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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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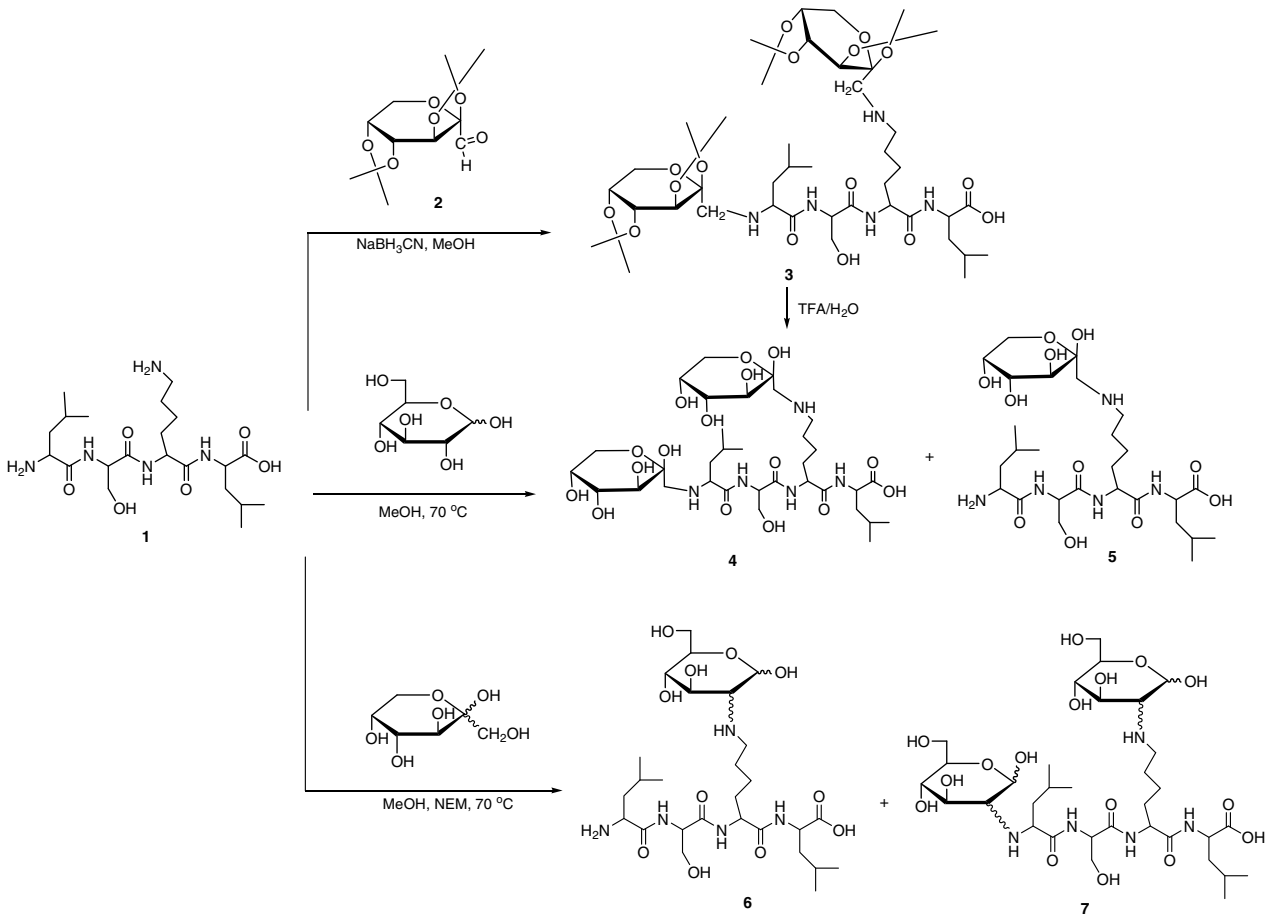

Scheme 1. Synthetic routes from Leu-Ser-Lys-Leu (**1**) to Amadori (**4**, **5**) and Heyns (**6**, **7**) glycation products (presented in pyranose forms).

