Formation of a Novel Colored Product during the Maillard Reaction of D-Glucose

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Reactions between reducing sugars and proteins or amino acids (Maillard reaction) lead to the formation of yellow to brown products (melanoidins) that are important for food preparation and processing, such as baking, roasting, or malt production. Thus far, the structures of the melanoidins have not been elucidated, although some structural insights have been gained from model reactions. In this study, D-glucose was heated with an amine and two colored compounds were detected by HPLC/UV−vis. After purification, the main product was identified as [(4a*S*,6*R*,7*S*,8*R*,8a*R*)-4,-4a,6,7,8,8a-hexahydro-7,8-dihydroxy-6-hydroxymethyl-1,4-dipropyl-1*H*-pyrano[2,3-*b*]pyrazine-2-yl]-1-hydroxy-3-buten-2-one (**1a**). For the minor compound (**2a**), some spectral data were obtained, but the structure was not fully characterized. **1a** and **2a** are the main colored compounds when the reaction is performed in alcoholic solution or on a cellulose surface. Thus, it was concluded that products with an analogous structure are important for the color formation of foodstuffs with low water activity.

Keywords: *Color formation; D-glucose; [(4aS,6R,7S,8R,8aR)-4,4a,6,7,8,8a-hexahydro-7,8-dihydroxy-6-hydroxymethyl-1,4-dipropyl-1H-pyrano[2,3-b]pyrazine-2-yl]-1-hydroxy-3-buten-2-one; melanoidins; Maillard reaction*

INTRODUCTION

The Maillard reaction between reducing sugars and compounds containing amino groups leads to the fast development of a yellow color, which turns dark brown during prolonged heating. This process is very important for food preparation and processing because it results in desirable browning, for example, during baking, roasting of meat, and malt production, but also in discoloration, which is observed in heat-treated milk products, among others. The structures of these browning products have therefore been intensively studied during the past decades. However, the major part of colored Maillard products in food products, the melanoidins, are of high molecular weight, up to 100 kDa, and have very complex molecular structures (*1*). Although numerous attempts have been undertaken to isolate and purify melanoidins from food products, such as coffee or dark beer, it has thus far not been possible to elucidate their chemical structure.

In model studies, on the other hand, reducing sugars or Maillard intermediates were used as starting materials and defined colored reaction products could be isolated and identified. It can be concluded that analogous structures and chromophores are part of more complex melanoidins and contribute to their color (*2*).

In early studies with pentoses, which are very potent browning precursors, 4-hydroxy-5-methyl-2,3-dihydrofuran-3-one was identified as a key intermediate that undergoes condensation reactions, leading to a range of yellow reaction products (*3*). D-Glucose and other hexoses cannot react in the same manner, but it was shown that D-glucose degradation products with a CH-acidic methyl group can undergo similar condensation reactions with carbonyl compounds, resulting in the formation of yellow products (*4*). Additionally, colored β-pyranone and furanone condensation products were isolated from reaction mixtures of D-glucose and pentoses (*5*). The presence of amines or amino acids yields a range of colored products from sugars or Maillard intermediates that have nitrogen incorporated in the chrompohore (*6*−*10*).

Here we report on the isolation and structural identification of a novel yellow Maillard product derived from D-glucose.

MATERIALS AND METHODS

Apparatus. ^{1}H NMR (500 or 400 MHz), ^{13}C NMR (125 or 100 MHz), H,H-COSY, and C,H-COSY (correlated spectroscopy) spectra were recorded with JEOL Eclipse + 500 or JEOL GSX 400 spectrometers. Chemical shifts are reported in parts per million relative to $(CH_3)_4Si$ as internal standard. Mass spectral analyses were obtained with an HP 5989 A MS engine, HR-MS spectra with a Finnigan MAT 95 S mass spectrometer, FTIR spectra with a Perkin-Elmer series 1600 in KBr, and UV−vis spectra with a Jasco V-530 device.

High-Performance Liquid Chromatography (HPLC). Analytical HPLC was performed with a Hitachi L6000 pump, equipped with a Hitachi L 4000 UV detector. The eluent was methanol/water = 1:1 with a flow rate of 0.4 mL/min, and the column was Nucleosil 100-5 C_{18} (Macherey and Nagel, Düren, Germany).

