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Pyridinium-carbaldehyde: active Maillard reaction product from the reaction of hexoses with lysine residues

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Abstract—Besides the formation of the aminotriazine N^6-[4-(3-amino-1,2,4-triazin-5-yl)-2,3-dihydroxybutyl]-L-lysine, the reaction of [1-^{13}C]D-glucose with lysine and aminoguanidine leads to the generation of 6-[2-({[amino(imino)methyl]hydrazono}methyl)-pyridinium-1-yl]-L-norleucine (**14**-$^{13}C_1$). The dideoxyosone N^6-(2,3-dihydroxy-5,6-dioxohexyl)-L-lysine was shown to be a precursor in the formation of **14**-$^{13}C_1$, which proceeds via the reactive carbonyl intermediate 6-(2-formylpyridinium-1-yl)-L-norleucine (**13**-$^{13}C_1$). In order to study the reactivity of **13**-$^{13}C_1$, the model compound 1-butyl-2-formylpyridinium (**18**) was prepared in a two-step procedure starting from 2-pyridinemethanol. The reaction of the pyridinium-carbaldehyde **18** with L-lysine yielded the Strecker analogous degradation product 2-(aminomethyl)-1-butylpyridinium and another compound, which was shown to be as 1-butyl-2-[(2-oxopiperidin-3-ylidene)methyl]pyridinium. Reaction of **18** with the C–H acidic 4-hydroxy-5-methylfuran-3(2*H*)-one leads to the formation of the condensation product 1-butyl-2-[hydroxy-(4-hydroxy-5-methyl-3-oxofuran-2(3*H*)-ylidene)methyl]-pyridinium.

Keywords: Maillard reaction; Pyridinium-carbaldehyde; Aminoguanidine; Glycation; Strecker degradation; Dideoxyosone; Glycotoxin

1. Introduction

The Maillard reaction or 'nonenzymatic browning' is a complex series of reactions between reducing carbohydrates and lysine side chains or N-terminal amino groups of proteins, respectively. The first step of this process is the formation of labile Schiff bases, which rearrange to the more stable Amadori products. The Amadori compounds are slowly degraded in complex reaction pathways via dicarbonyl intermediates such as 3-deoxyglucosone, glyoxal, and methylglyoxal to a plethora of compounds[1,2] subsumed summarily under the term 'advanced glycation end products' (AGEs). This overall reaction sequences proceed both in vitro and in vivo. A major consequence of the advanced Maillard reaction is the formation of covalently cross-linked proteins. In long-lived tissue proteins, such as collagen and eye lens crystallins, these chemical modifications accumulate with age and may contribute to pathophysiologies associated with aging and long-term complications of diabetes and atherosclerosis. Additionally, reactions leading to inter- or intramolecular protein cross-linking are of special importance for the nutritional and functional properties of various foods.[3] On the basis of various model reactions, different mechanisms for cross-linking of amino acid side chains in proteins have been discussed.[4–15] So far, pentosidine **7**,[16] fluorophore LM-1,[17,18] crossline,[19] MOLD **5**,[20] GOLD **6**,[21,22] carboxymethyllysine,[23] as well as the lysine–arginine cross-links glucosepane **1**, DOGDIC **2**,

Abbreviations: AGEs, advanced glycation end products; AGOEs, advanced glycoxidation end products; DOGDIC **2**, N^6-{2-{[(4*S*)-4-ammonio-5-oxido-5-oxopentyl]amino}-5-[(2*S*,3*R*)-2,3,4-trihydroxybutyl]-3,5-dihydro-4*H*-imidazol-4-ylidene}-L-lysinate; GODIC **4**, N^6-(2-{[(4-*S*)-4-ammonio-5-oxido-5-oxopentyl]amino}-3,5-dihydro-4*H*-imidazol-4-ylidene)-L-lysinate; GOLD **6**, 6-{1-[(5*S*)-5-ammonio-6-oxido-6-oxohexyl]-imidazolium-3-yl}-L-norleucinate; MODIC **3**, N^6-(2-{[(4*S*)-4-ammonio-5-oxido-5-oxopentyl]amino}-5-methyl-3,5-dihydro-4*H*-imidazol-4-ylidene)-L-lysinate; MOLD **5**, 6-{1-[(5*S*)-5-ammonio-6-oxido-6-oxohexyl]-4-methylimidazolium-3-yl}-L-norleucinate.

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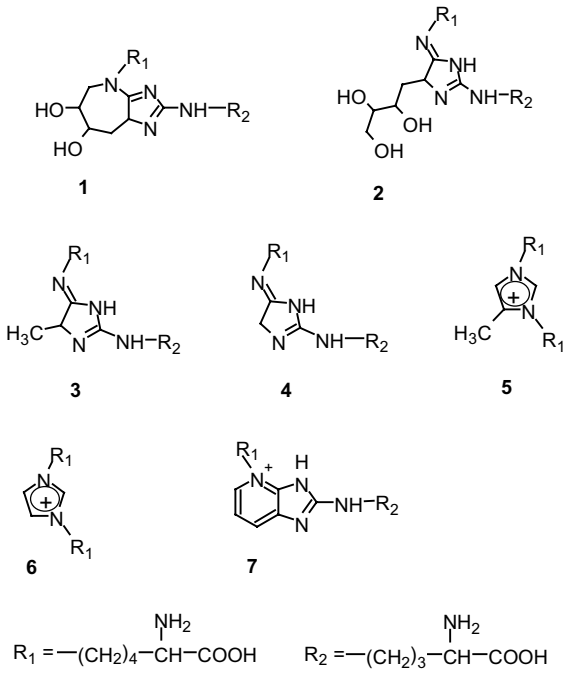


Figure 1. Structural formulas of major protein cross-links: glucosepane **1**, DOGDIC **2**, MODIC **3**, GODIC **4**, MOLD **5**, GOLD **6**, and pentosidine **7**.