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]

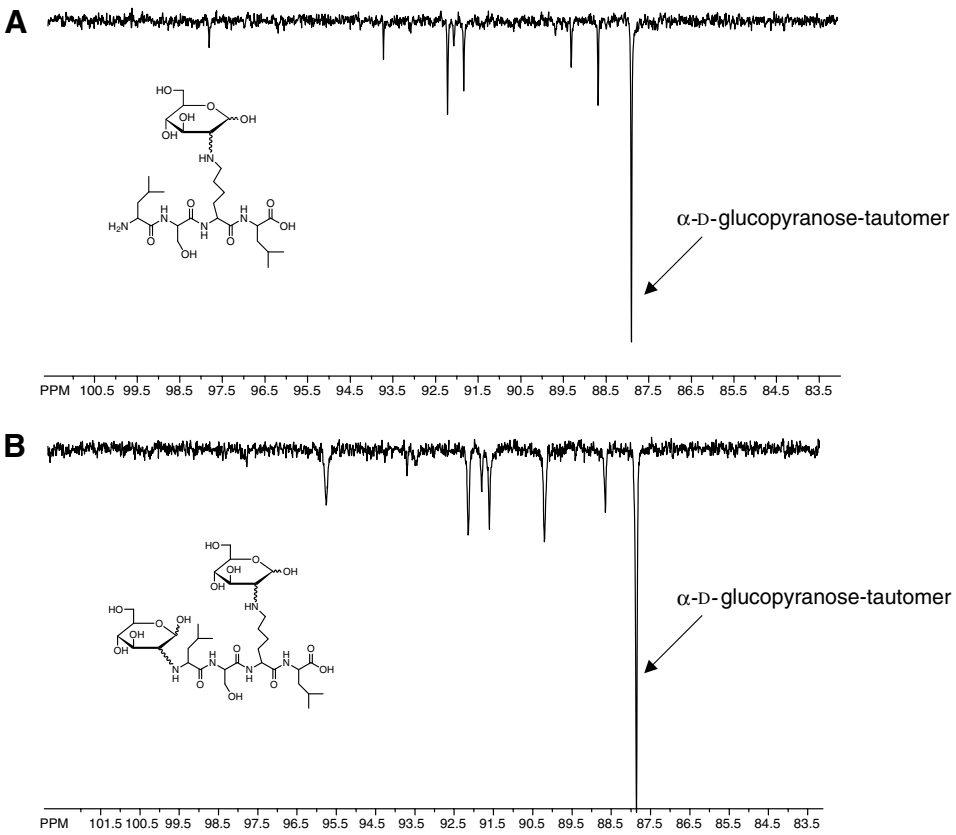

Figure 1. ^{13}C NMR spectra (anomeric region) of Heyns compounds **6** (A) and **7** (B) in D_2O solutions at 25 °C.

To determine the effect of pH and glucose or fructose concentrations on the formation of glycated product, the time course of the glycation of tetrapeptide **1** was monitored in phosphate buffers of pH 5.7, 7.4, and 8.0, at 37 °C. To evaluate the extent of glycation, 0.01 M Leu-Ser-Lys-Leu (**1**) was incubated under sterile conditions with glucose or fructose initially containing 0.01 M, 0.15 M, or 1.50 M of reducing sugar. The Amadori compounds **4** and **5** were detected in the glucose-containing samples and the Heyns compound **6** was detected in that with fructose. Identity of the products was confirmed by co-injection of sample and reference material in the HPLC system. According to the obtained results, besides the formation of the compounds **4**, **5**, or **6**, no degradation of these Maillard products was observed at the same time. The extent of glycation was calculated by comparing peak areas of **1**, **4**, **5**, or **6** with peak area of internal standard before and during incubation. Typical analytical HPLC profiles of **1**/glucose incubation mixtures are presented in Figure 2. As presented in Figure 3, the composition and yield of the

