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Characterization of precursors and elucidation of the reaction pathway leading to a novel coloured 2*H*,7*H*,8a*H*-pyrano[2,3-*b*]pyran-3-one from pentoses by quantitative studies and application of ^{13}C-labelling experiments

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Abstract

The intensely 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 (**1a**/**1b**) have recently been identified among the main coloured compounds formed in the presence of pentoses. To clarify its formation pathway, quantitative studies on the effectivity of certain carbohydrate degradation products as precursors of **1a**/**1b** were performed indicating the 3-deoxypentose-2-ulose, furan-2-carboxaldehyde and hydroxyacetaldehyde as the penultimate precursors of the colourant. In addition, a labelling experiment with [$^{13}C_1$]-xylose was performed to elucidate how these precursors are transformed into **1a**/**1b**. © 1998 Elsevier Science Ltd. All rights reserved.

Keywords: Maillard reaction; Coloured compounds; Non-enzymatic browning; Pentoses; ^{13}C-labelling experiment; Glyoxal; Glycolaldehyde; Deoxyaldosuloses

1. Introduction

Extensive studies have been performed in the last 30 years to obtain more detailed information on the structures of the chromophores involved in non-enzymatic browning reactions; however, only a very limited number of chromophoric structures could be characterized [1–7].

By application of colour dilution analysis [8], the intensely orange coloured, previously unknown (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 (**1a** and **1b** in Fig. 1) were identified among the main coloured compounds formed in an aqueous solution of xylose and L-alanine, which was thermally treated in the presence the pentose-degradation product furan-2-carboxaldehyde [9,10]. The precursors and the formation route leading to this type of chromophore are, however, as yet not clear. To disentangle the complex network of non-enzymatic browning reactions, it is a necessary prerequisite to clarify the precursors, and to elucidate the reaction pathway, as the carbohydrate is

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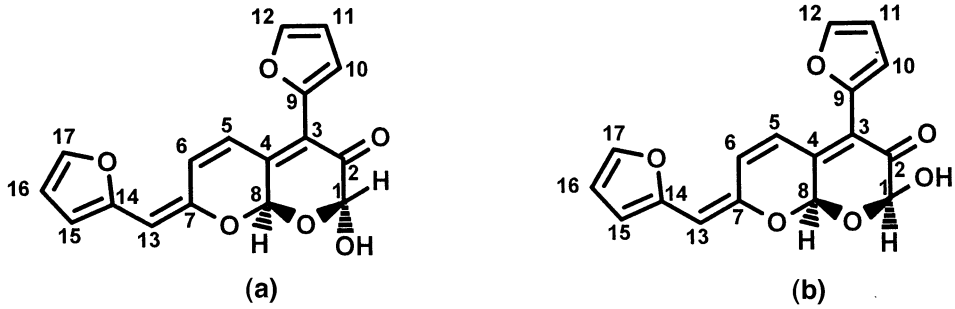

Fig. 1. Structures of (1*R*,8a*R*)- (**1a**) and (1*S*, 8a*R*)-4-(2-furyl)-7-[(2-furyl)methylidene]-2-hydroxy-2*H*,7*H*,8a*H*-pyrano[2,3-*b*]pyran-3-one (**1b**).

converted into the 2*H*,7*H*,8a*H*-pyrano[2,3-b]pyran-3-one chromophore in the course of the Maillard reaction.

To locate precursors of this chromophore, it might be a promising approach to react certain Maillard intermediates, which are most likely involved in its formation, with each other and to determine the effectiveness of these intermediates in generating **1a**/**1b** by means of quantitative measurements. This technique was successfully used to identify the precursors of several key-odorants ruling the overall aroma of heated carbohydrate/amino acid mixtures such as, e.g., 2-methyl-3-furanthiol [10], 2-furfurylthiol [10] and 2-acetyl-2-thiazoline [11].

When the precursors are then identified, it is a necessary further step to elucidate the mechanisms by which these precursors are converted into **1a**/**1b**. To meet this demand, the most promising technique is the use of labelled precursors offering the possibility to follow how the labelled atoms are shifted into the compound under investigation. Because of $^{2}H/^{1}H$ exchange in aqueous Maillard systems, deuterated precursors cannot be used and the labelling experiments have to be performed with ^{13}C isotopes. To detect the labelled positions in **1a**/**1b**, mass spectrometry might not definitely assign the labelled carbon atoms. ^{13}C NMR spectrosocopy, however, allows an unequivocal assignment of each labelled carbon site. Due to the low natural abundance (1.1%) of the ^{13}C nucleus, the site specific ^{13}C-enrichment of a specific carbon site has the advantage of increasing the ^{13}C NMR signal of the corresponding resonance up to 90-fold. A signal increase in the ^{13}C NMR spectrum, therefore, definitely indicates an incorporation of the label into the carbon position under investigation. This technique was very recently successfully used to clarify the formation pathway of red coloured 3(2*H*)-pyrrolinone chromophores formed from the Maillard reaction of furan-2-carboxaldehyde and L-alanine [12].

The objectives of the present investigation were, therefore, to identify the precursors of **1a**/**1b** by quantitative studies on certain Maillard reaction intermediates and, to clarify the reaction pathways governing the formation of the colourants **1a**/**1b** from pentoses by application of a ^{13}C-labelling experiment.