Isolation of [(4a*S*,6*R*,7*S*,8*R*,8a*R*)-4,4a,6,7,8,8a-Hexahydro-7,8-dihydroxy-6-hydroxymethyl-1,4-dipropyl-1*H*-pyr-

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ano[2,3-*b*]pyrazine-2-yl]-1-hydroxy-3-buten-2-one (1a). A mixture of D-glucose (45 g, 0.25 mol), propylamine (7.5 g, 0.125 mol), and acetic acid (6 g, 0.1 mol) in 250 mL of ethanol was heated for 20 min under reflux. One hundred and twenty-five milliliters of the solvent was removed under reduced pressure, and 125 mL of ethyl acetate was added. The viscous mixture was divided into two portions, and both portions were purified by column chromatography on silica gel (5.5 cm i.d. × 20 cm; eluent, ethanol/ethyl acetate = 1:1). Each time the first 500 mL was collected, and the solvent of the recombined fractions was removed under reduced pressure and again purified by chromatography on silica gel (5.5 cm i.d. × 20 cm; eluent, ethanol/ethyl acetate = 18:82). The first 400 mL was discarded, and the second 400 mL of the elution volume was used for further separation by thin-layer chromatography (TLC) (20 cm × 20 cm glass plates coated with a 0.5 mm thickness of silica gel 60 F_{254}; eluent, ethanol/ethyl acetate = 16:84; R_f value ≈ 0.4). Final purification was achieved by semipreparative HPLC on RP-18 (column, Hibar LiCHrosorb RP 18, 250 mm × 10 mm i.d., 7 μm particle size, Merck, Darmstadt, Germany; eluent, water/methanol = 1:1 with flow rate of 2.5 mL/min, detection at 371 nm), and **1a** was obtained as an orange-red oil (40 mg): ^{1}H NMR (400 MHz, COSY; $CDCl_3$) δ 7.05 (d, J = 15.4 Hz, 1H, H-4), 6.41 (s, 1H; H-3′), 5.95 (d, J = 15.4 Hz, 1H; H-3), 4.77 (d, J = 5.1 Hz, 1H; H-4a′), 4.37 (s, 2H; H-1), 3.86 (b s, 2H; H-15′), 3.63* (t, J = 9.2 Hz, 1H; H-7′), 3.41 (m, 1H; H-6′), 3.34 (ddd, J = 14, 8, 5 Hz, 1H; H-12′$_{(A)}$), 3.27* (t, J = 9.5, 1H; H-8′), 3.11 (dd, J = 10.3, 4.4 Hz, 1H; H-8a′), 2.94* (dt, J = 14, 8, 1H; H-12′$_{(B)}$), 2.79 (m, 1H; H-9′$_{(A)}$), 2.57 (m, 1H; H-9′$_{(B)}$), 1.62 (m, 2H; H-10′), 1.56 (m, 2H; H-13′), 0.92 (t, J = 7 Hz, 6H; H-11′, H-14′) (an asterisk indicates that the signal assignment is the apparent signal, which represents obviously a signal of higher order); ^{13}C NMR (DEPT, COSY; $CDCl_3$) δ 196.90 (s, C-2), 142.6 (d, C-4), 137.7 (d, C-3′), 117.8 (s, C-2′), 110.9 (d, C-3), 74.2 (d, C-4a′), 72.6 (C-6′), 70.2 (d, C-7′), 69.9 (d, C-8′), 66.0 (t, C-1), 61.7 (t, C-15′), 58.8 (d, C-8a′), 55.9 (t, C-9′), 50.1 (t, C-12′), 21.9 (t, C-10′), 21.0 (t, C-13′), 11.5 (q, 2C, C-14′,C-11′); HR-MS calculated for $C_{18}H_{30}N_2O_6$ 370.2104, found 370.2079; IR ν 3400 (br), 2962, 2929, 2873, 1559 (predominant), 1222, 1069 cm^{-1}; UV−vis (CH_3OH) λ_{max} (log ϵ = 4.18) 399 nm; MS (CI), m/z 371 (M + 1$^+$).

