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Synthesis and intramolecular reactions of Tyr-Gly and Tyr-Gly-Gly related 6-*O*-glucopyranose esters

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Abstract—6-*O*-(L-Tyrosylglycyl)- and 6-*O*-(L-tyrosylglycylglycyl)-D-glucopyranose were synthesized by condensation of the pentachlorophenyl esters of the respective di- and tripeptide with fully unprotected D-glucose. The intramolecular reactivity of the sugar conjugates was studied in pyridine–acetic acid and in dry methanol, at various temperatures and for various incubation times. The composition of the incubation mixtures was monitored by a reversed-phase HPLC method that permits simultaneous analysis of the disappearance of the starting material and the appearance of rearrangement and degradation products. To determine the influence of esterification of the peptide carboxy group on its amino group reactivity, parallel experiments were done in which free peptides were, under identical reaction conditions, incubated with D-glucose (molar ratios 1:1 and 1:5). Depending on the starting compound, different types of Amadori products (cyclic and bicyclic form), methyl ester of peptides, and Tyr-Gly-diketopiperazine were obtained.

Keywords: 6-*O*-(L-Tyrosylglycyl)-D-glucopyranose; 6-*O*-(L-Tyrosylglycylglycyl)-D-glucopyranose; D-Glucose; Peptide; Amadori rearrangement

1. Introduction

Glucose and other reducing sugars, during the early stage of the Maillard reaction, react in vivo nonenzymatically with free amino groups of proteins, glycoproteins, lipids, and nucleic acids to form labile Schiff bases which are then, by Amadori rearrangement, converted to more stable keto-amines.[1] Formed keto-amines, or Amadori products, undergo over time, a series of complex reactions leading to a class of permanently modified proteins implicated in the pathogenesis of diabetes mellitus,[2,3] normal processes of aging,[4,5] and Alzheimer's disease.[6] These reactions also occur during food processing or cooking, affecting flavors, color, and organoleptic properties of foods.[7]

In addition to numerous endogenous biologically active peptides, our recent interest has been focused on enkephalins, particularly leucine enkephalin (Tyr-Gly-Gly-Phe-Leu), an endogenous opioid pentapeptide involved in numerous physiological functions.[8] In vivo, leucine enkephalin is rapidly hydrolyzed, forming three tyrosine containing fragments: Tyr, Tyr-Gly, and Tyr-Gly-Gly.[9,10]

In our earlier studies on the synthesis of leucine enkephalin related 6-*O*-glycoconjugates, it was for the first time found that, depending on the employed solvent these glycoconjugates undergo different intramolecular rearrangement reactions. Thus, in pyridine–acetic acid (1:1) bicyclic Amadori compounds prevailed,[11] while in dry methanol solution cyclic sugar-related imidazolidinones were obtained (Fig. 1).[12–14]

To determine the influence of the peptide length on intramolecular reactivity, in the present paper we report the synthesis of Tyr-Gly and Tyr-Gly-Gly related 6-*O*-glucopyranose esters, on the first plane as model systems for glycoproteins, compounds involved in the innate and adaptive immune response[15] but also as compounds, which mimic the reactivity of sugar 6-phosphate esters.[16] Their behavior and reactivity were examined in pyridine–acetic acid and in dry methanol solution, at various temperatures and times of incubation and compared with

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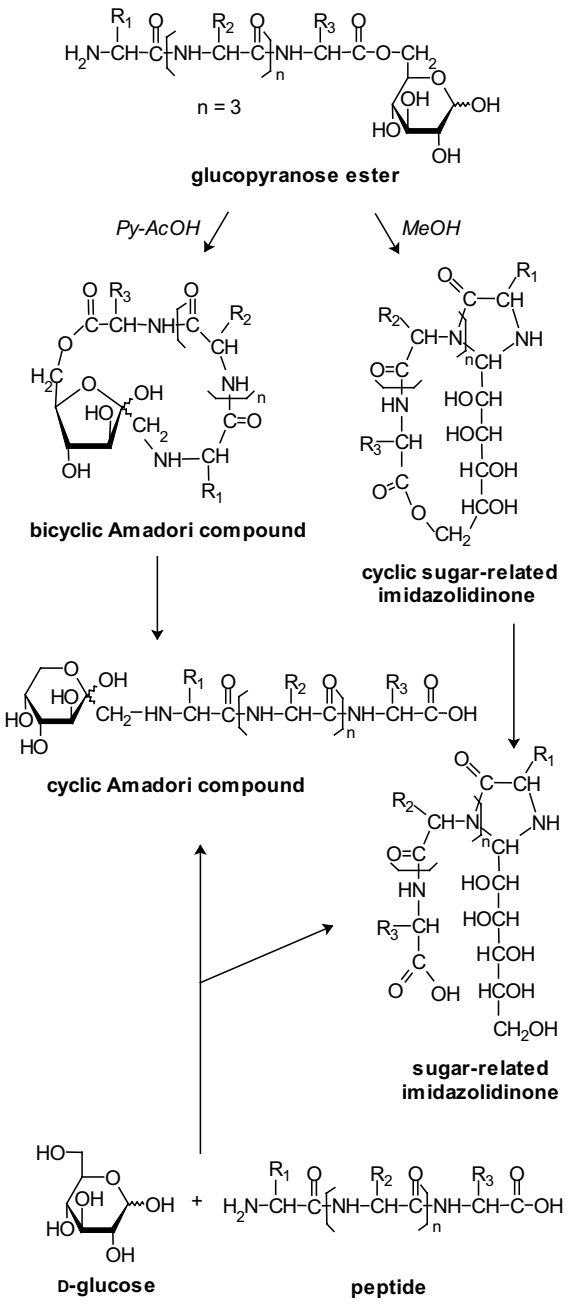


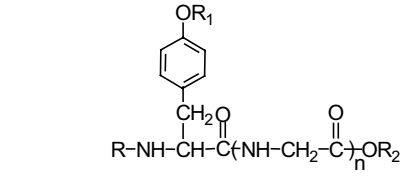
Figure 1. Possible routes of an intramolecular reactions of 6-*O*-pentapeptidyl-D-glucopyranose as a function of employed solvent.

those obtained when free Tyr-Gly and Tyr-Gly-Gly were incubated with D-glucose, under the same conditions.

2. Results and discussion

2.1. Synthesis of starting materials

6-O-(L-Tyrosylglycyl)- and *6-O*-(L-tyrosylglycylglycyl)-D-glucopyranose **3** and **4** were synthesized following a previously described procedure.[17] Di- and tripeptide containing glucoconjugate fractions were separated by column chromatography. ^{13}C NMR shift data for each of them, showed six signals at positions indicative for the C-1 atom and three signals in the region corresponding to the C-6 atom of the sugar moiety, thus suggesting a low primary hydroxyl group selectivity toward esterification with those peptides. Repeated chromatography gave compounds **3** and **4** (Fig. 2), as well as a mixture of glucopeptides in which the corresponding peptide molecule is located at C-3 or at C-2 of the sugar moiety. Attempts to separate those compounds were so far unsuccessful. Removal of the Boc-protecting groups (with TFA) from peptide conjugates **3** and **4**, resulted in the corresponding trifluoroacetate salts of **5** and **6**. Desalting on a Dowex (Ac) column and lyophilization afforded pure **5** and **6**, which were used in the subsequent incubation experiments.



