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Characterization of the most intense coloured compounds from Maillard reactions of pentoses by application of colour dilution analysis

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Abstract

Thermal treatment of an aqueous solution of xylose and L-alanine in the presence of furan-2-carboxaldehyde, one of the major pentose dehydration products, led to a rapid colorization of the reaction mixture. To characterize the key chromophores formed, a screening procedure, which is based on the determination of the visual threshold of coloured high-performance liquid chromatography (HPLC) fractions, was developed to select the most intense coloured compounds in the complex mixture of Maillard reaction products. This so-called colour dilution analysis (CDA) revealed 20 coloured fractions, amongst which four fractions were evaluated with by far the highest colour impacts. The identification experiments were therefore focused on the compounds evoking the intense colour of these four fractions. Compound **12** was characterized as a mixture of the previously unknown orange-coloured (1*R*,8a*R*)- and (1*S*,8a*R*)-4-(2-furyl)-7-[(2-furyl)methylidene]-2-hydroxy-2*H*,7*H*,8a*H*-pyrano[2,3-*b*]pyran-3-one (**12a**/**12b**) by several 1D- and 2D-NMR techniques, liquid chromatography–mass spectrometry (LC–MS) as well as UV–Vis spectroscopies. The other three key chromophores **7**, **17** and **9** were identified as the yellow coloured 2-[2-furyl)methylidene]-4-hydroxy-5-methyl-2*H*-furan-3-one (**7**), the red coloured 2-[(2-furyl)methylidene]-4-hydroxy-5-[(*E*)-(2-furyl)methylidene]-methyl-2*H*-furan-3-one (**17**) and the red coloured (*S*)-4-[(*E*)-1-formyl-2-(2-furyl)ethenyl]-5-(2-furyl)-2-[(*E*)-(2-furyl)methylidene]-2,3-dihydo-α-amino-3-oxo-1*H*-pyrrole-1-acetic acid (**9**). © 1998 Elsevier Science Ltd. All rights reserved.

Keywords: 4-(2-Furyl)-7-[(2-furyl)methylidene]-2-hydroxy-2*H*,7*H*,8a*H*-pyrano [2,3-*b*]pyran-3-one; Maillard reaction; Coloured compounds; Colour dilution analysis, Colour dilution factor

1. Introduction

The Maillard reaction between reducing carbohydrates and compounds bearing an amino group is chiefly responsible for the development of desirable colours and flavours that occurs, e.g., during thermal processing of foods, such as roasting of meat, baking of bread, kiln-drying of malt or roasting of coffee. In spite of extensive studies, surprisingly little is known about the structures of the compounds responsible for the typical brown colour due to the complexity and multiplicity of the non-volatile Maillard reaction products formed [1].

It is, therefore, helpful to study suitable model systems to obtain detailed information on the chemical species of the chromophoric compounds involved in colour formation. Severin and Krönig [2] isolated a yellow coloured compound from a heated mixture of

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xylose and isopropylamonium acetate and reported that this compound was formed by a condensation reaction between the methylene-active 4-hydroxy-5-methyl-3(2H)-furanone and furan-2-carboxaldehyde. Based on the results of model experiments, some years later Ledl and Severin [3,4] supposed that the condensation reaction between methylene-active compounds and carbonyl compounds, both formed during carbohydrate degradation, might be a general type of reaction leading to colour development during the Maillard reaction. Most model reactions have been carried out in organic solvent, rather than in aqueous solution and, also, using synthetically related amines instead of amino acids. Because these amines do not occur in foods, it is questionable whether these results can be transferred onto browning reactions in real foods.

In the hope of shedding more light onto the complex mechanisms of the non-enzymatic browning, aqueous solutions of a carbohydrate degradation product and an amino acid were recently heated under food-relevant conditions and the main coloured compounds formed were isolated [5]. Intense red coloured 1*H*-pyrrol-3(2*H*)-ones were identified as the main coloured compounds formed upon thermal treatment of aqueous solutions of furan-2-carboxaldehyde, a well-known degradation product of pentoses, in the presence of L-alanine [5,6] as well as protein [7]. Labelling experiments revealed that, after an amine-catalysed ring-opening of the furan-2-carboxaldehyde, several condensation reactions between methylene-active intermediates and carbonyl compounds were involved in the formation of the chromophores.

It is well accepted in the literature that the amino acid-catalysed conversion of reducing carbohydrates during the Maillard reaction results in a tremendous variety of reactive carbonyl compounds such as, e.g., furan-2-carboxaldehyde, acetaldehyde, glycerinaldehyde, 2-oxopropanal or 1- and 3-deoxyosones, as well as methylene-active compounds. The large variety of these reactive intermediates leads, therefore, to the multiplicity and the low yields of the coloured Maillard reaction products, making their identification a big challenge. To clarify general reaction types involved in Maillard-type browning, it might be promising to heat aqueous solutions of reducing carbohydrates and amino acids in the presence of an excess amount of one carbohydrate derived carbonyl compound. Using this strategy, all methylene-active colour precursors are forced to react with the same aldehyde, offering the possibility to characterize the key types of chromophores in the non-enzymatic browning.

The objective of the following studies was, therefore, to identify the most intense coloured compounds formed from solutions of pentoses and amino acids, which were heated in the presence of an additional Maillard-derived carbonyl compound. Because furan-2-carboxaldehyde is known as one of the predominant dehydration products of pentoses [8,9], it was chosen for these experiments.

2. Results

Thermal treatment of a neutral aqueous solution of xylose and L-alanine in the presence of furan-2-carboxaldehyde led to a rapid colorization of the reaction mixture. Coloured reaction products were registered after separation of the non-volatile fraction by reversed phase high-performance liquid chromatography (RP-HPLC) using a diode array detector (DAD) monitoring in the wavelength range between 220 and 500 nm. To characterize the key chromophores, mainly evoking the colour of the Maillard mixture, we first screened the reaction products for the most intense coloured compounds, and then focused the identification experiments on the compounds, which were evaluated with the highest colour activities.