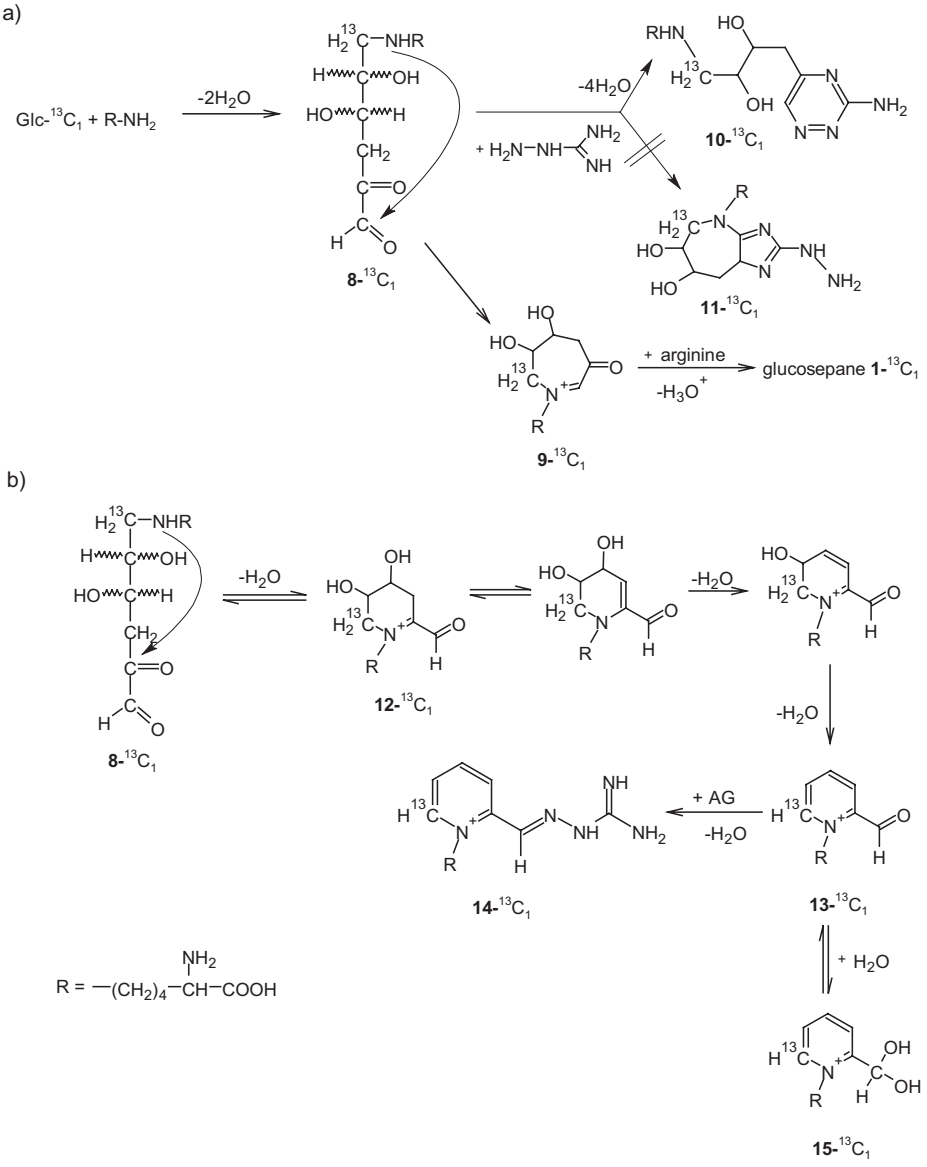
MODIC **3**, and GODIC **4** (Fig. 1) have been detected in vivo.[24] Some of the cross-links (e.g., fluorophore LM-1, **6**, and **4**) are formed via posttranslational modification of proteins by glycoxidation intermediates; they can therefore be designated as 'advanced glycoxidation end products' (AGOEs). Since most of the structurally identified AGEs are derived from α-dicarbonyl intermediates, it seems reasonable to expect that glycotoxins liberated from glycated proteins incorporate a similar highly reactive building block. Recently, it was shown that the major protein cross-link glucosepane **1** is derived from the regioisomeric structure N^6-(2,3-dihydroxy-5,6-dioxohexyl)-L-lysine (**8**).[24,25]

We now report on the structural characterization of 6-[2-({[amino(imino)methyl]hydrazono}methyl)pyridinium-1-yl]-L-norleucine (**14**-$^{13}C_1$), a Maillard reaction product formed as an follow-up product of the reactive pyridinium-carbaldehyde 6-(2-formylpyridinium-1-yl)-L-norleucine (**13**-$^{13}C_1$) (Scheme 1). The model compound for **13**-$^{13}C_1$, 1-butyl-2-formylpyridinium (**18**), was synthesized in order to gain more information about the glycating potential as the reactivity of the novel glycotoxin type toward lysine side chains and C–H acidic compounds.

2. Results and discussion

During incubations of ^{13}C-labeled glucose ([1-^{13}C]D-glucose), N^{α}-*t*-Boc-L-lysine, and aminoguanidine, Biemel et al.[25,26] identified the aminotriazine structure N^6-[4-(3-amino-1,2,4-triazin-5-yl)-2,3-dihydroxybutyl]-L-lysine (**10**-$^{13}C_1$), whereas the expected glucosepane-analogue **11**-$^{13}C_1$ could not be detected. Since aminoguanidine is an established trapping reagent for α-dicarbonyl compounds, the formation of the novel dideoxyosone N^6-(2,3-dihydroxy-5,6-dioxohexyl)-L-lysine (**8**-$^{13}C_1$) was proven (Scheme 1a). In this α-dicarbonyl the lysine N^{ε} is directly bonded to C-1 of the original carbohydrate; generation of **8**-$^{13}C_1$ requires carbonyl shifts along the entire sugar backbone. The intramolecular aldimine structure **9**-$^{13}C_1$ represents a plausible precursor in the formation of the major Maillard cross-link glucosepane **1**-$^{13}C_1$ (Scheme 1a).