Comp.	R	R_1	R_2	n
1	H	H	H	1
2	H	H	H	2
3	Boc	Boc	a	1
4	Boc	Boc	a	2
5	H	H	a	1
6	H	H	a	2
10	H	H	Me	1
11	H	H	Me	2
12	b-d	H	H	1
13	b-d	H	H	2

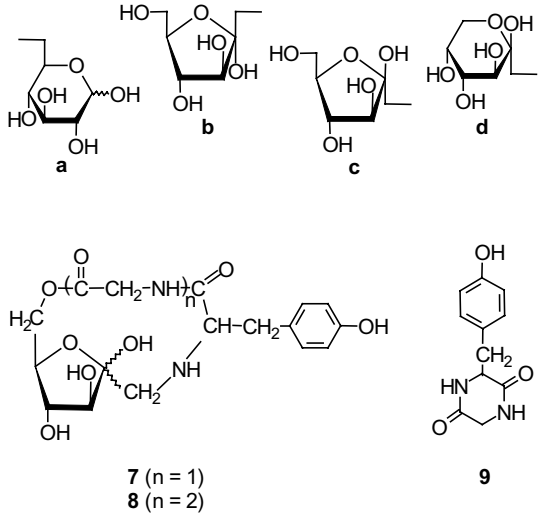


Figure 2. Structures of compounds **1**–**13**.

2.2. Incubation experiments

Tyr-Gly and Tyr-Gly-Gly related 6-*O*-glucopyranose esters **5** and **6** were incubated, at various temperatures and times of incubations. For that purpose two solvent systems were chosen pyridine–acetic acid (1:1) and dry methanol. Pyridine–acetic acid possesses the balance of acidity and basicity necessary for acid–base catalyzed reactions such as the Amadori rearrangement,[18] while methanol has been shown to be an ideal solvent for intramolecular rearrangement of enkephalin-related 6-*O*-glycoconjugates to the corresponding 4-imidazolidinone derivatives.[12–14]

Reversed-phase HPLC assay was used that permits simultaneous monitoring of the disappearance of the starting material and the appearance of rearrangement and degradation products. The structures of isolated incubation products **7**–**13** are presented in Figure 2. Figures 3–6 show their distribution and relative concentrations, depending on the starting compound and the incubation conditions, as estimated from peak areas in HPLC chromatograms.

The incubation of 6-*O*-(Tyr-Gly)-D-glucopyranose **5** in pyridine–acetic acid (1:1) gave bicyclic Amadori compound **7** and *cyclo*-(Tyr-Gly) **9**, which prevailed (Fig. 3A). The yield of **9** was positively correlated with temperature, not as a result of **7** instability, but as a result of intramolecular aminolysis of **5**. The optimum for **7** formation was a temperature of 37 °C and one day

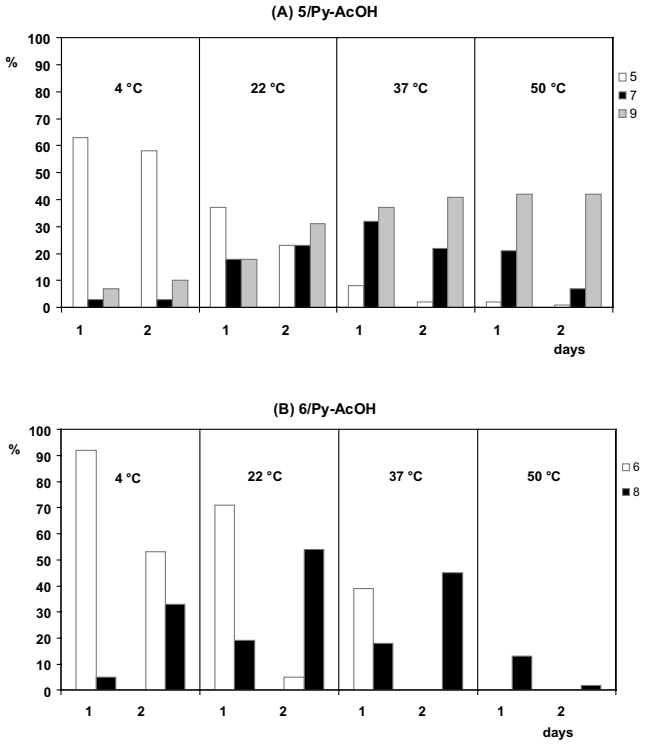


Figure 3. Relative amounts of products formed by incubations of 6-*O*-esters **5** (A) and **6** (B) in pyridine–acetic acid (1:1).

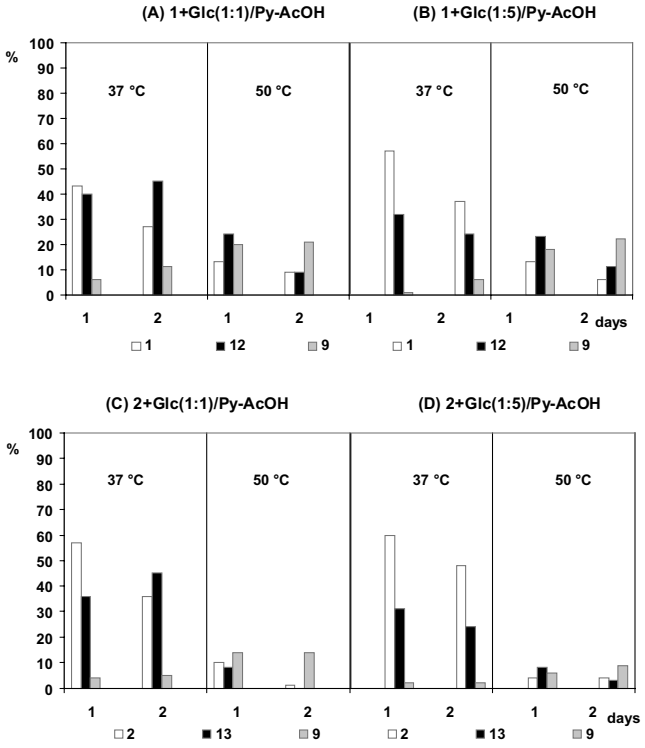


Figure 4. Relative amounts of products formed by incubations of D-glucose with Tyr-Gly (A, B), and D-glucose with Tyr-Gly-Gly (C, D) in pyridine–acetic acid (1:1).

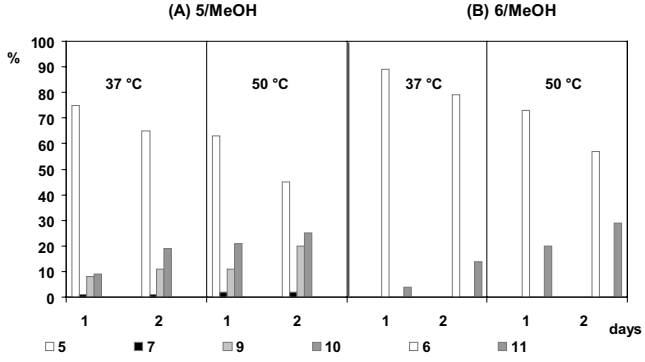


Figure 5. Relative amounts of products formed by incubations of 6-*O*-esters **5** and **6** in dry methanol.

incubation, while longer periods and higher temperature tend to favor its degradation.

