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Glycosylation of lysine-containing pentapeptides by glucuronic acid: new insights into the Maillard reaction

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ABSTRACT

The formation of glycosylation products in model systems consisting of D-glucuronic acid (GlcA) and lysine-containing peptides, such as Lys-Gly-Gly-Phe-Leu (**1**), Gly-Lys-Gly-Phe-Leu (**4**) and Ac-Gly-Lys-Gly-Phe-Leu (**6**), was examined to evaluate the site specificity as well as the extent and nature of the modification. Peptides were reacted with GlcA either in solution or under dry-heating conditions. From the incubations performed in solution (MeOH), the corresponding (1-deoxy-D-fructofuranos-1-yluronic acid)–peptide derivatives (Amadori compounds) were isolated. Whereas reaction of **1** resulted in the formation of mono-glycosylated Amadori compound **2** with the sugar moiety attached to the N^{ε}-amino group of the Lys residue and its di-glycosylated analogue **3**, exposure of **4** to GlcA afforded only di-glycosylated peptide **5**. From the incubation of GlcA with Ac-Gly-Lys-Gly-Phe-Leu (**6**) performed under mild dry-heating conditions (50 °C) in an environment of 75% relative humidity, besides Amadori compound **7**, two new Maillard reaction products were isolated that contained 3-hydroxypyridinium (**8**) and 3-hydroxy-picolinic acid moiety (**9**). The mechanism for the formation of pyridinium products is discussed.

1. Introduction

Nonenzymatic glycosylation (glycation) of proteins by reducing sugars (the Maillard reaction) is a common biological phenomenon that has been under active investigation for many years.[1,2] Most interest has been directed towards glycosylation of proteins by glucose and its potential role in diabetes and aging,[3] but an increasing number of studies have been conducted with other reducing sugars, providing evidence that they may also react with peptides or proteins through the glycosylation pathway.[4–6] In spite of its relatively low concentration compared to glucose in vivo, D-glucuronic acid (GlcA) is a reducing sugar of biological importance due to its participation in the metabolism of many drugs and endogenous compounds.[7] A major metabolic pathway for the biotransformation of exogenous and endogenous carboxylic acid substrates is conjugation with endogenous GlcA to yield acyl glucuronide metabolites implicated in a wide range of adverse drug effects.[8–10] Not unreactive as previously thought, acyl glucuronides are potentially reactive electrophilic species which can undergo hydrolysis, intramolecular rearrangement (isomerization via intramolecular acyl migration) and covalent binding to proteins, both in vivo and in vitro.[11–13] The covalent binding may occur via two different mechanisms.[14] The first is a transacylation mechanism, where a nucleophilic group (–NH_2, –OH, –SH) on a protein attacks the carbonyl group of the acyl glucuronide leading to the formation of an acylated protein and free GlcA. The second is a mechanism of Schiff base formation where condensation occurs between the GlcA aldehyde group and the amine group of the N-terminus and/or the ε-NH_2 of a lysine residue of a protein leading to the formation of a glycosylated protein. Both the intramolecular rearrangement of acyl glucuronides and their hydrolysis are of particular importance because they lead to re-exposure of the hemiacetal function of the glucuronic acid, thus allowing sugar–protein covalent adduct formation through a nonenzymatic glycation reaction. The investigation of the reactivity of these electrophilic metabolites in forming the corresponding acyl glucuronide–peptide adduct, carried out by trapping experiments in the presence of Lys-Phe dipeptide, demonstrates that the extent of Schiff base formation is proportional to the rearrangement rate of the parent glucuronide.[15] In addition, the reactivity is strongly dependent on the inherent electronic and steric properties of each specific aglycone. The imine formed between the glucuronic acid moiety and the primary amino groups of proteins could undergo Amadori rearrangement. Although structures of such glycation products have not yet been formally documented, previous studies have shown that GlcA, or reactive acyl glucuronide metabolites, can bind irreversibly to albumin, leading to the formation of fluorescent advanced glycation end products (AGEs) similar to those that are formed with glucose and fructose.[16,17]

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To gain more detailed insight into peptide/protein glycosylation processes under physiological conditions, model systems using mixtures of GlcA with peptides and proteins containing selected structural elements should be studied. In our previous study,[18] we characterized the glycosylation products generated from GlcA and endogenous opioid peptides (enkephalins) that did not contain lysine residues. Herein we focus on the small lysine-containing peptides, Lys-Gly-Gly-Phe-Leu (**1**), Gly-Lys-Gly-Phe-Leu (**4**) and Ac-Gly-Lys-Gly-Phe-Leu (**6**) as model peptides to examine their susceptibility to nonenzymatic glycation reaction and to characterize the products formed after being exposed to glucuronic acid.

2. Results and discussion

2.1. Synthesis and structure determination of Amadori products formed from glucuronic acid in MeOH

Systems containing GlcA and Lys-Gly-Gly-Phe-Leu (**1**), Gly-Lys-Gly-Phe-Leu (**4**) or Ac-Gly-Lys-Gly-Phe-Leu (**6**) were reacted in MeOH for three days at 50 °C. In all reaction systems, the corresponding 1-deoxy-D-fructofuranos-1-yluronic acid derivatives (Amadori compounds) (Scheme 1) were generated. The glycosylation products were isolated from the incubation mixtures by using preparative RP-HPLC. The structures of Amadori compounds were confirmed by MS and NMR spectroscopy. ^{1}H and ^{13}C resonances were assigned by homonuclear COSY experiments combined with ^{1}H–^{13}C correlation techniques through one-bond (HMQC) and multiple-bond (HMBC) 2D NMR methods.

Incubation of the Lys-Gly-Gly-Phe-Leu (**1**) with GlcA resulted in the formation of mono-glycosylated Amadori compound **2** (15%) with the sugar moiety attached to the N^{ε}-amino group of the Lys residue, and its di-glycosylated analogue **3** (38%). Amadori compounds **2** and **3** were unseparable by RP-HPLC. The assignment of the glycosylated lysine residue was possible following the observation of the tripled set of ε-CH_2 and α-CH resonances in the Lys^1 region of the ^{13}C NMR spectrum of the obtained mixture of Amadori compounds. Analysis of the ^{13}C chemical shift values for Lys^1 residue in both glycosylated and unmodified peptide **1** shows that ε-NH_2 group was preferentially glycosylated in the model peptide system. The population of products **2** and **3** was estimated by integration of the signal intensities of the α-CH lysine carbon atoms in the 50–61 ppm region of the spectrum. The ^{13}C NMR spectrum (DMSO-d_6) of the glycated product contained a complex «sugar box» region (70–104 ppm) corresponding to peaks derived from the attached 1-deoxy-D-fructofuranos-1-yluronic acid moieties present in the α- or β-furanose form.

