Intramolecular rearrangement of the monosaccharide esters of an opioid pentapeptide: formation and identification of novel Amadori compounds related to fructose and tagatose

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Intramolecular rearrangements leading to Amadori adducts 4 and 5 from monosaccharide esters 1–3 in which either D-glucose, D-mannose or D-galactose is linked through its C-6 hydroxy group to the C-terminal carboxy group of the endogenous opioid pentapeptide leucine-enkephalin (H-Tyr-Gly-Gly-Phe-Leu-OH) are reported. The formation of bicyclic compounds 4 and 5 from the corresponding monosaccharide esters is much faster than formation of the Amadori product from the parent free sugar and leucine-enkephalin. Bicyclic ketoses 4 and 5 are each transformed by hydrolysis into the corresponding 1-amino-1-deoxy-D-fructose (6) and -D-tagatose (7) Amadori products of leucine-enkephalin, indistinguishable by their physical and spectroscopic data from compounds 6 and 7 obtained by independent syntheses. The equilibrium compositions of the prepared Amadori compounds 4–7 in aqueous solutions have been determined by ^{13}C NMR spectroscopy.

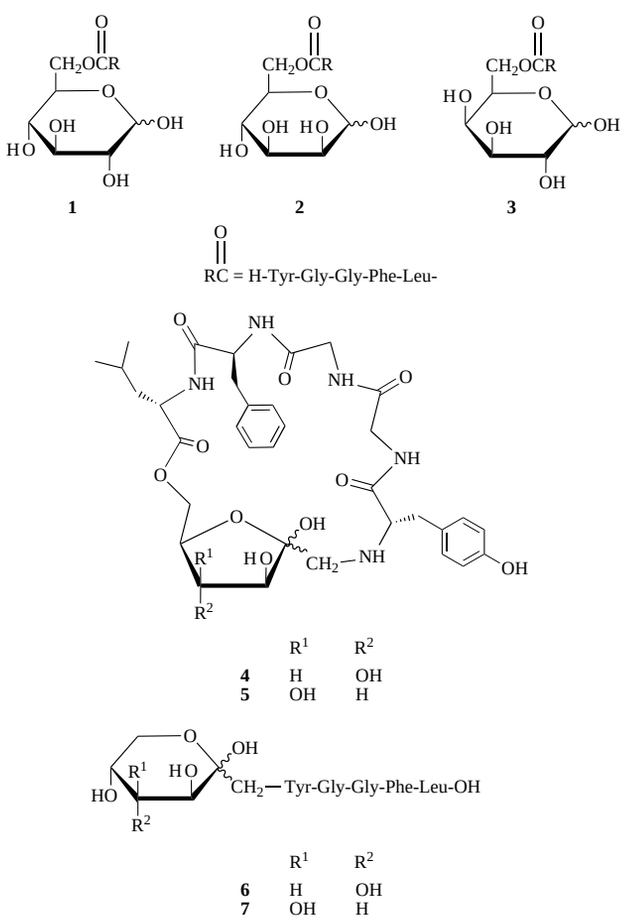
Introduction

Incorporation of saccharides into biologically active compounds is a promising field of bioorganic chemistry due to the manifold applications carbohydrate moieties offer, such as: carriers for specific delivery of therapeutic drugs and genes,[1] alteration of peptide backbone conformation,[2] and manipulation of membrane permeability and molecular recognition.[3]

We have recently described the synthesis of glycoconjugates of the opioid peptides, leucine- and methionine-enkephalin (H-Tyr-Gly-Gly-Phe-Leu/Met-OH), and demonstrated that both the type of linkage and the nature of the sugar moiety had a modifying influence on the opioid activity profile, antiviral activity and proliferative effects of the parent peptide compounds.[4] As part of these studies we have prepared and pharmacologically tested glycoconjugates **1**–**3** related to the endogenous opioid pentapeptide leucine-enkephalin in which either D-glucose (**1**), D-mannose (**2**) or D-galactose (**3**) was coupled through an ester linkage to the C-terminal carboxy group of the peptide *via* the C-6 hydroxy group of the sugar moiety.[5] Leucine-enkephalin belongs to an important group of opioid peptides producing a wide range of central and peripheral effects, including effects on gastrointestinal, cardiovascular and immune functions.[6]

