–C(19″)						5.36 (s)		2
H–C(14″)						5.58 (s)		2
H–C(4)	5.89 (dd)	5.89 (dd)	5.85 (dd)	5.85 (dd)	5.85 (dd)	5.86 (dd)	5.94 (dd)	1
H–C(17)	6.26 (d)	5.98 (d)	6.26 (d)	6.01 (d)	6.01 (d)	6.16 (d)	6.87 (dd)	1
H–C(18)	6.43 (dd)	6.30 (dd)	6.43 (dd)	6.10 (d)	6.10 (d)	6.22 (d)	6.98 (dd)	1
H–C(13)	6.78 (dd)	6.78 (dd)	6.41 (d)	6.78 (dd)	6.41 (d)	6.56 (d)	7.29 (dd)	1
H–C(12)	7.19 (d)	7.19 (d)	7.09 (d)	7.19 (d)	7.09 (d)	7.12 (d)	7.36 (dd)	1
H–C(10)	7.34 (d)	7.34 (d)	7.34 (d)	7.34 (d)	7.25 (d)	7.30 (d)	7.61 (d)	1
H–C(9)	7.45 (s)		7.45 (s)	7.45 (s)	7.42 (s)	7.47 (s)	7.72 (s)	1
H–C(19)	7.60 (d)	7.47 (d)	7.60 (d)				7.02 (dd)	1
H–C(6)	7.96 (s)	7.96 (s)	7.96 (s)	7.96 (s)	7.94 (s)	7.94 (s)	7.95 (s)	1
H–C(14)	8.05 (d)	8.04 (d)		8.05 (d)			8.00 (dd)	1

[a] Arbitrary numbering of carbon atoms refers to structures **1**–**7** in **Figure 2**. Signal assignment was confirmed by measuring homonuclear ^{1}H,^{1}H connectivities in DQF-COSY experiments. [b] The ^{1}H chemical shifts are given in relation to DMSO-d_6. [c] Determined from a 1D spectrum.



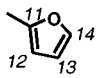
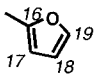
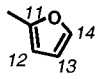
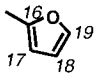
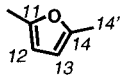
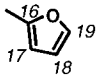
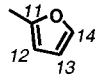
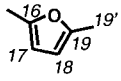
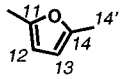
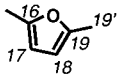
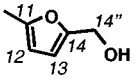
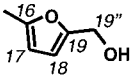
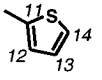
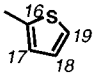
R_1:	R_2:	R_3:	compound
		-H	**1**
			**2**
		-H	**3**
		-H	**4**
		-H	**5**
		-H	**6**
		-H	**7**

Figure 2. Structures of bitter-tasting 1-oxo-2,3-dihydro-1*H*-indolizinium-6-olates **1**–**7** identified in the heated xylose/rhamnose/L-alanine mixture or synthesized from 3-deoxypentosulose, L-alanine, and aromatic aldehydes.

3: UV/vis (water) λ_{max} = 412, 335 nm; LC/MS (APCI$^+$) *m*/*z* 352 (100, [M + 1]$^+$), 322 (39); ^{1}H NMR data are given in **Table 1**. Compound **4**: UV/vis (water) λ_{max} = 412, 335 nm; LC/MS (APCI$^+$) *m*/*z* 352 (100, [M + 1]$^+$), 322 (42); ^{1}H NMR data are given in **Table 1**. Compound **5**: UV/vis (water) λ_{max} = 412, 335 nm; LC/MS (APCI$^+$) *m*/*z* 366 (100, [M + 1]$^+$), 336 (44); ^{1}H NMR data are given in **Table 1**.

