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Formation of pyridinium betaines by reaction of hexoses with primary amines

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Abstract

The reaction of glucose or fructose with primary amines and the thermal degradation of Amadori products of glucose leads to the formation of 6-formyl-1-alkylpyridinium-3-olates (**5**), of which 6-formyl-1-propylpyridinium-3-olate was isolated from a reaction mixture. A reference substance can be prepared in a two-step procedure from 5-(hydroxymethyl)-2-furaldehyde. Comparatively high yields of these betaines in relation to other UV-absorbing products are obtained under typical food processing and physiological conditions. With primary amines, the betaines readily form a Schiff base that undergoes Strecker-type degradation when heated in aqueous solution.

Keywords: Maillard reaction; D-glucose; D-fructose; Pyridinium betaine; 6-Formyl-1-alkylpyridinium-3-olate; Strecker degradation

1. Introduction

Reactions of reducing sugars with amino acids or proteins (Maillard reactions) are of great importance in food chemistry and have been thoroughly investigated. During food processing, Maillard-type reactions result in development of colour and flavour components, for example via Strecker degradation of amino acids in the presence of sugar-derived dicarbonyl compounds [1].

More recently, interactions between glucose and proteins have received significant attention due to their medical implications. There is substantial evidence that these processes contribute to pathophysiological changes associated with diabetes and arteriosclerosis [2]. Moreover, Maillard-type reactions are considered to be involved in biological aging [3].

The term Maillard reaction describes a complex process, which despite intensive investigations is only partially understood. Interaction of glucose or other reducing carbohydrates with lysine side-chains of proteins or with amino acids initially leads to the formation of the corresponding Schiff bases which can rearrange to the more-stable Amadori compounds. The latter degrade via different pathways to give a large variety of products.

In this communication we report on the isolation and reactions of a new substance with a pyridinium betaine structure, which must be considered as one of the main products of Maillard-type processes.

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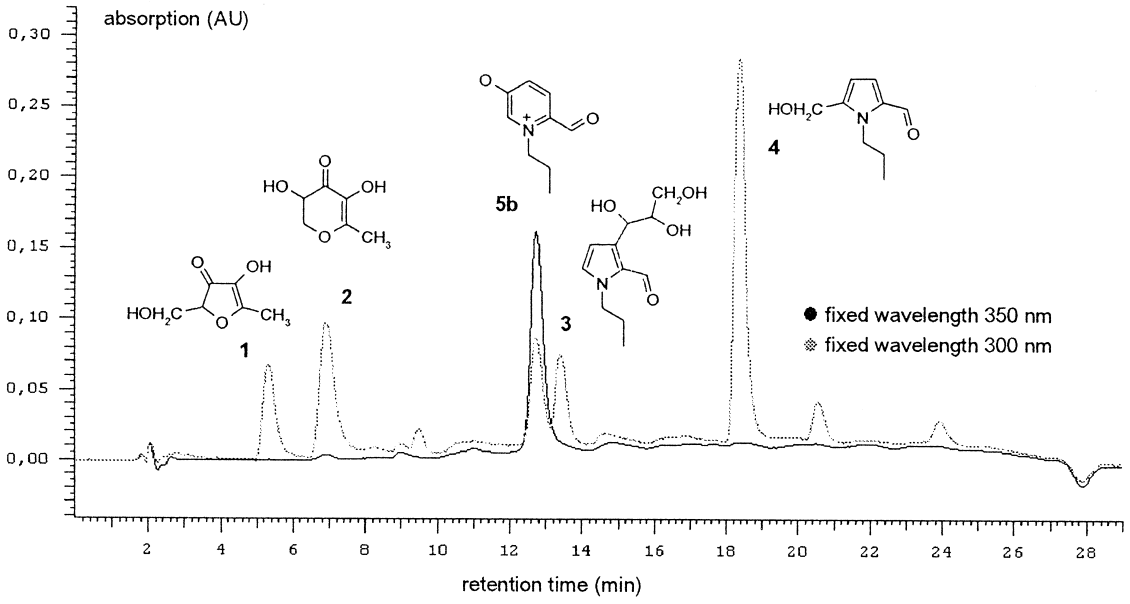


Fig. 1. Chromatogram of a reaction mixture with glucose and 1-propylamine, which had been heated at 100 °C and pK 5.0 for 60 min. UV-absorption is shown for two different wavelengths (300 and 350 nm).