2. Results and discussion

It is obvious from the structure **1a**/**1b** (Fig. 1) that the furan rings 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-one correspond to two molecules of furan-2-carboxaldehyde and that, besides five carbon atoms of the pentose skeleton, two further carbon atoms have to be incorporated in the colourant. This encouraged us to study, whether these carbon atoms originate from the C-2 compound hydroxyacetaldehyde or glyoxal, well-known reaction products derived from cleavage of the carbohydrate skeleton [13,14].

In the hope of shedding more light onto the precursors of the colourant, the xylose/L-alanine/furan-2-carboxaldehyde mixture was thermally treated in the presence of either hydroxyaldehyde or glyoxal, and the amounts

Table 1
Influence of C-2 carbonyl compounds on the amounts of **1a**/**1b** generated from xylose/L-alanine in the presence of furan-2-carboxaldehyde[a]

C-2 compound	Amount of **1a**/**1b**	
	(mg)	(%)
(No additive)	0.5	0.01
Glyoxal	3.1	0.06
Hydroxyacetaldehyde	5.6	0.11

[a] A mixture of xylose (16.5 mmol) and L-alanine (4 mmol) was heated in phosphate buffer (20 mL; 1 mmol/L, pH 7.0) under reflux for 10 min, then, the C-2 compound (0.75 mmol) and furan-2-carboxaldehyde (25 mmol) was added and heating was continued for another 60 min.

of **1a**/**1b** formed were compared with those generated in a control experiment, in which the mixture was heated without addition of a C-2 compound. The results, given in Table 1, showed that, in comparison with the control experiment, **1a**/**1b** were produced in the Maillard mixtures containing hydroxyaldehyde or glyoxal, by factors of 11 or 6 higher amounts, respectively. The favoured production of **1a**/**1b** in the presence of hydroxyacetaldehyde documented this C-2 compound as a penultimate precursor in the formation of the chromophore.

Because a redox equilibrium between hydroxyacetaldehyde and glyoxal is discussed in the literature [13], this encouraged us to study which of the two precursors is predominantly liberated from the pentose. The formation of glyoxal and hydroxyacetaldehyde in a heated aqueous solution of xylose and L-alanine was, therefore, followed by quantitative measurements during a time period of 60 min. The results, given in Fig. 2, showed that the amounts of glyoxal rapidly increased as soon as the mixture was heated. After running through a maximum of about 58 μg/mmol xylose at 3 min, the amount of glyoxal decreased again to about 10% after 10 min. Prolonging the reaction time led only to a slight decrease of the glyoxal amounts approximating to a concentration of about 3 μg/mmol xylose after 60 min. The concentrations of hydroxyaldehyde increased only slowly during the first 10 min and, after reaching a maximum at 15 min, decreased again to about 5 μg/mmol xylose after 60 min. These data revealed glyoxal as the primary reaction product from the pentose and demonstrated that the decrease of glyoxal after 3 min of heating runs in parallel with the increase of glycolaldehyde. It would, therefore, appear that the former intermediate might be reduced to the latter C-2 compound. This is well in line with the recent finding that, after an induction period of some minutes, the concentration of reducing substances formed in aqueous carbo-

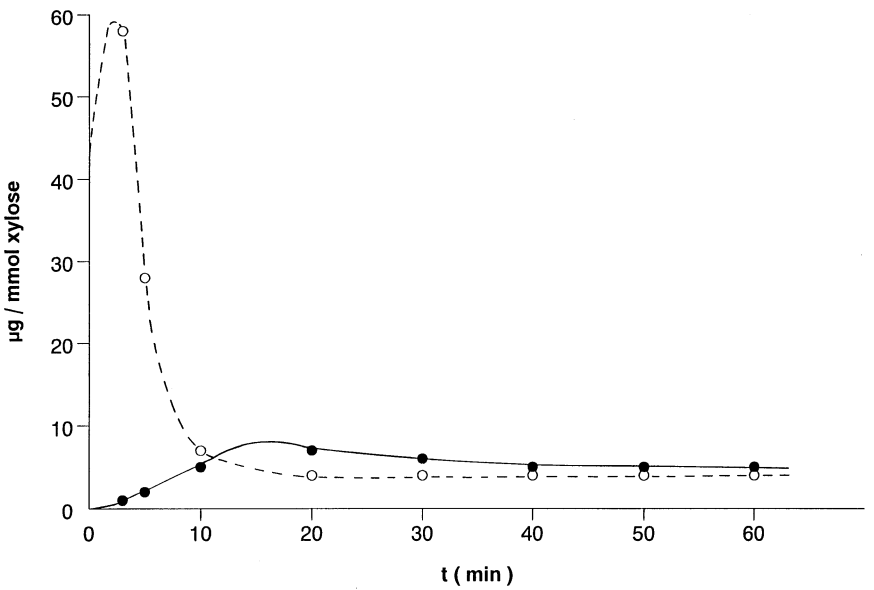

Fig. 2. Time course of the formation of glyoxal (- - -) and hydroxyacetaldehyde (—) in a heated neutral aqueous solution of xylose and L-alanine.