Exposure of Gly-Lys-Gly-Phe-Leu (**4**) to GlcA in MeOH afforded after purification di-glycosylated peptide **5** in 40% yield. A mono-substituted derivative was not detected after three days of incubation, suggesting high susceptibility of both N^{α}-Gly^1 and N^{ε}-Lys^2 amino groups to glycation. While MS analysis revealed the number of sugar moieties, their positions were unequivocally deduced from the observed large downfield shifts ($\Delta\delta \sim 10$ ppm) of the Gly^1 and Lys^2 CH_2–N carbons caused by the N-alkylation at these positions. The NMR spectra (DMSO-d_6) of Amadori compound **5** showed the presence of attached ketose moieties in α- or β-furanose form. The chemical shifts for these tautomers are summarized in Table 1. The population of tautomers in equilibrated DMSO solution of Amadori compound **5** was estimated by integration of the signal intensities of the anomeric carbon atoms (C-2) in the 100–105 ppm region of the ^{13}C NMR spectra. According to integrations, the α⇆β equilibrium was shifted to the α-furanose side with an 87:13 preference. When comparing the equilibrium composition of sugar moieties attached to the Gly^1 with those attached to the Lys^2 amino group, the apparent destabilization of the β anomer in favour of the α furanose tautomer was observed at Gly^1 residue (α:β = 95:5), whereas the ketose attached to Lys^2 residue established a 77:23 α⇆β equilibrium. These data indicate that interaction(s) between the 1-deoxy-D-fructofuranos-1-yluronic acid and peptide moiety in Amadori compound **5** may change the relative populations of tautomers by inducing effects that cannot be defined at present without more detailed conformational analysis.

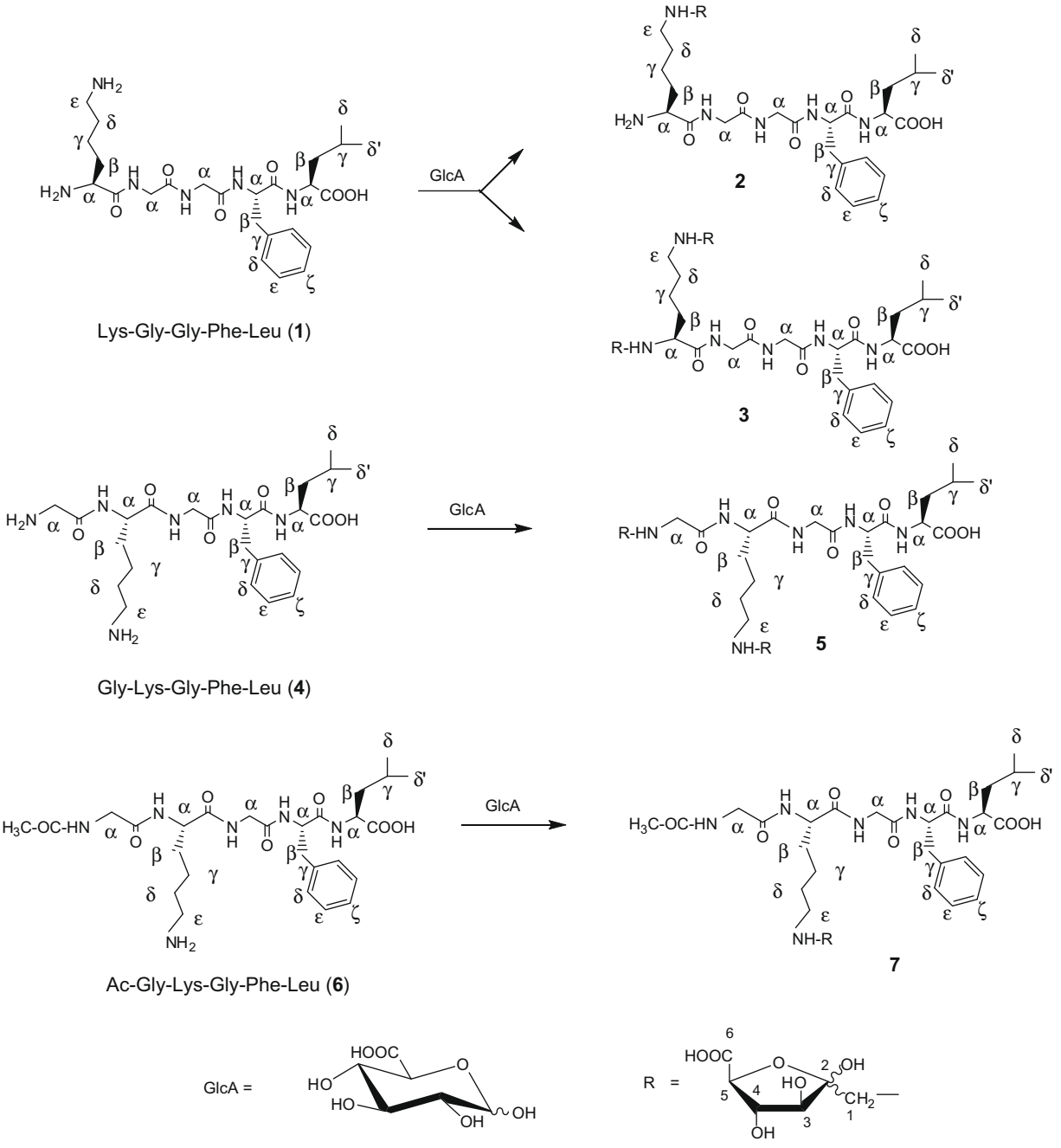
The peptide **6** was synthesized with the glycine N-terminal amino group blocked with an acetyl group to prevent glycosylation at this site in the synthetic peptide. Following incubation with GlcA and purification of the glycosylated peptide **7** (79%) from Ac-Gly-Lys-Gly-Phe-Leu (**6**), MS analysis confirmed that peptide **6** was glycated with one sugar residue per peptide molecule as indicated by an increase in the $[M+H]^+$ ion by 177 mass units from the parent peptide arising from 1-deoxy-D-fructofuranos-1-yluronic acid, and indicating that all carbons of the original sugar backbone (GlcA) are retained in Amadori compound **7**. The spectroscopic results, derived from experiments in DMSO solution, indicate that N^{ε}-Lys^2 amino group glycosylation of peptide **6** resulted in a similar distribution of tautomers at equilibrium as observed for ketose attached to the Lys^2 residue in compound **5** (**7**; α:β = 69:31). The chemical shifts for tautomers of Amadori compound **7** are listed in Table 1.