The studies have been performed with different aliphatic amines as model compounds for the lysine side chains in proteins.

2. Results and discussion

When D-glucose and 1-propylamine are heated in phosphate-buffered, weakly acidic aqueous solution at 100 °C, various substances are formed that can be separated by HPLC. A typical chromatogram of a reaction mixture is shown in Fig. 1. In addition to products **1**, **2**, **3** and **4** that were previously isolated and identified [4], a compound with UV-maxima at 238, 291, and 343 nm could be detected. The importance of this substance in relation to other main products of the Maillard reaction can be derived from the figure. When fructose was subjected to identical conditions, the same product was formed.

The new substance was isolated by preparative UPLC and purified by crystallization. The structure of the pyridinium betaine **5b** was established by spectral data and confirmed by X-ray crystallography[1] (Fig. 2).

Next, the reaction conditions under which betaine **5** is formed were investigated, and quantitative aspects taken into account. Quantitation was performed using caffeine as the internal standard.

The new product was also obtained by thermal degradation of the corresponding Amadori compound in aqueous solution. In this case, the maximal yield of 0.6% was observed after 4 h of heating.

Furthermore, the Maillard reaction of glucose under physiological conditions, namely, in dilute phosphate-buffered, neutral aqueous solution at 38 °C, furnishes betaine **5**. Remarkably, under these conditions, the new pyridinium aldehyde was

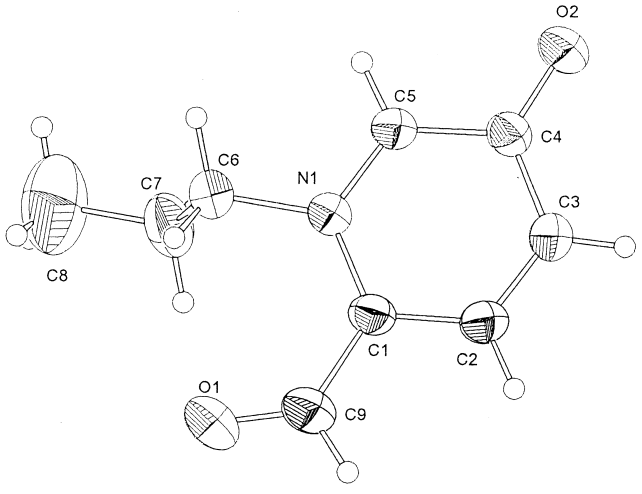
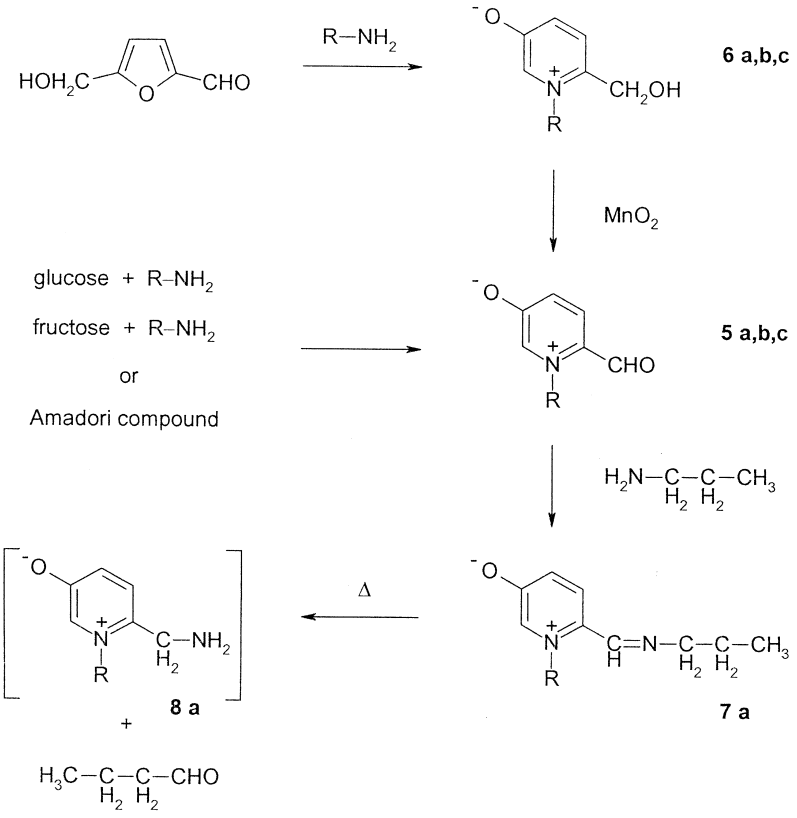


Fig. 2. X-ray structure of the isolated pyridinium betaine.

