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Fructose-induced *N*-terminal glycation of enkephalins and related peptides

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Abstract: The formation of glycation products in model systems consisting of fructose and the endogenous opioid peptides not containing lysine residue, such as Leu-enkephalin (Tyr-Gly-Gly-Phe-Leu) and Met-enkephalin (Tyr-Gly-Gly-Phe-Met), or of their fragments, Tyr-Gly-Gly-Phe and Tyr-Gly-Gly, was examined. *N*-(2-Deoxy-aldos-2-yl)-peptides (Heyns compounds) as well as diastereoisomeric imidazolidinone compounds were identified as reaction products of *N*-terminal amino group glycation for each of the peptides studied. The structure of the glycation products and relative configuration of C-2 substituents on the imidazolidinone ring in diastereoisomers were determined by NMR experiments. The chemical and enzymatic stability of the fructose-derived glycated products of Leu- and Met-enkephalin was studied in phosphate-buffered saline (pH 7.4) and in human serum at 37 °C. The obtained results revealed that glycation increases the stability of the parent peptide to enzymatic degradation. As a result of different configuration at the newly formed stereogenic center, large stability differences in the 2*S** and 2*R** isomers of the imidazolidinone compounds were observed. Copyright © 2008 European Peptide Society and John Wiley & Sons, Ltd.

Keywords: enkephalin; fructose; glycation; Heyns compound; imidazolidinone; Maillard reaction; peptide stability; reducing sugar

INTRODUCTION

The growing interest for the Maillard reaction can be attributed to its omnipresence in nature. The reaction starts with the interaction between the carbonyl group of a reducing sugar of aldo or keto type and the amino group of an amino acid, peptide or protein [1–3]. The glycated products can undergo further reactions involving dicarbonyl intermediates, such as 3-deoxyglucosones, giving raise to poorly characterized structures called advanced glycation end products (AGEs) [4–6]. The role of glucose as the most abundant sugar has received preferential attention in these reactions, but other sugars also deserve consideration. For example, the intake of the keto-sugar fructose, as 'healthy sugar', has greatly increased during the past years. This led to increased interest in the Maillard reaction with fructose [7–11]. Extensive studies on the chemistry and biochemistry of protein modification by sugars, which may lead to various complications in diabetes, suggest that fructose may be an active participant in the disease, along with glucose and other sugars [7]. The Heyns rearrangement represents one of the initial steps of the Maillard reaction when it involves the reaction of ketoses and amines [12]. Heyns compounds have been detected in liver extracts and in human ocular lens proteins in measurable amounts, suggesting that they have more important roles than realized so far [13].

Endogenous opioid peptides, such as enkephalins, participate in the regulation of a variety of physiological and behavioral functions [14]. In particular, they participate in glucoregulation and seem able to influence insulin and glucagon release [15]. Studies on circulating opioid peptide levels have shown that diabetes patients have significantly lowered β-endorphin and Met-enkephalin levels, a situation that contributes to a worsening of metabolic control under stress conditions [16]. For these reasons, the enkephalins were recognized as good models for glycation studies.

In this article the glycation products derived from fructose and the small bioactive peptides, Leu-enkephalin (Tyr-Gly-Gly-Phe-Leu) and Met-enkephalin (Tyr-Gly-Gly-Phe-Met), and their fragments (Tyr-Gly-Gly-Phe and Tyr-Gly-Gly), were studied, employing mainly NMR and MS techniques.

The stability of the prepared glycated Met- and Leu-enkephalin derivatives in phosphate buffer and in human serum was the second issue addressed. The main degradation products were also identified. The types of products formed from fructose and bioactive peptides, their relative amounts, and their stabilities, are of crucial importance for understanding the mechanisms involved in the formation of AGE products from keto-sugars.

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MATERIALS AND METHODS

General

Melting points were determined on a Tottoli (Büchi) apparatus and were 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, ROESY) 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 (150 × 4.5 mm ID, 5 μm) (flow rate: 0.5 ml/min) column under isocratic conditions using different concentrations of MeOH in 0.1% aqueous trifluoroacetic acid (TFA). UV detection was performed at 215, 254 and 280 nm using a Varian Pro Star 335 photodiode-array detector. The quantum-chemical calculations were performed on the Isabella cluster at the University Computing Center 'SRCE' Zagreb using the program Gaussian03 [17] with the implemented methods and basis sets.

Leu-enkephalin (**1**), Met-enkephalin (**2**), Tyr-Gly-Gly-Phe (**3**) and Tyr-Gly-Gly (**4**) were purchased from Bachem. Human serum was obtained from Sigma-Aldrich. *o*-Hydroxyphenylacetic acid was obtained from Fluka.

Synthesis of Glycation Products 5–12

Method A. D-Fructose (270 mg, 1.5 mmol), peptides **1**–**4** (0.1 mmol) and *N*-ethylmorpholine (NEM) (192 μl, 1.5 mmol) were dissolved in dry MeOH (20 ml), and the reaction mixture was stirred for 24 h at 70 °C. The solvent was evaporated and the residue was applied to a column (90 × 1.6 cm) of Sephadex G-15 eluted with 1% aqueous acetic acid. The fractions containing glycation products were combined and further purified by RP HPLC using 40% MeOH/0.1% TFA (for compounds **5**, **6**, **9** and **10**), 35% MeOH/0.1% TFA (for compounds **7** and **11**), or 15% MeOH/0.1% TFA (for compounds **8** and **12**) as eluents.

Method B. To a solution of D-fructose (810 mg, 4.5 mmol) in MeOH (10 ml), peptide **1** or **2** (0.06 mmol), KOH (3 mg, 0.06 mmol), $Na_2S_2O_5$ (0.4 mg, 0.0016 mmol), and glycerol (0.5 ml) were added, and the reaction mixture was stirred for 8 h at 70 °C. Further treatment of the reaction mixture was the same as in Method A.

Glycation Products Derived from the Reaction of Fructose with Leu-enkephalin (1)

Mixture of N-(2-deoxy-D-glucos-2-yl)-L-tyrosylglycylglycyl-L-phenylalanyl-L-leucine (5a) and N-(2-deoxy-D-mannos-2-yl)-L-tyrosylglycylglycyl-L-phenylalanyl-L-leucine (5b). Method A: 11 mg (15%); Method B: 14 mg (32%); analytical RP HPLC analysis: 40% MeOH/0.1%TFA $t_R = 14.21$ min. ^{1}H NMR and ^{13}C NMR data in D_2O for the major component, α-pyranose form of **5a**, are given in Table 2. MS-ES, *m/z* 718.43 $[M + H]^+$.

(2R*, 4S*)-N-{(2-(D-arabino-tetritol-1-yl)-2-hydroxymethyl-4-(4-hydroxybenzyl)-5-oxoimidazolidin-1-yl)acetyl}glycyl-L-phenylalanyl-L-leucine (9a). Method A: 10 mg (14%); Method B: 3 mg (7%); analytical RP HPLC analysis: 40% MeOH/0.1%TFA $t_R = 20.13$ min; mp 120–131 °C; $[\alpha]_D$ −37° (c 1.05, MeOH). ^{1}H NMR and ^{13}CNMR data in DMSO-d_6 are given in Table 4. MS-ES, *m/z* 718.44 $[M + H]^+$.