Table 2. Concentration, Detection Thresholds, and Taste Activity Values (TAV) of 1-Oxo-2,3-dihydro-1*H*-indolizinium-6-olates **1**–**5** and Their Contribution to the Overall Bitter Taste of the Heated Xylose/Rhamnose/Alanine Mixture

tastant[a]	concn [μmol/kg]	detection threshold[b] [μmol/(kg of water)]	TAV[c]	contribution to total bitterness[e] [%]
1	9.2	0.25	37	15
2	1.9	1.00	2	1
3	16.2	0.25	65	25
4	13.1	0.50	26	10
5	15.1	1.00	15	6
		$\sum(TAV_{1-5})$:	145	57
		TD_{total} factor:[d]	256	100

[a] The structures of the tastants are displayed in **Figure 2**. [b] The detection threshold was determined in water using a triangle test as reported earlier (*1*). [c] The taste activity value (TAV) was calculated from the ratio of the concentration to the detection threshold of a tastant as reported earlier (*6*). [d] The taste activity of the overall Maillard mixture was determined by measuring the taste dilution factor. [e] The taste contribution of each individual compound was calculated using the following equation: contribution (%) = (TAV/TD_{total} factor) × 100.

Quanitation of 1-Oxo-2,3-dihydro-1*H*-indolizinium-6-olates 1−5 from a Thermally Treated Mixture of Xylose, Rhamnose, and L-Alanine. An aliquot of the solvent extract isolated from the reacted mixture of xylose, rhamnose, and alanine was analyzed by analytical RP-HPLC. Quantification of the bitter compounds was performed by comparing the peak areas obtained at 412 nm with those of defined standard solutions of each reference compound in methanol. The quantitative data given in **Table 2** are the mean values of triplicates.

Synthetic Preparation of 1-Oxo-2,3-dihydro-1*H*-indolizinium-6-olates 1−5, (*E*)-2-[2-(5-Hydroxymethylfuryl)methylidene]-7-[2-(5-hydroxymethylfuryl)methyl]-3-hydroxymethyl-1-oxo-2,3-dihydro-1*H*-indolizinium-6-olate (6), and (*E*)-2-[(2-Thienyl)methylidene]-7-[(2-thienyl)methyl]-3-hydroxymethyl-1-oxo-2,3-dihydro-1*H*-indolizinium-6-olate (7). A solution of 3-deoxypentosulose (10 mmol) and L-alanine (5 mmol) in phosphate buffer (50 mL; 0.1 mol/L, pH 5.0) was refluxed in the presence of furan-2-aldehyde (2.5 mmol, for preparation of **1** and **2**), a binary mixture of furan-2-aldehyde and 5-methylfuran-2-aldehyde (1.25 mmol each, for preparation of **3** and **4**), 5-methylfuran-2-aldehyde (2.5 mmol, for preparation of **5**), 5-hydroxymethylfuran-2-aldehyde (2.5 mol, for preparation of **6**), or thiophene-2-aldehyde (2.5 mol, for preparation of **7**) for 90 min. After

the mixture was cooled to room temperature, the pH of the aqueous solutions was adjusted to 3.5 with aqueous hydrochloric acid (1 mmol/L), and the mixtures were then extracted with ethyl acetate (5 × 50 mL). The combined organic layers were dried over Na_2SO_4, then concentrated at 25 °C in vacuo (100 mbar) to 5 mL, and finally, fractionated by column chromatography (35 mm × 450 mm) on silica gel (200 g, silica gel 60, Merck, Darmstadt, Germany). After application of the crude material onto the top of the column conditioned with toluene/ethyl acetate (2/8, v/v), chromatography was performed using a sequence of ethyl acetate/methanol mixtures with increasing methanol content from 0% to 80% (v/v) in steps of 20%. After removal of the solvent from the fractions containing the intensely fluorescent target compounds **1**−**7**, the residue was taken up in methanol (2 mL). After membrane filtration, the target compounds **1**−**7** were isolated by semipreparative RP-HPLC. From the monitoring of the chromatographic separation at 412 nm, the effluent of the major peak was collected and freeze-dried, affording the target compounds **1**−**7** as yellow amorphous powders in purities of more than 98%. The spectroscopic data of compounds **1**−**5** were identical with those obtained for the compounds isolated from the reaction mixture of xylose, L-alanine, furan-2-aldehyde, and 5-methylfuran-2-aldehyde. Determination of melting points was not successful because compounds **6** and **7** decomposed rapidly on dry-heating. Compound **6** (**Figure 2**): UV/vis (water) λ_{max} = 412, 335 nm; LC/MS (APCI$^+$) *m/z* 398 (100, [M + 1]$^+$), 368 (40); ^{1}H NMR data are given in **Table 1**. Compound **7** (**Figure 2**): UV/vis (water) λ_{max} = 412, 337 nm; LC/MS (APCI$^+$) *m/z* 370 (100, [M + 1]$^+$), 340 (42); ^{1}H NMR data are given in **Table 1**.