Isolation of [(4a*S*,6*R*,7*S*,8*R*,8a*R*)-4,4a,6,7,8,8a-Hexahydro-7,8-diacetoxy-6-(acetoxymethyl)-1,4-dipropyl-1*H*-pyrano[2,3-*b*]pyrazine-2-yl]-1-acetoxy-3-buten-2-one (1b) and 2b. A mixture of d-glucose (2.5 g, 13.9 mmol), propylamine (1.44 g, 24 mmol), and acetic acid (1.47 g, 24.5 mmol) in 10 mL of methanol was heated for 60 min under reflux. After the reaction, 20 mL of water was added and the mixture was extracted with a 3-fold volume of ethyl acetate. The organic layer was dried over anhydrous sodium sulfate, concentrated under reduced pressure, and purified on a silica gel column (2 × 14 cm, silica gel 60, 0.040−0.063 mesh, Merck) using ethyl acetate/methanol (20:3) as eluent. The fractions were checked by TLC on silica gel using the same eluent as for the column chromatography, and fractions containing **1a** and **2a** (two yellow spots with R_f values of ∼0.5) were combined and acetylated: The solvent was removed from the fractions under reduced pressure, and the residue was dissolved in 2 mL of pyridine. After the addition of 1 mL of acetic anhydride, the mixture was reacted for 2 h at room temperature. Ice water was then added to quench unreacted acetic anhydride, and the mixture was extracted with the same volume of ethyl acetate. The ethyl acetate layer was washed four times with the same volume of water and dried over anhydrous sodium sulfate. The mixture was further purified on a silica gel column (10 cm × 5.5 cm, with ethyl acetate as an eluent) and by preparative TLC (20 cm × 20 cm glass plates coated with a 0.5 mm thickness of silica gel 60 F_{254}) using ethyl acetate as an eluent. The R_f values were 0.6 for **1b** and 0.7 for **2b**.

Compound **1b** was obtained as a yellow oil (1 mg): ^{1}H NMR (COSY, HMQC; $CDCl_3$) δ 7.01 (d, J = 15.0 Hz, 1H, H-4), 6.34 (s, 1H; H-3′), 5.87 (d, J = 15.0 Hz, 1H; H-3), 5.02 (dd, J = 10.2, 9.7 Hz, 1H; H-7′), 4.85 (dd, J = 10.5, 10,2 Hz, 1H; H-8′), 4.78 (d, J = 4.8 Hz, 1H; H-4a′), 4.77 (s, 2H; H-1), 4.29 (dd, J = 12.3, 5.1 Hz, 1H; H-15′$_{(A)}$), 4.06 (dd, J = 12.3, 2.3 Hz, 1H; H-15′$_{(B)}$), 3.77 (ddd, J = 9.7, 5.1, 2.3 Hz, 1H; H-6′), 3.36 (dd, J = 10.5, 4.8 Hz, 1H; H-8a′), 3.22 (ddd, J = 13.7, 8.5, 4.5 Hz, 1H; H-12′$_{(A)}$), 2.92 (ddd, J = 13.8, 8.5, 4.5 Hz, 1H; H-12′$_{(B)}$), 2.77 (ddd, J = 13.5, 7.2, 6.2 Hz, 1H; H-9′$_{(A)}$), 2.46 (ddd, J = 13.5, 8.9, 5.2 Hz, 1H; H-9′$_{(B)}$), 2.17, 2.10, 2.03, 1.99 (4 × 3H; 4 Ac), 1.7−1.4 (m, 4H; H-10′, H-13′), 0.92 (t, J = 7.4 Hz, 6H; H-11′, H-14′); ^{13}C NMR (DEPT, HMQC, HMBC; $CDCl_3$) δ 191.60 (C-2), 170.53, 170.42, 169.60, 169.51 (4 C=O, 4 Ac), 141.69 (C-4), 135.07 (C-3′), 119.38 (C-2′), 112.79 (C-3), 73.99 (C-4a′), 68.75 (C-7′), 68.70 (C-8′), 68.52 (C-6′), 67.27 (C-1), 62.18 (C-15′), 56.76 (C-8a′), 55.46 (C-9′), 49.93 (C-12′), 21.86 (C-10′), 20.73, 20.71, 20.67, 20.64 (4 CH_3, 4 Ac), 20.61 (C-13′), 11.49 (C-14′), 11.18 (C-11′); HR-MS calculated for $C_{26}H_{38}N_2O_{10}$ 538.2526, found 538.2522; UV−vis (CH_3OH) λ_{max} (log ϵ) 395 nm (4.46).