[1] Complete details of the structure investigations are available on request from the Fachinformationszentrum Karlesruhe, D-76344 Eggenstein-Leopoldshafen on quoting the depository number CSD-408594, the names of the authors, and the journal citation.



a R = CH_3

b R = $CH_2CH_2CH_3$

c R = $CH_2CH_2CH_2CH_2CH(NHCOCH_3)COOH$

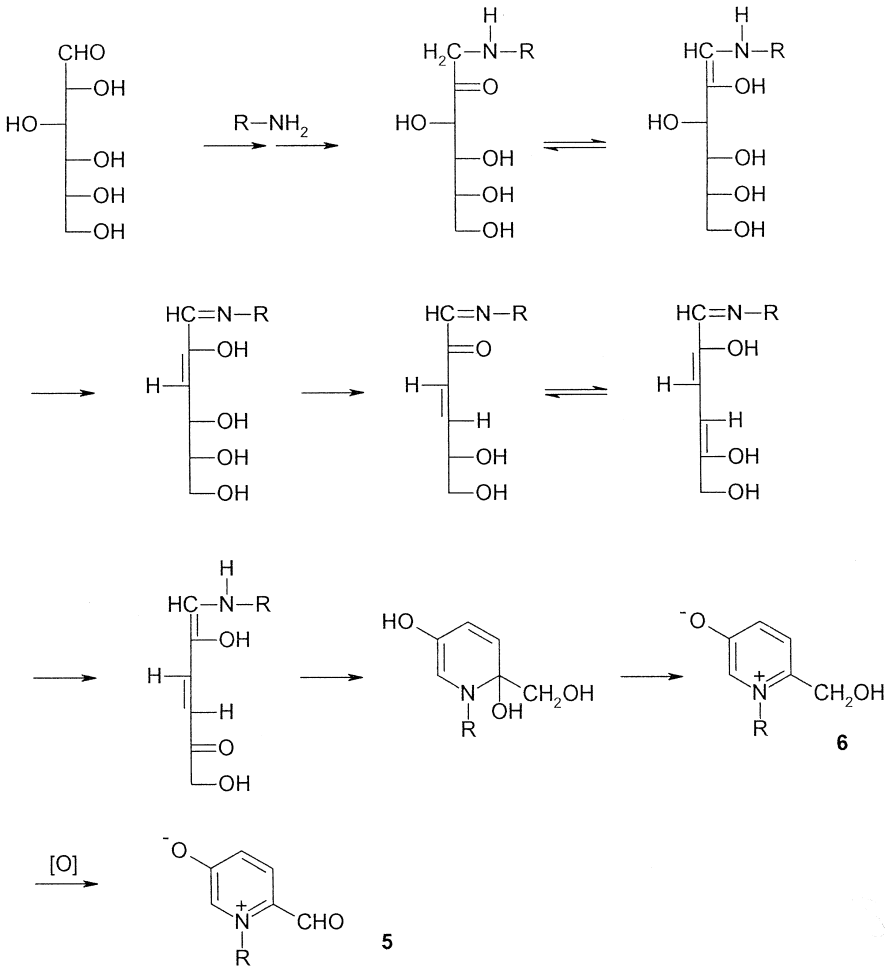
detected even as the main UV-absorbing product, which had the amine compound incorporated. After 10 days of incubation the yield exceeded 0.1%, and increased further upon prolongation of the reaction.

Conversion of glucose and 1-propylamine under slightly acidic conditions gave yields of up to 0.5% after 4 h. However, it should be recognized that **5b** is a labile substance, which readily degrades under the conditions used.

Besides aliphatic amines, lysine derivatives were also expected to react in the same way. Indeed, when glucose was heated with N^6-acetyl-L-lysine, a substance with the characteristic UV-maxima of **5** at 238, 291, and 343 nm could be detected in the sample. This product was not purified, however.