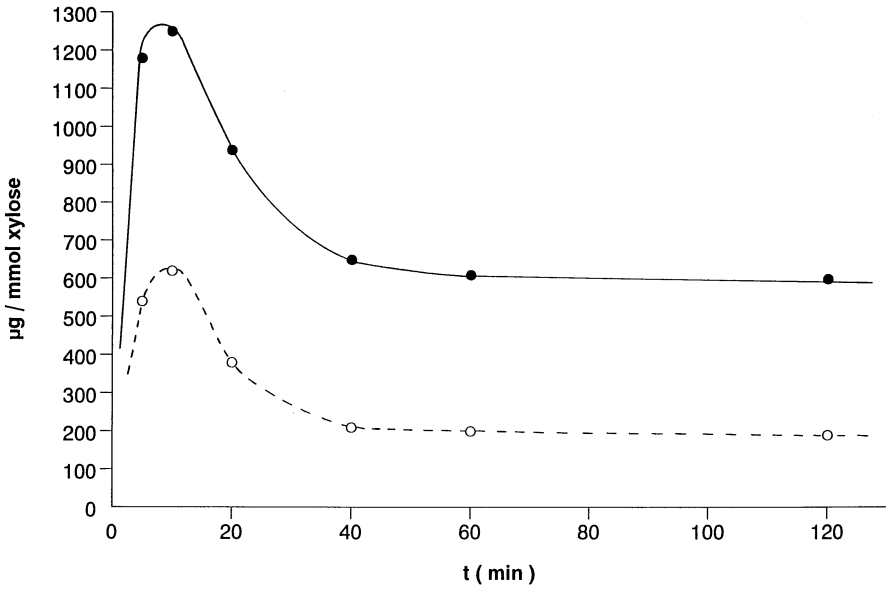

Fig. 3. Time course of the formation of 1-deoxypentose-2-ulose (- - -) and 3-deoxypentose-2-ulose (—) in a heated neutral aqueous solution of xylose and L-alanine.

hydrate/L-alanine solutions increased rapidly [14]. The fact that an additional reduction step is a necessary prerequisite for the formation of **1a**/**1b** from glyoxal is reflected by the higher precursor effectivity of hydroxyacetaldehyde in comparison with glyoxal.

Besides furan-2-carboxaldehyde and hydroxyacetaldehyde, the carbon skeleton of the pentose is most likely involved in the formation of **1a**/**1b**. Because 3-deoxypentose-2-ulose and 1-deoxy-2,3-pentodiulose are known as major pentose dehydration products with intact carbon chain, we followed their formation in the aqueous xylose/L-alanine solution over a time period of 120 min. The data, given in Fig. 3, demonstrated that the formation of 3-deoxypentose-2-ulose and 1-deoxy-2,3-pentodiulose increased rapidly in the first 10 min and, after running through a maximum, decreased again to about 50 and 30% after 120 min, respectively. Independent from the reaction time, the actual concentrations of 3-deoxypentose-2-ulose were a factor of two to three higher than those of the 1-deoxy-2,3-pentodiulose. Comparing the time course of the deoxyosone formation (Fig. 3) with the time course of the glyoxal formation (Fig. 2) showed that the glyoxal is liberated from the pentose skeleton at a very early stage of the Maillard reaction, prior to deoxyosone formation, fitting well with data found recently for hexoses [14].

The high concentrations of the 3-deoxypentose-2-ulose in the heated xylose/L-alanine solution prompted us to study, whether the pentose skeleton is transformed into **1a**/**1b** via the 3-deoxyosone pathway. Aqueous solutions of xylose/L-alanine, the corresponding Amadori rearrangement product *N*-(1-deoxy-D-xylulos-1-yl)-L-alanine and 3-deoxypentose-2-ulose, respectively, were therefore heated in the presence of furan-2-carboxaldehyde as well as hydroxyaldehyde, and the amounts of **1a**/**1b** generated were determined. The results, given in Table 2, showed that, compared with the Amadori rearrangement product or the xylose/L-alanine mixture, the formation of **1a**/

Table 2
Influence of the C-5 skeleton on the generation of **1a**/**1b**[a]

Precursors	Amount of **1a**/**1b**	
	(mg)	(%)
Xylose (16.5 mmol)/L-alanine (4 mmol)	5.6	0.11
N-(1-Deoxy-D-xylulos-1-yl)-L-alanine (16.5 mmol)	7.1	0.14
3-Deoxypentose-2-ulose (16.5 mmol)	14.8	0.29

[a] The precursor mixtures were heated in phosphate buffer (20 mL; 1 mmol/L, pH 7.0) under reflux for 10 min, then, a mixture of glycolaldehyde (0.75 mmol) and furan-2-carboxaldehyde (25 mmol) was added and heating was continued for another 60 min.

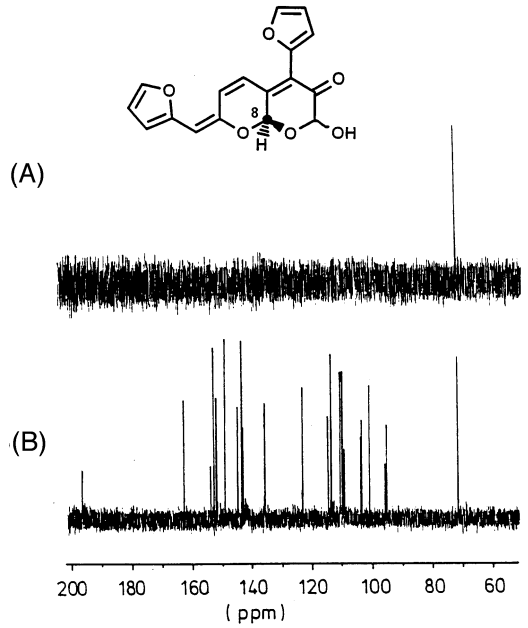

Fig. 4. Excerpt of ^{13}C NMR spectrum (900 MHz; Me_2SO-d_6) of **1a**/**1b** formed from (A) $^{13}C_1$-xylose and (B) xylose with natural ^{13}C abundance, respectively.

