Characterization of Key Chromophores Formed by Nonenzymatic Browning of Hexoses and L-Alanine by Using the Color Activity Concept

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Thermal treatment of an aqueous solution of D-glucose and L-alanine in the presence of the carbohydrate degradation product furan-2-aldehyde resulted in the formation of a variety of colored compounds, among which (*Z*)-2-[(2-furyl)methylidene]-5,6-di(2-furyl)-6*H*-pyran-3-one (**I**), [*E*]- and [*Z*]-1,2-bis(2-furyl)-1-pentene-3,4-dione (**IIa**/**IIb**), 4,5-bis(2-furyl)-2-methyl-3*H*-furan-2-one (**III**), and (*S*,*S*)- and (*S*,*R*)-2-[4,5-bis(2-furyl)-2-hydroxy-2-methyl-3(2*H*)-pyrrol-1-yl]propionic acid (**IVa**/**IVb**) as well as 2-[(2-furyl)methylidene]-4-hydroxy-5-[(*E*)-(2-furyl)methylidene]methyl-2*H*-furan-3-one (**V**) were successfully identified as the most intense by application of the color dilution analysis. To measure the contribution of these colorants to the overall color of the browned Maillard mixture, color activity values were calculated as the ratio of the concentration to the visual detection threshold of each colorant. By application of this color activity concept, 16.0% of the overall color of the Maillard mixture accounted for these five types of colorants, thus confirming them as key chromophores. On the basis of synthetic model experiments, the formation pathways leading to the chromophores **IIa**/**IIb**, **III**, and **IVa**/**IVb** were proposed.

Keywords: *Color activity concept; color dilution analysis; Maillard reaction; nonenzymatic browning*

INTRODUCTION

Besides the unique aroma, the typical brown color of thermally processed foods, such as roasted coffee, bread crust, roasted meat, or kiln-dried malt, is highly desirable and is intimately associated in consumers' minds with a delicious, high-grade product. This nonenzymatic browning mainly originates from reactions between reducing carbohydrates and amino compounds, known as the Maillard reaction. To further improve the quality of processed foods, for example, by controlling the nonenzymatic browning reaction more efficiently, a better understanding of the structures and the formation of chromophores from carbohydrates is required.

Several model experiments have been performed to provide more detailed information on the chromophores formed, indicating condensation reactions between methylene-active intermediates and carbonyl compounds as a general key reaction type in nonenzymatic browning [e.g., Severin and Krönig (1972), Ledl and Severin (1978, 1982), Arnoldi et al. (1997), Hofmann (1998a−c), and Ravagli et al. (1999)]. The variety of these reactive Maillard intermediates leads, consequently, to the multiplicity and the low yields of the reaction products formed.

Because the reaction of a certain methylene-active compound with different carbonyl components was found to generate the same type of chromophore, varying only in the substituents (Ledl and Severin, 1978, Hofmann et al., 1999), we, to reduce the product multiplicity from pentoses to the key chromophores, forced all methylene-active intermediates to react with the same carbonyl compound by heating D-xylose (Hofmann, 1998a,d,e) or D-glucose (Hofmann and Heuberger, 1999), respectively, with L-alanine in the presence of the carbohydrate degradation product furan-2-aldehyde. By application of the color dilution analysis (CDA) (Hofmann, 1998e), the most intense colorants could be identified and ranked in their color contribution on the basis of color activity values, which were calculated as the ratio of the concentration to the visual detection threshold of each colorant (Hofmann, 1998d; Hofmann et al., 1999). These studies revealed that 13.4% of the total color of the heated pentose mixture accounted for four key chromophores of known structures (Hofmann, 1998d).

In comparison, information on the key colorants formed from hexoses is amazingly scarce. By application of the color activity concept, only a single colored compound has as yet been clearly shown to contribute to the browning of a glucose-containing Maillard mixture (Hofmann and Heuberger, 1999). The characterization of additional types of chromophores in aqueous solutions of glucose and primary amino acids heated in the presence of a Maillard-derived carbonyl compound is, therefore, necessary to gain more detailed insights into the nonenzymatic browning chemistry of hexoses.

The objective of the present investigation was, therefore, to apply the color activity concept on the colorants formed in an aqueous D-glucose/L-alanine solution, which was heated in the presence of an additional carbonyl compound. Because furan-2-aldehyde was shown to be formed from carbohydrates (Hofmann, 1999) 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.

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Table 1. Assignment of ^{1}H NMR Signals (360 MHz, $CDCl_3$) of (*E*)- and (*Z*)-1,2-Bis(2-furyl)-1-pentene-3,4-dione (IIa/IIb)

H at relevant C atom[b]	δ[a] IIa	δ[a] IIb	I[c]	M[c]	J[c] (Hz)	connectivity[d] with
CH_3(1)	2.52	2.44	3	s		
H−C(11)	6.31	6.31	1	d	3.5	H−C(12), H−C(13)
H−C(12)	6.42	6.65	1	dd	3.5, 1.4	H−C(11), H−C(13)
H−C(8)	6.42	6.66	1	dd	3.5, 1.4	H−C(7), H−C(9)
H−C(7)	6.51	6.73	1	d	3.5	H−C(8), H−C(9)
H−C(5)	7.17	7.27	1	s		
H−C(13)	7.36	7.56	1	d	1.4	H−C(11), H−C(12)
H−C(9)	7.41	7.65	1	d	1.4	H−C(7), H−C(8)

[a] The ^{1}H chemical shifts (ppm) are given in relation to $CDCl_3$. [b] Numbering of carbon atoms refers to formula **IIa**/**IIb** in Figure 2. [c] Determined from 1D spectrum. [d] Observed homonuclear ^{1}H,^{1}H connectivities by DQF-COSY.

EXPERIMENTAL PROCEDURES

Chemicals. The following compounds were obtained commercially: D-glucose, L-alanine, furan-2-aldehyde, piperidinium acetate, 2-oxopropanal, and hydroxy-2-propanone (Aldrich, Steinheim, Germany). Furan-2-aldehyde was distilled at 30 °C in a high vacuum prior to use. Solvents were of HPLC grade (Aldrich, Steinheim, Germany). DMSO-d_6 and $CDCl_3$ were obtained from Isocom (Landshut, Germany).

The following compounds were prepared following closely the methods described in the literature given in parentheses: (*Z*)-2-[(2-furyl)methylidene]-5,6-di(2-furyl)-6*H*-pyran-3-one (**I**; Frank et al., 2000) and 2-[(2-furyl)methylidene]-4-hydroxy-5-[(*E*)-(2-furyl)methylidene]methyl-2*H*-furan-3-one (**V**; Hofmann, 1998e).