2.2. Synthesis and structure determination of products formed from glucuronic acid and peptides under dry-heating conditions

The formation of products was investigated by RP-HPLC in lyophilized model systems containing GlcA and Lys-Gly-Gly-Phe-Leu (**1**), Gly-Lys-Gly-Phe-Leu (**4**) or Ac-Gly-Lys-Gly-Phe-Leu (**6**) (15:1 molar ratio of sugar to peptide) as reactants, when exposed to 75% relative humidity environment at 50 °C, in the dark, for five days.

In contrast to the reaction performed in MeOH, when lyophilized GlcA/**1** sample was kept under above conditions, generation of the numerous new products was observed; however, no major product was detectable in the reaction mixture even after prolonged periods of time. Although the concentration of the parent peptide **1** (Lys-Gly-Gly-Phe-Leu) decreased fast in the reaction mixture reaching a value of 3% at the end of three days heating period, the RP-HPLC analysis revealed only trace amounts of Amadori compounds **2** and **3** present in the reaction mixture. This result, in combination with the fact that mono-glycosylated compound **2** was very unstable, while di-glycosylated Amadori compound **3** was very stable under identical dry-heating conditions (data not shown), indicates the rapid generation and decomposition of Amadori compound **2** by 1,2- and 2,3-enolization processes leading to reactive dicarbonyl compounds, which reacted readily with lysine amino groups in peptide **1**.

When Gly-Lys-Gly-Phe-Leu (**4**) was incubated with GlcA under dry-heating conditions, peptide **4** disappeared completely during the one-day incubation period. This was associated with the formation of the mono-substituted Amadori compounds (40%) having the sugar moiety attached either to N^{α}-Gly^1 or to N^{ε}-Lys^2 amino groups of peptide **4** and with generation of the di-substituted Amadori compound **5** (13%) as confirmed by NMR analysis of the isolated compounds. The concentrations of Amadori products decreased during incubation to reach after five days 7% and 8% for mono- and di-glycated compound, respectively. After five days, analysis of the reaction mixture by RP-HPLC revealed the presence of eight new peaks detected by UV absorbance at 215 nm. The peaks were isolated and analyzed by NMR spectroscopy. NMR data indicated that none of the isolated products was homogeneous.



Scheme 1. Glycosylation products derived in MeOH from lysine-containing peptides **1**, **4** and **6** in the presence of D-glucuronic acid.

However, the chemical shift differences between the peptide **4** and the products obtained suggested a dehydration process of the sugar residue(s) in the Amadori compounds generated, which leads to partially unsaturated or fully aromatized compounds.

The reaction of GlcA with Ac-Gly-Lys-Gly-Phe-Leu (**6**) under identical dry-heating conditions led to a multicomponent mixture from which three major products, compounds **7**–**9** (Scheme 2), were isolated in 5%, 9% and 13% yield, respectively. Novel glycation products **8** and **9** were characterized by MS and NMR spectroscopy. The mass spectrum of compound **8** gave an ion of *m/z* 641, while **9** gave an ion of *m/z* 685 in the positive-ion mode, showing an increase of 81 (C_5H_5O) and 125 ($C_6H_5O_3$) mass units, respectively, from the parent peptide **6**. This experiment indicated that only five carbons from GlcA are integrated into glycosylation product **8**, while a total of six carbons from the starting sugar were incorporated into compound **9**. The NMR data acquired in DMSO and compiled in Table 2 unequivocally proved the formation of 3-hydroxypyridinium derivative **8** and 3-hydroxypicolinic acid derivative **9** under dry-heating conditions in the GlcA/**6** model system. Hydrogen/carbon assignments were validated by COSY, HMQC and HMBC measurements. The chemical shifts of the pyridinium heterocycle core were in agreement with those reported for 3-hydroxypyridine. Comparison of the data listed in Table 2 for *N*-peptidyl 3-hydroxypyridinium derivative **8** with the NMR data given for peptide **6** revealed almost identical chemical shifts for all amino acid residues, except for those of the Lys^2 ε-CH_2 group, which were shifted downfield by ~22 ppm and 1.7 ppm in the ^{13}C and ^1H NMR spectra, respectively. Such a pronounced low-field shift can only be rationalized if the primary nitrogen of the lysine residue in compound **6** is converted to a tertiary one in **8**. The ^1H

Table 1
^{1}H and ^{13}C NMR spectral data (δ, ppm) for the Amadori compounds **5** and **7**[a]