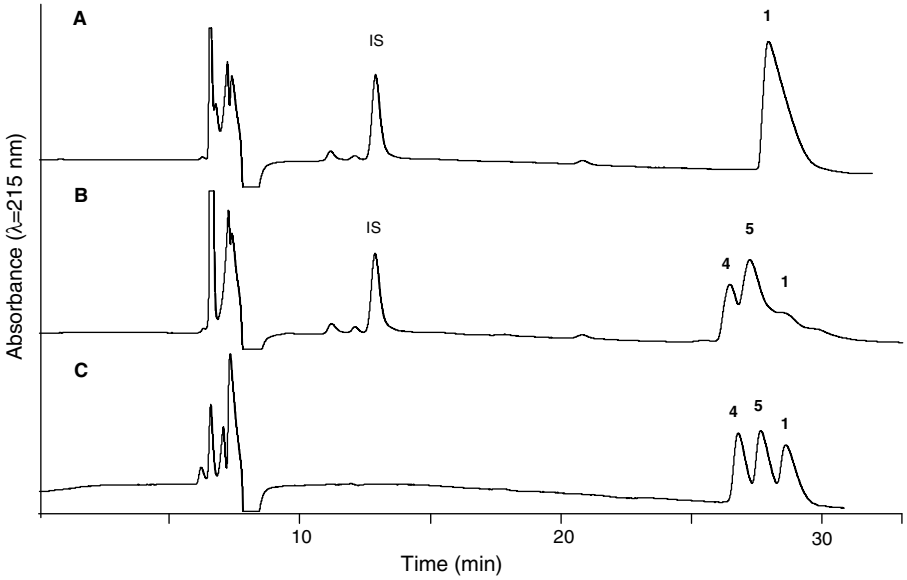

Figure 2. Typical analytical RP-HPLC elution profile of the solution of Leu-Ser-Lys-Leu (**1**) (0.01 M) in phosphate buffer (pH 8.0) containing D-glucose (1.50 M) and internal standard (IS) before (A) and after incubation at 37 °C for 28 days (B). Chromatogram (C) shows elution profile of the peptide **1** and purified compounds **4** and **5** dissolved in phosphate buffer (pH 8.0).

corresponding Amadori/Heyns products formed from the hexoses strongly depended on the reducing sugar used, on the concentration of the reactants, and on the pH of the reaction medium. With a sugar concentration of 0.01 M, no glycation was observed in any of the model systems investigated, not even after a month of incubation. A sugar concentration of 0.15 M was sufficient for glycation, but only in **1**/glucose model systems, and preferentially at neutral and alkaline pH (Fig. 3A). The product was, almost exclusively, mono-glycated Amadori compound **5**, with an approximate yield of 20% after one month of incubation. Increasing the hexose concentration to 1.50 M accelerated glycation in all **1**/sugar systems studied (Fig. 3B). After 28 days of incubation, the combined yields of Amadori compounds **4** and **5** were 15% (pH 5.7), 69% (pH 7.4), and 74% (pH 8.0) (Fig. 3B). Fructose reacted more slowly than glucose and only at the higher pH values tested. At pH 7.4, three weeks were required to produce 10% of the mono-glycated Heyns rearrangement product **6**. Generally, pH 5.7 was not favorable for glycation, while reaction rates at pH 7.4 and 8.0 were comparable. This observation is consistent with the chemical prediction that only uncharged amino groups in peptides/proteins can participate in addition reactions with reducing sugars.[22]

The obtained results suggest that, in model systems resembling physiological conditions (buffered aqueous solution, pH 7.4, 37 °C), D-glucose is more reactive than D-fructose with respect to the formation of *N*-glycated peptides. The obtained results also substantially support previous studies[7] showing the site specificity of the Maillard reaction with a preferential glycation on Lys residue. In contrast to low yields of Heyns compounds formed under the conditions used in the present study, high yields of the rearrangement products were obtained under the baking conditions used in the baked biscuits containing fructose or glucose.[12] Consequently, the baking conditions (175 °C) and pH 7.4 favor the formation of the rearrangement products. However, in agreement with our results, when compared with the Amadori compound, lower amounts of Heyns compounds were always formed at the same reaction conditions.

To conclude, because the model systems used in this study unequivocally showed the formation of Heyns compounds, similar glycation products are expected to be present under selected biological conditions, such as on comsuption of a high fructose diet. However, since the amount of rearrangement products in vivo is multifactorial, more studies on different peptides would be necessary to produce more breakthrough information.

