Acetylformoin—A Chemical Switch in the Formation of Colored Maillard Reaction Products from Hexoses and Primary and Secondary Amino Acids

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Thermal treatment of a methanolic solution of *N*-(1-deoxy-D-fructos-1-yl)-L-proline and furan-2-carboxaldehyde led to the rapid formation of colored compounds, among which 4-hydroxy-2-methoxy-2-methyl-5-[(*E*)-(2-furyl)methylidene]methyl-2*H*-furan-3-one (**1**) could be identified as one of the main colored compounds by NMR, LC/MS, and UV−vis spectroscopy. Studies on its formation revealed that condensation of the hexose dehydration product acetylformoin (**2a**/**2b**) with furan-2-carboxaldehyde formed the labile 2,4-hydroxy-2-methyl-5-[(*E*)-(2-furyl)methylidene]methyl-2*H*-furan-3-one (**3a**/**3b**), which then reacts with methanol to the more stable colorant **1**. Reacting furan-2-carboxaldehyde with *N*-(1-deoxy-D-fructos-1-yl)-L-alanine, however, completely stalled the formation of acetylformoin and, consequently, also of colorant **1**. Further model studies revealed acetylformoin as a chemical switch in the Maillard reaction determining different reaction pathways in the presence of primary and secondary amino acids; for example, the reaction with glycine methyl ester and glycine revealed 2,4-dihydroxy-2,5-dimethyl-1-methoxycarbonylmethyl- (**4**) and 2,4-dihydroxy-2,5-dimethyl-1-carboxymethyl -3-oxo-2*H*-pyrrole (**5**), respectively, whereas the reaction with L-proline resulted in the formation of pyrrolidino- (**6**) and bispyrrolidino-hexose-reductone (**7**). Dry heating of these amino reductones in the presence of furan-2-carboxaldehyde produced the colorants 2,4-dihydroxy-2-methyl-5-[(*E*)-(2-furyl)methylidene]methyl-1-methoxycarbonylmethyl-3-oxo-2*H*-pyrrole (**8**) from **4** and 3-hydroxy-4-[(*E*)-(2-furyl)methylidene] methyl-3-cyclopentene-1,2-dione (**9**) from **6** and **7**. Quantitative studies revealed 3-hydroxy-4-methyl-3-cyclopentene-1,2-dione (**10**) and methylene-reductinic acid (**11**) as the key intermediates in the formation of colorant **9**.

Keywords: *Acetylformoin; amino-hexose-reductones; pyrrolinone-reductone; colored compounds; Maillard reaction; nonenzymatic browning*

INTRODUCTION

The Maillard reaction between reducing carbohydrates and compounds bearing an amino group is chiefly responsible for the development of desirable colors and flavors occurring during the thermal processing of foods such as roasting of meat, baking of bread, kiln drying of malt, or roasting of coffee.

Despite extensive studies, due to the complexity and multiplicity of the nonvolatile Maillard reaction products formed, surprisingly little is known about the structures of the compounds responsible for the typical brown color. It is well-known from the literature that the amino acid-catalyzed conversion of reducing carbohydrates results in a tremendous variety of reactive aldehydes as well as methylene-active intermediates (Ledl and Schleicher, 1992). Results of model studies (Severin and Krönig, 1972; Hofmann, 1998a−c; Hofmann and Heuberger, 1998) revealed that indiscriminate condensation reactions between these reactive intermediates lead to the multiplicity and the low yields of colored reaction products.

To obtain more detailed information on the chemical species of the chromophoric compounds involved in color formation of pentoses, we, in recent investigations, reacted solutions of xylose and amino acids in the presence of a surplus amount of one carbohydrate-derived carbonyl compound, for example, furan-2-carboxaldehyde, forcing all methylene-active compounds to react with the same aldehyde and reducing the product multiplicity to the key chromophores (Hofmann, 1998a,b).

In comparison to pentoses, less information is as yet available regarding the color formation from hexoses. On the basis of the finding that aqueous conditions destabilize certain colorants in Maillard reactions, Ledl and Severin (1982) used alcohol instead of water to gain insights into the structures of possible labile colorants. The authors identified colored compounds in a heated ethanolic solution of *N*-(1-deoxy-D-fructos-1-yl)piperidine and furan-2-carboxaldehyde and proposed acetylformoin as a key intermediate in colorant formation. Because this model reaction has been carried out with the synthetically related piperidine instead of more food-related amino acids, it is not clear whether the results obtained can be transferred to real food systems.

Recent studies (Hofmann, 1998c), therefore, aimed at the characterization of colored compounds formed from hexoses and primary amino acids, among which 2*H*,7*H*,-8a*H*-pyrano[2,3-*b*]pyran-3-ones and 2*H*-pyran-3-ones were identified as the main colored compounds. Studies on their formation revealed the 3-deoxy-2-hexosulose as the key intermediate of these colorants (Hofmann, 1998d; Hofmann and Heuberger, 1998). However, colored compounds formed via the 1-deoxyosone path-

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way have as yet not been identified in model systems of glucose and food-related amino acids.

The objectives of the present investigation were, therefore, to characterize colored Maillard reaction products formed from methanolic solutions of *N*-(1-deoxy-D-fructos-1-yl)-L-proline and *N*-(1-deoxy-D-fructos-1-yl)-L-alanine, respectively, heated in the presence of an additional Maillard-derived carbonyl compound and to study certain acetylformoin-derived reaction intermediates as precursors in colorant formation. Because furan-2-carboxaldehyde was shown to be formed from hexoses (Hofmann and Schieberle, 1998) and also during thermal processing of foods such as wheat and rye bread crusts (Schieberle and Grosch, 1987), as well as roasted coffee, barley, and chicory (Kanjahn et al., 1996), it was chosen as a suitable carbonyl compound for these experiments.

EXPERIMENTAL PROCEDURES

Chemicals. The following compounds were obtained commercially: D-glucose, L-proline, L-alanine, glycine, furan-2-carboxaldehyde, piperidine, pyrrolidine, 2-oxopropanal (40% aqueous solution), potassium cyanide, glycine methyl ester hydrochloride (Aldrich, Steinheim, Germany). Furan-2-carboxaldehyde was freshly distilled at 30 °C in high vacuum prior to use. Solvents were of HPLC grade (Aldrich). Deuterated solvents were obtained from Isocom (Landshut, Germany).

The following Maillard reaction products were prepared as described in the literature given in parentheses: *N*-(1-deoxy-D-fructos-1-yl)-piperidine (Hofmann and Heuberger, 1998); *N*-(1-deoxy-D-fructos-1-yl)-L-alanine (Hofmann et al., unpublished results); *N*-(1-deoxy-D-fructos-1-yl)-proline (Hofmann and Schieberle, unpublished results).

Syntheses. *Acetylformoin [3,4-Dihydroxy-3-hexene-2,5-dione (**2a**) and 2,4-Dihydroxy-2,5-dimethyl-3(2H)-furanone (**2b**)].* Following a procedure of Goto et al. (1963) with major modifications, freshly distilled 2-oxopropanal (60 g) was diluted with water (180 mL) and cooled to 0 °C in an ice bath upon bubbling of argon. After dropwise addition of an ice-cooled solution of potassium cyanide (1.74 g) in water (60 mL), the pH was then adjusted to pH 7.0 with a satured aqueous $NaHCO_3$ solution. The mixture was then stirred at 2 °C while the pH was held between 7.1 and 7.3 by adding aqueous phosphoric acid (5 mol/L). After 60 min, the reaction was quenched by adjusting the pH to 4.5 with concentrated phosphoric acid. The water was evaporated in vacuo at 40 °C, the syrupy residue was mixed with ethanol (300 mL), and, after filtration, the solvent was removed in vacuo. The residue was taken up in diethyl ether (300 mL) and, after filtration, the solvent was again evaporated in vacuo. The yellowish syrup was then fractionated by high-vacuum sublimation (0.1 mbar; 90−100 °C) affording the target compound as intense yellow crystalls (3.9 mg; 16% in yield): MS/CI 145 (59), 131 (11), 127 (100), 103 (14); MS/EI 144 (33), 101 (72), 73 (28), 55 (100). NMR data of 3,4-dihydroxy-3-hexene-2,5-dione (**2a**):^{1}H NMR (360 MHz, $CDCl_3$; arbitrary numbering of the carbon atoms refers to structure **2a** in Figure 3) δ 2.45 [s, 2 × 3H, H−C(1) and H−C(6)], 10.83 [bs, 2 × 1H, HO−C(3) and HO−C(4)];^{13}C NMR (360 MHz, $CDCl_3$, HMQC, HMBC, DEPT; arbitrary numbering of the carbon atoms refers to structure **2a** in Figure 3) δ 25.7 [CH_3, C(1) and C(6)], 139.4 [C, C(3) and C(4)], 205.3 [C, C(2) and C(5)]. NMR data of 2,4-dihydroxy-2,5-dimethyl-3(2*H*)-furanone (**2b**): ^{1}H NMR (360 MHz, MeOD-d_3; arbitrary numbering of the carbon atoms refers to structure **2b** in Figure 3) δ 1.42 [s, 3H, H−C(1)], 2.18 [s, 3H, H−C(6)]; ^{13}C NMR (360 MHz, MeOD-d_3, HMQC, HMBC, DEPT; arbitrary numbering of the carbon atoms refers to structure **3b** in Figure 3) δ 13.4 [CH_3, C(1)], 22.5 [CH_3, C(6)], 102.6 [C, C(2)], 132.5 [C, C(5)], 174.8 [C, C(4)], 197.4 [C, C(3)].

*2,4-Dihydroxy-2-methyl-5-[(E)-(2-furyl)methylidene]methyl-2H-furan-3-one (**3a**/**3b**).* Following a method of Ledl and Severin (1982) with some modifications, a mixture of acetylformoin (5 mmol), furan-2-carboxaldehyde (15 mmol), piperidine (100 μL), and acetic acid (100 μL) in chloroform (20 mL) was heated for 90 min at 80 °C in a closed vial under an atmosphere of argon. After cooling, the solvent was removed in vacuo and the target compound was isolated by preparative thin-layer chromatography on silica gel (20 × 20 cm; 0.5 mm; Merck, Darmstadt, Germany) using toluene/ethyl acetate (80:20, v/v) as the mobile phase. An intense colored band at R_f = 0.25−0.32 was scraped off, suspended in ethyl acetate (50 mL), and filtered, affording a deep yellow residue of pure 2,4-dihydroxy-2-methyl-5-[(*E*)-(2-furyl)methylidene]methyl-2*H*-furan-3-one after evaporation of the solvent (3.6 mmol; ~72% in yield): LC/MS(APCI$^-$) 221 (100, [M + 1 − H_2]$^-$); UV−vis λ_{max} = 390 nm (methanol); ^{1}H and ^{13}C NMR data as well as signal assignments are given in Tables 1 and 2.