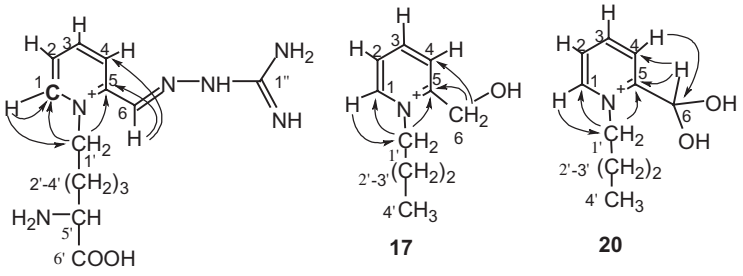
In our model studies [1-^{13}C]D-glucose and N^{α}-*t*-Boc-L-lysine were incubated with aminoguanidine in phosphate buffer pH 7.4, at 70 °C for 18 h. Beside the twin peak with the quasimolecular ion $[M+H]^+$ at m/z 430 (*t*-Boc-**10**-$^{13}C_1$) and the typical aminotriazine ultraviolet (UV) spectrum an additional product with $[M+H]^+$ at m/z 394 (*t*-Boc-**14**-$^{13}C_1$) and an UV maximum at 316 nm could be detected. The protective groups of the compound were cleaved off in acidic medium (3 M HCl) and **14**-$^{13}C_1$ and **10**-$^{13}C_1$ with $[M+H]^+$ at m/z 294 and m/z 330, respectively, were isolated by preparative HPLC. Accurate mass determination of **14**-$^{13}C_1$ gave $[M+H]^+$ at m/z 294.1762, corresponding to an elemental composition of $^{12}C_{12}^{13}CH_{22}N_6O_2$. The NMR data, compiled in Table 1, unequivocally prove formation of **14**-$^{13}C_1$, the ^{13}C label appears directly bonded to N^{ε} of the lysine moiety (Table 1). The formation pathway for **14**-$^{13}C_1$ starting from the dideoxyosone **8**-$^{13}C_1$ proceeds via the six-membered intramolecular ketimine **12**-$^{13}C_1$ as plausible intermediate followed by a series of tautomerizations and dehydrations, which lead to the formation of the pyridinium-carbaldehyde structure **13**-$^{13}C_1$ (Scheme 1). The highly reactive 6-(2-formylpyridinium-1-yl)-L-norleucine (**13**-$^{13}C_1$) finally condensates with the hydrazine moiety of aminoguanidine under the formation of 6-[2-({[amino(imino)methyl]hydrazono}methyl)pyridinium-1-yl]-L-norleucine (**14**-$^{13}C_1$). Since **14**-$^{13}C_1$ represents a Schiff base, it was surprising that this compound remains stable under acidic conditions. In the reaction mixture compound **13**-$^{13}C_1$ could only be detected in trace amounts by LC–(ESI)MS analysis. The reason may be the high reactivity of the carbonyl moiety as well as the fact that the aldehyde occurs mainly in the hydrated form 6-[2-(dihydroxymethyl)pyridinium-1-yl]-L-norleucine (**15**-$^{13}C_1$) (Scheme 1b) as shown below. Tessier et al.[27] tentatively identified the labile homologue 6-(3-formylpyridinium-1-yl)-L-norleucine (structure not shown) of **13**-$^{13}C_1$ in incubations of glyceraldehyde with N^{α}-acetyl-L-lysine by mass spectrometry experiments. The triosidine-carbaldehyde was assigned to the so-called 'triosidines' a novel group of Maillard reaction pyridinium compounds.



Scheme 1. (a) Reaction pathway as established for the formation of the aminotriazine N^6-[4-(3-amino-1,2,4-triazin-5-yl)-2,3-dihydroxybutyl]-L-lysine (**10**-$^{13}C_1$) and glucosepane **1**-$^{13}C_1$. (b) Reaction of [1-^{13}C]D-glucose with L-lysine and aminoguanidine yielded 6-[2-({[amino(imino)methyl]hydrazono}methyl)pyridinium-1-yl]-L-norleucine (**14**-$^{13}C_1$) via the pyridinium-carbaldehyde 6-[2-(dihydroxymethyl)pyridinium-1-yl]-L-norleucine (**13**-$^{13}C_1$).

To determine the glycating potential of such pyridinium-carbaldehydes, the model compound 1-butyl-2-formylpyridinium (**18**) was synthesized. 2-Pyridinemethanol (**16**) was incubated with 1-iodobutane at 37 °C for three weeks.[28] The reaction mixture was analyzed by LC–(ESI)MS and the expected signal with $[M+H]^+$ at m/z 166 was detected. The NMR data of the purified compound are given in Table 1, which establish the formation of 1-butyl-2-(hydroxymethyl)pyridinium (**17**). The transformation of the hydroxymethyl group of **17** into the corresponding aldehyde **18** was performed with oxalyl chloride in dimethyl sulfoxide according to the procedure described by Mancuso and Swern[29] (Scheme 2). LC–(ESI)MS analysis of the purified compound showed the expected peak with the quasimolecular ion $[M+H]^+$ at m/z 164 but also two further peaks with $[M+H]^+$ at m/z 182 and m/z 196. While the peak with $[M+H]^+$ at m/z 182 represents the hydrated form of the pyridinium-carbaldehyde **18**, the peak with $[M+H]^+$ at m/z 196 derives from the methanolic hemiacetal **19** formed during the purification process, which was performed by preparative HPLC using a water–methanol gradient (Scheme 2). Since the hydrated form of **18** predominates in aqueous solution, only for this product **20** the structure was established. Accurate mass determination (m/z 182.1184) proved the expected elemental composition $C_{10}H_{16}NO_2$. The NMR data acquired in D_2O (Table 1) also establish the formation of 1-butyl-2-(dihydroxymethyl)pyridinium (**20**). The chemical shifts of the pyridinium heterocycle core were in agreement

Table 1. ^{1}H and ^{13}C NMR data of **14**-$^{13}C_1$, **17**, and **20**[a]