1b was significantly favoured from the 3-deoxypentose-2-ulose, by a factor of 2.6 or 0.8, respectively. These quantitative data indicated the incorporation of the pentose skeleton into the colourant via the 3-deoxypentose-2-ulose as the key intermediate.

In the hope of shedding more light on how the 3-deoxypentose-2-ulose is incorporated into **1a**/**1b**, a mixture of [^{13}C]-labelled xylose and L-alanine was heated in the presence of the precursors furan-2-carboxaldehyde and hydroxyacetaldehyde in order to follow the fate of the anomeric carbon atom of the pentose during its incorporation into the colourant. After its isolation, the site specific ^{13}C-enriched isotopomer of the colourant (**e-1a**/**e-1b**) was analysed by ^1H broad band decoupled ^{13}C NMR spectroscopy (Fig. 4). Comparing the ^{13}C NMR spectrum of **e-1a**/**e-1b** (A in Fig. 4) with the spectrum of non-labelled **1a**/**1b** (B in Fig. 4), shows the signal at 71.4 ppm to be ^{13}C-enriched. As reported by Hofmann [8], heteronuclear correlation experiments (HMQC, HMBC, HMQC-DEPT) led to the assignment of the increased signal as C-8 in structure **1a**/**1b**. The position of the ^{13}C-enriched carbon atom at C-8 in **e-1a**/**e-1b** clearly demonstrates that the aldehyde function in the hemiacetal group C-1 cannot originate from the anomeric carbon atom of the pentose.

On the basis of the quantitative studies and the results of the ^{13}C-labelling experiments, the reaction pathway, displayed in Fig. 5, was proposed for the formation of **1a** and **1b**. To follow the fate of the ^{13}C label of the pentose throughout its incorporation into the colourant, the ^{13}C-enriched atom is marked in the structures outlined in Fig. 5. The reaction between the pentose and the amino acid results in the Schiff base (I) as an intermediate in the very beginning of the Maillard reaction. In course of the Amadori rearrangement this intermediate is converted into the *N*-(1-deoxy-D-xylulos-1-yl)-amino acid (II), which, upon dehydration at the 3-position and hydrolysis of the amino acid, reveals the 3-deoxypentose-2-ulose (IIa), found as a penultimate precursor of **1a**/**1b**. Quantitative studies revealed that glyoxal can be formed at a very early stage of the Maillard reaction, prior to deoxyosone formation. Because Maillard-derived α-aminoketones and α-iminoalcohols were shown to be easily oxidized into the corresponding 1,2-iminoketones [11,15] and glyoxal formation was found to be strongly oxygen-dependend [14], glyoxal (IIIa) might be formed by an oxidative breakdown of the Schiff base I. With increasing formation of reducing substances in the course of the Maillard reaction, hydroxyacetaldehyde (IIIb) is then formed from IIIa upon reduction [14]. Condensation between the methylene-active hydroxyacetaldehyde (III) and furan-2-carboxaldehyde (IIb), formed by cyclization and dehydration of 3-deoxy-2-pentosulose (IIa), yields the 2-(2-furyl)methylidene-2-hydroxyacetaldehyde (IV) showing strong nucleophilic characteristics at the methylidene group. Condensation with the cyclic hemi acetal of the 3-deoxypentose-2-ulose then gives rise to a α-ketoaldehyde intermediate (V). Due to the strong electron-withdrawing effect of the aldehyde function, this intermediate subsequently forms the 4-(2-furyl)-2,6-dihydroxy-2,5,6,7,8a-pentahydropyrano[2,3-*b*]pyran-3-one (VI) upon ring closure between the hydroxyl group of the hemiacetal and the aldehyde. Vinylogous keto–enol tautomerism, followed by water elimination leads to the 4-(2-furyl)-2,6-