*4-Hydroxy-2-methoxy-2-methyl-5-[(E)-(2-furyl)methylidene]-methyl-2H-furan-3-one (**1**).* A solution of 2,4-dihydroxy-2-methyl-5-[(*E*)-(2-furyl)methylidene]methyl-2*H*-furan-3-one (2 mmol) in methanol (10 mL) was heated in the presence of hydrochloric acid (20 μL) for 30 min at 60 °C. The mixture was then diluted with water (150 mL) and extracted with diethyl ether (3 × 50 mL). After drying over Na_2SO_4, the organic layer was concentrated to ~2 mL, and the target compound was isolated by thin-layer chromatography on silica gel (20 × 20 cm; 0.5 mm; Merck) using pentane/toluene/ethyl acetate (50:40:10, v/v/v) as the mobile phase. A yellow band at R_f = 0.15 was scraped off and suspended in ethyl acetate (20 mL). After filtration, the solvent was removed, affording an intense orange residue consisting of pure colorant **1** (1.8 mmol, ~90% in yield): ^{1}H NMR (360 MHz, DMSO-d_6; arbitrary

Table 1. Assignment of ^{1}H-NMR Signals (360 MHz) of Cyclic (3a; in MeOD-d_3) and Open-Chain Forms (3b; in $CDCl_3$) of 2,4-Dihydroxy-2-methyl-5-[(*E*)-(2-furyl)methylidene]methyl-2*H*-furan-3-one

H at relevant C atom[a]	δ[b] 3a	δ[b] 3b	I[c]	M[c]	J[c] (Hz)	COSY[d]
H−C (1)	1.49	2.47	3	s		
H−C(10)	6.54	6.55	1	dd	3.5, 1.4	H−C(9), H−C(11)
H−C(9)	6.71	6.84	1	d	3.5	H−C(10), H−C(11)
H−C(7)	6.94	7.64	1	d	15.92	H−C(6)
H−C(6)	7.19	7.45	1	d	15.92	H−C(7)
H−C(11)	7.62	7.58	1	d	1.4	H−C(9), H−C(10)
HO−C(3,4)		11.05/11.63	1	bs		

[a] Numbering of carbon atoms refers to structures **3a** and **3b** in Figure 3. [b] The ^{1}H chemical shifts are given in relation to the solvent signal. [c] Determined from 1D spectrum. [d] Observed homonuclear ^{1}H,^{1}H connectivities by TOCSY and DQF−COSY.

Table 2. Assignment of ^{13}C-NMR Signals (360 MHz) of Cyclic (3a; in MeOD-d_3) and Open-Chain Forms (3b; in $CDCl_3$) of 2,4-Dihydroxy-2-methyl-5-[(*E*)-(2-furyl)methylidene]methyl-2*H*-furan-3-one

H at relevant C atom[a]	δ[b] 3a	δ[b] 3b	DEPT[c]	heteronuclear ^{1}H,^{13}C multiple-quantum coherence[d] via ^1J(C,H)	via $^{2,3,4}J$(C,H)
C(1)	22.6	25.8	CH_3	H−C(1)	
C(2)	102.1	205.2	C		H−C(1)
C(6)	112.0	113.1	CH	H−C(6)	H−C(7)
C(10)	113.4	117.0	CH	H−C(10)	H−C(9), H−C(11)
C(9)	115.1	118.3	CH	H−C(9)	H−C(10), H−C(11)
C(7)	123.7	131.9	CH	H−C(7)	H−C(6)
C(5)	133.0	192.6	C		H−C(6)
C(11)	145.9	146.2	CH	H−C(11)	H−C(9), H−C(10)
C(8)	153.2	151.5	C		H−C(7), H−C(10),
C(4)	165.5	141.1	C		H−C(11)
C(3)	196.4	140.4	C		

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

numbering of the carbon atoms refers to structure **1** in Figure 3) δ 1.35 [s, 3H, H−C(1], 3.08 [s, 3H, H−C(12)], 6.59 [dd, 1H, $^3J_{10,9} = 3.5$ Hz, $^3J_{10,11} = 1.4$ Hz, H−C(10)], 6.78 [d, 1H, $^3J_{9,10} = 3.5$ Hz, H−C(9)], 6.99 [d, 1H, $^3J_{7,6} = 15.9$ Hz, H−C(7)], 7.04 [d, 1H, $^3J_{6,7} = 15.9$ Hz, H−C(6)], 7.76 [dd, 1H, $^3J_{11,10} = 1.4$ Hz, H−C(11)]; ^{13}C NMR (360 MHz, DMSO-d_6, HMQC, HMBC, DEPT; arbitrary numbering of the carbon atoms refers to structure **1** in Figure 3) 21.9 [(CH_3, C(1)], 50.9 [CH_3, C(12)], 102.3 [C, C(2)], 111.6 [CH, C(6)], 112.7 [CH, C(10)], 113.1 [CH, C(9], 118.6 [C, C(7)], 133.3 [C, C(5)], 144.7 [CH, C(11)], 152.1 [C, C(8)], 162.9 [C, C(4)], 196.3 [C, C(3)]; LC/MS (APCI$^-$) 235 (100, $[M + 1 - H_2]^-$), 220 (19, $[M + 1 - H_2 - CH_3]^+$); UV−vis (in water) $\lambda_{max} = 441$ nm, $\epsilon = 1.1 \times 10^4$ L mol^{-1} cm^{-1}.

*2,4-Dihydroxy-2,5-dimethyl-1-methoxycarbonylmethyl-3-oxo-2H-pyrrole (**4**).* Following a procedure of Ledl and Fritsch (1984) with major modifications, acetylformoin (30 mmol) and glycine methyl ester hydrochloride (30 mmol) were refluxed in phosphate buffer (150 mL; 0.1 mmol/L; pH 5.5) for 25 min. After cooling, the mixture was extracted with methylene chloride (6 × 50 mL) and the aqueous phase was mixed with silica gel (20 g) and then freeze-dried. The residue was applied onto the top of a column filled with a slurry of silica gel (200 g) in ethyl acetate. Chromatography was performed with ethyl acetate (400 mL), followed by ethyl acetate/methanol (90:10, v/v; 400 mL), affording an orange fraction. Thin-layer chromatography on RP-18 material using water/methanol (70:30) as the eluent revealed a yellow, fluorescent compound at $R_f = 0.61$. The solvent was removed in vacuo, and the residue was taken up in methanol (3 mL). Upon dropwise addition of ethyl acetate, the target compound was obtained as an orange powder. Recrystalliztation from methanol/ethyl acetate (80:20, v/v) affords compound **4** as yellow-orange crystals (4.5 mmol; 15% in yield): MS(EI) 43 (100), 56 (89), 215 (78, M^+), 172 (86, $M^+ - CH_3CO$), 84 (55), 149 (53), 115 (52), 86 (47), 156 (35), 140 (32), 144 (25), 199 (20, $M^+ - CH_3$): ^{1}H NMR (360 MHz, MeOD-d_3; arbitrary numbering of the carbon atoms refers to structure **4** in Figure 4) δ 1.26 [s, 3H, H−C(1)], 2.16 [s, 3H, H−C(6)], 3.73 [s, 3H, H−C(9)], 4.15 [d, 1H, $^2J_{7a,7b} = 18.13$ Hz, H_a-C(7)], 4.23 [d, 1H, $^2J_{7a,7b} = 18.13$ Hz, H_a-C(7)]; ^{13}C NMR (360 MHz, MeOD-d_3, HMQC, HMBC, DEPT; arbitrary numbering of the carbon atoms refers to structure **4** in Figure 4) δ 11.5 [CH_3, C(1)], 22.0 [CH_3, C(6)], 43.3 [CH_3, C(9)], 53.4 [CH_2, C(7)], 88.2 [C, C(2)], 128.2 [C, C(5)], 169.1 [C, C(4)], 172.7 [C, C(8)], 193.1 [C, C(3)]; UV−vis (water) $\lambda_{max} = 363$ nm, $\epsilon = 0.6 \times 10^4$ L mol^{-1} cm^{-1}.

*2,4-Dihydroxy-2,5-dimethyl-1-carboxymethyl-3-oxo-2H-pyrrole (**5**).* Compound **4** (1.0 mmol) was dissolved in phosphate buffer (10 mL, 0.2 mol/L, pH 7.5) and, after addition of porcine liver esterase (2000 units; Sigma, Deistenhofen, Germany), was stored for 12 h at 37 °C. The mixture was freeze-dried and the residue suspended in methanol/ethyl acetate (10 mL, 50:50, v/v). After filtration and concentration to 1 mL, the target compound was purified by flash chromatography on RP-18 material (15.0 g; Lichroprep 25−40 μm, Merck) using a mixture (20:80, v/v) of methanol and trifluoroacetic acid (0.2% TFA in water) as the mobile phase. After application of the crude material, chromatography with the same eluent afforded compound **5** in the effluent >70 mL. The combined eluates were freeze-dried, yielding the colored compound as an orange powder (0.44 mmol, ∼44% in yield): LC/MS(APCI$^-$) 200 (100, $[M + 1 - H_2]^-$);^{1}H NMR (360 MHz, MeOD-d_3; arbitrary numbering of the carbon atoms refers to structure **5** in Figure 4) δ 1.28 [s, 3H, H−C(1)], 2.17 [s, 3H, H−C(6)], 4.17 [d, 1H, $^2J_{7a,7b} = 18.2$ Hz, H_a-C(7)], 4.25 [d, 1H, $^2J_{7a,7b} = 18.2$ Hz, H_a-C(7)].

