Characterization of Natural "Cooling" Compounds Formed from Glucose and L-Proline in Dark Malt by Application of Taste Dilution Analysis

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Gel permeation chromatography of the solvent extractables isolated from a thermally treated glucose/L-proline mixture and sensory analysis of the fractions collected led to the discovery of the presence of "cooling" compounds in Maillard reactions. To characterize the key compounds imparting this cooling sensation to the oral cavity, a taste dilution analysis was performed by determining the taste threshold of reaction products in serial dilutions of HPLC fractions to select the most intense "cooling" compounds in the complex GPC fraction of the Maillard reaction mixture. Systematic ^{13}C-labeling experiments and GC-MS, LC-MS, and 1D- and 2D-NMR measurements, followed by synthesis, led to the unequivocal identification of 3-methyl-2-(1-pyrrolidinyl)-2-cyclopenten-1-one (3-MPC), 5-methyl-2-(1-pyrrolidinyl)-2-cyclopenten-1-one (5-MPC), and 2,5-dimethyl-4-(1-pyrrolidinyl)-3(2*H*)-furanone (DMPF) as the key compounds contributing the most to the cooling sensation. Although these structures were described earlier with regard to Maillard reactions, this is the first time that Maillard reaction products are reported to cause intense cooling sensations by degustation. Finally, the detection of 5-MPC (101.3 μg/kg), 3-MPC (9.4 μg/kg), and DMPF (11.5 μg/kg) in dark malt verified their natural occurrence in thermally processed foods.

INTRODUCTION

The Maillard reaction between reducing carbohydrates and amino acids is chiefly responsible for the development of unique aromas and typical tastes during thermal processing of foods, such as roasting of meat or coffee, baking of bread, or kiln-drying of malt. Although the consumer acceptance of a food product is strongly influenced by a balanced interplay between the aroma-active (perceived in the nose) and the taste-active components (detected on the tongue), the information available on taste compounds generated during thermal food processing is as yet very limited.

Although the reaction products formed from Maillard reactions involving the secondary amino acid L-proline, one of the dominant amino acids in cereals, have been extensively investigated in past decades, only a few bitter-tasting compounds have been identified so far, for example, pyrrolidino-hexose-reductones (*1–3*), pyrrolidines (*4*), and cyclopent[*b*]azepin-8-ones (*4–6*). One reason for this lack of information might be the fact that most of these identification experiments focused primarily on the major reaction products formed rather than target compounds selected with regard to taste activity.

To bridge the gap between pure structural chemistry and human taste perception, a more straightforward technique, the so-called taste dilution analysis (TDA), has recently been developed, which is based on the determination of the detection threshold of taste compounds in serial dilutions of HPLC fractions (*7*). This novel bioassay offers the possibility to rank food components according to their relative taste impact and has proved to be a powerful technique for the identification of key taste compounds; for example, the previously unknown 3-(2-furyl)-8-[(2-furyl)methyl]-4-hydroxymethyl-1-oxo-1*H*,4*H*-quinolizinium-7-olate, abbreviated as quinizolate, was successfully identified as the most intense bitter tasting compound formed during thermal treatment of pentoses and primary amino acids (*7*). This novel taste compound, exhibiting an extraordinarily low detection threshold of 0.00025 mmol/kg of water, was found to have a threshold concentration 2000- and 28-fold lower than that of the standard bitter compounds caffeine and quinine hydrochloride, respectively, and TDA successfully identified quinizolate as one of the most intense bitter compounds reported so far.

Until now, all of the taste-active Maillard reaction products reported in the literature showed bitter taste qualities. However, no information is available on the chemical structures of reaction products causing other, probably more desirable, taste sensations, such as sweetness or cooling. The aim of the present investigation was, therefore, to identify taste compounds exhibiting desirable sensory qualities in thermally treated mixtures of hexoses and L-proline and to verify their natural occurrence in processed cereals.

MATERIALS AND METHODS

Chemicals. The following compounds were obtained commercially: glucose, [^{13}C$_6$]glucose, L-proline, pyrrolidine, acetic acid, lactic acid, NaCl, caffeine, quinine hydrochloride, sodium

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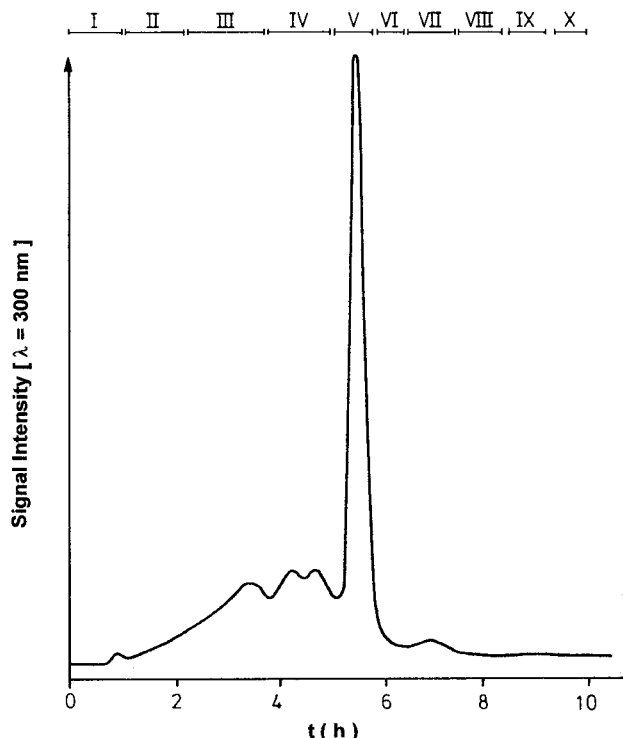
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Figure 1. GPC chromatogram of the solvent-extractable fraction of a dry-heated glucose/L-proline mixture.

Table 1. Taste Qualities and Yields of Fractions Obtained by GPC of the Solvent-Extractable Reaction Products Formed from Glucose and L-Proline

fraction[b]	yield[a]		taste quality[c]
	mg	%	
I	202	6.99	nd
II	428	14.81	bitter
III	720	24.91	bitter
IV	855	29.58	bitter
V	415	14.36	bitter, *cooling* effect
VI	120	4.15	bitter
VII	53	1.83	bitter
VIII	28	0.97	nd
IX	10	0.35	nd
X	<1	<0.01	nd

[a] The yields related to the amount of material (2.89 g) applied onto the column. [b] Number of GPC fraction refers to Figure 1. [c] The taste quality was determined by a trained sensory panel in three sessions. nd, no taste detectable.

Table 2. Taste Dilution Analysis of GPC Fraction V

no.[a]	taste quality[b]	TD factor[b]	no.[a]	taste quality[b]	TD factor[b]
V-1	salty	1	V-16	glutamate-like	16
V-2	salty	4	V-17	bitter	16
V-3	salty	4	V-18	bitter, astringent	256
V-4	soapy	8	V-19	bitter, astringent	64
V-5	bitter	4	V-20	bitter	128
V-6	bitter	8	V-21	*cooling*, bitter	128
V-7	salty	4	V-22	bitter, astringent	128
V-8	soapy	8	V-23	*cooling*, bitter	256
V-9	roasty	4	V-24	bitter	64
V-10	roasty	4	V-25	bitter, astringent	64
V-11	roasty	8	V-26	*cooling*, bitter	64
V-12	soapy	8	V-27	bitter	16
V-13	soapy	4	V-28	bitter	16
V-14	glutamate-like	4	V-29	bitter	4
V-15	bitter	2	V-30	astringent	2

[a] Number of HPLC fraction refers to Figure 2. [b] The taste qualities and TD factors were determined by a triangle test.

glutamate, tannin (gallustannic acid), (−)-menthol, 2-hydroxy-3-methyl-2-cyclopenten-1-one (cyclotene), and 2,5-dimethyl-4-hydroxy-3(2*H*)-furanone (Aldrich, Steinheim, Germany). Solvents were of HPLC grade (Aldrich). CDCl$_3$ was obtained from Isocom (Landshut, Germany).