(2S*, 4S*)-N-{(2-(D-arabino-tetritol-1-yl)-2-hydroxymethyl-4-(4-hydroxybenzyl)-5-oxoimidazolidin-1-yl)acetyl}glycyl-L-phenylalanyl-L-leucine (9b). Method A: 16 mg (23%); Method B: 3 mg (7%); analytical RP HPLC analysis: 40% MeOH/0.1%TFA $t_R = 22.60$ min; mp 120–143 °C; $[\alpha]_D$ −40° (c 1.03, MeOH). ^{1}H NMR and ^{13}CNMR data in DMSO-d_6 are given in Table 4. MS-ES, *m/z* 718.44 $[M + H]^+$.

Glycation Products Derived from the Reaction of Fructose with Met-enkephalin (2)

Mixture of N-(2-deoxy-D-glucos-2-yl)-L-tyrosylglycylglycyl-L-phenylalanyl-L-methionine (6a) and N-(2-deoxy-D-mannos-2-yl)-L-tyrosylglycylglycyl-L-phenylalanyl-L-methionine (6b). Method A: 7 mg (10%); Method B: 14 mg (32%); analytical RP HPLC analysis: 40% MeOH/0.1%TFA $t_R = 8.85$ min. ^{1}H NMR and ^{13}C NMR data in D_2O for the major component, α-pyranose form of **6a**, are given in Table 2. MS-ES, *m/z* 736.40 $[M + H]^+$.

(2R*, 4S*)-N-{(2-(D-arabino-tetritol-1-yl)-2-hydroxymethyl-4-(4-hydroxybenzyl)-5-oxoimidazolidin-1-yl)acetyl}glycyl-L-phenylalanyl-L-methionine (10a). Method A: 3 mg (7%); Method B: 16 mg (21%); analytical RP HPLC analysis: 40% MeOH/0.1%TFA $t_R = 11.57$ min; mp 120–131 °C; $[\alpha]_D$ −32° (c 1, MeOH). ^{1}H NMR and ^{13}CNMR data in DMSO-d_6 are given in Table 4. MS-ES, *m/z* 734.5 $[M - H]^-$.

(2S*, 4S*)-N-{(2-(D-arabino-tetritol-1-yl)-2-hydroxymethyl-4-(4-hydroxybenzyl)- 5-oxoimidazolidin-1-yl)acetyl}glycyl-L-phenylalanyl-L-methionine (10b). Method A: 2 mg (5%); Method B: 20 mg (27%); analytical RP HPLC analysis: 40% MeOH/0.1%TFA $t_R = 12.62$ min; mp 126–130 °C; $[\alpha]_D$ −30° (c 1, MeOH). ^{1}H NMR and ^{13}CNMR data in DMSO-d_6 are given in Table 4. MS-ES, *m/z* 734.5 $[M - H]^-$.

Glycation Products Derived from Reaction of Fructose and Tyr-Gly-Gly-Phe (3)

Mixture of N-(2-deoxy-D-glucos-2-yl)-L-tyrosylglycylglycyl-L-phenylalanine (7a) and N-(2-deoxy-D-mannos-2-yl)-L-tyrosylglycylglycyl-L-phenylalanine (7b). Method A: 4 mg (6%); analytical RP HPLC analysis: 30% MeOH/0.1%TFA $t_R = 10.64$ min. ^{13}C NMR (D_2O) (α-pyranose forms): δ (ppm) 36.79 (Tyr β), 36.79 (Phe β), 42.43 (Gly^2 α), 43.32 (Gly^3 α), 54.44 (Phe α), 62.91 (Glc C-6), 62.91 (Glc C-2), 62.91 (Tyr α), 69.10 (Glc C-4), 70.69 (Glc C-3), 70.69 (Glc C-5), 89.05 (Glc C-1), 93.40 (Man C-1), 115.80 (Tyr ε), 127.13 (Phe ζ), 127.14 (Tyr γ), 128.50 (Phe ε), 129.90 (Phe δ), 130.84 (Tyr δ), 136.17 (Phe γ), 151.80 (Tyr ζ). MS-ES, *m/z* 605.42 $[M + H]^+$.

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(2R*, 4S*)-N-{(2-(D-arabino-tetritol-1-yl)-2-hydroxymethyl-4-(4-hydroxybenzyl)-5-oxoimidazolidin-1-yl)acetyl}glycyl-L-phenylalanine (11a). Method A: 7 mg, (11%); analytical RP HPLC analysis: 30% MeOH/0.1%TFA $t_R = 13.37$ min; mp 132–137 °C; $[\alpha]_D$ −10° (c 1, MeOH). ^{13}C NMR (D_2O): δ (ppm) 33.62 (C-a), 36.07 (Phe β), 41.94 (Gly α), 43.24 (C-f), 53.79 (Phe α), 57.61 (C-4), 59.05 (CH_2OH), 62.05 (C-4′), 68.68 (C-1′), 68.70 (C-2′), 68.89 (C-3′), 83.96 (C-2), 115.33 (C-d), 126.67 (C-b), 128.29 (Phe ζ), 128.79 (Phe ε), 130.75 (Phe δ), 130.75 (C-c), 136.22 (Phe γ), 157.20 (C-e). MS-ES, m/z 605.42 $[M + H]^+$.

(2S*, 4S*)-N-{(2-(D-arabino-tetritol-1-yl)-2-hydroxymethyl-4-(4-hydroxybenzyl)-5-oxoimidazolidin-1-yl)acetyl}glycyl-L-phenylalanine (11b). Method A: 7 mg (11%); analytical RP HPLC analysis: 30% MeOH/0.1%TFA $t_R = 14.40$ min; mp 135–137 °C; $[\alpha]_D$ −27° (c 0.98, MeOH). ^{13}C NMR (D_2O): δ (ppm) 34.35 (C-a), 36.42 (Phe β), 41.95 (Gly α), 42.68 (C-f), 54.53 (Phe α), 58.84 (C-4), 61.12 (CH_2OH), 62.01 (C-4′), 64.68 (C-2′), 69.03 (C-1′), 69.95 (C-3′), 84.99 (C-2), 115.40 (C-d), 126.33 (C-b), 128.12 (Phe ζ), 128.77 (Phe δ), 130.57 (Phe ε), 130.59 (C-c), 136.30 (Phe γ), 154.38 (C-e). MS-ES, m/z 605.42 $[M + H]^+$.

Glycation Products Derived from Reaction of Fructose and Tyr-Gly-Gly (4)

Mixture of N-(2-deoxy-D-glucos-2-yl)-L-tyrosylglycylglycine (8a) and N-(2-deoxy-D-mannos-2-yl)-L-tyrosylglycylglycine (8b). Method A: 7 mg (15%); analytical RP HPLC analysis: 30% MeOH/0.1%TFA $t_R = 6.9$ min. ^{13}C NMR (D_2O) (α-pyranose forms): δ (ppm) 38.06 (Tyr β), 43.52 (Gly^2 α), 44.67 (Gly^3 α), 62.62 (Tyr α), 62.72 (Glc C-6), 64.48 (Glc C-2), 71.84 (Glc C-4), 72.53 (Glc C-3), 73.60 (Glc C-5), 91.53 (Glc C-1), 94.38 (Man C-1), 118.20 (Tyr ε), 127.85 (Tyr γ), 133.23 (Tyr δ), 157.57 (Tyr ζ). MS-ES, m/z 458.27 $[M + H]^+$.