*N-(1-Methyl-1,2,3-trihydroxy-2-cyclopentene-4-ylidene)pyrrolidinium Betaine (Pyrrolidino-hexose-reductone; **6**) and N-[1-Methyl-1,2-dihydroxy-3-(1-pyrrolidino)-2-cyclopenten-4-ylidene]-pyrrolidinium Betaine (Bispyrrolidino-hexose-reductone; **7**).* Following a procedure of Papst et al. (1984) with some modifications, a mixture of glucose (500 mmol) and pyrrolidine (500 mmol) in ethanol (150 mL) was refluxed for 90 min, acetic acid (500 mmol) was added, and heating was continued for additional 20 h. After cooling, the solvent was removed, and the syrupy residue was redissolved in water (300 mL) and then extracted with ethyl acetate (10 × 50 mL). After drying over Na_2SO_4, the organic layer was concentrated to ∼10 mL, and the crude material was fractionated by chromatography on silica gel (2 × 60 cm, silica gel 60, Merck), which was conditioned with toluene/ethyl acetate (10:90, v/v). After application of the crude material (in aliquots of 5 mL) onto the column, chromatography was performed using toluene/ethyl acetate (10:90, v/v; 500 mL; fraction A), followed by ethyl acetate (500 mL; fraction B), ethyl acetate/methanol (90:10, v/v; 500 mL; fraction C) and ethyl acetate/methanol (80:20, v/v; 500 mL; fraction D). Concentration of fractions D and C afforded compound **6** in fraction D (20 mmol, 4% in yield) and compound **7** in fraction C (35 mmol; 7% in yield) as white crystals after recrystallization from methanol. Spectroscopical data of compound **6**: LC/MS (ESI) 198 (100, $[M + 1]^+$), 220 (27, $[M + Na]^+$), 180 (18, $[M + 1 - H_2O]^+$);^{1}H NMR (360 MHz; DMSO-d_6, DQF−COSY; arbitrary numbering of the carbon atoms refers to structure **6** in Figure 5) δ 1.17 [s, 3H, H−C(1)], 1.80−1.84 [m, 4H, H−C(8,9)], 2.44 [d, 1H, $^2J_{6a,6b} = 15.92$ Hz, H_a-C(6)], 2.56 [m, 1H, $^2J_{6b,6a} = 15.92$ Hz, H_b-C(6)], 3.55−3.72 [m, 4H, H−C(7,10)], 4.91 [bs, 1H, HO−C(2)], 7.32 [bs, 1H, HO−C(4)]; ^{13}C NMR (360 MHz; DMSO-d_6, DEPT, HMQC, HMBC; arbitrary numbering of the carbon atoms refers to formula **6** in Figure 5): δ 24.6 [CH_3, C(1)], 25.7 [CH_2, C(8,9)], 48.2 [CH_2, C(7,10)], 70.2 [C, C(2)], 125.8 [C, C(5)], 152.2 [C, C(4)], 193.1 [C, C(3)]. Spectroscopical data of compound **7**: LC/MS (ESI) 251 (100, $[M + 1]^+$), 273 (11, $[M + Na]^+$), 233 (19, $[M + 1 - H_2O]^+$); ^{1}H NMR (360 MHz; DMSO-d_6, DQF−COSY; arbitrary numbering of the carbon atoms refers to structure **7** in Figure 5) δ 1.13 [s, 3H, H−C(1)], 1.76−1.78 [m, 4H, H−C(8′,9′)], 1.82−1.84 [m, 4H, H−C(8,9)], 2.44 [d, 1H, $^2J_{6a,6b} = 15.92$ Hz, H_a-C(6)], 2.57 [m, 1H, $^2J_{6b,6a} = 15.92$ Hz, H_b-C(6)], 2.89−3.00 [m, 4H, H−C(7′,10′)], 3.50−3.61 [m, 4H, H−C(7,10)], 4.82 [bs, 1H, HO−C(2)]; ^{13}C NMR (360 MHz; DMSO-d_6, DEPT, HMQC, HMBC; arbitrary numbering of the carbon atoms refers to structure **7** in Figure 5) δ 24.6 [CH_3, C(1)], 25.7 [CH_2, C(8,9)], 48.2 [CH_2, C(7,10)], 70.2 [C, C(2)], 125.8 [C, C(5)), 152.2 [C, C(4)], 193.1 [C, C(3)].

*2,4-Dihydroxy-2-methyl-5-[(E)-(2-furyl)methylidene]methyl-1-methoxycarbonylmethyl-3-oxo-2H-pyrrole (**8**).* A mixture of 2,4-dihydroxy-2,5-dimethyl-1-methoxycarbonylmethyl-3-oxo-2*H*-pyrrole (2 mmol) and furan-2-carboxaldehyde (5 mmol) in phosphate buffer (5 mL; pH 7.0; 0.5 mol/L) was heated for 10 min under reflux. After cooling, the mixture was extracted with ethyl acetate (4 × 10 mL) and the combined organic layers were dried over Na_2SO_4 and concentrated to ∼1 mL. The target compound was then isolated by thin-layer chromatography on silica gel (20 × 20 cm; 0.5 mm; Merck) with ethyl acetate/methanol (90:10, v/v) as the mobile phase. The band at $R_f = 0.2$ was scraped off, suspended in methanol, and filtered. Removing the solvent revealed the target compound as a yellow residue (0.25 mmol; ∼5% in yield):^{1}H NMR (360 MHz, MeOD-d_3; arbitrary numbering of the carbon atoms refers to formula **8** in Figure 6) δ 1.15 [s, 3H, H−C(1)], 3.70 [s, 3H, H−C(9)], 4.34 [d, 1H, $^2J_{7a,7b} = 18$ Hz, H_a-C(7)], 4.53 (d, 1H, $^2J_{7b,7a} = 18$ Hz, H_b-C(7)], 6.54 [dd, 1H, $^3J_{13,12} = 3.5$ Hz, $^3J_{13,14} = 1.7$ Hz, H−C(13)], 6.69 [d, 1H, $^3J_{12,13} = 3.5$ Hz, H−C(12)], 6.81 [d, 1H, $^3J_{10.6} = 15.9$ Hz, H−C(10)], 6.84 [d, 1H, $^2J_{6,10} = 15.9$ Hz, H−C(6)], 7.50 [d, 1H, $^3J_{14,13} = 1.7$ Hz, H−C(14)]; UV−vis $\lambda_{max} = 402$ nm, $\epsilon = 1.4 \times 10^4$ L mol^{-1} cm^{-1}.

N-(1-Methyl-1,2,3-trihydroxy-2-cyclopentene-4-ylidene)piperidinium Betaine (Piperidino-hexose-reductone). A mixture of *N*-(1-deoxy-D-fructos-1-yl)piperidine (200 mmol) and acetic acid (200 mmol) in ethanol (150 mL) was refluxed for 20 h. After cooling, the mixture was concentrated to ∼50 mL in vacuo and then cooled at −20 °C. The crystals formed were filtered off and recrystallized from methanol, affording the piperidino-hexose-reductone as a white solid (39 mmol, ∼20% in yield): LC/MS (ESI) 212 (100, $[M + 1]^+$), 234 (38, $[M + Na]^+$), 194 (12, $[M + 1 - H_2O]^+$); ^{1}H NMR (360 MHz; DMSO-d_6, DQF−COSY) δ 1.17 (s, 3H, $-CH_3$), 1.51−1.61 (m, 6H, 3 × $-CH_2-$), 2.40 [d, 1H, $^2J_{6a,6b} = 15.92$ Hz, $-CH_aH_b$−C(OH)], 2.52 [m, 1H, $^2J_{6b,6a} = 15.92$ Hz, $-CH_aH_b$−C(OH)], 3.55−3.63 (m, 4H, 2 × $-CH_2-$N), 4.94 [bs, 1H, $HO-C(CH_3)-$], 7.64 (bs, 1H, *HO*−C); ^{13}C NMR (360 MHz; DMSO-d_6, DEPT, HMQC, HMBC) δ 23.9

Table 3. Assignment of ^{1}H-NMR Signals (360 MHz, DMSO-d_6) of 3-Hydroxy-4-[(*E*)-(2-furyl)methylene]methyl-3-cyclopentene-1,2-dione (9)

H at relevant C atom[a]	δ[b]	I[c]	M[c]	J[c] (Hz)	connectivity[d] with
H−C(6)	3.12	2	s		
H−C(10)	6.65	1	dd	3.5, 1.8	H−C(9), H−C(11)
H−C(9)	6.85	1	d	3.5	H−C(10)
H−C(7)	7.00	1	d	15.9	H−C(1)
H−C(1)	7.30	1	d	15.9	H−C(7)
H−C(11)	7.86	1	d	1.8	H−C(10)
HO−C(3)	10.85	1	bs		

[a] Numbering of carbon atoms refers to structure **9** in Figure 7. [b] The ^{1}H chemical shifts are given in relation to DMSO-d_6. [c] Determined from 1D spectrum. [d] Observed homonuclear ^{1}H,^{1}H connectivities by DQF−COSY.

(−*C*H$_2$−), 25.7 (−*C*H$_3$), 26.0 (2 × −*C*H$_2$−), 41.2 [−*C*H$_2$−C(OH)], 48.1 (2 × −*C*H$_2$−N), 69.5 [-−(OH)−*C*H$_3$], 125.8 [N−*C*(CH$_2$)−], 150.8 [−*C*(OH)−], 194.1 (−*C*O−).