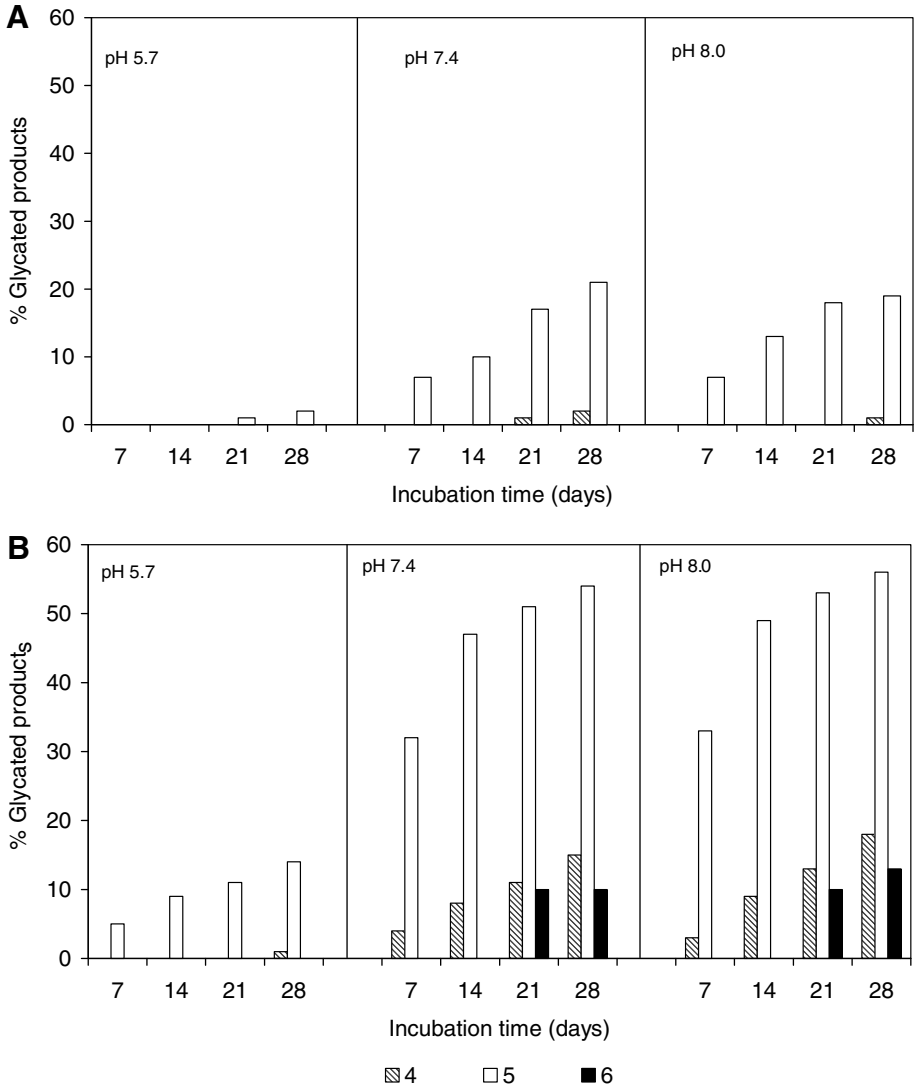

Figure 3. Comparison of glycation product (Amadori and Heyns compounds) formation from Leu-Ser-Lys-Leu (**1**) in the presence of D-glucose (**4** ▨, **5** □) or D-fructose (**6** ■) in phosphate buffered solutions (pH 5.7, 7.4, and 8.0) at 37 °C. The molar ratio of the reactants was as follows: (A) peptide–sugar = 1:15; (B) peptide:sugar = 1:150. The columns represent three independent sets of experiments. The concentration of glycated products was determined by RP-HPLC.

1. Experimental

1.1. General methods

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 D_2O and DMSO-d_6 solutions at 25 °C. Chemical shifts in parts per million were referenced to TMS (DMSO-d_6 solutions) or to dioxane (D_2O solutions). Spectra were assigned based on 2D homonuclear (COSY, NOESY) and heteronuclear (HMQC, HMBC) experiments. Mass spectra were recorded on a TermoFinnigan Deca ion trap mass spectrometer operating in electrospray ionization (ESI) mode. 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 (250 × 4 mm ID, 5 μm) (flow rate: 0.5 mL/min) column under isocratic and gradient conditions using different concentrations of MeOH in 0.1% aqueous trifluoroacetic acid (TFA). UV detection was performed at 215 nm using a Varian Pro Star 335 photodiode-array detector. Leu-Ser-Lys-Leu (**1**) was purchased from Bachem AG. *N*-Acetyl-L-tyrosine amide was purchased from Sigma. 2,3:4,5-Di-*O*-isopropylidene-*aldehydo*-β-D-*arabino*-hexosulo-2,6-pyranose (**2**) was prepared following a published procedure.[20]