14-$^{13}C_1$ **17** **20**

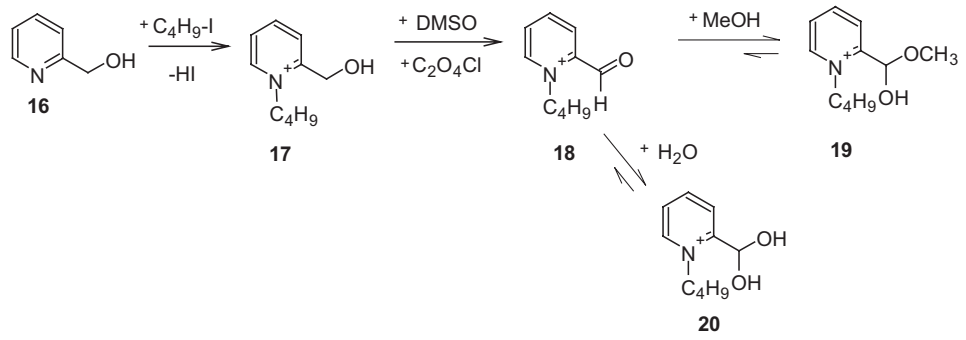
^{1}H NMR	δ (ppm)[b]		
	14–$^{13}C_1$	**17**	**20**
H-1	8.71	8.70	8.74
H-2	7.93	7.86	7.93
H-3	8.42	8.42	8.47
H-4	8.51	8.10	8.26
H-6	8.46		6.36
H_2-6		4.98	
H_2-1′	4.64	4.43	4.59
H_2-2′	1.92	1.85	1.89
H_2-3′	1.42	1.34	1.35
H_2-4′	1.81		
H_3-4′		0.86	0.85
H-5′	3.63		
	J (Hz)[c]		
$^{1}J_{1H,C1}$	192.0		
$^{3}J_{1,2}$	5.9	6.2	5.8
$^{3}J_{2,3}$	7.5	7.8	7.6
$^{3}J_{3,4}$	8.6	8.0	8.2
$^{3}J_{1',2'}$	7.5	7.8	8.0
$^{3}J_{2',3'}$		7.7	7.7
$^{3}J_{3',4'}$		7.4	7.4
$^{3}J_{4',5'}$	6.6		
^{13}C NMR	δ (ppm)[b]		
C-1	**146.5**	145.9	145.8
C-2	129.4	126.9	127.7
C-3	146.3	145.5	146.5
C-4	127.8	126.7	125.6
C-5	150.0	156.4	154.4
C-6	137.1	59.4	85.0
C-1′	58.0	57.3	57.8
C-2′	30.0	32.2	32.8
C-3′	22.1	19.3	19.0
C-4′	30.5	12.9	12.7
C-5′	55.5		
C-6′	175.5		
C-1″	—[d]		

[a]The arrows in the structural formulas indicate the characteristic carbon–proton long-range coupling connectivities from the gs-HMBC spectra. Hydrogen/carbon assignment has been validated by ^{1}H, ^{1}H-COSY, TOCSY 1D, gs-HSQC, and gs-HMBC measurements. The numbering C-1 to C-6, in structure **14**-$^{13}C_1$, refers to the original sugar backbone and does not follow IUPAC rules. The bold number for C-1 in **14**-$^{13}C_1$ signify that this carbon is derived from C-1 of D-glucose as proven by experiments with [1-^{13}C]D-glucose. The numbering of the respective C atoms in structures **17** and **20** does not follow IUPAC rules.

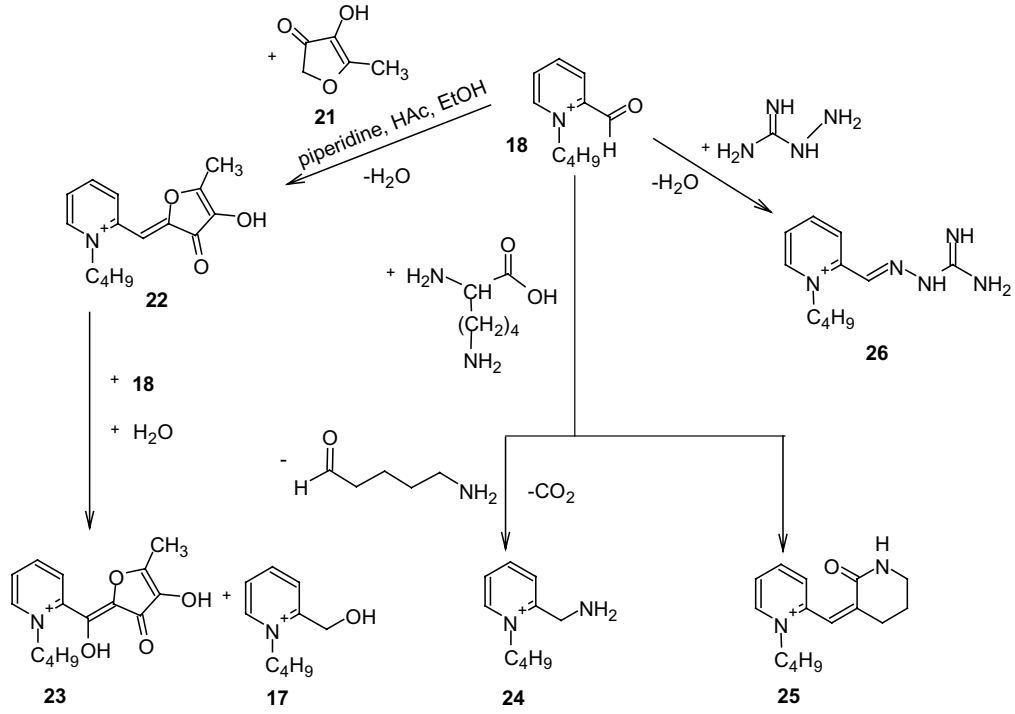
[b]δ (ppm), chemical shift for the indicated hydrogen/carbon.

[c]J (Hz), coupling constant between the indicated protons.

[d]Chemical shift for C-1″ could not be determined due to the lacking connectivity (^{4}J) of H-6 with C-1″ in the gs-HMBC spectra and the low amount of **14**-$^{13}C_1$.