*3-Hydroxy-4-methyl-3-cyclopentene-1,2-dione (**10**) and 2,3-Dihydroxy-4-methylene-2-cyclopenten-1-one (Methylene-reductinic Acid, **11**).* Following a procedure of Ledl et al. (1982), a mixture of piperidino-hexose-reductone (60 mmol) and concentrated hydrochloric acid (8 mL) in water (140 mL) was refluxed for 5 min. After cooling, the solution was extracted with diethyl ether (6 × 30 mL), the organic layer was dried over Na_2SO_4, and the solvent was removed to ~4 mL. Cooling to −30 °C afforded the methylene-reductinic acid (**11**) as yellow crystals (16.2 mmol; ~27% in yield). The mother liquor was fractionated by column chromatography on silica gel (50 g), which was conditioned with ethyl acetate. After elution with ethyl acetate (400 mL), the solvent was removed under vacuo and the residue was fractionated by high-vacuum sublimation (0.1 Torr) affording yellow crystalls of 3-hydroxy-4-methyl-3-cyclopentene-1,2-dione (**10**) between 60 and 80 °C (1.2 mmol; 2% in yield). The spectroscopical data of 3-hydroxy-4-methyl-3-cyclopentene-1,2-dione (**10**) are well in line with those reported by Ledl et al. (1982): ^{1}H NMR (360 MHz; $CDCl_3$, TOCSY; arbitrary numbering of the carbon atoms refers to structure **10** in Figure 8) δ 2.30 [t, 3H, 0.9 Hz, H−C(1)], 3.05 [q, 2H, 0.9 Hz; H−C(6)]; MS(EI) 126 (100, M$^+$), 98 (71), 70 (76), 69 (64), 55 (81), 43 (51), 42(38), 41(57), 39(55). Spectroscopical data of methylene-reductinic acid (**11**): MS(EI) 126 (100, M$^+$), 69 (50), 55 (29), 52 (38), 43 (50), 42 (25), 41 (31), 39 (37); ^{1}H NMR (360 MHz; DMSO-d_6, TOCSY; arbitrary numbering of the carbon atoms refers to structure **11** in Figure 8) δ 2.83 [s, 2H, H−C(1)], 4.93 [s, 1H, H$_a$-C(1)], 5.34 [s, 1H, H$_b$-C(1)], 9.29 [bs, 1H, HO−C(4)], 10.95 [bs, 1H, HO−C(3)]; ^{13}C NMR (360 MHz; DMSO-d_6, DEPT, HMQC, HMBC; arbitrary numbering of the carbon atoms refers to structure **11** in Figure 8) δ 35.8 [CH$_2$, C(6)], 104.5 [CH$_2$, C(1)], 128.8 [C, C(4)], 136.2 [C, C(3)], 157.2 [C, C(2)], 194.3 [C, C(5)].

*3-Hydroxy-4-[(E)-(2-furyl)methylidene]methyl-3-cyclopentene-1,2-dione (**9**).* A mixture of methylene-reductinic acid (5 mmol), furan-2-carboxaldehyde (10 mmol), and concentrated hydrochloric acid (0.1 mL) in ethanol/water (2:1; 20 mL) was heated for 1 h at 80 °C. The organic solvent was removed in vacuo, the pH of the aqueous layer was adjusted to 3.0 with aqueous hydrochloric acid (1 mol/L), and the aqueous phase was then extracted with ethyl acetate (5 × 20 mL). The organic layer was dried over Na_2SO_4, concentrated to ~2 mL, and then fractionated by column chromatography on silica gel (2 × 60 cm, silica gel 60, Merck), which was conditioned with toluene. After application of the raw material onto the column, chromatography was performed using toluene/ethyl acetate (80:20, v/v; 400 mL). Chromatography with ethyl acetate (800 mL) affords compound **9** as deep red crystals upon concentration (1.9 mmol, ~38% in yield): LC/MS 187 (100, [M + 1 − H$_2$O]$^+$), 177 (87), 205 (43, [M + 1]$^+$), 149 (37), 159 (27); UV−vis (water, pH 6.0) λ_{max} = 441 nm, $\epsilon = 1.8 \times 10^4$ L mol^{-1} cm^{-1}; ^{1}H and ^{13}C NMR data as well as signal assignments are given in Tables 3 and 4.

Table 4. Assignment of ^{13}C-NMR Signals (360 MHz, DMSO-d_6) of 3-Hydroxy-4-[(*E*)-(2-furyl)methylene]methyl-3-cyclopentene-1,2-dione (9)

			heteronuclear ^{1}H,^{13}C multiple-quantum coherence[d]	
relevant C atom[a]	δ[b]	DEPT[c]	via ^1J(C,H)	via $^{2,3}J$(C,H)
C(6)	33.5	CH$_2$	H−C(6)	H−C(1)
C(10)	113.0	CH	H−C(10)	H−C(9), H−C(11)
C(9)	114.3	CH	H−C(9)	H−C(7), H−C(10), H−C(11)
C(1)	117.8	CH	H−C(1)	H−C(6), H−C(7)
C(7)	123.6	CH	H−C(7)	H−C(1)
C(2)	138.3	C	H−C(2)	H−C(1), H−C(6), H−C(7)
C(11)	145.6	CH	H−C(11)	H−C(9), H−C(11)
C(8)	152.3	C	H−C(8)	H−C(9), H−C(10), H−C(11)
C(3)	155.1	C	H−C(3)	H−C(1), H−C(6)
C(4)	181.9	C	H−C(4)	
C(5)	196.9	C	H−C(5)	H−C(6)

[a] Numbering of carbon atoms refers to structure **9** in Figure 7. [b] The ^{13}C chemical shifts are given in relation to DMSO-d_6. [c] DEPT-135 spectroscopy. [d] Assignments based on HMQC (^1J) and HMBC ($^{2,3}J$) experiments.

Reaction of *N*-(1-Deoxy-D-fructos-1-yl)-L-alanine or *N*-(1-Deoxy-D-fructos-1-yl)-L-proline, Respectively, with Furan-2-carboxaldehyde. A mixture of *N*-(1-deoxy-D-fructos-1-yl)-L-alanine (10 mmol) or *N*-(1-deoxy-D-fructos-1-yl)-L-proline (10 mmol), respectively, in methanol (30 mL) was refluxed for 5 h in the presence of furan-2-carboxaldehyde (40 mmol). After cooling, the solvent was removed in vacuo, and the residue was dissolved in water (200 mL) and extracted with ethyl acetate (8 × 50 mL). The combined organic layers were dried over Na_2SO_4, concentrated to ~100 mL, and then distilled under high vacuum at 35 °C to remove the volatile fraction. The residue was dissolved in ethyl acetate (5 mL) and then fractionated by column chromatography (35 × 450 mm) on silica gel (150 g, silica gel 60, Merck), which was conditioned with toluene/ethyl acetate (90:10, v/v). Chromatography was performed using toluene/ethyl acetate (90:10; 300 mL; fraction A), toluene/ethyl acetate (70:30; 300 mL; fraction B), toluene/ethyl acetate (60:40; 300 mL; fraction C), and toluene/ethyl acetate (50:50; 300 mL; fraction D). The latter two fractions were collected and freed from solvent under vacuum at 25 °C; the colored residues were taken up in ethyl acetate (1 mL) and were then further fractionated by thin-layer chromatography on silica gel (20 × 20 cm; 0.5 mm; Merck) using toluene/ethyl acetate (60:40, v/v) as the mobile phase. In the reaction mixture containing *N*-(1-deoxy-D-fructos-1-yl)-L-proline, a yellow band at R_f = 0.44 was scraped off and suspended in ethyl acetate (20 mL). After filtration, the solvent was removed, affording an intense yellow residue consisting of 4-hydroxy-2-methoxy-2-methyl-5-[(*E*)-(2-furyl)-methylidene]methyl-2*H*-furan-3-one (**1**; 0.05 mmol, ~0.5% in yield) exclusively in the mixture containing *N*-(1-deoxy-D-fructos-1-yl)-L-alanine.

Detection of Acetylformoin (2a/2b) in Dry-Heated Mixtures of Amadori Rearrangement Products. *N*-(1-Deoxy-D-fructos-1-yl)-L-alanine (10 mmol) or *N*-(1-deoxy-D-fructos-1-yl)-L-proline (10 mmol) was dry-heated for 15 min at 160 °C. After cooling, the reaction mixtures were suspended in ethyl acetate/methanol (95:5, v/v; 30 mL), filtrated, concentrated in vacuo to ~300 μL, and analyzed by GC/MS. A peak, showing the same retention time and the identical MS spectrum as synthetic acetylformoin, was detected in the heated *N*-(1-deoxy-D-fructos-1-yl)-L-proline mixture in a yield of ~2 μg/mmol. In the *N*-(1-deoxy-D-fructos-1-yl)-L-alanine mixture, acetylformoine was, however, not detected. GC/MS-(EI): 101 (100), 43 (96), 144 (67), 73 (65), 55 (41). GC/MS(CI, isobutane): 145 (100, [M + 1]$^+$).

Formation of 2,4-Dihydroxy-2,5-dimethyl-1-methoxycarbonylmethyl- (4) and 2,4-Dihydroxy-2,5-dimethyl-1-carboxymethyl-3-oxo-2*H*-pyrrole (5) upon Dry Heating of Acetylformoin (2a/2b) with Glycine Methyl Ester and Glycine, Respectively. Acetylformoin (1 mmol) was inti-

mately mixed with glycine methyl ester (1 mmol) or glycine (1 mmol), respectively, and then dry-heated for 4 min at 150 °C. For identification of compound **4**, the reaction mixture taken up in methanol (2 mL) and separated by preparative thin-layer chromatography on silica gel (20 × 20 cm; 0.5 mm; Merck) using ethyl acetate/methanol (90:10, v/v) as the mobile phase. A band with $R_f = 0.46-0.57$ was scraped off, suspended in methanol, filtered, and concentrated to ~1 mL prior to HPLC analysis. For identification of compound **5**, the reaction mixture was dissolved in water (5 mL) and extracted with ethyl acetate (3 × 5 mL), and the aqueous phase was freeze-dried and taken up in methanol (2 mL). After membrane filtration, both reaction mixtures were analyzed by RP-HPLC starting with a mixture (10:90, v/v) of acetonitrile and aqueous ammonium formiate buffer (20 mmol/L; pH 3.5) and increasing the acetonitrile content within 50 min to 30%. When the effluent was monitored at $\lambda = 360$ nm, a peak of a fluorescent compound was detected at 9.2 and 4.8 min, respectively, showing UV−vis and LC/MS data as well as retention times identical with those of synthetic **4** and **5** (~6% in yield).

Formation of Pyrrolidino-hexose-reductone (6) and Bispyrrolidino-hexose-reductone (7) from Acetylformoin (2a/2b) and L-Proline. Acetylformoin (1 mmol) and L-proline (1 mmol) were intimately mixed and then dry-heated for 15 min at 180 °C. After cooling, the mixture was dissolved in methanol (2 mL), filtered, and separated by preparative thin-layer chromatography on silica gel (20 × 20 cm; 0.5 mm; Merck) using ethyl acetate/methanol (95:5, v/v) as the mobile phase. Two bands at $R_f = 0.30$ and 0.65 were scraped off and dissolved in methanol. After filtration and concentration, analysis of both fractions by HPLC revealed the pyrrolidino-hexose-reductone (band with $R_f = 0.30$; ~1.2% in yield) and the bispyrrolidino-hexose-reductone (band at $R_f = 0.65$; ~0.5% in yield) by comparison of the retention times as well as the UV−vis and the LC/MS data with those obtained for the synthetic reference compounds.