1.2. Synthesis of Amadori products 4 and 5

1.2.1. Procedure (a)

1.2.1.1. *N*-(1-Deoxy-2,3:4,5-di-*O*-isopropylidene-β-D-fructopyranos-1-yl)-L-leucyl-L-seryl-N^{ε}-(1-deoxy-2,3:4,5-di-*O*-isopropylidene-β-D-fructopyranos-1-yl)-L-lysyl-L-leucine (3). To a solution of Leu-Ser-Lys-Leu (**1**) (46 mg; 0.1 mmol) in MeOH (10 mL) were added 2,3:4,5-di-*O*-isopropylidene-*aldehydo*-β-D-*arabino*-hexosulo-2,6-pyranose (**2**) (52 mg; 0.2 mmol) and sodium cyanoborohydride (10 mg; 0.16 mmol). The resulting solution was stirred for 5 h at 70 °C. The solvent was evaporated and the residue was purified by silica gel column chromatography eluting with ethyl acetate, followed by MeOH. Trituration of the residue with diisopropyl ether gave amorphous crude compound **3** (55 mg, 58%); R_f 0.67 (7:1:1:1, EtOAc–EtOH–AcOH–water); mp 110–116 °C; $[\alpha]_D$ −28 (*c* 1, MeOH). ^{13}C NMR (150 MHz, DMSO-d_6): δ 22.03, 22.40, 22.78, 23.16 (Leu δ,δ′); 23.39 (Lys γ); 24.20, 24.42 (Leu γ); 25.25, 25.64, 26.27 (CH_3 isopropylidene); 29.22 (Lys β); 30.84 (Lys δ); 41.69, 42.30 (Leu β); 49.67 (Lys ε); 52.74, 54.25 (C-1); 52.64 (Leu^4 α); 52.80 (Lys α); 54.25 (Ser α); 60.08 (Leu^1 α); 60.21 (Ser β); 63.04 (C-6); 69.62 (C-5); 70.16, 70.32 (C-3); 70.70 (C-4); 102.97, 103.32 (C-2); 107.23, 107.28, 107.86, 107.92 (C isopropylidene); 170.02 (Leu, Ser CO); 173.86 (Lys CO); 175.05 (Leu COOH). ESIMS found *m*/*z* 966.68 $[M+Na]^+$. Calcd for $C_{45}H_{46}N_5O_{16}Na$, 966.53.

1.2.1.2. *N*-(1-Deoxy-D-fructos-1-yl)-L-leucyl-L-seryl-N^{ε}-(1-deoxy-D-fructos-1-yl)-L-lysyl-L-leucine (4). Protected compound **3** (72 mg; 0.08 mmol) was treated with 9:1 TFA–water (1 mL) in the presence of anisole (0.2 mL) for 30 min at room temperature. After addition of diethyl ether at 0 °C, the precipitate was collected by centrifugation and triturated several times with diethyl ether. The product was purified by semipreparative RP-HPLC using 30% MeOH/0.1% TFA as the eluent. Trituration several times with diisopropyl ether gave di-glycated compound **4** as TFA salt (32 mg; 40%); RP-HPLC analysis: 30% MeOH/0.1% TFA t_R = 17.6 min; mp 109 °C (decomp); $[\alpha]_D$ −25 (*c* 1, MeOH). ^{1}H NMR (600 MHz, D_2O) (β-pyranose form): δ 0.84, 0.92 (12H, Leu^1,Leu^4 δ,δ′); 1.41 (2H, Lys γ); 1.61, 1.81 (4H, Leu^1,Leu^4 β); 1.63 (2H, Lys δ); 1.64 (2H, Leu γ); 1.70 (2H, Lys β); 3.07 (2H, Lys ε); 3.16, 3.25, 3.63 (4H, H-1); 3.73 (2H, H-5); 3.74, 3.95 (4H, H-6); 3.85 (2H, H-3); 3.94 (2H, Ser β); 3.95 (2H, H-4); 4.15, 4.31 (2H, Leu^1,Leu^4 α); 4.36 (1H, Lys α); 4.57 (1H, Ser α). ^{13}C NMR (150 MHz, D_2O) (β-pyranose form): δ 20.61, 20.05, 24.15, 24.50 (Leu^1,Leu^4 δ, δ′); 22.06 (Lys γ); 22.21, 22.35 (Leu^1,Leu^4 γ); 24.80 (Lys β); 30.53 (Lys δ); 38.82, 39.28 (Leu^1,Leu^4 β); 48.20 (Lys ε); 51.47 (Leu^4 α); 51.78, 52.90 (C-1); 53.51 (Lys α) 55.47 (Ser α); 60.01 (Leu^1 α); 61.11 (Ser β); 64.00 (C-6); 69.31, 69.36 (C-5); 69.68, 69.71 (C-3); 95.39, 95.48 (C-2); 168.98 (Leu^{1}CO); 170.66 (Ser CO); 173.43 (Lys CO); 176.25 (Leu^4 COOH). ESIMS found *m*/*z* 784.54 $[M+H]^+$. Calcd for $C_{33}H_{62}N_5O_{16}$, 784.42. Anal. Calcd for $C_{33}H_{61}N_5O_{16} \times 2TFA$, 1011.91: C, 43.92; H, 6.28; N, 6.92. Found: C, 43.36; H, 6.67; N, 7.18.