It was earlier determined that intramolecular reaction of the L-tyrosine-related 6-*O*-glucopyranose ester gave less than 1% of an unexpected cyclic Amadori compound (although a bicyclic compound would be expected, Fig. 1) indicating that, in the mentioned molecule, rearrangement and ester bond hydrolysis occurred simultaneously.[19] Our result shows that addition of only one simple amino acid, such as glycine (compound **5**), stabilized Amadori product **7** to such an extent that it could easily be isolated, and no ester bond hydrolysis was detected.

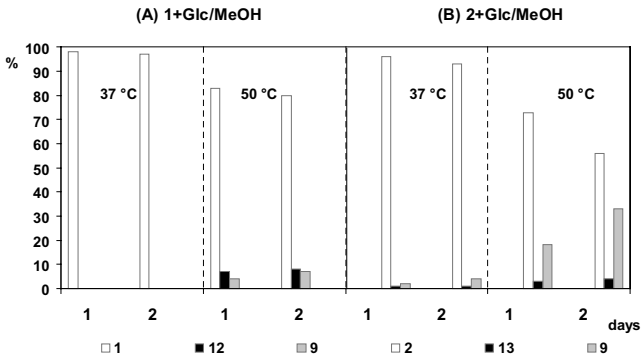


Figure 6. Relative amounts of products formed by incubation of Tyr-Gly and Tyr-Gly-Gly with D-glucose in dry methanol.

Introduction of an additional glycine residue (compound **6**) further stabilized Amadori compound **8** which was in that case, isolated as a main product. At the same time esterification with D-glucose obstructed cyclization of the peptide to diketopiperazine **9** (Fig. 3B).

The remaining levels of starting **5** and **6**, after a one day incubation period, demonstrate that although **5** reacts more readily than **6** do, the amounts of **7** formed were lower than those of **8**. Moreover, while the amounts of **7** were, at different temperatures, after two days, negligibly higher or even lower than after one day, the amounts of **8** were several times larger. Incubation of **5** and **6** at 50 °C gave, in addition to the compounds mentioned, a broad spectrum of degradation products.

Both dipeptide and tripeptide related 6-*O*-glucoconjugates **5** and **6** gave bicyclic Amadori compounds **7** and **8** in relatively good yields. Comparing that result with the previous finding that intramolecular reaction of Tyr-Pro related 6-*O*-glucopyranose ester gave *cyclo*-(Tyr-Pro) as the only product, while the rearrangement of Tyr-Pro-Phe related 6-*O*-glucopyranose ester was stopped when a glycosylamine was formed,[20] we deduced that the intramolecular rearrangement of the peptide-related glycoconjugates depends, not only on the length of the peptide, but also on the nature of the incorporated amino acids.

Parallel experiments were done in which Tyr-Gly (**1**) and Tyr-Gly-Gly (**2**) were incubated with D-glucose in pyridine–acetic acid (1:1) (Fig. 4A and C). An amino group of **1** and **2** has great affinity, not only for the C-1 atom of D-glucose (resulting in Amadori compounds **12** and **13**), but also toward the Gly^2 carbonyl group (affording diketopiperazine **9**). An excess of D-glucose (Fig. 4B and D) caused the significant lowering of peptides reactivity and thereby the yields of Amadori products **12** and **13** were lower. That is in agreement with the results of the Maillard reaction of L-leucine and D-glucose.[21]

From the reaction pathways of free (**1**, **2**) and esterified peptides (**5**, **6**), it could be concluded that this type of esterification influences the reactivity of the peptides free amino groups. While Amadori compound **12** was the main product obtained from **1** (exception: two days at 50 °C) (Fig. 4A), esterification of **1** (compound **5**) (Fig. 3A) shifts the reaction pathway in the direction of intramolecular aminolysis giving diketopiperazine **9** as the main product. At the same time, esterification of tripeptide (compound **6**) (Fig. 3B) completely obstructed intramolecular aminolysis. Reaction of free peptides **1** and **2** with D-glucose afforded slightly larger amount of Amadori compounds **12** and **13** (Fig. 4A and C), than were those of **7** and **8** obtained from 6-*O*-glucopyranose esters **5** and **6** (Fig. 3A and B). That may be explained by the fact that an unbonded small peptide can more easily approach the C-1 atom of free D-glucose, than the same peptide esterified to C-6 of the same sugar molecule.

The tautomer equilibrium composition of Amadori compounds was determined from relative peak intensities in ^{13}C NMR (Me_2SO-d_6) spectra and is presented in Section 3. Drawing a parallel between our results with previously published ones,[11] it was deduced that bicyclic Amadori compound **7** was obtained exclusively in its β-furanose form, while **8**, on which the addition of one more glycine residue facilitated twisting of the peptide molecule, opened the possibility for some α-furanose formation (α/β 14:86). **12** and **13**, obtained by hydrolysis of the ester linkage in **7** and **8**, gave much more heterogeneous mixtures of tautomers in solution: both α,β-furanose and β-pyranose forms were detected, but not a trace of α-pyranose.

It is well known that, under appropriate conditions, compounds containing an α-aminoamide moiety, such as peptides, condense with aldehydes and ketones giving 4-imidazolidinone derivatives.[22] Although sugars, in their open form, are aldehydes or ketones, no studies were done on the reaction of free sugars with peptides in terms of peptide-related 4-imidazolidinone formation. As our earlier studies showed that intramolecular cyclization of leucine enkephalin 6-*O*-monosaccharide esters in methanol solution resulted in the formation of novel glycation products containing a 4-imidazolidinone ring[12–14] (Fig. 1), we incubated esters **5** and **6** in methanol at both 37 and 50 °C (Fig. 5). The rates of disappearance of starting compounds **5** and **6** were linearly dependent on reaction time for both temperatures, but slower than in pyridine–acetic acid (1:1). Moreover, during these incubations alcoholysis took place giving the corresponding peptide methyl esters **10** and **11**, respectively. While **11** was the only isolable product generated from 6-*O*-ester **6**, the dipeptide moiety in **5** is sufficiently activated to give, in addition to Tyr-Gly-OMe **10**, diketopiperazine **9** as a result of intramolecular aminolysis. Although, in those incubations, not a trace of the desired 4-imidazolidinone type of compounds was obtained, the low yield of bicyclic Amadori compound **7** (Fig. 5A) suggested that, in methanol, the N-terminal amino group of compound **5** possesses affinity to attach

to the C-1 sugar atom and, at the same time, the intramolecular nucleophilic addition of the Gly^2 nitrogen to the already formed Schiff base was not possible.