Identification of Pyrrolidino-hexose-reductone (6) and Bispyrrolidino-hexose-reductone (7) in a Dry-Heated Mixture of L-Proline and Glucose. L-Proline (20 mmol) and glucose (10 mmol) were powdered in a mortar and then dry-heated for 15 min at 180 °C. The dry-heated mixture was dissolved in methanol (30 mL), filtered, concentrated to ~10 mL, and then ultracentrifugated with a cutoff of 1000 Da (Amicon, Witten, Germany). The filtrate was further fractionated by column chromatography on silica gel (2 × 60 cm, silica gel 60, Merck), which was conditioned with toluene/ethyl acetate (50:50, v/v). Chromatography was performed with toluene/ethyl acetate (50:50; 300 mL; fraction A) and ethyl acetate (300 mL; fraction B), followed by ethyl acetate/methanol (90:10, v/v; 300 mL; fraction C) and ethyl acetate/methanol (80:20, v/v; 300 mL; fraction D). Fractions C and D were further fractionated by thin-layer chromatography on silica gel (20 × 20 cm; 0.5 mm; Merck) using ethyl acetate/methanol (95:5, v/v) as the mobile phase. Two bands at $R_f = 0.30$ and 0.65 were scraped off and dissolved in methanol. After filtration and concentration, HPLC analysis coupled to a diode array detector (DAD) or an LC/MS revealed the pyrrolidino-hexose-reductone (band with $R_f = 0.30$; ~0.2% in yield) and the bispyrrolidino-hexose-reductone (band at $R_f = 0.65$; ~0.05% in yield).

Dry Heating of *N*-(1-Deoxy-D-fructos-1-yl)-L-proline with Glycine. *N*-(1-Deoxy-D-fructos-1-yl)-L-proline (10 mmol) and glycine (10 mmol) were intimately mixed and then dry-heated at 160 °C for 30 min. After cooling, the mixture was dissolved in methanol (100 mL), filtered, concentrated to ~4 mL, and fractionated by flash chromatography on RP-18 material (15.0 g; Lichroprep 25−40 μm, Merck) using a mixture of (10:90, v/v) of methanol and trifluoroacetic acid (0.2% TFA in water) as the mobile phase. After application of the crude material, chromatography with the same eluent afforded compound **5** in the effluent >100 mL. The combined eluates were freeze-dried, and the residue was taken up in methanol and then analyzed by RP-HPLC starting with a mixture (10:90, v/v) of acetonitrile and aqueous ammonium formiate buffer (20 mmol/L; pH 3.5) and increasing the acetonitrile content within 50 min to 30%. When the effluent was monitored at $\lambda = 360$ nm, a peak of a fluorescent compound was detected at 4.8 min showing UV−vis and LC/MS data and retention time identical with those of the synthetic 2,4-dihydroxy-2,5-dimethyl-1-carboxymethyl-3-oxo-2*H*-pyrrole. Quantification, performed by using the synthetic pyrrolinone **5** as the external standard, revealed a yield of ~0.3%.

Formation of 2,4-Dihydroxy-2-methyl-5-[(*E*)-(2-furyl)-methylidene]methyl-1-methoxycarbonylmethyl-3-oxo-2*H*-pyrrole (8) in a Mixture of Acetylformoin/Glycine Methyl Ester Heated in the Presence of Furan-2-carboxaldehyde. Acetylformoin (2 mmol) and glycine methyl ester (2 mmol) were intimately mixed and then dry-heated for 4 min at 150 °C. After addition of furan-2-carboxaldehyde (4 mmol), heating was continued for another 2 min. The reaction mixture was cooled, suspended in methanol (5 mL), and filtered. After concentration, the mixture was separated by preparative thin-layer chromatography on silica gel (20 × 20 cm; 0.5 mm; Merck) using ethyl acetate/methanol (90:10, v/v) as the mobile phase. A yellow band at $R_f = 0.4-0.5$ was scraped off, dissolved in methanol, and, after filtration and concentration, analyzed by HPLC connected to a DAD as well as an LC/MS. Spectroscopical data as well as retention time were identical with those obtained for the synthetic reference compound **8**.

Identification and Quantification of 3-Hydroxy-4-[(*E*)-(2-furyl)methylidene]methyl-3-cyclopentene-1,2-dione (9) in Several Precursor Systems. After the reaction mixtures had cooled, detailed in Figure 5, the mixtures were suspended in water (20 mL) and filtered, the pH was adjusted to 5.0, and the mixtures were then extracted with ethyl acetate (3 × 20 mL). After drying of the organic layer with Na_2SO_4, the solvent was evaporated in vacuo and the residue was taken up in methanol (1 mL). After membrane filtration, the mixtures were analyzed by RP-HPLC starting with a mixture (90:10, v/v) of acetonitrile and aqueous ammonium formiate buffer (20 mmol/L; pH 3.5) and increasing the acetonitrile content within 50 min to 80%. When the effluent was monitored at $\lambda = 440$ nm, a peak of a colored compound was detected at 25.4 min, showing UV−vis and LC/MS data as well retention time identical with those of synthetic 3-hydroxy-4-[(*E*)-(2-furyl)methylidene]methyl-3-cyclopentene-1,2-dione. Quantitation was performed by using the synthetic colorant as the external standard.

Gas Chromatography/Mass Spectroscopy (GC/MS). HRGC was performed with a type 5160 gas chromatograph (Fisons Instruments, Mainz, Germany) using an SE−54 capillary (30 m × 0.32 mm, 0.25 μm; J&W Scientific, Fisons Instruments) coupled with an MD-800 mass spectrometer (Fisons Instruments); sample application (0.5 μL) was done by on-column injection at 40 °C.

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 440) 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 preparative scale (10 × 250 mm, flow rate = 1.8 mL/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) or negative atmospheric chemical pressure ionization ($APCI^-$). After injection of the sample (2.0 μL), analysis was performed using a gradient starting with a mixture (10:90, v/v) of acetonitrile and water and increasing the acetonitrile content to 100% within 15 min.

UV−Vis Spectroscopy. UV−vis spectra were obtained by means of a U-2000 spectrometer (Colora Messtechnik Gmbh, Lorch, Germany).

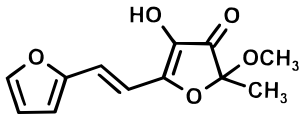

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Figure 1. Structure of the yellow 4-hydroxy-2-methoxy-2-methyl-5-[(*E*)-(2-furyl)methylidene]methyl-2*H*-furan-3-one (**1**).

Nuclear Magnetic Resonance Spectroscopy (NMR). ^{1}H, ^{13}C, DEPT-135, DQF−COSY, TOCSY, HMQC, and HMBC experiments were performed on a Bruker AC-200 and a Bruker AM-360 spectrometer (Bruker, Rheinstetten, Germany) using the acquisition parameters described recently (Hofmann, 1997). Tetramethylsilane (TMS) was used as the internal standard.

RESULTS AND DISCUSSION

On the basis of the finding that aqueous conditions destabilize certain colorants in Maillard reactions, Ledl et al. (1983) used methanol instead of water to clarify the structures of possible labile colorants. To gain an insight into the formation of such compounds from hexoses, methanolic solutions of *N*-(1-deoxy-D-fructos-1-yl)-L-alanine and *N*-(1-deoxy-D-fructos-1-yl)-L-proline, respectively, were heated in the presence of furan-2-carboxaldehyde, leading to a rapid colorization of both mixtures. After separation of the reaction mixtures, the colorants generated were registered by RP-HPLC using either a diode array detector (DAD), monitoring in the wavelength range between 220 and 500 nm, or an LC/MS.

A yellow compound, exhibiting an absorption maximum at 441 nm, was detected in the mixture containing *N*-(1-deoxy-D-fructos-1-yl)-L-proline; however, it was lacking in a heated solution of *N*-(1-deoxy-D-fructos-1-yl)-L-alanine, indicating that its formation was strongly dependent on the amino acid. LC/MS measurements with negative atmospheric pressure chemical ionization revealed an $[M + 1 - H_2]^-$ ion at *m/z* 235 (100%). A loss of 15 to a signal at *m/z* 220, most likely corresponding to the cleavage of a methyl group, and a methyl signal at 3.7 ppm in the ^{1}H NMR spectrum made a methyl acetal structure very likely. Because, in addition, a furan ring, two *E*-configured vicinal olefinic protons showing a coupling constant of 18 Hz, and an additional methyl group were observed in the ^{1}H NMR spectrum, the structure of the colorant was assumed as 4-hydroxy-2-methoxy-2-methyl-5-[(*E*)-(2-furyl)methylidene]methyl-2H-furan-3-one (**1**, Figure 1). The corresponding 2-ethoxy derivative was reported by Ledl and Severin (1982) to be formed via the hexose dehydration product acetylformoin (**2a**/**2b**), which is well-known to be formed by dehydration of 1-deoxyosone (**I**) at the 6-position as outlined in Figure 2. For further confirmation of the assumed structure, synthetic acetylformoin, which could be shown to consist of a mixture of 3,4-dihydroxy-3-hexene-2,5-dione (**2a**, in Figure 3) and 2,4-dihydroxy-2,5-dimethyl-3(2*H*)-furanone (**2b**, in Figure 3) was, therefore, condensed with furan-2-carboxaldehyde, affording the labile colorants 3,4-dihydroxy-6-[(*E*)-(2-furyl)methylidene]-3-hexene-2,5-dione (**3a**, Figure 3) and 2,4-dihydroxy-2-methyl-5-[(*E*)-(2-furyl)-methylidene]methyl-2H-furan-3-one (**3b**, Figure 3). The equilibrium between the forms **3a** and **3b** was unequivocally deduced from the ^{1}H- and the ^{13}C-NMR data. Although the existence of two forms was supposed earlier by Ledl and Severin (1982), it was as yet not confirmed by NMR spectroscopic data, which are summarized in Tables 1 and 2. Reacting **3a**/**3b** with methanol revealed the corresponding stable methyl acetal, showing spectroscopic data identical with those of the colorant isolated from the Maillard mixture, thereby confirming the proposed structure **1** (Figure 3). The corresponding 2-ethoxy derivative was reported by Ledl and Severin (1982) to be formed from *N*-(1-deoxy-D-fructos-1-yl)piperidine, which does, however, not occur in foods. The formation of **1** from glucose in the presence of the food-related secondary amino acid L-proline demonstrated that compound **3a**/**3b** might be involved in color formation during the thermal processing of foods.