The following compounds were synthesized following the procedures reported recently: 2,4-dihydroxy-2,5-dimethyl-3(2*H*)-furanone (*8*), 3-deoxy-2-hexosulose (*9*), and 3,5-dihydroxy-2-methyl-5,6-dihydropyran-4-one (*10*).

Thermally Treated Mixture of Glucose/L-Proline. A mixture of glucose (200 mmol) and L-proline (200 mmol) was intimately mixed in a mortar and then dry-heated for 20 min at 190 °C in a cabinet drier. After cooling to room temperature, the reaction mixture was suspended in hot water (1.5 L) and filtered. The aqueous filtrate was extracted with dichloromethane (10 × 500 mL), and the combined organic layers were dried over Na$_2$SO$_4$. After removal of the solvent in vacuo (45 mbar), the residue (2.89 g) was used for gel permeation chromatography (GPC) as well as for sensory analysis.

Gel Permeation Chromatography. The solvent extractables of the glucose/L-proline mixture (2.89 g) were taken up in a mixture (75:25, v/v) of methanol and an aqueous ammonium formate solution (50 mmol/L; pH 3.5) and then applied onto a water-cooled glass column (750 mm × 55 mm, Pharmacia, Uppsala, Sweden), filled with a slurry of Sephadex LH-20 (Pharmacia) in the same solvent mixture. Chromatography was performed with a flow rate of 3 mL/min and monitored by means of a UV−vis detector (UV−vis-151, Gilson) operating at 320 nm. Ten fractions (fractions I−X detailed in Figure 1) were collected by a fraction collector, the solvent was removed at 30 °C in vacuo (45 mbar), and the fractions were then freeze-dried twice. The material of each fraction was used for sensory analysis (Table 1). In addition, taste dilution analysis was applied to fraction V (Table 2; Figure 2).

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 (*11*): saccharose (50 mmol/L) for sweet taste; lactic acid (20 mmol/L) for sour taste; NaCl (12 mmol/L) for salty taste; caffeine (1 mmol/L) for bitter taste; sodium glutamate (8 mmol/

L, pH 5.7) for umami taste; tannin (gallustannic acid; 0.05%) for astringency; L-menthol (0.1%) for cooling sensation. Sensory analyses were performed in a sensory panel room at 22−25 °C in three different sessions.

Determination of Taste Thresholds. The taste thresholds were determined in a triangle test using tap water (pH 6.5) as the solvent. The samples (5 mL) were presented in order of increasing concentrations (serial 1:1 dilutions), and the threshold values evaluated in three different sessions were averaged. The values between 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.

Determination of Time/Intensity Courses. Aqueous solutions containing 3-methyl-2-(1-pyrrolidinyl)-2-cyclopenten-1-one (1800 mg/kg), 5-methyl-2-(1-pyrrolidinyl)-2-cyclopenten-1-one (338 mg/kg), or L-menthol (70 mg/kg), respectively, in concentrations of ∼50-fold above their "cooling" detection threshold were presented to six panelists, who were asked to evaluate the intensity of the cooling effect perceived in the oral cavity on a scale from 0 (absent) to 5 (strong) over a period of 25 min. To achieve this, the aqueous solutions (4 mL) were "chewed" for exactly 60 s, and the "cooling" intensities were assessed after each 10 s. After 1 min of chewing, the material was expectorated and the "cooling" intensities were assessed after an additional 2, 3, 5, 10, 15, 20, and 25 min. The time/intensity courses determined in a sensory panel room at 22−25 °C in three different sessions were averaged. The intensity values between individuals and separate sessions differed by not more than 0.5 unit.

Thermally Treated Mixture of Cyclotene/L-Proline. A mixture of cyclotene (50 mmol), L-proline (50 mmol), and Al$_2$O$_3$
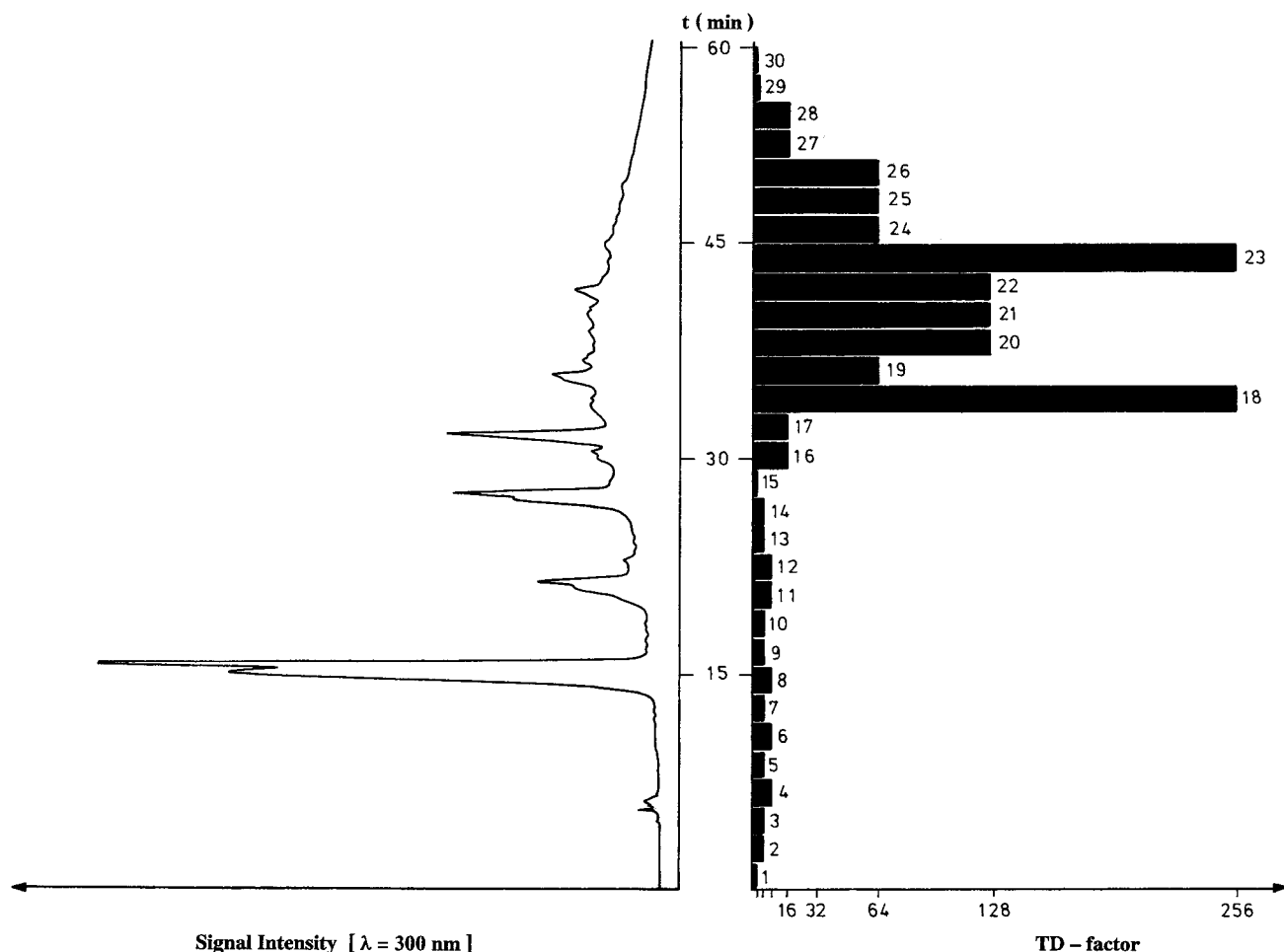
Figure 2. RP-HPLC chromatogram (left side) and TD chromatogram (right side) of fraction V obtained by GPC of the glucose/ L-proline mixture.

