Synthesis and ^{13}C NMR investigation of novel Amadori compounds (1-amino-1-deoxy-D-fructose derivatives) related to the opioid peptide, leucine–enkephalin

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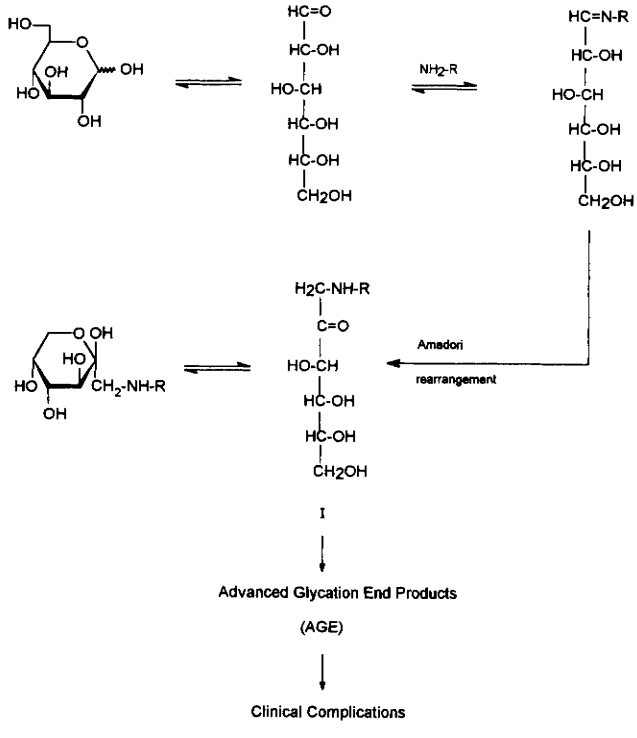
The *N*-(1-deoxy-D-fructos-1-yl) derivatives (Amadori compounds) of the endogenous opioid pentapeptide, leucine–enkephalin (11), leucine–enkephalin methyl ester (12) and of structurally related peptides (9, 10) are synthesized. The equilibrium compositions of the prepared Amadori compounds 9–12 in D_2O and [$^2H_6$]DMSO are determined using ^{13}C NMR spectroscopy. In water, the β-pyranose, α-furanose and β-furanose forms are detected, the β-pyranose tautomer being the most abundant at equilibrium (67–75%). The α-pyranose form and open-chain keto form are not detected. In dimethyl sulfoxide, the equilibrium compositions of 9–12 are markedly shifted towards a higher proportion of furanose forms, amounting to two-thirds of the mixture. In addition to the α- and β-furanoses and β-pyranose tautomers, DMSO solutions of compounds 9–12 contain at equilibrium a relatively high proportion of the acyclic hydrate (*gem*-diol) form (*ca.* 10%).

Introduction

Amadori compounds (1-amino-1-deoxy-D-fructose derivatives) play an important role in biological processes and as such are the subject of much current research interest.[1,2] How do Amadori compounds form? Monosaccharides react with N-terminal amino groups of proteins (the Maillard reaction) under physiological conditions to form glycated proteins.[3] Such reactions do not require an enzyme for catalysis and virtually any protein (including glycoproteins) can react slowly with D-glucose, a reducing sugar which is present in relatively high concentration, to yield a covalently linked reaction product. The reaction requires the lone pair electrons of the nitrogen atom of an amino group to donate to C-1 of the open-chain form of D-glucose to yield a Schiff base. This unstable aldimine undergoes an Amadori rearrangement to give a ketoamine derivative **I** (Scheme 1). Protein fructosamines slowly oxidize and rearrange under physiological conditions leading to the formation of advanced glycation end products (AGE) which participate in further protein modification (crosslinking), the production of fluorescent materials and in a variety of other reactions which directly alter protein function in target tissues.[4] Recent results provided evidence that these perturbations play a central role in the pathogenesis of diabetic complications,[5] as well as in normal aging,[6] and are also associated with a variety of diseases, including Alzheimer's disease.[7]

Although excellent studies dealing with the syntheses,[1,8–11] biological activity[12] and solution properties[2,8,11,13] of Amadori compounds derived from aliphatic and aromatic amino acids have been published, to the best of our knowledge there are only a few reports referring to the synthesis of Amadori compounds of peptides.[14–16] In an effort to better understand the role that Amadori compounds play in biologically relevant interactions, we describe herein the synthesis and solution properties of well-defined Amadori compounds related to the endogenous peptide, leucine–enkephalin[17] (Tyr-Gly-Gly-Phe-Leu) and of some structurally related peptides, as models suitable for studying the physicochemical properties and bioactivity of fructosamines formed under physiological conditions.