Herein we report studies on the intramolecular Amadori rearrangement of monosaccharide esters **1**–**3** to the novel bicyclic compounds **4** and **5**, related to the furanose tautomers of 1-deoxy-D-fructose and -D-tagatose, respectively, covering the areas of synthesis, chromatographic analysis by reversed-phase high-performance liquid chromatography (RP-HPLC), and characterization of products by NMR spectroscopy. The purpose of the present investigation was to determine the influence of the structure of the sugar moiety in compounds **1**–**3** on the overall course of these reactions and factors that contribute to the formation of the Amadori rearrangement product, the keto amine.

In view of the current research interest on non-enzymic glycation reactions, known to occur widely in foods and biological systems (for reviews see refs. 7 and 8), and in an effort to elucidate also the properties of the parent free sugars, another objective of this study was to investigate the formation of leucine-enkephalin-related Amadori adducts **6** and **7** from either D-glucose, D-mannose or D-galactose under identical reaction conditions. In particular, knowledge about products from the reaction of D-galactose with endogenous peptides could provide a deeper insight into the mechanism of pathophysiology in hereditary galactosaemia suggested to be related to the non-enzymic galactose-dependent modification of proteins.[9]

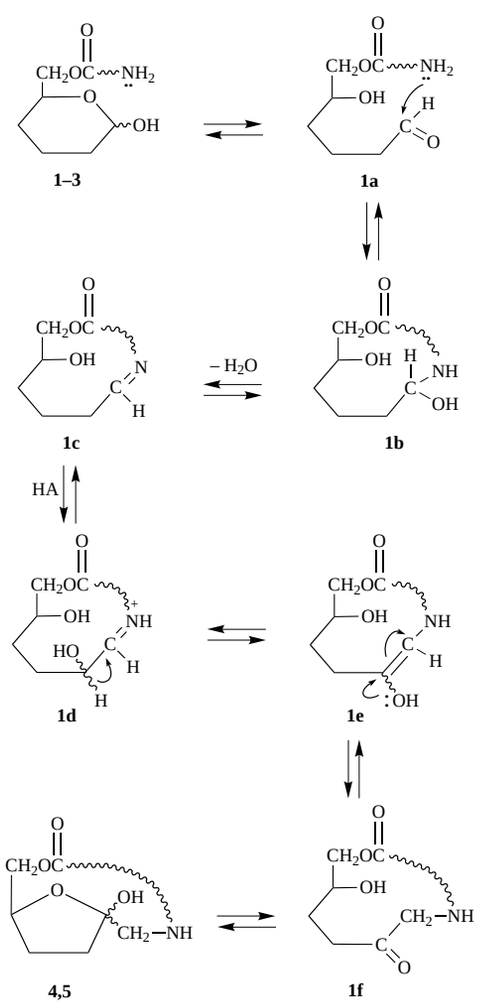


Results and discussion

Syntheses of Amadori compounds

Incubation of either 6-*O*-(H-Tyr-Gly-Gly-Phe-Leu-)-D-glucopyranose **1** or 6-*O*-(H-Tyr-Gly-Gly-Phe-Leu-)-D-mannopyranose **2** in pyridine–acetic acid (1 : 1) mixture at ambient temperature for 24 h afforded, after purification by semipreparative RP-HPLC, the D-fructose-related bicyclic Amadori product **4** in 58 and 50% yield, respectively. The rearrangement of 6-*O*-(H-Tyr-Gly-Gly-Phe-Leu-)-D-galactopyranose **3** carried out in the same solvent system at 4 °C for 24 h afforded D-tagatose-related compound **5** in 20% yield. The structures of products **4** and **5** were confirmed by NMR analysis. The spectroscopic data are discussed in detail below.

