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Transformations of bioactive peptides in the presence of sugars—Characterization and stability studies of the adducts generated via the Maillard reaction

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Abstract—Glycation of biomolecules, such as proteins, peptide hormones, nucleic acids, and lipids, may be a major contributor to the pathological manifestations of aging and diabetes mellitus. These nonenzymatic reactions, also termed the Maillard reaction, alter the biological and chemical properties of biomolecules. In order to investigate the effect of various reducing sugars on the products formed from small bioactive peptides (Tyr-Gly-Gly-Phe-Leu, Tyr-Gly-Gly-Phe-Leu-NH_2, Tyr-Gly-Gly-Phe-Leu-OMe, Tyr-Gly-Gly-Phe, and Tyr-Gly-Gly), model systems were prepared with glucose, mannose or galactose. Peptide-sugar mixtures were incubated at 37 or 50 °C in phosphate-buffered saline, pH 7.4, or in methanol. The extent of glycation was determined periodically by RP HPLC. All sugar–peptide mixtures generated two different types of glycation products: *N*-(1-deoxy-ketos-1-yl)-peptide (Amadori compound) and the imidazolidinone compound substituted by sugar pentitol and peptide residue. The amount and distribution of peptide glycation products depended on the structure of the reactants, and increased in both concentration- and time-dependent manner in relation to exposure to sugar. Additionally, the rate of hydrolysis of glucose-derived imidazolidinone compounds, obtained either from leucine–enkephalin (**1**) or its shorter N-terminal fragments **2** and **3**, was determined by incubation at 37 °C in human serum. These results revealed that imidazolidinones obtained from glucose and small peptides are almost completely protected from the action of enzymes in serum, the predominant route of degradation being spontaneous hydrolysis to initial sugar and peptide compound.
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1. Introduction

Increasing evidence in both experimental and clinical studies suggests that nonenzymatic glycation (the Maillard reaction) plays a major role in the pathogenesis of diabetes mellitus. Reaction of glucose with proteins or endogenous regulatory peptides results in Amadori product formation followed by degradation to advanced glycation end products (AGEs), which are involved in the genesis of many of the irreversible complications of diabetes, including expanded extracellular matrix, cellular hypertrophy, hyperplasia, and vascular complications.[1] In addition, a growing body of data supports the role of insulin glycation as a feature of β-cell dysfunction and insulin resistance.[2] The degree of hyperglycemia also appears to affect glucagon-like peptide-1(7–36)amide (tGLP-1), with 24–71% of tGLP-1 found in the glycated form in the intestines of the diabetic model.[3] The altered bioactivity of glycated functional peptides is partly due to their resistance to in vivo degradation, but effects on receptor binding may also be involved, as suggested by observations with glucose-dependent insulinotropic polypeptide (GIP).[4] The findings that glycation of insulin and tGLP-1 occurs naturally in a glucose-rich environment suggest the potential value of further studies on bioactive peptides to assess the physiological significance of their glycated products.

Endogenous opioid peptides, such as enkephalins, participate in the regulation of a variety of physiological and behavioral functions.[5] It has also been suggested that opioids may participate in the effect of stress on the pancreatic islets and insulin release.[6] Studies concerned with circulating opioid peptide levels in diabetes have shown that patients have significantly lower β-endorphin[7] and methionine–enkephalin[8] levels, a situation which contributes to a worsening of metabolic control under stress conditions.

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The aim of the present investigation was to examine susceptibility of small bioactive peptides, leucine–enkephalin (LE) (H-Tyr-Gly-Gly-Phe-Leu-OH), and structurally related bioactive fragments, leucine–enkephalin amide, leucine–enkephalin methyl ester, H-Tyr-Gly-Gly-Phe-OH, and H-Tyr-Gly-Gly-OH, to glycation reaction after being exposed to different reducing sugars. Although glucose is assumed to be the major source of glycation based on its abundance and association with diabetic complications, the evaluation of the reactivity of other reducing sugars in the Maillard reaction, such as galactose or mannose, is interesting. Galactose is an essential sugar found in abundance in the everyday's diet, especially in dairy products, whereas oral mannose is increasingly used for treatment of bladder infection and correction of different disorders.[9]

In a first set of experiments, we analyze the glycation products formed from small bioactive peptides in the presence of glucose, mannose or galactose and propose new pathways of glycation in the Maillard reaction. In a second step, information about the chemical stability and metabolic fate of glucose-derived imidazolidinone-ring-containing products in human serum is provided.

2. Results and discussion

2.1. Formation of hexose-derived glycation products from leucine–enkephalin and related fragments

Model systems containing leucine–enkephalin (**1**) and glucose, mannose or galactose were reacted in phosphate (0.05 M, pH 7.4)-buffered 0.1 M saline (PBS) or in methanol as detailed under Section 4. In all model systems, two different types of glycation products were generated. The products were isolated from the incubation mixtures under the conditions giving the best yields of the glycated peptides by using preparative RP HPLC. Mass determination afforded for all isolated products, irrespective of the starting sugar, $[M+H]^+$ ions at *m*/*z* 718 corresponding to the elemental composition $C_{34}H_{47}N_5O_{12}$, indicating that all carbons of the original sugar backbone are retained in N-glycated pentapeptide. NMR data unequivocally proved that Amadori compounds with either D-fructose (**6**; obtained from D-glucose or D-mannose) or D-tagatose (**17**; obtained from D-galactose) ketohexose moieties as well as the imidazolidinone-ring-containing compounds **7**, **16** or **18** are simultaneously formed from all three reducing hexoses and leucine–enkephalin (Fig. 1). The imidazolidinone compounds with D-*gluco* (**7**), D-*manno* (**16**) or D-*galacto* (**18**) sugar pentitol structures were obtained as diastereoisomers with *trans* or *cis* relative geometry of the carbon substituents at the imidazolidinone ring moiety.

In the classical view of the nonenzymatic glycation reaction (the Maillard reaction), the primarily formed Schiff base adduct (Fig. 2) yields, after protonation, the iminium cation. Deprotonation at C-2 results in the formation of a 1,2-enaminol which rearranges to an aminoketose or Amadori product. Herein, we propose a novel mechanism for the concomitant generation of hexose-derived imidazolidinones as glycation products from reducing sugars and peptides. In this pathway, the Schiff base undergoes, instead of Amadori rearrangement to the corresponding keto-sugar, attack by the nitrogen of the second amino acid residue (Gly in leucine–enkephalin) to yield the *trans*- and *cis*-isomers of imidazolidinone compounds in which C-1 of the sugar moiety forms a bridge between the amino group of the N-terminal tyrosine residue and the amide nitrogen of the next peptide bond.