High-Speed Countercurrent Chromatography (HSCCC). The system was a multilayer coil countercurrent chromatograph by P.C. Inc. (Potomac, MD) equipped with a single coil (tubing: 2.4 mm i.d., 390 mL volume) and an HPLC pump (Beckman, Germany). The separations were run at a revolution speed of 800 rpm with a solvent system (1/5/5, v/v/v) consisting of *n*-hexane, ethyl acetate, and phosphate buffer (0.1 mol/L, pH 3.5) at a flow rate of 2.5 mL/min. The elution mode was tail-to-head with the less dense phase as the mobile phase. The extract of the Maillard mixture was dissolved in a mixture (1/1, v/v, 5 mL) of light and heavy phases and then injected into the system by loop injection. Stationary-phase retention was about 75%. Twelve milliliter subfractions were collected by a fraction collector (LKB Bromma 7000 Ultrarac) and pooled to give fractions I−V. The effluent was monitored by means of a UV/vis detector (Gilson 115, Abimed, Germany) operating at λ = 405 nm, and the chromatogram was recorded on a LEM Servogor 124 plotter.

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 a 250 mm × 4.6 mm i.d. analytical (flow rate 0.8 mL/min) or a 150 mm × 10 mm i.d. semipreparative scale (flow rate 1.6 mL/min). After injection of the sample (20−100 μL), analysis was performed using a gradient starting with a mixture (35/65, v/v) of water and methanol and increasing the methanol content to 80% within 65 min and then to 100% within 70 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 atmospheric pressure chemical ionization in the positive mode. 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 COSY and DQF-COSY spectroscopy measurements were performed on a Bruker-AMX-400 spectrometer (^{1}H frequency, 400.13 MHz) (Bruker, Rheinstetten, Germany) using the aquisition parameters described recently. Chemical shifts were measured from residual DMSO-d_5 (2.49 ppm) in the proton dimension.

RESULTS AND DISCUSSION

Sensory analysis of an aqueous solution of the solvent-extractable compounds isolated from a thermally treated aqueous solution of xylose, rhamnose, and L-alanine revealed an intense bitter taste of the hydrophobic fraction of reaction products. To locate the taste-active compounds formed, first, odor-active volatiles were removed by high-vacuum distillation, and then the nonvolatile materials 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 tastants responsible for the bitter taste of the xylose/rhamnose/alanine reaction mixture, we first screened the reaction products for the most intense taste compounds by means of the taste dilution analysis and then focused on the structure determination of the compounds that were evaluated with the highest taste impact.

Screening for Taste-Active Compounds by Taste Dilution Analysis. Aimed at ranking the reaction products in their relative taste impact by application of the taste dilution analysis, an aliquot of the reaction mixture was chromatographed by RP-HPLC (**Figure 1**, left side) and the effluent was separated into 26 fractions, which were freeze-dried and then made up with water to the same volume. Each fraction was then stepwise 1 + 1 diluted 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. Because the dilution at which a taste difference between the diluted fraction and two blanks could just be detected (the so-called taste dilution (TD) factor) is proportional to the taste activity of the individual fractions in water, the TD factor ranked the 26 HPLC fractions according to their relative taste intensity, which is outlined in **Figure 1** (right side).

All fractions were evaluated as bitter tasting. Owing to the high TD factor of 64, fractions F12 and F15 were evaluated with by far the highest bitter activity, therefore mainly contributing to the overall bitter taste of the Maillard mixture. Fraction F20, which was also judged as intensely bitter tasting, was found to have an 2-fold lower taste impact, followed by the bitter tasting fractions F5, F9, F17, and F22 showing a TD factor of 16. The other fractions, all of which were judged with TD factors of 4 and 8, respectively, showed comparatively lower taste impacts and, in consequence, should not contribute significantly to the overall taste of the Maillard mixture (**Figure 1**).