Reaction of Acetylformoin with Primary and Secondary Amino Acids. The data indicated that compound **1** was exclusively generated from hexoses in the presence of the secondary amino acid L-proline. To gain more detailed information on the influence of primary and secondary amino acids on the formation of the colorants **1** and **3a**/**3b**, *N*-(1-deoxy-D-fructos-1-yl)-L-alanine or *N*-(1-deoxy-D-fructos-1-yl)-L-proline, respectively, was dry-heated, and the amounts of acetylformoin (**2a**/**2b**) formed were determined. In the presence of the latter, the Amadori rearrangement product acetylformoin was determined in amounts of ~2 μg/mmol. In contrast, acetylformoin could not be detected in the heated *N*-(1-deoxy-D-fructos-1-yl)-L-alanine mixture, thereby explaining the lack of colorant **1** in Maillard mixtures of primary amino acids.

Ledl and Fritsch (1984) reported on the pyrrolinone formation in aqueous solutions of acetylformoin and primary amines. To study whether this type of reaction might be the reason for the absence of acetylformoin in

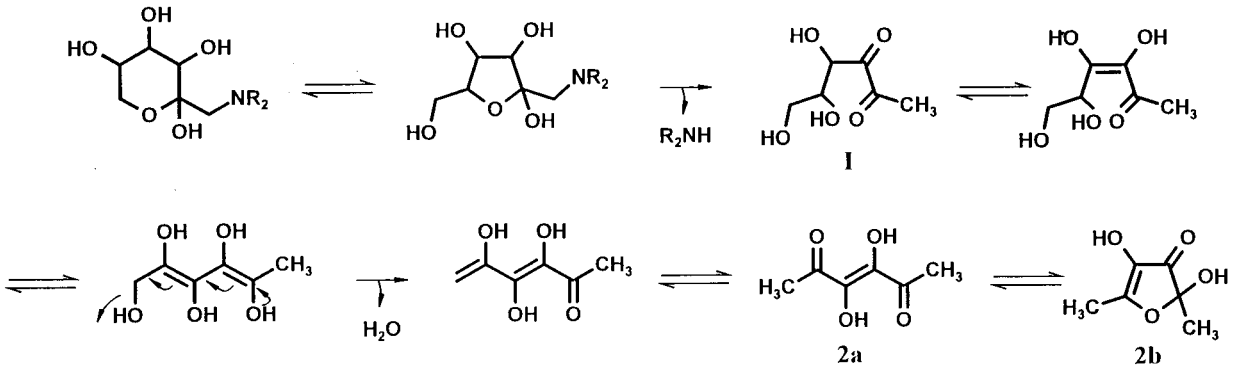


Figure 2. Formation of acetylformoin [3,4-dihydroxy-3-hexene-2,5-dione (**2a**) and 2,4-dihydroxy-2,5-dimethyl-3(2*H*)-furanone (**2b**)] via 1-deoxy-2,3-hexodiulose as the key intermediate.

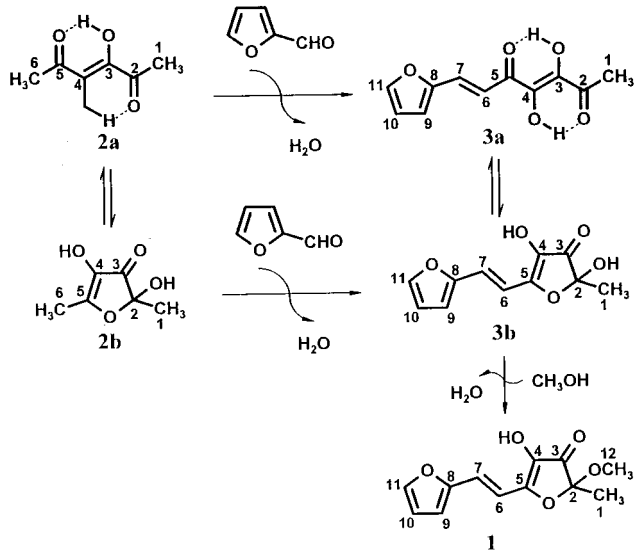


Figure 3. Formation of 3,4-dihydroxy-6-[(*E*)-(2-furyl)methylidene]-3-hexene-2,5-dione (**3a**), 2,4-dihydroxy-2-methyl-5-[(*E*)-(2-furyl)methylidene]methyl-2*H*-furan-3-one (**3b**), and 4-hydroxy-2-methoxy-2-methyl-5-[(*E*)-(2-furyl)methylidene]methyl-2*H*-furan-3-one (**1**).

the dry-heated *N*-(1-deoxy-D-fructos-1-yl)-L-alanine mixture, acetylformoin was heated in the presence of the primary amine glycine methyl ester, the primary amino acid glycine, and, in comparison, the secondary amino acid L-proline. HPLC analyses of these reaction mixtures showed that in the presence of glycine methyl ester and glycine the acetylformoin was rapidly decomposed to a variety of products, among which 2,4-dihydroxy-2,5-dimethyl-1-methoxycarbonylmethyl- (**4**, Figure 4) and 2,4-dihydroxy-2,5-dimethyl-1-carboxymethyl-3-oxo-2*H*-pyrrole (**5**, Figure 4), respectively, could be identified in yields of ~6% by comparison of the UV−vis and the LC/MS data as well as the retention times (RP-18) with those obtained for the synthetic reference compounds. These results clearly demonstrated that pyrrolinone-reductones were rapidly formed under roasting conditions from acetylformoin and primary amino acids, thereby explaining the absence of detectable amounts of acetylformoin in heated *N*-(1-deoxy-D-fructos-1-yl)-L-alanine. As proposed in Figure 4, the formation of the pyrrolinone-reductones **4** and **5** might run via Schiff base formation of the amino acid with the open-chain form of acetylformoin (**2a**), followed by cyclization. However, the amine-induced ring opening of the cyclic hemiacetal form of acetylformoin (**2b**), followed by cyclic hemi aminal formation, might also result in compounds **4** and **5** (Figure 4).

Also in the presence of L-proline, the acetylformoin was converted to several secondary reaction products. *N*-(1-Methyl-1,2,3-trihydroxy-2-cyclopentene-4-ylidene)-pyrrolidinium betaine (pyrrolidino-hexose-reductone; **6** in Figure 5) and *N*-[1-methyl-1,2-dihydroxy-3-(1-pyrrolidino)-2-cyclopenten-4-ylidene]pyrrolidinium betaine (bispyrrolidino-hexose-reductone; **7** in Figure 5) were identified in yields of about 1.2 or 0.5%, respectively, by comparison of the retention times (RP-18) and UV−vis as well as LC/MS data with those obtained for the synthetic reference compounds. On the basis of the results of ^{14}C-labeling experiments applied to the corresponding piperidino-hexose-reductone (Simon, 1962), the formation pathways leading from acetylformoin (**2a**/**2b**) and proline to the amino-hexose-reductones **6** and **7** are displayed in Figure 5. Proline-induced ring opening of acetylformoin (**2b**) results in 4-hydroxy-5-[1-(2-carboxy)pyrrolidino]-4-hexene-2,3-dione (**I**), which, upon decarboxylation and enolization, gives rise to the intermediate **II**. Ring closure via the nucleophilic enamine group forms the pyrrolidino-hexose-reductone (**6**). Schiff base formation of intermediate **II** with a molecule of pyrrolidine, which is formed by decarboxylation of proline under roasting conditions (data not shown), followed by enolization and nucleophilic ring closure, yields the corresponding bispyrrolidino-hexose-reductone (**7**). To study whether the amino-hexose-reductones **6** and **7** were also formed from carbohydrates and secondary amino acids, we reacted glucose and proline under dry-heating conditions. After chromatographical purification, we identified the amino-hexose-reductones **6** and **7** by comparison of the UV−vis and

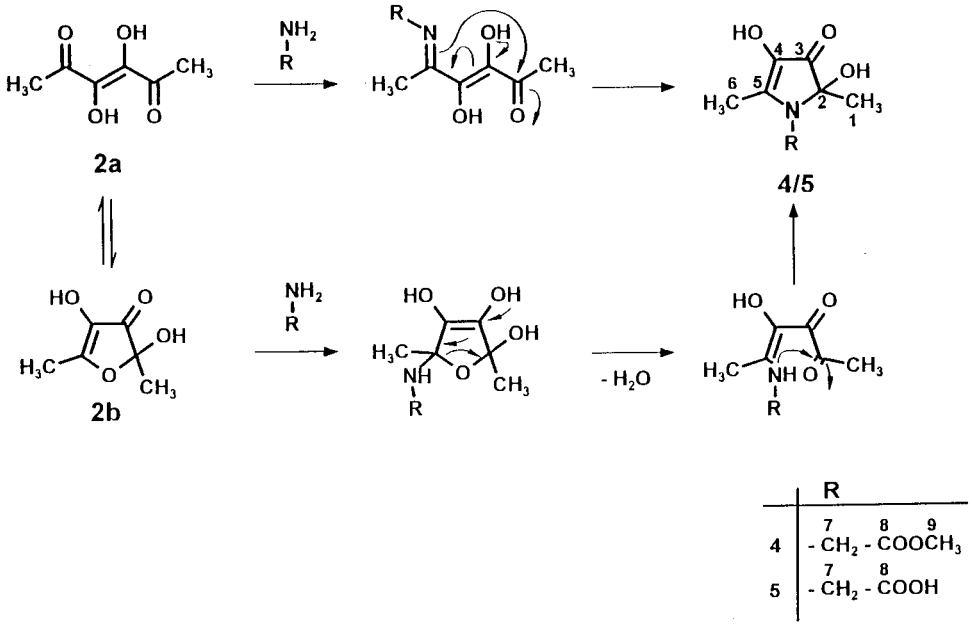


Figure 4. Formation of 2,4-dihydroxy-2,5-dimethyl-1-methoxycarbonylmethyl- (**4**) or 2,4-dihydroxy-2,5-dimethyl-1-carboxymethyl-3-oxo-2*H*-pyrrole (**5**) from the reaction of acetylformoin (**2a**/**2b**) with glycine methyl ester or glycine, respectively.