Screening for the most-intense coloured reaction products.—The non-volatile fraction of the heated pentose/L-alanine mixture consisted of a large variety of different reaction products, of which only a limited number of key chromophores were expected to evoke the colour of the Maillard mixture. To focus the identification experiments on these key colourants, it was necessary to distinguish between strongly colour-active compounds from

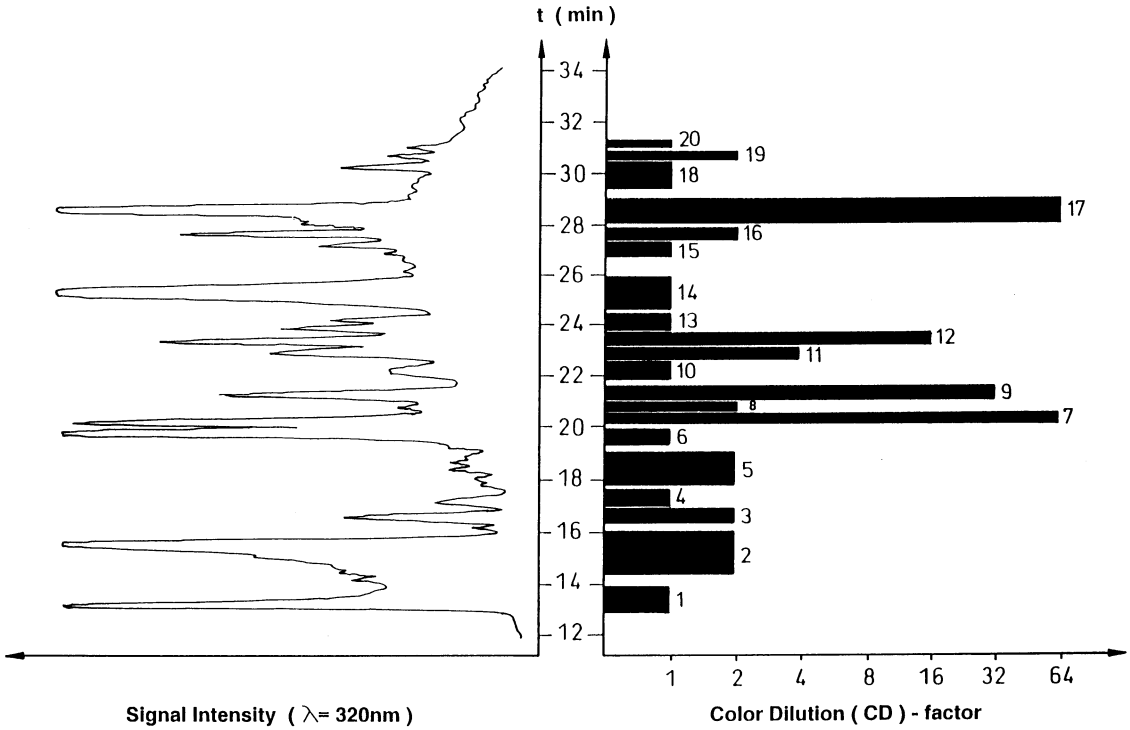


Fig. 1. RP-HPLC chromatogram (left side) and CD chromatogram (right side) of the solvent-extractable fraction of the Maillard mixture.

the less colour-active or colourless substances. Separation of the reaction products by HPLC and monitoring the effluent with diode array detection was, however, insufficient to meet this demand, because the UV–Vis absorption spectra obtained did not allow an evaluation of the colour impact of the reaction products formed.

To rank the coloured compounds in their colour impact, we therefore developed a screening technique, which we call colour dilution analysis (CDA). An aliquot of the reaction mixture was separated by RP-HPLC (Fig. 1, left side), the effluents of peaks exhibiting UV–Vis absorption above 320 nm being collected separately in one HPLC run. Twenty coloured fractions were obtained and diluted stepwise 1:1 with water. The dilution, at which a colour difference between the diluted fraction and two blanks (tap water) could just be visually detected, was defined as the colour dilution (CD) factor (Fig. 1; right side). The yellow coloured fraction no. **7** and the red coloured fraction no. **17** were evaluated with the highest colour impacts, followed by the red and orange coloured fraction no. **9** and no. **12**, respectively, showing somewhat lower colour activities (Fig. 1). The coloured compounds in these four fractions, therefore, mainly contribute to the colour of the Maillard mixture. The other 16 coloured fractions showed significantly lower CD factors and should, therefore, be only of minor importance for the colour of the reaction mixture. The other compounds detected at 320 nm in the HPLC chromatogram were colourless, because their concentration levels were lower than their visual thresholds (Fig. 1).

The striking advantage of this screening procedure is that the key chromophores can be located in complex Maillard mixtures without knowledge of their structures. The following experiments were then focused on the coloured compounds **12**, **7**, **17** and **9** mainly contributing to the colour of the heated carbohydrate/amino acid mixture.

Identification of the most-intense coloured reaction products.—For isolation of colourant no. **12**, exhibiting an absorption maximum at 460 nm, the coloured non-volatile fraction was separated in several chromatographic steps using silica gel and RP-18 material as the stationary phase. The determination of its chemical structure was performed by several

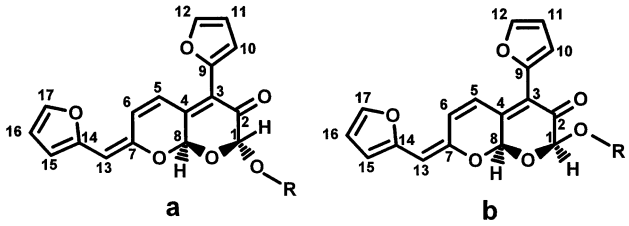


Fig. 2. Structures of (1*R*,8a*R*)- (**12a**) and (1*S*,8a*R*)-4-(2-furyl)-7-[(2-furyl)methylidene]-2-hydroxy-2H,7H,8aH-pyrano[2,3-*b*]-pyran-3-one (**12b**) as well as (1*R*,8a*R*)- (**12′a**) and (1*S*,8a*R*)-4-(2-furyl)-7-[(2-furyl)methylidene]-2-ethoxy-2H,7H,8aH-pyrano[2,3-*b*]pyran-3-one (**12′b**).

1D- and 2D-NMR techniques, and, in addition, by liquid chromatography–mass spectrometry (LC–MS), and UV–Vis spectroscopy. The spectroscopic data were consistent with structure **12**, given in Fig. 2, existing in the two diastereomers **12a** and **12b**.