Previously, we have synthesized 1-alkyl-2-hydroxymethyl-5-oxypyridinium betaines **6a** and **6c** by heating 5-(hydroxymethyl)-2-furaldehyde with primary amines (Scheme 1) [5]. In contrast to our expectations, betaines with the structure of **6** are formed only in minor amount under the conditions of the Maillard reaction just described. In a synthetic procedure, the alcohol **6** is readily transformed into the corresponding aldehyde **5** with activated manganese dioxide (Scheme 1).

A reaction mechanism explaining the formation of the pyridinium betaines, is suggested in Scheme 2. Dehydration and cyclization of the Amadori compound may lead to betaine **6**, which is further oxidized to the aldehyde **5**. So far, it is not clear whether the latter step is directly induced by



oxygen or by oxidizing intermediates that are formed during the course of the Maillard reaction. However, it is well established that numerous redox processes can take place in Maillard reaction mixtures [6].

Under appropriate reaction conditions, namely, weakly acidic phosphate-buffered aqueous solution, the pyridinium betaine **5b** can be isolated in comparatively high yield from heated samples. The compound, though, is not a stable end-product of the Maillard reaction, as can be expected from its structure. With a slight excess of primary amine, the aldehyde **5b** readily forms the corresponding Schiff base **7b**, which was characterized by its NMR spectrum, although the elemental analysis indicated that the product was not obtained as a completely pure substance.

Upon heating at 70 °C in aqueous solution, the imine **7b** undergoes a Strecker-type degradation. In this way, the volatile Strecker aldehyde and probably betaine **8b** (not isolated) are formed. The aldehyde was extracted from the heated sample and transformed into the *O*-benzyloxime, which could be detected by GC–MS.

In summary it may be stated that the new pyridinium betaine **5** is a major Maillard product, which has the amino component incorporated. The substance is formed in comparatively high yields (up to 0.6%) under conditions that are typical for food processing, such as cooking, baking, and boiling at slightly acidic pH. Thus, it can be assumed that compounds of type **5** can be formed in various foodstuffs, where further reactions with amino acids may occur. By the formation of volatile aldehydes in a Strecker degradation process, the pyridinium betaine is capable of contributing to the flavour, which is generated by the Maillard reaction. On the other hand, compound **5** is also

obtained by incubation at 38 °C and neutral pH. Although initial yields are low in comparison to conversion under acidic conditions at elevated temperature, formylpyridiniumbetaine (**5**) is still the main LTV-absorbing product under physiological conditions. It can be expected that glucose reacts in vivo with lysine side chains or N-terminal amino groups of proteins to give protein-bound pyridinium betaine **5**. This product should be capable of binding to the lysine side-chain of a second molecule of proteins. The resulting Schiff bases of type **8** can be responsible, at least partially, for protein crosslinks, which are formed in vivo and attributed to the Maillard reaction.

3. Experimental

General methods.—^{1}H NMR and ^{13}C NMR spectra were recorded with a Jeol 400 GSX spectrometer. Chemical shifts are reported in ppm and referenced to $(CH_3)_4Si$ in CD_3OD, or to acetone in D_2O as the internal or external standard, respectively. Chemical-shift correlations were made via ^{1}H–^{1}H COSY and ^{1}H–^{13}C COSY spectra. Mass-spectral analyses were obtained with an HP 5989 A MS Engine (CI with CH_4). The GC–MS system consists of a Hewlett–Packard (Waldbronn, Germany) gas chromatograph BP 5890 series II coupled with a Hewlett–Packard BP 5971A msd mass spectrometer. Separations were performed on an Optima fused silica capillary column 1701–0.25 μm (0.25 mm×25 m, Macherey–Nagel, Düren, Germany) with a helium flow of 0.5 mL/min (split: 1/10). The oven temperature was programmed as follows: $\theta_1 = 60\ ^\circ C$; isotime 1 = 4 min; ramp rate 1 = 2 °C/min. $\theta_2 = 80\ ^\circ C$; isotime 2 = 0 min; ramp rate 2 = 20 °C/min. $\theta_3 = 260\ ^\circ C$; isotime 3 = 15 min. The mass spectrometer was operated in scan mode for compound identification, and mass spectra were recorded in the electron-impact mode. Analytical HPLC was performed with a Merck L-6200A gradient pump and a Merck L-4500 photodiode array detector (DAD) including Merck–Hitachi Model D-6500 Chromatography Data Station software (Merck, Darmstadt, Germany). UV spectra were directly taken from this system (λ in nm). For preparative HPLC a Merck L-6250 pump, a Merck L-4000 UV detector, and a Merck D-2500 chromatointegrator were used. The water used for HPLC was deionized and distilled prior to use. HPLC-grade solvent (MeOH) was used without further purification. System 1 (analytical): column: Nucleosil RP 18, 125×3 mm i.d., 5 μm particle size with guard cartridge; UV-detection between 220 and 415 nm (DAD); eluent: gradient elution starting with 100% A (0.05 M triethylammonium acetate buffer, pH 7.0), ending within 25 min with A-methanol (2:8) at a flow rate of 0.4 mL/min. System 2 (preparative): column: Nucleosil RP 18, 250×21 mm i.d., 7 μm particle size; LRI-detection at 343 nm; eluent: MeOH–water (3:7).