Table 3. Taste Dilution Analysis of a Heated Cyclotene/ L-Proline Mixture

fraction[a]	taste quality[b]	TD factor[b]	fraction[a]	taste quality[b]	TD factor[b]
1	bitter	2	9	sour	16
2	nd	<1	10	bitter	128
3	nd	<1	11	soapy	64
4	bitter	8	12	nd	<1
5	*cooling*	32	13	bitter	128
6	bitter	8	14	pungent	16
7	nd	<1	15	sour	4
8	*cooling*	512	16	astringent	4

[a] Number of HPLC fraction refers to Figure 3. [b] The taste quality and TD factor were determined by a triangle test. nd, no taste detectable.

(10 g, neutral), which was used as a "solvent matrix", was intimately mixed in a mortar and then dry-heated for 10 min at 180 °C in a cabinet drier. After cooling to room temperature, the reaction mixture was taken up in hot water (500 mL), filtered, and adjusted to pH 8 by using an aqueous sodium hydroxide solution (1 mol/L). The aqueous solution was extracted with dichloromethane (5 × 200 mL), and the combined organic layers were dried over Na_2SO_4. After removal of the solvent in vacuo (45 mbar), the solvent extractables were used for taste dilution analysis (Table 3) as well as for the identification experiments.

HPLC/Taste Dilution Analysis (HPLC/TDA). GPC fraction V (200 mg) of the glucose/L-proline mixture or the solvent-extractable fraction of the cyclotene/L-proline mixture, respectively, was dissolved in methanol (3 mL) and, after membrane filtration, aliquots (100 μL) were analyzed by RP-HPLC. The effluent was separated into 30 (GPC fraction V; Figure 2) or

16 fractions (cyclotene/L-proline mixture; Figure 4), respectively, which were collected in glass vials by means of a fraction collector. The corresponding fractions obtained from 12 HPLC runs were collected, combined, and freeze-dried twice. The residues obtained from these pooled HPLC fractions were taken up in exactly 1.0 mL of water and, then, diluted stepwise 1+1 with tap water. The serial dilutions of each of these fractions were then presented to the sensory panel in order of increasing concentrations, and each dilution was sensorily assessed 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. The TD factors evaluated by four different assessors in three different sessions were averaged. The TD factors between individuals and separate sessions did not differ by more than one dilution step.

$^{12}C/^{13}C$ Labeling Experiment. Glucose (0.5 mmol), [$^{13}C_6$]-glucose (0.5 mmol), and L-proline (1.0 mmol) were intimately mixed with silica gel (500 mg), which was used as a carrier, and dry-heated for 15 min at 190 °C in a cabinet drier. After cooling to room temperature, the reaction mixture was suspended in hot water (20 mL) and filtered, the filtrate was extracted with dichloromethane (4 × 10 mL), and the combined organic layers were dried over Na_2SO_4. After concentration to ~1 mL, the solvent extract was applied onto a water-cooled glass column (5 × 100 mm), filled with a slurry of Al_2O_3 (basic activity III−IV, Merck, Darmstadt, Germany) in pentane. Chromatography was performed using sequentially pentane (20 mL; fraction A), pentane/diethyl ether (8:2, v/v; 20 mL; fraction B), and pentane/diethyl ether (6:4, v/v; 20 mL; fraction C), pentane/diethyl ether (4:6, v/v; 20 mL; fraction D), followed by pentane/diethyl ether (2:8, v/v; 20 mL, fraction E). HRGC-MS of these fractions revealed the isotopomeric patterns of
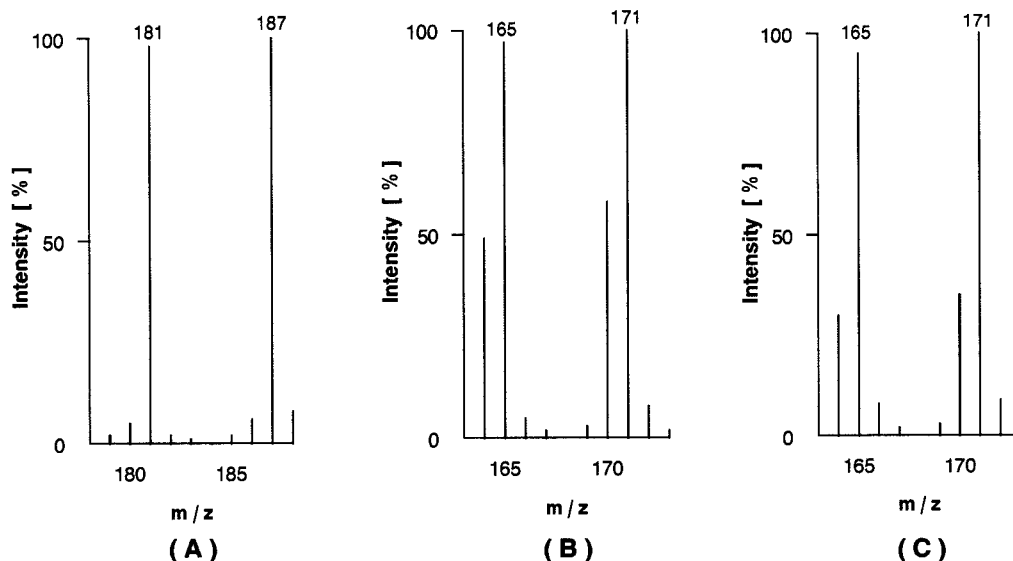
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Figure 3. Isotopomeric pattern of the molecular ions (MS-EI) of "cooling" compounds in HPLC fractions (A) V-21, (B) V-23, and (C) V-26 obtained from a thermally treated mixture (1:1:2) of natural abundant [^{13}C]glucose, [^{13}C$_6$]glucose, and L-proline.
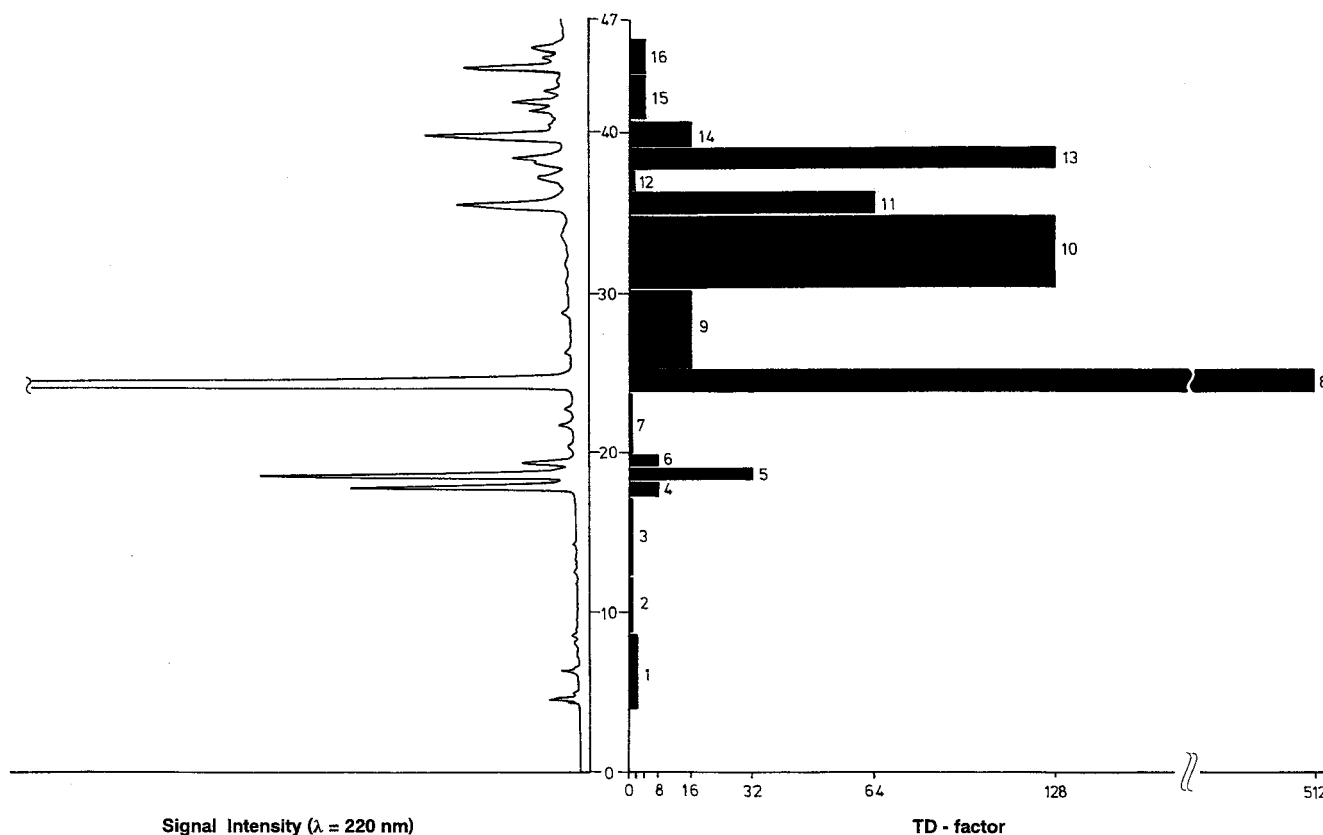