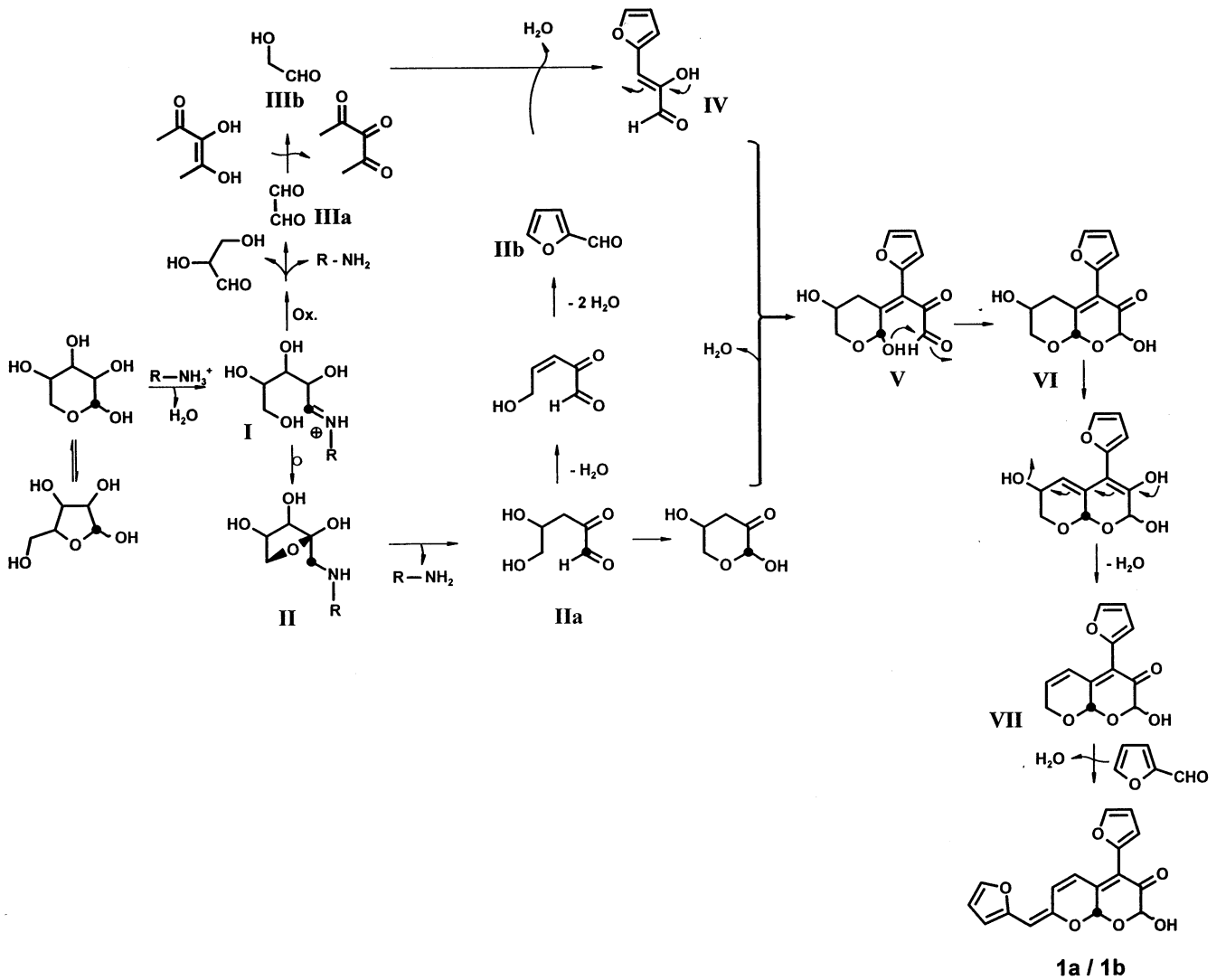

Fig. 5. Formation pathway leading to **1a**/**1b** from pentoses and amino acids (●, ^{13}C-labelled carbon atom).

dihydroxy - 2*H*,7*H*,8a*H* - pyrano[2,3 - *b*]pyran-3-one (VII), which, upon condensation with an additional molecule of furan-2-carboxaldehyde then gives rise to the (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 (**1a**/**1b**).

These data demonstrate that the identification of precursors by quantitative studies, followed by the elucidation of reaction pathways using ^{13}C-labelling experiments, offers the possibility to disentangle the complex network of non-enzymatic browning reactions. Details, obtained thereof, will help to construct a route map of reactions leading to non-enzymatic browning during thermal processing of foods.

3. Experimental

General methods.—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 as the mobile phase: (A) MeCN–water, 9:1; (B) EtOAc; (C) toluene–EtOAc, 1:1.

Flash chromatography (16 × 200 mm) was performed using a RP-18 stationary phase (15.0 g; Lichroprep 25–40 μm, E. Merck, Darmstadt, Germany) using MeOH–water (7:3) as the mobile phase.

The high-performance liquid chromatography (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 an analytical scale (4.6 × 250 mm, flow rate 0.8 mL/min). The following solvent gradients were used: (A) starting with a mixture (1:9) of MeCN and aqueous trifluoracetic acid (0.1% TFA in water), the MeCN content was raised to 60% after 50 min; (B) starting with a mixture (1:9) of MeCN and aqueous ammonium formiate buffer (pH 3.5; 20 mmol/L), the MeCN content was increased to 30% within 50 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 (9:1) of MeCN and water and increasing the MeCN content to 100% within 15 min.

Gas chromatography–mass spectroscopy (GC–MS) was performed with a type 5160 gas chromatograph (Fisons Instruments, Mainz, FRG) using an SE-54 capillary (30 m × 0.32 mm, 0.25 μm; J&W Scientific, Fisons Instruments, Mainz, FRG) coupled with an MD-800 mass spectrometer (Fisons Instruments, Mainz, FRG) running in chemical ionization (CI) mode with sample application (0.5 μL) by on-column injection at 40 °C. After 2 min, the temperature was raised at 40 °C/min to 50 min, held for 1 min isothermally, and then raised at 6 °C/min to 230 °C and held for 10 min.