The peptide hormone leucine–enkephalin belongs to an important group of opioid peptides producing a wide range of central and peripheral effects, which, in addition to analgesia, include tolerance and physical dependence, respiratory depression, effects on gastrointestinal motility, cardiovascular and immune functions.[18]



Scheme 1

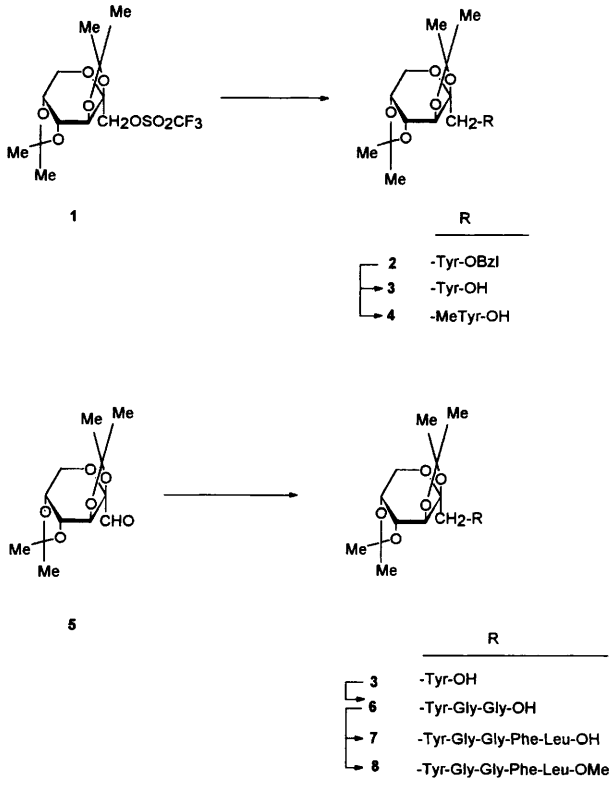
Results and discussion

Synthesis of Amadori compounds 9–12

The synthetic approach to Amadori compounds in this study was based on organic synthesis with blocked starting materials. This avoided the formation of undesirable by-products from the thermal degradation of amino acids and the Amadori compounds themselves, and also avoided the participation of the free sugar in many side-reactions which are observed in the

preparation of Amadori compounds when the amino acid in question is heated with an excess of D-glucose in alcohol–water mixtures.[2,13] The elaborate purification procedures required generally result in very poor yields of products (for fructose–tyrosine conjugate **9** the yield was $< 2\%$[13]).

Our overall strategy for the preparation of the desired compounds **9–12** included first the synthesis of the fully protected *N*-(1-deoxy-D-fructos-1-yl)tyrosine **3** and its incorporation into a solution–phase peptide synthesis. In order to prepare larger quantities of **3** it became necessary to evaluate critically previously reported methods. Thus, for the synthesis of **3** we explored two approaches as shown in Scheme 2.



Scheme 2

One route involved reaction of triflate (trifluoromethanesulfonate) derivative **1**[10] with tyrosine benzyl ester to give the corresponding blocked Amadori compound **2** in 56% yield. When the tyrosine derivative **2** was hydrogenated over Pd/C in 2-methoxyethanol (used for solubility reasons) overnight, to cleave the protection from the amino acid carboxy function, unexpectedly *N*-methylation of the tyrosine moiety occurred yielding **4** almost quantitatively. The structure of **4** was determined by NMR analysis and electron-impact mass spectrometry (MS). In the mass spectra of **4** in positive ion mode, a significant base peak (100% relative intensity) was clearly detected at *m/z* 438 ($[M + H]^+$) with a molecular weight of $C_{22}H_{31}NO_8$ (437.20) that corresponds to *N*-(1-deoxy-2,3:4,5-di-*O*-isopropylidene-β-D-fructopyranos-1-yl)-*N*-methyl-L-tyrosine (**4**). Other research groups[19–21] have reported a side-reaction of *N*-methylation during Pd-catalysed hydrogenolytic cleavage of *N*-protected amino acid moieties in methanol. It was concluded that the incomplete removal of atmospheric oxygen from the hydrogenation apparatus and the presence of Pd/C catalyst readily convert methanol to formaldehyde followed by Schiff base formation and subsequent reduction to the *N*-methylated product.

To investigate palladium catalysed *N*-methylation of compound **2** we undertook a series of experiments to discover the origin of the formaldehyde in the 2-methoxyethanol used as the solvent in the hydrogenation reaction. Quantitative estimation by a chromotropic acid method[22] revealed 1.8 mg cm^{-3} of formaldehyde in the used solvent, formed by molecular fragmentation of this molecule by prolonged standing.[23] It appears that no additional conversion of 2-methoxyethanol to formaldehyde takes place either in the presence of Pd/C catalyst and air or under hydrogenation conditions. A shorter hydrogenation time (2–3 h) for **2** provided the desired Amadori derivative **3** as the major product (80%).

The second route involved reductive amination of 2,3:4,5-di-*O*-isopropylidene-*aldehydo*-β-D-*arabino*-hexos-2-ulo-2,6-pyranose (**5**) by tyrosine in the presence of sodium cyanoborohydride[8] giving **3** in relatively good yield (56%). Despite using the rather unstable aldehyde **5** as the starting material we found this route suitable for a large scale preparation since building block **3** was obtained in one step and can be directly used for the elongation of the peptide chain.