Synthetic Experiments. *[E]- and [Z]-1,2-Bis(2-furyl)-1-pentene-3,4-dione (**IIa/IIb**) and 4,5-Bis(2-furyl)-2-methyl-3H-furan-2-one (**III**) from Hydroxy-2-propanone and Furan-2-aldehyde.* The target compound **IIa**/**IIb** was prepared following a procedure described by Ledl (1982) with major modifications. A solution of furan-2-aldehyde (1.2 mol) and hydroxy-2-propanone (0.5 mol) in water/ethanol (2:3, v/v; 250 mL) was refluxed for 35 min in the presence of piperidinium acetate (20 mmol). After cooling, the solvent was removed in vacuo, water (100 mL) was added, and the reaction mixture was extracted with ethyl acetate (10 × 100 mL). The combined organic layers were dried over Na_2SO_4, concentrated to ∼150 mL at 25 °C in vacuo (100 mbar), and then distilled in a high vacuum (0.04 mbar) at 35 °C to remove the volatiles. The intensely colored residue was dissolved in pentane/ethyl ether (9:1, v/v; 10 mL), and aliquots (5 mL) were then applied onto a column (30 × 500 mm) filled with a slurry of silica gel (200 g, silica gel 60, Merck, Darmstadt, Germany) in pentane/diethyl ether (9:1, v/v). Chromatography was performed using pentane/diethyl ether (9:1, v/v; 200 mL; fraction A), pentane/diethyl ether (8:2, v/v; 400 mL; fraction B), and pentane/diethyl ether (7:3, v/v; 400 mL; fraction C), followed by pentane/diethyl ether (2:8, v/v; 400 mL, fraction D).

Fractions B and C containing an intense orange-yellow compound were stored at −18 °C affording [*E*]- and [*Z*]-1,2-bis(2-furyl)-1-pentene-3,4-dione (**IIa**/**IIb**) as red crystals (20 mmol, about 2% in yield): GC-MS (EI) 230 (40, $[M]^+$), 187 (100), 159 (98), 131 (80), 103 (18), 77 (28), 77 (28); UV (water) $\lambda_{max} = 322$ nm, $\epsilon = 0.3 \times 10^4$ L mol^{-1} cm^{-1}; ^{1}H and ^{13}C NMR data of **IIa**/**IIb** are given in Tables 1 and 2.

Fraction D was further separated by preparative thin-layer chromatography (TLC) on silica gel (20 × 20 cm; 0.5 mm; Merck) using toluene/ethyl acetate (9:1, v/v) as the eluent. A yellow, strongly fluorescent band at $R_f = 0.35$ was scraped off and dissolved in ethyl acetate (15 mL). After filtration, the solvent was evaporated to dryness affording 4,5-bis(2-furyl)-2-methyl-3*H*-furan-2-one (**III**) in 95% purity: GC-MS *m*/*z* 230 (100, $[M]^+$), 201 (5), 173 (10), 158 (38), 145 (5), 102 (38), 95 (42); UV (water) $\lambda_{max} = 325$ nm, $\epsilon = 2.1 \times 10^4$ L mol^{-1} cm^{-1}; ^{1}H and ^{13}C NMR data of **III** are listed in Tables 3 and 4.

Table 2. Assignment of ^{13}C NMR Signals (360 MHz, $CDCl_3$) of (*E*)-1,2-Bis(2-furyl)-1-pentene-3,4-dione (IIa)

H at relevant C atom[b]	δ[c]	DEPT[d]	heteronuclear ^{1}H,^{13}C multiple-quantum coherence[a] via ^1J(C,H)	via $^{2,3,4}J$(C,H)
C(1)	23.6	CH_3	CH_3(1)	
C(12)	109.3	CH	H−C(12)	H−C(11), H−C(13)
C(11)	112.0	CH	H−C(11)	H−C(12), H−C(13)
C(8)	112.8	CH	H−C(8)	H−C(7), H−C(9)
C(7)	113.8	CH	H−C(7)	H−C(8), H−C(9)
C(5)	116.4	CH	H−C(5)	
C(4)	118.9	C		H−C(5)
C(13)	142.8	CH	H−C(13)	H−C(11), H−C(12)
C(9)	143.8	CH	H−C(9)	H−C(7), H−C(8)
C(10)	149.9	C		H−C(11), H−C(12), H−C(13)
C(6)	150.8	C		H−C(5), H−C(7), H−C(8), H−C(9)
C(2)	193.2	C		H−C(1)
C(3)	196.2	C		H−C(1), H−C(5)

[a] Assignments based on HMQC (^1J) and HMBC ($^{2,3,4}J$) experiments. [b] Numbering of carbon atoms refers to formula **IIa**/**IIb** in Figure 2. [c] The ^{13}C chemical shifts (ppm) are given in relation to $CDCl_3$. [d] DEPT-135 spectroscopy.

Table 3. Assignment of ^{1}H NMR Signals (360 MHz, $CDCl_3$) of 4,5-Bis(2-furyl)-2-methyl-3*H*-furan-2-one (III)

H at relevant C atom[a]	δ[b]	I[c]	M[c]	J[c] (Hz)	connectivity[d] with
CH_3(1)	1.52	3	d	7.0	H−C(2)
H−C(2)	4.71	1	q	7.0	H−C(1)
H−C(12)	6.53	1	dd	3.1, 1.7	H−C(11), H−C(13)
H−C(8)	6.60	1	dd	3.1, 1.7	H−C(7), H−C(9)
H−C(11)	6.94	1	dd	3.1	H−C(12), H−C(13)
H−C(13)	7.51	1	dd	1.7	H−C(11), H−C(12)
H−C(7)	7.53	1	d	3.1	H−C(8), H−C(9)
H−C(9)	7.70	1	d	1.7	H−C(7), H−C(8)

[a] Numbering of carbon atoms refers to formula **III** in Figure 2. [b] The ^{1}H chemical shifts (ppm) are given in relation to $CDCl_3$. [c] Determined from 1D spectrum. [d] Observed homonuclear ^{1}H, ^{1}H connectivities by TOCSY.

Table 4. Assignment of ^{13}C NMR Signals (360 MHz, $CDCl_3$) of 4,5-Bis(2-furyl)-2-methyl-3*H*-furan-2-one (III)

H at relevant C atom[b]	δ[c]	DEPT[d]	heteronuclear ^{1}H,^{13}C multiple-quantum coherence[a] via ^1J(C,H)	via $^{2,3}J$(C,H)
C(1)	16.7	CH_3	H−C(1)	H−C(2)
C(2)	80.9	CH	H−C(2)	H−C(1)
C(11)	110.5	CH	H−C(11)	H−C(12), H−C(13)
C(12)	111.0	CH	H−C(12)	H−C(11), H−C(13)
C(8)	112.6	CH	H−C(8)	H−C(7), H−C(9)
C(7)	118.6	CH	H−C(7)	H−C(8), H−C(9)
C(13)	142.0	CH	H−C(13)	H−C(11), H−C(12)
C(10)	143.3	C		H−C(11), H−C(12), H−C(13)
C(6)	144.7	C		H−C(7), H−C(8), H−C(9)
C(9)	146.5	CH	H−C(9)	H−C(7), H−C(8)
C(4)	167.8	C		H−C(2)
C(5)	195.0	C		H−C(2)
C(3)	200.9	C		H−C(1), H−C(2)

[a] Assignments based on HMQC (^1J) and HMBC ($^{2,3}J$) experiments. [b] Numbering of carbon atoms refers to Formula **III** in Figure 2. [c] The ^{13}C chemical shifts are given in relation to $CDCl_3$. [d] DEPT-135 spectroscopy.