LC–MS measurements gave an $[M^+ + 1]$ ion at m/z 313 and a base peak at m/z 295. The loss of 18, most likely corresponding to the cleavage of a molecule of H_2O, fitted well with the proposed hemi acetal structure **1**.

The ^{1}H NMR spectrum, measured in Me_2SO-d_6, showed two sets of 12 resonance signals each in a ratio of about 2:1, indicating the existence of two diastereomeric forms. Further NMR data, fitting well with the assignment of structures **12a** and **12b**, are given in Table 1. In the following, the structure determination of the predominating diastereomer **12a** is reported in more detail. Two furan rings substituted at the 2-position were deduced from the characteristic coupling pattern of the hydrogens resonating at 6.48, 6.75 and 7.64 ppm as well as at 6.50, 6.65 and 7.79 ppm. This was confirmed by gradient enhanced double-quantum-filtered δ/δ-correlation spectroscopy (GRASP DQF-COSY) as well as total correlated spectroscopy (TOCSY) indicating the expected strongly coupled ^{1}H-spin system in both the furan rings. In addition, these homonuclear δ/δ-correlation techniques revealed a coupling of 5.4 Hz between the duplets resonating at 7.47 and 7.56 ppm, which were assigned as the vicinal olefinic hydrogen atoms H–C(5) and H–C(6) incorporated in a cyclohexene system. Also, the chemical shift of a signal at 6.53 ppm was in the range expected for olefinic hydrogen atoms, but no coupling with other hydrogens could be observed. Heteronuclear multiple-bond coherence experiments (HMBC) optimized for ^2J(C,H) and ^3J(C,H) coupling

Table 1
Assignment of ^{1}H NMR signals (360 MHz, Me_2SO-d_6) of (1*R*,8a*R*)- and (1*S*, 8a*R*)-4-(2-furyl)-7-[(2-furyl)methylidene]-2-hydroxy-2*H*,7*H*,8a*H*-pyrano[2,3-*b*]pyran-3-ones (**12a**/**12b**)

H at relevant C atom[a]	δ (ppm)[b]		I[c]	M[c]	J (Hz)[c]	Connectivity[d] with
	1a	**1b**				
H–C-1	5.25	5.22	1	d	7.5/6.6	H–(OH)
H–C-8	6.22	6.10	1	s		
H–C-11	6.48	6.46	1	dd	3.5, 1.8	H–C(10), H–C(12)
H–C-15	6.50	6.50	1	d	3.5	H–C(16)
H–C-13	6.53	6.54	1	s		
H–C-16	6.65	6.66	1	dd	3.5,1.8	H–C(15), H–C(17)
H–C-10	6.75	6.63	1	d	3.5	H–C(11)
H–OH	7.14	7.18	1	d	7.5/6.6	H–C(1)
H–C-6	7.47	7.47	1	d	5.4	H–C(5)
H–C-5	7.56	7.56	1	d	5.4	H–C(6)
H–C-12	7.64	7.64	1	d	1.8	H–C(11)
H–C-17	7.79	7.79	1	d	1.8	H–C(16)

[a] Numbering of carbon atoms refers to formulae **12a** and **12b** in Fig. 2.
[b] The ^{1}H chemical shifts are given in relation to Me_2SO-d_6.
[c] Determined from 1D spectrum.
[d] Observed homonuclear ^{1}H, ^{1}H connectivities by GRASP-DQF-COSY and TOCSY.

Table 2
Assignment of ^{13}C NMR signals (360 MHz, Me_2SO-d_6) of (1*R*,8a*R*)- and (1*S*, 8a*R*)-4-(2-furyl)-7-[(2-furyl)methylidene]-2-hydroxy-2*H*,7*H*,8a*H*-pyrano[2,3-*b*]pyran-3-ones (**12a**/**12b**)

H at relevant C atom[a]	δ (ppm)[b]		DEPT[c]	Heteronuclear $^{1}H,^{13}C$ multiple-quantum coherence[d]	
	1a	**1b**		via ^{1}J(C, H)	via $^{2,3,4}J$(C, H)
C-8	71.4	71.4	CH	H–C(8)	H–C(1), H–C(5)
C-1	94.9	95.5	CH	H–C(1)	H–(OH), H–C(8)
C-13	100.6	100.6	CH	H–C(13)	H–C(6)
C-3	103.3	103.6	C		H–C(1), H–C(5), H–C(8)
C-10	109.7	109.0	CH	H–C(10)	H–C(11), H–C(12)
C-11	110.4	110.5	CH	H–C(11)	H–C(10), H–C(12)
C-16	113.3	113.3	CH	H–C(16)	H–C(15), H–C(17)
C-15	114.4	114.3	CH	H–C(15)	H–C(13), H–C(16), H–C(17)
C-5	122.9	123.0	CH	H–C(5)	H–C(6), H–C(8)
C-6	135.8	135.7	CH	H–C(6)	H–C(5), H–C(13)
C-12	143.3	142.8	CH	H–C(12)	H–C(10), H–C(11)
C-17	144.7	144.7	CH	H–C(17)	H–C(15), H–C(16)
C-14	149.0	149.1	C		H–C(13), H–C(15), H–C(16), H–C(17)
C-7	152.0	152.0	C		H–C(5), H–C(6), H–C(13)
C-9	152.8	153.9	C		H–C(10), H–C(11), H–C(12)
C-4	163.0	163.0	C		H–C(5), H–C(6), H–C(8)
C-2	196.6	196.6	C		H–C(1)

[a] Numbering of carbon atoms refers to formulae **12a** and **12b** in Fig. 2.
[b] The ^{13}C chemical shifts are given in relation to Me_2SO-d_6.
[c] DEPT-135 spectroscopy.
[d] Assignments based on HMQC (^{1}J) and HMBC ($^{2,3}J$) experiments.

constants (Table 2), however, revealed a correlation between this olefinic hydrogen assigned as H–C(13) and the furan carbon atoms C(14) and C(15). These data demonstrate clearly that one furan ring is directly linked to an methylidene group, as proposed for structure **12**.