The formation of Amadori compounds **4** and **5** from the monosaccharide esters **1**–**3** can be explained as follows (Scheme 1). The Amadori rearrangement is initiated by protonation of the ring oxygen, resulting in acyclic sugar form **1a** which is nucleophilically attacked by the amino terminus of the peptide moiety. After dehydration of the tetrahedral intermediate **1b**, protonation of the Schiff base **1c** yields iminium cation **1d**. Deprotonation of C-2 results in the formation of the cyclic enamine adduct **1e** from which keto-enol tautomerism generates C=O moiety **1f**. This species upon nucleophilic attack by the C-5 hydroxy group gives rise to the bicyclic ketoses **4** and **5** in the α- and β-furanose forms.



Scheme 1

Hydrolysis of compounds **4** and **5** (0.1 M NH_4OH) resulted in *N*-(1-deoxy-D-fructos-1-yl) (**6**, 55%) and *N*-(1-deoxy-D-tagatos-1-yl) (**7**, 39%) derivatives of the leucine-enkephalin, indistinguishable by their spectroscopic data and optical rotations from compounds **6** and **7** obtained by the chemical synthesis described below. Amadori compound **6** was obtained by following the reaction sequence described by Jakas and Horvat.[10] For the preparation of stereoisomer **7**, D-galactose was treated with the leucine-enkephalin acetate salt in dry methanol for 3 days at 50 °C. Purification by RP-HPLC afforded the desired Amadori compound **7** in moderate yield (32%). It appeared that compound **7** could not be detected in the reaction mixture when leucine-enkephalin in the free-base form was used. This observation strongly supports the Amadori rearrangement mechanism proposed by de Kok and Rosing[11] according to which acid catalysts (phosphate ion, acetate, citrate) dramatically accelerate the rate of the reaction by passing the proton from the C-terminal carboxy group of the peptide onto the reactive site of the sugar/peptide adduct. The structures of products **6** and **7** were confirmed by NMR spectroscopy (see Structural analyses section).

Table 1 Relative abundance of the products formed by Amadori rearrangement of monosaccharide esters **1**–**3** as well as from free sugars[a]

Entry	Starting compound	Amadori product	Yield (%) Reaction time (*t*/h) 3	24
1	**1**	**4**	37	70
2	**2**	**4**	26	67
3	**3**	**5**	31 (37)[b]	29 (56)[b]
4	Glc	**6**	4	13
5	Man	**6**	7	19
6	Gal	**7**	6	23

[a] Determined by RP-HPLC (*cf*. Experimental for details). [b] The value in parenthesis presents the yield corrected for decomposition of compound **5** under the same reaction conditions.

HPLC Studies

Once chromatographic and spectroscopic data were established for compounds **4**–**7**, we studied the Amadori rearrangement of esters **1**–**3**, as well as of their parent sugars, by RP-HPLC. The starting compounds (see Table 1) were incubated in pyridine–acetic acid (1 : 1) at 22 °C. Chromatograms were obtained directly from the lyophilized samples taken from the reaction mixtures after 3 h and 24 h of incubation. The relative intensities of the HPLC peaks clearly show (Table 1) that the formation of bicyclic products **4** and **5** from the esters **1**–**3** is favoured compared with Amadori-product formation from glucose, mannose or galactose and leucine-enkephalin. It appears that the esterified carboxy group of leucine-enkephalin *per se* does not account for the increased reactivity of compounds **1**–**3** since leucine-enkephalin methyl ester incubated with D-glucose under identical reaction conditions showed similar reactivity as the carboxy-unprotected pentapeptide (data not shown). The behaviour of esters **1**–**3** closely resembled that found for D-glucose 6-phosphate which reacted considerably faster with amines than did D-glucose itself.[12] Formation of the Amadori compounds requires the open-chain form of the reducing sugar, and it can be assumed that, similarly to D-glucose 6-phosphate,[13] the acyclic forms of compounds **1**–**3** are either more abundant in solution or more efficiently trapped, whereafter the equilibria producing the acyclic forms are rapidly restored. Among the Amadori compounds studied, the tagatose-related product **5** is the most unstable, giving rise to a significant amount of degradation products after longer incubation times (Table 1).