The composition and yield of the corresponding Amadori and imidazolidinone products formed from the hexoses strongly depended upon the reducing sugar used, concentration of the reactants, reaction medium, and temperature. To evaluate the extent of glycation, 0.01 M leucine–enkephalin (**1**) was incubated under sterile conditions with glucose, mannose or galactose in PBS at pH 7.4 and 37 °C, initially containing either 0.15 M (Fig. 3A) or 1.50 M (Fig. 3B) of reducing sugar. Under these conditions, the amount of both glycated products slowly increased over the period studied and positively correlated with the concentration of the sugar in the incubation mixture. The yield of glycation products reached, after one month, 11%, 21%, and 20% in reaction mixtures containing 0.15 M glucose, mannose or galactose, respectively. The degree of glycation increased markedly with higher hexose concentration and reached, after two weeks, ~30% in all LE–hexose model systems investigated. As shown in Figure 3A, among the hexoses, mannose and galactose showed comparable reactivity, while glucose was the least reactive sugar. At higher concentrations of hexoses (Fig. 3B) the individual sugars showed similar tendencies to produce early glycation products. However, under these conditions, accelerated degradation of sugars into different reactive carbonyl compounds was observed resulting in a greatly diminished proportion of free LE in the incubation mixtures. The catalytic effect of phosphate ions on sugar degradation is documented in the literature of the Maillard chemistry.[10]

It is noteworthy that, among the generated glycation products, the Amadori compound was the major product of glycation of LE with glucose, while incubation with mannose or galactose resulted in a significant accumulation of the corresponding diastereoisomers of imidazolidinone compounds (Figs. 3A and B). Comparison with reference compounds, obtained by different synthetic procedures,[11] revealed that the major isomer formed in the equilibrated LE/glucose and LE/galactose model systems has the *trans* orientation of substituents attached to the imidazolidinone-ring moiety, amounting to 87% **7**-*trans* and 90% **18**-*trans*, respectively, in the obtained diastereoisomeric mixture. On the contrary, the LE/mannose system contained only 45% **16**-*trans*, indicating that the ring closure occurred with almost equal ease to either the *Re* or the *Si* face of the initially formed Schiff base.

In order to better understand the influence of the medium, the effect of methanol as the solvent was evaluated on the reactivity of sugars in the glycation reaction.

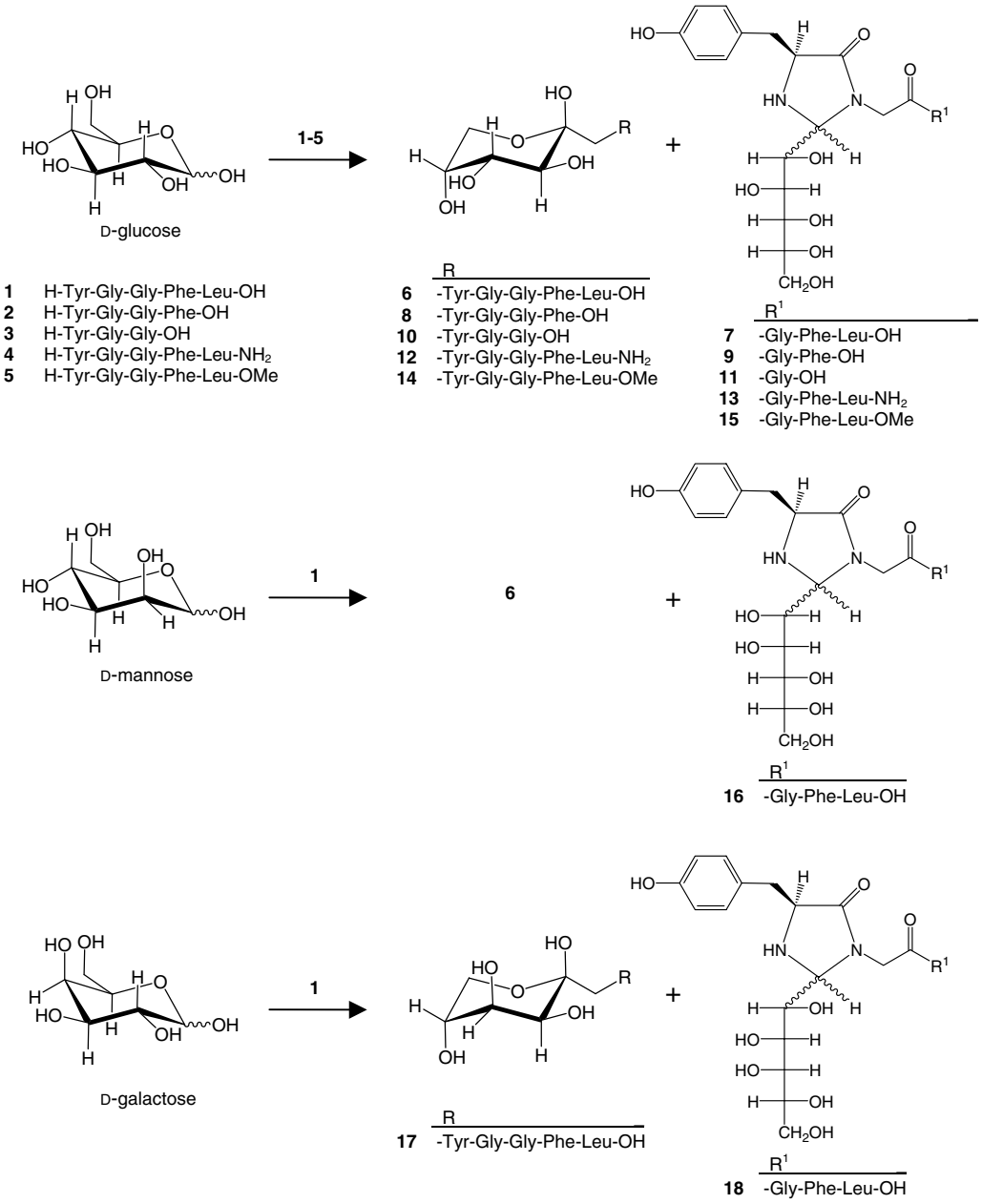

Figure 1. Glycation products derived from leucine–enkephalin (**1**), N-terminal tetrapeptide (**2**) and tripeptide (**3**) fragments as well as leucine–enkephalin amide (**4**) and methyl ester (**5**) in the presence of the reducing sugars, D-glucose, D-mannose or D-galactose.

Although there has not been unanimous agreement on a single mode of action by which alcohols modify the structure of peptides and proteins, it was assumed that alcohols act as strong hydrogen-bond donors at peptide carbonyl groups, inducing a twist in the peptide backbone.[12]

Figure 3C shows the relative amounts of glycation products obtained at 37 °C with different sugar species in the leucine–enkephalin (0.001 M)—sugar (0.015 M) system containing methanol as the solvent. Regardless of the monosaccharide used, the extent of glycation was more pronounced in methanolic than that in phosphate-buffered aqueous solution. It appears that, under these conditions, mannose was the most reactive among the sugars tested, whereas glucose and galactose showed almost equal reactivity, producing after six days of incubation, the corresponding Amadori compound (**6** or **17**) in 72%, 54%, and 51% yield, respectively. Evidence also shows that, in methanol, the formation of imidazolidinones was significantly inhibited when compared to aqueous model systems (Fig. 3A), even in reactions conducted at 50 °C (Fig. 3D).