Figure 4. RP-HPLC chromatogram (left side) and TD chromatogram (right side) of the solvent-extractable fraction of the dry-heated cyclotene/L-proline mixture.

the molecular ions of two "cooling" compounds in fraction B and another in fraction E, each of which was identical with the "cooling" compounds detected by the taste dilution analysis of GPC fraction V. Portions of the mass spectra of the isotopomeric mixtures are shown in Figure 3.

Isolation of 3-Methyl-2-(1-pyrrolidinyl)-2-cyclopenten-1-one (3-MPC) and 5-Methyl-2-(1-pyrrolidinyl)-2-cyclo-penten-1-one (5-MPC) from the Cyclotene/L-Proline Mixture. The solvent extractable fraction of the cyclotene/L-proline mixture was dissolved in pentane/ethyl ether (6:4, v/v; 5 mL) and then applied onto a water-cooled glass column (30 × 500 mm) filled with a slurry of Al$_2$O$_3$ (basic activity III−IV, Merck) in pentane. Chromatography was performed using pentane

(300 mL; fraction A), pentane/diethyl ether (9:1, v/v; 150 mL; fraction B), pentane/diethyl ether (8:2, v/v; 300 mL; fraction C), and pentane/diethyl ether (7:3, v/v; 150 mL; fraction D), followed by pentane/diethyl ether (5:5, v/v; 300 mL, fraction E). Fractions B, C, and D, showing intense cooling activity upon degustation, were collected separately and freed from solvent in vacuo to afford 5-methyl-2-(1-pyrrolidinyl)-2-cyclo-penten-1-one (5-MPC; 120 mg) from fraction B and 3-methyl-2-(1-pyrrolidinyl)-2-cyclopenten-1-one (3-MPC; 33 mg) from fractions C and D as pale yellow oils in a purity of >98%. HPLC analysis of an aqueous solution of these substances, followed by tasting of the HPLC fractions collected, confirmed 3-MPC and 5-MPC, respectively, to cause the cooling sensation
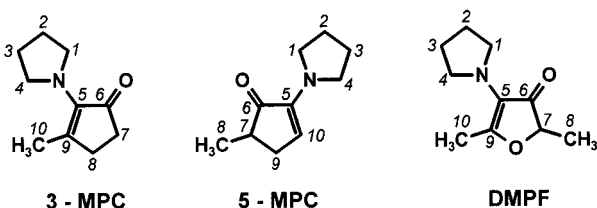
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Figure 5. Structures of the "cooling" compounds, 3-methyl-2-(1-pyrrolidinyl)-2-cyclopenten-1-one (3-MPC), 5-methyl-2-(1-pyrrolidinyl)-2-cyclopenten-1-one (5-MPC), and 2,5-dimethyl-4-(1-pyrrolidinyl)-3(2*H*)-furanone (DMPF).

of the HPLC fractions V-23 and V-26, detected by taste dilution analysis. 3-MPC (Figure 5): LC-MS (ESI) 166 (100, [M + 1]$^+$); HRGC-MS (EI) 165 (100; [M]$^+$), 164 (47), 137 (34), 136 (38), 122 (53), 109 (136), 108 (43), 94 (27), 81 (26), 67 (21), 41 (27); HRGC-MS (CI) 166 (100, [M + 1]$^+$). ^{1}H and ^{13}C NMR data are summarized in Tables 4 and 5. 5-MPC (Figure 5): LC-MS (ESI) 166 (100, [M + 1]$^+$); GC-MS (EI) 165 (100; [M]$^+$), 164 (32), 150 (26), 137 (22), 136 (37), 122 (87), 108 (34), 95 (34), 94 (31), 70 (21), 67 (24), 54 (24), 41 (25); HRGC-MS (CI) 166 (100, [M + 1]$^+$). ^{1}H and ^{13}C NMR data are given in Tables 4 and 5.