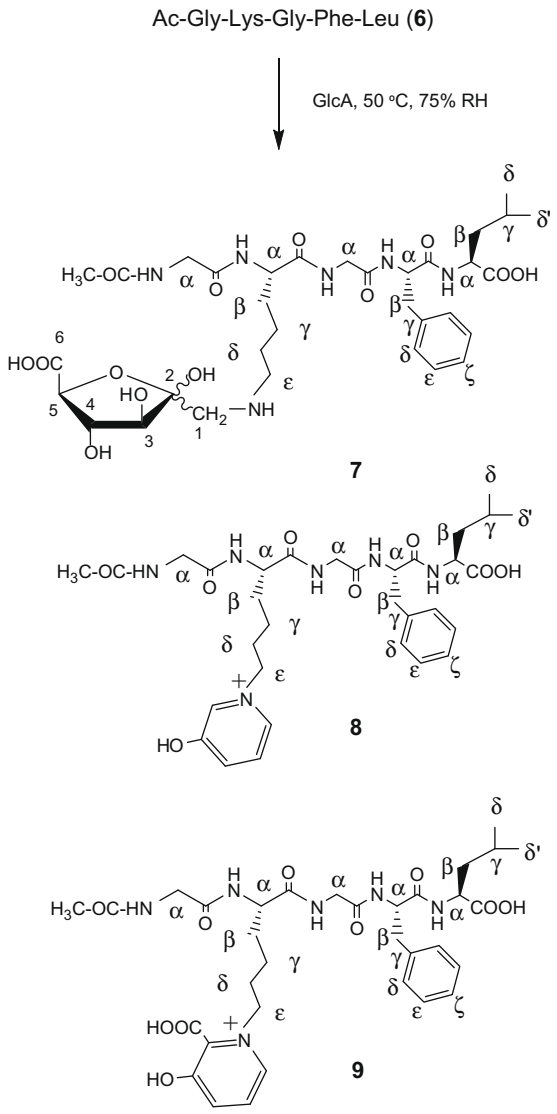
Residue[b]	Atom	**5** Anomeric form of the keto-sugar						**7** Anomeric form of the keto-sugar			
		α-furanose at Gly^{1} residue[c]		α-furanose at Lys^{2} residue		β-furanose at Lys^{2} residue		α-furanose at Lys^{2} residue		β-furanose at Lys^{2} residue	
		δ_H	δ_C	δ_H	δ_C	δ_H	δ_C	δ_H	δ_C	δ_H	δ_C
Ketose	1	3.01/3.12	50.28	3.06/3.12	50.28	3.62	51.59	3.11	50.47	3.68	52.30
	2	—	103.41	—	104.24	—	101.13	—	104.26	—	101.15
	3	3.74	80.11	3.92	81.41[d]	3.74	77.07	3.92	81.34[d]	3.74	77.06
	4	3.85	83.54	4.09	79.21	4.26	77.39	4.09	79.21	4.25	77.37
	5	3.98	81.85	4.37	81.33[d]	4.09	79.67	4.37	81.36[d]	4.09	79.61
	6	NO	167.08	NO	172.59	NO	171.87	NO	172.74	NO	172.01
Gly^{1}	α	3.31/3.45	**50.63**					3.71	42.07		
	NH	NO	—					8.08	—		
	CO	—	167.79					—	169.13		
Lys^{2}	α	4.23	52.35					4.22	52.30		
	β	1.45/1.64	31.13					1.52/1.64	31.26		
	γ	1.26	22.09				22.21	1.25	22.29		22.32
	δ	1.55	24.55[e]		24.79[e]			1.55	24.67		24.25
	ε	2.91	**47.83**[f]	2.91	**47.89**[f]	2.86	**47.68**	2.91	**47.85**	2.87	**47.68**
	NH$^{\alpha}$	8.14	—		—			8.02	—		
	CO	—	171.38		171.43			—	171.64		
Gly^{3}	α	3.59/3.72	41.70					3.55/3.71	41.72		
	NH	8.13	—					8.10	—		
	CO	—	168.27					—	168.27		
Phe^{4}	α	4.56	53.46					4.55	53.52		
	β	2.74/3.04	37.62					2.75/3.04	37.67		
	γ	—	137.61					—	137.71		
	δ	7.25	129.13					7.24	129.22		
	ε	7.25	127.92					7.24	127.98		
	ζ	7.20	126.15					7.18	126.21		
	NH	8.01	—					7.96	—		
	CO	—	170.90					—	171.05		
Leu^{5}	α	4.23	50.28					4.22	50.31		
	β	1.55	39.89					1.52	39.92		
	γ	1.55	24.20					1.64	24.25		
	δ,δ′	0.85/0.91	21.34/22.72					0.85/0.90	21.35/22.82		
	NH	8.29	—					8.27	—		
	COOH	—	173.70					—	173.85		
Ac	Me							1.84	22.39		
	CO							—	169.71		

NO = not observed.
[a] In DMSO-d_6 at 25 °C. Those chemical shifts that show significant differences when compared with parent peptide value are indicated in bold.
[b] One set of resonances detected for Gly^{1}, Gly^{3}, Phe^{4} and Leu^{5} amino acid residues.
[c] Keto-sugar moiety in β-furanose form at Gly^{1} residue present in traces.
[d,e,f] Assignment of signals could be interchangeable.

and ^{13}C chemical shift differences between the compounds **8** and **9** were found to be very small with the exception of the C-2 chemical shift value for the 3-hydroxypiridinium moiety observed in picolinic acid derivative **9** ($\Delta\delta \sim 25$ ppm) (Table 2).

The general profile for the formation of pyridinium compounds **8** and **9** in the GlcA/**6** model system is shown in Figure 1 as a function of time. In the initial phase of the reaction, the concentration of the parent peptide **6** decreased in the reaction mixture, reaching a value of 40% after a heating period of one day. Under the same conditions, the formation of Amadori compound **7** was slow to reach the maximum concentration (24%) after three days of incubation, and then it started to decrease. As presented in Figure 1, the exposure of peptide **6** to GlcA under dry-heating conditions resulted in almost equal amounts of glycation products **8** and **9**. After five days of incubation, the relative amounts of pyridinium compound **8** and picolinic acid derivative **9** reached 25% and 32%, respectively.

The results thus obtained revealed that under mild dry-heating conditions (50 °C) and in an environment of 75% relative humidity, the conjugation between the lysine residue in peptide **6** and glucuronic acid occurred readily, modifying up to 80% of the lysine amino group within 48 h. The data obtained are consistent with the previously reported observation for lysine/sugar systems showing increased rates of the Maillard reaction in a high relative humidity (65.5%) environment, due to increased mobility of the molecules in the matrix.[19] The formation of Amadori product **7** in the initial phase of the dry-heating process and almost linear increases of the pyridinium derivatives **8** and **9** as a function of time suggested compound **7** as their precursor. In support of this hypothesis, it was shown that enkephalin/GlcA-derived Amadori compounds decompose readily to 3-hydroxypyridinium compounds under similar dry-heating conditions.[18] Ring-substituted pyridinium compounds were also previously obtained by reaction of either triose sugars or glucose with lysine and arginine residues in phosphate buffer solu-



Scheme 2. Glycosylation products derived from Ac-Gly-Lys-Gly-Phe-Leu (**6**) and D-glucuronic acid under dry-heating conditions (50 °C, 75% relative humidity).

tions.[20,21] An interesting case was the formation of a conjugated enol–keto-immonium derivative in the reaction of sodium glucuronate with the lysine-containing tripeptide, Ac-Tyr-Lys-Gly-NH_2.[22]

A possible mechanism of formation of the two novel structures **8** and **9** is shown in Scheme 3. The reaction cascade starts with the condensation of the glucuronic acid in its open-chain form with the free amino group of the peptide moiety to give Schiff base, which then undergoes rearrangement to the keto-sugar peptide derivative or Amadori product **7**. It can be assumed that the extra COOH group of the sugar residue effectively catalyzes the rate-determining step in the rearrangement reaction by intramolecular protonation of the imine nitrogen atom in intermediate Schiff base leading to the immonium ion required for Amadori compound formation. Water initiates and/or participates in further keto–enol tautomerizations and carbonyl group migrations[23,18] along the carbohydrate backbone leading to β- and α-keto acid intermediates. Unstable β-keto acid intermediate eliminates CO_2 at C-6 resulting in a 4-ketopentose. Carbonyl group migration to C-5, followed by an intramolecular amino–carbonyl reaction and a series of dehydrations, yield the final 3-hydroxypyridinium compound **8**. On the other hand, the α-keto acid intermediate leads, after intramolecular reaction and dehydrations, to the picolinic acid derivative **9**.