It can be assumed that, in methanolic solution, sugar-ring opening takes place simultaneously with the proton transfer from the in situ formed $CH_3OH_2^+$ ion to the ring oxygen atom leading to higher amounts of the acyclic sugar form, which is required for the glycation reaction. Furthermore, the high Amadori product content suggests that reaction with leucine–enkephalin proceeds mainly through an acyclic immonium ion (Fig. 2, path

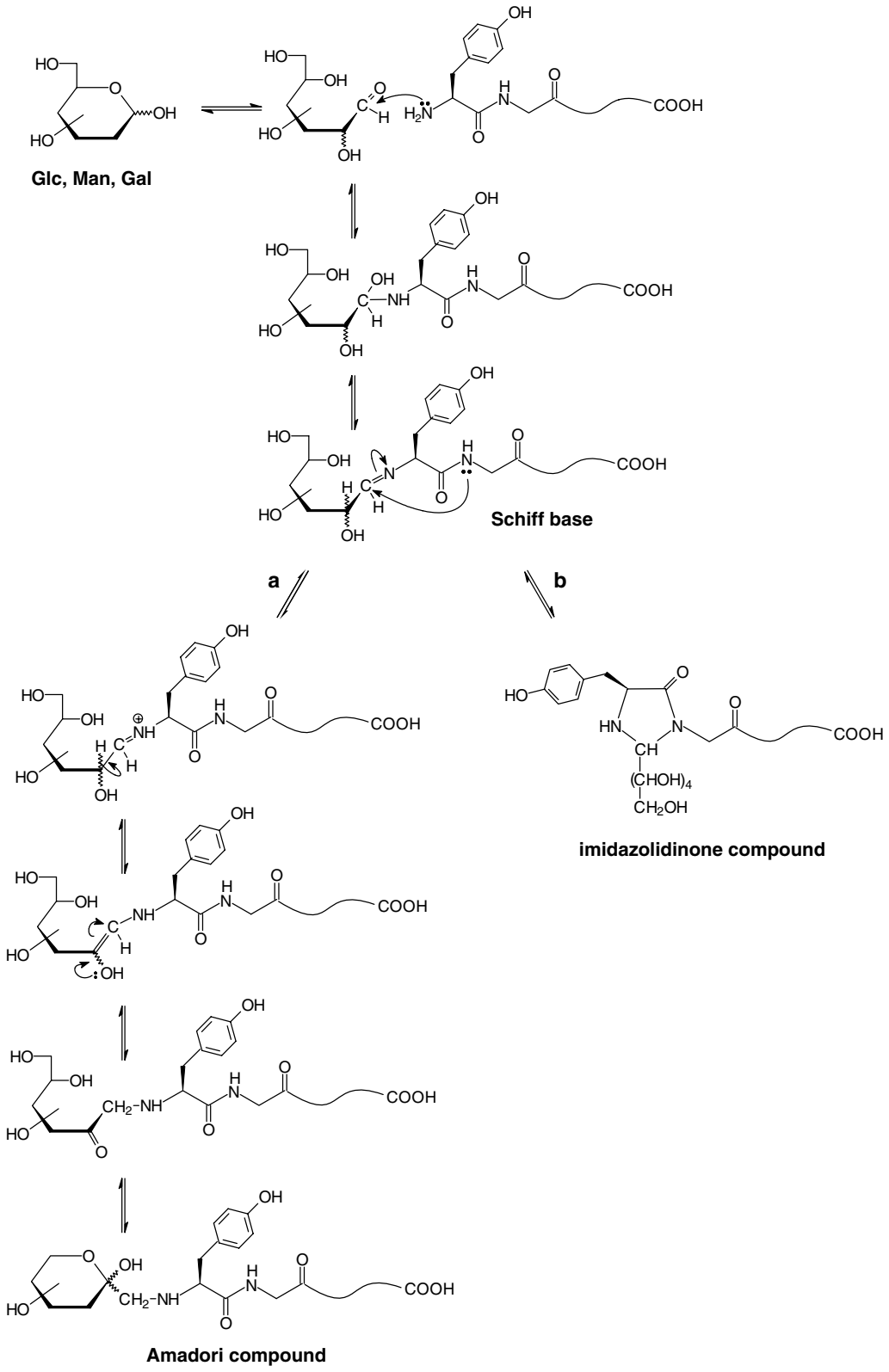

Figure 2. Pathways for chemical transformations of peptides **1**–**5** in the presence of reducing sugars.

a), found to be relatively stable in methanolic solution.[13] With respect to imidazolidinone compound formation, it is obvious that, in methanol, the equilibrium has been shifted in the direction of Schiff base and/or starting compounds. The reaction sequence presented in Figure 2 implies that imidazolidinone ring formation involves intramolecular attack of a neighboring amido group onto the adjacent Schiff base, suggesting that deprotonation of this peptide bond nitrogen is necessary for nucleophilic attack on the carbon atom of the Schiff

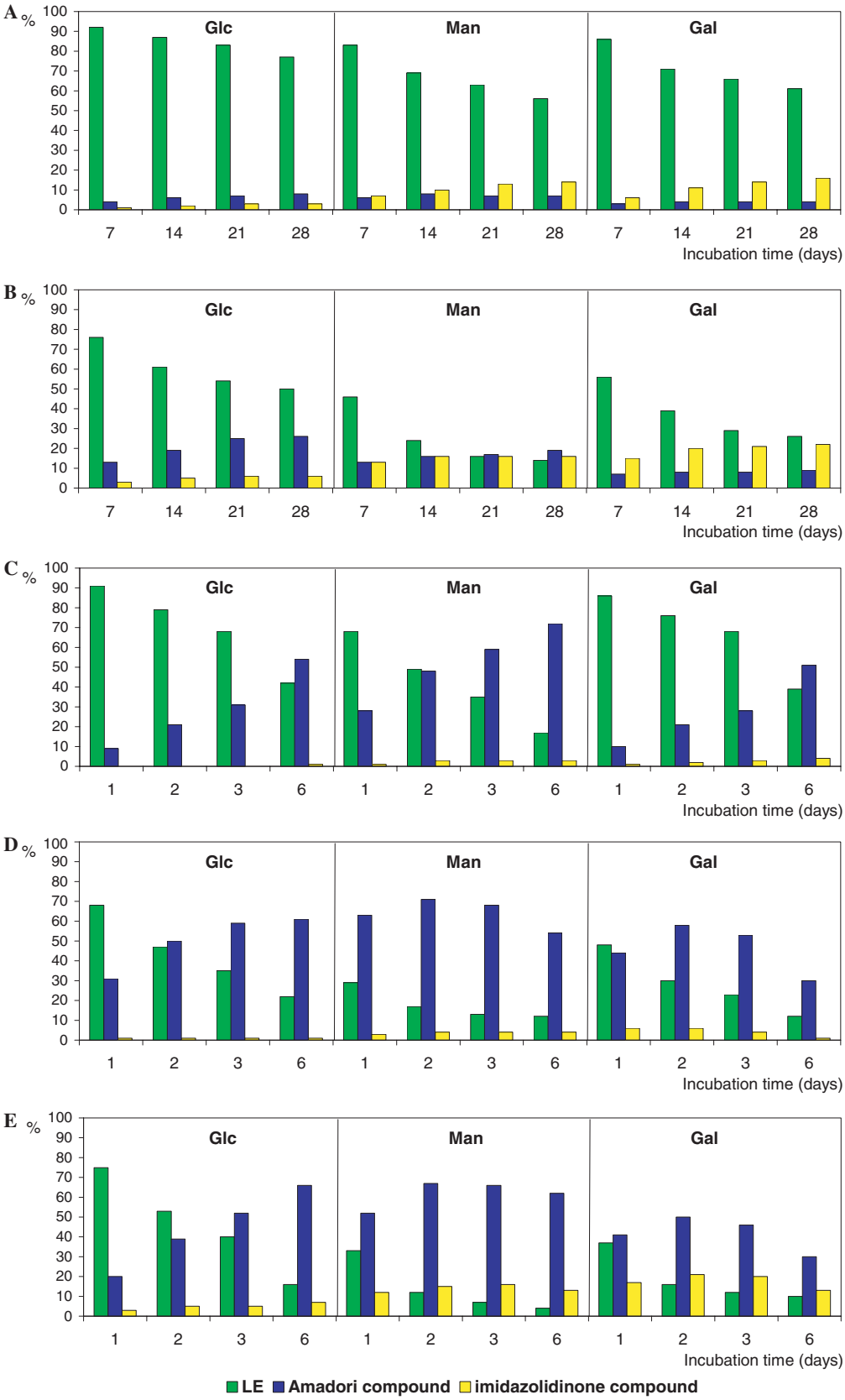

Figure 3. Effects of reducing sugars, glucose (Glc), mannose (Man), and galactose (Gal), on glycation product (Amadori and imidazolidinone compounds) formation from leucine–enkephalin (**1**) (LE). LE was incubated with the corresponding sugar either in phosphate-buffered saline, pH 7.4 (PBS), or in methanol (MeOH) in the absence or presence of *N*-ethylmorpholine (NEM). The molar ratio of the reactants and the reaction conditions were as follows: (A) LE:sugar = 1:15 (PBS, 37 °C); (B) LE:sugar = 1:150 (PBS, 37 °C); (C) LE:sugar = 1:15 (MeOH, 37 °C); (D) LE:sugar = 1:15 (MeOH, 50 °C); (E) LE:sugar:NEM = 1:15:5 (MeOH, 50 °C). The columns represent three independent sets of experiments. The concentration of glycated and nonglycated LE was measured by RP HPLC.