Although the taste dilution analysis is based on the simplified assumption that taste intensity is a linear function of concentration, the striking advantage of this technique 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 key compounds causing the intense bitter taste of fractions F9, F12, F15, F17, and F20, and in consequence, contributing mainly to the taste of the heated carbohydrate/amino acid mixture.

Identification of Bitter Compounds in Fractions F9, F12, F15, F17, and F20. After preparative HPLC isolation of compounds **1** and **2** from fractions F12 and F9, respectively, 1D and 2D NMR experiments as well as LC/MS measurements showing an intense [M + 1]$^+$ ion at *m/z* 338 and 368, respectively, led to the identification of these compounds as (*E*)-2-[(2-furyl)methylidene]-7-[(2-furyl)methyl]-3-hydroxymethyl-1-oxo-2,3-dihydro-1*H*-indolizinium-6-olate (**1**; **Figure 2**)

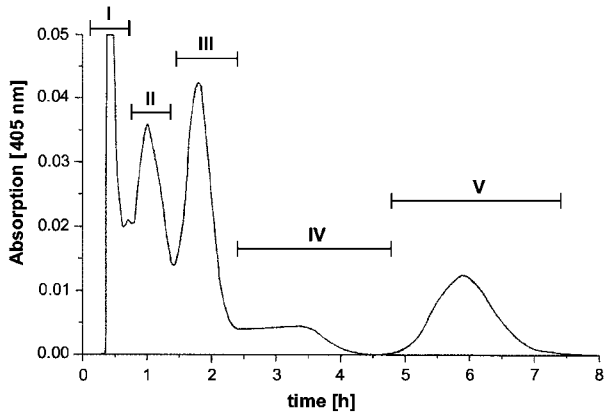


Figure 3. Separation of a reaction mixture of xylose, L-alanine, furan-2-aldehyde, and 5-methylfuran-2-aldehyde by means of high-speed countercurrent chromatography (HSCCC).

and (*E*)-2-[(2-furyl)methylidene]-7-[(2-furyl)methyl]-3,8-bis-(hydroxymethyl)-1-oxo-2,3-dihydro-1*H*-indolizinium-6-olate (**2**; **Figure 2**), showing the identical spectroscopic and chromatographic data as the purified reference compounds quinizolate and homoquinizolate isolated recently as the bitter key compounds in a heated xylose/alanine mixture (*1*, *3*).

HPLC with diode array detection revealed that the compounds eluting in fractions F15, F17, and F20 showed identical UV/vis spectra with absorption maxima at 412 and 335 nm as observed for the taste compounds **1** and **2**. In addition, LC/MS analysis revealed $[M + 1]^+$ ions of *m*/*z* 352 for the compounds present in fractions F15 and F17, and $[M + 1]^+$ ions with *m*/*z* 366 for the major compound isolated from fraction F20. Because these pseudomolecular ions differ by 14 and 28 amu, respectively, from the $[M + 1]^+$ ion detected for compound **1**, we assumed that the bitter compounds in fractions F15, F17, and F20 might be structurally related 1-oxo-2,3-dihydro-1*H*-indolizinium-6-olates varying in the number and position of additional methyl groups. Because recent ^{13}C labeling studies on the formation of compounds **1** and **2** revealed pentose-derived furan-2-aldehyde as an efficient precursor of both 1-oxo-2,3-dihydro-1*H*-indolizinium-6-olates, this prompted us to study whether the reaction of xylose and L-alanine in the presence of furan-2-aldehyde and 5-methylfuran-2-aldehyde, which is well-known to be formed from rhamnose (*5*), is able to generate the bitter compounds present in fractions F15, F17, and F20 in amounts enabling an unequivocal structure elucidation. A comparison of retention times, UV/vis spectra, and LC/MS data of the reaction products formed from xylose/alanine in the presence of furan-2-aldehyde and 5-methylfuran-2-aldehyde with those of the Maillard mixture revealed that the identical bitter compounds had been formed in fractions F15, F17, and F20 but in significantly higher concentrations than in the xylose/rhamnose/alanine mixture.