Table 2
^{1}H and ^{13}C NMR spectral data (δ, ppm) for the pyridinium–peptide derivatives **8** and **9**[a]

Residue	Atom	8		9	
		δ_H	δ_C	δ_H	δ_C
Aryl[b]	2	8.58	132.88	—	157.63[c]
	3	—	157.09	—	157.13[c]
	4	7.91	128.46	8.19	130.41
	5	7.93	131.21	7.92	131.00
	6	8.50	135.42	8.59	132.90
	OH	12.27		12.27	
	COOH			NO	161.29
Gly^1	α	3.69	42.03	3.71	42.05
	NH	8.06		8.08	
	CO	—	169.06	—	169.15
Lys^2	α	4.25	52.02	4.24	52.20
	β	1.53/1.68	31.08	1.52/1.69	31.03
	γ	1.25	21.66	1.32	22.04
	δ	**1.86**	**30.14**	**1.84**	**30.57**
	ε	**4.46**	**60.52**	**4.79**	**59.01**
	NH^{α}	8.02		8.04	
	CO	—	171.59	—	171.72
Gly^3	α	3.57/3.72	41.68	3.59/3.70	41.79
	NH	8.16		8.21	
	CO	—	168.26	—	168.32
Phe^4	α	4.56	53.49	4.56	53.56
	β	2.76/3.04	37.68	2.76/3.04	37.66
	γ	—	137.69	—	137.73
	δ	7.24	129.24	7.24	129.25
	ε	7.24	127.99	7.24	128.01
	ζ	7.18	126.22	7.17	126.23
	NH	7.95		7.96	
	CO	—	171.06	—	171.06
Leu^5	α	4.22	50.32	4.22	50.34
	β	1.53	39.93	1.63	39.87
	γ	1.63	24.25	1.63	24.26
	δ,δ′	0.84/0.89	21.34/22.81	0.84/0.89	21.37/22.83
	NH	12.60		8.26	
	COOH	NO	173.85	NO	173.86
Ac	Me	1.83	22.40	1.84	22.41
	CO	—	169.67		169.72

NO = not observed.
[a] In DMSO-d_6 at 25 °C. Those chemical shifts that show significant differences when compared with parent peptide value are indicated in bold.
[b] Aryl = 3-hydroxypyridinium residue for compound **8** and 3-hydroxypicolinic acid residue for compound **9**.
[c] Assignment of signals could be interchangeable.

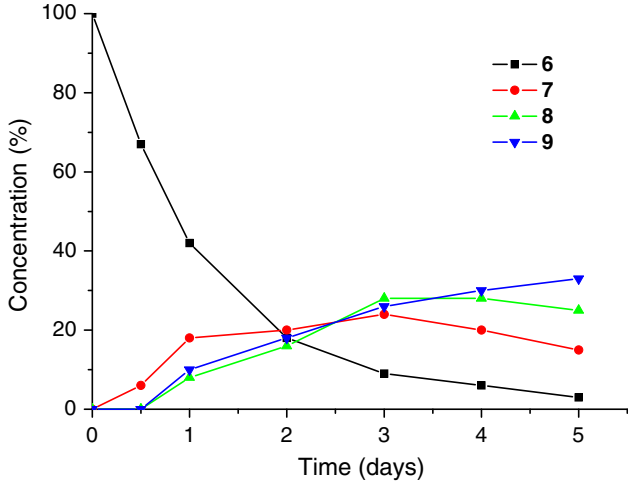
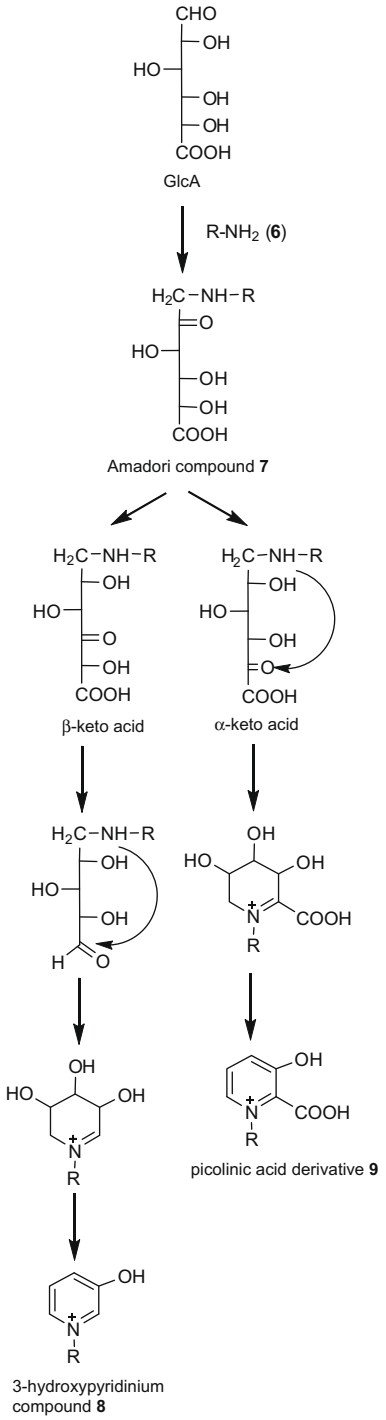


Figure 1. Kinetics of the formation of pyridinium compounds **8** and **9** during dry-heating in the dark, at 50 °C, in the environment of 75% relative humidity.



Scheme 3. Proposed mechanism of formation of 3-hydroxypyridinium compound **8** and picolinic acid derivative **9**.