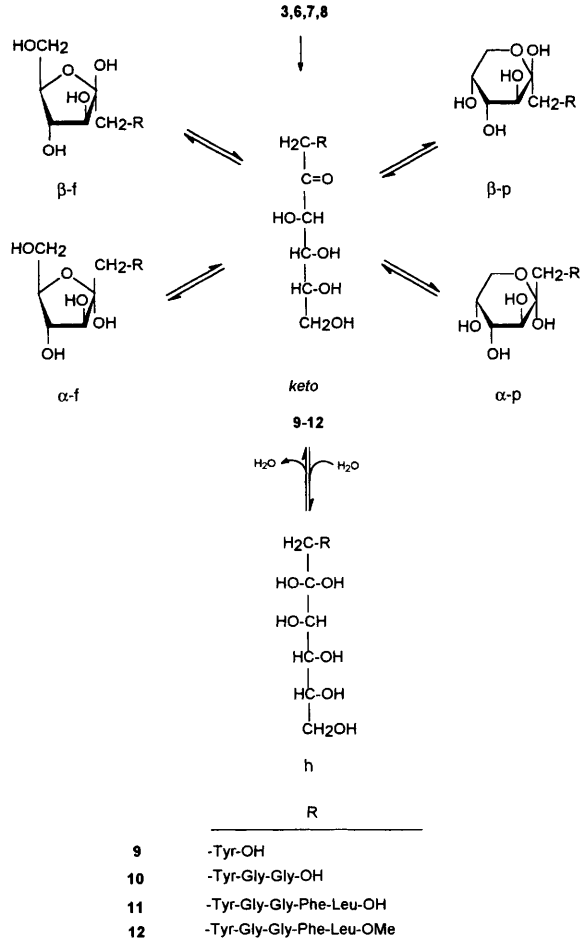
Next, coupling of the *N*-hydroxysuccinimide ester of the tyrosine derivative **3** prepared *in situ* with free glycylglycine in partially aqueous solution gave tripeptide **6** in 62% yield. To construct protected pentapeptide derivatives **7** and **8**, the Amadori tripeptide **6** was converted to the mixed anhydride by treatment with isobutyl chloroformate and allowed to react with the corresponding dipeptides (H-Phe-Leu-OH/OMe) to provide **7** and **8** in 41% and 38% yield, respectively.

Removal of the acetonide protecting groups from **3**, **6**, **7** and **8** with aq. TFA (90%) at room temperature and subsequent purification by semipreparative reversed phase HPLC (RP-HPLC) furnished target Amadori compounds **9–12** in 60–84% yield. The purity and identity of the glycoconjugates prepared were assessed by RP-HPLC, TLC, NMR and elemental analysis. The Amadori compounds prepared are relatively stable and can be stored at −20 °C for periods of several months with minimal loss of purity.

^{13}C NMR study of compounds 9–12

^{13}C NMR spectroscopy was used to determine the purity of the Amadori compounds **9–12** and the proportions of the various ring structures present after mutarotation in aqueous solution, namely, α-pyranose (α-p), β-pyranose (β-p), α-furanose (α-f) and β-furanose (β-f) (Scheme 3). The ^{1}H spectra of unprotected Amadori derivatives **9–12** were complex, the signals for the ring protons of the various structures appearing within a narrow range (*ca.* 0.2–0.3 ppm) together with the signals of the amino acid or peptide moieties, thus making the assignments ambiguous, because of second-order effects and peak overlap. The initial mutarotation required several hours to reach equilibrium after dissolving the compounds in deuteriated solvents.

The NMR spectra of Amadori compounds of various amino acids[2,8,11,13] were studied extensively and assignments of the ^{13}C chemical shifts to specific carbons of **9–12** were made on the basis of literature data or from the heteronuclear shift correlation experiments (HETCOR). All of the compounds studied show a predominance of the β-pyranose form in water in accordance with the view that the pyranose chair conformation is well accommodated into the tridimyte structure of water, particularly when the hydroxy groups are equatorially oriented.[24] ^{13}C Values for this major tautomer of **9–12** are summarized in Table 1. The ^{13}C chemical shifts of the amino acid residues were not altered significantly by *N*-glycation with the D-fructose moiety, except for that of C^{α} of tyrosine residues in **9–12** which were shifted downfield by *ca.* 10 ppm upon glycation. As seen from Table 1, the ^{13}C resonances for identical amino acid residues in compounds **9–12** are generally very similar and were not markedly affected by the pH of the solution and are in accord with those of enkephalins reported in the literature.[25,26] Regarding β-D-fructopyranose residues in **9–12**, the chemical shifts for the ring carbon signals (Table 1) were in excellent agreement with the published data



Scheme 3

Table 1 ^{13}C NMR chemical shifts (δ) of Amadori compounds **9**–**12**[a]