Considerably low reactivity was observed in methanolic solutions of D-glucose and peptides **1** or **2**, in which even at 50 °C more than 50% of the unreacted peptide remained (Fig. 6A and B). From neither **1** nor **2** not a trace of a 4-imidazolidinone related rearrangement product was obtained. Moreover, although **1** and **2** followed similar reaction pathways, giving diketopiperazine **9** and Amadori products **12** or **13**, either of them prefers a particular product. While **1** gave low yields of **12** and next to nothing of **9**, tripeptide **2** as the main product, particularly at higher temperatures, gave **9** and only traces of Amadori product **13**. The same reactivity order (dipeptide > tripeptide) in terms of Amadori product formation, was obtained when different di- and tripeptides were incubated with D-glucose in phosphate buffer.[23] A very good example how solvent and the aldehyde employed influence the reaction pathway is the incubation of Tyr-Gly-Gly (**2**) with D-glucose in methanol in which case not a trace of a 4-imidazolidinone related product was detected, while just Tyr-Gly-Gly is the shortest N-terminal amino acid sequence of enkephalin, which with acetaldehyde leads to rapid 4-imidazolidinone formation.[24]

It could be summarized that, under precisely controlled incubation conditions, either Tyr-Gly or Tyr-Gly-Gly related 6-*O*-glucopyranose esters **5** and **6**, or model systems with free peptides **1** or **2** and D-glucose gave Amadori rearrangement products. It is obvious that esterification through the primary hydroxyl group of D-glucose influences the reactivity of the peptide amino group. While esterification of Tyr-Gly, in pyridine–acetic acid, accelerated aminolysis, esterification of Tyr-Gly-Gly made it impossible. In methanol solution esterification of both peptides opened a new reaction pathway giving their methyl esters as main products. The results reported here should facilitate understanding of the in vivo behavior of those small endogenous opioid metabolites.

3. Experimental

3.1. General methods

Trifluoroacetic acid (TFA) was of spectroscopic grade (Uvasol, Merck, Darmstadt, Germany). All other solvents were distilled at the appropriate pressure. Mps were determined in open capillaries and are uncorrected. Optical rotations were measured at room temperature using an Optical Activity LTD automatic AA-10 polarimeter. Reactions were monitored by TLC on glass plates (Silica Gel 60 F_{254}, Merck, Darmstadt, Germany) using detection with ninhydrin, the chlorine–iodine reagent, or heating with H_2SO_4. Column chromatography was performed on silica gel (Merck; 0.040–0.063 mm). The following solvents were used: (A) petroleum ether–ethyl acetate–acetic acid (14:10:1), (B) ethyl acetate–acetone–water (4:5:0.25), (C) ethyl acetate–ethanol–acetic acid–water (7:1:1:1). RP HPLC was performed on a Varian 9010 HPLC system with Eurospher 100 reversed-phase C-18, 5 μm, analytical (250 × 4 mm) and semipreparative (250 × 8 mm) columns (flow rate 0.7 mL min^{-1} for analytical and 1.1 mL min^{-1} for semipreparative separations) under isocratic conditions using the following solvents: (D) 21% methanol in 0.1% aq TFA and (E) 15% methanol in 0.1% aq TFA. RP HPLC column eluates were monitored by their UV absorbance at 280 nm. The trifluoroacetate salts of the obtained compounds were desalted on a Dowex 1X2 200 (Ac) column (Aldrich, Milwaukee, WI; 10 × 0.8 cm, eluent water), lyophilized and as such used in the incubation experiments. The optical purity of all prepared compounds was examined on Chiral Plates (Aldrich) in methanol–water–acetonitrile (1:1:4) with ninhydrin detection. The ^1H and ^{13}C NMR spectra (one- and two-dimensional), in Me_2SO-d_6 solution (at 20 °C in 5 mm NMR tubes) were recorded with Varian Gemini 300 spectrometer, operating at 75.5 MHz (^{13}C) and 300.1 MHz (^1H). Chemical shifts, in ppm, are referred to Me_4Si. The tautomers equilibrium composition (%) was determined from the relative peak intensities in ^{13}C NMR spectra. Elemental analyses were carried out at the Microanalytical Laboratory, Ruđer Bošković Institute.

3.2. Synthesis of starting materials

3.2.1. *N*,*O*-Di-*tert*-butyloxycarbonyl-L-tyrosylglycine. To an ice-cold solution of *N*,*O*-di-*tert*-butyloxycarbonyl-L-tyrosine[25] (11.60 g, 30.4 mmol) and *N*,*N*′-dicyclohexylcarbodiimide (DCC) (6.27 g, 30.4 mmol) in *N*,*N*-dimethylformamide (160 mL), *N*-hydroxysuccinimide (3.50 g, 30.4 mmol) was added. The reaction mixture was stirred for 1 h at 0 °C and 2 h at room temperature, and left overnight at +4 °C. The precipitated DCHU was filtered off and the filtrate added dropwise to the stirred slurry of glycine (2.28 g, 30.4 mmol), 1 M NaOH (60 mL), and $NaHCO_3$ (5.46 g, 65 mmol). The mixture was stirred for 1 h at room temperature and filtered. The ice-cold filtrate was acidified to pH 3.5% with 10% citric acid solution and extracted with chloroform (3 × 100 mL). Combined extracts were washed with water (100 mL), dried (Na_2SO_4), and evaporated. The residue was crystallized (ethanol–water) to give product (8.15 g), which was purified by stirring overnight with benzene (200 mL). Filtration afforded pure *title compound* (5.56 g, 42%); R_f (A) 0.34; mp 152–154 °C. Anal. calcd for $C_{21}H_{30}N_2O_8$: C, 57.52; H, 6.90; N, 6.39. Found: C, 57.40; H, 6.95; N,

6.38. ^{1}H NMR: δ 1.29 and 1.48 (Boc CH_3), 2.73/3.02 (Tyr β/β′CH_2), 3.81 (Gly CH_2), 4.20 (Tyr αC*H*), 6.95 (Tyr N*H*), 7.07–7.33 (Tyr arom.), 8.29 (Gly N*H*), 12.65 (Gly COO*H*). ^{13}C NMR: δ 27.33 and 28.22 (Boc *C*H$_3$), 36.89 (Tyr β*C*H$_2$), 40.80 (Gly *C*H$_2$), 55.65 (Tyr α*C*H), 78.21 and 83.15 (Boc C_q), 121.12 (Tyr ε*C*H), 130.47 (Tyr δ*C*H), 136.19 (Tyr γ*C*), 149.44 and 151.64 (Boc *C*O), 155.59 (Tyr ζ*C*), 171.53 and 172.37 (Tyr, Gly *C*O).

3.2.2. *N*,*O*-Di-*tert*-butyloxycarbonyl-L-tyrosylglycine pentachlorophenyl ester. To a solution of *N*,*O*-di-*tert*-butyloxycarbonyl-L-tyrosylglycine (4.38 g, 10 mmol) in dichloromethane (100 mL), DCC–pentachlorophenol complex[26] (10.05 g, 10 mmol) was added, stirred for 2 h at room temperature and left overnight at +4 °C. The precipitated DCHU was filtered off and the filtrate evaporated. The oily residue was precipitated with diisopropyl ether. After two recrystallizations from dichloromethane–diisopropyl ether pure *title compound* was obtained (6.60 g, 96%); R_f (A) 0.79; mp 156–157 °C. Anal. calcd for $C_{27}H_{29}N_2O_8Cl_5$: C, 47.22; H, 4.25; N, 4.08. Found: C, 47.36; H, 4.11; N, 4.15.