Scheme 2. Reaction scheme for the synthesis of the model compound 1-butyl-2-formylpyridinium (**18**) with the hydrated form 1-butyl-2-(dihydroxymethyl)pyridinium (**20**) and the hemiacetal **19**.



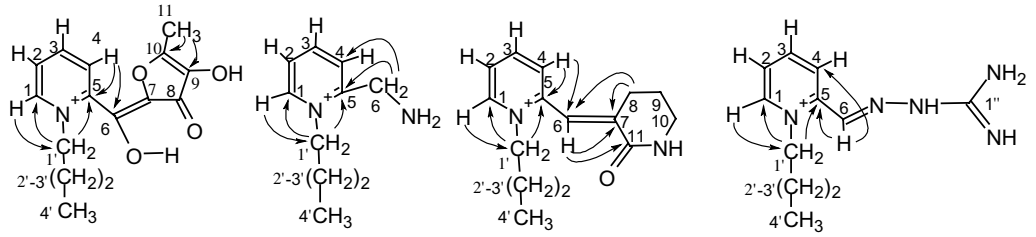
Scheme 3. Reaction of **18** with 4-hydroxy-5-methylfuran-3(2*H*)-one (**21**), L-lysine, and aminoguanidine, yielded 1-butyl-2-[hydroxy-(4-hydroxy-5-methyl-3-oxofuran-2(3*H*)-ylidene)methyl]pyridinium (**23**), 2-(aminomethyl)-1-butyl-pyridinium (**24**), 1-butyl-2-[(2-oxopiperidin-3-ylidene)methyl]-pyridinium (**25**), and 2-({[amino(imino)methyl]hydrazono}methyl)-1-butylpyridinium (**26**), respectively.

with those reported for **14**-$^{13}C_1$; the chemical shift of 85 ppm for C-6 prove the formation of the aldehyde hydrate. Although the hydrate form **20** dominates in the equilibrium with the free aldehyde **18**, the last one is responsible for reactions.

The incubation of **18** with aminoguanidine was performed under similar conditions as reported for the synthesis of **14**-$^{13}C_1$ (Scheme 3). The crude reaction mixture was analyzed by LC–(ESI)MS and an intensive peak with $[M+H]^+$ at m/z 220 could be detected. After isolation by preparative HPLC, accurate mass determination gave $[M+H]^+$ at m/z 220.1563, corresponding to an elemental composition of $C_{11}H_{18}N_5$. The NMR data (Table 2) confirmed the formation of 2-({[amino(imino)methyl]hydrazono}methyl)1-butylpyridinium (**26**) an analogous product to **14**-$^{13}C_1$ (Table 1). These findings prove the pyridinium-carbaldehyde **13**-$^{13}C_1$ as precursor in the formation of **14**-$^{13}C_1$.

Incubations of **18** with L-lysine were performed to gain information about the glycating potential of such structures. LC–(ESI)MS analysis of the reaction mixture showed two peaks with $[M+H]^+$ at m/z 165 and m/z 245. Accurate mass determination of the main compound gave $[M+H]^+$ at m/z 165.1397, corresponding to an elemental composition of $C_{10}H_{17}N_2$. NMR spectroscopic analysis clearly proved the structure 2-(aminomethyl)-1-butylpyridinium (**24**). The results obtained indicate that **24** is formed by the reaction of the

Table 2. ^{1}H and ^{13}C NMR data of **23**–**26**[a]



^{1}H NMR	δ (ppm)[b]			
	23	**24**	**25**	**26**
H-1	8.78	8.92	8.82	8.67
H-2	7.96	8.06	7.94	7.88
H-3	8.46	5.58	8.45	8.39
H-4	7.88	8.09	7.91	8.01
H-6			7.60	8.43
H_2-6		4.59		
H_2-8			2.59	
H_2-9			1.81	
H_2-10			3.38	
H_3-11	2.18			
H_2-1′	4.46	4.59	4.49	4.63
H_2-2′	1.81	1.88	1.80	1.85
H_2-3′	1.22	1.38	1.28	1.32
H-4′	0.75	0.90	0.84	0.85
			J (Hz)[c]	
$^{2}J_{1,2}$	5.8	6.3	6.3	6.3
$^{2}J_{2,3}$	6.4	7.8	7.8	6.6
$^{3}J_{3,4}$	7.9	8.2	8.2	8.1
$^{3}J_{9,10}$			5.7	
$^{3}J_{1',2'}$	7.9	7.8	7.6	7.6
$^{3}J_{2',3'}$	7.6	7.5	7.5	7.5
$^{3}J_{3',4'}$	7.3	7.3	7.3	7.3
^{13}C NMR			δ (ppm)[b]	
C-1	145.4	147.0	145.6	147.7
C-2	128.0	127.7	127.2	130.4
C-3	146.4	146.6	145.4	148.1
C-4	128.6	127.3	129.8	129.7
C-5	152.2	150.2	150.5	150.5
C-6	157.2	38.2	124.0	138.7
C-7	—[d]		141.0	
C-8	—[d]		25.0	
C-9	157.0[e]		21.1	
C-10	134.0[e]		41.1	
C-11	11.0		165.8	
C-1′	58.5	58.2	58.2	61.3
C-2′	32.0	31.2	31.2	34.9
C-3′	18.5	18.3	18.3	21.7
C-4′	12.2	12.1	12.1	15.5
C-1″				—[f]

Hydrogen/carbon assignment has been validated by ^{1}H,^{1}H-COSY, TOCSY 1D, gs-HSQC, and gs-HMBC measurements.

[a]The arrows in the structural formulas indicate the characteristic carbon–proton long-range coupling connectivities from the gs-HMBC spectra. The numbering of the respective C atoms does not follow IUPAC rules. Hydrogen/carbon assignment has been validated by ^{1}H,^{1}H-COSY, TOCSY 1D, gs-HSQC, and gs-HMBC measurements.

[b]δ (ppm), chemical shift for the indicated hydrogen/carbon.

[c]J (Hz), coupling constant between the indicated protons.

[d]Chemical shifts for C-7 and C-8 could not be determined due to the lacking connectivity (^{4}J and ^{5}J) of H-4 with C-7 and C-8, respectively, in the gs-HMBC spectra and the low amount available for **23**.