Residue	Carbon(s)	Compound (pH of solution) **9** (6.0)	**10** (5.8)	**11** (5.5)	**12** (5.6)
Tyr-1	α	63.1	63.8	62.9	64.3
	β	35.1	37.1	37.9	38.1
	γ	126.2	127.5	125.9	128.1
	δ	131.6	131.5	131.7	131.6
	ε	116.7	116.5	116.7	116.4
	ζ	156.0	155.7	156.1	155.5
Gly-2	α[b]		43.2	43.0	43.0
Gly-3	α[b]		44.0	43.2	43.1
Phe-4	α			55.6	53.9
	β			35.9	37.7
	γ			136.9	137.0
	δ			130.1	131.6
	ε			129.6	130.2
	ζ			128.0	129.7
Leu-5	α			52.8	53.0
	β			40.6	40.1
	γ			25.2	24.9
	δ			21.6	21.4
	δ′			23.0	22.8
CO		171.8	171.2	169.3	171.6
			172.7	171.4	172.5
			177.0	171.6	173.9
				173.6	175.4
				176.5	
D-Fru	C–1	53.4	53.0	52.8	53.7
	C–2	95.9	97.2	96.1	98.2
	C–3	71.0	70.3	70.6	70.4
	C–4	70.1	70.3	70.1	70.1
	C–5	69.6	69.8	69.7	69.9
	C–6	64.7	64.5	64.8	64.2
OMe					52.1

[a] For the major tautomeric form (β-D-pyranose) at 75.5 MHz; δ values (ppm) were determined for solutions in D_2O (25 °C) with 1,4-dioxane as internal standard (δ 66.6). [b] Assignments of signals can be interchangeable.

concerning Amadori compounds derived from aliphatic amino acids.[2]

The ^{13}C NMR spectrum of each compound in D_2O showed the presence of α- and β-furanose forms and the β-pyranose form. The ratio of the isomers (Table 2) was deduced from the distribution of the relative peak intensities. The estimated equilibrium composition of the tautomeric forms in water solution revealed that the β-pyranose form is by far the major tautomer (67–75%) in Amadori compounds **9**–**12**. No signals were observed which could be attributed either to C=O of the open chain form or to the α-pyranose form of compounds **9**–**12**, although published information concerning the tautomeric equilibrium of various Amadori compounds stated that these forms were present in amounts ranging from traces to about 6%.[2,13] Röper and co-workers[13] have also reported on the tautomeric composition for our Amadori compound **9** derived from tyrosine, and have found 10% of sugar moiety in α-pyranose form possibly due to the fact that the mutarotation equilibrium was reached at elevated temperature (65 °C). However, comparison of data listed in Table 2 with published results[13] gave the same (3:1) pyranose:furanose ratio for **9** in water. It is worth noting that in the tautomerization of tripeptide **10** and pentapeptide Amadori derivatives **11** and **12** in water, the pyranose:furanose ratio decreased to 2:1, reflecting the influence of the length of the peptide chain on the pyranose $\rightleftharpoons$ furanose equilibrium. Although, the data obtained for aqueous solutions of **10**–**12** reveal an almost constant proportion of furanose forms (31–33%), the α $\rightleftharpoons$ β furanose equilibrium was influenced by the nature of the peptide moiety (Table 2). The apparent increase in the proportion of the β-furanose form, as a result of esterification of **12**, suggests the participation of the free carboxy group in ring-opening and -closing reactions of Amadori compounds studied.

Continuing the spectroscopic studies of the tautomeric composition of Amadori compounds, we have studied the distribution of pyranose and furanose tautomers of **9**–**12** in [$^2H_6$]dimethyl sulfoxide ([$^2H_6$]DMSO) solution (Table 2). In contrast to many excellent contributions concerning the tautomeric distributions of Amadori compounds in aqueous solution,[2,8,11,13] we know of no previous NMR studies of Amadori compounds structurally related to amino acids and peptides in DMSO solution. However, the composition of D-fructose in DMSO has been extensively studied and is well documented.[24,27,28] Thus, the assignment of the signals was made by comparison with data for D-fructose[24] and for *N*-(1-deoxy-D-fructos-1-yl) derivatives of aliphatic and aromatic amines[29,30] in DMSO solution. According to ^{1}H and ^{13}C NMR spectroscopic observations, β-furanose becomes the major form (45–55%) in a DMSO solution of D-fructose[24,28] (see also Table 2). It was established[28] that intramolecular hydrogen bonding and the influence of the medium on the free energy of the β-pyranose form contribute to the relative stability of the β-furanose form of D-fructose in DMSO. It is also noteworthy that the proportion of the α-furanose tautomer increased from *ca.* 5% in water to *ca.* 20% in DMSO, whereas the α-pyranose tautomer is a trace component in either solvent.

As seen from Table 2, the observed compositions at equilibrium of the solutions of Amadori compounds **9**–**12** in [$^2H_6$]DMSO are distinctly different from those of D-fructose. The ^{13}C NMR spectra of compounds **9**–**12** showed, for each compound, the presence of the α- and β-furanose forms and the β-pyranose form in almost equal proportions whereas the α-pyranose form and the open-chain keto form were not

Table 2 ^{13}C NMR chemical shifts (ppm) of anomeric carbon resonances (δ_{C-2}) and estimated equilibrium composition of tautomeric forms of Amadori compounds **9**–**12** and D-fructose in D_2O and [$^2H_6$]DMSO solutions at 25 °C[a]