(2R*, 4S*)-N-{(2-(D-arabino-tetritol-1-yl)-2-hydroxymethyl-4-(4-hydroxybenzyl)-5-oxoimidazolidin-1-yl)acetyl}glycine (12a). Method A: 12 mg (26%); analytical RP HPLC analysis: 30% MeOH/0.1%TFA $t_R = 8.44$ min; mp 135–139 °C; $[\alpha]_D$ +24° (c 1, MeOH). ^{1}H NMR (DMSO-d_6/CD_3CN 3/1): δ (ppm) 2.70/3.00 (H-a), 3.36 (H-3′), 3.40 (H-2′), 3.44/3.57 (H-4′), 3.49/3.62 (CH_2OH), 3.67 (H-4), 3.80 (H-1′), 3.84 (Gly α), 4.00/4.08 (H-f), 6.70 (H-d), 7.04 (H-c). ^{13}C NMR (DMSO-d_6/CD_3CN 3/1): δ (ppm) 33.00 (C-a), 41.95 (Gly α), 44.62 (C-f), 58.08 (C-4), 59.63 (CH_2OH), 64.18 (C-4′), 70.40 (C-2′), 71.12 (C-1′), 72.41 (C-3′), 83.75 (C-2), 116.06 (C-d), 128.94 (C-b), 131.42 (C-c), 157.06 (C-e). MS-ES, m/z 458.33 $[M + H]^+$.

(2S*, 4S*)-N-{(2-(D-arabino-tetritol-1-yl)-2-hydroxymethyl-4-(4-hydroxybenzyl)-5-oxoimidazolidin-1-yl)acetyl}glycine (12b). Method A: 14 mg (31%); analytical RP HPLC analysis: 30% MeOH/0.1%TFA $t_R = 9.02$ min; mp 137–140 °C; $[\alpha]_D$ −31° (c 1, MeOH). ^{1}H NMR (DMSO-d_6/CD_3CN 3/1): δ (ppm) 2.72/2.90 (H-a), 3.45 (H-2′), 3.43/3.61 (H-4′), 3.49/3.59 (CH_2OH), 3.53 (H-3′), 3.70 (H-4), 3.76 (H-1′), 3.83 (Gly α), 3.92/4.00 (H-f), 6.67 (H-d), δ 7.05 (H-c). ^{13}C NMR (DMSO-d_6/CD_3CN 3/1): δ (ppm) 39.08 (C-a), 42.06 (Gly α), 43.8 (C-f), 61.09 (C-4), 63.88 (CH_2OH), 64.26 (C-4′), 66.59 (C-1′), 71.10 (C-2′), 72.31 (C-3′), 85.22 (C-2), 116.01 (C-d), 130.34 (C-b), 131.12 (C-c), 156.75 (C-e). MS-ES, m/z 458.33 $[M + H]^+$.

Stability of the Heyns and Imidazolidinone Compounds Obtained from D-Fructose and Pentapeptides 1 and 2 in Phosphate Buffer (pH 7.4) and Human Serum

For the determination of the stability, solutions (7×10^{-4} M) of the Heyns compounds (**5** and **6**) or imidazolidinone compounds (**9a, 9b, 10a** and **10b**) were prepared in PBS (0.05 M phosphate buffer/0.1 M NaCl, pH 7.4) containing NaN_3 (0.02%) and an internal standard [(*o*-hydroxyphenylacetic acid (40 μg/ml)]. The solutions 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 analyzed by RP HPLC.

For serum stability studies, a mixture of human serum (3 ml, diluted with water, final concentration 80%), the Heyns compounds (**5** and **6**) or the imidazolidinone compounds (**9a, 9b, 10a** and **10b**) (7×10^{-4} M), and an internal standard [(*o*-hydroxyphenylycetic acid (40 μg/ml)] were kept at 37 °C in teflon-lined screw-cap test tubes. Aliquots (0.1 ml) were sampled in triplicate at appropriate time intervals and deproteinized by addition of 48% aqueous TFA (0.02 ml). The samples were briefly vortexed and frozen. The thawed samples were centrifuged for 10 min (15 000*g*), and the supernatants were analyzed by RP HPLC.

The concentrations of the starting compounds and metabolites in the incubation mixtures were monitored on RP HPLC (by using UV/Vis detector) on an analytical column at a flow rate of 0.5 ml/min with 43.5% MeOH/0.1% TFA for **5**, **9a** and **9b** and 40% MeOH/0.1% TFA for **6**, **10a** and **10b**. The degradation products were identified by coelution with standards.

RESULTS AND DISCUSSION

A glycation reaction between D-fructose and the endogenous opioid peptides Leu- and Met-enkephalin was studied. Two bases were tested as catalysts with methanol as solvent in the reaction: the organic base, NEM, and the inorganic base, KOH. The best results were obtained in the reactions with NEM when the molar ratio sugar-peptide-base was 15 : 1 : 15 (Method A). In the reactions with KOH the modified procedure of Mossine *et al.* [18] was applied (Method B). The glycation resulted in parallel formation of the Heyns compounds, *N*-(2-deoxy-D-glucopyranos-2-yl)- and *N*-(2-deoxy-D-mannopyranos-2-yl)-Tyr-Gly-Gly-Phe-Leu/Met (**5** and **6**), and imidazolidinone compounds **9** and **10** for both peptides studied (Figure 1). The amounts of Heyns or imidazolidinone compounds formed depend on the nature of the base used as a catalyst. Addition of NEM leads preferably to the formation of imidazolidinone compounds, while Heyns compounds were the major products in the presence of KOH (Table 1). The Heyns compounds were accompanied by 5% of Amadori compound **13** [*N*-(1-deoxy-D-fructos-1-yl)-Tyr-Gly-Gly-Phe-Leu] (Figure 2) in the reaction of fructose with Leu-enkephalin using KOH as the catalyst. This was detected by NMR analysis, since attempts