^{1}H broad-band decoupled ^{13}C NMR spectroscopy was performed on a Bruker-AM-360 spectrometer using the following aquisition parameters: transmitter frequency 90.56 MHz, spectral width 25,000 Hz, pulse length 10.4 μs, recorded with 64 K data points, repetition time 2.5 s, 1 Hz line broadening, 20,000 scans; processing was done by multiplication with a Lorentz–Gaussian function prior to transformation. The sample was dissolved in Me_2SO-d_6, chemical shifts expressed in ppm were measured from residual Me_2SO-d_6 (39.5 ppm).

Methods.—D-Xylose, [^{13}C-1]-xylose, L-alanine, glyoxal (30% solution in water), hydroxyacetaldehyde (glycolaldehyde), 1-hydroxycyclo-hexan-2-one, ethoxamine hydrochloride, 1,2-diaminobenzene, furan-2-carboxaldehyde, trifluoracetic acid (TFA) were obtained from Aldrich (Steinheim, Germany). Furan-2-carboxaldehyde was freshly distilled prior to use. 1,2-Diaminobenzene was recrystallized twice from MeOH prior to use. Me_2SO-d_6 was from Isochom (Landshut, Germany). Solvents were HPLC grade (Aldrich, Steinheim, Germany).

The following compounds were synthesized as described in the literature: (1*S*,8a*R*)- and (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 (**1a**/**1b** in Fig. 1) [8], 3-deoxypentos-2-ulose [10], [$^{13}C_4$]-butane-2,3-dione [16].

Syntheses.—*N-(1-Deoxy-*D*-xylulos-1-yl)-*L*-alanine*. A mixture of powdered anhydrous D-xylose (300 mmol) and L-alanine (500 mmol) was refluxed in anhydrous MeOH (400 mL), whilst stirring. After heating for 3 h, malonic acid (90 mmol) was added and the solution was refluxed for additional 2 h. The mixture was cooled to room temperature and concentrated to about 200 mL under vacuum. Unreacted L-alanine was filtered off and the filtrate was then cooled to −20 °C. Dropwise addition of acetone yielded the Amadori rearrangement product as a brown hygroscopic solid. Concentration of the mother liquor, recooling and addition of acetone yielded the additional raw product. Both crops were combined, dissolved in a mixture of water (80 mL) and EtOH (200 mL) and applied to cation-exchange chromatography. Non-reacted carbohydrate was eluted with a mixture of water (120 mL) and EtOH (280 mL), followed by water (100 mL). After elution with an aqueous ammonium hydroxide solution (0.2 mol/L) and freeze-drying, the *N*-(1-deoxy-D-xylulos-1-yl)-L-alanine was obtained as a white powder with a purity of about 90% (24 mmol; yield: 8%). LC–MS: 222 (100, $[M+1]^+$), 204 (25, $[M+1-H_2O]^+$); ^{1}H NMR (360 MHz in D_2O; DQF-COSY): δ 1.50 (d, 3H, $^3J = 7.1$ Hz, $-CH_3$), 3.10 (d, 1H, $^2J = 10.0$ Hz, $-CH_aH_b-N$), 3.14 (d, 1H, $^2J = 10.0$ Hz, $-CH_aH_b-N$), 3.70–4.50

(m, 4H, 2 × –C*H*(OH)–, –*CH*$_2$–O), 3.80 (d, 1H, $^3J = 7.1$ Hz, N–*CH*(COOH)–CH$_3$).

2-(2,3-Dihydroxypropyl) quinoxaline. A mixture of 3-deoxypentose-2-ulose (2 mmol) and 1,2-diaminobenzene (2.2 mmol) in phosphate buffer (5 mL; 0.2 mol/L; pH 7.0) was kept under argon in the dark at 40 °C for 3 h, and was then extracted with CH_2Cl_2 (5 × 10 mL). The combined organic layers were dried over Na_2SO_4, concentrated under vacuum to about 1 mL and the target compound was then isolated by preparative TLC on silica gel using solvent system A as the mobile phase. The band with $R_f = 0.4–0.5$ was scraped off and suspended in MeOH. After filtration and concentration, the 2-(2,3-dihydroxypropyl) quinoxaline (118 mg; yield: 58%) was obtained as a colourless oil. LC–MS: 205 (100; $[M + 1]^+$), 187 (21, $[M + 1 - H_2O]^+$), 237 (12; $[M + Na]^+$); ^{1}H NMR (360 MHz; CD_3OD): δ 3.08 (dd, 1H, $^2J = 15.04$, $^3J = 4.87$, Hz, –*CH*$_a$H$_b$–C(OH)–), 3.12 (dd, 1H, $^2J = 15.04$ Hz, $^3J = 7.96$ Hz, –*C*H$_a$*H*$_b$–C(OH)–), 3.62–3.74 (m, 2H, –*CH*$_2$–OH), 4.27 (m, 1H, –*CH*(OH)–), 7.59 (m, 2H, 2 × =*CH*–), 7.84 (m, 1H, =*CH*–), 7.92 (m, 1H, =*CH*–), 8.69 (s, 1H, N=*CH*–);^{13}C NMR (360 MHz; CD_3OD): δ 38.8 (–*C*H$_2$), 65.9 (–*C*H$_2$–OH), 71.2 (–*C*H(OH)–), 128.3 (=*C*H–), 128.9 (=*C*H–), 129.3 (=*C*H–), 130.1 (=*C*H–), 140.9 (=*C*(C)–), 141.3 (=*C*(C)–), 146.2 (=*C*(C)–), 154.7 (=*C*(C)–).