*4,5-Bis(2-furyl)-2-methyl-3H-furan-2-one (**III**) by Cyclization of **IIa**/**IIb**.* A solution of 1,2-bis(2-furyl)-1-pentene-3,4-dione (**IIa**/**IIb**; 1.0 mmol) in water/methanol (1:1, v/v; 6 mL) was heated for 1 h at 80 °C in a closed vial. After cooling, water (50 mL) was added and the solution was extracted with ethyl

Table 5. Assignment of ^{1}H NMR Signals (360 MHz, DMSO-d_6) of (*S*,*S*)- and (*S*,*R*)-2-[4,5-Bis(2-furyl)-2-hydroxy-2-methyl-3(2*H*)-pyrrol-1-yl]propionic Acid (IVa/IVb)

H at relevant C atom[b]	δ[a] IVa	δ[a] IVb	I[c]	M[c]	*J*[c] (Hz)	connectivity[d] with
CH_3-(5)	0.99	0.99	3	s		
CH_3-(15)	1.26	1.26	3	d	7.1	H−C(14)
H−C(14)	4.57	4.67	1	q	7.1	H−C(15)
H−C(11)	6.40	6.36	1	d	3.1	H−C(12), H−C(13)
H−C(12)	6.49	6.48	1	dd	3.1, 1.7	H−C(11), H−C(13)
H−C(7)	6.58	6.60	1	d	3.1	H−C(8), H−C(9)
H−C(8)	6.71	6.71	1	dd	3.1, 1.7	H−C(7), H−C(9)
H−C(13)	7.54	7.54	1	d	1.7	H−C(11), H−C(12)
H−C(9)	7.95	7.96	1	d	1.7	H−C(7), H−C(9)

[a] The ^{1}H chemical shifts (ppm) are given in relation to DMSO-d_6. [b] Numbering of carbon atoms refers to formula **IVa**/**IVb** in Figure 2. [c] Determined from 1D spectrum. [d] Observed homonuclear ^{1}H, ^{1}H connectivities by TOCSY.

acetate (5 × 50 mL). The combined organic layer was dried over Na_2SO_4 and fractionated by preparative TLC on silica gel as described above, affording 4,5-bis(2-furyl)-2-methyl-3*H*-furan-2-one (**III**) in 98% purity (0.15 mmol; ∼15% in yield). Heating **IIa**/**IIb** in the presence of catalytic amounts of piperidinium acetate (0.05 μmol) drastically increased the yield of **III** to ∼60%. The GC-MS as well as the ^{1}H and ^{13}C NMR data were identical to those of compound **III** isolated from the model mixture of furan-2-aldehyde and hydroxy-2-propanone.

*(S,S)- and (S,R)-2-[4,5-Bis(2-furyl)-2-hydroxy-2-methyl-3(2H)-pyrrol-1-yl]propionic Acid (**IVa**/**IVb**).* A mixture of 1,2-bis(2-furyl)-1-pentene-3,4-dione (**IIa**/**IIb**; 1.0 mmol) and L-alanine (5 mmol) was dissolved in water/ethanol (4:6, v/v; 8 mL), the pH was adjusted with aqueous sodium hydroxide (1 mol/L) to 7.5, and the solution was then heated for 1 h at 70 °C in a closed vial. After cooling, the solvent was removed in vacuo, water (50 mL) was added, the pH was adjusted to 4.0 with aqueous hydrochloric acid (1 mol/L), and the solution was extracted with ethyl acetate (10 × 50 mL). The organic layer was dried over Na_2SO_4, the solvent was removed in vacuo, and the residue was taken up in methanol/water (6:4, v/v; 3 mL). The colored target compounds were then isolated by flash chromatography (20 × 1.6 cm) using an RP-18 stationary phase (15.0 g; Lichroprep 25−40 μm, Merck), which was conditioned with methanol/water (6:4, v/v). Flushing with the same solvent mixture afforded a fraction containing the red-orange colorants. After the solvent had been removed, the pH was adjusted to 4.0 and the aqueous phase was extracted with ethyl acetate (5 × 20 mL). After drying over Na_2SO_4, the orange colorant was isolated in 96% purity by preparative TLC on silica gel (20 × 20 cm; 0.5 mm; Merck) using methanol/ethyl acetate (20:80, v/v) as the eluent. The red-orange bands at R_f = 0.13 and 0.20 containing the target compounds **IVa** and **IVb** were scraped off and dissolved in methanol/ethyl acetate (30:70, v/v; 20 mL). After filtration, the solvent was evaporated to dryness affording (*S*,*S*)- and (*S*,*R*)-2-[4,5-bis(2-furyl)-2-hydroxy-2-methyl-3(2*H*)-pyrrol-1-yl]propionic acid (**IVa**/**IVb**) as intense red-orange oils (0.44 mmol; ∼44% in yield): LC-MS(APCI$^+$) of **IVa** *m*/*z* 318 (100, $[M + 1]^+$), 300 (64, $[M + 1 - H_2O]^+$); UV (in water) λ_{max1} = 393 nm ($\epsilon = 0.7 \times 10^4$ L mol^{-1} cm^{-1}), λ_{max2} = 298 nm ($\epsilon = 1.2 \times 10^4$ L mol^{-1} cm^{-1}); ^{1}H and ^{13}C NMR data of **IVa**/**IVb** are listed in Tables 5 and 6.

*[Z]-1-Hydroxy-1-[(2-furyl)methylidene]propan-2-one (**VI**).* A solution of furan-2-aldehyde (10 mmol) and hydroxy-2-propanone (15 mmol) in water/ethanol (2:3, v/v; 20 mL) was refluxed for 10 min in the presence of piperidinium acetate (0.5 mmol). After cooling, the solvent was removed in vacuo, water (50 mL) was added, and the reaction mixture was extracted with ethyl acetate (10 × 50 mL). The combined organic layer was dried over Na_2SO_4, concentrated to ∼150 mL at 25 °C in vacuo (100 mbar), and then distilled in a high vacuum (0.04 mbar; 35 °C) to remove the volatiles. The residue was dissolved in pentane/diethyl ether (9:1, v/v; 10 mL) and

Table 6. Assignment of ^{13}C NMR Signals (360 MHz, DMSO-d_6) of (*S*,*S*)- and (*S*,*R*)-2-[4,5-Bis(2-furyl)-2-hydroxy-2-methyl-3(2*H*)-pyrrol-1-yl]propionic Acid (IVa/IVb)