[e]The respective assignments may have to be reserved.

[f]Chemical shift for C-1″ could not be determined due to the lacking connectivity (^{4}J) of H-6 with C-1″ in the gs-HMBC spectra and the low amount of **26**.

aldehyde **18** with the α-amino group of L-lysine via a Strecker-type degradation process. This assumption is supported by the finding that the reaction of **18** with N^{α}-*t*-Boc-L-lysine does not lead to the formation of **24**. Instead of this, the Schiff base (structure not shown) could be detected by LC–(ESI)MS, the isolation of which was not practicable due to the instability toward acidic agents needed for the cleavage of the *t*-Boc group (data not shown). As compared to **18**, the 26 nm bathochromic shift of the second detected compound with $[M+H]^+$ at *m/z* 245 can only be rationalized by extension of the chromophoric system as shown for **25** in Scheme 3. Accurate mass determination (*m/z* 245.1660) prove the elemental composition $C_{15}H_{21}N_2O$, the structure of the corresponding product 1-butyl-2-[(2-oxopiperidin-3-ylidene)methyl]pyridinium (**25**) was finally established by NMR (Table 2).

To obtain more evidences of **18** compared to C–H acidic structures, **18** was incubated with 4-hydroxy-5-methylfuran-3(2*H*)-one (**21**) for 3 days at room temperature in ethanol containing piperidine and acetic acid (Scheme 3). The reaction conditions were in accordance with those reported for the Knoevenagel condensation reaction.[30] However, the expected product 1-butyl-2-[(4-hydroxy-5-methyl-3-oxofuran-2(3*H*)-ylidene)methyl]pyridinium (**22**) with $[M+H]^+$ at *m/z* 260 could not be detected in the reaction mixture by LC–(ESI)MS analysis; but two new peaks appeared with the quasimolecular ions $[M+H]^+$ at *m/z* 166 and *m/z* 276 (Scheme 3). Since the peak with $[M+H]^+$ at *m/z* 166 had the same retention time and UV spectra reported for **17** (Scheme 2) the product could be identified as 1-butyl-2-(hydroxymethyl)pyridinium (**17**). The second compound with $[M+H]^+$ at *m/z* 276, that is, 16 Da higher than the expected product **22**, suggest that an oxidation process took place, even in the presence of diethylenetriaminepentaacetic acid (DTPA). The reaction mixture obviously provide a redox system, which enables the oxidation of Knoevenagel product **22** to **23**, while the excess of **18** is reduced to **17**. The elemental composition $C_{15}H_{18}NO_4$ of **23** was confirmed by accurate mass determination showing $[M+H]^+$ at *m/z* 276.1238. The NMR data, combined in Table 2, unequivocally prove the formation of 1-butyl-2-[hydroxy-(4-hydroxy-5-methyl-3-oxofuran-2(3*H*)ylidene)methyl]pyridinium (**23**).

In conclusion, the present study has clearly established 6-(2-formylpyridinium-1-yl)-L-norleucine (**13**) as an important posttranslational protein modification, since it derives from the lysine linked dideoxyosone **8**, which shows lysine derivatization quota in vitro ranging from 3% to 7.5%.[31] Since only a small amount of **8** is involved in the formation of **1**, it must be concluded that the major follow-up products are yet unknown. The identification of **13** may, thus, contribute to the detection of major Maillard reaction products, the reactivity clearly point to the potential of **13** as exogenous or endogenous glycotoxin.

3. Experimental

3.1. General methods

^1H NMR (500 MHz), $^1H,^1H$-COSY (correlation spectroscopy), $^1H,^1H$-TOCSY (total correlation spectroscopy), TOCSY 1D (one-dimensional), gs-HSQC (gradient-selected heteronuclear single quantum coherence), and gs-HMBC (gradient-selected heteronuclear multiple bond correlation) spectra were recorded at 25 °C on a Varian (Darmstadt, Germany) Unity Inova 500 spectrometer in D_2O. The LC–(ESI; electrospray ionization)MS analyses were run on an HP1100 (Hewlett–Packard, Waldbronn, Germany) high-performance liquid chromatography (HPLC) system coupled to a Micromass (Manchester, UK) VG platform II quadrupole mass spectrometer equipped with an ESI interface. The HPLC system consists of an HP1100 autosampler, HP1100 gradient pump, HP1100 thermoregulator, and HP1100 diode array detector (DAD) module. Column: 150×4.6 mm i.d., 5 μm, YMC-Pack Pro C 18; 10×4.6 mm i.d. guard column (YMC Europe, Schermbeck, Germany); column temperature, 25 °C; flow rate, 1.0 mL/min; injection volume, 20 μL. Gradients: 10 mM ammonium formate buffer (pH 4.0)–MeOH. (A) 5% MeOH, 0 min; 95% MeOH, 30–40 min; 5% MeOH, 40–45 min; 10 mM *n*-heptafluorobutyric acid (HFBA)–MeOH. (B) 5% MeOH, 0 min; 95% MeOH, 30–35 min; 5% MeOH, 40–45 min. MS parameters: ESI^+; source temperature, 120 °C; capillary, 3.5 kV; HV lens, 0.5 kV; cone, 55 V. For LC analyses, the MS system was operated in the full scan mode (*m/z* 150–1000). For accurate mass determination, data were collected in the multichannel acquisition (MCA) mode with 128 channels per *m/z* unit using 12 scans (6 s) with 0.1 s reset time. The resolution was 1060–1110 (10% valley definition). The sample was dissolved in water/MeCN (1:1) containing reference material (0.1 μg/μL, see below), ammonium formate (0.1%), and formic acid (1%); the sample concentration was similar to that of the reference compound. The solution was introduced into the ESI source (80 °C) at a flow rate of 5 μL/min. The following scan ranges and reference peaks were used for calibration: **14**-$^{13}C_1$, *m/z* 250–350; poly(ethylene glycol) 200, *m/z* 256.1747, 283.1849, 300.2068, 327.2031, 344.2284; **20**, *m/z* 130–240; poly(ethylene glycol) 200, *m/z* 133.0865, 177.1116, 195.1259, 212.1496, 239.1502; **23**, *m/z* 220–320; poly(ethylene glycol) 350 monomethyl ether, *m/z* 226.1662, 253.1651, 270.1929, 297.1915, 314.2184; **24**, *m/z* 130–240; poly(ethylene glycol) 200, *m/z* 133.0876, 177.1107, 195.1278, 212.1378, 239.1503; **25**, *m/z* 190–290; poly(ethylene glycol) 200, *m/z* 195.1244, 212.1511,