Compound **2b** yielded 0.5 mg of a yellow oil: ^{1}H NMR (COSY; $CDCl_3$) δ 7.10 (d, J = 14.7 Hz, 1H, H-4),6.49 (s, H-3′), 6.06 (d, J = 14.7, 1H, H-3), 4.87 (dd, J = 5.0, 0.9 Hz, 1 H, H-9′), 4.83 (d, J = 15.8 Hz, 1H, H-1$_{(A)}$), 4.78 (d, J = 15.8 Hz, 1 H, H-1$_{(B)}$), 4.78 (d, J = 0.9 Hz, 1 H, H-10′), 4.41 (dd, J = 11.5, 3.9 Hz, 1 H, H-11′$_{(A)}$), 4.34 (ddd, J = 5.7, 5.0, 3.9 Hz, 1 H, H-8′), 4.22 (dd, J = 11.6 Hz, 1 H, H-11′$_{(B)}$), 3.32 (d, J = 12.5 Hz, 1 H, H-6′$_{(A)}$), 3.19 (d, J = 12.5 Hz, 1 H, H-6′$_{(A)}$), 3.13, 2.96, 2.81, 2.52 (4 m, 4 H, 2 N−CH_2, propyl), 2.18, 2.11, 2.10, 2.07 (4 s, 3 CH_3, 4 Ac), 1.58−1.54 (m, 4 H, 2 CH_2, propyl), 0.88, 0.84 (2 t, J = 7.2 Hz, 6 H, 2 CH_3, propyl); MS (EI), m/z 538 (M$^+$); UV−vis (CH_3OH) λ_{max} = 399.5 nm.

Browning Reaction on a Cellulose Surface. A cellulose filter paper (Schleicher and Schüll) was soaked in a concentrated solution of 2.5 g of D-glucose, 2 mL of *n*-propylamine, 1.4 mL of acetic acid, and 2.5 mL of water. It was then removed from the solution and kept for 30 min at 85 °C. After cooling, the products were eluted from the filter by a mixture of methanol/ethyl acetate (1:4) and analyzed by HPLC or TLC as described above.

RESULTS AND DISCUSSION

Compared to a diluted aqueous solution, Maillard browning reactions are highly favored at low water activity (*11*). Therefore, alcoholic solutions are widely used (*5*, *9*) to model Maillard reactions at low water activity, as, for example, present in bread crust or during coffee roasting.

D-Glucose was therefore reacted with amines, such as propylamine, in methanolic or ethanolic solution under reflux, and after different time points, the mixtures were analyzed by HPLC with UV detection. In the visible wavelength range two main products were detected, the formation of which increased during prolonged heating.

The main product could be isolated by the subsequent application of column chromatography on silica gel, preparative TLC, and semipreparative RP-HPLC. Both products were rather labile and slowly degraded, but it was possible to stabilize the structures by acetylation. Thus, higher quantities of the peracetylated derivatives were obtained for further spectral analyses.

The major product was identified by 2D NMR, MS, and UV−vis spectroscopy as [(4a*S*,6*R*,7*S*,8*R*,8a*R*)-4,4a,6,7,8,8a-hexahydro-7,8-dihydroxy-6-(hydroxymethyl)-1,4-dipropyl-1*H*-pyrano[2,3-*b*]pyrazine-2-yl]-1-hydroxy-3-buten-2-one (**1a**) (Figure 1). The molecular weight of 370, which was obtained by CI-MS and the HR-MS of the acetylated derivative, suggested that in the formation of **1a** two molecules of D-glucose and two molecules of propylamine are involved, with the loss of six molecules of water. A signal at δ_C 196.9 (s) in the ^{13}C NMR was assigned to an unsaturated ketone, which is consistent with the fact that three olefinic protons are detected, with chemical shifts of δ_H 7.05 (d), 6.41 (s),

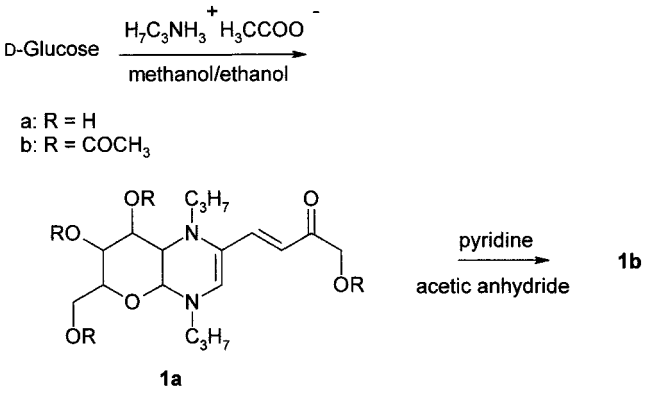

Figure 1. Formation of the colored compound **1a** from D-glucose and propylamine in alcoholic solution.