For a preparative isolation of these bitter compounds from the reacted mixture of xylose, L-alanine, furan-2-aldehyde, and 5-methyl-2-furanaldehyde, the reaction products were isolated by solvent extraction and were then separated by means of high-speed countercurrent chromatography (HSCCC) using a multilayer coil countercurrent chromatograph operating with a ternary solvent system of *n*-hexane, ethyl acetate, and phosphate buffer. From the monitoring of the effluent at 405 nm by means of a UV/vis detector, 12 mL fractions were collected, pooled to give fractions I−V (**Figure 3**), and analyzed by RP-HPLC for the presence of the bitter compounds in fractions F15, F17, and F20. In HSCCC fraction II, three bitter tastants (compounds **3**−**5**) were detected, which were identical with the key tastants evoking the bitter taste of the RP-HPLC fractions F15, F17, and F20. In addition, compounds **1** and **2** were eluted as pure compounds in HSCCC fractions III and V, respectively.

The HSCCC fraction II containing high amounts of the unknown compounds **3**−**5** was then further separated by semipreparative RP-HPLC. LC/MS analysis of compound **3**, which was identical with the bitter taste compound in fraction F15 of the TDA, revealed an $[M + 1]^+$ ion of *m*/*z* 352 and a loss of 30 amu to give *m*/*z* 322, most likely corresponding to the cleavage of one molecule of formaldehyde. These LC/MS data as well as the ^1H NMR spectroscopic data were consistent with structure **3** displayed in **Figure 3**. The ^1H NMR spectrum showed major similarities to the spectroscopic data observed for quinizolate (**1**), but in addition to compound **1**, the spectrum of compound **3** showed an additional signal integrating for three protons and resonating in the chemical shift range expected for a methyl group linked to a furan ring (**Table 1**). Furthermore, the proton H−C(14) resonating at 8.05 ppm for compound **1** was missing in the spectrum of compound **3** (**Table 1**). Double-quantum-filtered homonuclear H,H correlation spectroscopy (DQF-COSY) revealed strong coupling between the protons H−C(12) and H−C(13) but not to H−C(14) (data not shown), thus confirming the finding that the furan ring in compound **1** is substituted by a 5-methylfuran group in compound **3**. Taking all these data into consideration, the structure of compound **3** was unequivocally identified as (*E*)-2-[2-(5-methylfuryl)methylidene]-7-[(2-furyl)methyl]-3-hydroxymethyl-1-oxo-2,3-dihydro-1*H*-indolizinium-6-olate (**3**; **Figure 2**). To the best of our knowledge, this bitter compound has not been previously reported in the literature.

In addition to **3**, compound **4** was also isolated from HSCCC fraction II by means of semipreparative HPLC. LC/MS analysis revealed a quasi molecular ion of *m*/*z* 352 and a cleavage of 30 amu as already found for compound **3**, thus indicating that compound **4** might differ from compound **3** only by the position of the methyl group. ^1H NMR spectroscoypy as well as a DQF-COSY experiment revealed that compound **4** exhibits an additional methyl group resonating at 2.23 ppm, whereas the proton H−C(19) at 7.60 ppm detected for compound **1** was missing (**Table 1**). The lack of homonuclear couplings between H−C(18) and H−C(19) showed that the (2-furyl)methyl group linked to the pyridine ring in compound **1** is substituted by a (5-methyl-2-furyl)methyl group in compound **4**. On the basis of these observations, the structure of compound **4** was identified as the previously unknown (*E*)-2-[(2-furyl)methylidene]-7-[2-(5-methylfuryl)methyl]-3-hydroxymethyl-1-oxo-2,3-dihydro-1*H*-indolizinium-6-olate (**Figure 2**).