	D_2O		[$^2H_6$]DMSO	
Compound[b]	δ_{C-2}	Relative %	δ_{C-2}	Relative %
9 β-Pyranose	95.9	75	96.6	28
β-Furanose	99.6	13	101.1	27
α-Furanose	102.1	12	102.4	34
Hydrate			98.6	11
10 β-Pyranose	97.2	67	97.8	30
β-Furanose	101.0	16	101.7	26
α-Furanose	103.2	17	103.9	33
Hydrate			98.6	11
11 β-Pyranose	96.1	69	97.6	32
β-Furanose	99.8	13	101.6	24
α-Furanose	102.3	18	103.6	33
Hydrate			98.6	11
12 β-Pyranose	98.2	67	97.8	34
β-Furanose	102.0	21	101.7	25
α-Furanose	104.4	12	103.8	31
Hydrate			103.0	10
D-Fructose				
α-Pyranose			97.5	6
β-Pyranose	99.1	75	98.2	28
β-Furanose	102.8	20	102.2	45
α-Furanose	105.7	5	104.3	21

[a] For equilibrium of the tautomeric mixtures the solutions were kept at 25 °C for 8 h. [b] α-D-Fructopyranose form in compounds **9**–**12** was below the detection limit (< 3%).

observed. As reported earlier,[27] the side chain attached to the anomeric carbon atom in ketoses affects the proportion of one furanose form at equilibrium. In accordance with this observation, the results obtained with 1-deoxy-1-[(2,2-diacylvinyl)amino]-D-fructoses showed that, in contrast to D-fructose, there was no great preponderance of the β- over the α-furanose form.[30] Since the formation of two intramolecular hydrogen bonds was claimed to stabilize the β-fructofuranose form in DMSO (HO-3 with HO-6 and HO-4 with HO-1)[24] it seems that nearly equal proportions of β- and α-furanose tautomers found for Amadori compounds **9**–**12** in DMSO are possibly the consequence of the involvement of the amino group in a hydrogen bond with C=O of the *N*-substituent, that is an amino acid or peptide moiety. It has been recently suggested that the slow rotation around the C-1–C-2 bond observed for aqueous solutions of Amadori compounds of α- and β-amino acids is possibly a result of an intramolecular hydrogen bond involving the carboxy group.[2] It is interesting to note that in DMSO, compound **9** adopts equilibria with a 2.2:1 furanose:pyranose ratio, whereas compound **12** establishes a 1.6:1 equilibrium, thus demonstrating the decrease in the proportion of furanose tautomers with increasing length of peptide chain.

The ^{13}C NMR spectra in DMSO of Amadori compounds **9**–**12** (Table 2) contained in the anomeric region, besides the three C-2 signals of the α,β-furanose and β-pyranose tautomers, a fourth signal at 98.6 ppm in **9**–**11** and at 103.0 ppm in the spectra of methyl ester **12**. This resonance was assigned to C-2 of the open-chain hydrate (*gem*-diol) tautomer of compounds **9**–**12** (*h*, Scheme 3). The assignment was made on the basis of the ^{13}C NMR data for other monosaccharides, where the corresponding resonance of the open-chain hydrate lies between 90 and 104 ppm.[31,32] At the mutarotational equilibrium in DMSO, compounds **9**–**12** exist to the extent of *ca.* 10% in the acyclic hydrate form (Table 2). In order to exclude the presence of decomposition products in DMSO solutions of **9**–**12**, after obtaining NMR spectra each compound was examined by RP-HPLC and TLC, but no degradation products were observed.

A report by Baynes and co-workers[33] describes one unidentified signal in the anomeric region of the ^{13}C NMR spectra of Amadori compounds derived from poly-lysine (97.8 ppm) and RNase A (98.1 ppm) taken in D_2O–potassium phosphate buffer. No comparable resonances were observed in the spectra of simple Amadori compounds related to amino acids under the same conditions.[33] Although external phosphate ions, in contrast to internal phosphate ester groups, have no appreciable effect on the equilibrium concentration of the acyclic keto form of D-fructose,[34] NMR studies of the same sugar have shown a shift in the β-furanose ⇌ β-pyranose equilibrium when aqueous DNA is added, indicating strong hydrogen bonding of the sugar moiety with the phosphate groups of DNA.[35] These results suggest that in glycated protein molecules, due to the specific microenvironment, external phosphate ions may cause changes in tautomeric distributions in aqueous solution. Thus, by comparing our ^{13}C NMR data of compounds **9**–**12** in DMSO with that of glycated RNase A in phosphate buffer solution,[33] we assume that the 'unknown' signal observed in the latter compound could be assigned to C-2 of the open-chain hydrate form.

In conclusion, members of a new class of the Amadori compounds, related to biologically active opioid pentapeptide, leucine–enkephalin, were synthesized and their equilibrium compositions have been determined by ^{13}C NMR spectroscopy in aqueous and DMSO solutions. The information reported here on the behaviour of these compounds is of importance for a better understanding of the chemical and biochemical reactivities of Amadori compounds formed *in vivo*, particularly those that involve the free ketone forms in degradative reactions leading to protein modification and thus affecting the properties of the protein (peptide) molecule.