6-Formyl-1-methylpyridinium-3-olate (**5a**).—5-Hydroxymethyl-2-furaldehyde (3.2 g, 25 mmol, Roth, Karlsruhe, Germany) was dissolved in a mixture of 20 mL of water and 40 mL of an ethanolic solution of $MeNH_2$ (33%, Fluka, Neu-Ul, Germany). After heating at reflux for 3 days, the mixture was evaporated and lyophilized. Flash-chromatography of the residue on silica gel, eluting with MeOH, was performed twice to give **6a**. The crude product was dissolved in 50 mL of MeOH and heated with activated MnO_2 (5 g, 57.5 mmol, Fluka, Neu-Ulm, Germany) at reflux for 3 h. Subsequent to evaporation, flash-chromatography of the residue on silica gel, eluting with MeOH, and crystallisation (EtOAc, CH_2Cl_2, acetone) gave 1.3 g (9.5 mmol, 38%) of **5a** as yellow needles: mp 178 °C; t_{ret}, (HPLC) 6.1 min; UV_{max} 238, 291, 343 nm ^{1}H NMR (Me_2SO-d_6): δ 4.31 (s, 3 H, CH_3), 6.86 (dd, 1 H, H-4), 7.69 (d, 1 H, H-2), 7.84 (d, 1 H, H-5), 9.70 (s, 1 H, CHO); ^{13}C NMR (COSY, Me_2SO-d_6): 45.23 (CH_3), 124.38 (C-3), 128.63 (C-4), 132.63 (C-5), 141.10 (C-2), 171.22 (C-6), 181.62 (CHO); MS-CI: m/z 138 (100%, $M+1^+$). Anal. Calcd for $C_7H_7NO_2$: C, 61.31; H, 5.11; N, 10.22. Found: C, 61.41; H, 5.15; N, 10.05.

6-Hydroxymethyl-1-propylpyridinium-3-olate (**6b**).—5-(hydroxymethyl)-2-furaldehyde (3.2 g, 25 mmol, Roth, Karlsruhe, Germany) was dissolved in a mixture of 20 mL EtOH and 20 mL, water. 1-Propylamine (1.77 g, 30 mmol) was added and the pH of the solution adjusted to 9.4 with NaOH. After heating at reflux for three days, the mixture was evaporated and lyophilized. Flash-chromatography of the residue on silica gel, eluting with MeOH was performed twice and followed by crystallisation (EtOAc, EtOH) to give 1.8 g (10.8 mmol, 43%) of **6b** as colourless needles: mp 149 °C; t_{ret} (HPLC) 8.1 min; UV_{max} 252, 322 nm; ^{1}H NMR (CD_3OD): δ 1.04 (t, 3 H, CH_3), 1.97 (m, 2 H, CH_2CH_3), 4.36 (t, 2 H, NCH_2), 4.71 (s, 2 H, CH_2OH), 7.32 (dd, 1 H, H-4), 7.51 (d, 1 H, H-5), 7.68 (d, 1 H, H-2). ^{13}C NMR (COSY, CD_3OD):

11.08 (CH_3), 25.87 (CH_2–CH_3), 59.16 (N–CH_2), 60.43 (CH_2OH), 129.77 (C-5), 135.43 (C-4), 136.28 (C-2), 138.45 (C-3), 168.67 (C-6); MS-CI: *m*/*z* 168 (100%, $M+1^+$). Anal. Calcd for $C_9H_{13}NO_2$: C, 64.65; H, 7.84; N, 8.38. Found: C, 64.22; H, 8.27; N, 8.34.