3.2.3. L-Tyrosylglycine (1). *N*,*O*-Di-*tert*-butyloxycarbonyl-L-tyrosylglycine (200 mg, 0.46 mmol) was dissolved in 90% trifluoroacetic acid solution (1 mL) containing anisole (0.2 mL). The solution was stirred at room temperature for 30 min. Addition of dry diisopropyl ether (0 °C) and subsequent centrifugation gave the pure **TFA×1** (144 mg, 89%); RP HPLC t_R (D) 15.65 min. ^{1}H NMR: δ 2.83/3.01 (Tyr β/β′CH_2), 3.61 (Tyr αC*H*), 3.91 (Gly CH_2), 6.70 (Tyr δC*H*), 7.08 (Tyr ε*CH*), 8.79 (Gly N*H*), 9.23 (Tyr O*H*). ^{13}C NMR: δ 36.52 (Tyr β*C*H$_2$), 41.12 (Gly *C*H$_2$), 53.82 (Tyr α*C*H), 115.60 (Tyr ε*C*H), 125.13 (Tyr γ*C*), 130.87 (Tyr δ*C*H), 156.93 (Tyr ζ*C*), 169.08 and 171.14 (Tyr, Gly *C*O).

3.2.4. L-Tyrosylglycylglycine (2). This compound was obtained starting from *N*,*O*-di-*tert*-butyloxycarbonyl-L-tyrosylglycylglycine[27] (496 mg, 1 mmol), dissolved in 90% trifluoroacetic acid solution (2 mL) containing anisole (0.3 mL) and using the same procedure as described for **1**. Yield: 377 mg (92%) of **TFA×2**; RP HPLC t_R (E) 15.47 min. ^{1}H NMR: δ 2.50/2.66 (Tyr β/β′CH_2), 3.39/3.50 (Gly^3 CH_2), 3.50/3.56 (Gly^2 CH_2), 3.66 (Tyr αC*H*), 6.36 (Tyr ε*CH*), 6.71 (Tyr δC*H*), 7.82 (Tyr N*H*), 7.92 (Gly^3 N*H*), 8.48 (Gly^2 N*H*), 9.15 (Tyr O*H*). ^{13}C NMR: δ 36.32 (Tyr β*C*H$_2$), 40.72 (Gly^2 *C*H$_2$), 41.89 (Gly^3 *C*H$_2$), 53.87 (Tyr α*C*H), 115.47 (Tyr ε*C*H), 124.91 (Tyr γ*C*), 130.61 (Tyr δ*C*H), 156.72 (Tyr ζ*C*), 168.66 and 171.14 (Tyr, Gly^2, Gly^3 *C*O).

3.2.5. 6-*O*-(*N*,*O*-Di-*tert*-butyloxycarbonyl-L-tyrosylglycyl)-D-glucopyranose (3). To a stirred solution of D-glucose (1.62 g, 9 mmol) in dry pyridine (60 mL), *N*,*O*-di-*tert*-butyloxycarbonyl-L-tyrosylglycine pentachlorophenyl ester (2.06 g, 3 mmol) and imidazole (1.02 g, 15 mmol) were added. The resulting mixture was stirred overnight at room temperature. After evaporation of the solvent ethyl acetate (100 mL) was added, extracted with 10% citric acid (2×100 mL) and water (100 mL), dried (Na_2SO_4), and evaporated. The residue (1.85 g) was purified by column chromatography (solvent B) to yield esterificated D-glucose (mixture of 6-*O*-, 3-*O*-, and 2-*O*-esters) (860 mg, 48%) [R_f (B) 0.53 and 0.58]. Repeated column chromatography (solvent B) gave the pure *title compound* **3** (405 mg, 22%); R_f (B) 0.53; mp 129 °C; $[\alpha]_D^{22}$ +21 (*c* 1.0, MeOH). Anal. calcd for $C_{27}H_{40}N_2O_{13}$: C, 53.99; H, 6.71; N, 4.66. Found: C, 53.95; H, 6.48; N, 4.76. ^{1}H NMR: δ 1.29 and 1.48 (Boc CH_3), 2.74/3.02 (Tyr β/β′CH_2), 2.94 (βGlc *H*-2), 3.00 (αGlc *H*-4), 3.05 (βGlc *H*-4), 3.12 (βGlc *H*-3), 3.15 (αGlc *H*-2), 3.34 (βGlc *H*-5), 3.44 (αGlc *H*-3), 3.82 (αGlc *H*-5), 3.93 (Gly CH_2), 4.00/4.21 (α,βGlc *H*-6/6′), 4.08 (Tyr αC*H*), 4.34 (βGlc *H*-1), 4.58 (αGlc 2-O*H*), 4.80 (αGlc 3-O*H*), 4.92 (αGlc *H*-1), 4.97 (βGlc 2-O*H*), 5.00 (βGlc 4-O*H*), 5.10 (αGlc 4-O*H*), 5.16 (βGlc 3-O*H*), 6.41 (αGlc 1-O*H*), 6.73 (βGlc 1-O*H*), 6.99 (Tyr N*H*), 7.08 (Tyr εC*H*), 7.34 (Tyr δC*H*), 8.44 (Gly N*H*). ^{13}C NMR: δ 27.33 and 28.22 (Boc *C*H$_3$), 36.85 (Tyr β*C*H$_2$), 40.65 (Gly *C*H$_2$), 55.67 (Tyr α*C*H), 64.88 (α,βGlc *C*-6), 69.25 (αGlc *C*-5), 70.26 (βGlc *C*-4), 70.66 (αGlc *C*-4), 72.31 (αGlc *C*-2), 73.02 (αGlc *C*-3), 73.61 (βGlc *C*-5), 78.21 and 83.18 (Boc C_q), 92.51 (αGlc *C*-1), 97.13 (βGlc *C*-1), 121.16 (Tyr ε*C*H), 130.45 (Tyr δ*C*H), 136.21 (Tyr γ*C*), 151.62 (Tyr ζ*C*), 149.43 and 151.64 (Boc *C*O), 170.23 and 172.66 (Tyr, Gly *C*O); anomer ratio α-*p*/β-*p* 58:42.