1.2.2. Procedure (b)

D-Glucose (234 mg; 1.30 mmol) and Leu-Ser-Lys-Leu (40 mg; 0.087 mmol) were dissolved in MeOH (40 mL), and the reaction mixture was stirred for 10 h at 70 °C. The solvent was evaporated and the residue was redissolved in water and passed through a C-18 solid-phase extraction (SPE) cartridge (500 mg). Elution with water removed the unreacted sugar. The glycated peptide compounds were then recovered with methanol. The effluent was evaporated and the residue was purified by semipreparative RP-HPLC eluting with 30% MeOH/0.1% TFA. After evaporation of the solvent and trituration with diisopropyl ether di-glycated compound **4** (5 mg, 6%) and mono-glycated L-leucyl-L-seryl-N^{ε}-(1-deoxy-D-fructos-1-yl)-L-lysyl-L-leucine (**5**) (17 mg, 23%) were obtained as TFA salts.

1.2.2.1. L-Leucyl-L-seryl-N^{ε}-(1-deoxy-D-fructos-1-yl)-L-lysyl-L-leucine (5). RP-HPLC analysis: 30% MeOH/0.1% TFA t_R = 18.6 min; mp 110–116 °C; $[\alpha]_D$ −26 (*c* 1, MeOH); ^{1}H NMR (600 MHz, D_2O) (β-pyranose form): δ 0.84, 0.94 (12H, Leu^1,Leu^4 δ, δ′); 1.42 (2H, Lys γ); 1.64 (4H, Leu^1,Leu^4 β); 1.65 (2H, Lys δ); 1.68 (2H, Lys β); 1.78 (2H, Leu^1,Leu^4 γ); 3.06 (2H, Lys ε); 3.25 (2H, H-1); 3.69, 3.95 (2H, H-6); 3.72 (1H, H-3); 3.84 (1H, H-4); 3.95 (2H, Ser β); 3.97 (1H, H-5); 4.04 (1H, Leu^1 α), 4.32 (1H, Leu^4 α); 4.35 (1H, Lys α); 4.51 (1H, Ser α). ^{13}C NMR (150 MHz, D_2O) (β-pyranose form): δ 21.63, 21.94, 23.92, 24.50 (Leu^1,Leu^4 δ, δ′); 21.24, 22.20 (Leu^1,Leu^4 γ); 22.04 (Lys γ); 24.78 (Lys β); 30.52 (Lys δ); 39.30, 39.89 (Leu^1, Leu^4 β); 48.23 (Lys ε); 51.46 (Leu^1 α); 51.92 (Leu^4 α); 52.91 (C-1); 53.47 (Lys α); 55.44 (Ser α); 61.13 (Ser β); 64.01 (C-6); 68.95 (C-5); 69.37 (C-4); 69.69 (C-3); 95.47 (C-2); 170.62 (Leu^1 CO); 170.94 (Ser CO); 173.42 (Lys CO); 176.20 (Leu^4 COOH). ESIMS found *m*/*z* 622.45 $[M+H]^+$. Calcd for $C_{27}H_{52}N_5O_{11}$, 622.37. Anal. Calcd for $C_{27}H_{51}N_5O_{11} \times 2TFA \times H_2O$, 867.78: C, 42.91; H, 6.39; N, 8.07. Found: C, 43.12; H, 6.49; N, 8.30.

1.3. Synthesis of Heyns products 6 and 7

D-Fructose (1350 mg; 7.5 mmol), Leu-Ser-Lys-Leu (46 mg; 0.1 mmol), and *N*-ethylmorpholine (NEM) (192 μl, 1.50 mmol) were dissolved in dry MeOH (10 mL), and the reaction mixture was stirred for 24 h at 70 °C. Further treatment of the reaction mixture was the same as described in Section 1.2.2. The obtained residue was purified by semipreparative RP-HPLC using 30% MeOH/0.1% TFA and after evaporation of the solvent residue was triturated with diisopropyl ether to yield mono-glycated L-leucyl-L-seryl-N^{ε}-(2-deoxy-D-glucos-2-yl/2-deoxy-D-mannos-2-yl)-L-lysyl-L-leucine (**6**) (6 mg, 7%) and di-glycated *N*-(2-deoxy-D-glucos-2-yl/2-deoxy-D-mannos-2-yl)-L-leucyl-L-seryl-N^{ε}-(2-deoxy-D-glucos-2-yl/2-deoxy-D-mannos-2-yl)-L-lysyl-L-leucine (**7**) (6 mg, 6%) as TFA salts.