H at relevant C atom[c]	δ[a] IVa	δ[a] IVb	DEPT[d]	heteronuclear ^{1}H, ^{13}C multiple-quantum coherence[b] via ^1J(C,H)	via $^{2,3}J$(C,H)
C(5)	17.1	17.1	CH_3	CH_3(5)	
C(15)	19.9	19.9	CH_3	CH_3(15)	H−C(14)
C(14)	50.9	50.9	CH	H−C(14)	CH_3(15)
C(4)	89.0	88.6	C		CH_3(5), H−C(14)
C(11)	107.8	107.8	CH	H−C(11)	H−C(12), H−C(13)
C(12)	111.0	111.0	CH	H−C(12)	H−C(11), H−C(13)
C(8)	112.6	112.3	CH	H−C(8)	H−C(7), H−C(9)
C(7)	117.6	117.1	CH	H−C(7)	H−C(8), H−C(9)
C(13)	140.9	140.9	CH	H−C(13)	H−C(11), H−C(12)
C(6)	142.8	142.8	C		H−C(7), H−C(8), H−C(9)
C(2)	143.9	143.9	C		
C(9)	145.7	145.7	CH	H−C(9)	H−C(7), H−C(8)
C(10)	146.0	146.0	C		H−C(11), H−C(12), H−C(13)
C(1)	157.1	157.9	C		H−C(14)
C(16)	173.1	173.1	COOH		H−C(14)
C(3)	195.7	195.6	C		

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

was then applied onto a column (15 × 250 mm) filled with a slurry of silica gel (100 g, silica gel 60, Merck) in pentane/diethyl ether (9:1, v/v). Chromatography was performed using pentane/diethyl ether (9:1, v/v; 200 mL; fraction A), pentane/diethyl ether (5:5, v/v; 400 mL; fraction B), and diethyl ether (400 mL, fraction C). The target compound was isolated from fraction B by preparative TLC on silica gel (20 × 20 cm; 0.5 mm; Merck) using toluene/ethyl acetate (9:1, v/v) as the eluent. The band at R_f = 0.85 was scraped off and dissolved in ethyl acetate (20 mL). After filtration, [*Z*]-1-hydroxy-1-[(2-furyl)-methylidene]propan-2-one (**VI**) was obtained as a colorless oil (0.3 mmol, ∼0.3% in yield): GC-MS (EI) 152 (53; $[M]^+$), 82 (38), 81 (100), 53 (70), 43 (91); ^{1}H NMR (360 MHz; $CDCl_3$) δ 2.21 (s, 3H, CH_3), 6.51 (s, 1H), 6.53 (dd, 1H, 3.5 Hz, 1.7 Hz), 7.01 (d, 1H, 3.5 Hz), 7.52 (d, 1H, 1.7 Hz).

Maillard Reaction Mixture. A mixture of D-glucose (0.1 mol) and L-alanine (0.02 mol) dissolved in phosphate buffer (140 mL; 0.5 mol/L, pH 7.0) was refluxed for 1 h, and then furan-2-aldehyde (0.2 mmol) was added and heating was continued for another 3 h.

Preparation of a Color Extract. After the mixture had cooled to room temperature, the pH was adjusted to 4.0, and the aqueous reaction mixture was extracted with ethyl acetate (20 × 25 mL); the organic layer was dried over Na_2SO_4 and concentrated at 25 °C in vacuo (100 mbar) to 100 mL. To remove volatiles, the extract was distilled in a high vacuum (0.04 mbar) at 35 °C, yielding the solvent extract as an intensely colored powder.

Color Dilution Analysis. The color extract was dissolved in methanol (35 mL), and an aliquot (20 μL) of this solution was analyzed by HPLC. The effluents of peaks exhibiting absorption maxima above 320 nm were separately collected in glass vials and were then added with water to exactly 1 mL. The colored fractions were then stepwise 1+1-diluted with water, and each dilution was judged by color until a color difference between the diluted fraction in a glass vial (10 mm i.d.) and two blanks (tap water) could just be detected visually. This dilution was defined as the color dilution (CD) factor (Hofmann, 1998e).

Identification and Quantification of Colorants in the Maillard Reaction Mixture. The color extract was dissolved in ethyl acetate (5 mL) and was applied onto the top of a glass column (500 × 30 mm) filled with silica gel (200 g, silica gel 60, Merck), which was conditioned with toluene. Chromatog-

raphy was performed using toluene (800 mL; fraction A), followed by toluene/ethyl acetate (80:20, v/v; 400 mL; fraction B), toluene/ethyl acetate (50:50, v/v; 400 mL; fraction C), toluene/ethyl acetate (30:70, v/v; 400 mL; fraction D), ethyl acetate (500 mL, fraction E), methanol/ethyl acetate (20:80, v/v; 400 mL; fraction F), and methanol/ethyl acetate (50:50, v/v; 400 mL; fraction G). The fractions, containing the colorants given in parentheses, A+B (**I**, **IIa**/**IIb**, **III**), C (**V**), and F+G (**IVa**/**IVb**) were collected, freed from solvent under vacuum at 25 °C, and separately taken up in methanol (50 mL). After membrane filtration, the fractions were analyzed by RP-HPLC. Identification of the colorants was performed by comparison of the LC-MS and the UV−vis spectra as well as the retention times (RP-HPLC; **I**, 44.4 min; **IIa**/**IIb**, 38.8−39.0 min; **III**, 38.0 min; **IVa**/**IVb**, 8.4/13.4 min; **V**, 43.6 min) with those obtained for the synthetic reference compounds. Quantification of the colorants was performed by comparing the peak areas obtained at the absorption maximum of each colorant (**I**, 430 nm; **IIa**/**IIb**, 322 nm; **III**, 325 nm; **IVa**/**IVb**, 393 nm; **V**, 426 nm) with those of defined standard solutions of each reference compound in methanol.

Determination of Color Dilution (CD_{total}) Factor of the Maillard Mixture. The Maillard mixture was diluted with water until a color difference between an aliquot (5 mL) and two blanks (tap water; 5 mL) in a glass vial (1 cm i.d.) could just be detected visually using a triangle test. Using this procedure, a color dilution (CD_{total}) factor of the Maillard mixture was determined (Hofmann, 1998d).

Determination of Visual Detection Thresholds. An aqueous solution, containing known amounts of the colorant, was diluted with water until no color difference between the diluted sample (5 mL) and two blanks containing tap water (5 mL) in a glass vial (1 cm i. d.) could be detected visually using a triangle test. The concentration of the colorant at which a difference between the diluted sample and the two blanks could just be detected visually is defined as the detection threshold (Hofmann, 1998d).

Gas Chromatography—Mass Spectrometry (GC-MS). HRGC was performed with a type 5890 gas chromatograph (Hewlett-Packard, Heilbronn, Germany) using an SE-54 capillary (30 m × 0.32 mm, 0.25 μm; J&W Scientific, Fisons Instruments, Mainz, Germany) coupled with a MAT 95 S mass spectrometer (Finnigan MAT, Bremen, Germany) running in the electron impact (EI) ionization mode (115 eV); sample application (0.5 μL) was done by on-column injection at 40 °C. High-resolution mass spectrometry was performed with perfluor cerosine as the internal standard.

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, Shandon, Frankfurt, Germany) in an analytical scale (4.6 × 250 mm, flow rate = 0.6 mL/min). For quantification of colored Maillard reaction products, the following solvent gradient was used: starting with a mixture (10:90, v/v) of methanol and water, the methanol content was increased to 100% within 55 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) using electrospray ionization (ESI). After injection of the sample (5.0 μL), analysis was performed using a gradient starting with a 10:90 (v/v) mixture of methanol and water and increasing the methanol content to 100% within 55 min.