The formation of ring-substituted pyridinium compounds as a novel class of Maillard reaction products is expected to offer new insights of biomedical importance for disorders of glucuronidation processes as a major detoxification route for endobiotic and xenobiotic substances in humans. Increasing experimental evidence indicates that 3-hydroxypyridinium epitopes are also formed during tissue glycosylation under physiological conditions.[20,24–26] Importantly, *N*-alkyl 3-hydroxypyridinium derivatives were identified as the minimum phototoxic chromophores contained in endogenous hydroxypyridine photosensitizers capable of inducing macromolecular damage.[27]

3. Experimental

3.1. General methods

Dry-heating experiments were performed in a CLIMACELL 111 Cooling incubator with controlled humidity. Melting points were determined on a Tottoli (Büchi) apparatus and are uncorrected. Optical rotations were measured at 25 °C using an Optical Activity LTD automatic AA-10 polarimeter. NMR spectra were recorded on a Bruker AV 600 spectrometer, operating at 150.91 MHz for ^{13}C and 600.13 MHZ for ^{1}H nuclei. The spectra were measured in DMSO-d_6 solution at 25 °C. Spectra were assigned based on 2D homonuclear (COSY, NOESY and ROESY) and heteronuclear (HMQC and HMBC) experiments. Reversed-phase high-performance liquid chromatography (RP-HPLC) was performed on a Varian Pro Star 230 HPLC system using a Eurospher 100 reversed-phase C-18 semipreparative (250 × 8 mm ID, 5 μm) (flow rate: 1.0 ml/min) or analytical (150 × 4.5 mm ID, 5 μm) (flow rate: 0.5 mL/min) column under isocratic or gradient conditions using different concentrations of MeOH in 0.1% aq trifluoroacetic acid (TFA). UV detection was performed at 215 and 254 nm using a Varian Pro Star 335 photodiode-array detector. Mass spectrometry measurements were performed on a Agilent Technologies 1200 HPLC system consisting of a binary pump, a degasser, an autosampler and a diode-array detector and coupled with a 6410 Triple Quadrupole mass spectrometer operating in a positive-ion electrospray ionization (ESI) mode. Lys-Gly-Gly, Gly-Lys-Gly and Phe-Leu were purchased from Bachem. D-Glucuronic acid (GlcA) was purchased from Sigma Chemical Co.

3.2. Synthesis of pentapeptides

The N-terminally protected pentapeptides **1** and **4** were prepared by classical solution-phase methods of peptide synthesis with Boc-Lys(Boc)-Gly-Gly or Boc-Gly-Lys(Boc)-Gly and Phe-Leu as the starting compounds. The coupling of the peptide segments was achieved by using the mixed anhydride method with isobutyl chloroformate. Boc protecting groups were cleaved by trifluoroacetic acid (TFA), and the crude peptides were purified by RP-HPLC.

3.2.1. Lys-Gly-Gly-Phe-Leu (1)

This compound was prepared from Boc-Lys(Boc)-Gly-Gly (197 mg, 0.43 mmol) and TFA × Phe-Leu (200 mg, 0.51 mmol). Purification by semipreparative RP-HPLC using 40% MeOH/0.1% TFA as the eluent afforded title compound **1**. Yield: 112 mg, 50%; white solid, mp 120–130 °C; $[\alpha]_D$ −7 (*c* 1.0, MeOH). Analytical RP-HPLC: 37% MeOH/0.1% TFA t_R 17.3 min. ^{1}H NMR: δ Lys^{1}: 3.59 (α-CH), 1.58/1.67 (β-CH_2), 1.37 (γ-CH_2), 1.52 (δ-CH_2), 2.75 (ε-CH_2); Gly^{2}: 3.78 (α-CH_2), 8.78 (NH); Gly^{3}: 3.59/3.72 (α-CH_2), 8.17 (NH); Phe^{4}: 4.42 (α-CH), 2.75/3.07 (β-CH_2), 7.25 (δ-CH), 7.25 (ε-CH), 7.18 (ζ-CH), 8.29 (NH); Leu^{5}: 4.06 (α-CH), 1.50 (β-CH_2), 1.61 (γ-CH), 0.85/0.88 (δ,δ′-CH_3), 7.82 (NH). ^{13}C NMR: Lys^{1}: 52.85 (α-CH), 31.75 (β-CH_2), 21.35 (γ-CH_2), 26.37 (δ-CH_2), 38.34 (ε-CH_2), 171.60 (CO); Gly^{2}: 42.01 (α-CH_2), 168.73 (CO); Gly^{3}: 42.01 (α-CH_2), 168.49 (CO); Phe^{4}: 54.37 (α-CH), 37.26 (β-CH_2), 137.97 (γ-C), 129.03 (δ-CH), 127.98 (ε-CH), 126.15 (ζ-CH), 170.30 (CO); Leu^{5}: 51.53 (α-CH), 41.11 (β-CH_2), 24.34 (γ-CH), 21.86/22.92 (δ,δ′-CH_3), 174.64 (COOH). ESIMS *m/z*: $[M+H]^+$ calcd for $C_{25}H_{41}N_6O_6$: 521.31; found: 521.30.

3.2.2. Gly-Lys-Gly-Phe-Leu (4)

This compound was prepared from Boc-Gly-Lys(Boc)-Gly (150 mg, 0.33 mmol) and TFA × Phe-Leu (150 mg, 0.36 mmol). Purification by semipreparative RP-HPLC using 40% MeOH/0.1% TFA as the eluent afforded title compound **4**. Yield: 137 mg, 80%; white solid, mp 165–175 °C; $[\alpha]_D$ −35 (*c* 1.0, MeOH). Analytical

RP-HPLC: 37% MeOH/0.1% TFA t_R 15.8 min. ^{1}H NMR: δ Gly^1: 3.43 (α-CH_2); Lys^2: 4.31 (α-CH), 1.49/1.69 (β-CH_2), 1.30 (γ-CH_2), 1.52 (δ-CH_2), 2.73 (ε-CH_2), 8.44 (NH); Gly^3: 3.63/3.72 (α-CH_2), 8.21 (NH); Phe^4: 4.39 (α-CH), 2.76/3.07 (β-CH_2), 7.24 (δ-CH), 7.24 (ε-CH), 7.18 (ζ-CH), 8.25 (NH); Leu^5: 4.02 (α-CH), 1.44/1.54 (β-CH_2), 1.62 (γ-CH), 0.86/0.88 (δ,δ′-CH_3), 7.74 (NH). ^{13}C NMR: Gly^1: 41.53 (α-CH_2), 168.25 (CO); Lys^2: 51.97 (α-CH), 31.29 (β-CH_2), 21.89 (γ-CH_2), 26.46 (δ-CH_2), 38.45 (ε-CH_2), 171.37 (CO); Gly^3: 42.09 (α-CH_2), 168.63 (CO); Phe^4: 54.70 (α-CH), 37.13 (β-CH_2), 137.95 (γ-C), 129.07 (δ-CH), 128.07 (ε-CH), 126.22 (ζ-CH), 170.22 (CO); Leu^5: 51.71 (α-CH), 41.53 (β-CH_2), 24.41 (γ-CH), 21.93/23.08 (δ,δ′-CH_3), 174.87 (COOH). ESIMS *m/z*: $[M+H]^+$ calcd for $C_{25}H_{41}N_6O_6$: 521.31; found: 521.52.