6-Formyl-1-propylpyridinium-3-olate (**5b**).—6-hydroxymethyl-1-propylpyridinium-3-olate (1.67 g, 10 mmol) was dissolved in 50 mL, of MeOH, activated MnO_2 (5 g, 57.5 mmol, Fluka, Neu-Ulm, Germany) was added and the mixture was heated at reflux for 3 h. Subsequent to filtration and evaporation, the residue was purified by flash chromatography on silica gel, eluting with MeOH and crystallisation (Et_2O THF) to give 1.16 g (7 mmol, 70%) of **5b** as yellow crystals: mp 121 °C; t_{ret} (HPLC) 12.6 min; UV_{max} 238, 291, 343 nm; ^1H NMR (Me_2SO-d_6): δ 0.91 (t, 3 H, CH_3), 1.81 (m, 2H, CH_2CH_3), 4.62 (t, 2 H, NCH_2), 6.88 (dd, 1 H, H-4), 7.77 (d, 1 H, H-2), 7.89 (d, 1 H, H-5), 9.61 (s, 1 H, CHO); MS-CI: *m*/*z* 166 (100%, $M+1^+$). Anal. Calcd for $C_9H_{11}NO_2$: C, 65.44; H, 6.71; N, 8.48. Found: C, 65.13; H, 7.17; N, 8.28. Spectral data were identical to those of the isolated compound.

*6-(*N*-Propyl)-carbimino-1-methylpyridinium-3-olate* (**7b**).—6-Formyl-1-methylpyridinium-3-olate (137 mg, 1 mmol) was dissolved in 1-propylamine (2.36 g, 40 mmol) and the excess of amine was evaporated in vacuo at room temperature and in high vacuo to give 176 mg (99%) of **8b** as a yellow solid: mp 126 °C; t_{ret} (HPLC) 13.2 min; UV_{max} 236, 337 nm; ^1H NMR ($CDCl_3$): δ 0.90 (t, 3 H, CH_2CH_3), 1.65 (m, 2 H, CH_2CH_3), 3.52 (t, 2 H, NCH_2), 4.19 (s, 3 H, NCH_3), 7.14 (dd, 1 H, H-4), 7.32 (d, 1 H, H-2), 7.56 (d, 1 H, H-6), 8.17 (s, 1 H, CHN); ^{13}C NMR (COSY, $CDCl_3$): 11.70 (CH_2CH_3), 23.98 (CH_2CH_3), 46.43 (NCH_3), 64.09 (NCH_2), 126.60 (C-3), 130.03 (C-5), 133.28 (C-4), 137.79 (C-2) 152.20 (CHN), 170.39 (C-6); MS-CI: *m*/*z* 179 (100%, $M+1^+$).

*Propanal-*O*-benzyloxime (syn and anti isomers).*—Propanal (58 mg, 1 mmol) was added to a solution of *O*-benzylhydroxylamine hydrochloride (160 mg, 1 mmol) and Na_2CO_3 (53 mg, 0.5 mmol) in 10 mL of water. After shaking the mixture, the product was extracted with diethyl ether and the organic layer dried over anhydrous Na_2SO_4, followed by evaporation in vacuo. Subsequent purification on silica gel, eluting with EtOAc, gave 130 mg (80%) of colourless liquid. NMR data are given for the *anti* (a) and *syn* (b) isomers. (a) ^1H NMR ($CDCl_3$): δ 1.05 (t, 3 H, CH_3), 2.18 (m, 2 H, CH_2CH_3), 5.04 (s, 2 H, OCH_2), 7.33 (m, 5 H, C_6H_5), 7.43 (t, 1 H, CHN); ^{13}C NMR (COSY, $CDCl_3$): 11.04 (CH_3), 23.06 (CH_2CH_3), 75.50 (CH_2O), 128.22 (C-2), 128.33 (C-4), 128.34 (C-3), 138.18 (C-1), 152.28 (CHN); (b) ^1H NMR ($CDCl_3$): δ 1.02 (t, 3 H, CH_3),2.36 (m, 2H, CH_2CH_3), 5.09 (s, 2 H, OCH_2), 6.64 (t, 1 H, CHN), 7.27 (m, 5 H, C_6H_5); ^{13}C NMR (COSY, $CDCl_3$):10.61 (CH_3), 19.28 (CH_2CH_3), 75.67 (CH_2O), 127.68 (C-4), 127.76 (C-3), 127.86 (C-2), 137.78 (C-1), 153.50 (CHN); MS-CI: *m*/*z* 164 (100%, $M+1^+$); GC–MS data: t_{ret} 17.3 min; *m*/*z* (rel. intensity) 163 (1%, M^+), 146 (2),133 (4),105 (2),92 (8),91 (100),77 (12),65 (9),51 (8). Anal. Calcd for $C_9H_{11}NO$: C, 73.57; H, 8.03; N, 8.59. Found: C, 73.19; H, 8.08; N, 8.49.