A comparison of the ^{13}C NMR spectrum, in which 17 signals appeared, with the results of the DEPT-135 experiment revealed six signals corresponding to quarternary carbon atoms (Table 2). Unequivocal assignment of these quarternary carbon atoms could be successfully achieved by means of HMBC optimized for ^{2}J(C,H) and ^{3}J(C,H) coupling constants (Table 2). Heteronuclear correlations between the quarternary carbon atom resonating at 152.0 ppm with C(5), C(6) and C(13) led to its unequivocal assignment as C(7). Further correlations were observed between H–C(6) and C(5) as well as the quarternary carbon atom C(4).

CH-Coupling between the olefinic hydrogen H–C(5) and the quarternary atom C(3) connected with the second furan ring, as well as between H–C(5) and a carbon atom resonating at 71.4 ppm [C(8)] in the ^{13}C NMR spectrum, enabled a more detailed insight into the structure of **12a**. Beside the coupling between C(8) and H–C(5), an additional correlation was found with a signal resonating at 5.25 ppm in the ^{1}H NMR spectrum. Signals in this ppm range were reported in the literature for acetal protons in 2-methoxy-2*H*-pyran-3-one derivatives [10]. Because the signal at 5.25 ppm showed homonuclear coupling with a hydroxyl proton resonating at 7.14 ppm, and, in addition, a weak heteronuclear coupling with the carbonyl carbon atom C-2, it was assigned as the proton H–C-1 of the hemi acetal proposed as partial structure in **12a**. The disappearance of the duplet at 7.14 ppm of HO–C-1 and, in addition, the reduction of the doublet for H–C-1 to a singlet upon H/D-exchange upon adding trace amounts of D_2O confirmed the proposed hemi acetal structure in **12a**. The finding that the atoms C-1 and C-8 are bridged by an oxygen atom fitted well with the absence of a homonuclear coupling between the adjacent hydrogens H–C-1 and H–C-8.

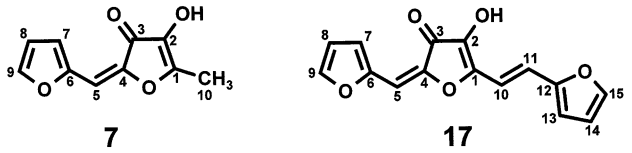
Both the chemical shift of the carbon C-8 and the adjacent proton H–C-8, are, however, not in the range expected for an acetal group. The strong high-field shift for C(8) and, in contrast, the down-field shift for the H–C-8 might be due to the steric environment of the acetal group in the stressed ring system of the proposed structure **12a**. Such a destabilization of a ring system, e.g., in the sugar moiety of a glycoside, was found by Perlin et al. [11] to promote a bond polarization leading to an increased shielding of the ^{13}C nucleus accompanied by a decrease in the shielding of the appended proton, e.g., ^{13}C and ^{1}H shifts are affected inversely. The proposed (*R*)-configuration at C-8 was evidenced by molecular mechanics calculations revealing that the (*S*)-configuration is, due to its higher energy, not very likely.

The assignment of the chirality at C-1 was performed from the NMR data using the findings of Perlin et al. [11] that the ^{1}H and the ^{13}C chemical shifts of the anomeric centre in carbohydrates are strongly influenced by configurational and conformational influences. Perlin et al. [11] could show that inversion of an equatorial into an axial anomer leads to a destabilization of the system resulting in a strong high-field shift of the adjacent carbon atom and an opposite down-field shift of the acetal hydrogen. Thus, the conversion of the equatorial (**12b**) into the axial position (**12a**) of the hydroxyl group in structure **12** is evidenced at the anomeric centre C(1) by an increase in the shielding of the carbon atom [$\Delta\delta(^{13}C) = +0.6$ ppm] and a decrease in shielding of the adjacent proton [$\Delta\delta(^{1}H) = -0.03$ ppm]. This is also well in line with the fact that axial protons are usually more shielded than is an equatorial one [12]. Also the C-1–O bond is affected by the changes and, in turn, the O–H bond [($\Delta\delta(^{1}H) = +0.04$ ppm]. The axial C-1–O bond becomes less polarized, because the carbon nucleus C-1 is less positive contributing to the fact that the difference in hydroxyl proton shifts [13,14] is such that the axial C–O bond bears the more shielded (less positive) proton. On the basis of these data, the (*R*)- and the (*S*)-configurations were assigned for C-1 in **12a** and **12b**, respectively. The predominance of the (1*R*,8a*R*)-diastereomer **12a**, in addition, was confirmed by molecular mechanics calculations.

The proposed hemi acetal structure was further confirmed by heating an ethanolic solution of **12a**/**12b** in the presence of trace amounts of hydrochloric acid leading to the corresponding 4-(2-furyl)-7-[(2-furyl)methylidene]-2-ethoxy-2*H*,7*H*,8a*H*-pyrano[2,3-*b*]pyran-3-ones (**12′a**/**12′b** in Fig. 2), which were identified as a 3:1 mixture of two diastereomers by ^{1}H NMR, MS and UV–Vis spectroscopy.

In summary, the obtained spectroscopical data are consistent with the proposed structure of **12a** and **12b** as (1*R*,8a*R*)- and (1*S*,8a*R*)-4-(2-furyl)-7-[(2-furyl)methylidene]-2-hydroxy-2*H*,7*H*,8a*H*-pyrano[2,3-*b*]pyran-3-one (Fig. 2). To our knowledge, this intense orange coloured compound **12a**/**12b** showing an extinction coefficient of 1.0×10^4 l mol^{-1} cm^{-1} (in water, pH 7.0) has not been previously described in the literature.