base. In this respect it was assumed that addition of a tertiary base to the reactants in methanol might increase the nucleophilicity of the attacking amide bond nitrogen by formation of a hydrogen-bonded complex between the amide and the base, and lead to enhanced rates of imidazolidinone product formation. To test this hypothesis, leucine–enkephalin was incubated with sugars in methanol at 50 °C in the presence of *N*-ethylmorpholine (NEM). The added base increased the content of imidazolidinone products almost three times, after six days at 50 °C (Glc → **7**, 7%; Man → **16**, 13%; Gal → **18**, 13%), while the amount of Amadori products remained unchanged (Fig. 3E).

To probe further the proposed mechanisms of glycation, leucine–enkephalin analogues or its fragments were evaluated in the reaction with glucose. These analogues were either the smaller N-terminal tetrapeptide (**2**, H-Tyr-Gly-Gly-Phe-OH) and tripeptide (**3**, H-Tyr-Gly-Gly-OH) fragments or they were the C-terminally protected amide (**4**, H-Tyr-Gly-Gly-Phe-Leu-NH_2) and methyl ester (**5**, H-Tyr-Gly-Gly-Phe-Leu-OMe) derivatives of leucine–enkephalin. The reactivity of peptides **2**–**5** and leucine–enkephalin (**1**) with glucose was compared at 50 °C in aqueous (PBS) and methanolic medium containing added base (NEM). As illustrated in Figure 4A, glycation of peptides **1**–**5** in PBS resulted in the parallel formation of the corresponding Amadori and imidazolidinone-type compounds. It should be noted that, in phosphate-buffered solution, the least reactive was tripeptide **3**, while amide **4** showed similar reactivity as leucine–enkephalin. Interestingly, however, amide derivative **4** afforded, after six days of incubation, the highest amount of imidazolidinone products as compared to the other peptides (e.g., **4** → **13**, 14%; **1** → **7**, 7%). Unexpectedly, and in contrast to the results obtained in PBS, exposure of peptides **1**–**5** to glucose in methanol–NEM medium resulted in highly different amounts of glycation products (Fig. 4B). The conversion varied (after six days at 50 °C) between 1% and 66% for the Amadori products and between 6% and 17% for the imidazolidinone products, depending upon the peptide. Pentapeptide **1** and tetrapeptide **2** showed comparable reactivity, yielding mainly Amadori products **6** (66%) and **8** (64%), whereas the corresponding imidazolidinones were formed in much lower yields (**1** → **7**, 7%; **2** → **9**, 6%). Under identical conditions, tripeptide **3** and carboxy-protected pentapeptide derivatives **4** and **5** gave only traces of the corresponding Amadori products and imidazolidinones in 10–17% yields. It is generally believed that the Amadori rearrangement is a complex acid–base catalyzed reaction in which the balance of the acidity and basicity in the reaction system controls the simultaneous and consecutive reactions.[14] Therefore, the shift of the reaction equilibrium toward Amadori product formation from

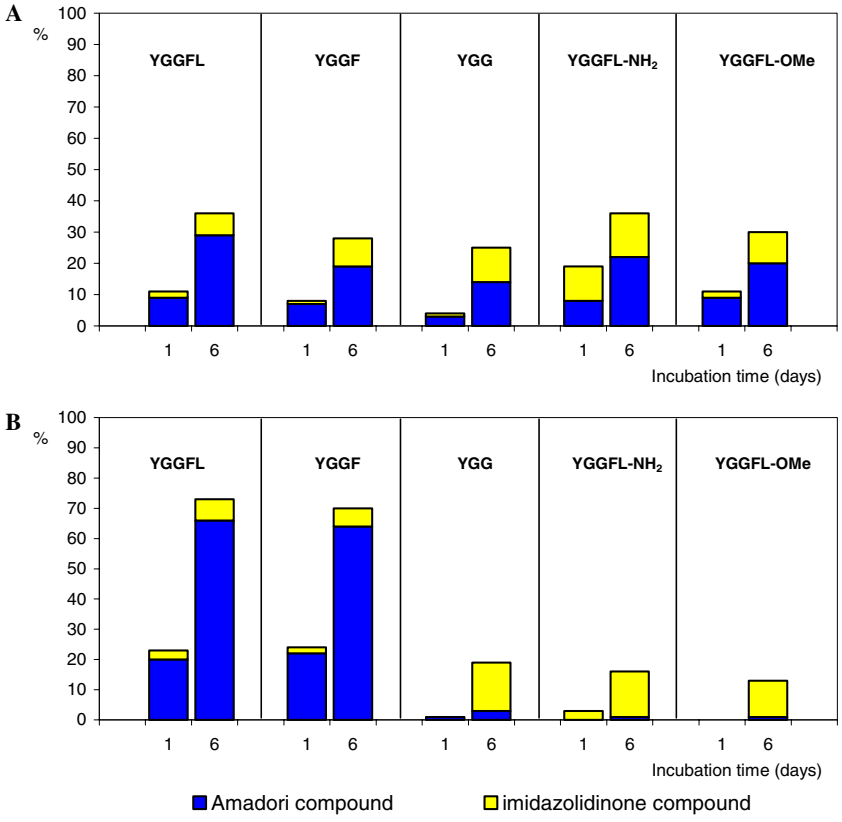

Figure 4. Comparison of the glycation product formation from YGGFL (**1**), YGGF (**2**), YGG (**3**), YGGFL-NH_2 (**4**), and YGGFL-OMe (**5**) in the presence of glucose under identical reaction conditions. The molar ratio of the reactants and reaction conditions were as follows: (A) peptide:glucose = 1:150 (PBS, 50 °C); (B) peptide:glucose:NEM = 1:15:5 (MeOH, 50 °C). The columns represent three independent sets of experiments. The concentration of glycated products was determined by RP HPLC.

peptides **1** and **2**, in methanol as the solvent, suggests intramolecular participation of the free carboxyl group in the rearrangement reaction by formation of a proton-transferring transition state. Consequently, the absence of Amadori product formation from peptides **4** and **5**, which lack a free carboxyl group, has been attributed to their inability for such cooperative interaction. However, if the catalytic mechanism involves participation of the peptide carboxyl group in Amadori rearrangement, then the model system containing H-Tyr-Gly-Gly-OH (**3**) should also lead to Amadori product formation. Unexpectedly, in the case of peptide **3** only 3% conversion to Amadori compound **10** was observed, whereas the other products formed were imidazolidinone **11** (16%) and diketopiperazine (12%), formed by cyclization of the N-terminal dipeptide fragment in **3**. Previous studies[15] have shown that heating of peptide **3** alone in methanol afforded diketopiperazine [*cyclo*-(Tyr-Gly)] as the major product (62%). It is worth noting that, because of the small size of the diketopiperazine ring, only peptides with the first peptide bond in the *cis* conformation can react. Taking these facts into account, it is rational to presume that, for conformational reasons, in the **3**/glucose model system, in methanol as the solvent, following Schiff base formation the Gly^2 nitrogen atom is adjacent to the –CH=N– unit thus increasing the probability of imidazolidinone ring closure and making the Amadori rearrangement less favorable.