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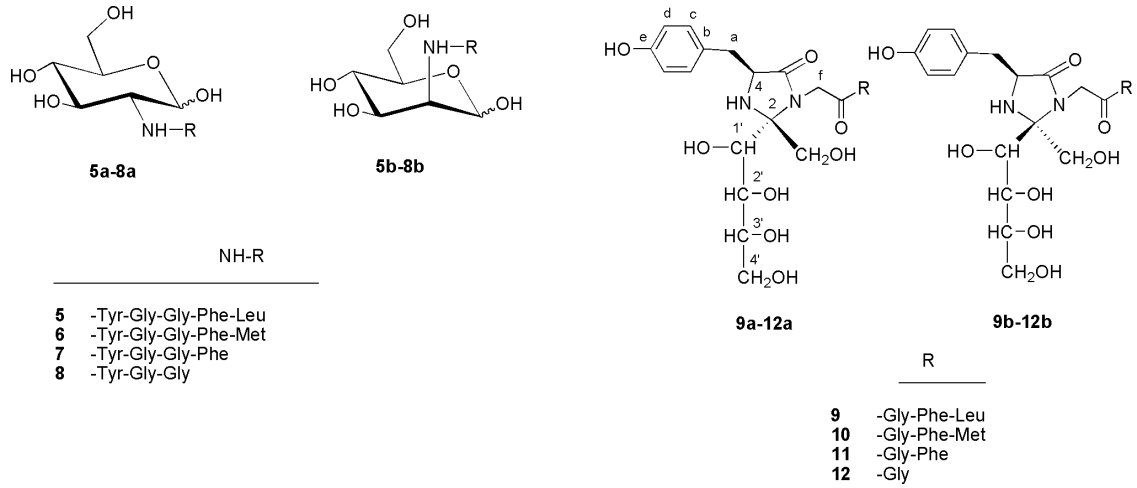

Figure 1 Glycation products derived from Leu-enkephalin (**1**), Met-enkephalin (**2**), Tyr-Gly-Gly-Phe (**3**) and Tyr-Gly-Gly (**4**) in the presence of D-fructose.

Table 1 Comparison of the glycation product formation from peptides **1**–**4** in the presence of D-fructose[a]

Peptide	Method	Relative amounts of glycation products (%)		
		Heyns compounds	Imidazolidinone compounds	
			(2*R**, 4*S**)-isomer	(2*S**, 4*S**)-isomer
Tyr-Gly-Gly-Phe-Leu (**1**)	A	17	22	25
	B	35	11	9
Tyr-Gly-Gly-Phe-Met (**2**)	A	15	23	29
	B	35	12	10
Tyr-Gly-Gly-Phe (**3**)	A	7	12	17
Tyr-Gly-Gly (**4**)	A	15	26	31

[a] The relative amounts of the glycation products in the reaction mixtures were determined by RP HPLC.

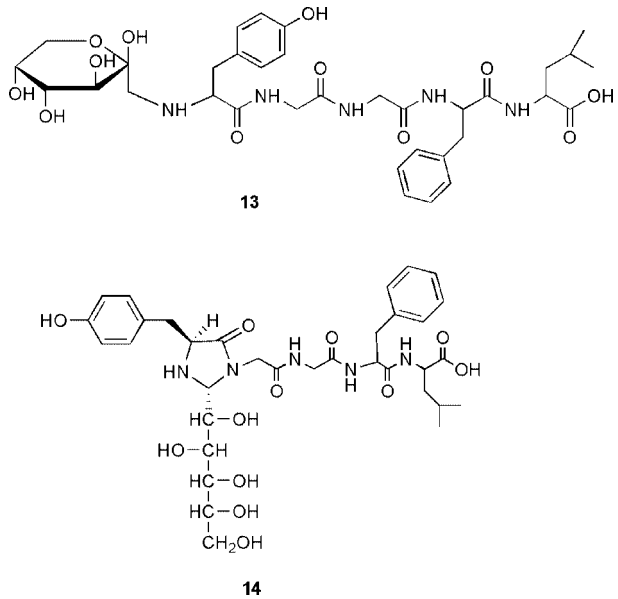

Figure 2 Glycation products derived from Leu-enkephalin and D-glucose.

to separate the Heyns and Amadori compounds by RP HPLC failed. To avoid the formation of Amadori compounds in the reactions of peptides **3** or **4** with fructose, only Method A was applied.

While it is known that Heyns compounds with *gluco* or *manno* sugar configuration can be expected as products of glycation by fructose [12,18–20], the formation of imidazolidinone derivatives from ketoses was not previously observed. However, the parallel formation of Amadori compounds and the corresponding imidazolidinones was recently observed in the reaction of Leu-enkephalin (**1**) with various aldoses [21]. It is understood that similar imidazolidinone rings could not be formed after addition of a sugar aldehyde or ketone to the ε-amino group of lysine residues in proteins. Even though there is no evidence yet of sugar-derived imidazolidinone compounds formation at the α-amino group at the *N*-terminus of proteins, imidazolidinone adduct was detected at the *N*-terminus of insulin after incubation with simple aldehyde compound [22]. While the formation of Amadori compounds in the reaction with

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aldoses does not require base or acid initiation, a base is necessary for the formation of Heyns compounds and imidazolidinones in the reaction of keto-sugars (such as fructose) with Leu- (**1**) and Met-enkephalin (**2**). The proposed mechanism for the formation of fructose-derived imidazolidinones and Heyns compounds is presented in Figure 3. Glycation of the peptide starts with nucleophilic attack of the terminal (tyrosine) amino group free nitrogen electronic pair at C-2 (carbonyl) of fructose in its acyclic form. Dehydration of the tetrahedral intermediate produces a Schiff base, which is susceptible to further chemical rearrangements. Its protonation leads to an intermediate, which via keto-enol tautomerization affords a mixture of *N*-alkylated glucosamine and *N*-alkylated mannosamine (Heyns compounds). Alternatively, the Schiff base may undergo nucleophilic