Isolation of 2,5-Dimethyl-4-(1-pyrrolidinyl)-3(2*H*)-furanone (DMPF) from a Dry-Heated Mixture of 2,5-Dimethyl-4-hydroxy-3(2*H*)-furanone and L-Proline. 2,5-Dimethyl-4-hydroxy-3(2*H*)-furanone (100 mmol), L-proline (100 mmol), and Al$_2$O$_3$ (20 g, basic, activity III–IV) were intimately mixed in a mortar and dry-heated for 15 min at 180 °C. The mixture was suspended in hot water (100 mL), filtered, and then extracted with diethyl ether (5 × 50 mL); the combined organic layers were dried over Na$_2$SO$_4$ and then freed from solvent in vacuo (45 mbar). The residual oil was dissolved in pentane/diethyl ether (2:8, v/v; 5 mL) and then applied onto a water-cooled glass column (30 × 500 mm) filled with a slurry of Al$_2$O$_3$ (basic activity III–IV, Merck) in pentane. After the column had been flushed with pentane/diethyl ether (8:2, v/v; 300 mL), pentane/diethyl ether (6:4, v/v; 300 mL), pentane/diethyl ether (4:6 v/v; 300 mL), and pentane/diethyl ether (3:7, v/v; 300 mL), a fraction was eluted with pentane/diethyl ether (2:8, v/v; 300 mL), in which a cooling compound was detected by HPLC–degustation. This fraction was freed from solvent under vacuum affording 2,5-dimethyl-4-(1-pyrrolidinyl)-3(2*H*)-furanone (DMPF; 29 mg) as a pure pale yellow oil: LC-MS (ESI) 182 (100, [M + 1]$^+$); HRGC-MS (EI) 181 (100; [M]$^+$), 180 (13), 166 (17), 152 (14), 138 (45), 125 (14), 124 (72), 110 (51), 83 (12), 82 (20), 70 (10), 55 (36), 54 (18); HRGC-MS (CI) 182 (100, [M + 1]$^+$); ^{1}H NMR (360 MHz; CDCl$_3$, DQF-COSY; arbitrary numbering of the carbon atoms refers to DMPF in Figure 5) δ 1.40 [d, 3H, *J* = 6.6 Hz, H–C(8)], 1.83–1.86 [m, 2 × 2H, H–C(2), H–C(3)], 2.23 [s, 3H, H–C(10)], 3.04–3.17 [m, 2 × 2H, H–C(1), H–C(4)], 4.34 [q, 1H, *J* = 7.1 Hz, H–C(7)]; ^{13}C NMR (360 MHz; CDCl$_3$; DEPT-135, HMQC, HMBC; arbitrary numbering of the carbon atoms refers to DMPF in Figure 5) δ 14.8 [CH$_3$, C(8)], 16.4 [CH$_3$, C(10)], 25.1 [CH$_2$, C(2), C(3)], 51.0 [CH$_2$, C(1), C(4)], 80.0 [CH, C(7)], 125.5 [C, C(9)], 182.0 [C, C(5)], 202.4 [C, C(6)].

Syntheses. *5-Methyl-2-(1-pyrrolidinyl)-2-cyclopenten-1-one (5-MPC) and 3-Methyl-2-(1-pyrrolidinyl)-2-cyclopenten-1-one (3-MPC).* A solution of cyclotene (100 mmol), pyrrolidine (400 mmol), and acetic acid (400 mmol) in ethanol (600 mL) was refluxed for 4 h. After cooling to room temperature, the solvent was removed in vacuo (45 mbar), the residue taken up in water (300 mL) and the pH adjusted to 10 with sodium hydroxide solution (30% in water). The solution was then extracted with diethyl ether (5 × 150 mL), and the combined organic layers were washed with an aqueous solution of Na$_2$CO$_3$ (200 mL; 0.5 mol/L) and dried over Na$_2$SO$_4$. After removal of the solvent in vacuo, the residual oil was dissolved in pentane/ethyl ether (6:4, v/v; 10 mL) and applied onto a water-cooled glass column (30 × 500 mm), filled with a slurry of Al$_2$O$_3$ (basic activity III–IV, Merck) in pentane. Chromatography was performed using the same solvents as detailed above under isolation from the cyclotene/L-proline mixture, affording 5-MPC (2.64 g, ~17%

yield) and 3-MPC (1.98 g, ~13% yield) as pale yellow oils with a purity of >99.5%. The spectroscopic data (MS, NMR) and sensory characteristics of synthetic 3-MPC and 5-MPC were identical to those obtained for the "cooling" compounds isolated from the glucose/L-proline and cyclotene/L-proline mixtures.

2,5-Dimethyl-4-(1-pyrrolidinyl)-3(2H)-furanone (DMPF). A solution of 2,5-dimethyl-4-hydroxy-3(2*H*)-furanone (100 mmol), pyrrolidine (500 mmol), and acetic acid (500 mmol) in ethanol (600 mL) was refluxed for 3.5 h. After cooling to room temperature, the solvent was removed in vacuo and the residue was taken up in water (300 mL). The solution was then extracted with diethyl ether (5 × 150 mL), and the combined organic layers were dried over Na$_2$SO$_4$ and then freed from solvent in vacuo. Following the procedure described above for the chromatographic isolation of the cooling compound from 2,5-dimethyl-4-hydroxy-3(2*H*)-furanone/L-proline, the target compound DMPF was isolated as a pale yellow oil (0.8 g, ~4.4% yield) with a purity of >99.5%. The spectroscopic data (MS, NMR) and sensory properties of synthetic DMPF were identical to those obtained for the "cooling" compound isolated from the glucose/L-proline and 2,5-dimethyl-4-hydroxy-3(2*H*)-furanone/L-proline mixture.

Identification of 3-MPC, 5-MPC, and DMPF in Dark Malt. Dark malt (50 g, Caraffa special; Weihenstephan, Germany) was frozen in liquid nitrogen and then ground in a mortar. The powder was stirred overnight with dichloromethane (2 × 400 mL). The combined organic layers were concentrated to ~50 mL in vacuo (45 mbar), and the volatile components were isolated by SAFE distillation (*12*) in high vacuum at 35 °C. The distillate obtained was concentrated to ~1 mL and then fractionated by column chromatography (0.9 × 100 mm) on Al$_2$O$_3$ (basic activity III–IV, Merck), which was conditioned in pentane. Chromatography was performed using sequentially pentane (100 mL; fraction A), pentane/diethyl ether (9:1, v/v; 100 mL; fraction B), pentane/diethyl ether (8:2, v/v; 100 mL; fraction C), pentane/diethyl ether (7:3, v/v; 100 mL; fraction D), pentane/diethyl ether (6:4, v/v; 100 mL; fraction E), pentane/diethyl ether (4:6, v/v; 100 mL; fraction F), pentane/diethyl ether (2:8, v/v; 100 mL; fraction G), and diethyl ether (100 mL, fraction H). Fractions B, D, and G were separately collected and analyzed by GC-MS. By comparison of the retention times as well as mass spectra with those obtained for the synthetic reference compounds, 5-MPC (101.3 μg/kg) could be identified in fraction B, 3-MPC (9.4 μg/kg) in fraction D, and DMPF (11.5 μg/kg) in fraction G.

High-Resolution Gas Chromatography—Mass Spectrometry (HRGC-MS). HRGC was performed with a type 5160 gas chromatograph (Fisons Instruments, Mainz, Germany) using SE-54 (30 m × 0.32 mm fused silica capillary, DB-5, 0.25 μm; J&W Scientific, Fisons, Mainz, Germany) by on-column injection at 40 °C. After 2 min, the temperature of the oven was raised at 40 °C/min to 50 °C and held for 2 min isothermally, then raised at 6 °C/min to 230 °C, and held for 5 min. The flow of the carrier gas, helium, was 2.5 mL/min. MS analysis was performed with an MS 95 S (Finnigan, Bremen, Germany) in tandem with the HRGC. Mass chromatography in the electron-impact mode (MS/EI) was performed at 70 eV and in the chemical ionization mode (MS/CI) at 115 eV with isobutane as the reactant gas.