These results imply that the reaction equilibrium toward the Amadori and imidazolidinone adduct depends upon the length, amino acid sequence, and possible C-terminal substituents in the peptide. Comparison of the amount and distribution of the compounds generated in sugar–peptide mixtures suggests that, in addition to the reactivity of individual sugar, also the solvent is an important criterion for the conversion of peptides to glycation products.

2.2. Stability of glucose-derived imidazolidinones in human serum and PBS

The kinetics as well as the mechanism of proteolytic degradation of glucose-derived imidazolidinone compounds **7**, **9**, and **11** (*trans*-isomers only), obtained either from leucine–enkephalin (**1**), or from the shorter N-terminal tetrapeptide **2** and tripeptide fragment **3**, were determined by incubation at 37 °C in 80% human serum. The degradation profiles are presented in Figure 5 and the half-life data are given in Table 1. The exact mechanism of degradation in serum was monitored via RP HPLC analysis of aliquots that allows unambiguous identification and precise quantification of the degradation products resulting from proteolysis of the imidazolidinone compounds. As shown in Figure 5A, the leucine–enkephalin-derived **7**-*trans* was degraded very slowly with a half-life of 6.5 days, while native peptide **1** was fully decomposed under identical conditions within 1 h. As a consequence of the imidazolidinonyl moiety at the N-terminus of the peptide, compound **7** is not susceptible to amino-terminal degradation by aminopeptidases and is more available for carboxypeptidase(s)

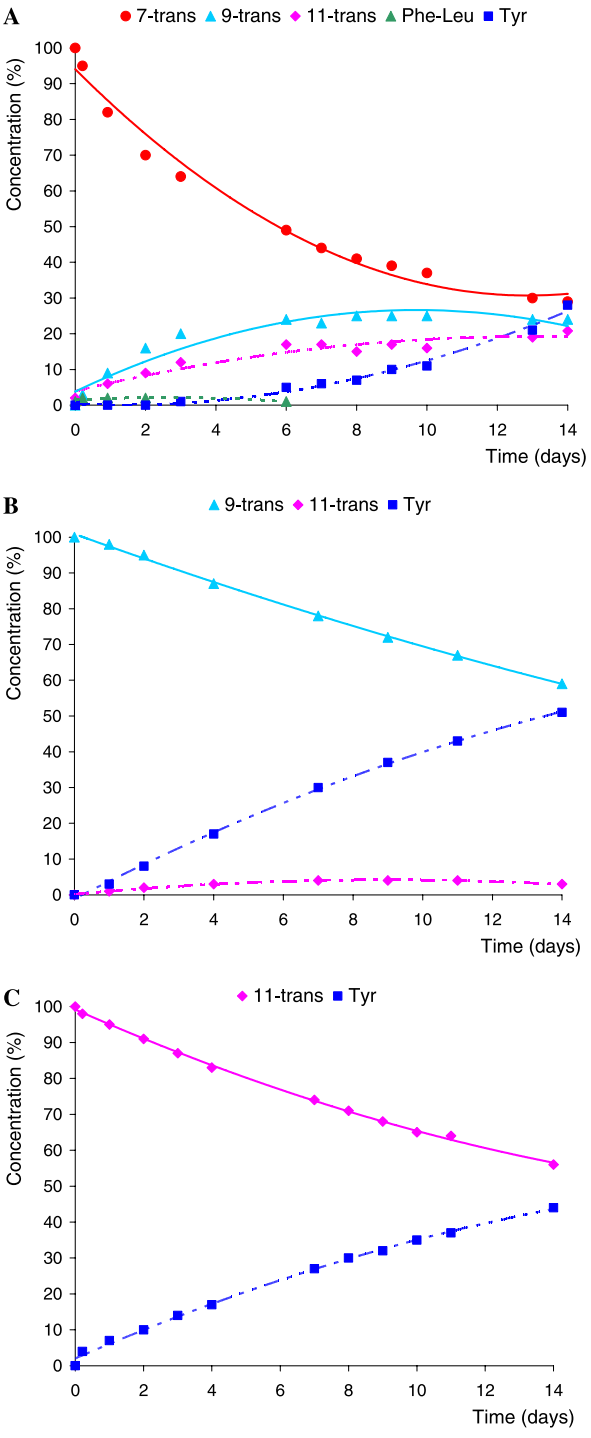

Figure 5. Time course of the change in the concentration of glucose-derived imidazolidinone compounds **7**-*trans* (A), **9**-*trans* (B), and **11**-*trans* (C), and their hydrolysis products, during incubation in 80% human serum at 37 °C. The initial concentration of imidazolidinone compounds **7**, **9** or **11** was 7×10^{-4} M. Samples were collected in triplicate, deproteinized with trifluoroacetic acid, and centrifuged. The supernatant was analyzed by RP HPLC.

activity. Thus, the degradation process of **7**-*trans* in human serum followed two main pathways involving dipeptidyl carboxypeptidase cleavage of the Gly-Phe peptide bond as well as carboxypeptidase hydrolysis of the Phe-Leu bond as shown by the simultaneous presence of two main imidazolidinone metabolites, **9**

Table 1. Comparison of half-lives of hydrolysis of *trans*-isomers of imidazolidinones **7**, **9** and **11** in human serum and in phosphate (0.05 M, pH 7.4) buffered saline (PBS) at 37 °C with enzymatic and chemical stability of leucine–enkephalin (**1**) and Amadori compounds **6** and **10**

Compound	Parent peptide	$t_{1/2}$	
		80% human serum	PBS
1	YGGFL (**1**)	14.8 min	Stable
7-*trans*	YGGFL (**1**)	6.5 days	20.5 days
9-*trans*	YGGF (**2**)	18.5 days	21.7 days
11-*trans*	YGG (**3**)	16.6 days	15.4 days
6[a]	YGGFL (**1**)	14.0 h	8.7 days
10[a]	YGG (**3**)	Stable	2.2 days

[a] Data taken from Ref. 16.

and **11**, in the course of the degradation. The very slow increase in tyrosine formation over time reflects the bioreversibility of **7** and its metabolites **9** and **11** to parent peptides by spontaneous hydrolysis followed by degradation at the Tyr-Gly bond by serum aminopeptidase(s).

Next, the kinetics of the processing of imidazolidinones **9**-*trans* and **11**-*trans* with shorter peptide chains were examined in human serum. As presented in Figures 5B and C, both compounds displayed very high stability in serum showing almost identical trends of degradation. Glycated metabolite **11** was detected in the incubation mixture of compound **9**. The amount of degradation product **11** slowly increased with time reaching the maximal value (4%) after 7 days of incubation (Fig. 5B). The appearance of this metabolite may result from the action of peptidases that exhibit carboxypeptidase-like activity on the Gly-Phe bond of **9**. Nevertheless, as exemplified in Figures 5B and C, the predominant route for degradation of imidazolidinones **9** and **11** in serum included spontaneous hydrolysis to the parent peptide compounds, as demonstrated by the parallel appearance of tyrosine as aminopeptidase hydrolysis by-product. In fact, a comparison of the stability of these compounds in human serum with their stability in phosphate buffer (pH 7.4) at 37 °C revealed almost identical rates of hydrolysis (Table 1).