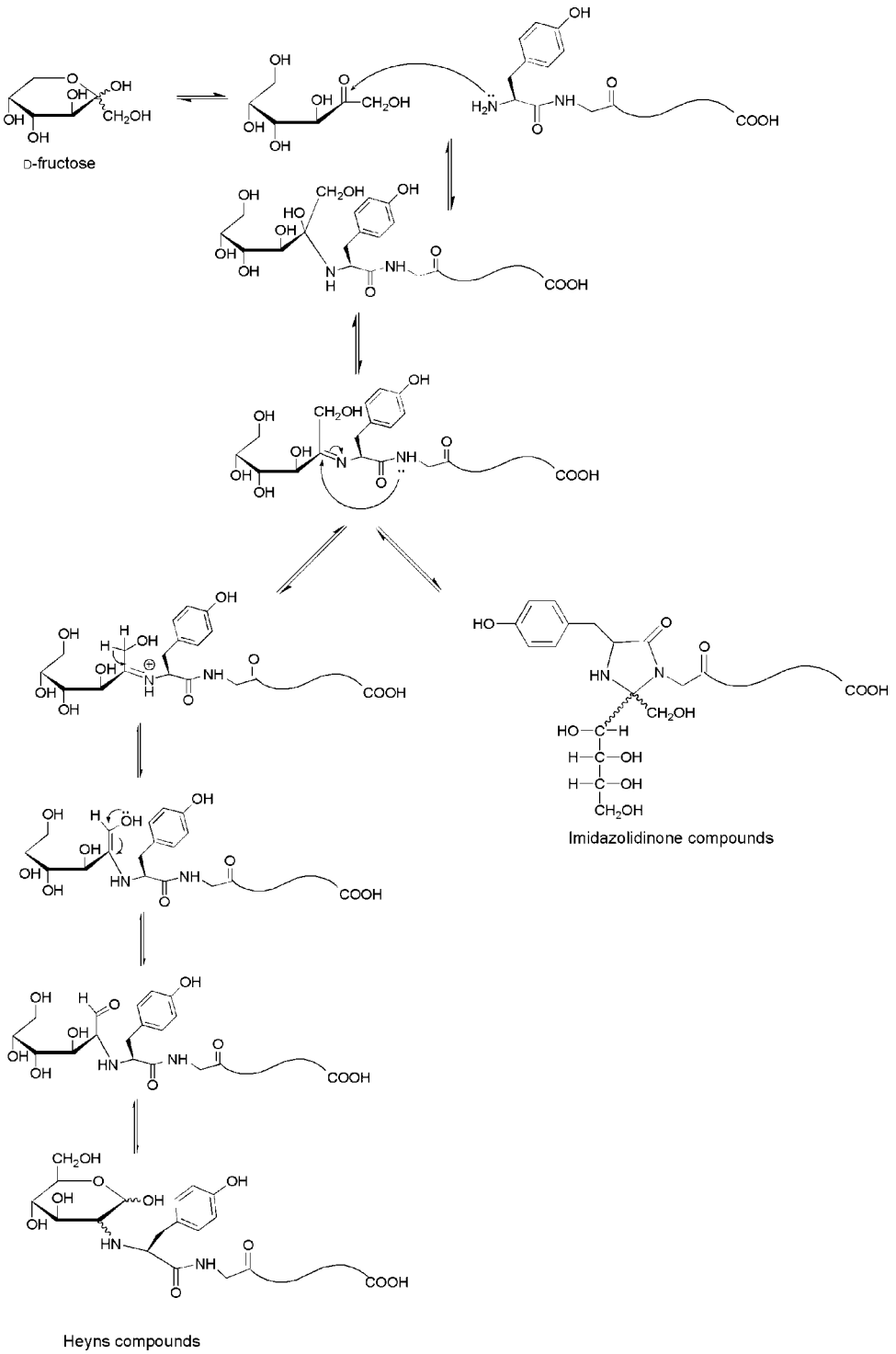

Figure 3 Pathways for chemical transformation of peptides **1**–**4** in the presence of D-fructose.

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attack by the Gly^2 amide nitrogen, which results in the formation of an imidazolidinone ring containing a new chiral center at its 2-position. The nucleophilicity of the Gly^2 amide nitrogen should be increased through formation of a –CO–NH–base complex in the presence of an organic base NEM, leading preferentially to imidazolidinone formation. An inorganic base KOH cannot form this kind of complex and thus only accelerates the keto-enol tautomerization, which leads to the formation of Heyns compounds. The latter were isolated as inseparable mixtures of *gluco* and *manno* derivatives **5a**/**5b** and **6a**/**6b**. The structure and the population of the isomers present in the mixtures of Heyns compounds were determined by NMR analysis (Tables 2 and 3). The *gluco* derivatives were the major products (74–80%) in the isolated Heyns compounds of Leu- and Met-enkephalin, as can be seen from data presented in Table 3. The estimated equilibrium composition of the tautomeric forms in aqueous solution reveals that the *gluco* compound in α-pyranose form is the major tautomer by far (~60%) in the mixture of Heyns compounds **5** and **6**. NMR data obtained for DMSO solution indicated almost equal distribution between the detected forms of compounds **5** and **6**. Only the α-pyranose form was detected in D_2O and in DMSO-d_6 solutions at equilibrium for *manno* derivatives **5b** and **6b** (Table 3).

On the basis of NMR analysis the second main products isolated from the reaction of fructose with peptides **1** and **2** were identified as diastereoisomeric imidazolidinone compounds **9** and **10**. Diastereoisomers **9a** and **9b**, as well as **10a** and **10b** were separated by RP HPLC. Table 4 shows ^1H and ^{13}C NMR chemical shifts for imidazolidinones **9a, 9b, 10a** and **10b**. The formation of imidazolidinone ring results in an absence of the Gly^2 amide proton in ^1H spectra of compounds **9** and **10**. Another consequence of the imidazolidinone ring formation is downfield shift by approximately 8 ppm of the respective ^{13}C resonance of the C-α of tyrosine residue, which becomes a part of imidazolidinone ring (C-4). In ^{13}C NMR spectra of compounds **9** and **10,** the signals at ~83 ppm were observed and assigned to the quaternary C-2 of the imidazolidinone moiety, originating from the C-2 atom of the sugar moiety. Depending on the configuration of this new asymmetric center, a difference in the C-2 chemical shifts of **9a** and **9b**, as well as **10a** and **10b** was observed, amounting to

Table 2 NMR chemical shift data (ppm) of the major component (2-deoxy-α-D-glucopyranos-2-yl derivative) in the mixture of Heyns compounds derived from Leu- and Met-enkephalin[a]

Residue	Atom	**5a** (α-p)		**6a** (α-p)	
		δ_H	δ_C	δ_H	δ_C
Glc	1	5.42	89.39	5.24	88.97
	2	3.14	60.43	3.56	60.43
	3	4.04	70.38	3.85	70.01
	4	3.39	69.99	3.42	69.46
	5	3.83	71.33	3.70	70.82
	6	3.06/3.26	60.43	3.62	60.22
Tyr	α	4.52	62.52	4.10	62.01
	β	3.06/3.95	35.88	2.84/2.96	35.49
	γ		125.75		125.44
	δ	7.12	130.89	6.96	130.39
	ε	6.83	115.89	6.66	115.24
	ζ		155.23		154.65
Gly^2	α	3.65/3.90	42.21	3.64	41.71
Gly^3	α	3.65/3.90	42.46	3.64	41.92
Phe	α	4.60	54.83	4.48	54.58
	β	2.97/3.13	37.08	2.84/2.96	36.47
	γ		136.43		135.95
	δ	7.32	129.33	7.08	128.79
	ε	7.23	128.81	7.18	128.31
	ζ	7.27	127.23	7.12	126.72
Leu/Met	α	4.24	59.91	4.10	53.41
	β	1.53	40.32	1.76	30.60
	γ	1.53	24.50	2.25/2.29	29.02
	δ/S-Me	0.82/ 0.86	20.93/ 22.37	1.88	13.67

[a] In D_2O at RT.

Table 3 Chemical shifts of anomeric atoms (C-1, H-1) and tautomeric composition of the Heyns compounds **5** and **6** in D_2O and DMSO-d_6 solutions estimated from the NMR data

Compound[a]	Sugar type	D_2O			DMSO-d_6		
		δ_{C-1} (ppm)	δ_{H-1} (ppm)[b]	%	δ_{C-1} (ppm)	δ_{H-1} (ppm)	%
5a α-p	*Gluco*	89.39	5.42 (2.8)	61	91.18	4.88	29
5a β-p	*Gluco*	94.33	4.80 (8.5)	19	97.48	4.28	38
5b α-p	*Manno*	90.43	5.27 (1.4)	20	91.83	4.49	33
6a α-p	*Gluco*	88.97	5.24 (3.6)	56	91.18	4.95	29
6a β-p	*Gluco*	94.57	4.98 (8.4)	18	97.45	4.32	32
6b α-p	*Manno*	90.41	5.02 (1.3)	26	91.83	4.58	39

[a] α-p, α-anomer in pyranose form; β-p, β-anomer in pyranose form.
[b] Values for the $J_{1,2}$ constants in Hz are given in parenthesis.