LC/MS analysis of compound **5** detected in HSCCC fraction II revealed an $[M + 1]^+$ ion at *m*/*z* 366 and a loss of 30 amu, most likely corresponding to the cleavage of one molecule of formaldehyde, thus comfirming that compound **5** differed from compound **1** by the presence of two methyl groups. This was then confirmed by ^1H NMR (**Table 1**) and DQF-COSY spectroscopy showing that both furan rings in compound **1** are substituted by 5-methylfuran groups in (*E*)-2-[2-(5-methylfuryl)-methylidene]-7-[2-(5-methylfuryl)methyl]-3-hydroxymethyl-1-oxo-2,3-dihydro-1*H*-indolizinium-6-olate (**5**; **Figure 2**). To the best of our knowledge, this bitter tastant has previously not been reported in the literature.

In summary, the taste dilution analysis in combination with LC/MS and NMR measurements led us to the unequivocal identification of the 1-oxo-2,3-dihydro-1*H*-indolizinium-6-olates

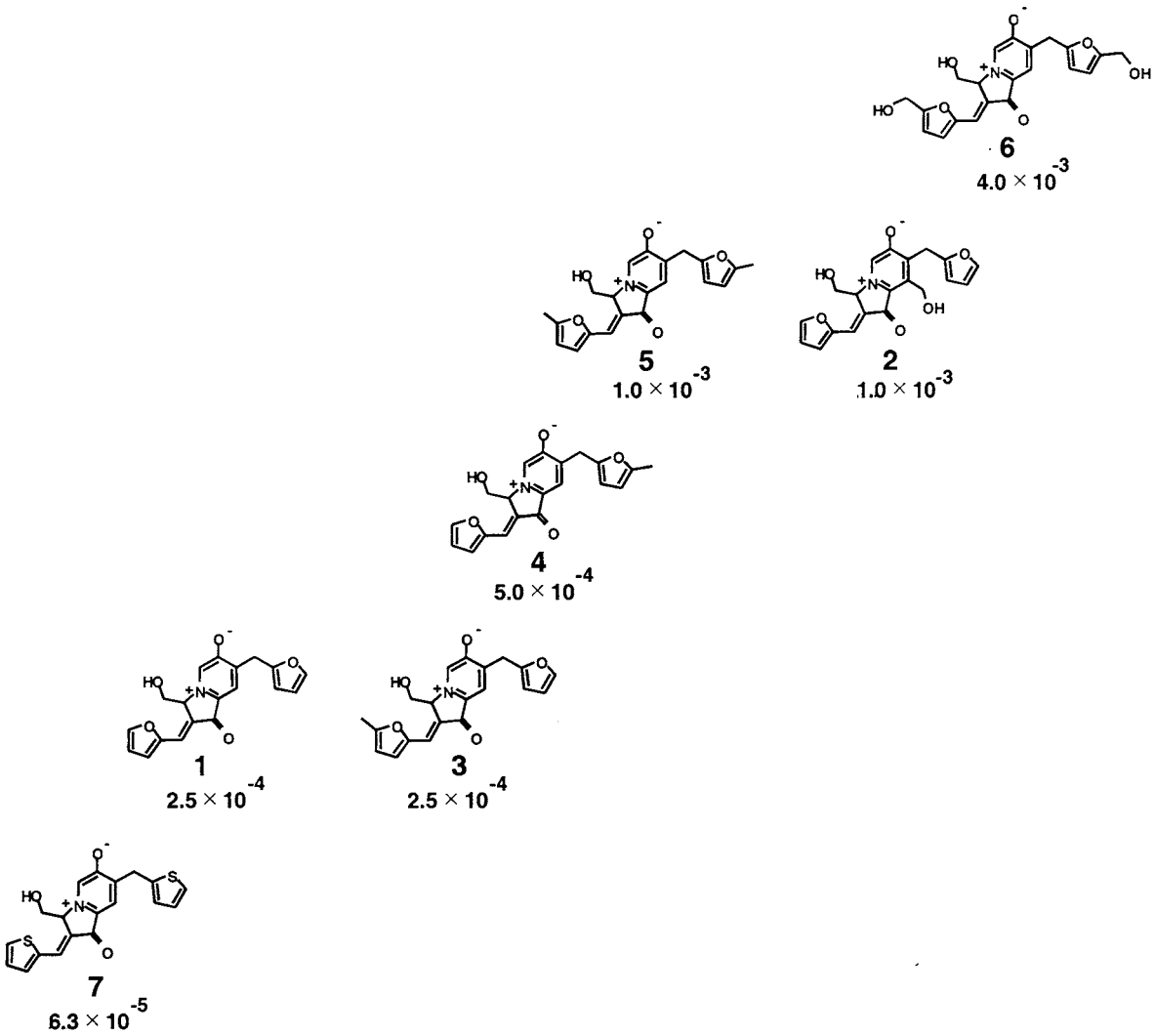


Figure 4. Structures and human bitter taste thresholds of 1-oxo-2,3-dihydro-1*H*-indolizinium-6-olates **1**–**7**.

1−**5** (**Figure 2**) as potent bitter compounds in a heated aqueous solution of xylose, rhamnose, and L-alanine.

Taste Activity of 1-Oxo-2,3-dihydro-1*H*-indolizinium-6-olates 1−**5.** To determine the taste contribution of these 1-oxo-2,3-dihydro-1*H*-indolizinium-6-olates more accurately, compounds **1**−**5** were quantified in the heated mixture of xylose, rhamnose, and alanine by HPLC/DAD using the pure reference compounds as external standards. As given in **Table 2**, the highest amounts were found for the methylated derivatives, among which compound **3** was predominant with a concentration of 16.2 μmol/kg. Quinizolate (**1**) was detected in about half the concentration, whereas homoquinizolate (**2**) reached an amount of 1.9 μmol/kg only.

Since the quantitative data alone does not allow an estimation of the importance of these tastants in evoking the overall bitter taste of the xylose/rhamnose/alanine solution, their taste activity values (TAVs) were calculated from the quotient of the actual concentration and the human taste detection thresholds as reported recently (*6*). To achieve this, first, the detection thresholds of the tastants were determined using a triangle test. Quinizolate (**1**) and compound **3** were evaluated with the extraordinarily low bitter detection threshold of 0.25 μmol/kg water, followed by compound **4** showing a 3 times higher threshold concentration (**Table 2**). Compounds **2** and **5** were 4-fold above the threshold of compound **1**. Relating the concentration with the detection threshold of the tastants then revealed