Interestingly, the data obtained in a previous study,[16] in which the enzymatic and chemical stability of leucine–enkephalin-related Amadori product **6** was investigated, showed much faster degradation for **6** in human serum ($t_{1/2}$ 14 h) when compared with imidazolidinone compound **7** (Table 1). Additionally, these experiments revealed that **6** was almost exclusively hydrolyzed by dipeptidyl carboxypeptidase(s) in human serum yielding Tyr-Gly-Gly-related Amadori compound **10** as the only metabolite. Metabolite **10** showed no sign of further degradation in serum nor hydrolysis to the parent peptide over the time span investigated. Experiments indicated that binding to human serum albumin was responsible for the protection of the low molecular weight Amadori compound **10** from hydrolysis.[16]

It should be noted that the chemical hydrolysis of *N*-(1-deoxy-ketos-1-yl)-peptides (Amadori compounds)[15] and the peptide-derived imidazolidinones proceeds via different pathways. Although both types of compounds produce by hydrolysis a parent peptide, the pathway of Amadori compound degradation results after 1,2- and 2,3-enolizations in reactive dicarbonyl compounds[14] whereas during hydrolysis of imidazolidinones unchanged hexose sugar is generated.

3. Conclusion

In conclusion, the described results and interpretations should contribute to our knowledge of the underlying chemistry in the Maillard reaction and to eventual physiological consequences of peptide-sugar reactions. With respect to utilizing small bioactive peptides as a tool in studying the glycation reaction mechanisms, we noted that in addition to the well-known Amadori rearrangement, peptide-derived imidazolidinone compounds are also formed from different reducing sugars. The occurrence of glycation products appears to be determined primarily by the reacting sugar, and by peptide sequence and structure. Additionally, significant differences were observed in the rate of chemical and enzymatic degradation of both types of glycation products derived from glucose-peptide model reactions.

From this study, it cannot be concluded whether glycated products **6** and **7** obtained from glucose and endogenous opioid peptide leucine–enkephalin (**1**) could have any biological relevance to in vivo pathological conditions associated with diabetes mellitus. For example, one might argue that if endogenous (self-produced) insulin has a half-life of about 4 min in the bloodstream, no glycation of this regulatory hormone would be observed. However, circulating glycated insulin was readily detected in control and Type 2 diabetic subjects.[2] Further studies are therefore required to evaluate physiological significance of products generated in bioactive peptide–sugar mixtures in vitro.

4. Experimental

4.1. 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 in DMSO-d_6 and to dioxane in D_2O. Spectra were assigned based on 2D homonuclear (COSY) and heteronuclear (HMQC, HMBC) experiments. Mass spectra were recorded on a ThermoFinnigan Deca ion trap mass spectrometer operating in Electrospray ionization (ESI) mode. Reverse-phase high-performance liquid chromatography (RP HPLC) was performed on a Varian Pro Star 230 or HP 1090 HPLC systems 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 conditions by using different concentrations of MeOH in 0.1% aqueous trifluoroacetic acid (TFA). UV detection was performed at 215, 254 and 280 nm by using a HP 1090 diode-array detector.

4.2. Chemicals

Leucine–enkephalin (**1**), L-tyrosylglycylglycyl-L-phenylalanine (**2**), L-tyrosylglycylglycine (**3**), and L-tyrosylglycylglycyl-L-phenylalanyl-L-leucine amide (**4**) were purchased from Bachem. L-Tyrosylglycylglycyl-L-phenylalanyl-L-leucine methyl ester (**5**) was synthesized as described previously.[11a] Human serum and *p*-nitro-L-phenylalanine were obtained from Sigma–Aldrich Chemical. *o*-Hydroxyphenylacetic acid was obtained from Fluka. Literature procedures were used for the synthesis of the referential Amadori compounds, *N*-(1-deoxy-D-fructos-1-yl)-L-tyrosylglycylglycyl-L-phenylalanyl-L-leucine (**6**),[17a] *N*-(1-deoxy-D-fructos-1-yl)-L-tyrosylglycylglycine (**10**),[17a] *N*-(1-deoxy-D-fructos-1-yl)-L-tyrosylglycylglycyl-L-phenylalanyl-L-leucine methyl ester (**14**),[17a] *N*-(1-deoxy-D-tagatos-1-yl)-L-tyrosylglycylglycine (**17**)[17b] as well as the referential imidazolidinone derivatives, *N*-{[2-(D-*gluco*-pentitol-1-yl)-4-(4-hydroxybenzyl)-5-oxoimidazolidin-1-yl]}acetylglycyl-L-phenylalanyl-L-leucine (**7**-*cis*, **7**-*trans*),[11a] *N*-{[2-(D-*gluco*-pentitol-1-yl)-4-(4-hydroxybenzyl)-5-oxoimidazolidin-1- yl]}acetylglycyl-L-phenylalanyl-L-leucine methyl ester (**15**),[11a] *N*-{[2-(D-*manno*-pentitol-1-yl)-4-(4-hydroxybenzyl)-5-oxoimidazolidin-1-yl]}acetylglycyl-L-phenylalanyl-L-leucine (**16**),[17c] and *N*-{[2-(D-*galacto*-pentitol-1-yl)-4-(4-hydroxybenzyl)-5-oxoimidazolidin-1-yl]}acetylglycyl-L-phenylalanyl-L-leucine (**18**).[11a]

4.3. Synthesis of compounds

4.3.1. *N*-(1-Deoxy-D-fructos-1-yl)-L-tyrosylglycylglycyl-L-phenylalanine (8) and *N*-{[2-(D-*gluco*-pentitol-1-yl)-4-(4-hydroxybenzyl)-5-oxoimidazolidin-1-yl]}acetylglycyl-L-phenylalanine (9-*trans*). L-Tyrosylglycylglycyl-L-phenylalanine (**2**) as acetate salt (150 mg, 0.3 mmol), D-glucose (810 mg, 4.5 mmol), and *N*-ethylmorpholine (NEM) (1.9 mL, 15.0 mmol) were dissolved in dry MeOH (300 mL) and kept in a closed round-bottomed flask for 10 days at 50 °C. The solvent was evaporated and the residue was applied to a Sephadex G-15 column (90 × 1.6 cm) and eluted with 1% aq acetic acid. Fractions containing compounds **8** and **9** were combined and purified by semipreparative RP HPLC using 35% MeOH/0.1% TFA as the eluent to give pure title compounds **8** (RP HPLC t_R = 19.9 min) and **9** (RP HPLC t_R = 28.2 min).

N-(1-Deoxy-D-fructos-1-yl)-L-tyrosylglycylglycyl-L-phenylalanine (**8**): 63 mg, 35%; analytical RP HPLC analysis: 35% MeOH/0.1% TFA t_R = 14.4 min; 40% MeOH/0.1% TFA t_R = 10.5 min; mp 110–120 °C; $[\alpha]_D$ +28 (*c* 1, MeOH). ^{1}H NMR (600 MHz, D_2O) (β-pyranose form): δ 2.83/3.03 (Phe β,β′), 2.90/3.12 (Tyr β,β′), 3.09/3.17 (Fru H-1,1′), 3.50–3.70 (Gly^2,Gly^3 α,α′), 3.53 (Fru H-3), 3.56/3.81 (Fru H-6,6′), 3.68 (Fru H-4), 3.81 (Fru H-5), 4.10 (Tyr α), 4.45 (Phe α), 6.68 (Tyr ε), 6.95 (Tyr δ), 7.07 (Phe δ), 7.12 (Phe ζ), 7.16 (Phe ε). ^{13}C NMR (150 MHz, D_2O) (β-pyranose form): δ 35.9 (Tyr β), 37.6 (Phe β), 42.9 (Gly^2 α), 43.2 (Gly^3 α), 52.8 (Fru C-1), 55.3 (Phe α), 63.0 (Tyr α), 64.7 (Fru C-6), 69.7 (Fru C-5), 70.1 (Fru, C-4), 70.6 (Fru C-3), 96.1 (Fru C-2), 116.7 (Tyr ε), 125.9 (Tyr γ), 127.9 (Phe ζ), 129.5 (Phe ε), 130.1 (Phe δ), 131.6 (Tyr δ), 137.5 (Phe γ), 156.0 (Tyr ζ), 169.2 (Tyr CO), 171.4 (Gly^2 CO), 171.5 (Gly^3 CO), 176.1 (Phe CO). TOF MS-ES, *m*/*z* 605 $[M+H]^+$.