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~1.5 ppm. Considering the relative amounts of imidazolidinone diastereoisomers obtained from peptides **1** and **2** (Table 1), it can be assumed that ring closure occurred with almost equal facility to either *Re* or *Si* face of the initially formed Schiff base (Figure 3).

NMR spectroscopic data, retention times on RP HPLC and optical rotations of the diastereoisomeric imidazolidinones **9**–**12** were examined to find general trends that could be used to determine the relative configuration of the C-substituents on the imidazolidinone ring, i.e. to distinguish the isomers in which the D-*arabino*-tetritolyl and hydroxybenzyl groups point in opposite (2*R**, 4*S**- or *trans*-isomers) or same (2*S**, 4*S**- or *cis*-isomers) direction with respect to the plane defined by the heterocyclic ring system (Figure 1). In all imidazolidinone isomers with shorter retention times, the ^{13}C NMR shifts of the imidazolidinone C-2 atom and of the attached hydroxymethyl carbon atom were more upfield than that in the corresponding isomers with longer retention times (see Materials and Methods and Table 4). For example, C-2 in **12a** and **12b** appeared at 83.75 and 85.22 ppm, while the corresponding CH_2OH resonances were observed at 59.63 and 61.09 ppm.

The observed NOE interaction between the imidazolidinone H-4 and the tetritol H-2′ in the **12** isomer with the shorter retention time suggests *trans*-configuration for the two bulky C-substituents at the imidazolidinone ring (Figure 4(a)). If free rotation about (C-1′)–(C-2′) bond is allowed after DFT calculations on optimized geometries of **12a** and **12b**, one can follow the corresponding change in H-4-H-2′ internuclear distance. Thus, in *cis*-isomer (**12b**) the minimal H-4-H-2′ distance was ~4.1Å, while in *trans*-isomer (**12a**) it was ~2.3Å. On the basis of these findings and NOESY spectra, one can assign the isomer with shorter retention time, for which an NOE contact between H-4 and H-2′ was observed, as *trans*-isomer (**12a**). On the other hand, the observed H-d-H-1′, H-2′, H-4′ and H-c-H-3′, H-4′ NOE correlations (Figure 4(b)) in the isomer with the longer retention time are in agreement with *cis* arrangement of the two bulky substituents at the heterocyclic ring moiety (**12b**). Attempts to observe corresponding NOE interactions in the diastereoisomers of compounds **9**–**11** failed due to severe peak overlapping. However, the observed upfield shifts of the CH_2OH carbons in all imidazolidinone isomers first eluted from the RP HPLC column are in accordance

Table 4 NMR chemical shift data (ppm) of the fructose-derived imidazolidinone compounds of Leu- (**9a, 9b**) and Met-enkephalin (**10a, 10b**)[a,b]

Residue	Atom	**9a**		**9b**		**10a**		**10b**	
		δ_H	δ_C	δ_H	δ_C	δ_H	δ_C	δ_H	δ_C
Imidazolidinone ring	C-2		82.58		83.95		82.60		84.06
	CH-4	3.62	58.46	3.64	60.05	3.61	58.46	3.61	60.07
p-Hydroxybenzyl	a/a′	2.60/2.94	37.22	2.67/2.86	37.21	2.63/2.94	37.15	2.66/2.86	37.63
	b		127.98		129.26		127.87		129.28
	c	7.02	130.23	7.03	129.83	7.01	130.04	7.01	129.85
	d	6.66	115.09	6.65	114.99	6.64	115.09	6.63	115.01
	e		155.82		155.58		155.89		155.59
	f/f′	3.93/4.01	43.83	3.84/4.10	43.42	3.92/4.01	43.85	3.88/3.94	43.20
Hydroxymethyl	CH_2	3.45/3.63	60.56	3.43/3.56	63.18	3.45/3.80	58.46	3.44/3.52	63.16
Sugar moiety	1′	3.81	69.79	3.82	65.08	3.82	69.20	3.77	65.31
	2′	3.34	69.25	3.50	70.11		69.85	3.44	71.23
	3′	3.34	71.22	3.50	71.25		71.12	3.49	70.39
	4′/4″	3.38/3.54	63.14	3.40	63.18	3.54	63.13	3.60	63.20
Gly	α/α′	3.58	42.64	3.57/3,60	41.99	3.58/3.80	41.95	3.62/3.82	41.95
Phe	α	4.55	53.86	4.49	54.39	4.50	54.07	3.53	53.99
	β/β′	2.78/3.03	37.40	2.80/3.06	38.08	2.80/3.05	37.30	2.78/3.05	37.89
	γ		137.85		137.91		137.87		137.81
	δ	7.26	129.19	7.29	129.09	7.35	129.17	7.19	129.16
	ε	7.25	128.01	7.24	128.00	7.29	128.06	7.26	128.05
	ζ	7.18	126.21	7.18	126.17	7.19	126.24	7.22	126.25
Leu/Met	α	4.19	50.61	4.10	50.38	4.21	51.59	4.26	51.33
	β/β′	1.54	40.19	1.46/1.51	40.04	1.86/1.99	31.06	1.87/1.99	29.69
	γ	1.64	24.27	1.63	24.22	2.44	29.67	2.46/2.58	28.98
	δ/δ′/S-Me	0.85/0.90	21.50	0.80/0.85	21.61/22.95	2.03	14.58	2.02	14.57

[a] In DMSO-d_6 at RT.
[b] The enumeration of the individual atoms is given in Figure 1.

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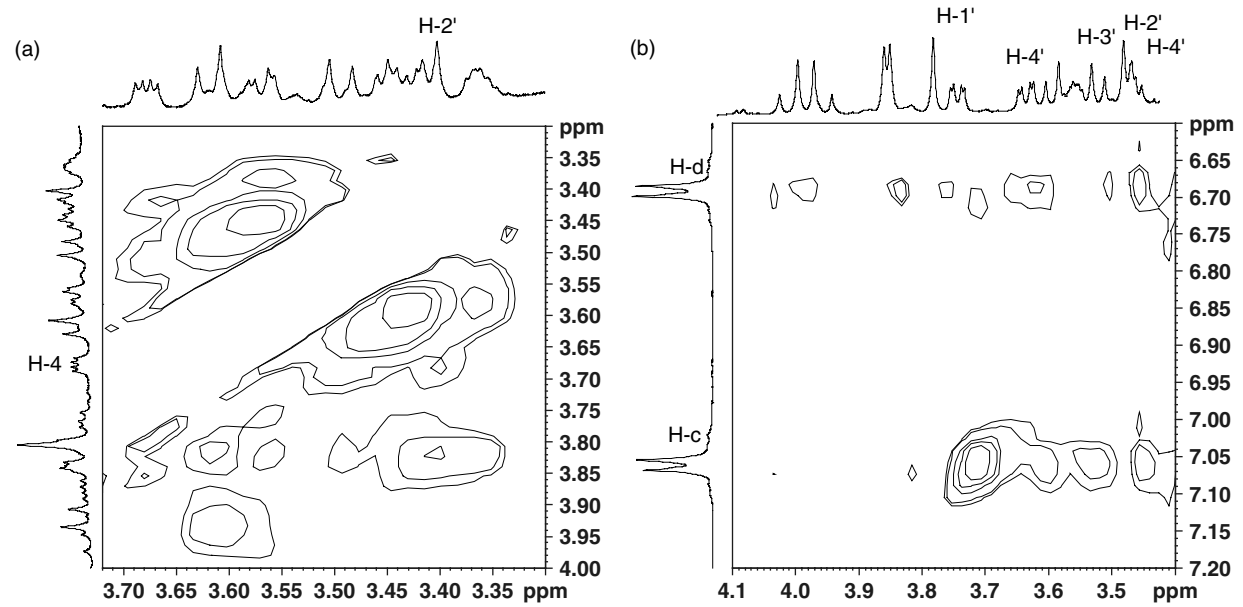