and 5.95 (d). The large coupling constant (15.4 Hz) of the two doublets and the difference in their chemical shift of ~1 ppm suggest a double bond in a trans configuration, with an electron-withdrawing group. Together with the fact that a fourth quaternary olefinic C-atom appears at δ_C 117.8, an $\alpha,\beta,\gamma,\delta$-unsaturated ketone was assumed to be part of the chromophore. Additionally, the ^{1}H NMR shows six signals between 3.0 and 5.0 ppm that belong to seven protons in a carbon chain. The coupling constant (5.1 Hz) of the first proton in the chain reveals that it is a derivative of α-glucopyranose. However, the signal of the proton and the carbon in position 2 of the chain is shifted to a higher field (3.11 and 58.8 ppm), so it was concluded that the hydroxy group of the sugar is substituted by an amine group. At a higher field (3.34 and ≤2.94 ppm) the protons of two *N*-propyl groups can be seen. All four protons of both methylene groups that are bound to the nitrogen show different chemical shifts, leading to spectra of higher order. This observation is usually made when the nitrogen is incorporated into a ring system, particularly when chiral centers are involved. Finally, a singlet can be detected in the ^{1}H NMR at δ_H 4.4 (δ_C 66.0) which does not belong to the carbon chain of the sugar. In summary, these observations are in accordance with structure **1a**. Similar NMR data were obtained for the acetylated derivative **1b**. For further structural confirmation, HMBC experiments were performed with **1b**. The long-range coupling signals obtained are summarized in Table 1 and clearly support the proposed structure.

A possible reaction mechanism that explains the formation of **1a** is presented in Figure 2.

The second product (**2b**) was formed in minor concentrations. After acetylation, a small quantity was purified, of which ^{1}H NMR, MS, and UV spectra were recorded. The ^{1}H NMR spectrum showed very similar signals for the chromophore to those of **1a**, but differences in the sugar residue. Together with the fact that **1b** and **2b** possess the same molecular mass, it has to be assumed that they possess a similar structure. However, ^{13}C NMR spectra are required for unambiguous structure assignment.

Browning reactions of D-glucose were also performed on a cellulose filter paper, which should mimic Maillard reactions on surfaces with low water activity, for example, the bread crust during baking. After the reaction, products were eluted from the paper, and **1a** and **2a** were identified as the main products by TLC and HPLC.

Table 1. Selected CH Long-Range Couplings That Support the Structure of Compound 1b Obtained by HMBC Experiment (Atom Numbering Refers to Figure 4)

		CH coupling in HMBC with	
signal	chemical shift (ppm)	signal	chemical shift (ppm)
C-11′	11.18	H-10′	1.4−1.7
C-14′	11.49	H-12′	2.92
C-13′	20.61	H-12′	2.92
		H-14′	0.92
C-10′	20.86	H-9′	2.77
		H-11′	0.92
C-12′	49.93	H-13′	1.4−1.7
		H-14′	0.92
		H-3′	6.34
C-9′	55.46	H-10′	1.4−1.7
		H-11′	0.92
C-8a′	56.76	H-9′$_{(A)}$ and H-9′$_{(B)}$	2.77 and 2.46
		H-8′	4.85
C-15	62.18	H-7′	5.02
C-6′,[a] C-8′,[a] C7′[a]	68.52, 68.70, 68.75	H-8′,[a] H-7′,[a] H-6′[a]	4.85, 5.02, 3.77
C-4a′	73.99	H-3′	6.34
		H-12′	2.92
C-2′	119.38	H-4	7.01
		H-3′	6.34
		H-3	5.87
		H-8a′	3.36
		H-9′	2.77
C-3′	135.07	H-4	7.01
		H-12′$_{(A)}$ and H-12′$_{(B)}$	3.22 and 2.92
C-4	141.69	H-3	5.87
C=O(Ac)[a]	169.51	H-8′ or H-7′	4.85 or 5.02
		CH_3(Ac)	2.03 or 1.99
C=O(Ac)[a]	169.6	H-8′ or H-7′	4.85 or 5.02
		CH_3(Ac)	2.03 or 1.99
C=O(Ac)[a]	170.42	H-15′ or H-1	4.06 or 4.77
		CH_3(Ac)	2.17
C=O(Ac)[a]	170.53	H-15′ or H-1	4.06 or 4.77
		CH_3(Ac)	2.10
C-2	191.6	H-4	7.01
		H-3	6.34
		H-1	4.77

[a] Differentiation not possible.