Experimental

Materials and methods

All solvents were distilled at the appropriate pressure. Distillation of 2-methoxyethanol did not remove formaldehyde present in this solvent. Reagents for peptide synthesis were purchased from Aldrich. Melting points were determined in capillaries and are uncorrected. Optical rotations were measured at room temperature using an Optical Activity LTD automatic AA-10 Polarimeter and $[\alpha]_D$ values are given in units of 10^{-1} deg cm^2 g^{-1}. Column chromatography was performed on silica gel (Merck, 0.040–0.063 mm) and TLC on silica gel 60 with detection with ninhydrin, the chlorine–iodine reagent, or charring with H_2SO_4. 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 cm^3 min^{-1} for analytical and 1 cm^3 min^{-1} for semipreparative separations) under isocratic conditions using buffer A [15% methanol in 0.1% trifluoroacetic acid (TFA)], buffer B (45% methanol in 0.1% TFA) and buffer C (75% methanol in 0.1% TFA). ^{13}C NMR spectra were recorded on a Varian Gemini 300 spectrometer operating at 75.5 MHz (^{13}C) and 300.1 MHz (^1H); δ values are given in ppm relative to internal dioxane standard. Mass spectra were recorded on an FT MS 2001 DD Fourier transform mass spectrometer (FTMS, Madison, WI, USA) equipped with a 3T superconducting magnet. Elemental analyses were carried out at Microanalytical Laboratory, Ruđer Bošković Institute.

N-(1-Deoxy-2,3:4,5-di-*O*-isopropylidene-β-D-fructopyranos-1-yl)-L-tyrosine benzyl ester 2

A solution of the triflate (trifluoromethanesulfonate) **1**[10] (365 mg, 0.93 mmol) in *N*,*N*-dimethylformamide (DMF) (20 cm^3) was refluxed with tyrosine benzyl ester (1020 mg, 3.7 mmol) in the presence of activated 4 Å molecular sieves (200 mg) for 30 min under nitrogen. The reaction mixture was worked up by

pouring into water (25 cm^3) and extraction with dichloromethane (8 × 10 cm^3). The organic phase was washed with water (6 × 10 cm^3), dried ($CaCl_2$) and concentrated. Chromatography of the residue on a silica gel column with benzene–ethyl acetate (3:2) as eluent, and crystallization from ethanol–water gave *title compound* **2** (266 mg, 56%), mp 106–108 °C, $[\alpha]_D$ −26 (*c* 1, $CHCl_3$); $\delta_C(CD_3OD)$ 24.6, 26.1, 26.6, 27.0 (CH_3 isopropylidene), 39.5 (Tyr β-C), 54.8 (C-1), 62.5 (C-6), 65.1 (Tyr α-C), 67.7 (CH_2 benzyl), 71.9 (C-5), 72.6 (C-3), 72.9 (C-4), 104.7 (C-2), 109.7, 110.4 (C isopropylidene), 116.7 (Tyr ε-C), 129.2 (Tyr γ-C), 129.5, 129.7, 129.8 (ar. C benzyl), 131.6 (Tyr δ-C), 137.5 (ar. C benzyl), 157.6 (Tyr ζ-C), 175.8 (Tyr C=O) (Found: C, 65.3; H, 6.8; N, 2.9. $C_{28}H_{35}NO_8$ requires C, 65.5; H, 6.9; N, 2.7%).

N-(1-Deoxy-2,3:4,5-di-*O*-isopropylidene-β-D-fructopyranos-1-yl)-L-tyrosine 3

To the solution of L-tyrosine (524 mg, 2.9 mmol) in methanol (15 cm^3) and water (15 cm^3) were added 2,3:4,5-di-*O*-isopropylidene-*aldehydo*-β-D-*arabino*-hexos-2-ulo-2,6-pyranose [8] (**5**) (1500 mg, 5.8 mmol) and sodium cyanoborohydride (365 mg, 5.8 mmol). The resulting solution was stirred for 5 h at 70 °C. The solution was cooled to room temperature, adjusted to pH 5 with 4 M HCl and evaporated. The residue was purified by silica gel column chromatography with ethyl acetate–ethanol–acetic acid–water (70:10:2:2) as eluent, to give *compound* **3** which crystallized from ethanol (680 mg, 56%), mp 202–208 °C, $[\alpha]_D$ −25 (*c* 1, methanol); $\delta_C([^2H_6]DMSO)$ 24.0, 25.4, 25.8, 26.4 (CH_3 isopropylidene), 37.3 (Tyr β-C), 52.3 (C-1), 60.5 (C-6), 63.0 (Tyr α-C), 69.7 (C-5), 70.3 (C-3), 70.4 (C-4), 103.0 (C-2), 107.5, 108.3 (C isopropylidene), 115.0 (Tyr ε-C), 127.7 (Tyr γ-C), 130.1 (Tyr δ-C), 155.9 (Tyr ζ-C), 174.6 (Tyr C=O) (Found: C, 59.4; H, 6.9; N, 3.1. $C_{21}H_{29}NO_8$ requires C, 59.6; H, 6.9; N, 3.3%).