2-Methyl-3-(1,2-dihydroxyethyl)-quinoxaline. A mixture of D-xylose (19.6 mmol), L-alanine (18.3 mmol), 1,2-diaminobenzene (14.8 mmol) in phosphate buffer (80 mL; 0.5 mol/L, pH 6.8) was refluxed for 12 h. After cooling to room temperature, the reaction mixture was extracted with CH_2Cl_2 (5 × 20 mL), the combined organic layers were dried over Na_2SO_4, and, after concentration, separated by column chromatography (35 × 400 mm) on silica gel (150 g, Silica Gel 60, E. Merck, Darmstadt, Germany), which was conditioned with EtOAc. After application of the crude material onto the column, chromatography was performed with EtOAc (400 mL), followed by EtOAc–MeOH (1:1; 500 mL) affording the target compound as a crude product. Further fractionation by preparative TLC on silica gel using solvent system B as the mobile phase revealed the target compound in a band at $R_f = 0.3$, which was scraped off and suspended in MeOH. Filtration and concentration afforded the target compound as white crystals (6.4 mmol; 33% in yield). LC–MS: 205 (100; $[M + 1]^+$), 187 (18, $[M + 1 - H_2O]^+$), 237 (19; $[M + Na]^+$); ^{1}H NMR (360 MHz; CD_3OD): δ 2.80 (s, 3H, –*CH*$_3$), 3.86 (m, 1H, –*CH*$_a$H$_b$–OH), 4.04 (m, 1H, –*C*H$_a$*H*$_b$–OH), 5.12 (m, 1H; =C–*CH*(OH)–), 7.72 (m, 2H, 2 × =*CH*–), 8.01 (m, 2H, 2 × =*CH*–); ^{13}C NMR (360 MHz; CD_3OD): δ 21.9 (–*C*H$_3$), 65.8 (–*C*H$_2$–OH), 71.0 (–*C*H(OH)–), 128.3 (=*C*H–), 128.4 (=*C*H–), 129.4 (=*C*H–), 130.0 (=*C*H–), 139.3 (=*C*(C)-), 141.7 (=*C*(C)–), 151.9 (=*C*(C)–), 152.9 (=*C*(C)–).

*Quantification 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-one* (**1a/1b**) *in precursor mixtures*.—After cooling the reaction mixture, detailed in Table 1, the aqueous solution was extracted with EtOAc (5 × 10 mL), the combined organic layers were dried over Na_2SO_4 and then distilled under high vacuum (0.04 mbar) at 35 °C. The residue was taken up in MeCN, membrane filtered and then analysed by RP-18 HPLC using the solvent system A. Monitoring the effluent at $\lambda_{max} = 460$ nm, colourant **1a**/**1b** ($R_t = 21.6$ min) was quantified by using the pure reference compound as external standard. The results given in Tables 1 and 2 are the mean of duplicates.

Quantification of hydroxyacetaldehyde after derivatization with ethoxamine.—Mixtures of xylose (6 mmol) and L-alanine (6 mmol) were refluxed in phosphate buffer (4 mL). At the reaction times, given in Fig. 2, the mixtures were rapidly cooled to room temperature, the internal standard 1-hydroxycyclohexan-2-one, dissolved in water, and, ethoxamine hydrochloride (5 mmol) were added. The pH was adjusted to 7.5 with aq sodium hydroxide (0.1 mol/L) and the mixtures were maintained for 3 h at 30 °C. The pH was then adjusted to 6.0 with hydrochloric acid (0.1 mol/L) and the mixtures were extracted with diethyl ether (3 × 15 mL). After drying over Na_2SO_4, the organic layer was concentrated to about 3 mL and applied to GC–MS analyses scanning the mass traces of the ethoxamine derivatives of

hydroxyacetaldehyde (*m/z* 104) and 1-hydroxy-cyclohexan-2-one (*m/z* 158). For the determination of the response factor (0.5), solutions containing defined amounts of hydroxyacetaldehyde and 1-hydroxycyclohexan-2-one in a ratio of 3:1 to 1:3 were treated as described above for the Maillard mixtures. GC–MS–CI of *O*-ethyl hydroxyacetaldehyde oxime ($R_t = 6.09/6.50$ min): 104 (100, $[M+1]^+$); GC–MS–CI of *O*-ethyl 1-hydroxycyclohexan-2-one oxime ($R_t = 15.25$ min): 158 (100, $[M+1]^+$).

Quantification of glyoxal after derivatization with 1,2-diaminobenzene.—Mixtures of xylose (6 mmol) and L-alanine (6 mmol) were refluxed in phosphate buffer (4 mL). At the reaction times, given in Fig. 2, a quarter of the reaction mixture was withdrawn, rapidly cooled to room temperature and dissolved with water (5 mL). The solution was adjusted to pH 6.5 with hydrochloric acid (0.1 mol/L), the internal standard [13C-4]-butane-2,3-dione, dissolved in diethyl ether, and 1,2-diaminobenzene (2 mmol), dissolved in MeOH, was added. After maintaining the mixture for 3 h at 30 °C, the pH was adjusted to 5.0 with hydrochloric acid (0.1 mol/L) and the mixtures were extracted with diethyl ether (3×15 mL). After drying over Na_2SO_4, the organic layer was concentrated to about 3 mL and was applied to GC–MS analyses scanning the mass trace of *m/z* 131 for the derivative of the glyoxal and *m/z* 163 for the derivative of the internal standard. For the determination of the response factor (0.95), solutions containing definite amounts of glyoxal and [13C-4]-butane-2,3-dione in a ratio of 1:3 to 3:1 were treated as described above for the Maillard mixtures. GC–MS–CI of quinoxaline ($R_t =$ 15.38 min): 131 (100, $[M+1]^+$); GC–MS–CI of $[^{13}C_4]$-2,3-dimethylchinoxaline ($R_t = 19.70$ min): 163 (100, $[M+1]^+$).