When D-glucose or other reducing sugars are heated with amino acids or alkylamines in neutral aqueous or alcoholic solution, the formation of a yellow color can be observed, which turns, during prolonged heating, dark brown. Thus far, the reaction mechanisms underlying this process are not fully understood.

Maillard reaction of pentoses, for example, xylose, yields 4-hydroxy-5-methyl-2,3-dihydrofuran-3-one (**3**) in very high concentrations. Because **3** possesses a CH-acidic methylene group, it was suggested that it undergoes an aldol-type reaction with other intermediates containing an aldehyde group. This process could then lead to a variety of colored products, of which, for example, **4**, and its precursors **5** and **3**, were isolated from a Maillard reaction mixture of xylose (Figure 3; *3*). From Maillard mixtures of D-glucose, several yellow or orange products have been isolated (*5*, *10*). Furthermore, degradation of D-glucose leads also to the formation of products with CH-acidic groups and of products with reactive aldehyde groups. It was therefore suggested that, similar to pentoses, aldol-type reactions of these intermediates can lead to the formation of colored compounds. Therefore, furfural, as a model for a reactive aldehyde, was added to Maillard mixtures or heated with amino acids, and indeed several colored products were isolated from the reaction mixtures (*1*, *4*, *6*−*9*).

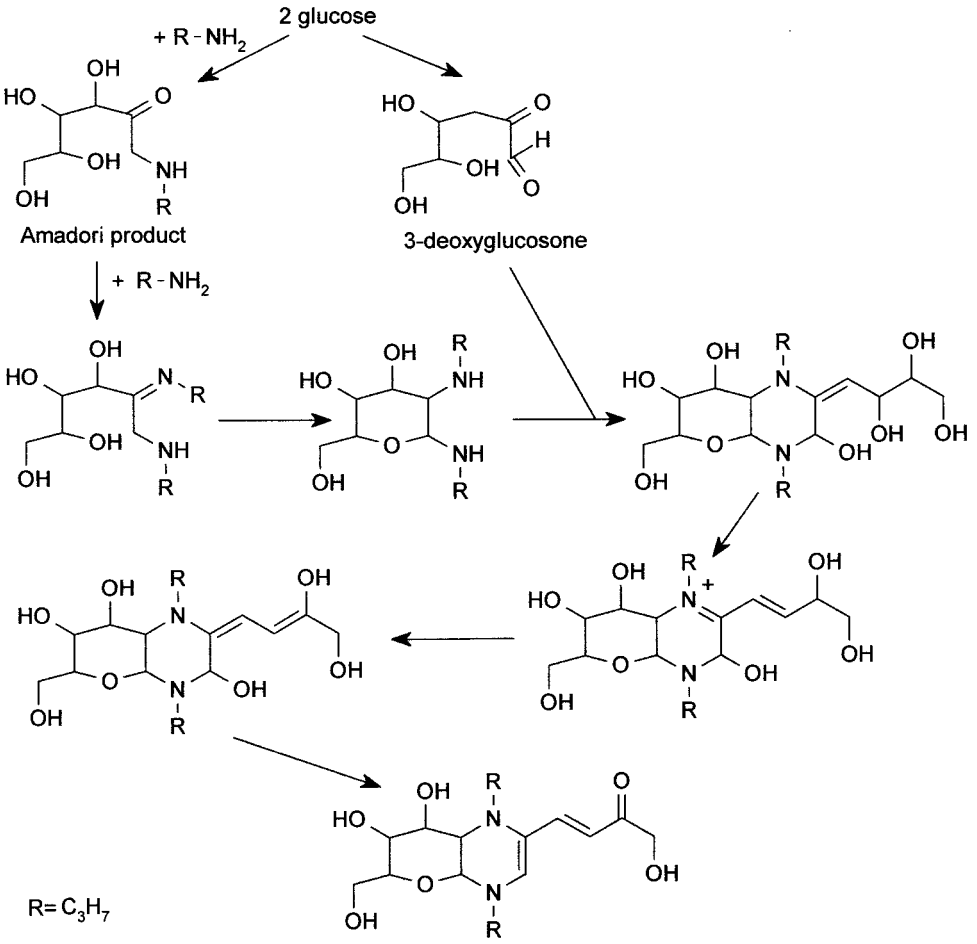

Figure 2. Proposed reaction mechanism leading from D-glucose to **1a**.