For the identification of the colourants no. **7** and no. **17**, exhibiting an absorption maximum at 360 and 426 nm, respectively, the coloured non-volatile fraction was separated by column chromatography on silica gel or flash chromatography on RP-18 material. The determination of their chemical structures was performed by NMR, LC–MS, and UV–Vis spectroscopy. The ^{1}H NMR spectrum of compound no. **7** measured in Me_2SO-d_6 showed five resonance signals. Double-quantum-filtered homonuclear δ,δ-correlation spectroscopy (DQF-COSY) revealed a strongly coupled ^{1}H spin systems, indicating the presence of one furan ring system in **7**. The singlets at 2.29 and 6.64 ppm were in the chemical shift range of a methyl group and an olefinic hydrogen, which could be assigned as H–C-10 and H–C-5 by heteronuclear correlation experiments (HMQC, HMBC). The ^{13}C NMR spectrum showed ten signals, which could be unequivocally assigned by an HMBC experiment corroborating the proposed (2-furyl)methylidene ring connected to the 2*H*-furan-3-one structure in Fig. 4. These data, as well as the results of LC–MS measurements, showing a molecular ion at m/z 193, were consistent with structure **7** in Fig. 3. The proposed structure was further confirmed by



7 17

Fig. 3. Structures of 2-[(2-furyl]methylidene]-4-hydroxy-5-methyl-2*H*-furan-3-one (**7**) and 2-[(2-furyl)methylidene]-4-hydroxy-5-[(E)-(2-furyl)methylidene]-methyl-2*H*-furan-3-one (**17**).

synthesis from 4-hydroxy-5-methyl-2*H*-furan-3-one and furan-2-carboxaldehyde. Colourant no. **17**, which was isolated and purified by chromatographic procedures, was unequivocally identified as 2-[(2-furyl)methylidene]-4-hydroxy-5-[(*E*)-(2-furyl)methylidene]-methyl-2*H*-furan-3-one (Fig. 3) by comparison of the spectroscopic data and the retention times with those obtained from the synthetic reference compound prepared by reacting colourant no. **7** with additional furan-2-carboxaldehyde. Both colourants, **7** and **17**, were described earlier in the literature [3], however, neither their importance in evoking the colour of a Maillard mixture has as yet been documented, nor the ^{13}C NMR data and signal assignments of the ^{1}H NMR reported.

In addition, colourant no. **9**, exhibiting absorption maxima at 330, 414 and 480 nm, was identified as the red coloured (*S*)-4-[(*E*)-1-formyl-2-(2-furyl)ethenyl]-5-(2-furyl)-2-[(*E*)-(2-furyl)methylidene]-2,3-dihydo-α-amino-3-oxo-1*H*-pyrrole-1-acetic acid (Fig. 4). Details on the isolation and identification will be reported elsewhere [15].

3. Discussion

CDA has been demonstrated as a suitable screening technique to locate coloured compounds in complex Maillard reaction mixtures and to rank them in their colour impacts, thus offering the possibility to evaluate the most intense colourants, on which the identification experiments can then be focused. Using this strategy, compounds **12a**/**12b** (Fig. 2), **7** (Fig. 3), **17** (Fig. 3) and **9** (Fig. 4) have been established as the main coloured compounds evoking the colour of the solvent-extractable fraction of the xylose/L-alanine mixture heated in the presence of furan-2-carboxaldehyde.

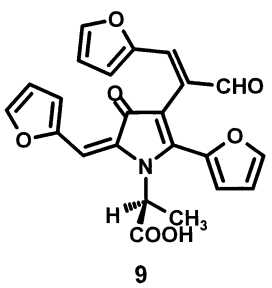


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Fig. 4. Structures of (*S*)-4-[(*E*)-1-formyl-2-(2-furyl)ethenyl]-5-(2-furyl)-2-[(*E*)-(2-furyl) methylidene]-2,3-dihydo-α-amino-3-oxo-1*H*-pyrrole-1-acetic acid (**9**).

Synthetic studies could show that methylene-active compounds such as 4-hydroxy-5-methyl-2*H*-furan-3-one react with several carbonyl compounds to form a variety of coloured reaction products with the same type of chromophore, differing only in the substituents [3]. The furan rings in **12a**/**12b**, **7**, **17** and **9** might be, therefore, also substituted by other alkyl or aryl residues of carbonyl compounds derived from carbohydrate degradation, e.g., acetaldehyde, glycerinaldehyde, 2-oxopropanal or deoxyosones. Thus, also a wide range of coloured 2-hydroxy-2*H*,7*H*,8a*H*-pyrano[2,3-*b*]pyran-3-ones can be assumed to be formed from pentoses.

The colorants **7** and **17** could be shown to be formed from the condensation reaction of the carbohydrate degradation products furan-2-carboxaldehyde and 4-hydroxy-5-methyl-2*H*-furan-3-one fitting well with data of the literature [8]. As displayed in Fig. 5, the amino acid-induced dehydration of the pentose (I) via the Amadori product (II) as the key intermediate results in the formation of the 1-deoxy-2,3-pentodiulose (IIIa) and the isomeric 3-deoxy-2-pentosulose (VIa). Ring closure and water elimination then give rise to 4-hydroxy-5-methyl-2*H*-furan-3-one (IIIb) as well as furan-2-carboxaldehyde (IVb). A condensation reaction between the methylene-active intermediate IIIb and the carbonyl function of IVb then produces the 2-[(2-furyl]methylidene]-4-hydroxy-5-methyl-2*H*-furan-3-one (**7**). Condensation of the methyl group of **7** with an additional molecule of IVb leads to the formation of 2-[(2-furyl)methylidene]-4-hydroxy-5-[(*E*)-(2-furyl)methylidene]-methyl-2*H*-furan-3-one (**17**).

Earlier model studies revealed that the colourant **9** was formed from the reaction