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

Nuclear Magnetic Resonance Spectroscopy (NMR). ^{1}H, ^{13}C, DEPT-135, DQF-COSY, TOCSY, HMQC, and HMBC 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

Thermal treatment of a neutral aqueous solution of D-glucose and L-alanine in the presence of furan-2-aldehyde led to a rapid coloration of the reaction mixture. After separation of the nonvolatile, solvent-extractable fraction by RP-HPLC, reaction products were registered using a diode array detector monitoring in the wavelength range between 220 and 500 nm. Among the multiplicity of reaction products detected, the most intensely colored compounds were located in the HPLC chromatogram by application of the CDA, which was recently developed as a screening procedure to rank colored compounds in their relative color impact (Hofmann, 1998e).

Screening for the Most Intense Colored Reaction Products (CDA). To detect the key chromophores contributing the most to the browning of the Maillard mixture, an aliquot of the solution was separated by RP-HPLC (Figure 1, left side) and the effluents of peaks exhibiting UV−vis absorption above 320 nm were separately collected in one HPLC run. Forty-two fractions were obtained, which were then diluted with water to 1 mL. Each fraction was then stepwise 1+1-diluted with water, and each dilution was judged by color. We recently defined the CD factor as the dilution at which a color difference between the diluted fraction and two blanks (tap water) could just be detected visually (Hofmann, 1998e). As the CD factor obtained for each compound is proportional to its color activity in water, the CD factor, therefore, ranks the 42 fractions in their relative color intensities (Figure 1, right side). On the basis of the high CD factors of 64 and 32, the orange fraction 37 and the yellow fractions 31 and 32, respectively, were evaluated with the highest color impacts, followed by fractions 23, 26, 30, 33−36, 38, and 40 showing somewhat lower color activities (Figure 1). The other fractions showed significantly lower CD factors and should, therefore, be only of minor importance for the color of the browned solution. The identification experiments were, therefore, focused on selected compounds judged with the highest CD factors and, as a consequence, contributing the most to the color of the heated Maillard mixture.

Identification of Intensely Colored Reaction Products. For identification of the most intense chromophore in the orange fraction 37, exhibiting absorption maxima at 430 and 379 nm, the colored nonvolatile fraction was separated by column chromatography on silica gel and RP-18 material. Several one- and two-dimensional NMR measurements, LC-MS and UV−vis spectroscopy, and quantitative precursor studies as well as ^{13}C labeling experiments led to the unequivocal identification of the orange Maillard reaction product as (*Z*)-2-[(2-furyl)methylidene]-5,6-di(2-furyl)-6*H*-pyran-3-one (**I** in Figure 2). Details of the isolation and structure determination of the previously unknown compound **I** as well as studies on its formation pathways will be published elsewhere (Frank et al., 2000).

In fraction 32, which was evaluated with a CD factor of 32, an intensely yellow compound was detected exhibiting an absorption maximum at 322 nm. After chromatographic separation on silica gel, the colorant was analyzed by HRGC-MS revealing a molecular mass

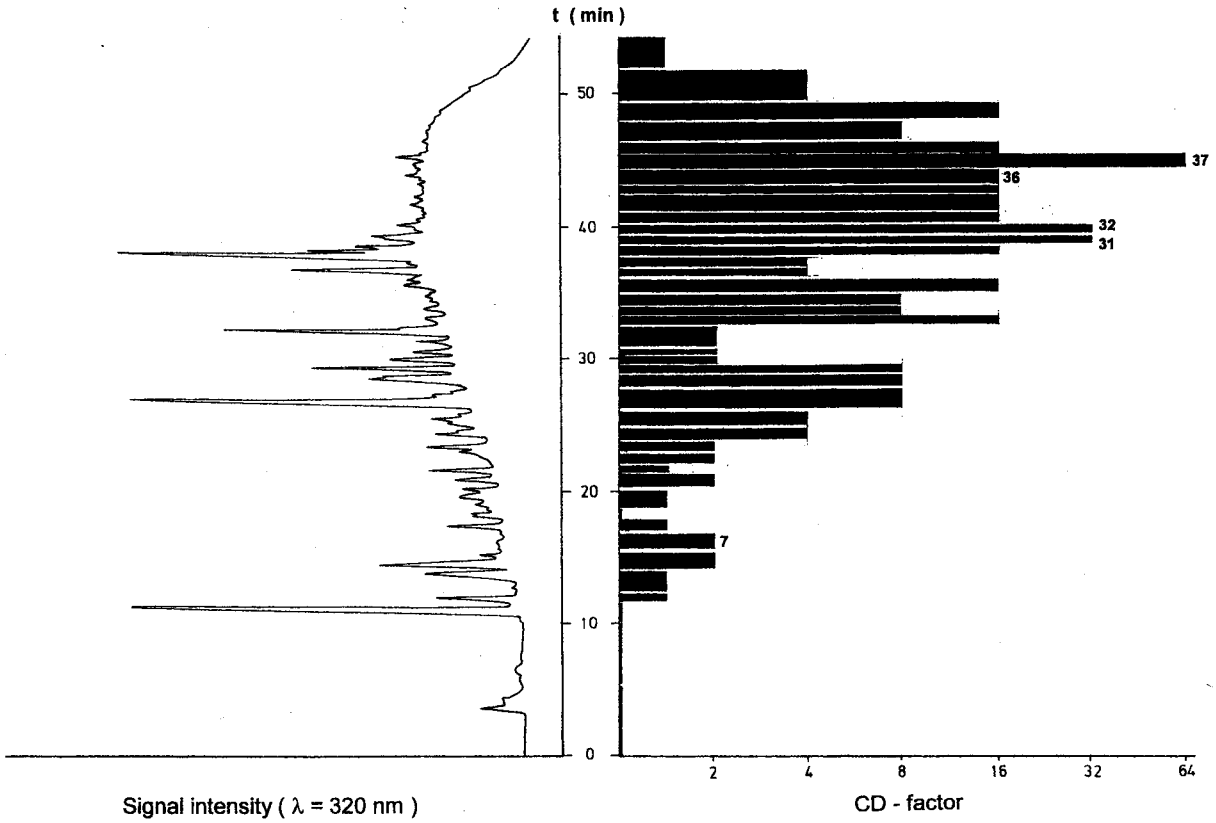

Figure 1. RP-HPLC chromatogram ($\lambda = 320$ nm; left side) and color dilution chromatogram (right side) of the solvent-extractable fraction of the Maillard mixture.

