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Note

Glycation of a lysine-containing tetrapeptide by D-glucose and D-fructose—influence of different reaction conditions on the formation of Amadori/Heyns products

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ABSTRACT

The site specificity, extent, and nature of modification of the tetrapeptide, Leu-Ser-Lys-Leu (**1**), incubated with D-glucose or D-fructose in methanol, or in phosphate buffer of pH 5.7, 7.4, and 8.0 were investigated. The generated mono- and di-glycated Amadori (1-deoxy-D-fructosyl derivatives) and Heyns rearrangement products (N-alkylated glucosamine/mannosamine derivatives) were isolated and characterized by NMR and mass spectrometry. The results identified the ε-amino group of the Lys residue as the preferential glycation site in tetrapeptide **1**. Under all conditions investigated, glucose afforded higher yields of glycation products than fructose. In the reactions carried out in buffer, glycation at pH 7.4 and 8.0 was much faster than at pH 5.7.

The Maillard reaction involves the carbonyl of a reducing carbohydrate in reaction with free amino groups in peptides/proteins resulting, in the first step, in reversible formation of a Schiff base which can then undergo irreversible Amadori or Heyns rearrangements.[1–3] Further reactions lead to the formation of advanced glycation end (AGE) products, assumed to be responsible for a number of pathophysiological syndromes accompanying diabetes, aging, endothelial dysfunction, and vascular diseases.[4–6] Although the Maillard reaction is not an enzymatic reaction, a certain degree of specificity at the glycation site was observed. This has been rationalized by selective effects of the microenvironment on the isomerization of the protein-bound sugar to a protein-bound ketose or aldose.[7–10] To gain more detailed insight into peptide/protein glycation processes under physiological conditions, model systems using mixtures of sugars with peptides and proteins containing selected structural elements should be studied. While there has been enormous advance in understanding the general chemistry and biochemistry of AGE formation, the glycation adducts themselves have only rarely been isolated and rigorously purified.[1,3,11–13] In our previous studies,[14–16] we characterized the glycation products generated from endogenous opioid peptides (enkephalins), which did not contain lysine residues. Here, we focus on the lysine-containing peptide Leu-Ser-Lys-Leu (**1**) and its reaction with D-glucose and D-fructose. The tetrapeptide sequence Leu-Ser-Lys-Leu occurs in the inactive form of the transforming growth factor β (TGF-β), as part of the latency-associated protein (LAP).[17] Peptide **1** contains two amino groups available for glycation: at the N-terminal leucine residue and at the lysine side chain. The aim of the research presented was (a) to isolate and to characterize the products formed by condensation of D-glucose or D-fructose with tetrapeptide **1**, (b) to study the influence of pH and sugar concentration on the kinetics of glycation product formation, and (c) to compare the reactivities of glucose and fructose in these reactions. The findings that excessive fructose consumption may have a major role in the present epidemic of metabolic syndrome and obesity, due to its ability to raise uric acid,[18,19] suggest the potential value of studies on lysine-containing peptides to assess the physiological significance of their glycated products.

The strategy for the preparation of glucose-derived Amadori products **4** and **5** included two synthetic approaches (Scheme 1). The fully protected di-glycated compound **3** was prepared in 58% yield by reductive amination of aldoketose **2**[20] with tetrapeptide **1** in the presence of sodium cyanoborohydride. Removal of the acetonide protecting groups from **3** with aq TFA (90%) and subsequent purification by semipreparative RP-HPLC furnished di-glycated Amadori compound **4** in 40% yield. The second route involved the reaction of glucose and tetrapeptide **1** in MeOH at 70 °C to afford, after initial Schiff base formation, followed by Amadori

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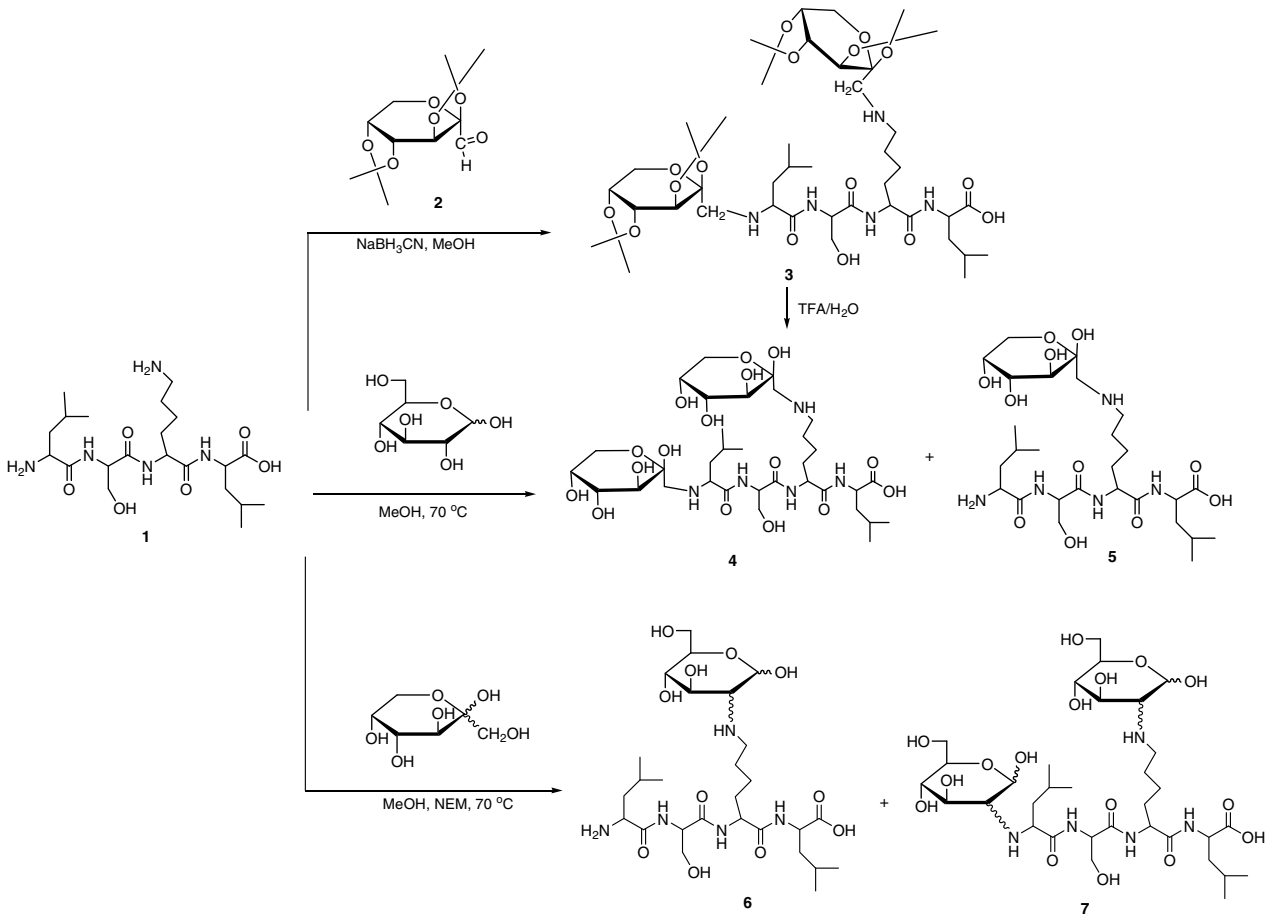