Structural analyses

^{13}C NMR spectroscopy was used to confirm the structures of Amadori compounds **4**–**7** and the proportions of the various tautomers present after mutarotation in solution. The assignment of the signals was made by comparison with those reported previously for the *N*-(1-deoxy-D-fructos-1-yl) deriv-

ative of leucine-enkephalin (**6**)[10] or for Amadori compounds of amino acids,[14] on the basis of literature data for D-fructose[15] and D-tagatose,[15,16] and by using chemical-shift-correlation spectroscopy (COSY) and ^{1}H-^{13}C COSY experiments.

The ^{13}C NMR spectra of a solution of bicyclic compounds **4** and **5** in D_2O showed, for each compound, the presence of the α- (α-*f*) and β-furanose (β-*f*) form. The major constituents of the D-tagatose-related Amadori compound **7** in D_2O solution were the α- (α-*p*) and β-pyranose (β-*p*) forms as well as the α-furanose (α-*f*) form. The ^{13}C chemical shifts for these tautomers of **4**, **5** and **7** are summarized in Table 2.

According to integrations of the ^{13}C signals (average of the respective sets), for compound **4** the β-*f* $\rightleftharpoons$ α-*f* equilibrium was shifted to the β-*f* side with an 80 : 20 preference, whereas compound **5** established a 47 : 53 β-*f* $\rightleftharpoons$ α-*f* equilibrium. When comparing the equilibrium composition of compound **4** with those of D-fructose[17] and D-fructose 6-phosphate[18] in aqueous solution, close resemblance in their α : β furanose ratio (~1 : 4) was noted. It is noteworthy, however, that the data reported for the 1-deoxy-D-fructose residue in Amadori compounds derived from amino acids showed approximately equal proportions of the α,β-furanose forms present at equilibrium in aqueous solution (61% β-pyranose, 16% α-furanose, 15% β-furanose and 6% α-pyranose).[14] The apparent increase in the proportion of the β-furanose form in compound **4**, as a result of C-6 esterification of the sugar moiety with the *C*-terminal carboxy group of the leucine-enkephalin suggests the participation of the free carboxy group in ring-opening and -closing reactions in D-fructose-related Amadori compounds. The perturbation of the equilibrium tautomeric composition in water, such that the α-*f* form is destabilized in favour of the β-*f* tautomer, was also observed as a result of carboxy-group esterification of the *N*-(1-deoxy-D-fructos-1-yl) derivative of leucine-enkephalin (compound **6**).[10]