1.3.1. L-Leucyl-L-seryl-N^{ε}-(2-deoxy-D-glucos-2-yl/2-deoxy-D-mannos-2-yl)-L-lysyl-L-leucine (6)

RP-HPLC analysis: 30% MeOH/0.1% TFA t_R = 18.6 min; mp 113–125 °C; $[\alpha]_D$ −4 (*c* 1, MeOH); ^{1}H NMR (600 MHz, D_2O) (α-D-glucopyranose form): δ 0.86, 0.96 (12H, Leu^1,Leu^4 δ, δ′); 1.44 (2H, Lys γ); 1.62 (6H, Leu^1,Leu^4 β, γ); 1.67 (2H, Lys β); 1.76, 1.83 (2H, Lys δ); 3.17 (2H, Lys ε); 3.26 (1H, H-2); 3.43 (1H, H-4); 3.75, 3.85 (2H, H-6); 3.82 (2H, Ser β); 3.86 (1H, H-5); 3.91 (1H, H-3); 4.04, 4.26 (2H, Leu^1,Leu^4 α); 4.35 (1H, Lys α); 4.53 (1H, Ser α); 5.53 (d, 1H, $J_{1,2}$ 3.3 Hz, H-1). ^{13}C NMR (150 MHz, D_2O) (α-pyranose form): δ 20.81, 21.22, 23.93, 24.64 (Leu^1,Leu^4 δ, δ′); 21.83, 22.34 (Leu^1,Leu^4 γ); 22.09 (Lys γ); 24.51 (Lys β); 30.49 (Lys δ); 39.90, 40.11 (Leu^1, Leu^4 β); 45.52 (Lys ε); 51.88 (Leu^1 α); 52.94 (Leu^4 α); 53.56 (Lys α); 55.47 (Ser α); 59.36 (C-2); 60.38 (Ser β); 61.15 (C-6); 69.62 (C-4); 69.76 (C-3); 71.39 (C-5); 87.90 (C-1); 170.64 (Leu^1 CO); 170.92 (Ser CO); 172.92 (Lys CO); 178.18 (Leu^4 COOH). ESIMS found *m*/*z* 622.45 $[M + H]^+$. Calcd for $C_{27}H_{52}N_5O_{11}$, 622.37. Anal. Calcd for $C_{27}H_{51}N_5O_{11} \times 2$TFA, 849.34: C, 43.82; H, 6.29; N, 8.24. Found: C, 44.15; H, 6.78; N, 7.81.

1.3.2. *N*-(2-Deoxy-D-glucos-2-yl/2-deoxy-D-mannos-2-yl)-L-leucyl-L-seryl-N^{ε}-(2-deoxy-D-glucos-2-yl/2-deoxy-D-mannos-2-yl)-L-lysyl-L-leucine (7)

RP-HPLC analysis: 30% MeOH/0.1% TFA t_R = 17.6 min; mp 118–131 °C; $[\alpha]_D$ −4 (*c* 1, MeOH); ^{1}H NMR (600 MHz, D_2O) (α-D-glucopyranose form): δ 0.87, 1.01 (12H, Leu^1,Leu^4 δ, δ′); 1.42, 1.62 (4H, Leu^1,Leu^4 β); 1.50 (2H, Leu γ); 1.62 (2H, Lys γ); 1.73, 1.92 (2H, Lys δ); 1.79 (2H, Lys β); 3.23 (2H, Lys ε); 3.53 (2H, H-4); 3.75 (2H, Ser β); 3.89 (2H, H-5); 3.94 (4H, H-6); 3.96, 4.25 (2H, Leu^1,Leu^4 α); 3.98 (2H, H-3); 4.44 (1H, Lys α); 4.57 (1H, Ser α); 5.60 (2H, H-1). ^{13}C NMR (150 MHz, D_2O) (α-pyranose form): δ 20.93, 21.94 (Leu^1,Leu^4 δ, δ′); 22.18 (Lys γ); 24.69 (Leu^1,Leu^4 γ); 25.09 (Lys β); 30.36, 30.50 (Lys δ); 40.69 (Leu^1,Leu^4 β); 45.54 (Lys ε); 53.58 (Leu^4 α); 53.96 (Lys α); 55.47 (Ser α); 59.42 (Leu^1 α); 60.00 (C-2); 60.41 (Ser β); 61.16 (C-6); 69.77 (C-4); 69.90 (C-3); 71.49 (C-5); 87.91 (C-1); 170.96 (Leu^1 CO); 171.58 (Ser CO); 172.60 (Lys CO); 179.66 (Leu^4 COOH). ESIMS found *m*/*z* 784.54 $[M + H]^+$. Calcd for $C_{33}H_{62}N_5O_{16}$, 784.42. Anal. Calcd for $C_{33}H_{61}N_5O_{16} \times 1.5$TFA $\times$ $4H_2O$, 933.35: C, 44.24; H, 7.52; N, 7.50. Found: C, 44.24; H, 7.07; N, 7.53.