3.2.3. Ac-Gly-Lys-Gly-Phe-Leu (6)

This peptide was synthesized manually from its C- to N-terminal end by the solid-phase Fmoc method on a commercially available preloaded Fmoc-Leu Wang resin (Bachem, *p*-alkoxybenzyl alcohol resin, 200–400 mesh, 0.8 mmol/g loading) on a 0.1 mmol scale. The consecutive steps in the solid-phase peptide synthesis performed in each cycle were (i) deprotection of the Fmoc group by two treatments (1 and 30 min) with 20% piperidine in DMF (v/v); (ii) coupling by applying HBTU/HOBt/DIPEA activation and a threefold excess of the appropriate Fmoc-amino acid for 30 min, and in the last step, by applying the Ac-Gly for 1 h and (iii) removal of the peptide from the resin by treatment with a mixture of TFA:TIS:H_2O in a ratio of 9.5:0.25:0.25 (v/v/v) for 1 h. Successive deprotection and coupling steps were monitored by the positive and negative Kaiser (ninhydrin) test, respectively. The peptide **6** was obtained as a filtrate in TFA, and it was precipitated with cold dry diisopropyl ether. Purification by semipreparative RP-HPLC using 40% MeOH/0.1% TFA as the eluent afforded title compound **6**. Yield: 31 mg, 56%; $[\alpha]_D$ −21 (*c* 1.0, MeOH). Analytical RP-HPLC: 37% MeOH/0.1% TFA t_R 27.78 min. ^{1}H NMR: δ Ac-Gly^1: 1.84 (Ac, CH_3), 3.70 (α-CH_2), 8.16 (NH); Lys^2: 4.23 (α-CH), 1.52/1.64 (β-CH_2), 1.29 (γ-CH_2), 1.52 (δ-CH_2), 2.73 (ε-CH_2), 8.07 (NH); Gly^3: 3.55/3.72 (α-CH_2), 8.17 (NH); Phe^4: 4.49 (α-CH), 2.77/3.05 (β-CH_2), 7.24 (δ-CH), 7.24 (ε-CH), 7.18 (ζ-CH), 8.04 (NH); Leu^5: 4.13 (α-CH), 1.52 (β-CH_2), 1.64 (γ-CH), 0.85/0.89 (δ,δ′-CH_3), 8.03 (NH). ^{13}C NMR: Ac-Gly^1: 22.41 (Ac, CH_3), 42.01 (α-CH_2), 169.16 (CO), 169.75 (Ac, CO); Lys^2: 52.21 (α-CH), 31.14 (β-CH_2), 21.98 (γ-CH_2), 26.43 (δ-CH_2), 38.53 (ε-CH_2), 171.75 (CO); Gly^3: 42.11 (α-CH_2), 168.45 (CO); Phe^4: 54.00 (α-CH), 37.48 (β-CH_2), 137.81 (γ-C), 129.20 (δ-CH), 128.03 (ε-CH), 126.22 (ζ-CH), 170.60 (CO); Leu^5: 51.13 (α-CH), 40.66 (β-CH_2), 24.35 (γ-CH), 21.66/22.97 (δ,δ′-CH_3), 174.41 (COOH). ESIMS *m/z*: $[M+H]^+$ calcd for $C_{27}H_{43}N_6O_7$: 563.32; found: 563.40.

3.3. Synthesis of D-glucuronic acid-derived Amadori compounds

3.3.1. N^{ε}-(1-Deoxy-α,β-D-fructofuranos-1-yluronic acid)-L-lysyl-glycyl-glycyl-L-phenylalanyl-L-leucine (2) and N^{α},N^{ε}-di-(1-deoxy-α,β-D-fructofuranos-1-yluronic acid)-L-lysyl-glycyl-glycyl-L-phenylalanyl-L-leucine (3)

D-Glucuronic acid (145 mg, 0.75 mmol) and Lys-Gly-Gly-Phe-Leu (**1**), as diacetate salt, (32 mg, 0.05 mmol) were dissolved in dry MeOH (30 mL), and the reaction mixture was kept in a closed round-bottomed flask for three days at 50 °C. The solvent was evaporated, and the excess of sugar from the reaction mixture was removed by using an C-18 solid-phase extraction (SPE) cartridge. The cartridge was first eluted with water to remove the sugar. The peptide material was then recovered with MeOH. The effluent was evaporated, and the residue obtained was purified by semipreparative RP-HPLC using 33% MeOH/0.1% TFA and lyophilized to yield an inseparable mixture of mono-glycated N^{ε}-(1-deoxy-α,β-D-fructofuranos-1-yluronic acid)-L-lysyl-glycyl-glycyl-L-phenylalanyl-L-leucine (**2**) and di-glycated N^{α},N^{ε}-di-(1-deoxy-α,β-D-fructofuranos-1-yluronic acid)-L-lysyl-glycyl-glycyl-L-phenylalanyl-L-leucine (**3**). Analytical RP-HPLC: 37% MeOH/0.1% TFA **2**: t_R 16.97 min; **3**: t_R 17.58 min. ^{1}H NMR (**2**+**3**): δ 1-deoxy-α,β-D-fructofuranos-1-yluronic acid: 3.08/3.23, 3.03/3.12 (1-CH_2), 3.74, 3.87, 3.92, 3.96, 4.09, 4.24, 4.28, 4.30, 4.36, 4.41 (3-, 4-, 5-CH); Lys^1: 3.65, 3.87, 3.96 (α-CH), 1.71, 1.82 (β-CH_2), 1.34 (γ-CH_2), 1.62 (δ-CH_2), 2.88, 2.92 (ε-CH_2); Gly^2: 3.74/3.82 (α-CH_2), 8.73, 8.78, 8.81 (NH); Gly^3: 3.63/3.78 (α-CH_2), 8.18 (NH); Phe^4: 4.58 (α-CH), 2.74/3.03 (β-CH_2), 7.25 (δ-CH), 7.17, 7.25 (ε-CH), 7.20, 7.25 (ζ-CH), 8.05, 8.12 (NH); Leu^5: 4.23 (α-CH), 1.54 (β-CH_2), 1.62 (γ-CH), 0.85/0.91 (δ,δ′-CH_3), 8.34, 8.37 (NH). ^{13}C NMR (**2**+**3**): 1-deoxy-α,β-D-fructofuranos-1-yluronic acid: 50.27, 50.44, 51.60 (1-CH_2), 101.18, 104.20, 104.44 (2-C), 70.63, 73.11, 75,48, 76.95, 77.11, 77.40, 79.23, 79.27, 79.40, 79.72, 79.84, 81.34, 81.41, 82.11, 82.22 (3-, 4-, 5-CH), 171.84, 171.87 (6-COOH); Lys^1: 51.97, 59.84, 60.13 (α-CH), 28.94, 30.39 (β-CH_2), 20.98, 21.15 (γ-CH_2), 24.48, 24.58 (δ-CH_2), 47.38, 47.49, 47.57 (ε-CH_2), 167.56, 168.70 (CO); Gly^2: 41.79 (α-CH_2), 168.03 (CO); Gly^3: 41.57 (α-CH_2), 168.15 (CO); Phe^4: 53.46 (α-CH), 37.61 (β-CH_2), 137.61 (γ-C), 129.13 (δ-CH), 127.89 (ε-CH), 126.15 (ζ-CH), 170.96, 171.02 (CO); Leu^5: 51.42 (α-CH), 39.47 (β-CH_2), 24.34 (γ-CH), 20.57/22.24 (δ,δ′-CH_3), 173.68, 174.01 (COOH). ESIMS *m/z*: $[M+H]^+$ calcd for $C_{31}H_{49}N_6O_{12}$: 697.34; found: 697.10; *m/z*: $[M+2H]^+$ calcd for $C_{37}H_{58}N_6O_{18}$: 874.89; found: 874.26.