Quantification of 1-deoxy-2,3-pentodiulose and 3-deoxypentos-2-ulose after derivatization with 1,2-diaminobenzene.—Mixtures of xylose (6 mmol) and L-alanine (6 mmol) were refluxed in phosphate buffer (4 mL). At the reaction times given in Fig. 3, a quarter of the reaction mixture was withdrawn, cooled to room temperature and dissolved with water (1 mL). After addition of 1,2-diaminobenzene (2 mmol), dissolved in MeOH, the mixture was maintained at 30 °C for 3 h. The derivatized reaction mixture was then diluted with water and analyzed by RP-HPLC using the solvent gradient B. Monitoring the effluent at $\lambda = 320$ nm, 2-methyl-3-(1,2-dihydroxyethyl)-quinoxaline, the derivative of 1-deoxy-2,3-pentodiulose, and 2-(2,3-dihydroxypropyl)-quinoxaline, the derivative of 3-deoxypentose-2-ulose were detected at the retention times of 14.3 and 15.9 min, respectively. Quantification of these derivatives was performed by using the synthetic compounds as external standards.

^{13}C stable isotope-labelling experiment.—A solution of $[^{13}C_1]$-D-xylose (2.0 mmol) and L-alanine (0.48 mmol) in phosphate buffer (2.4 mL; 1 mmol/L, pH 7.0) was refluxed for 10 min in a closed vial. A mixture of hydroxyacetaldehyde (0.1 mmol) and furan-2-carboxaldehyde (3.0 mmol) in water (0.4 mL) was added and heating was continued for another 50 min.

*Isolation of ^{13}C-enriched (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* (**e-1a/e-1b**).—The reacted mixture was cooled to room temperature, the pH was adjusted to 5.0 and the aqueous solution was extracted with diethyl ether (5×4 mL), the organic layer was dried over Na_2SO_4 and the orange colourant was isolated by preparative thin layer chromatography using solvent system C. An orange coloured band at $R_f = 0.4$ was scraped off and dissolved in MeOH (20 mL). After filtration and concentration, the coloured compound was further purified by RP-18 flash chromatography. The coloured fraction was collected and freed from MeOH under vacuum. The aqueous phase was extracted with EtOAc (3×20 mL) and, after drying over Na_2SO_4, the solvent was removed under vacuum yielding a red residue, which was dissolved in Me_2SO-d_6 and then analyzed by ^1H broad band decoupled ^{13}C NMR spectroscopy and LC–MS. The comparison of the LC–MS data with those of natural ^{13}C abundant **1a**/**1b** [8] demonstrates the incorporation of one ^{13}C atom in **1a**/**1b**. LC–MS: 296 (100, $[M+1-H_2O]^+$), 314 (29, $[M+1]^+$).

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References

[1] Th. Severin, U. Krönig, *Chem. Mikrobiol. Technol. Lebensm.*, 1 (1972) 156–157.
[2] F. Ledl, Th. Severin, *Z. Lebensm. Unters. Forsch.*, 167 (1978) 410–413.
[3] F. Ledl, Th. Severin, *Z. Lebensm. Unters. Forsch.*, 175 (1982) 262–265.
[4] A. Arnoldi, E.A. Corain, L. Scaglioni, J.M. Ames, *J. Agric. Food Chem.*, 45 (1997) 650–655.
[5] T. Hofmann, *J. Agric. Food Chem.*, 46 (1998) 932–940.
[6] T. Hofmann, *Helv. Chim. Acta.*, 80 (1997) 1843–1856.
[7] T. Hofmann, *Z. Lebensm. Unters. Forsch.*, 206 (1998) 251–258.
[8] T. Hofmann, *Carbohydr. Res.*, 313 (1998) 203–213.
[9] F. Ledl, E. Schleicher, *Angew. Chem.*, 102 (1990) 597–734.
[10] T. Hofmann, P. Schieberle, *J. Agric. Food Chem.*, 46 (1998) 235–241.
[11] T. Hofmann, P. Schieberle, *J. Agric. Food Chem.*, 43 (1995) 2946–2950.
[12] T. Hofmann, *J. Agric. Food Chem.*, 46 (1998) 914–945.
[13] T. Hayashi, M. Namiki, *Agric. Biol. Chem.*, 44 (1980) 2575–2580.
[14] T. Hofmann, K. Stettmeier, W. Bors, *J. Agric. Food Chem.*, 47 (1999) in press.
[15] T. Hofmann, P. Schieberle, *J. Agric. Food Chem.*, 46 (1998) 616–619.
[16] T. Hofmann, P. Schieberle, *J. Agric. Food Chem.*, 45 (1997) 227–232.