239.1501, 256.1782, 283.1779; **26**, m/z 175–280; poly(ethylene glycol) 350 monomethyl ether, m/z 182.1392, 209.1393, 226.1649, 253.1654, 270.1915. MassLynx 3.2 software was used for data acquisition and processing. The preparative HPLC system consisted of a Kronlab (Sinsheim, Germany) KD200/100SS gradient pump system combined with a Knauer (Berlin, Germany) A0293 variable wavelength detector and a 250×20 mm i.d., 7 μm, Nucleosil C 18 column with 50×20 mm i.d. guard column (Kronlab); injection volume, 1.5 mL; flow rate, 18 mL/min. Gradients were applied as follows: ammonium formate buffer (10 mM, pH 4.0)–MeOH. (A) 5% MeOH, 0 min; 50% MeOH, 20 min; 100% MeOH, 22–25 min; 5% MeOH, 28–35 min. (B) 0% MeOH, 0 min; 10% MeOH, 6 min; 0% MeOH, 11–16 min. (C) 5% MeOH, 0 min; 70% MeOH, 20–23 min; 100% MeOH, 25 min; 5% MeOH, 30 min; water (pH 4.0 with acetic acid)–MeOH. (D) 5% MeOH, 0 min; 70% MeOH, 20–23 min; 100% MeOH, 25 min; 5% MeOH, 30 min; *n*-heptafluorobutyric acid (HFBA, 10 mM)–MeOH. (E) 5% MeOH, 0 min; 70% MeOH, 20–23 min; 100% MeOH, 25 min; 5% MeOH, 30 min.

3.2. Materials

Milli-Q water, purified to 18 MΩ/cm^2 (Millipore, Eschborn, Germany) was used in the preparation of all solutions. HPLC grade MeOH was employed for LC and LC–MS. For preparative HPLC, solvents were degassed by flushing with helium. N^{α}-*t*-Boc-L-lysine, aminoguanidine hydrochloride (AG), 2-pyridinemethanol, 1-iodobutane, L-lysine, and *n*-heptafluorobutyric acid (HFBA) were purchased from Fluka (Neu-Ulm, Germany); oxalyl chloride, dimethyl sulfoxide, and Et_3N were from Merck (Darmstadt, Germany); diethylenetriaminepentaacetic acid (DTPA) was from Sigma (Steinheim, Germany); poly(ethylene glycol) 350 monomethyl ether and poly(ethylene glycol) 200 were from Aldrich (Steinheim, Germany), and [1-^{13}C]D-glucose was from Cambridge Isotope Laboratories (Andover, USA). For a phosphate buffer salt mixture giving solutions with pH 7.4, KH_2PO_4 (2.68 g; 20 mmol) and $Na_2HPO_4{\cdot}2H_2O$ (14.3 g; 80 mmol) were mixed vigorously. 4-Hydroxy-5-methylfuran-3(2*H*)-one (**21**) was generously supplied by Marcus A. Glomb (Technical University of Berlin, Germany).

3.3. Synthesis and purification of the stable isotope labeled compounds N^6-[4-(3-amino-1,2,4-triazin-5-yl)-2,3-dihydroxybutyl]-L-lysine (10-$^{13}C_1$) and 6-[2-({[amino(imino)methyl]hydrazono}methyl)pyridinium-1-yl]-L-norleucine (14-$^{13}C_1$)

[1-$^{13}C_1$]D-Glucose (175 mg; 1 mmol), aminoguanidine hydrochloride (110 mg; 1 mmol), N^{α}-*t*-Boc-L-lysine (760 mg; 3 mmol), and phosphate buffer, pH 7.4 (800 mg; 5 mmol), were dissolved in water (5 mL). The mixture was kept at 70 °C for 18 h and purified by preparative HPLC (gradient A; detection wavelength, 320 nm). Fractions with retention time of 17.4 min yielded, after lyophilization a mixture of N^6-[4-(3-amino-1,2,4-triazin-5-yl)-2,3-dihydroxybutyl]-N^2-(*tert*-butoxycarbonyl)-L-lysine (*t*-Boc-**10**-$^{13}C_1$) and 6-[2-({[amino(imino)methyl]-hydrazono}methyl)pyridinium-1-yl]-*N*-(*tert*-butoxycarbonyl)-L-norleucine (*t*-Boc-**14**-$^{13}C_1$). LC–(ESI)MS (gradient A): *t*-Boc-**10**-$^{13}C_1$, retention time 11.00 min, m/z 430 $[M+H]^+$; *t*-Boc-**14**-$^{13}C_1$, retention time 9.61 min, m/z 394 $[M+H]^+$. The crude mixture was dissolved in aqueous 3 M HCl (2 mL) and kept at ambient temperature for 30 min. The pH was adjusted to 7 by slowly adding solid $NaHCO_3$, the volume finally filled up to 4 mL, and the mixture subjected to preparative HPLC (gradient B; detection wavelength, 320 nm). Fractions with retention time of 7.7 and 9.3 min yielded, after lyophilization, **10**-$^{13}C_1$ (10.7 mg; 0.033 mmol; 3.3%) and **14**-$^{13}C_1$ (1.5 mg; 0.005 mmol; 0.51%), respectively; LC–(ESI)MS (gradient B): **10**-$^{13}C_1$, retention time 15.62 min, m/z 330 $[M+H]^+$; **14**-$^{13}C_1$, retention time 16.15 min, m/z 294 $[M+H]^+$. Accurate mass: **14**-$^{13}C_1$ $[M+H]^+$ calcd for $^{12}C_{12}^{13}CH_{22}N_6O_2$, 294.1760; found, 294.1762 ± 0.0003 (mean of 11 measurements ±SD). For NMR data, see Table 1. In addition, these compounds were also synthesized with D-glucose using the same protocol leading to the formation of **10** with m/z 329 $[M+H]^+$[24,25] and **14** with m/z 293 $[M+H]^+$ (data not shown).