3.2.6. 6-*O*-(*N*,*O*-Di-*tert*-butyloxycarbonyl-L-tyrosylglycylglycyl)-D-glucopyranose (4). Compound **4** was obtained starting from D-glucose (1.62 g, 9 mmol), *N*,*O*-di-*tert*-butyloxycarbonyl-L-tyrosylglycylglycine pentachlorophenyl ester[27] (2.26 g, 3 mmol) and imidazole (1.02 g, 15 mmol) in dry pyridine (60 mL) and using the same procedure as described for compound **3**. The residue obtained after extraction (1.37 g) was purified by column chromatography (solvent B) to yield esterificated D-glucose (mixture of 6-*O*-, 3-*O*-, and 2-*O*-esters) (970 mg, 49%) [R_f (B) 0.36 and 0.43]. Repeated column chromatography (solvent B) gave the pure *title compound* **4** (393 mg, 20%); R_f (B) 0.36; mp 153–154 °C; $[\alpha]_D^{22}$ +26 (*c* 1.0, MeOH). Anal. calcd for $C_{29}H_{43}N_3O_{14}$: C, 52.96; H, 6.59; N, 6.39. Found: C, 53.21; H, 6.66; N, 6.33. ^{1}H NMR: δ 1.29 and 1.48 (Boc CH_3), 2.73/3.05 (Tyr β/β′CH_2), 2.92 (βGlc *H*-2), 3.00 (αGlc *H*-4), 3.04 (βGlc *H*-4), 3.13 (βGlc *H*-4), 3.17 (αGlc *H*-2), 3.35 (βGlc *H*-5), 3.47 (αGlc *H*-3), 3.77/3.79 (Gly^2 CH_2), 3.86/3.89 (Gly^{3}CH_2), 3.91 (αGlc *H*-5), 4.00/4.21 (α,βGlc *H*-6/6′), 4.16 (Tyr αC*H*), 4.33 (βGlc *H*-1), 4.91 (αGlc *H*-1), 7.01 (Tyr N*H*), 7.07 (Tyr εC*H*), 7.29 (Tyr δC*H*), 8.25 (Gly^2 N*H*), 8.29 (Gly^3 N*H*), 9.20 (Tyr O*H*). ^{13}C NMR: δ 27.4 and 28.2 (Boc *C*H$_3$), 36.8 (Tyr β*C*H$_2$), 40.6 (Gly^2 *C*H$_2$),

42.0 (Gly^3 CH_2), 55.9 (Tyr αCH), 64.9 (α,βGlc C-6), 69.3 (αGlc C-5), 70.3 (βGlc C-4), 70.7 (αGlc C-4), 72.3 (αGlc C-2), 73.1 (αGlc C-3), 73.6 (βGlc C-5), 74.9 (βGlc C-2), 76.6 (βGlc C-3), 78.4 and 83.3 (Boc C_q), 92.6 (αGlc C-1), 97.1 (βGlc C-1), 121.2 (Tyr εCH), 130.5 (Tyr γC), 136.2 (Tyr δCH), 149.5 and 155.8 (Boc CO), 151.7 (Tyr ζC), 169.7, 170.1, and 172.4 (Tyr, Gly^2, Gly^3 CO); anomer ratio α-*p*/β-*p* 51:49.

3.2.7. 6-*O*-(L-Tyrosylglycyl)-D-glucopyranose (5). Compound **3** (200 mg, 0.34 mmol) was dissolved in 90% trifluoroacetic acid solution (2 mL) containing anisole (0.3 mL). The solution was stirred at room temperature for 30 min. Addition of dry diethyl ether (0 °C) and subsequent centrifugation gave the pure **TFA×5** (174 mg, 99%); R_f (C) 0.16; RP HPLC t_R (D) 12.73 and 13.29 (**5**α and **5**β); mp 106–125 °C (dec); $[\alpha]_D^{22}$ +45.3 (*c* 2.0, EtOH). Anal. calcd for $C_{17}H_{24}N_2O_9 \times CF_3COOH \times 2H_2O$: C, 41.46; H, 5.31; N, 5.09. Found: C, 41.71; H, 5.79; N, 5.07. ^{1}H NMR: δ 2.85/3.02 (Tyr β/β′CH_2), 2.93 (βGlc H-2), 3.01 (βGlc H-3), 3.04 (βGlc H-4), 3.07 (αGlc H-4), 3.13 (αGlc H-2), 3.17 (βGlc H-5), 3.35 (αGlc H-5), 3.45 (αGlc H-3), 3.96/3.98 (Gly CH_2), 4.05 (Tyr αCH), 4.09/4.37 (α,βGlc H-6/6′), 4.31 (βGlc H-1), 4.91 (αGlc H-1), 6.71 (Tyr εCH), 7.08 (Tyr δCH), 8.14 (Tyr NH), 9.00 (Gly NH), 9.50 (Tyr OH). ^{13}C NMR: δ 38.48 (Tyr βCH_2), 40.43 (Gly CH_2), 53.52 (Tyr αCH), 64.77 (α,βGlc C-6), 68.99 (βGlc C-4), 70.02 (αGlc C-4), 70.42 (αGlc C-5), 72.08 (αGlc C-2), 72.77 (αGlc C-3), 73.34 (βGlc C-2), 74.60 (βGlc C-3), 76.31 (βGlc C-5), 92.28 (αGlc C-1), 96.88 (βGlc C-1), 115.38 (Tyr εCH), 124.67 (Tyr γC), 130.59 (Tyr δCH), 156.70 (Tyr ζC), 168.94 and 169.53 (Tyr, Gly CO); anomer ratio α-*p*/β-*p* 51:49.

3.2.8. 6-*O*-(L-Tyrosylglycylglycyl)-D-glucopyranose (6). Compound **4** (150 mg, 0.22 mmol) was deprotected by using 90% trifluoroacetic acid solution as described in the preparation of **5**, affording **TFA×6** (134 mg, 98%); R_f (C) 0.13; RP HPLC t_R (E) 12.27 and 12.95 (**6**α and **6**β); mp 127–135 °C (dec); $[\alpha]_D^{22}$ +72 (*c* 1.0, EtOH). Anal. calcd for $C_{19}H_{27}N_3O_{10} \times CF_3COOH \times 3H_2O$: C, 40.32; H, 5.48; N, 6.72. Found: C, 40.93; H, 5.51; N, 6.83. ^{1}H NMR: δ 2.84/2.99 (Tyr β/β′CH_2), 2.92 (βGlc H-2), 3.04 (αGlc H-4), 3.08 (Glc H-4), 3.13 (βGlc H-3), 3.15 (αGlc H-2), 3.36 (βGlc H-5), 3.46 (αGlc H-3), 3.77 (αGlc H-5), 3.82 (Gly^3 CH_2), 3.88 (Gly^2 CH_2), 4.01 (Tyr αCH), 4.06/4.30 (α/βGlc H-6/6′), 4.33 (βGlc H-1), 4.91 (αGlc H-1), 6.70 (Tyr εCH), 7.05 (Tyr δCH), 8.11 (Tyr NH), 8.41 (Gly^2 NH), 8.81 (Gly^3 NH), 9.42 (Tyr OH). ^{13}C NMR: δ 36.31 (Tyr βCH_2), 40.55 (Gly^2 CH_2), 41.80 (Gly^3 CH_2), 53.85 (Tyr αCH), 64.88 (α,βGlc C-6), 69.26 (αGlc C-5), 70.25 (βGlc C-4), 70.65 (αGlc C-4), 72.31 (αGlc C-2), 73.02 (αGlc C-3), 73.60 (βGlc C-5), 74.83 (βGlc C-2), 76.55 (βGlc C-3), 92.51 (αGlc C-1), 97.11 (βGlc C-1), 115.61 (Tyr εCH), 125.05 (Tyr γC), 130.77 (Tyr δCH), 156.90 (Tyr ζC), 168.86, 169.06, and 170.11 (Tyr, Gly^2, Gly^3 CO); anomer ratio α-*p*/β-*p* 55:45.