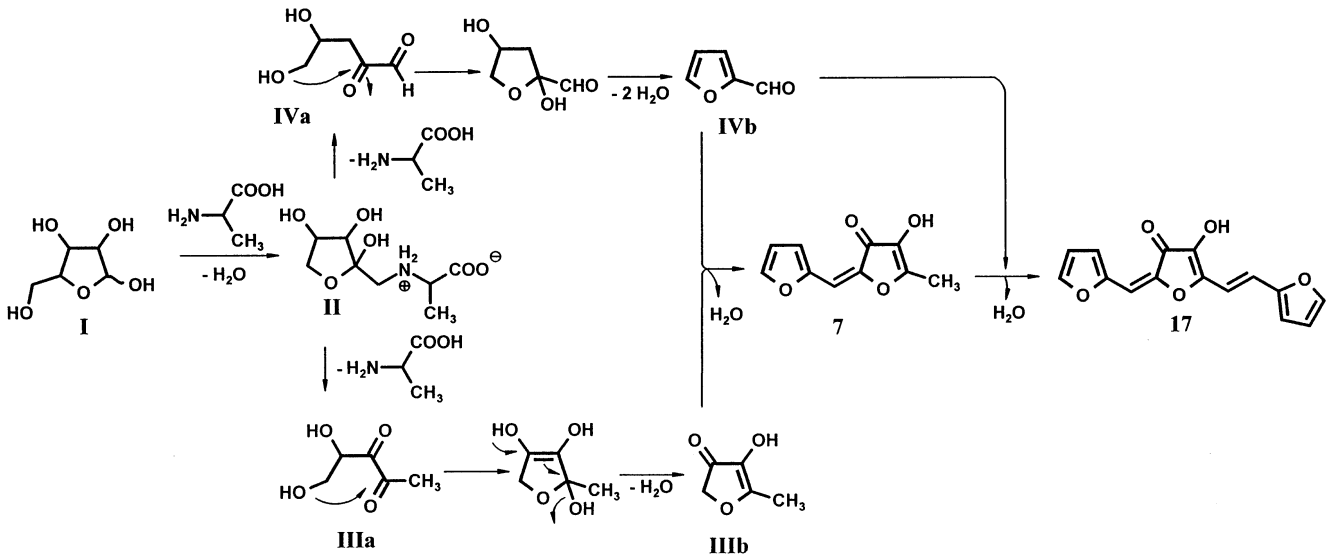

Fig. 5. Formation pathways leading to coloured compounds **7** and **17** from pentoses and L-alanine.

between furan-2-carboxaldehyde and L-alanine [5], however, very recent investigations indicated clearly that, in the presence of L-alanine, **9** is not exclusively formed via furan-2-carboxaldehyde as the precursor, but even more effectively from another intermediate of the 3-deoxyosone pathway. The results of quantitative studies on the formation of this colourant will be published elsewhere [15].

The precursors of the previously unknown colourant **12a**/**12b** are not obvious from its structure, however, due to its high colour activity, have to be clarified. Quantitative measurements determining the effectivity of several precursors, which are very likely involved in the formation of **12a**/**12b** and, in addition, ^{13}C-labelling experiments revealed 3-deoxy-2-pentosulose, hydroxyacetaldehyde and furan-2-carboxaldehyde as the penultimate precursors. Details on these studies are published in the following paper [16].

4. Experimental

General methods.—Column chromatography was performed with a water-cooled glass column (35 × 450 mm) filled with silica gel (200 g, Silica Gel 60, E. Merck, Darmstadt, Germany). Flash chromatography (16 × 200 mm) was performed using an RP-18 stationary phase (15.0 g; Lichroprep 25–40 μm, E. Merck, Darmstadt, Germany) using the following solvent systems: (A) 7:3 MeOH–water; (B) 4:1 MeCN–aq trifluoracetic acid (TFA) (0.1% TFA in water). Preparative thin layer chromatography (TLC) was performed on silica gel (20 × 20 cm; 0.5 mm; E. Merck, Darmstadt, Germany) using the following solvent systems: (A) 1:1 toluene–EtOAc; (B) 4:1 toluene–EtOAc.

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 material (ODS-Hypersil, 5 μm, 10 nm, Shandon, Frankfurt, FRG) in an analytical scale (4.6 × 250 mm, flow rate 0.6 mL/min) using the following solvent gradient: starting with a mixture (1:9) of MeCN and aq ammonium formiate buffer (20 mmol/L; pH 3.5), the MeCN content was increased to 100% within 40 min.

An analytical HPLC column (Nucleosil 100-5C18, Macherey and Nagel, Dürren, Germany) was coupled to an LCQ–MS (Finnigan MAT GmbH, Bremen, Germany) using electrospray ionization (ESI). After injection of the sample (2.0 μL) analysis was performed using a gradient starting with a mixture (1:9) of MeCN and water and increasing the acetonitrile content to 100% within 15 min.

UV–Vis spectra were obtained using a U-2000 spectrometer (Colora Messtechnik Gmbh, Lorch, Germany).

^{1}H, ^{13}C, DEPT-135, DQF-COSY, TOCSY, HMQC and HMBC NMR experiments were performed on a Bruker-AC-200 and a Bruker-AM-360 spectrometer (Bruker, Rheinstetten, Germany) at 297 K using the aquisition parameters described recently [6]. Tetramethylsilane (TMS) was used as the internal standard.

Molecular mechanics calculations were performed using an MM3 force field (Alchemy III).

Materials.—D-Xylose, L-alanine, furan-2-carboxaldehyde, piperidine, TFA, acetic acid were obtained from Aldrich (Steinheim, Germany). Furan-2-carboxaldehyde was freshly distilled prior to use. Solvents were HPLC-grade (Aldrich, Steinheim, Germany). Me_2SO-d_6 and D_2O were obtained from Isocom (Landshut, Germany). 4-Hydroxy-5-methyl-2*H*-furan-3-one was prepared following closely a procedure described recently [9].

Maillard reaction mixture.—A solution of D-xylose (1.34 mol) and L-alanine (0.32 mol) in phosphate buffer (1600 mL; 1 mmol/L, pH 7.0) was heated under reflux for 15 min, then, furan-2-carboxaldehyde (2 mol) was added and heating was continued for another 60 min. After cooling to room temperature, the aqueous solution was extracted with EtOAc (10×300 mL), the organic layer was dried over Na_2SO_4 and concentrated at 25 °C under vacuum (100 mbar) to 100 mL. This colour extract was used for the CDA as well as for the identification experiments.

Screening for intense coloured compounds by colour dilution analysis.—An aliquot (2 mL) of the colour extract of the Maillard reaction mixture was freed from the volatile fraction by high vacuum distillation (0.04 mbar, 30 °C), the residue was dissolved in MeOH (10 mL) and an aliquot (100 μL) was analysed by HPLC. The effluents of peaks exhibiting absorption maxima above 320 nm were collected separately in glass vials and were then made up with water to exactly 1 mL. The coloured fractions were then stepwise 1:1-diluted with water and each dilution was visually judged until a colour difference between the diluted fraction in a glass vial (10 mm i.d.) and two blanks (tap water) could just be visually detected. This dilution was defined as the CD factor.