3.4. Synthesis and purification of 1-butyl-2-(dihydroxymethyl)pyridinium (20)

2-Pyridinemethanol (**16**; 5.46 g; 0.05 mol) and 1-iodobutane (13.8 g; 0.075 mol) were pooled and kept at 37 °C with gentle shaking.[28] After three weeks, isopropanol (1.5 mL) was added to precipitate the reaction product. This compound in the form of nearly colorless crystals was filtered and washed with isopropanol. Two recrystallization processes in isopropanol yielded 1-butyl-2-(hydroxymethyl)pyridinium (**17**; 5 g; 0.03 mol, 60%) in the form of colorless crystals. UV (H_2O): λ_{max} (nm) (lg ε) **17**, 260 (3.51); LC–(ESI)MS (gradient B): retention time 12.34 min, m/z 166 $[M+H]^+$. For NMR data, see Table 1.

In a second step **17** was transformed to the corresponding formyl compound according to the procedure described by Mancuso and Swern.[29] To a mixture of dichloromethane (6.3 mL) and oxalyl chloride (250 μL; 2.75 mmol), dimethyl sulfoxide (425 μL; 5.5 mmol) in 1.25 mL dichloromethane were added at −50 to −60 °C. Compound **17** (415 mg; 2.5 mmol) dissolved in 2.5 mL dichloromethane containing some dimethyl sulfoxide was added to the stirring solution and the reaction performed for 15 min. To the reaction mixture Et_3N (1.5 mL; 10.7 mmol) was added, stirred for another

5 min and then allowed to warm to room temperature. The desired product was extracted twice with 20 mL water, the pH adjusted to 6.3 with 1 N HCl, concentrated to nearly 10 mL by lyophilization, and finally subjected to preparative HPLC (gradient D; detection wavelength, 260 nm). Fractions with retention time of 7.6 min yielded, after lyophilization, 1-butyl-2-(dihydroxymethyl)pyridinium (**20**; 311.8 mg; 1.7 mmol; 68.5%). LC–(ESI)MS (gradient B): t_R 14.34 min, m/z 182 $[M+H]^+$. Accurate mass: $[M+H]^+$ calcd for $C_{10}H_{16}NO_2$, 182.1181; found, 182.1184 ± 0.0006 (mean of 10 measurements ±SD). For NMR data, see Table 1.

3.5. Formation of 1-butyl-2-[hydroxy-(4-hydroxy-5-methyl-3-oxofuran-2(3H)- ylidene)methyl]pyridinium (23)

Compound **18** (60.4 mg; 0.37 mmol) and 4-hydroxy-5-methylfuran-3(2*H*)-one (**21**; 37.9 mg; 0.33 mmol) were dissolved in 3.3 mL ethanol and 6.8 μL piperidine; to the solution 6.8 μL acetic acid and diethylenetriaminepentaacetic acid (DTPA, 1 mg, 2.5 μmol) were added. The mixture was flushed with argon, kept at room temperature for 3 d, and finally purified by preparative HPLC (gradient D; detection wavelength, 312 nm). Fractions with retention time of 13.6 min yielded, after lyophilization, **23** (2.8 mg; 0.01 mmol; 3.1%); LC–(ESI)MS (gradient A): **23**, retention time 9.95 min, m/z 276 $[M+H]^+$. Accurate mass: $[M+H]^+$ calcd for $C_{15}H_{18}NO_4$, 276.1236; found, 276.1238 ± 0.0009 (mean of 10 measurements ±SD). For NMR data, see Table 2.

3.6. Formation of 2-(aminomethyl)-1-butylpyridinium (24) and 1-butyl-2-[(2-oxopiperidin-3-ylidene)methyl]pyridinium (25)

Compound **18** (58.9 mg; 0.36 mmol), L-lysine (46 mg; 0.31 mmol), DTPA (1 mg; 2.5 μmol), and phosphate buffer, pH 7.4 (120 mg; 0.71 mmol), were dissolved in water (10 mL). The mixture was flushed with argon, kept at 70 °C for 18 h, and purified by preparative HPLC (gradient E; detection wavelength, 220 nm). Fractions with retention time of 17.1 min yielded, after lyophilization, a mixture of the products **24** and **25** (15.5 mg); LC–(ESI)MS (gradient B): **24**, retention time 15.33 min, m/z 165 $[M+H]^+$; **25**, retention time 13.69 min, m/z 245 $[M+H]^+$. Accurate mass: **24** $[M+H]^+$ calcd for $C_{10}H_{17}N_2$, 165.1392; found, 165.1397 ± 0.0009; **25** $[M+H]^+$ calcd for $C_{15}H_{21}N_2O$, 245.1654; found, 245.1660 0.0006 (mean of nine measurements ± SD). For NMR data, see Table 2.

3.7. Formation of 2-({[amino(imino)methyl]hydrazono}methyl)-1-butylpyridinium (26)

Compound **18** (12.6 mg; 0.08 mmol), aminoguanidine hydrochloride (8.8 mg; 0.08 mmol), and phosphate buffer, pH 7.4 (85 mg; 0.5 mmol), were dissolved in water (4 mL). The mixture was kept at 50 °C for 6 h and purified by preparative HPLC (gradient C; detection wavelength, 316 nm). Fractions with retention time of 10.7 min yielded, after lyophilization, **26** (5.5 mg; 0.03 mmol; 43%); LC–(ESI)MS (gradient B): **26**, retention time 17.02 min, m/z 220 $[M+H]^+$. Accurate mass: $[M+H]^+$ calcd for $C_{11}H_{18}N_5$, 220.1562; found, 220.1563 ± 0.0001 (mean of eight measurements ±SD). For NMR data, see Table 2.

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