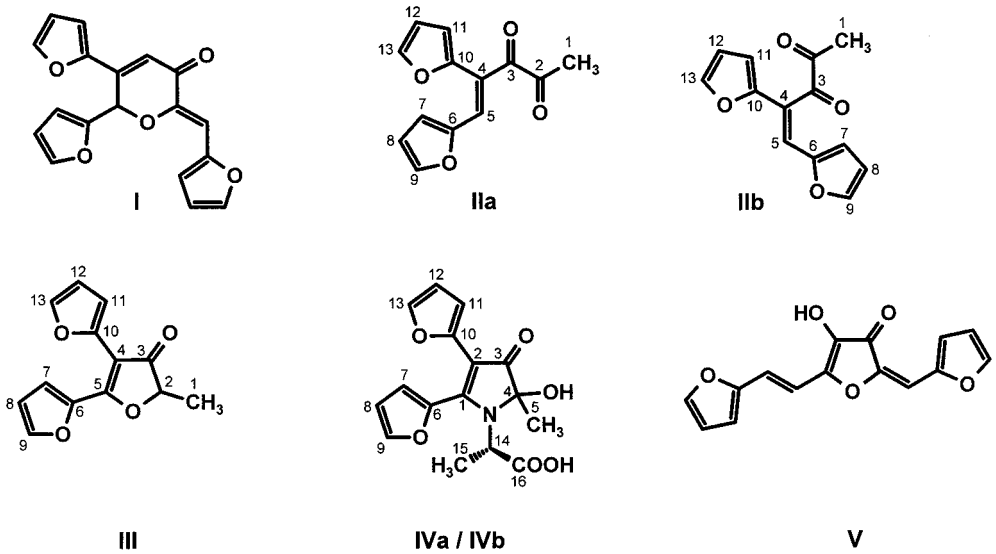

Figure 2. Structure of key chromophores identified: (*Z*)-2-[(2-furyl)methylidene]-5,6-di(2-furyl)-6*H*-pyran-3-one (**I**, in fraction 37), [*E*]- and [*Z*]-1,2-bis(2-furyl)-1-pentene-3,4-dione (**IIa**/**IIb**, in fraction 32), and 4,5-bis(2-furyl)-2-methyl-3*H*-furan-2-one (**III**, in fraction **31**) as well as (*S*,*S*)- and (*S*,*R*)-2-[4,5-bis(2-furyl)-2-hydroxy-2-methyl-3(2*H*)-pyrrol-1-yl]propionic acid (**IVa**/**IVb**, in fraction 7) 2-[(2-furyl)methylidene]-4-hydroxy-5-[(*E*)-(2-furyl)methylidene]methyl-2*H*-furan-3-one (**V**, in fraction 36).

of 230 Da. On the basis of the elementary composition ($C_{13}H_{10}O_4$), which was obtained by HRGC-MS operating in the high-resolution mode, two molecules of furan-2-aldehyde and a carbohydrate degradation product with a C_3 carbon skeleton were assumed to be involved in its formation. Because the amount of the colorant was too low to obtain NMR data, furan-2-aldehyde was reacted with the C_3 compound hydroxy-2-propanone or 2-oxopropanal, respectively, both of which were recently identified as major C_3 carbohydrate cleavage products (Hofmann, 1999), and the reacted mixtures were analyzed for the yellow compound detected in fraction 32 (data not shown). A yellow colorant could be isolated from the furan-2-aldehyde/hydroxy-2-propanone mixture and purified by crystallization showing the identical retention times (HPLC, HRGC), UV−vis, and GC-MS data as the colorant detected in the Maillard mixture. The determination of its chemical structure was performed by one- and two-dimensional NMR spectroscopy. The ^{1}H NMR spectrum of the colorant measured in $CDCl_3$ showed two sets of eight resonance signals each in a ratio of about 7:1, demonstrating the

existence of two isomers. Further NMR data, fitting well with the structures **IIa** and **IIb**, are given in Tables 1 and 2. In the following, the structure determination of the predominating diastereomer **IIa** is explained in more detail.

Double-quantum-filtered homonuclear δ,δ-correlation spectroscopy (DQF-COSY) revealed two strongly coupled ^{1}H spin systems, confirming the presence of two furan ring systems in **IIa**. The singlets at 2.52 and 7.17 ppm were in the chemical shift range of an activated methyl group and an olefinic hydrogen, which could be assigned as H−C(1) and H−C(5) by heteronuclear multiquantum correlation (HMQC) and heteronuclear multiple-bond coherence experiments (HMBC). The ^{13}C NMR spectrum showed 13 signals (Table 2), which could be unequivocally assigned by an HMBC experiment corroborating the proposed structure of [*E*]- and [*Z*]-1,2-bis(2-furyl)-1-pentene-3,4-dione (**IIa**/**IIb**). The configuration of the C(4)═C(5) double bond was deduced from the chemical shift differences of the olefinic proton H−C(5), which was downfield shifted by 0.10 ppm in the (*Z*)-configured isomer **IIb**. Although the major isomer **IIa** was described earlier in the literature (Ledl, 1982), neither its importance in evoking the color of a Maillard mixture nor the ^{13}C NMR data and signal assignments of the ^{1}H NMR were documented earlier.

From fraction 31 a strongly fluorescent yellow compound was isolated by column chromatography, exhibiting an absorption maximum of 325 nm. This compound showed the identical retention times (RP-HPLC, HRGC), UV−vis, and MS data as found for a byproduct of the synthesis of **IIa**/**IIb** from furan-2-aldehyde and hydroxy-2-propanone. After isolation of this byproduct, its chemical structure was determined as 4,5-bis(2-furyl)-2-methyl-3*H*-furan-2-one (**III** in Figure 2) by ^{1}H NMR and MS spectroscopic measurements. For further confirmation of the proposed structure, 4,5-bis(2-furyl)-2-methyl-3*H*-furan-2-one was synthetically prepared by cyclization of 1,2-bis(2-furyl)-1-pentene-3,4-dione (**IIa**/**IIb**). A fluorescent yellow compound showing chromatographic and spectroscopic data identical to those of colorant **III** could be isolated from the Maillard mixture. To our knowledge, the structure of **III** has as yet not been reported in the literature.