*Isolation of (1*R,*8a*R*)-4-(2-furyl)-7-[(2-furyl)methylidene] - 2 - hydroxy - 2*H,*7*H,*8a*H-*pyrano[2,3*-b*]pyran-3-one* (**12a**) *and its (1*S,*8a*R*)-diastereomer* (**12b**) *from the Maillard mixture.*—After removing the volatile fraction of an aliquot (98 mL) of the colour extract by high vacuum distillation (0.04 mbar, 35 °C), the intense coloured residue was dissolved in toluene and aliquots were then fractionated by column chromatography on silica gel. After application of the raw material onto the column conditioned with toluene, chromatography was performed using toluene (Fraction A; 500 mL), followed by toluene–EtOAc (4:1; Fraction B; 300 mL), toluene–EtOAc (7:3; Fraction C, 300 mL), toluene–EtOAc (3:2; Fraction D; 300 mL) and toluene–EtOAc (1:1; Fraction E; 300 mL). Fraction E was collected and further subfractionated by column chromatography on silica gel. The fraction was concentrated and applied onto water-cooled silica gel conditioned with toluene. After flushing with toluene (200 mL), followed by toluene–EtOAc (9:1, 200 mL), elution with toluene–EtOAc (1:1, 400 mL) afforded a fraction containing a deep orange-coloured compound, which was freed from solvent under vacuum and dissolved in MeOH (1 mL). The coloured compound was further purified by RP-18 flash chromatography using the solvent system B. An orange coloured fraction was collected, freed from MeOH under vacuum, the aqueous phase was extracted with EtOAc (3×20 mL) and, after drying over Na_2SO_4, the orange colorant was isolated in 98% purity by preparative TLC using solvent system A as the eluent. An orange coloured band at $R_f = 0.4$ was scraped off, dissolved in MeOH (20 mL), filtered and the

solvent was evaporated to dryness affording **12** as an intense red–orange solid (0.19 mmol). The NMR data are given in Tables 1 and 2; LC–MS: *m/z* 295 (100, $[M+1-H_2O]^+$), 313 (19, $[M+1]^+$); UV–Vis (water, pH 7.0): $\lambda_{max}=460$ nm, $\varepsilon=1.2\times10^4$ L mol^{-1} cm^{-1}. ^{1}H NMR (360 MHz; Me_2SO-d_6 containing 5% D_2O; DQF-COSY; arbitrary numbering of the carbon atoms refers to formulae **12a** and **12b** in Fig. 2) of **12a**/**12b**: 5.26/5.23 (s, 1H, H–C(1)), 6.23/6.10 (s, 1H, H–C(8)), 6.48/6.46 (dd, 1H, $^3J_{11,10}=3.54$ Hz, $^3J_{11,12}=1.77$ Hz, H–C(11)), 6.50/6.50 (d, 1H, $^3J_{15,16}=3.54$ Hz, H–C(15)), 6.54/6.55 (s, 1H, H–C(13)), 6.65/6.67 (dd, 1H, $^3J_{16,15}=3.54$ Hz, $^3J_{16,17}=1.77$ Hz, H–C(16)), 6.73/6.64 (d, 1H, $^3J_{10,11}=3.54$ Hz, H–C(10)); 7.48/7.48 (d, 1H, $^3J_{6,5}=5.4$ Hz, H–C(6)), 7.57/7.57 (d, 1H, $^3J_{5,6}=5.4$ Hz, H–C(5)), 7.64 (d, 1H, $^3J_{12,11}=1.77$ Hz, H–C(12)), 7.80 (d, 1H, $^3J_{17,16}=1.77$ Hz, H–C(17)).

*Preparation of (1*R,*8a*R*) - 4 - (2 - furyl) - 7 - [(2-furyl)methylidene] - 2 - ethoxy - 2*H,*7*H,*8a*H-*pyrano[2,3*-b*]pyran-3-one* (**12′a**) *and its (1*S,*8a*R*)-diastereomer* (**12′b**).—**12a**/**12b** (0.1 mmol) was dissolved in EtOH (10 mL), concd HCl (5 μL) was added and the mixture was then refluxed for 15 min. The coloured compound formed was isolated by TLC on silica gel using solvent system B. The yellow band at $R_f=0.55$–0.65 was scraped off and dissolved in EtOAc. After filtration, the solvent was removed in vacuo affording **12′a**/**12′b** as a red–orange crystalline residue (0.08 mmol, 88% in yield). ^{1}H NMR (360 MHz; Me_2SO-d_6; DQF-COSY; arbitrary numbering of the carbon atoms refers to formulae **12′a** and **12′b** in Fig. 2) of **12′a**/**12′b**: 1.19/1.15 (t, 3H, $^3J_{19,18}=7.07$ Hz, H–C(19)), 4.03/3.96 (q, 3H, $^3J_{18,19}=7.07$ Hz, H–C(18)), 5.19/5.11 (s, 1H, H–C(1)), 6.26/6.20 (s, 1H, H–C(8)), 6.49/6.47 (dd, 1H, $^3J_{11,10}=3.54$ Hz, $^3J_{11,12}=1.77$ Hz, H–C(11)), 6.50/6.54 (d, 1H, $^3J_{15,16}=3.54$ Hz, H–C(15)), 6.55/6.57 (s, 1H, H–C(13)), 6.66/6.67 (dd, 1H, $^3J_{16,15}=3.54$ Hz, $^3J_{16,17}=1.77$ Hz, H–C(16)), 6.78/6.62 (d, 1H, $^3J_{10,11}=3.54$ Hz, H–C(10)); 7.51/7.51 (d, 1H, $^3J_{6,5}=5.4$ Hz, H–C(6)), 7.55/7.55 (d, 1H, $^3J_{5,6}=5.4$ Hz, H–C(5)), 7.65/7.66 (d, 1H, $^3J_{12,11}=1.77$ Hz, H–C(12)), 7.80/7.81 (d, 1H, $^3J_{17,16}=1.77$ Hz, H–C(17)); LC–MS: *m/z* 295 (100, $[M+1-EtOH]^+$), 341 (32, $[M+1]^+$); UV–Vis (water, pH 7.0): $\lambda_{max}=460$ nm, $\varepsilon=1.1\times10^4$ L mol^{-1} cm^{-1}.