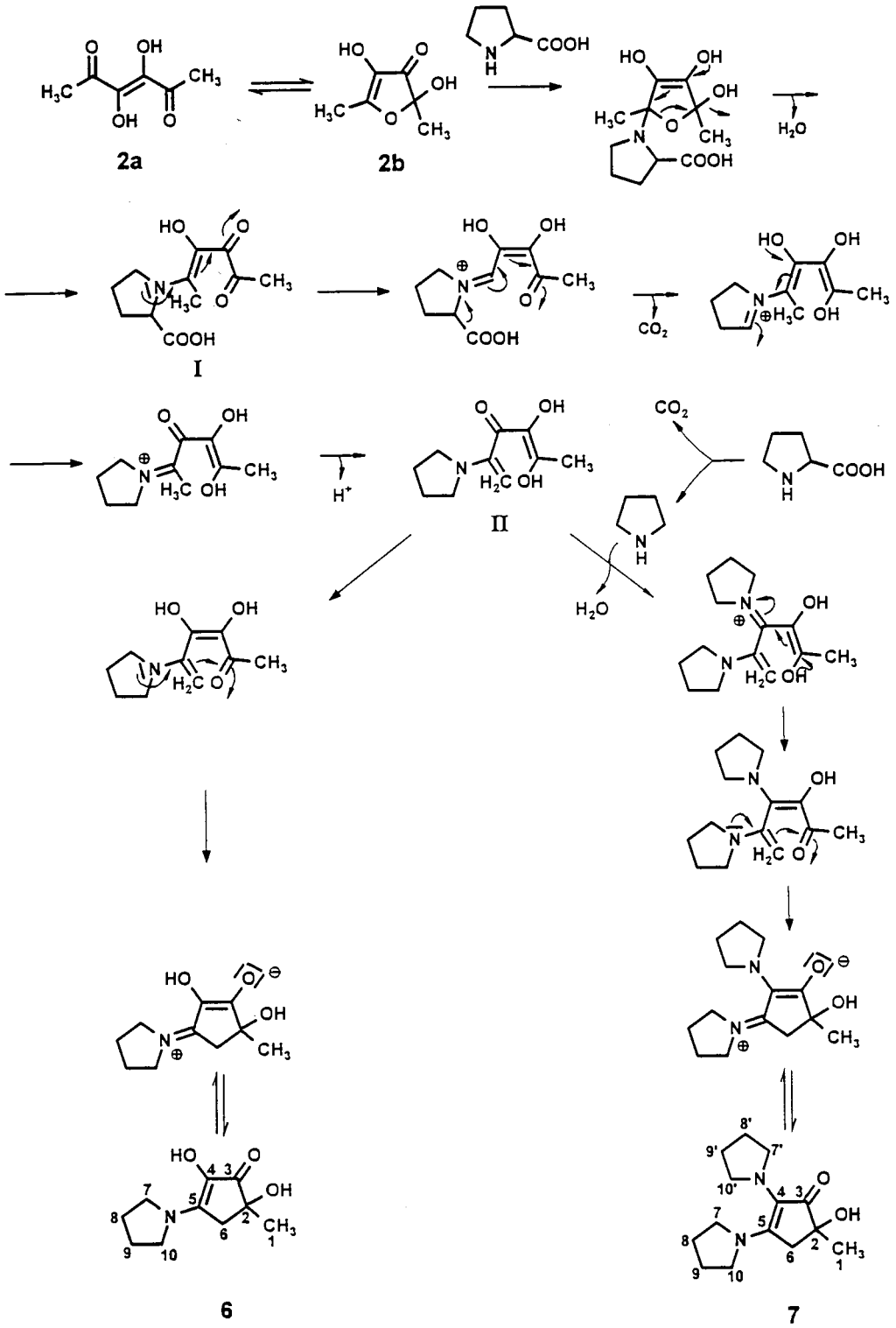
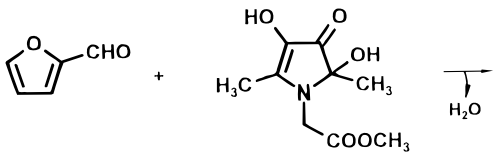


Figure 5. Reaction pathways leading to pyrrolidino-hexose-reductone (**6**) and bispyrrolidino-hexose-reductone (**7**) from acetylformoin and L-proline.

the LC/MS data as well as the retention times (RP-HPLC) with those obtained for the synthetic reference compounds. To our knowledge, this is the first time that the pyrrolidino-hexose-reductone (**6**) was identified in a carbohydrate/proline mixture, whereas the bispyrrolidinyl-hexose-reductone (**7**) was identified by Papst et al. (1984) in a heated sucrose/proline mixture. Both amino-hexose-reductones were, however, found as bitter-tasting Maillard reaction products in a heated ethanolic solution of glucose and pyrrolidine (Papst et al., 1984).

The data presented indicate that the formation of either pyrrolinone-reductones (**4**, **5**) or amino-hexose-reductones (**6**, **7**) from acetylformoin is determined by the amino acid. Because in foods primary and secondary amino acids occur side by side, we studied whether the formation of either the pyrrolinone **5** or the amino-hexose-reductones **6** and **7** from glucose is favored in the presence of primary and secondary amino acid. A mixture of *N*-(1-deoxy-D-fructos-1-yl)-L-proline was, therefore, dry-heated in the presence of glycine. HPLC analysis of the products formed revealed that the



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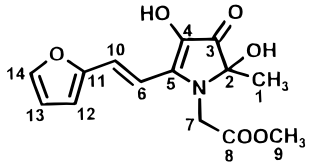

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Figure 6. Structure of 2,4-dihydroxy-2-methyl-5-[(*E*)-(2-furyl)-methylidene]methyl-1-methoxycarbonylmethyl-3-oxo-2*H*-pyrrole (**8**).

pyrrolinone **5** was preferentially formed with a yield of ~0.3%, whereas the formation of the amino-hexose-reductones **6** and **7** was nearly stalled. Ledl and Fritsch (1984) obtained similar results in heated aqueous solutions of glycine ethyl ester and the synthetically related *N*-(1-deoxy-D-fructos-1-yl)-piperidine. However, this is the first time that the key role of acetylformoin as a chemical switch in the Maillard reaction determining the formation of pyrrolinone reductones (**5**) and amino-hexose-reductones (**6**, **7**) could be demonstrated with food-related amino acids, thereby implying that similar reactions can be expected to participate in nonenzymatic browning during the thermal processing of foods.

Formation of Colored Compounds from Acetylformoin in the Presence of Primary and Secondary Amino Acids. To study the influence of primary and secondary amino acids on the formation of colored Maillard reaction products, a mixture of acetylformoin (**2a**/**2b**) and glycine methyl ester or L-proline, respectively, was dry-heated in the presence of furan-2-carboxaldehyde. In the deeply colored mixture containing glycine methyl ester, a colored compound was detected, which could be identified as 2,4-dihydroxy-2-methyl-5-[(*E*)-(2-furyl)methylidene]methyl-1-methoxycarbonyl-methyl-3-oxo-2*H*-pyrrole (**8**, Figure 6) by comparison of the UV−vis and LC/MS data as well as the retention time (RP-18) with those obtained for the synthesized reference compound. A homologous compound formed from 1-ethoxycarbonylmethyl derivative and 5-(hydroxymethyl)furan-2-carboxaldehyde in aqueous solution was earlier reported by Ledl and Fritsch (1984).

Also, dry-heating of the acetylformoin/L-proline mixture in the presence of furan-2-carboxaldehyde led to an intense colorization of the reaction mixture. A colored compound was registered by HPLC/DAD exhibiting an absorption maximum at 441 nm. LC/MS measurements revealed an $[M + 1]^+$ ion at *m*/*z* 205 with a loss of 18 to the base peak at *m*/*z* 187 and a loss of 28 to *m*/*z* 177, most likely corresponding to the cleavage of H_2O and CO, respectively. The UV−vis and MS data were well in line with those of 3-hydroxy-4-[(*E*)-(2-furyl)-methylidene]methyl-3-cyclopentene-1,2-dione (**9**, Figure 7), which was prepared by Ledl and Severin (1982) from piperidino-hexose-reductone, furan-2-carboxaldehyde, and concentrated hydrochloric acid. To confirm our supposition, colorant **9** was synthesized and characterized by several NMR measurements. Because neither the signal assignment nor the ^{13}C NMR data of compound **9** were as yet available in the literature, the NMR data are listed in Tables 3 and 4.

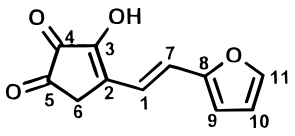

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Figure 7. Structure of 3-hydroxy-4-[(*E*)-(2-furyl)methylidene]-methyl-3-cyclopentene-1,2-dione (**9**).

Table 5. Formation of 3-Hydroxy-4-[(*E*)-(2-furyl)methylidene]methyl-3-cyclopentene-1,2-dione (9) upon Dry Heating of Several Precursors[a]

precursors reacted in the presence of furan-2-carboxaldehyde	amount of **9**	
	mg	%
bispyrrolidino-hexose-reductone (**7**)	0.9	0.9
pyrrolidino-hexose-reductone (**6**)	3.6	3.5
3-hydroxy-4-methyl-3-cyclopentene-1,2-dione (**10**)	23.1	22.7
methylene-reductinic acid (**11**)	28.7	28.1

[a] The precursor (0.5 mmol) and furan-2-carboxaldehyde (1.5 mmol) were intimately mixed with silica gel (1 g) containing phosphate buffer (150 μL; pH 5.0, 0.5 mol/L) and then dry-heated for 10 min at 180 °C.

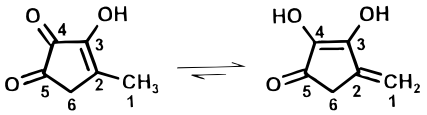

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Figure 8. Structures of 3-hydroxy-4-methyl-3-cyclopentene-1,2-dione (**10**) and methylene-reductinic acid (**11**).