These data imply that the colored [*E*]- and [*Z*]-1,2-bis(2-furyl)-1-pentene-3,4-dione (**IIa**/**IIb**) is not a stable end product of the Maillard reaction but can undergo further reactions to form additional types of chromophores. It was, therefore, assumed that in the reaction with the amino acid L-alanine, **IIa**/**IIb** might form additional chromophores potentially contributing to the overall color of the glucose/alanine mixture. To check this assumption, an aqueous solution of **IIa**/**IIb** was heated in the presence of L-alanine and the spectrum of colored reaction products was compared to that of the CDA of the Maillard mixture (Figure 1). Already after a short heating period, an orange compound was formed showing UV−vis and LC-MS data identical to those of an orange chromophore detected in fraction 7 (Figure 1) in the Maillard mixture. This colorant, exhibiting absorption maxima at 298 and 393 nm, was accompanied by a minor isomer with a nearly identical UV−vis spectrum. LC-MS measurements using negative APCI ionization showed an $[M + 1 - H_2]^-$ ion at *m*/*z* 318 (100%) and a loss of 18, most likely corresponding to the cleavage of a molecule of water, to an ion at *m*/*z* 300. The molecular mass of 317 Da clearly demonstrated the presence of one nitrogen atom in the colorant. These data and, in addition, the polarity of the colorant, led us to the suggestion that the amino acid moiety of L-alanine was incorporated into both isomers of the chromophore. After chromatographic isolation, their chemical structures were unequivocally determined by several 1D and 2D NMR experiments. The ^{1}H NMR spectra measured in DMSO-d_6 showed nine resonance signals each, confirming the existence of two diastereomeric forms. Further NMR data, fitting well with the structures **IVa** and **IVb**, are given in Tables 5 and 6. In the following, the structure determination of the predominating diastereomer **IVa** is explained in more detail. A total of two furan rings, each substituted at the 2-position, was deduced from the characteristic coupling pattern of the hydrogens H−C(7)/H−C(8)/H−C(9) and H−C(11)/H−C(12)/H−C(13). This was further confirmed by DQF-COSY as well as total correlated spectroscopy (TOCSY), indicating the expected strongly coupled ^{1}H spin system in the furan rings. In addition, these homonuclear δ/δ correlation techniques revealed a coupling of 7.1 Hz between the doublet at 1.26 ppm and a quartet at 4.57 ppm being consistent with the chemical shifts expected for the methyl group CH_3-(15) and the methine proton H−C(14) of the L-alanine moiety. In addition, a singlet resonating at 0.99 ppm was detected integrating for three protons and demonstrating the presence of the methyl group CH_3-(5). HMBC experiments optimized for $^2J_{C,H}$ and $^3J_{C,H}$ coupling constants (Table 6) revealed a correlation between this methyl group and a quaternary carbon atom assigned as C(4). A comparison of the ^{13}C NMR spectrum of the predominant isomer **IVa**, in which 20 signals appeared, with the results of the DEPT-135 experiment revealed 6 quarternary carbon atoms, which could be unequivocally assigned by means of HMBC spectroscopy (Table 6). In summary, the obtained spectroscopical data are consistent with the proposed structure of **IVa**/**IVb** as (*S*,*S*)- and (*S*,*R*)-2-[4,5-bis(2-furyl)-2-hydroxy-2-methyl-3(2*H*)-pyrrol-1-yl]propionic acid (Figure 2). To our knowledge, the intensely orange compound **IVa**/**IVb**, showing an extinction coefficient of 0.7×10^4 L mol^{-1} cm^{-1} at 393 nm (in water, pH 7.0), has previously not been reported in the literature.

For the identification of the key colorant in fraction 36 exhibiting an absorption maximum at 426 nm, the colored nonvolatile fraction was separated by column chromatography. Comparison of the spectroscopical data (LC-MS, UV−vis) and the retention times with those obtained from the synthetic reference compound (Hofmann, 1998) led to the unequivocal identification of the colorant as 2-[(2-furyl)methylidene]-4-hydroxy-5-[(*E*)-(2-furyl)methylidene]methyl-2*H*-furan-3-one (**V** in Figure 2). This colorant was reported earlier as a pentose-typical carbohydrate degradation product (Hofmann, 1998e), but its importance in contributing to the color of a hexose-containing Maillard mixture has as yet not been documented.

Although the most intensely colored compounds in fractions 7, 31, 32, 36, and 37 of the CDA (Figure 1) have been identified, it is possible that additional colorants of unknown structure coeluted in the effluents of these HPLC fractions. This would result in an overestimation of the CD factor and, consequently, also in the color contribution of the compounds **I**−**V**. To

Table 7. Concentrations, Detection Thresholds, and Color Activity Values (CAV) of Selected Colored Compounds in the Maillard Mixture

colorant[a]	concn (mg/kg)	detection threshold[b] (mg/kg of water)	CAV[c]
I	52.9	0.9	59
IIa/IIb	42.1	0.6	69
III	48.9	5.8	8
IVa/IVb	11.6	0.7	17
V	18.0	2.5	7

[a] The structures of the colorants are given in Figure 2. [b] The structures of the colorant are given in Figure 2. [c] CAV is calculated from the ratio of the concentration to the visual threshold (in water).

determine the contribution of the colorants more accurately, the amounts of compounds **I**−**V** were quantified in the browned Maillard reaction mixture.

Quantification of Colorants and Calculation of Color Activity Values (CAVs). To gain more detailed insights into the amounts of the color-active compounds and their color contribution, the reaction mixture was separated by HPLC and the colorants **I**−**V** were quantified by means of diode array detection using the reference compounds as external standards. The data, given in Table 7, showed that compound **I** was formed with 52.9 mg/kg in the highest concentrations, closely followed by the yellow compounds **IIa**/**IIb** and **III**. Colorant **IVa**/**IVb** was found in somewhat lower amounts; for example, 4.6-fold lower amounts were determined compared to colorant **I**.

Because the quantitative data alone do not allow an estimation of the importance of these colorants in evoking the total color of the browned Maillard mixtures, we, to gain insights into their color contribution, recently defined the CAV of a colorant as the ratio of its concentration to its detection threshold (Hofmann, 1998). To calculate the CAVs of the colorants **I**−**V** in the reaction mixture, first, the detection threshold of each colorant was determined in water using a triangle test (Table 7). As given in Table 7, the lowest threshold was found for colorant **IIa**/**IIb** with 0.6 mg/kg (water), followed by **IVa**/**IVb** with 0.7 mg/kg (water). Colorant **III** showed a nealy 10-fold higher detection threshold concentration compared to compound **IIa**/**IIb**.

Using the color activity concept, the colorants **I**−**V** were then ranked in their color activities as given in Table 7. The highest color activity was found for colorants **IIa**/**IIb**, closely followed by 3(6*H*)-pyranone **I** showing a 1.2-fold lower CAV. Also, colorant **IVa**/**IVb** was evaluated with a high color activity, because its concentration was 17-fold above its detection threshold. Despite the 4-fold higher concentration of colorant **III** in comparison to **IVa**/**IVb**, this colorant contributed not as much as **IVa**/**IVb** to the color, because its detection threshold was >8-fold higher.

Evaluation of the Color Contribution of Compounds I−V. To evaluate the percent contribution of each colorant in the overall color, the total color impact was measured by determination of the CD_{total} factor (Hofmann, 1998d). The complete Maillard mixture was, therefore, diluted step by step with water until no color difference between the diluted sample and two blanks containing tap water could just be detected visually using a triangle test. As given in Table 8, the reaction mixture was evaluated with a CD_{total} factor of 1000, which means that the color of the nondiluted mixture

Table 8. Contribution of Selected Colored Compounds to the Overall Color of the Browned Maillard Mixture

colorant[a]	color effectivity	contribution to total color (%)
I	59	5.9
IIa/IIb	69	6.9
III	8	0.8
IVa/IVb	17	1.7
V	7	0.7
$\sum(CAV_{I}-CAV_{V})$[b]	160	16.0
CD_{total} factor[c]	1000	100.0

[a] The structure of the colorant is given in Figure 2. [b] The color effectivity of the colorants **I**−**V** was calculated as the sum of their CAVs. [c] The color effectivity of the complete Maillard mixture was determined as the color dilution factor. [d] The color contribution of a compound x was calculated by using the following equation: color contribution (%) = $(CAV_x/CD_{total}) \times 100$ (Hofmann, 1998d).

was 1000-fold above its detection threshold. Because the CAV of a single colorant corresponds, by definition, to the factor to which the actual concentration is above the detection threshold, the percent color contribution of a single colorant can be estimated, as given in Table 8, from the CAV of the colorant and the CD_{total} factor of the Maillard mixture accounting for 100% color activity (Hofmann, 1998d). The data, given in Table 8, showed that colorants **IIa**/**IIb** and **I** contributed 6.9 and 5.9%, respectively, at most to the total color of the Maillard mixture. Also, compounds **III**, **IVa**/**IVb**, and **V** are significantly involved in evoking the color of the browned Maillard mixture because color contributions of 1.7, 0.8, and 0.7%, respectively, have been determined for these chromophores. Taking all of these data into consideration, it can be estimated that ∼16.0% of the overall color of the Maillard mixture accounted for only five types of key chromophores.