Scheme 1. Synthetic routes from Leu-Ser-Lys-Leu (**1**) to Amadori (**4**, **5**) and Heyns (**6**, **7**) glycation products (presented in pyranose forms).

rearrangement and purification, di-glycated peptide **4** in 6% yield and mono-glycated Amadori product **5** in 23% yield. In spite of the large excess of glucose used in the glycation reaction (sugar to peptide molar ratio 15:1), di-glycated derivative **4** was the minor product formed. A monosubstituted derivative with the ketose moiety attached to the N-terminal Leu residue was not detected suggesting much higher susceptibility of the N^{ε}-Lys amino group to glycation. Glucose-derived glycation products **4** and **5** were characterized by NMR spectroscopy, MS, and elemental analysis. 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 Lys^3 CH_2–N (**4** and **5**) and Leu^1 CH–N (**4** only) carbons caused by the N-alkylation at these positions. The NMR spectra (D_2O) of Amadori compounds **4** and **5** showed three sets of sugar resonances attributable to the 1-deoxy-D-fructosyl moiety in its α- and β-furanose and β-pyranose forms. The β-pyranose form was the major tautomer (66–69%) in both Amadori compounds. The α- and β-furanose forms were present in almost equal proportions.

The glycation of Leu-Ser-Lys-Leu (**1**) with D-fructose was conducted in MeOH at 70 °C in the presence of the organic base, *N*-ethylmorpholine (NEM), as the catalyst. While the formation of Amadori compounds **4** and **5** in the reaction with glucose did not require base or acid initiation, a base was necessary for the formation of Heyns compounds **6** and **7**. The best results were obtained using a sugar-peptide-base molar ratio of 75:1:15. The reaction (Scheme 1) resulted in parallel formation of mono-glycated Heyns compound **6** (7%) with the sugar moiety attached to the N^{ε}-amino group of the Lys residue, and its di-glycated analogue **7** (6%). Both products were isolated as mixtures of *N*-peptidyl-glucosamine and -mannosamine derivatives which were HPLC inseparable. If, instead of NEM, inorganic base (KOH) was used in the glycation reaction,[21] Amadori compound **5** (28%) was formed, in addition to **6** and **7**, as a result of extensive epimerization of fructose to glucose (data not shown). The structures of compounds **6** and **7** were confirmed by MS, NMR spectroscopy, and elemental analysis. The NMR spectra were extremely complex, showing at least eight sets of resonances in D_2O solution originating from 2-deoxy-D-glucos/mannos-2-yl moieties present in α,β-furanose and α,β-pyranose forms. The anomeric regions (87–99 ppm) in the ^{13}C NMR spectra of compounds **6** and **7** in D_2O are shown in Figure 1. Earlier published NMR data on glycine-derived Heyns compounds were helpful in proton and carbon atom assignments.[21] According to the NMR analysis 2-deoxy-D-glucopyranos-2-yl residue in α-pyranose form is the most abundant tautomer (~50 %) in the D_2O solutions of fructose-derived Heyns compounds **6** and **7**.

In previous studies, we demonstrated parallel formation of Amadori or Heyns compounds and the corresponding imidazolidinones by condensation of opioid peptides not containing lysine residues (e.g., Tyr-Gly-Gly-Phe-Leu) with glucose or fructose in NEM-containing MeOH solution.[15,16] No imidazolidinones were isolated in the present study. Their formation would have required preponderant addition of the glucose or fructose moiety to the α-amino group at the N-terminus of Leu-Ser-Lys-Leu (**1**), as an obligatory preceding step. The results presented above indicate, however, that the ε-amino group of the Lys residue is the primary glycation site. These data support studies showing strongly increased lysine reactivity in dipeptides containing hydrophobic amino acid residues such as Lys-Leu, Lys-Ile, and Lys-Phe.[10]