The piperidino-hexose-reductone used by Ledl and Severin (1982) for the synthesis of **9** is not a food-related compound, and also the reaction conditions chosen are not suitable to simulate the thermal processing of foods. It was, therefore, as yet not clear whether the formation of **9** might also occur under food-relevant conditions. We therefore studied whether the pyrrolidino- and bispyrrolidino-hexose-reductones **6** and **7**, formed from the more food-related glucose and L-proline, might generate colorant **9** under conditions used in roasting processes. To elucidate the effectiveness of the amino-hexose-reductones **6** and **7** in the formation of colorant **9**, we heated the pyrrolidino- as well as the bispyrrolidino-hexose-reductone in the presence of furan-2-carboxaldehyde under dry-heating conditions and determined the amounts of compound **9** formed (Table 5). Because studies of Ledl et al. (1982) revealed 3-hydroxy-4-methyl-3-cyclopentene-1,2-dione and methylene-reductinic acid as the major hydrolysis products of piperidino-hexose-reductones, we, in comparison, reacted synthetic 3-hydroxy-4-methyl-3-cyclopentene-1,2-dione (**10**, Figure 8) and methylene-reductinic acid (**11**, Figure 8) with furan-2-carboxaldehyde. The results, given in Table 5, showed that the methylene-reductinic acid (**11**) and 3-hydroxy-4-methyl-3-cyclopentene-1,2-dione (**10**) generated colorant **9** most effectively with yields of 28.1 and 22.7%, respectively. Also, **6** and **7** were found as precursors of **9**; however, 31- or 8-fold lower amounts of the colorant were formed compared to the methylene-reductinic acid. These data indicate that the formation of colorant **9** from glucose and L-proline via amino-hexose-reductones as intermediates is possible under dry-heating conditions, implying that similar reactions

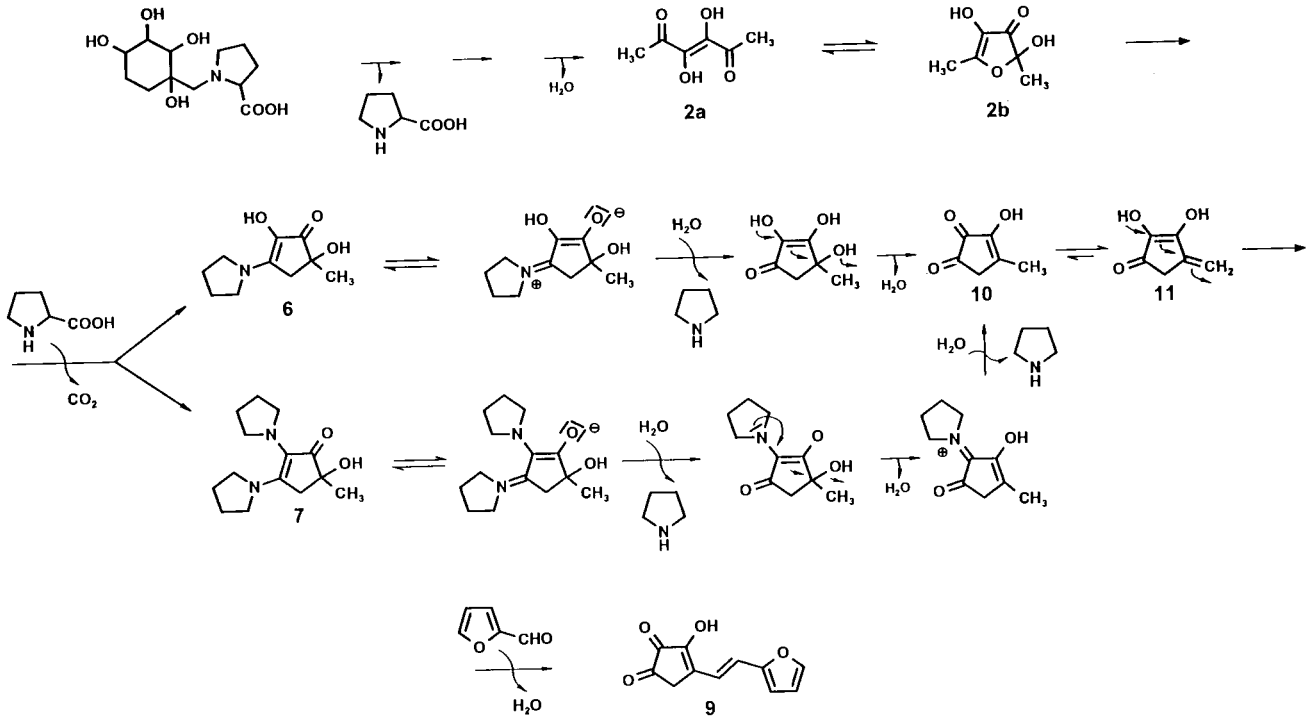

Figure 9. Formation pathways leading to 3-hydroxy-4-[(*E*)-(2-furyl)methylidene]methyl-3-cyclopentene-1,2-dione (**9**) from *N*-(1-deoxy-D-fructos-1-yl)-L-proline.

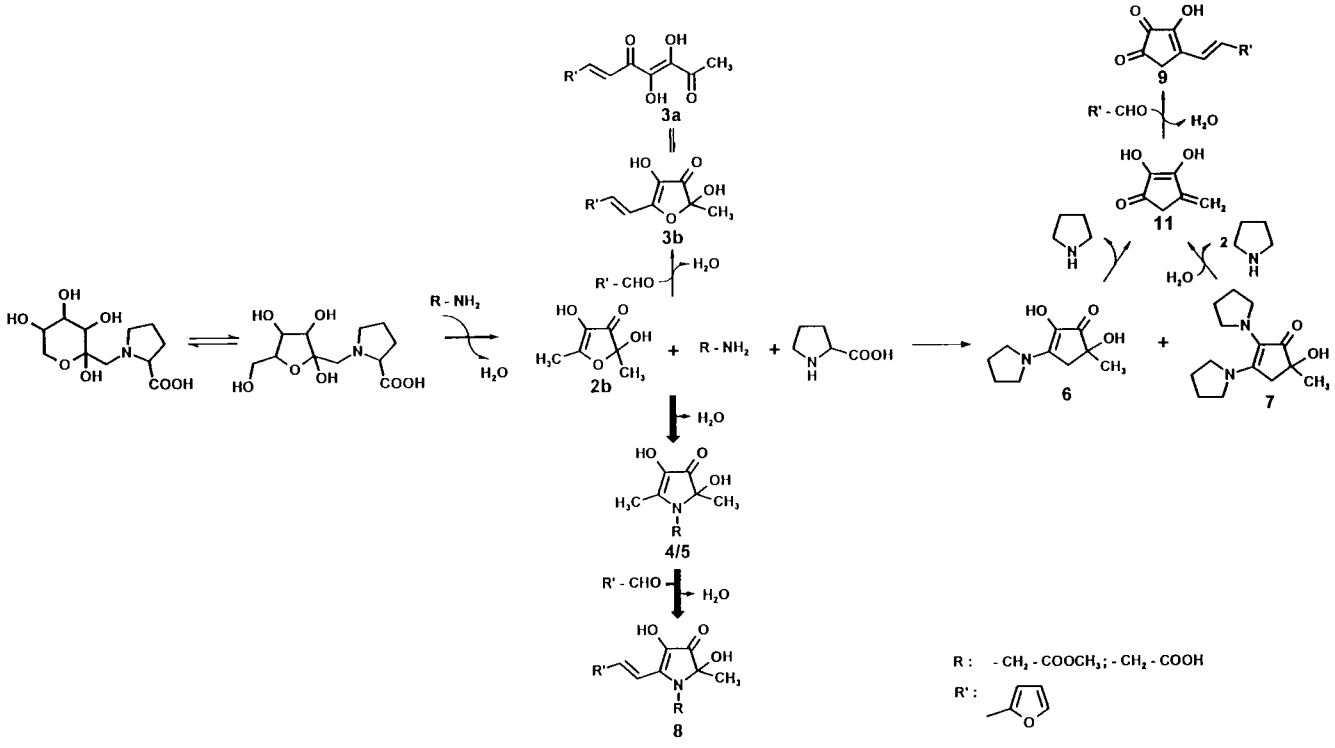

Figure 10. Role of acetylformoin (**2b**) as a switch determining the formation of the colorants **3a**/**3b**, **8**, and **9** in the presence of primary and secondary amino acids.

might participate in color formation during, for example, roasting of coffee or baking of bread.

On the basis of these quantitative data, the following reaction pathway leading to **9** from *N*-(1-deoxy-D-fructos-1-yl)-L-proline is displayed in Figure 9. Decomposition of *N*-(1-deoxy-D-fructos-1-yl)-L-proline leads to acetylformoin (**2a**/**2b**), which, upon reaction with L-proline, gives rise to pyrrolidino-hexose-reductone (**6**) and bispyrrolidino-hexose-reductone (**7**) as detailed in Figure 5. Hydrolysis of **6** and **7** yields 3-hydroxy-4-methyl-3-cyclopentene-1,2-dione (**10**) and methylene-reductinic acid (**11**), which were demonstrated by quantitative measurements to form colorant **9** very effectively in the presence of furan-2-carboxaldehyde.

Conclusions. The data presented indicate that in the presence of primary and secondary amino acids, the hexose-derived Maillard intermediate acetylformoin (**2a**/**2b**) predominantly reacts with the primary amino acid, leading to pyrrolinone-reductones (**4**, **5**) and, in the presence of a carbonyl compound, to the corresponding

chromophore of type **8** (Figure 10). The condensation of acetylformoin with a carbonyl compound to the corresponding colorant of type **3a**/**3b**, the proline-induced formation of the amino-hexose-reductones **6** and **7** and, consequently, the formation of colored compounds of type **9**, are, therefore, mostly suppressed.

Because in foods primary amino groups of amino acids as well as lysine residues of proteins were predominant in comparison to secondary amino groups, the formation of pyrrolinone-reductones should be favored. Nevertheless, the thermal processing of foods with a high content of L-proline, such as malt, might lead to the formation of colored compounds with chromophores of type **9**.

The results of the present investigation on the reactions of certain Maillard intermediates provide useful information to extend the knowledge on chromophores generated by nonenzymatic browning during food processing and will help to construct a route map of reactions leading to color development in heated foodstuffs.

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