N-(1-Deoxy-2,3:4,5-di-*O*-isopropylidene-β-D-fructopyranos-1-yl)-*N*-methyl-L-tyrosine 4

The benzyl ester **2** (100 mg, 0.195 mmol) was dissolved in 2-methoxyethanol (BDH, used for solubility reasons) (10 cm^3) and hydrogenated in the presence of acetic acid (0.5 cm^3) and 10% Pd/C (50 mg) for 24 h. The catalyst was filtered off and the filtrate evaporated. Chromatography of the residue on a silica gel column with ethyl acetate–acetic acid–water (70:2:2) afforded *title compound* **4** (44 mg, 53%), mp 70–75 °C, $[\alpha]_D$ −18 (*c* 1, methanol); $\delta_C(CD_3OD)$ 24.2, 25.4, 26.4, 26.5 (CH_3 isopropylidene), 34.2 (Tyr β-C), 42.1 (*N*-CH_3), 61.8 (C-1), 62.6 (C-6), 71.2 (C-5), 71.9 (C-3), 72.6 (Tyr α-C), 73.2 (C-4), 102.5 (C-2), 110.4, 110.7 (C isopropylidene), 116.6 (Tyr ε-C), 128.9 (Tyr γ-C), 131.2 (Tyr δ-C), 157.5 (Tyr ζ-C), 172.7 (Tyr C=O); *m/z* (EI) (rel. intensity) 438 ($[M + H]^+$, 100%) (Found: C, 60.2; H, 7.4; N, 3.2. $C_{22}H_{31}NO_8$ requires C, 60.4; H, 7.1; N, 3.2%).

N-(1-Deoxy-2,3:4,5-di-*O*-isopropylidene-β-D-fructopyranos-1-yl)-L-tyrosylglycylglycine 6

To a cool (0 °C) solution of compound **3** (423 mg, 1 mmol) in DMF (20 cm^3) *N*-hydroxysuccinimide (115 mg, 1 mmol) and 1,3-dicyclohexylcarbodiimide (DCC) (206 mg, 1 mmol) were added and the reaction mixture was stirred for 1 h at 0 °C and then overnight at room temperature. The precipitated dicyclohexylurea (DCU) was filtered off and the filtrate was added to a solution of glycylglycine (198 mg, 1.5 mmol) and Na_2CO_3 (159 mg, 1.5 mmol) in water (5 cm^3). After stirring for 1 h the mixture was cooled (0 °C) and adjusted to pH 5 with 4 M HCl and the solvent evaporated. Chromatography on silica gel with chloroform–ethanol–acetic acid (12:3:1) as eluent, and crystallization from dioxane–diethyl ether gave *compound* **6** (333 mg, 62%), mp 162–168 °C, $[\alpha]_D$ −47 (*c* 1, methanol); $\delta_C(CD_3OD)$ 24.6, 26.2, 26.5, 27.0 (CH_3 isopropylidene), 39.3 (Tyr β-C), 43.9, 44.5 (Gly,Gly CH_2), 55.4 (C-1), 62.5 (C-6), 65.7 (Tyr α-C), 71.8 (C-5), 72.6 (C-3), 72.9 (C-4), 104.3 (C-2), 109.8, 110.4 (C isopropylidene), 116.8 (Tyr ε-C), 129.2 (Tyr γ-C), 131.6 (Tyr δ-C), 157.8 (Tyr ζ-C), 171.4, 177.2 (Tyr,Gly,Gly C=O) (Found: C, 55.9; H, 6.7; N, 7.8. $C_{25}H_{35}N_3O_{10}$ requires C, 55.9; H, 6.6; N, 7.8%).

N-(1-Deoxy-2,3:4,5-di-*O*-isopropylidene-β-D-fructopyranos-1-yl)-L-tyrosylglycylglycyl-L-phenylalanyl-L-leucine 7

To a chilled solution (−15 °C) of tripeptide **6** (165 mg, 0.31 mmol) in THF (8 cm^3), *N*-methylmorpholine (NMM) (0.034 cm^3, 0.31 mmol) and isobutyl chloroformate (0.041 cm^3, 0.31 mmol) were added. The reaction mixture was stirred for 2 min at the same temperature and a precooled solution of TFA·H-Phe-Leu-OH (120 mg, 0.31 mmol) in THF (12 cm^3) containing NMM (0.068 cm^3, 0.62 mmol) was then added. The reaction mixture was stirred for 20 min at −15 °C and for 3 h at room temperature. The solvent was evaporated and the residue was purified by silica gel chromatography with ethyl acetate–ethanol–acetic acid–water (70:10:2:2) as eluent to yield *title compound* **7** (100 mg, 41%), mp 135–145 °C, $[\alpha]_D$ −25 (*c* 1, methanol); $\delta_c(CD_3OD)$ 22.4, 23.9 (Leu δ,δ′-C), 24.6 (Leu γ-C), 26.0, 26.3, 26.5, 26.9 (CH_3 isopropylidene), 38.8 (Phe β-C), 38.9 (Tyr β-C), 42.4 (Leu β-C), 43.6, 43.9 (Gly,Gly CH_2), 53.5 (Leu α-C), 55.4 (C-1), 56.4 (Phe α-C), 62.5 (C-6), 65.3 (Tyr α-C), 71.7 (C-5), 72.5 (C-3), 73.1 (C-4), 103.9 (C-2), 110.1, 110.4 (C isopropylidene), 116.8 (Tyr ε-C), 128.0 (Tyr γ-C), 128.8 (Phe ζ-C), 129.8 (Phe ε-C), 130.6 (Phe δ-C), 131.7 (Tyr δ-C), 138.8 (Phe γ-C), 157.9 (Tyr ζ-C), 171.6, 171.9, 176.0, 176.2 (Tyr,Gly,Gly,Phe,Leu C=O) (Found: C, 60.3; H, 7.0; N, 8.6. $C_{40}H_{55}N_5O_{12}$ requires C, 60.2; H, 6.9; N, 8.8%).