3.3. Incubation experiments and reversed-phase HPLC analysis

6-*O*-(L-Tyrosylglycyl)-D-glucopyranose (**5**) (0.8 mg, 1.9 μmol) or 6-*O*-(L-tyrosylglycylglycyl)-D-glucopyranose (**6**) (0.9 mg, 1.9 μmol) were incubated in pyridine–acetic acid (1:1, 1 mL) at 4, 22, 37, and 50 °C and in dry methanol (1 mL) at both 37 and 50 °C. For comparison, the reactivity of free peptides and D-glucose, in the same solvents, were examined at both 37 and 50 °C. In experiments with peptide–sugar molar ratio 1:1, L-tyrosylglycine (**1**) (0.7 mg, 2.8 μmol) or L-tyrosylglycylglycine (**2**) (0.8 mg, 2.8 μmol) were incubated separately with D-glucose (0.5 mg, 2.8 μmol) either in pyridine–acetic acid (1:1, 1.5 mL) or in dry methanol (1.5 mL). When the molar ratio peptide–sugar was 1:5, the peptide (2.8 μmol) and D-glucose (2.5 mg, 14 μmol) were dissolved in pyridine–acetic acid (1:1, 4.5 mL). The progress of the reaction was monitored by RP HPLC. Aliquots were withdrawn from the incubation mixtures, immediately frozen, and lyophilized. The respective samples were directly analyzed by RP HPLC (for details see Section 3.1).

3.4. Isolation of products formed during incubations

3.4.1. *cyclo*-{*N*-[-6)-1-Deoxy-β-D-fructofuranos-1-yl]-L-tyrosylglycyl-(1 → O} (7). 6-*O*-(L-Tyrosylglycyl)-D-glucopyranose (**5**) (50 mg, 0.12 mmol) was dissolved in pyridine–acetic acid (1:1, 50 mL) and the solution was stirred for 17 h at 37 °C. The solvent was evaporated off, the residue was dissolved in water (1 mL), applied to a short (10×0.8 cm) Dowex 1X2 200 (Ac) column, and eluted with water. Fractions containing *title compound* **7** were pooled, evaporated, and the residue purified by semipreparative RP HPLC (solvent D). Lyophilization and crystallization from methanol–diethyl ether gave pure *title compound* **7** (7.8 mg, 17%); R_f (C) 0.41; RP HPLC t_R (D) 15.19 min; mp 130–132 °C (dec); $[\alpha]_D$ +33 (*c* 1.0, MeOH). Anal. calcd for $C_{17}H_{22}N_2O_8 \times CF_3COOH \times H_2O$: C, 44.36; H, 4.90; N, 5.44. Found: C, 44.95; H, 5.00; N, 4.92. ^{13}C NMR for **7** (**β-*furanose***): δ 34.0 (Tyr βCH_2), 43.0 (Gly CH_2), 51.0 (Fru C-1), 61.8 (Tyr αCH), 62.9 (Fru C-6), 72.6 (Fru C-4), 77.0 (Fru C-3), 80.8 (Fru C-5), 101.6 (Fru C-2), 115.6 (Tyr εCH), 125.5 (Tyr γC), 130.2 (Tyr δCH), 156.6 (Tyr ζC), 168.2 and 169.2 (Tyr, Gly CO); only β-*f*.

3.4.2. *cyclo*-{*N*-[-6)-1-Deoxy-α,β-D-fructofuranos-1-yl]-L-tyrosylglycylglycyl-(1 → O} (8). 6-*O*-(L-Tyrosylglycylglycyl)-D-glucopyranose (**6**) (87 mg, 0.19 mmol) was dissolved in pyridine–acetic acid (1:1, 87 mL) and the solution was stirred for 48 h at room temperature. The

solvent was evaporated off, and following the isolation procedure described for compound **7**, but using solvent E, the *title compound* **8** was obtained (15 mg, 19%); R_f (C) 0.34; RP HPLC t_R (E) 14.32 min; mp 128–139 °C (dec); $[\alpha]_D^{22}$ +54.7 (*c* 0.7, MeOH). Anal. calcd for $C_{19}H_{25}N_3O_9 \times 1/2\ CF_3COOH \times H_2O$: C, 46.69; H, 5.39; N, 8.17. Found: C, 46.34; H, 5.29; N, 8.89. ^{13}C NMR for **8** (**α-*furanose***): δ 34.8 (Tyr β), 42.3 (Gly^3 *C*H_2), 43.8 (Gly^2 *C*H_2), 48.4 (Fru *C*-1), 61.9 (Tyr α*C*H_2), 62.2 (Fru *C*-6), 72.4 (Fru *C*-4), 78.2 (Fru *C*-5), 83.5 (Fru *C*-3), 103.6 (Fru *C*-2), 115.5 (Tyr ε*C*H), 124.5 (Tyr γ*C*), 130.7 (Tyr δ*C*H), 156.7 (Tyr ζ*C*); for **8** (**β-*furanose***): δ 34.6 (Tyr β*C*H_2), 41.6 (Gly^3 *C*H_2), 43.2 (Gly^2 *C*H_2), 51.8 (Fru *C*-1), 61.3 (Fru *C*-6), 61.9 (Tyr α*C*H_2), 71.8 (Fru *C*-4), 77.6 (Fru *C*-3), 82.5 (Fru *C*-5), 100.7 (Fru *C*-2), 115.5 (Tyr ε*C*H), 125.1 (Tyr γ*C*), 130.2 (Tyr δ*C*H), 156.5 (Tyr ζ*C*). For both of them 167.3, 167.5, 169.0, and 169.3 (Tyr, Gly^2, Gly^3 *C*O); anomer ratio α-*f*/β-*f* 14:86.

3.4.3. *cyclo*-(L-Tyrosylglycyl) (9). 6-*O*-(L-Tyrosylglycyl)-D-glucopyranose (**5**) (50 mg, 0.12 mmol) was dissolved in pyridine–acetic acid (1:1, 50 mL) and the solution was incubated for 17 h at 50 °C. Following the procedure described for the isolation of compound **7**, the *title compound* **9**, being identical with formerly prepared one,[28] was obtained (8 mg, 33%); R_f (C) 0.75; RP HPLC t_R (D) 18.80 and t_R (E) 22.11 min. ^{13}C NMR: δ 38.28 (Tyr β*C*H_2), 43.75 (Gly *C*H_2), 55.88 (Tyr α*C*H), 115.20 (Tyr ε*C*H), 125.98 (Tyr γ*C*), 131.30 (Tyr δ*C*H), 156.58 (Tyr ζ*C*), 166.04 and 167.68 (Tyr, Gly *C*O).