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 between 220 and 500 nm. Separations were performed on a stainless steel column packed with RP-18 (ODS-Hypersil, 5 μm, 10 nm, Shandon, Frankfurt, Germany) in either an analytical (4.6 × 250 mm, flow rate = 0.8 mL/min) or a semipreparative scale (10 × 250 mm, flow rate = 1.6 mL/min). For HPLC separation of GPC fraction V, after injection of the sample (20–100 μL), analysis was performed using a linear gradient starting with an aqueous solution of ammonium formate (10 mmol/L; pH 8.2), increasing the methanol content to 80% within 50 min, and then to 100% within 5 min. For HPLC separation of the cyclotene/L-proline mixture, after injection of the sample (20–

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Table 4. Assignment of ^{1}H NMR Signals (360 MHz, CDCl$_3$) of 5-Methyl-2-(1-pyrrolidinyl)-2-cyclopenten-1-one (5-MPC) and 3-Methyl-2-(1-pyrrolidinyl)-2-cyclopenten-1-one (3-MPC)

compd	H at relevant C atom[a]	δ[b]	(multiplicity and coupling)[c]	DQF-COSY[d]
5-MPC	H−C(8)	1.17	(3H, d, 7.5 Hz)	H−C(7)
	H−C(2), H−C(3)	1.82−1.88	(2 × 2H, m)	H−C(1), H−C(4)
	H$_a$−C(9)	2.08	(1H, m, 17.7, 3.1, 2.2 Hz)	H$_b$−C(9), H−C(10), H−C(7)
	H−C(7)	2.38	(1H, m, 7.5, 2.2 Hz)	H−C(8), H$_{a/b}$−C(9)
	H$_b$−C(9)	2.75	(1H, m, 17.7, 7.0, 3.1 Hz)	H$_b$−C(9), H−C(7), H−C(10)
	H−C(1), H−C(4)	3.26	(2 × 2H, m)	H−C(2), H−C(3)
	H−C(10)	5.82	(1H, t, 3.1 Hz)	H$_{a/b}$−C(9), H−C(15)
3-MPC	H−C(2), H−C(3)	1.77−1.81	(2 × 2H, m)	H−C(1), H−C(4)
	H−C(10)	2.13	(3H, s)	
	H−C(8)	2.34	(2H, m)	H−C(7)
	H−C(7)	2.40	(2H, m)	H−C(8)
	H−C(1), H−C(4)	3.40−3.44	(2 × 2H, m)	H−C(2), H−C(3)

[a] Arbitrary numbering of carbon atoms refers to structures given in Figure 5. [b] The ^{1}H chemical shifts are given in relation to CDCl$_3$. [c] Determined from 1D spectrum. [d] Homonuclear ^{1}H,^{1}H connectivities observed by DQF-COSY.

Table 5. Assignment of ^{13}C NMR Signals (360 MHz, CDCl$_3$) of 5-Methyl-2-(1-pyrrolidinyl)-2-cyclopenten-1-one (5-MPC) and 3-Methyl-2-(1-pyrrolidinyl)-2-cyclopenten-1-one (3-MPC)

compd	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)
5-MPC	C(8)	16.5	CH$_3$	H−C(8)	H−C(7), H−C(9)
	C(2), C(3)	24.8	CH$_2$	H−C(2/3)	H−C(1), H−C(4)
	C(9)	32.6	CH$_2$	H−C(9)	H−C(7), H−C(8), H−C(10)
	C(7)	40.2	CH	H−C(7)	H−C(8), H−C(9)
	C(1), C(4)	48.1	CH$_2$	H−C(1/4)	H−C(2), H−C(3)
	C(10)	123.6	CH	H−C(10)	H−C(7), H−C(9)
	C(5)	146.7	C		H−C(10)
	C(6)	207.4	C		H−C(7), H−C(8), H−C(10)
3-MPC	C(10)	17.8	CH$_3$	H−C(10)	H−C(8)
	C(2), C(3)	24.9	CH$_2$	H−C(2/3)	H−C(1), H−C(4)
	C(8)	30.0	CH$_2$	H−C(8)	H−C(7), H−C(10)
	C(7)	34.1	CH$_2$	H−C(7)	H−C(8)
	C(1), C(4)	49.5	CH$_2$	H−C(1/4)	H−C(2), H−C(3)
	C(9)	143.7	C		H−C(8), H−C(10)
	C(5)	145.9	C		H−C(10)
	C(6)	205.9	C		H−C(7), H−C(8)

[a] Arbitrary numbering of carbon atoms refers to structures given in Figure 5. [b] The ^{13}C chemical shifts are given in relation to CDCl$_3$. [c] DEPT-135 spectroscopy. [d] Assignments based on HMQC (^1J) and HMBC ($^{2,3}J$) experiments.

100 μL), analysis was performed using a linear gradient starting with a mixture (1:1, v/v) of aqueous ammonium formate (10 mmol/L; pH 8.2) and methanol, increasing the methanol content to 90% 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 solution of aqueous ammonium formate (10 mmol/L; pH 8.2) and increasing the methanol content to 80% within 50 min and then to 100% within 5 min.

Nuclear Magnetic Resonance Spectroscopy (NMR). ^{1}H, ^{13}C, DEPT-135, DQF-COSY, HMQC, and HMBC spectroscopies were performed on Bruker-AC-200 and Bruker-AM-360 spectrometers (Bruker, Rheinstetten, Germany) using the acquisition parameters described recently (*13*).

RESULTS AND DISCUSSION

HPLC analysis of the solvent-extractable fraction isolated from a thermally treated glucose/L-proline mixture demonstrated that a tremendous variety of different reaction products were formed. Because only a limited number of compounds were expected to contribute significantly to the taste of this mixture, it was necessary, first, to sort out the strongly taste-active compounds from the bulk of less taste-active or tasteless substances. To achieve this, the reaction products were

separated from the high molecular weight, melanoidin-type material by means of GPC, using Sephadex LH-20 as the stationary phase and a mixture of methanol and aqueous ammonium formate as the mobile phase. The GPC chromatogram displayed in Figure 1 was recorded with the effluent monitored at 300 nm, and 10 fractions (fractions I−X) were collected separately. To evaluate their taste activity, these fractions were freeze-dried, and the odorless residues were taken up in tap water and then presented to the sensory panel to judge the taste qualities of these fractions by gustation in a triangle test. Whereas fraction I and fractions VII−X did not show any taste impact, fractions II−VI tasted bitter (Table 1). It was, however, interesting to notice that fraction V, showing the most intense absorption at 300 nm (Figure 1), in addition to the bitter taste, imparted a significant cooling effect to the tongue of the panelists. Because the presence of "cooling" compounds in Maillard reactions had not been previously reported, the following identification experiments focused on fraction V.

Taste Dilution Analysis of GPC Fraction V. To locate the taste-active compounds and to rank them in their relative taste impacts, we applied the GPC fraction V. To achieve this, an aliquot of this fraction was chromatographed by RP-HPLC (Figure 2, left side), and the effluent was separated into 30 fractions, which were freeze-dried and then made up with water to a

volume of 1 mL. Each fraction was stepwise (1 + 1) diluted with water, and the dilutions were then presented in order of increasing concentration to trained sensory panelists, who were asked to evaluate the taste quality and to determine the dilution at which a taste difference between the diluted fraction and two blanks (tap water) could just be detected. This so-called taste dilution (TD) factor, obtained for each fraction, is related to its taste activity in water, and so the 30 HPLC fractions (fractions V-1 to V-30) were ranked in their relative taste intensity as given in Figure 2 (right side).

As fractions V-18 and V-23 had TD factors of 256, they had by far the highest taste impact. The taste qualities of the fractions are listed in Table 2. Both fractions V-18 and V-23 exhibited a bitter taste, but fraction V-23, in addition, imparted an intense cooling sensation to the oral cavity. Cooling activity was also detectable in fractions V-21 and V-26, whereas the other fractions evaluated with higher TD factors, that is, V-19, V-20, V-24, or V-25, were judged only as bitter tasting with or without astringency (Table 2). In addition, Maillard products with glutamate-like (fractions V-14 and V-16) or roasty taste (fractions V-9 to V-11) were detected, but, according to their low TD factors, these compounds can be expected to show only a weak taste impact.