N-(1-Deoxy-2,3:4,5-di-*O*-isopropylidene-β-D-fructopyranos-1-yl)-L-tyrosylglycylglycyl-L-phenylalanyl-L-leucine methyl ester 8

Compound **8** was prepared by the mixed anhydride method from tripeptide **6** (326 mg, 0.61 mmol) and TFA·H-Phe-Leu-OMe (246 mg, 0.61 mmol) in the same way as described for **7**. Silica gel column chromatography with ethyl acetate–acetic acid–water (70:2:2) and crystallization from dioxane–diethyl ether afforded *compound* **8** (187 mg, 38%), mp 109–111 °C, $[\alpha]_D$ −41 (*c* 1, methanol); $\delta_c(CD_3OD)$ 22.2, 23.6 (Leu δ,δ′-C), 24.5 (Leu γ-C), 26.1, 26.5, 27.0 (CH_3 isopropylidene), 38.2 (Phe β-C), 38.9 (Tyr β-C), 41.6 (Leu β-C), 43.6, 43.8 (Gly,Gly CH_2), 52.5 ($COOCH_3$), 53.1 (Leu α-C), 55.5 (C-1), 56.1 (Phe α-C), 62.5 (C-6), 65.6 (Tyr α-C), 71.7 (C-5), 72.5 (C-3), 72.9 (C-4), 104.3 (C-2), 109.9, 110.4 (C isopropylidene), 116.8 (Tyr ε-C), 128.1 (Tyr γ-C), 129.3 (Phe ζ-C), 129.7 (Phe ε-C), 130.6 (Phe δ-C), 131.6 (Tyr δ-C), 138.5 (Phe γ-C), 157.7 (Tyr ζ-C), 171.5, 172.1, 173.8, 174.7, 177.1 (Tyr,Gly,Gly,Phe,Leu C=O) (Found: C, 60.5; H, 7.3; N, 8.6. $C_{41}H_{57}N_5O_{12}$ requires C, 60.6; H, 7.1; N, 8.6%).

General procedure for the preparation of deprotected Amadori compounds 9–12

Protected compounds **3**, **6**, **7** or **8** (0.1 mmol) were treated with TFA–water (9:1, 1 cm^3) in the presence of anisole (0.2 cm^3) for 24 h at room temperature. After addition of diethyl ether at 0 °C, the precipitate was collected by centrifugation and triturated several times with diethyl ether. The products were purified by semipreparative RP-HPLC [buffer A for **9**; buffer B–buffer C (1:1) for **11** and **12**] or gel chromatography on Sephadex G-10 (eluent water) (for **10**) and crystallized from methanol–diethyl ether to give pure *compounds* **9**–**12**. ^{13}C NMR data are given in Table 1.

***N*-(1-Deoxy-D-fructos-1-yl)-L-tyrosine 9.** Compound **9** (22 mg, 65%), mp 145–147 °C, $[\alpha]_D$ −32 (*c* 0.7, water); [lit.,[13] (dihydrate), mp 115 °C (decomp.), $[\alpha]_D$ −16 (*c* 0.5, DMSO)].

***N*-(1-Deoxy-D-fructos-1-yl)-L-tyrosylglycylglycine 10.** Compound **10** (35 mg, 77%), mp 105–110 °C (decomp.), $[\alpha]_D$ +14

(*c* 1, water) (Found: C, 49.8; H, 6.2; N, 9.1. $C_{19}H_{27}N_3O_{10}$ requires C, 49.9; H, 6.0; N, 9.2%).

***N*-(1-Deoxy-D-fructos-1-yl)-L-tyrosylglycylglycyl-L-phenylalanyl-L-leucine 11.** Compound **11** (70 mg, 84%), mp 120–125 °C (decomp.), $[\alpha]_D$ −4 (*c* 0.8, water) (Found: C, 52.0; H, 5.8; N, 8.4. $C_{34}H_{47}N_5O_{12}\cdot CF_3COOH$ requires C, 52.0; H, 5.8; N, 8.4%).

***N*-(1-Deoxy-D-fructos-1-yl)-L-tyrosylglycylglycyl-L-phenylalanyl-L-leucine methyl ester 12.** Compound **12** (53 mg, 60%), mp 112–114 °C (decomp.), $[\alpha]_D$ −13 (*c* 1, water) (Found: C, 50.3; H, 5.8; N, 7.9. $C_{35}H_{49}N_5O_{12}\cdot CF_3COOH\cdot 2H_2O$ requires C, 50.4; H, 6.2; N, 7.9%).

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