3.4.4. L-Tyrosylglycine methyl ester (10). 6-*O*-(L-Tyrosylglycyl)-D-glucopyranose (**5**) (75 mg, 0.19 mmol) was dissolved in dry methanol (90 mL) and the solution was incubated for three days at 50 °C. The solvent was evaporated off and the residue was purified by semipreparative RP HPLC (solvent D) giving the *title compound* **10** (15 mg, 37%); R_f (C) 0.46; RP HPLC t_R (D) 21.97 min. ^{13}C NMR: δ 36.29 (Tyr β*C*H_2), 40.71 (Gly *C*H_2), 51.99 (*C*H_3), 53.67 (Tyr α*C*H), 115.43 (Tyr ε*C*H), 124.67 (Tyr γ*C*), 130.62 (Tyr δ*C*H), 156.63 (Tyr ζ*C*), 168.83 and 169.86 (Tyr, Gly *C*O).

3.4.5. L-Tyrosylglycylglycine methyl ester (11). 6-*O*-(L-Tyrosylglycylglycyl)-D-glucopyranose (**6**) (100 mg, 0.22 mmol) was dissolved in dry methanol (80 mL). Incubation and purification was performed as described for **10**, but using solvent E, yielding the *title compound* **11** (23 mg, 41%); R_f (C) 0.36; RP HPLC t_R (E) 23.05 min.

3.4.6. *N*-(1-Deoxy-D-fructos-1-yl)-L-tyrosylglycine (12).

3.4.6.1. Method a: Hydrolysis of compound 7. Bicyclic Amadori compound **7** (68 mg, 0.18 mmol) was dissolved in 0.1 M NH_4OH (28 mL) and the solution was stirred at room temperature for 15 min. The solvent was removed and the residue was purified by semipreparative RP HPLC (solvent D) to give the pure *title compound* **12** (20 mg, 27%); R_f (C) 0.14; RP HPLC t_R (D) 15.59 min; mp 116–120 °C (dec); $[\alpha]_D^{22}$ +9 (*c* 1.0, MeOH). Anal. calcd for $C_{17}H_{24}N_2O_9 \times CF_3COOH$: C, 44.36; H, 4.90; N, 5.40. Found: C, 44.58; H, 4.71; N, 5.37. ^{13}C NMR for **12** (**α-*furanose***): δ 35.2 (Tyr β*C*H_2), 41.0 (Gly *C*H_2), 50.5 (Fru *C*-1), 60.7 (Fru *C*-6), 61.6 (Tyr α*C*H), 75.2 (Fru *C*-4), 81.7 (Fru *C*-5), 83.1 (Fru *C*-3), 101.0 (Fru *C*-2), 115.5 (Tyr ε*C*H), 124.9 (Tyr γ*C*), 130.6 (Tyr δ*C*H), 156.6 (Tyr ζ*C*); for **12** (**β-*furanose***): δ 35.0 (Tyr β*C*H_2), 41.0 (Gly *C*H_2), 50.9 (Fru *C*-1), 61.5 (Tyr α*C*H), 62.5 (Fru *C*-6), 74.6 (Fru *C*-4), 78.2 (Fru *C*-3), 82.4 (Fru *C*-5), 99.5 (Fru *C*-2), 115.4 (Tyr ε*C*H), 124.7 (Tyr γ*C*), 130.5 (Tyr δ*C*), 156.6 (Tyr ζ*C*); for **12** (**β-*pyranose***): δ 35.1 (Tyr β*C*H), 41.0 (Gly *C*H_2), 52.3 (Fru *C*-1), 61.6 (Tyr α*C*H), 64.0 (Fru *C*-6), 68.7 (Fru *C*-5), 69.1 (Fru *C*-4), 70.3 (Fru *C*-3), 95.4 (Fru *C*-2), 115.4 (Tyr ε*C*H), 124.7 (Tyr γ*C*), 130.5 (Tyr δ*C*H), 156.6 (Tyr ζ*C*). For all of them 167.4, 167.5, 167.6, and 170.6 (Tyr, Gly^2, Gly^3 *C*O); anomer ratio β-*p*/α-*f*/β-*f* 40:40:20.

3.4.6.2. Method b: Incubation of L-tyrosylglycine (1) and D-glucose. L-Tyrosylglycine (**1**) (120 mg, 0.5 mmol) and D-glucose (68 mg, 0.38 mmol) were dissolved in pyridine–acetic acid (1:1, 170 mL) and the solution was incubated for two days at 37 °C. The solvent was evaporated off and the residue was purified by semipreparative RP HPLC (solvent D). Crystallization from methanol–diethyl ether gave the *title compound* **12** (23 mg, 15%), which was identical (mp, optical rotation, RP HPLC retention times, and NMR data) with compound **12** obtained by Method a.

3.4.7. *N*-(1-Deoxy-D-fructos-1-yl)-L-tyrosylglycylglycine (13). Bicyclic Amadori compound **8** (42 mg, 0.1 mmol) was dissolved in 0.1 M NH_4OH (21 mL) and the solution was stirred at room temperature for 1 h. The solvent was removed and the residue was purified by semipreparative RP HPLC, using solvent E, to give the pure *title compound* **13** (20 mg, 44%), which was found to be identical (comparison of mp, optical rotation, RP HPLC retention time, and NMR data) with formerly prepared Amadori compound **13**;[29] R_f (C) 0.11; RP HPLC t_R (E) 14.47 min. ^{13}C NMR for **13** (**α-*furanose***): δ 35.2 (Tyr β*C*H_2), 41.1 (Gly^2 *C*H_2), 42.0 (Gly^3 *C*H_2), 50.3 (Fru *C*-1), 60.7 (Fru *C*-6), 61.5 (Tyr α*C*H_2), 75.2 (Fru *C*-4), 81.8 (Fru *C*-5), 83.1 (Fru *C*-3), 101.0 (Fru *C*-2), 115.5 (Tyr ε*C*H), 124.7 (Tyr γ*C*), 130.5 (Tyr δ*C* H), 156.6 (Tyr ζ*C*); for **13** (**β-*furanose***): δ 34.9 (Tyr β*C*H_2), 41.1 (Gly^2 *C*H_2), 42.0 (Gly^3 *C*H_2), 50.8 (Fru *C*-1), 61.3 (Tyr α*C*H_2), 62.6 (Fru *C*-6), 74.6 (Fru *C*-4), 78.2 (Fru *C*-3), 82.6 (Fru *C*-5), 99.5 (Fru *C*-2), 115.5 (Tyr ε*C*H), 124.7 (Tyr γ*C*), 130.5 (Tyr δ*C*H), 156.6 (Tyr ζ*C*); for **13** (**β-*pyranose***): δ 35.1 (Tyr β*C*H_2), 41.1 (Gly^2 *C*H_2), 42.0 (Gly^3 *C*H_2), 51.9 (Fru *C*-1), 61.5 (Tyr α*C*H_2), 64.0 (Fru *C*-6), 68.7 (Fru *C*-5), 69.1 (Fru *C*-4), 70.1 (Fru *C*-3), 95.6 (Fru *C*-2),

115.6 (Tyr εCH), 124.9 (Tyr γC), 130.5 (Tyr δCH), 156.6 (Tyr ζC). For all of them 167.2, 167.3, 168.4, and 171.2 (Tyr, Gly^2, Gly^3 CO); anomer ratio β-*p*/α-*f*/β-*f* 44:42:14.

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