Structure Determination of "Cooling" Compounds. To characterize the Maillard reaction products causing the cooling sensation, fractions V-21, V-23, and V-26 were separately collected and analyzed by LC-MS and HRGC-MS. In fraction V-21, a reaction product was detected exhibiting cooling activity upon degustation and showing a molecular mass of 181 Da. Analysis of fraction V-23 revealed two major reaction products, one of which had a molecular mass of 165 Da and showed cooling activity and the other tasting bitter. In fraction V-26, a compound with a molecular mass of 165 Da was also found to cause a cooling sensation in the oral cavity. The amounts of these "cooling" compounds were, however, too low for an unequivocal structure determination by ^{1}H and ^{13}C NMR analysis. Therefore, the following experiments aimed to generate these "cooling" compounds in higher amounts by reacting L-proline with certain carbohydrate-derived intermediates, which might be involved in their formation. To characterize such potential intermediates, a labeling experiment was performed with a mixture (1:1) of glucose with natural abundance of [^{13}C]- and [$^{13}C_6$]glucose. After chromatographic preseparation, HRGC-MS analysis of the "cooling" compounds in fractions V-21, V-23, and V-26 revealed a 1 + 1 mixture of the nonlabeled and 6-fold-labeled isotopomers; that is, molecular ions with m/z 181 and with a shift of six and a molecular ion with m/z 187 were found for the "cooling" compound in fraction V-21 (A in Figure 3). The isotopomeric mixture of the "cooling" compounds in fractions V-23 (B in Figure 3) and V-26 (C in Figure 3), respectively, showed an [M]$^+$ ion with m/z 165, together with an [M − 1]$^+$ ion of somewhat lower intensity with m/z 164, and the [M]$^+$ and [M − 1]$^+$ ions of the corresponding 6-fold ^{13}C-enriched isotopomers. These data clearly demonstrate that six carbohydrate-derived carbon atoms are involved in the formation of the target compounds. In addition, the lack of isotopomeric mixing, which is expected when the C$_6$ skeleton of the "cooling" compounds is generated from recombination of short-chain C$_2$ or C$_3$ carbohydrate cleavage products, indicated that the target compounds are formed via precursors possessing the original C$_6$

carbon chain of the hexose. To identify these precursors and to generate the "cooling" compounds in higher yields, L-proline was dry-heated in the presence of various well-known carbohydrate intermediates with a C$_6$ backbone, namely, 3-deoxy-2-hexosulose, 2,4-dihydroxy-2,5-dimethyl-3(2H)-furanone (acetylformoin), 3,5-dihydroxy-2-methyl-5,6-dihydropyran-4-one, 2,5-dimethyl-4-hydroxy-3(2H)-furanone, and 2-hydroxy-3-methyl-2-cyclopenten-1-one (cyclotene), respectively. HPLC analysis and degustation of the fractions collected revealed that the "cooling" compounds detected in fractions V-23 and V-26 were exclusively formed in the reaction mixture containing the C$_6$ intermediate cyclotene, whereas the "cooling" compound detected in fraction V-21 was formed in the presence of 2,5-dimethyl-4-hydroxy-3(2H)-furanone.

Because cyclotene was found as the precursor for the two "cooling" compounds in fractions V-23 and V-26, the following identification experiments focused on the cyclotene/L-proline mixture. Application of the TDA revealed 16 HPLC fractions, among which 12 fractions were judged with TD factors >1 (Figure 4). The high TD factor of 512 shows fraction 8 to have by far the highest cooling activity (Table 3). Whereas fractions 10 and 13 were judged with high TD factors as bitter tasting (Table 3), fraction 5 caused also a cooling sensation, even when the original HPLC fraction was diluted 1:32. From a comparison of the retention times, the sensory characteristics, and the mass spectra, the "cooling" compounds in fractions 5 and 8 of the cyclotene/L-proline mixture were found to be identical to those detected in fractions V-23 and V-26 of the glucose/L-proline mixture.

For isolation of these compounds from fractions 5 and 8, the cyclotene/L-proline reaction mixture was separated chromatographically on aluminum oxide, and the fractions obtained were evaluated by HPLC−degustation. The "cooling" compounds were obtained as pale yellow oils in a purity of >99.5%. The spectroscopic data obtained by 1D- and 2D-NMR techniques, LC-MS, and HRGC-MS were consistent with the structures displayed in Figure 5. The NMR data of the major isomer present in fraction 8 are reported in detail as follows. The ^{1}H NMR spectrum showed seven resonance signals, among which one integrated for the three protons of a methyl group and two signals integrated for four protons each (Table 4). A pyrrolidine ring was deduced from the characteristic coupling pattern of the hydrogens H−C(1)/H−C(2)/H−C(3)/H−C(4) and was further confirmed by a double-quantum filtered homonuclear δ,δ correlation (DQF-COSY) experiment (Table 4). In addition, the DQF-COSY experiment revealed a coupling of 17.7 Hz between the geminal hydrogen atoms H$_a$−C(9) and H$_b$−C(9), resonating at 2.08 and 2.75 ppm, and, in addition, a coupling between these protons and the triplet observed at 5.82 ppm and the multiplet detected at 2.38 ppm, respectively. These signals were assigned as the olefinic proton H−C(10) and the proton H−C(7). A comparison of the ^{13}C NMR spectrum, in which eight signals appeared, with the results of the DEPT-135 experiment showing six signals revealed two signals corresponding to quaternary carbon atoms (Table 5). Unequivocal assignment of these quaternary carbon atoms as C(5) and C(6) 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 $^3J_{C,H}$

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coupling constants and heteronuclear multiquantum correlation spectroscopy (HMQC) optimized for $^1J_{C,H}$ coupling constants (Table 5). The HMBC experiment revealed a correlation between the methyl protons H−C(8) and neighboring carbon atoms, for example, with the carbons C(7) and C(9), as well as the carbonyl group C(6), or between the olefinic proton H−C(10) and the quaternary carbons C(5) and C(6), thus confirming the cyclopentene ring in the 5-methyl-2-(1-pyrrolidinyl)-2-cyclopenten-1-one as proposed in Figure 5. 1D- and 2D-NMR studies (Tables 4 and 5) on the "cooling" compound present in fraction 5 led to its identification as the isomeric 3-methyl-2-(1-pyrrolidinyl)-2-cyclopenten-1-one. For a final structure confirmation, both compounds were synthesized by a hydroxy/amine exchange from 2-hydroxy-3-methyl-2-cyclopenten-1-one and pyrrolidinium acetate. Two reaction products were formed in high yields and were chromatographically purified and analyzed by ^1H and ^{13}C NMR as well as mass spectrometry. These synthetic products gave identical spectroscopic data as well as "cooling" characteristics, as did 3-methyl- and 5-methyl-2-(1-pyrrolidinyl)-2-cyclopenten-1-one isolated from glucose/L-proline or cyclotene/L-proline, respectively.

In summary, TDA, ^{13}C labeling experiments, 1D- and 2D-NMR measurements, mass spectrometry, and synthesis led to the unequivocal identification of 3-methyl-2-(1-pyrrolidinyl)-2-cyclopenten-1-one (3-MPC in Figure 5) and 5-methyl-2-(1-pyrrolidinyl)-2-cyclopenten-1-one (5-MPC in Figure 5) as the first Maillard reaction products causing an intense cooling sensation upon degustation. Although these structures were described earlier to be formed from glucose and L-proline (*14*) as well as 1-[(2′-carboxy)pyrrolidinyl]-1-deoxy-D-fructose (*15*), neither their strong cooling activity nor the ^{13}C NMR spectroscopic data have been reported earlier.