the highest TAV of 65 for compound **3**, indicating that the concentration of that tastant in the Maillard mixture is 65-fold above the threshold (**Table 2**). In comparison, quinizolate (**1**) and compound **4** were evaluated with somewhat lower TAVs of 37 and 26, respectively. The taste activity of the bismethyl derivative **5** was by a factor of 4.3 below the taste impact evaluated for compound **3**. Homoquinizolate (**2**) was judged only with a TAV of 2, indicating that the concentration of this compound is only 2-fold above its taste threshold and demonstrating that this compound is only of minor importance for the overall bitter taste of the Maillard mixture.

To estimate the percent taste contribution of an individual compound, the measurement of the overall bitterness of the Maillard solution is a necessary prerequisite. To achieve this, the taste dilution factor of the complete reaction mixture, the TD_{total} factor, was determined. The complete Maillard solution was, therefore, diluted with water until no bitter taste difference between a diluted aliquot and two blanks containing tap water could be detected using a triangle test. A TD_{total} factor of 256 was found for the total mixture, indicating that the taste of the undiluted, original reaction mixture was 256-fold above its detection threshold (**Table 2**). Also, the TAV of a tastant corresponds, by definition, to the factor for which the actual concentration is above the detection threshold. The percent taste contribution of a single tastant could, therefore, be estimated from the TDA of the compound and the TD_{total} factor of the

complete Maillard mixture, which was defined as possessing a taste activity of 100% (**Table 2**). A TAV of 65 for compound **3** means that the actual concentration of **3** is 65-fold above its detection threshold (**Table 2**). Since the complete Maillard mixture, accounting for 100% of the bitterness, is 256-fold above its detection threshold, it can be estimated that about 25.4% of the overall bitterness is caused by compound **3**. Also, compounds **1** and **4** showed major contributions to the overall bitter taste and accounted for 15% and 10%, respectively, to the overall bitter taste. In contrast, compounds **5** and **2** showed only a lower taste contribution of 6% and 1%, respectively. In summary, these data demonstrate that the five key compounds **1**−**5** accounted for 57% of the total bitterness and covered more than the half of the bitter impact of all compounds formed by the complex network of Maillard reactions in the heated solution of xylose, rhamnose, and L-alanine (**Table 2**).