Figure 4 Part of the NOESY spectra of imidazolidinones **12a** (a) and **12b** (b).

with arrangements bringing the hydroxybenzyl and CH_2OH substituents into close proximity, while the tetritol moiety is located on the opposite side. Therefore, based on the above observations and following the findings from the literature on similar imidazolidinone compounds derived from aldose sugars [23,24], the imidazolidinone isomers with shorter retention times were anticipated as 2*R**, 4*S**-diastereoisomers (**9a**–**12a**), and compounds with longer retention times as 2*S**, 4*S**-isomers (**9b**–**12b**).

The stability of Heyns compounds **5** and **6**, and of imidazolidinones **9a, 9b, 10a** and **10b**, was investigated at 37 °C in 0.05 M PBS (pH 7.4) and in human serum (Table 5). The Heyns compounds derived from Leu- or Met-enkephalin were very stable in PBS, with 60–70% of the starting compound being present in solution after 4 weeks of incubation. Even though the parent 2-amino-2-deoxyaldoses are very unstable in neutral aqueous solution, it appears that *N*-peptidyl glucosamine and mannosamine glycation products are greatly stabilized toward autoxidative rearrangements and degradation. Interestingly, under identical conditions, Amadori product **13** (Figure 2), derived from glucose and Leu-enkephalin, undergoes faster degradation [25,26] through 1,2- or 2,3-enolization reactions (Table 5). Surprisingly, the 2*R**, 4*S**-isomers of imidazolidinone compounds of Leu- (**9a**) and Met-enkephalin (**10a**) are less stable than the

Table 5 The half-lives of hydrolysis of glycation products in PBS and human serum at 37 °C

Compound	Original sugar	Peptide	Structure of the compound	$t_{1/2}$	
				PBS	human serum
1		YGGFL	Peptide	Stable	14.8 min
5	Fru	YGGFL	Heyns	—[a]	13.5 h
6	Fru	YGGFM	Heyns	—[b]	4.7 h
9a	Fru	YGGFL	Imidazolidinone	12.8 days	1.8 days
9b	Fru	YGGFL	Imidazolidinone	—[a]	5.9 days
10a	Fru	YGGFM	Imidazolidinone	14.7 days	1.8 days
10b	Fru	YGGFM	Imidazolidinone	—[b]	3.0 days
13[c]	Glc	YGGFL	Amadori	8.7 days	14.0 h
14[d]	Glc	YGGFL	Imidazolidinone	20.5 days	6.5 days

[a] 70% of starting compound still present after 27 days.
[b] 60% of starting compound still present after 27 days.
[c] *N*-(1-deoxy-D-fructos-1-yl)-L-tyrosylglycylglycyl-L-phenylalanyl-L-leucine (data taken from Refs. 25,26).
[d] (2*R*, 4*S*)-*N*-{[2-(D-*gluco*-pentitol-1-yl)-4-(4-hydroxybenzyl)-5-oxoimidazolidin-1-yl]acetyl}glycyl-L-phenylalanyl-L-leucine (data taken from Ref. 21).

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corresponding 2*S**, 4*S**-isomers (**9b** and **10b**) having bulky benzyl and the tetritol moieties on the same side of the ring.

In human serum, all glycated products derived from either Leu- or Met-enkephalin were more stable than the native peptides. In general, the most susceptible to enzymatic degradation among the glycated products were Heyns compounds (especially **6**, Table 5). The degradation of Leu- and Met-enkephalins in human serum is primarily mediated by aminopeptidases. These enzymes cleave Leu- and Met-enkephalin at the Tyr^1-Gly^2 bond. It was already reported that Amadori compound **13** [25] and Leu-enkephalin-derived imidazolidinone compound **14** [21] (Figure 2) are stable against degradation mediated by aminopeptidases. It was found that both compounds were degraded preferably by dipeptidylcarboxypeptidase, which cleaves the Gly^3-Phe^4 bond. Imidazolidinone **14** was also a substrate of carboxypeptidase, which targets the Phe^4–Leu^5 bond.

Similarly, the Heyns compounds of Leu- (**5**) and Met-enkephalin (**6**) were degraded in human serum by dipeptidylcarboxypeptidase to the same product – the Heyns compound of tripeptide **8**. The estimated half-life for Heyns compound **5** in human serum was almost the same as that found for Amadori compound **13**.

The 2*S** isomers of the imidazolidinone compounds (**9b** and **10b**) showed higher stability, in human serum, than the 2*R** isomers (**9a, 10a**), presumably as a result of steric hindrance in *cis*-isomers (Table 5). Leu-enkephalin derivative **9a** is approximately 3 times less stable than imidazolidinone compound **14**, indicating that the glucose-derived imidazolidinone is more stable than its fructose-derived analog. The main degradation product, as a result of the dipeptidylcarboxypeptidase activity on imidazolidinone compounds **9** and **10**, was imidazolidinone compound **12** having 2*R** (**12a**) or 2*S** (**12b**) configuration, depending on the starting isomer. In addition to metabolite **12**, as products of carboxypeptidase activity, imidazolidinones **11a** or **11b** were also found in the incubation mixtures of the imidazolidinone compounds of Leu-enkephalin **9**.

CONCLUSIONS

Heyns and imidazolidinone compounds were simultaneously formed from fructose and several bioactive peptides, presenting first model compounds for ketose-derived glycation products of peptides. Whether the findings of imidazolidinone compounds formation reached in our model studies in any way represent reality is open to question. NMR analysis was used to identify compounds formed and to determine tautomeric composition of the Heyns compounds. DFT calculations combined with NMR analysis were performed to distinguish the two imidazolidinone isomers formed in the glycation reaction.

The stability of the Heyns and imidazolidinone compounds derived from Leu- and Met-enkephalin was evaluated in phosphate buffer and human serum at 37 °C. Significant differences in stability between both groups of glycation products were observed, Heyns compounds being more stable in buffer whereas imidazolidinones were more stable toward proteolytic cleavage in serum. The observed higher stability of *cis*- compared to *trans*-isomers of diastereomeric imidazolidinones is probably the result of subtle unfavorable steric hindrance present in one configuration (2*S**-isomers) but not in the other (2*R**-isomers).