Application of TDA on the dry-heated mixture of 2,5-dimethyl-4-hydroxy-3(2*H*)-furanone and L-proline (data not shown) generated a reaction product causing the cooling sensation of the GPC fraction V-21. LC-MS, HRGC-MS, and 1D- and 2D-NMR experiments led to the identification of that "cooling" compound as 2,5-dimethyl-4-(1-pyrrolidinyl)-3(2*H*)-furanone (DMPF in Figure 5), the structure of which was further confirmed by synthesis. Although this structure was described earlier as a proline-specific Maillard reaction product (*14*, *15*), neither its cooling properties nor the ^{13}C NMR spectroscopic data were as yet reported in the literature.

To prove the "naturalness" of these "cooling" Maillard compounds, the following investigations were aimed at identifying 3-MPC, 5-MPC, and DMPF in dark malt. A solvent extract was prepared from dark malt, and the volatile fraction of the malt components was then isolated by SAFE distillation in high vacuum. The distillate obtained was fractionated by column chromatography, and the fractions containing the target compounds were collected and analyzed by HRGC-MS. By comparison of the retention times as well as mass spectra in the electron impact as well as the chemical ionization mode with those obtained from the synthetic reference compounds, 5-MPC (101.3 μg/kg), 3-MPC (9.4 μg/kg), and DMPF (11.5 μg/kg) were identified in dark malt, thus verifying their natural occurrence in thermally processed foods.

Sensory Properties of "Cooling" Compounds. The effect that DMPF, 3-MPC, and 5-MPC impart to the oral cavity was described by the sensory panel as

Table 6. Comparison of "Cooling" and Odor Thresholds of Selected Compounds

| compd | threshold concn (mg/kg)[a] of | | odor quality | ratio (cooling/ odor) |
	cooling activity	odor activity		
DMPF	100.0−140.0	30.0−60.0	nutty, roasty	2.7
3-MPC	29.0−43.5	43.5−72.5	faintly amine-like	0.8
5-MPC	4.5−9.0	2.6−5.2	faintly mint-like	1.7
(−)-menthol	0.95−1.85	0.10−0.20	strongly mint-like	9.5

[a] Threshold concentrations were determined in tap water by using a triangle test following the procedure reported earlier (*11*).
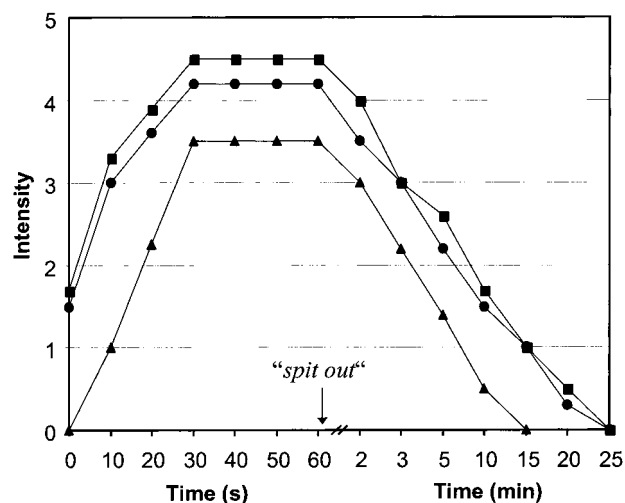

Figure 6. Time/intensity course of "cooling" perception measured for (■) 3-methyl-2-(1-pyrrolidinyl)-2-cyclopenten-1-one (3-MPC), (●) 5-methyl-2-(1-pyrrolidinyl)-2-cyclopenten-1-one (5-MPC), and (▲) (−)-menthol.

"cooling" or "fresh". To compare the sensory characteristics of these Maillard reaction products with that of (−)-menthol, the most common natural "cooling" agent, the "cooling" thresholds of 3-MPC, 5-MPC, and DMPF were determined in water by using a triangle test (Table 6). DMPF was evaluated with the highest detection theshold, causing a cooling sensation at a level of >100−140 mg/kg. Also, the cyclotene-derived compounds 3-MPC and 5-MPC were virtually tasteless but exhibited a pleasant, long-lasting cooling effect. In comparison to DMPF, 3-MPC and 5-MPC were found to have 3-fold and 17-fold lower "cooling" thresholds, respectively, but these were still higher by factors of 5 and 25, respectively, than the threshold concentration determined for (−)-menthol. Contrary to the literature (*14*), a bitter taste could not be detected of 3-MPC by the sensory panel.

To gain insights into the dynamics of the "cooling" perception, time/intensity courses were determined for the Maillard compounds, 3-MPC and 5-MPC, and for (−)-menthol. Aqueous solutions containing these compounds in concentrations of 50-fold above the corresponding "cooling" threshold (Table 6) were presented to a sensory panel, which was asked to evaluate the intensity of the cooling effect perceived in the oral cavity on a scale from 0 (absent) to 5 (strong) over a period of 25 min. These solutions were chewed for exactly 60 s, and the intensity of the cooling sensation was determined each 10 s (Figure 6). After 1 min of chewing, the material was spat out, and the "cooling" intensity in the aftertaste was evaluated after an additional 2, 3, 5, 10, 15, 20, and 25 min. For both the Maillard compounds,

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3-MPC (■ in Figure 6) and 5-MPC (● in Figure 6), an intense cooling effect was perceived instantaneously after the solutions had been taken into the oral cavity. Chewing the material led to an increase of the intensity within 30 s from about 1.5 to 4.5, after which the cooling sensation remained constant in intensity (Figure 6). After the material had been spat out, the intensity diminished by 50% after 5 min. After 20 min, a significant cooling effect was still perceivable, which, however, could not be detected after an additional 5 min. In comparison, sensory evaluation of the aqueous solution of (−)-menthol (▲ in Figure 6) revealed a significant cooling sensation at first after 10 s. After 30 s, the (−)-menthol also caused the maximum cooling effect, but with somewhat lower intensity compared to 3-MPC and 5-MPC. After the solution had been spat out, the intensity of the cooling effect diminished again and was still perceivable after 10 min. However, after 15 min, a significant "cooling" effect could no longer be detected. These data indicate that the "cooling" effect provided by the Maillard compounds starts more rapidly and is longer lasting than that of (−)-menthol.

Because all of the compounds investigated are volatile, we also were interested in their odor thresholds. The lowest odor threshold concentration of 0.1−0.2 mg/kg was found for menthol, eliciting a strong mint-like odor. In contrast, the "cooling" Maillard compounds 5-MPC, 3-MPC, and DMPF were evaluated with significantly higher odor thresholds (Table 6). Calculating the ratio of cooling threshold to odor threshold clearly demonstrated a value of <1 for 3-MPC, exhibiting only a very faint amine-like odor at a threshold concentration of 43.5−72.5 mg/kg. This indicates that 3-MPC might be used as a "cooling" compound without having a strong odor. In comparison, the ratio for (−)-menthol is 9.5; that is, the odor threshold is well below the cooling threshold, implying that menthol cannot be used to provide cooling effects to food applications without imparting a predominant mint-like odor, which would be difficult to mask. In contrast, natural "cooling" Maillard compounds, in particular 3-MPC, offer new possibilities of imparting a pleasant "freshness" and "cooling" effect to the oral cavity during consumption of non-mint food compositions, such as confectionery products, malted beverages, or fruity flavorings.

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