3.3.2. *N*-(1-Deoxy-α,β-D-fructofuranos-1-yluronic acid)-glycyl-N^{ε}-(1-deoxy-α,β-D-fructofuranos-1-yluronic acid)-L-lysyl-glycyl-L-phenylalanyl-L-leucine (5)

Compound **5** was obtained starting from the diacetate salt of Gly-Lys-Gly-Phe-Leu (**4**) (32 mg, 0.05 mmol) and D-glucuronic acid (145 mg, 0.75 mmol) by using the same procedure as described for compounds **2** and **3**. Purification by semipreparative RP-HPLC using 36% MeOH/0.1% TFA as the eluent afforded the pure title compound **5**. Yield: 17 mg, 40%; white hygroscopic solid; $[\alpha]_D$ −3 (*c* 1.0, MeOH). Analytical RP-HPLC: 33% MeOH/0.1% TFA t_R 25.0 min. ^{1}H and ^{13}C NMR data are given in Table 1. ESIMS *m/z*: $[M-H_2O+H]^+$ calcd for $C_{37}H_{55}N_6O_{17}$: 855.36; found: 855.24.

3.3.3. *N*-Acetyl-glycyl-N^{ε}-(1-deoxy-α,β-D-fructofuranos-1-yluronic acid)-L-lysyl-glycyl-L-phenylalanyl-L-leucine (7)

Compound **7** was obtained from the acetate salt of Ac-Gly-Lys-Gly-Phe-Leu (**6**) (31 mg, 0.05 mmol) and D-glucuronic acid (145 mg, 0.75 mmol) by using the same procedure as described for compound **5**. Purification by semipreparative RP-HPLC using 40% MeOH/0.1% TFA as the eluent afforded the pure title compound **7**. Yield: 29 mg, 79%; white hygroscopic solid; $[\alpha]_D$ −2 (*c* 1.0, MeOH). Analytical RP-HPLC: 37% MeOH/0.1% TFA t_R 25.1 min. ^{1}H and ^{13}C NMR data are given in Table 1. ESIMS *m/z*: $[M+H]^+$ calcd for $C_{33}H_{51}N_6O_{13}$: 739.35; found: 739.12.

3.4. Synthesis of D-glucuronic acid-derived pyridinium compounds 8 and 9

D-Glucuronic acid (582 mg, 3.0 mmol) and Ac-Gly-Lys-Gly-Phe-Leu (**6**), as acetate salt (124 mg, 0.2 mmol), were dissolved in water (3 mL) and were lyophilized to give a friable glassy material with a water content of 9%. The reactants were then exposed to 75% relative humidity at 50 °C, in the dark, for five days. The dark brown solid mixture was dissolved in water and was applied to an SPE catridge to remove the excess sugar. Purification of the peptide-containing fraction, recovered by MeOH, by semipreparative RP-HPLC using 37% MeOH/0.1% TFA as the eluent afforded Amadori

compound **7** (8 mg, 5%), 3-hydroxypyridinium derivative **8** (12 mg, 9%) and 3-hydroxypicolinic acid derivative **9** (18 mg, 13%).

3.4.1. 3-Hydroxypyridinium derivative (8)

White hygroscopic solid; $[\alpha]_D$ −13 (*c* 0.9, MeOH). Analytical RP–HPLC: 37% MeOH/0.1% TFA t_R 34.5 min. ^1H and ^{13}C NMR data are given in Table 2. ESIMS *m/z*: $[M+H]^+$ calcd for $C_{32}H_{45}N_6O_8$: 641.33; found: 641.08.

3.4.2. 3-Hydroxypicolinic acid derivative (9)

White hygroscopic solid; $[\alpha]_D$ −12 (*c* 1.0, MeOH). Analytical RP–HPLC: 37% MeOH/0.1% TFA t_R 32.7 min. ^1H and ^{13}C NMR data are given in Table 2. ESIMS *m/z*: $[M+H]^+$ calcd for $C_{33}H_{45}N_6O_{10}$: 685.32; found: 685.07.

3.5. Kinetics of pyridinium compounds formation under dry-heating conditions

D-Glucuronic acid (14.5 mg, 0.075 mmol) and Ac-Gly-Lys-Gly-Phe-Leu (**6**), as acetate salt (3 mg, 0.005 mmol), were dissolved in water (0.25 mL) and were lyophilized. The peptide–carbohydrate sample was then exposed to 75% relative humidity at 50 °C, in the dark, for various periods up to five days. The concentrations of the starting peptide and glycation products in the incubation mixture were determined by RP-HPLC on an analytical column at a flow rate of 0.5 mL/min with 37% MeOH/0.1% TFA by using an external standard method. UV detection was performed at 215 and 254 nm.

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