N-{[2-(D-*gluco*-Pentitol-1-yl)-4-(4-hydroxybenzyl)-5-oxoimidazolidin-1-yl]}acetylglycyl-L-phenylalanine (**9**-*trans*): 10 mg, 6%; analytical RP HPLC analysis: 35% MeOH/0.1% TFA t_R = 19.5 min; 40% MeOH/0.1% TFA t_R = 12.8 min. ^{1}H NMR (600 MHz, DMSO-d_6) (for designation of particular atoms in imidazolidinone compounds, see Ref. 17): δ 2.74/3.01 (HO-Bzl CH_2), 2.87/3.05 (Phe β,β′), 3.38/3.56 (pentitol H-5′,5″), 3.50 (pentitol H-3′,4′), 3.73 (Gly α,α′), 3.84/4.15 (N^1–CH_2), 3.92 (pentitol H-2′), 3.98 (pentitol H-1′), 4.02 (Im-ring H-4), 4.43 (Phe α), 4.93 (Im-ring H-2), 6.68 (HO-Bzl CH), 7.10 (HO-Bzl CH), 7.21 (Phe δ,ζ), 7.28 (Phe ε), 8.34 (Phe NH), 8.48 (Gly NH), 9.33 (HO-Bzl OH). ^{13}C NMR (150 MHz, DMSO-d_6): δ 36.8 (Phe β), 41.6 (Gly α), 42.8 (N^1–CH_2), 53.5 (Phe α), 59.2 (Im-ring C-4), 63.2 (pentitol C-5′), 68.5 (pentitol C-1′), 69.1 (pentitol C-2′), 71.0 (pentitol C-3′,4′), 73.0 (Im-ring C-2), 115.1 (HO-Bzl CH), 126.4 (Phe ζ), 128.2 (Phe ε), 129.1 (Phe δ), 130.0 (HO-Bzl CH), 137.4 (Phe γ), 156.1 (HO-Bzl C), 167.8 (N^1–CH_2–CO CO), 168.3 (Gly CO), 172.7 (Phe CO). TOF MS-ES, *m*/*z* 605 $[M+H]^+$.

4.3.2. *N*-{[2-(D-*gluco*-Pentitol-1-yl)-4-(4-hydroxybenzyl)-5-oxoimidazolidin-1-yl]}acetylglycine (11-*trans*). This compound was obtained starting from the acetate salt of L-tyrosylglycylglycine (**3**) (355 mg, 1.0 mmol), D-glucose (2700 mg, 15.0 mmol), and NEM (0.64 mL, 5.0 mmol) in MeOH by using the same procedure as described for compound **9**. Purification by semipreparative RP HPLC using 15% MeOH/0.1% TFA as the eluent afforded the pure title compound **11** (RP HPLC t_R = 25.1 min) (60 mg, 13%); analytical RP HPLC analysis: 15% MeOH/0.1% TFA t_R = 17.3 min; 10% MeOH/0.1% TFA t_R = 35.5 min; mp 115–125 °C; $[\alpha]_D$ − 38 (*c* 1, MeOH). ^{1}H NMR (600 MHz, DMSO-d_6): δ 2.79/3.05 (HO-Bzl CH_2), 3.39/3.57 (pentitol H-5′,5″), 3.50 (pentitol H-4′), 3.53 (pentitol H-3′), 3.87/4.21 (N^1–CH_2), 3.81/3.86 (Gly α,α′), 3.95 (pentitol H-2′), 4.03 (pentitol H-1′), 4.09 (Im-ring H-4), 5.01 (Im-ring H-2), 6.70 (HO-Bzl CH), 7.12 (HO-Bzl CH), 8.62 (Gly NH), 9.38 (HO-Bzl OH). ^{13}C NMR (150 MHz, DMSO-d_6): δ 34.7 (HO-Bzl CH_2), 40.7 (Gly α), 42.6 (N^1–CH_2), 59.0 (Im-ring C-4), 63.3 (pentitol C-5′), 68.4 (pentitol C-1′), 69.1 (pentitol C-2′), 70.9 (pentitol C-4′), 71.0 (pentitol C-3′), 72.9 (Im-ring C-2), 115.2 (HO-Bzl CH), 130.1 (HO-Bzl CH), 156.2 (HO-Bzl C), 167.8 (Im-ring CO), 170.8 (Gly CO). TOF MS-ES, *m*/*z* 458 $[M+H]^+$.

4.3.3. *N*-(1-Deoxy-D-fructos-1-yl)-L-tyrosylglycylglycyl-L-phenylalanyl-L-leucine amide (12). To a chilled solution (−15 °C) of *N*-(1-deoxy-D-fructos-1-yl)-L-tyrosylglycyl-

glycyl-L-phenylalanyl-L-leucine (**6**)[17a] (40 mg, 0.056 mmol) in DMF (1 mL), NEM (0.006 mL, 0.056 mmol) and isobutyl chloroformate (0.007 mL, 0.056 mmol) were added. The reaction mixture was stirred for 2 min at the same temperature and a precooled NH_4OH solution (0.080 mL) was then added. The reaction mixture was stirred for 20 min at −15 °C and for 24 h at room temperature. The solvent was evaporated and the residue was purified by semipreparative RP HPLC using 43.5% MeOH/0.1% TFA as the eluent to give pure compound **12** (RP HPLC t_R = 15.7 min) (25 mg, 63%); analytical RP HPLC analysis: 43.5% MeOH/0.1% TFA t_R = 11.7 min; 40% MeOH/0.1% TFA t_R = 13.7 min; mp 105–120 °C; $[\alpha]_D$ +6 (*c* 0.8, MeOH). ^{1}H NMR (600 MHz, D_2O) (β-pyranose form): δ 0.63/0.70 (Leu δ,δ′), 1.34 (Leu γ), 1.37 (Leu β,β′), 2.88 (Phe β,β′), 2.91/3.13 (Tyr β,β′), 3.10/3.17 (Fru H-1,1′), 3.53 (Fru H-3), 3.56/3.80 (Fru H-6,6′), 3.60–3.73 (Gly^2,Gly^3 α,α′), 3.69 (Fru H-4), 3.80 (Fru H-5), 4.06 (Leu α), 4.11 (Tyr α), 4.41 (Phe α), 6.68 (Tyr ε), 6.96 (Tyr δ), 7.08 (Phe δ), 7.13 (Phe ζ), 7.18 (Phe ε). ^{13}C NMR (150 MHz, D_2O) (β-pyranose form): δ 21.4, 23.0 (Leu δ), 25.0 (Leu γ), 36.0 (Tyr β), 37.7 (Phe β), 40.5 (Leu β), 43.0 (Gly^2 α), 43.2 (Gly^3 α), 52.8 (Fru C-1), 52.9 (Leu α), 55.9 (Phe α), 63.0 (Tyr α), 64.7 (Fru C-6), 69.7 (Fru C-5), 70.1 (Fru, C-4), 70.6 (Fru C-3), 96.2 (Fru C-2), 116.7 (Tyr ε), 126.0 (Tyr γ), 128.2 (Phe ζ), 129.7 (Phe ε), 130.1 (Phe δ), 131.7 (Tyr δ), 136.9 (Phe γ), 156.1 (Tyr ζ), 169.6 (Tyr CO), 171.7 (Gly^2, Gly^3 CO), 173.8 (Phe CO), 177.7 (Leu CO). TOF MS-ES, *m*/*z* 717 $[M+H]^+$.