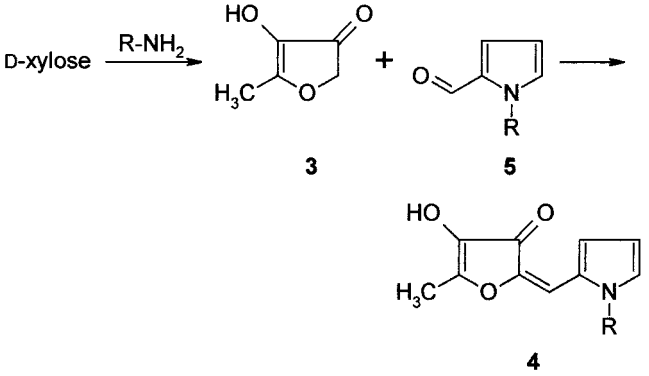

Figure 3. Formation of colored Maillard products from pentoses.

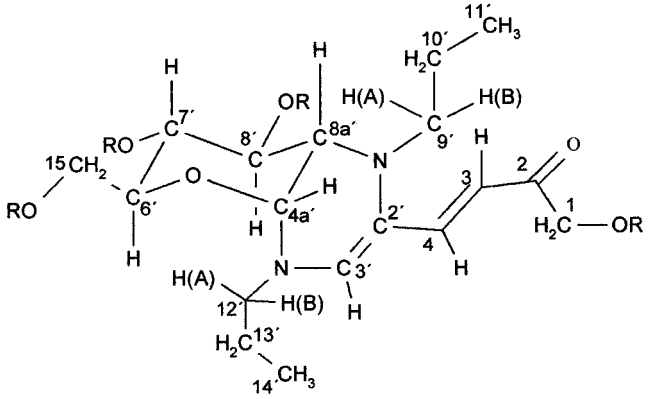

Figure 4. Proposed 3D structure of **1a**.

However, furfural and (hydroxymethyl)furfural are formed only in low concentrations during sugar degradation, so that most of these products have been detected so far only in Maillard mixtures with large amounts of furfural added. It can still be assumed that other intermediates with reactive carbonyl groups which are more relevant to sugar degradation can react in a similar way to furfural.

Very recently, two compounds were isolated from reaction mixtures of D-glucose or xylose and glycine having maximum UV absorbance at 371 nm and for which a 5,6-dihydropyridone and an azepinone structure were discussed (*12*).

Propylamine was used in these experiments as a model compound for primary amino groups, but it has been previously shown that amino acids or L-lysine side chains of proteins react in a similar way to alkylamines (e.g. ref *8*).

Under the conditions applied here, **1a** and **2a** are the products with highest color intensity at 400 nm when D-glucose and propylammonium acetate are heated in concentrated methanolic solution and the mixture is analyzed by HPLC/UV−vis. In the mixtures, peaks were identified by coelution with the purified standard. Both products are also formed when mixtures of methanol and water are used as solvent. In aqueous solution, however, other colored products are predominant, which are currently under investigation. Alcoholic solutions have often been applied to mimic foodstuffs with low water content, particularly those containing hydroxy

groups, for example, from starch or cellulose. It is known that under these conditions browning reactions are highly favored on bread crust or in roasted coffee. To evaluate the model system, the reaction was carried out not only in alcoholic solutions but also on the surface of a cellulose filter paper. After elution, a similar product profile was obtained as in the alcoholic solution. However, it still has to be investigated if products analogous to the structures **1a** and **2a** are formed during food processing and under harsher conditions as applied during baking or roasting.

ABBREVIATIONS USED

COSY, correlated spectroscopy; FTIR, Fourier transformation infrared spectroscopy; HMBC, heteronuclear multiple-bond correlation; HMQC, heteronuclear multiple-quantum coherence; HPLC, high-performance liquid chromatography; MS, mass spectroscopy; NMR, nuclear magnetic resonance spectroscopy; RP, reversed phase; TLC, thin-layer chromatography.

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