1.4. RP-HPLC monitoring of the glycation of Leu-Ser-Lys-Leu by incubation with reducing sugars in phosphate buffer (pH 5.7, 7.4 and 8.0)

Solutions of Leu-Ser-Lys-Leu (0.01 M) in 0.05 M phosphate buffer (pH 5.7, 7.4 or 8.0) containing D-glucose or D-fructose (0.01 M, 0.15 M, or 1.50 M), the internal standard *N*-acetyl-L-tyrosine amide (40 μg/mL) and NaN_3 (0.02%) were sterilized by passage through a 0.45 μm nylon filter and incubated in the dark, at 37 °C. Aliquots were withdrawn from the incubation mixtures at appropriate time points and immediately frozen. The relative concentrations of the respective Amadori and Heyns glycation products in the incubation mixtures were monitored by RP-HPLC using a Eurospher 100 reversed-phase C-18 analytical (250 × 4 mm ID, 5 μm) column. A linear gradient from 25% to 40% MeOH/0.1% TFA in 50 min at a flow rate of 0.5 mL/min was used. UV absorbance was monitored at 215 nm. The concentrations of the starting peptide and the rearrangement products were determined by electronic integration of the peak areas and calculation of the analyte/internal standard peak-ratios.

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References

1. Ledl, F.; Schleicher, E. *Angew. Chem., Int. Ed.* **1990**, *29*, 565–706.
2. Heyns, K.; Paulsen, H.; Eichstedt, R.; Rolle, M. *Chem. Ber.* **1957**, *90*, 2039–2049.
3. Gerrard, J. A. *Aust. J. Chem.* **2002**, *55*, 299–310.
4. Ahmed, N.; Thornalley, P. J. *Diabetes Obes. Metab.* **2007**, *9*, 233–245.
5. Nass, N.; Bartling, B.; Navarrete Santos, A.; Scheubel, R. J.; Börgerman, J.; Silber, R. E.; Simm, A. *Z. Gerontol. Geriat.* **2007**, *40*, 349–356.
6. Mostafa, A. A.; Randell, E. W.; Vasdev, S. C.; Gill, V. D.; Han, Y.; Gadag, V.; Raouf, A. A.; El Said, H. *Mol. Cell Biochem.* **2007**, *302*, 35–42.
7. Frolov, A.; Singer, D.; Hoffmann, R. *J. Peptide Sci.* **2006**, *12*, 389–395.
8. Brock, J. W. C.; Hinton, D. J. S.; Cotham, W. E.; Metz, T. O.; Thorpe, S. R.; Baynes, J. W.; Ames, J. M. *J. Proteome Res.* **2003**, *2*, 506–513.
9. Venkatraman, J.; Aggarwal, K.; Balarman, P. *Chem. Biol.* **2001**, *8*, 611–625.
10. Mennella, C.; Visciano, M.; Napolitano, A.; del Castillo, M. D.; Fogliano, F. *J. Pept. Sci.* **2006**, *12*, 291–296.
11. Thorpe, S. R.; Baynes, J. W. *Amino Acids* **2003**, *25*, 275–281.
12. Krause, R.; Schlegel, K.; Schwarzer, E.; Henle, T. *J. Agric. Food Chem.* **2008**, *56*, 2522–2527.
13. Hinton, D. J. S.; Ames, J. M. *Amino Acids* **2006**, *30*, 425–433.
14. Horvat, Š.; Jakas, A. *J. Peptide Sci.* **2004**, *10*, 119–137.
15. Roščić, M.; Horvat, Š. *Bioorg. Med. Chem.* **2006**, *14*, 4933–4943.
16. Jakas, A.; Horvat, Š. *Amino Acids* **2008**, *34*, 329–332.
17. Massagué, J.; Chen, Y. G. *Genes Dev.* **2000**, *14*, 627–644.
18. Nakagawa, T.; Hu, H.; Zharikov, S.; Tuttle, K. R.; Short, R. A.; Glushakova, O.; Ouyang, X.; Feig, D. I.; Block, E. R.; Herrera-Acosta, J.; Patel, J. M.; Johnson, R. J. *Am. J. Physiol.–Renal Physiol.* **2006**, *290*, F625–F631.
19. Eavel, P. J. *Nutr. Rev.* **2005**, *63*, 133–157.
20. Arrick, R. E.; Baker, D. C.; Horton, D. *Carbohydr. Res.* **1973**, *26*, 441–447.
21. Mossine, V.; Barens, C. L.; Glinsky, G. V.; Feather, M. S. *Carbohydr. Res.* **1996**, *284*, 11–24.
22. Lowrey, C. H.; Lyness, S. J.; Soeldner, J. S. *J. Biol. Chem.* **1985**, *260*, 1161–11618.