Isolation of 6-formyl-l-propylpyridinium-3-olate (**5b**).—1-Propylamine (9 g, 152.2 mmol) and KH_2PO_4, (40 g, 296 mmol) were dissolved in 150 mL of water and the pH adjusted to 4.8 with phosphoric acid. Glucose (27 g, 150 mmol) was added and the mixture was refluxed for 100 min. After cooling, the solution was extracted with EtOAc several times. The pH was then adjusted to 7.5 and the solution again extracted with EtOAc. The latter organic layers were subjected to solid-phase extraction on silica and eluted with MeOH. The eluate was evaporated, dissolved in water and purified by preparative HPLC. Collected fractions were evaporated, lyophilized and crystallized, giving ~40 mg (0.24 mmol, 0.16%) of **5b** as crystals. The structure was determined by X-ray crystallography (Fig. 1). Spectral data were identical to those of the synthesized reference compound.

Formation of **5b**,**c** *under acidic conditions.*—Glucose (90 mg, 0.5 mmol) or fructose (90 mg, 0.5 mmol) and 1-propylamine (59 mg, 1 mmol) or N^6-acetyl-L-lysine (188 mg, 1 mmol) were dissolved in 1 mL of water and KH_2PO_4 (136 mg, 1 mmol) was added. The pH was adjusted to 5.0 with phosphoric acid or NaOH, respectively. The mixture was heated at 100 °C in a sealed vessel. The sample was diluted and analyzed by HPLC–DAD.

Formation of **5b** *under physiological conditions.*—Glucose (90 mg, 0.5 mmol) and 1-propylamine (59 mg, 1 mmol) were heated in 5 mL of 1 M phosphate buffer (pH 7.4) at 38 °C. The mixture was analyzed daily by HPLC–DAD.

Degradation of the Amadori compound.—1-Deoxy-1-(1-propylammonium)fructose, oxalate salt

(120 mg, 0.39 mmol), synthesized according to [7], and KH_2PO_4 (200 mg, 1.47 mmol) were dissolved in 2 mL of water and the pH was adjusted to 5.0 with NaOH. The solution was heated in a sealed vessel at 100 °C, diluted and analyzed by HPLC–DAD.

Quantitation standard.—Standard correction factors were calculated from injections of a solution of 38.8 mg/L of caffeine and 33.0 mg/L of **5b**.

Reaction of **5a** *with 1-propylamine to give 6-(*N*-propyl)-carbimino-1-methylpyridinium-3-olate* (**7a**).—6-Formyl-1-methylpyridinium-3-olate (6.9 mg, 0.05 mmol) and 1-propylamine (3 mg, 0.051 mmol) were dissolved in 0.5 mL of water and heated at 38 °C. The sample was analyzed daily by HPLC–DAD.

*Strecker degradation of 6-(*N*-propyl)-carbimino-1-methylpyridinium-3-olate* (**7a**).—6-Formyl-1-methylpyridinium-3-olate (27.4 mg, 0.2 mmol) and 1-propylamine (11.8 mg, 0.2 mmol) were dissolved in 0.5 mL of water and heated for 24 h at 70 °C in a sealed vessel. The mixture was extracted twice with Et_2O and the organic layers were dried over anhydrous Na_2SO_4. An aqueous solution of *O*-benzylhydroxylamine hydrochloride (159.6 mg, 1 mmol) and Na_2CO_3 (63 mg, 0.5 mmol) was shaken vigorously with the dried ether phase. The organic layer was dried, filtered, evaporated, and analyzed by GC–MS.

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