Structure and Sensory Activity of 1-Oxo-2,3-dihydro-1*H*-indolizinium-6-olates. To gain first insights into the relationship between the chemical structure and the human psychobiological activity of 1-oxo-2,3-dihydro-1*H*-indolizinium-6-olates, compounds **1**−**5** and two other derivatives (**6** and **7**) were synthesized, varying in the substitution of the furan moiety by a thiophene moiety and a 5-hydroxymethyl-2-furyl moiety. To achieve this, 3-deoxypentosulose and L-alanine were reacted in the presence of either furan-2-aldehyde or a binary mixture of furan-2-aldehyde, and 5-methylfuran-2-aldehyde, 5-methylfuran-2-aldehyde, 5-hydroxymethyl-furan-2-aldehyde, or thiophene-2-aldehyde, respectively. Compounds **1**−**5** (**Figure 2**) showed identical spectroscopic data as found for the bitter compounds isolated from the mixture of xylose/alanine and 5-methylfuran-2-aldehyde (**Table 1**). After final HPLC purification, the chemical structures of the previously unknown compounds (*E*)-2-[2-(5-hydroxymethylfuryl)methylidene]-7-[2-(5-hydroxymethylfuryl)methyl]-3-hydroxymethyl-1-oxo-1*H*,2*H*,3*H*-indolizinium-6-olate (**6**; **Figure 2**) and (*E*)-2-[(2-thienyl)methylidene]-7-[(2-thienyl)methyl]-3-hydroxymethyl-1-oxo-1*H*,2*H*,3*H*-indolizinium-6-olate (**7**; **Figure 2**) were confirmed by LC/MS, ^{1}H NMR, and UV/vis spectroscopy, and the human bitter taste thresholds were determined by means of a triangle test with a trained sensory panel.

The highest taste activity was evaluated for the thiophene derivative **7**, which showed an extraordinarily low bitter detection threshold of 6.3×10^{-5} mmol/kg (water), followed by quinizolate (**1**), which showed a 4-fold higher detection threshold concentration (**Figure 4**). The incorporation of a methyl group into the (2-furan)methylidene system in compound **3** did not influence the bitter threshold of the 1-oxo-2,3-dihydro-1*H*-indolizinium-6-olate (**Figure 4**), whereas the presence of a methyl group in the upper furan ring of compound **4** led to an increase of the detection threshold by a factor of 2. The substitution of both furan rings in **1** by a 5-methyl-2-furyl moiety increased the threshold 4-fold to 1.0×10^{-3} mmol/kg (water). The same threshold was determined for homoquinizolate (**2**) bearing an additional hydroxymethyl group at the pyridinium ring (**Figure 4**). The attachment of two hydroxymethyl groups to the furan rings of **1** induced a 6-fold increase in the taste threshold; for example, a threshold concentration of 4.0×10^{-3} mmol/kg (water) was determined for **6**. Taking all these data into consideration, it might be concluded that the incorporation of polar groups such as, for example, the hydroxymethyl groups led to a pronounced increase in detection threshold. Additional methyl groups at the furan rings of quinizolate (**1**) led to a somewhat lower effect on threshold increase. In contrast, the substitution of the oxygen atoms in the furan rings of quinizolate (**1**) by sulfur atoms induced a significant decrease of the detection threshold of the 1-oxo-2,3-dihydro-1*H*-indolizinium-6-olate **7**. Most likely, the more "voluminous" sulfur atoms in **7** are favored by the corresponding taste receptor in comparison to the oxygen analogon **1** (*1*,*3*). Systematic studies on verifying this hypothesis on a receptor level are ongoing.

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