*Isolation of 2-[(2-furyl)methylidene]-4-hydroxy-5-methyl-2*H*-furan-3-one* (**7**) *from the Maillard mixture.*—Removing the solvent from fraction C obtained from the column chromatography described above for the isolation of compound **12** revealed red–orange crystals of compound **7** (1.3 mmol; 0.1% in yield). ^{1}H NMR (360 MHz; Me_2SO-d_6; DQF-COSY; arbitrary numbering of the carbon atoms refers to formula **7** in Fig. 3): 2.29 (s, 3H, H–C(10)), 6.64 (s, 1H, H–C(5)), 6.72 (dd, 1H, $^3J_{7,8}=3.54$ Hz, $^3J_{7,9}=1.77$ Hz, H–C(8)), 7.07 (d, 1H, $^3J_{7,8}=3.54$ Hz, H–C(7)), 7.95 (d, 1H, $^3J_{9,8}=1.77$ Hz, H–C(9)); ^{13}C NMR (360 MHz; Me_2SO-d_6; DEPT, HMQC, HMBC): δ 12.2 (CH_3, C(10)), 99.4 (CH, C(5)), 113.2 (CH, C(8)), 117.6 (CH, C(7)), 135.4 (C, C(1)), 141.8 (C, C(4)), 146.4 (CH, C(9)), 147.8 (C, C(6)), 162.0 (C, C(2)), 180.5 (C, C(3)); LC–MS: 193 (100; $[M+1]^+$); UV–Vis (water): $\lambda_{max}=360$ nm, $\varepsilon=0.5\times10^4$ L mol^{-1} cm^{-1}.

*Isolation of 2-[(2-furyl)methylidene]-4-hydroxy - 5 - [(*E*) - (2 - furyl)methylidene] - methyl-2*H*-furan-3-one* (**17**) *from the Maillard mixture.*—Fraction D obtained from the column chromatography described above for the isolation of compound **12** was subfractionated by RP-18 flash chromatography using solvent system B as the mobile phase. After 90 mL, the effluent containing a deep-red colourant was collected, freed from acetonitrile under vacuum and was then extracted with EtOAc (3×20 mL). The combined organic layers were dried over Na_2SO_4, the solvent was evaporated and the residue was taken up in a minimum amount of MeOH and the solution was then stored at -30 °C, affording colourant **17** as dark red crystals (2.6 mmol; 0.2% in yield). ^{1}H NMR (360 MHz; Me_2SO-d_6; DQF-COSY; arbitrary numbering of the carbon atoms refers to Formula **17** in Fig. 3): 6.54 (s, 1H, H–C(5)), 6.61 (dd, 1H, $^3J_{14,13}=3.54$ Hz, $^3J_{14,15}=1.77$ Hz, H–C(14)),)), 6.76 (dd, 1H, $^3J_{8,7}=3.54$ Hz, $^3J_{8,9}=1.77$ Hz, H–C(8)), 6.79 (d, 1H, $^3J_{13,14}=3.54$ Hz, H–C(13)), 7.06 (d, 1H, $^3J_{10,11}=15.93$ Hz, H–C(10)); 7.11 (d, 1H, $^3J_{11,10}=15.93$ Hz, H–C(11)), 7.19 (d, 1H, $^3J_{7,8}=3.54$ Hz, H–C(7)),)), 7.76 (d, 1H, $^3J_{15,14}=1.77$ Hz,

H–C(15)), 7.94 (d, 1H, $^3J_{9,8} = 1.77$ Hz, H–C(9)); ^{13}C NMR (360 MHz; Me_2SO-d_6; DEPT, HMQC, HMBC): δ 97.9 (CH, C(5)), 111.5 (C, C(10)), 112.3 (CH, C(14)), 112.7 (CH, C(13)), 113.4 (CH, C(8)), 116.5 (CH, C(7)), 116.7 (CH, C(11)), 142.5 (C, C(1)), 143.3 (C, C(4)), 144.4 (CH, C(15)), 145.7 (CH, C(9)), 148.4 (C, C(6)), 152.6 (C, C(12)), 155.2 (C, C(2)), 180.4 (C, C(3)); LC–MS: 271 (100; $[M+1]^+$); UV–Vis (water): $\lambda_{max} = 426$ nm, $\varepsilon = 1.1 \times 10^4$ L mol^{-1} cm^{-1}.

*Synthesis of 2-[(2-furyl)methylidene]-4-hydroxy-5-methyl-2*H*-furan-3-one* (**7**).—According to a procedure in Ref. [3] a solution of 4-hydroxy-5-methyl-2*H*-furan-3-one (10 mmol), furan-2-carboxaldehyde (10 mmol), piperidine (0.2 mL) and acetic acid (0.2 mL) in an EtOH–water mixture (30 mL; 1:1) was heated for 30 min at 50 °C. After evaporation of the ethanol under vacuum (30 mbar) at 35 °C, the reaction mixture was adjusted to pH 5.0 with aq HCl (0.1 mol/L) and then extracted with EtOAc (3×10 mL). After drying over Na_2SO_4, the organic layer was concentrated in vacuo to about 1 mL. Storing at −30 °C afforded red–orange crystals showing identical spectroscopical data as colourant **7** isolated from the Maillard mixture (2.1 mmol; about 21% in yield).

*Synthesis of 2-[(2-furyl)methylidene]-4-hydroxy-5-[(*E*)-(2-furyl)methylidene]-methyl-2*H*-furan-3-one* (**17**).—A mixture of **7** (2 mmol), furan-2-carboxaldehyde (2 mmol), piperidine (0.05 mL) and acetic acid (0.05 mL) in an EtOH–water mixture (10 mL; 1:1) was heated for 30 min at 50 °C. After removing the solvent in vacuo (30 mbar) at 35 °C, the reaction mixture was adjusted to pH 5.0 with aq HCl (0.1 mol/L) and then extracted with EtOAc (3×10 mL). After drying over Na_2SO_4, the compound **17** was isolated, purified and crystallized as described above for its isolation from the Maillard mixture (0.3 mmol; about 15% in yield).

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