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REFERENCES

1. Gerrard JA. New Aspects of an AGEing chemistry – Recent developments concerning the Maillard reaction. *Aust. J. Chem.* 2002; **55**: 299–310.
2. Ledl F, Schleicher E. New aspects of the Maillard reaction in foods and in the human body. *Angew. Chem., Int. Ed. Engl.* 1990; **29**: 565–594.
3. Horvat Š, Jakas A. Peptide and amino acid glycation: New insights into the Maillard reaction. *J. Pept. Sci.* 2004; **10**: 119–137.
4. Ahmed N. Advanced glycation endproducts – role in pathology of diabetic complications. *Diabetes Res. Clin. Pract.* 2005; **67**: 3–21.
5. Brownlee M. The pathobiology of diabetic complications: a unifying mechanism. *Diabetes* 2005; **54**: 1615–1625.
6. Vlassara H, Palace MR. Diabetes and advanced glycation endproducts. *J. Int. Med.* 2002; **251**: 87–101.
7. Gaby AR. Adverse effects of dietary fructose. *Altern. Med. Rev.* 2005; **10**: 294–306.
8. Schalkwijk CG, Stehouwer CDA, van Hinsbergh VWM. Fructose-mediated non-enzymatic glycation: sweet coupling or bad modification. *Diabet. Metab. Res. Rev.* 2004; **20**: 369–382.
9. Hinton DJS, Ames JM. Site specificity of glycation and carboxymethylation of bovine serum albumin by fructose. *Amino Acids* 2006; **30**: 425–433.
10. Shipar MAH. Formation of the Heyns rearrangement products in dihydroxyacetone and glycine Maillard reaction: a computational study. *Food Chem.* 2006; **97**: 231–243.
11. Suarez G, Rajaram R, Oronsky AL, Gawinowicz MA. Nonenzymatic glycation of bovine serum albumin by fructose (fructation). Comparison with the Maillard reaction initiated by glucose. *J. Biol. Chem.* 1989; **264**: 3674–3679.
12. Heyns K, Paulsen H, Eichstedt R, Rolle M. Uber die Gewinnung von 2-Amino-aldosen durch Umlagerung von Ketosylaminen. *Chem. Ber.* 1957; **90**: 2039–2049.
13. Ahmed N, Furth AJ. Failure of common glycation assays to detect glycation by fructose. *Clin. Chem.* 1992; **38**: 1301–1303.
14. Horvat Š. Opioid peptides and their glycoconjugates: structure-activity relationships. *Curr. Med. Chem. CNS Agents* 2001; **1**: 133–154.

15. Reid RL, Yen SSC. β-Endorphin stimulates the secretion of insulin and glucagon in humans. *J. Clin. Endocrinol. Metab.* 1981; **52**: 592–594.
16. Fallucca F, Tonnarini G, Dibiase N, Dalessandro M, Negri M. Plasma Met-enkephalin levels in diabetic patients – influence of autonomic neuropathy. *Metab. Clin. Exp.* 1996; **45**: 1065–1068.
17. Frisch MJ, Trucks GW, Schlegel HB, Scuseria GE, Robb MA, Cheeseman JR, Montgomery JA Jr, Vreven T, Kudin KN, Burant JC, Millam JM, Iyengar SS, Tomasi J, Barone V, Mennucci B, Cossi M, Scalmani G, Rega N, Petersson GA, Nakatsuji H, Hada M, Ehara M, Toyota K, Fukuda R, Hasegawa J, Ishida M, Nakajima T, Honda Y, Kitao O, Nakai H, Klene M, Li X, Knox JE, Hratchian HP, Cross JB, Bakken V, Adamo C, Jaramillo J, Gomperts R, Stratmann RE, Yazyev O, Austin AJ, Cammi R, Pomelli C, Ochterski JW, Ayala PY, Morokuma K, Voth GA, Salvador P, Dannenberg JJ, Zakrzewski VG, Dapprich S, Daniels AD, Strain MC, Farkas O, Malick DK, Rabuck AD, Raghavachari K, Foresman JB, Ortiz JV, Cui Q, Baboul AG, Clifford S, Cioslowski J, Stefanov BB, Liu G, Liashenko A, Piskorz P, Komaromi I, Martin RL, Fox DJ, Keith T, Al-Laham MA, Peng CY, Nanayakkara A, Challacombe M, Gill PMW, Johnson B, Chen W, Wong MW, Gonzalez C, Pople JA. *Gaussian 03, Revision D.02*. Gaussian, Inc.: Wallingford, CT, 2004.
18. Mossine V, Barens CL, Glinsky GV, Feather MS. Molecular and crystal structure of *N*-(2-deoxy-D-aldohexos-2-yl)-glycine (Heyns compounds). *Carbohydr. Res.* 1996; **284**: 11–24.
19. Frolov A, Hoffmann P, Hoffmann R. Fragmentation behavior of glycated peptides derived from D-glucose, D-fructose and D-ribose in tandem mass spectrometry. *J. Mass Spectrom.* 2006; **41**: 1459–1469.
20. Ardestani A, Yazdanparast R. Cyperus rotundus suppresses AGE formation and protein oxidation in a model of fructose-mediated protein glycoxidation. *Int. J. Biol. Macromol.* 2007; **41**: 572–578.
21. Roščić M, Horvat Š. Transformations of bioactive peptides in the presence of sugars-Characterization and stability studies of the adducts generated via the Maillard reaction. *Bioorg. Med. Chem.* 2006; **14**: 4933–4943.
22. Metz B, Kersten GFA, Baart GJE, de Jong A, Meiring H, ten Hove J, van Steenbergen MJ, Hennink WE, Crommelin DJA, Jiskoot W. Identification of formaldehyde-induced modifications in proteins: reactions with insulin. *Bioconjugate Chem.* 2006; **17**: 815–822.
23. Varga-Defterdarović L, Vikić-Topić D, Horvat Š. Synthesis and characterization of mannose-related imidazolidinone formed by the intramolecular rearrangement of the mannopyranose ester of leucine-enkephalin. *J. Chem. Soc., Perkin Trans. 1* 1999; 2829–2834.
24. Roščić M, Eklund R, Nordmark E-L, Horvat Š, Widmalm G. Stereochemical assignment of diastereomeric imidazolidinone-ring-containing bicyclic sugar-peptide adducts: NMR spectroscopy and molecular calculations. *Eur. J. Org. Chem.* 2004; 4641–4647.
25. Jakas A, Horvat Š. The effect of glycation on the chemical and enzymatic stabilitiy of the endogenous opioid peptide, leucine-enkephalin, and related fragments. *Bioorg. Chem.* 2004; **32**: 516–526.
26. Jakas A, Horvat Š. Study of degradation pathways of Amadori compounds obtained by glycation of opioid pentapeptide and related smaller fragments: Stability, reactions, and spectroscopic properties. *Biopolymers* 2003; **69**: 421–431.