Formation of Colorants IIa/IIb, III, and IVa/IVb. These results demonstrate that the color activity concept, correlating quantitative data with the color detection threshold of a compound, is a powerful tool to identify the key chromophores contributing the most to the color of browned Maillard mixtures. The fact that only four key colorants account for 16.0% of the overall color of the reaction mixture indicates that the variety of precursors involved in chromophore formation might be limited in the nonenzymatic browning reaction involving carbohydrates and amino acids. This is well reflected by the finding that the carbohydrate degradation products furan-2-aldehyde and hydroxy-2-propanone are involved in the formation of the three colorants **IIa**/**IIb**, **III**, and **IVa**/**IVb**. As demonstrated by model experiments, colorant **IIa**/**IIb** was formed upon Aldol-type condensation reactions between furan-2-aldehyde and hydroxy-2-propanone. Also, the colorless monocondensation product **VI** (Figure 3), namely [*Z*]-1-hydroxy-1-[(2-furyl)methylidene]propan-2-one, could be successfully identified in a heated aqueous solution of these carbohydrate degradation products. On the basis of the experimental data, the reaction routes displayed in Figure 3 were proposed for the formation of **IIa**/**IIb**, **III**, and **IVa**/**IVb**. Upon thermal treatment in aqueous solution, Michael addition of one molecule of water, subsequent hemiketal formation, followed by a dehydration reaction gives rise to the intensely fluorescent yellow chromophore **III** (Figure 3). In the presence of primary amino acids such as L-alanine, Michael addition and cyclization form a colorless leuco base, which undergoes subsequent oxidation yielding the orange (*S*,*S*)- and (*S*,*R*)-2-[4,5-bis(2-furyl)-2-hydroxy-2-methyl-

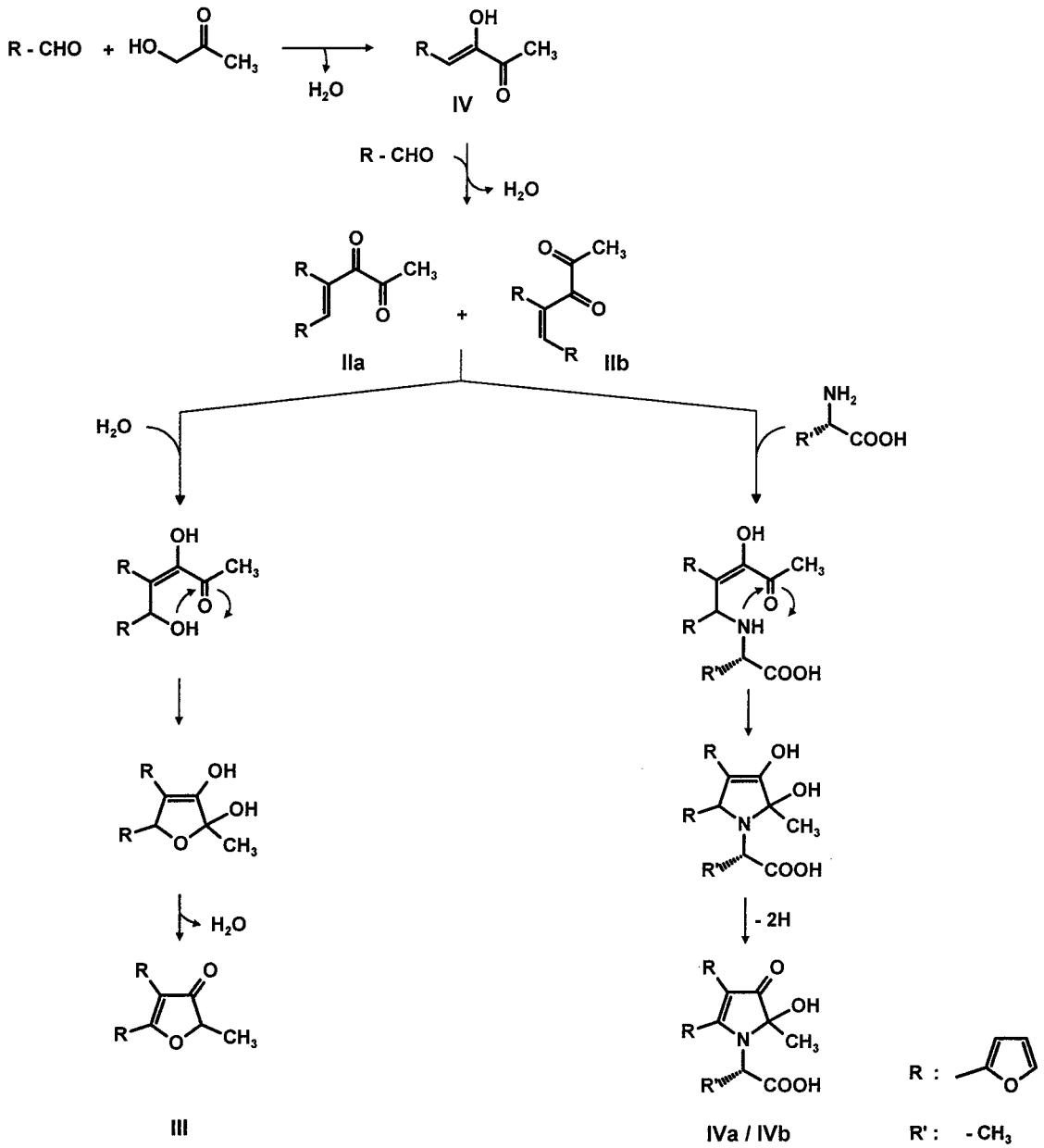

Figure 3. Propsed reaction pathways leading to the formation of colorants **IIa**/**IIb**, **III**, and **IVa**/**IVb**.

3(2H)-pyrrol-1-yl]propionic acid (**IVa**/**IVb**). Quantitative studies on model reactions with **IIa**/**IIb** and L-alanine performed in the absence or presence of air oxygen and/or copper ions, respectively, clearly pointed out that oxygen and/or transition metal ions are not involved in this oxidation step (data not shown). Either reactive Maillard reaction intermediates such as di- or tricarbonyls acting as redox partners or disproportionation reactions have, therefore, to be taken into consideration for this oxidation step, but this oxidation mechanism has to be clarified in future investigations.

Conclusions. Such systematic studies provide important information on nonenzymatic browning chemistry and will help to construct a route map of chromogenic reactions, on the basis of which color development during food processing might be understood in more detail. In future investigations, the characterization of additional chromophores and the determination of their color activities will gain further insights into the puzzling network of browning reactions of carbohydrates and amino acids.

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