4.3.4. *N*-{[2-(D-*gluco*-Pentitol-1-yl)-4-(4-hydroxybenzyl)-5-oxoimidazolidin-1-yl]}acetylglycyl-L-phenylalanyl-L-leucine amide (13-*trans*). This compound was obtained starting from the acetate salt of L-tyrosylglycylglycyl-L-phenylalanyl-L-leucine amide (**4**) (184 mg, 0.3 mmol), D-glucose (810 mg, 4.5 mmol), and NEM (0.192 mL, 1.5 mmol) in MeOH (300 mL) by using the same procedure as described for compound **9**, except that the reaction mixture was kept at 50 °C for 19 days. Purification by semipreparative RP HPLC using 40% MeOH/0.1% TFA as the eluent afforded the pure *trans*-isomer of compound **13** (**13**-*trans* RP HPLC t_R = 31.2 min) (31 mg, 14%); analytical RP HPLC analysis: 43.5% MeOH/0.1% TFA t_R = 15.6 min; 40% MeOH/0.1% TFA t_R = 18.9 min; mp 110–125 °C; $[\alpha]_D$ −42 (*c* 1, MeOH). ^{1}H NMR (600 MHz, DMSO-d_6): δ 0.84/0.89 (Leu δ,δ′), 1.46 (Leu β,β′), 1.57 (Leu γ), 2.75/3.04 (Phe β,β′), 2.81/3.06 (HO-Bzl CH_2), 3.38/3.56 (pentitol H-5′,5″), 3.50 (pentitol H-4′), 3.56 (pentitol H-3′), 3.69/3.78 (Gly α,α′), 3.83/4.21 (N^1–CH_2), 3.97 (pentitol H-2′), 4.05 (pentitol H-1′), 4.12 (Im-ring H-4), 4.22 (Leu α), 4.54 (Phe α), 5.05 (Im-ring H-2), 6.70 (HO-Bzl CH), 7.12 (HO-Bzl CH), 7.18 (Phe ζ), 7.25 (Phe δ,ε), 8.08 (Leu NH), 8.25 (Phe NH), 8.50 (Gly NH), 9.38 (HO-Bzl OH). ^{13}C NMR (150 MHz, DMSO-d_6): δ 21.6, 23.0 (Leu δ), 24.3 (Leu γ), 34.4 (HO-Bzl CH_2), 37.5 (Phe β), 40.9 (Leu β), 41.9 (Gly α), 42.6 (N^1-CH_2), 51.0 (Leu α), 54.0 (Phe α), 58.9 (Im-ring C-4), 63.2 (pentitol C-5′), 68.2 (pentitol C-1′), 69.1 (pentitol C-2′), 70.8 (pentitol C-4′), 71.0 (pentitol C-3′), 72.7 (Im-ring C-2), 115.3 (HO-Bzl CH), 126.1 (HO-Bzl CH), 126.3 (Phe ζ), 128.1 (Phe ε), 129.2 (Phe δ), 130.1 (HO-Bzl CH), 137.8 (Phe γ), 156.3 (HO-Bzl C), 167.6 (N^1-CH_2-CO CO), 168.2 (Gly CO), 170.7 (Phe CO), 174.0 (Leu CO). TOF MS-ES, *m*/*z* 717 $[M+H]^+$.

4.4. RP HPLC analysis of glycation products formed by incubation of peptides with reducing sugars

(a) Incubations in phosphate buffer. Solutions of the acetate salts of peptides **1**–**5** (0.01 M) and reducing sugars, D-glucose, D-mannose or D-galactose (0.15 M or 1.50 M), were prepared in 0.05 M phosphate buffer/0.1 M NaCl (pH 7.4) (PBS) containing NaN_3 (0.02%). The sterile solutions, obtained by passage through a 0.45 μm nylon filter, were incubated in the dark at 37 or 50 °C. Aliquots were withdrawn from the incubation mixtures at appropriate time intervals, immediately frozen and lyophilized. The relative concentrations of the respective Amadori and imidazolidinone glycation products in the incubation mixtures were determined by analytical RP HPLC at a flow rate of 0.5 mL/min (for details, see Section 4.1). The mobile phases used for the analysis were: 45.2% MeOH/0.1% TFA (for **1**), 35% MeOH/0.1% TFA (for **2**), 15% MeOH/0.1% TFA (for **3**), and 43.5% MeOH/0.1% TFA (for **4** and **5**). The relative amounts of the glycation products formed are presented in Figures 3 and 4.

(b) Incubations in methanol. Solutions of the acetate salts of peptides **1**–**5** (0.001 M) and reducing sugars, D-glucose, D-mannose or D-galactose (0.015 M), were prepared in methanol or in methanol containing *N*-ethylmorpholine (NEM) (0.025 M). The reaction mixtures were kept at either 37 or 50 °C. The sampling procedure and analysis of products was performed as described above. The relative amounts of the glycation products formed are presented in Figures 3 and 4.

4.5. Stability of the glucose-derived imidazolidinones in human serum and phosphate buffer (pH 7.4)

For serum stability studies, a mixture of 80% human serum (2 mL, diluted with water), the imidazolidinone compound, **7**-*trans*, **9**-*trans* or **11**-*trans*, or leucine–enkephalin (**1**) (7×10^{-4} M), and an internal standard [*o*-hydroxyphenylacetic acid (40 μg/mL) for compounds **7** and **9**; or *p*-nitro-L-phenylalanine (40 μg/mL) for compound **11**] was kept at 37 °C in a Teflon-lined screw-cap test tube. Three samples (0.1 mL) were removed at appropriate intervals and deproteinized by the 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.

For the determination of the stability in phosphate buffer, solutions of **7**-*trans*, **9**-*trans* or **11**-*trans* (7×10^{-4} M) and the internal standard [*o*-hydroxyphenylacetic acid (40 μg/mL) for compounds **7** and **9**; or *p*-nitro-L-phenylalanine (40 μg/mL) for compound **11**] were prepared in PBS containing NaN_3 (0.02%). The sterile solutions, obtained by passage through a 0.45 μm nylon filter, were incubated in the dark at 37 °C. At appropriate times,

samples of the reaction mixtures (in triplicate) were collected and chromatographed immediately.

The concentration of the starting compounds and metabolites in the incubation mixtures was monitored by RP HPLC on an analytical column at a flow rate of 0.5 mL/min with 43.5% MeOH/0.1% TFA for **7**-*trans*, 47% MeOH/0.1% TFA for **9**-*trans*, and 19.5% MeOH/0.1% TFA for **11**-*trans* using a HP 1090 system equipped with a diode-array detector. UV detection was performed at 280 and 215 nm. The imidazolidinone compound or peptide concentration of samples (in triplicate) was determined by electronic integration of peak areas and calculation of analyte/internal standard peak-areas.

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