In contrast to compound **4**, D-tagatofuranose-related Amadori compound **5** shows a marked increase in the content of the α-*f* form (53%). It is interesting to note that the amount of the α-*f* form in aqueous solutions of the structurally related D-tagatose 6-phosphate or D-tagatose 1,6-bisphosphate is notably smaller (20%)[19] than that found in D_2O solution of compound **5**. The contrathermodynamic arrangement of the ring supstituents in compound **5** strongly destabilizes the cyclic furanose forms, and the ^{13}C spectrum of compound **5** revealed, in addition to two dominant sets of signals, the presence of three additional minor forms (in almost equal proportions), accounting for ~20% of the total mixture. The Amadori compound **5** was rigorously purified before recording of the NMR spectra, thus on the basis of the established instability of compound **5** (Table 1) we assume that the additional signals observed may originate from the degradation products. In the anomeric region, in addition to the **5α** and **5β** C-2 signals at δ_C 104.8 and 101.6, three new resonances of the minor forms were observed, namely at δ_C 103.7, 105.3 and 105.8 (data not shown in Table 2). The chemical shifts of the signals at δ_C 103.7 and 105.8 parallel those of previously reported data for the OH-1 unprotected β- and α-furanose isomers of either D-tagatose[15,16] or D-tagatose 6-phosphate.[19] Since no signals attributable to the α- and β-pyranose forms of Amadori compound **7**, eventually formed by ester bond hydrolysis of **5**, were observed, the two minor forms present in the solution of compound **5** were presumably formed by hydrolysis of the C-1 CH_2NH bond while the C-6 ester bond of the tagatose moiety remained intact. The signal of the third minor form in the anomeric region at δ_C 105.3 was assigned to C-2 of the open-chain hydrate (*gem*-diol) tautomer of compound **5**. The open-chain *keto* form was not detected within the sensitivity limits of the measurement (~2%). The assignment was made on the basis of ^{13}C NMR studies on furanose ring anomerization[20] as well as on the basis of the NMR data obtained for the *N*-(D-fructosyl) derivative of leucine-enkephalin (compound **6**) and for the corresponding methyl ester, which at the mutarotational equilibrium in dimethyl sulfoxide (DMSO) exist, to the extent of ~10% in the acyclic hydrate form.[10] Evidence for the presence of the acyclic *keto* form in the solution of compound **6** has recently been provided by Fourier transform infra-red (FTIR) spectroscopy,[21] as well.

The estimated equilibrium of the *N*-(1-deoxy-D-tagatos-1-yl) derivative of leucine-enkephalin (compound **7**) revealed that the α-pyranose form is the major tautomer (58%) in aqueous solution, whereas the β-pyranose and α-furanose forms account for 30 and 12%, respectively. No signals were observed which could be attributed to either the β-furanose or to the open-chain form of compound **7**. As we are not aware of previous NMR studies of Amadori compounds related to 1-amino-1-deoxy-D-tagatose derivatives of amino acids or peptides, we compared the results obtained for compound **7** with those published for the structurally analogous D-tagatose. It appears that the composition of compound **7** at equilibrium is distinctly different from those reported for either D-tagatose (α-*p* 78%, β-*p* 14%, β-*f* 5%, α-*f* 2%, acyclic form <0.5%)[16] or 1-deoxy-D-tagatose (α-*p* 73.5%, β-*p* 6.3%, α-*f* 20.2%)[22] in aqueous solution. Although the all-*cis* substitution pattern is expected to destabilize the α-furanose form of D-tagatose derivatives strongly, it is conceivable that interaction(s) between the ketose and peptide moiety in Amadori compounds **5** and **7** may change the relative populations of tautomers by inducing effects that cannot at present be defined without more detailed conformational analysis.

To conclude, in this study we examined the susceptibility of the *gluco*, *manno* and *galacto* 6-*O*-peptidyl-D-glycopyranoses toward intramolecular rearrangement leading to novel bicyclic Amadori compounds. The results show that esterification of the primary hydroxy group of the monosaccharide moieties greatly accelerates the formation of the Amadori rearrangement products, presumably due to the increased abundance of the acyclic *aldehydo* form in solution. Unequivocal structural characterization for the novel bicyclic Amadori compounds was obtained *via* independent synthesis of the related 1-amino-1-deoxy-D-fructose and -D-tagatose derivatives of the leucine-enkephalin. The tautomeric compositions of the Amadori compounds studied, determined by ^{13}C NMR spectroscopy in aqueous solution, suggest that the attached pentapeptide may be responsible for the perturbation of equilibrium tautomeric mixtures of the parent ketose sugars.

Experimental

Materials and methods

All solvents were distilled at the appropriate pressure. Mps were determined in capillaries and are uncorrected. Optical rotations were measured at room temp. using an Optical Activity LTD automatic AA-10 Polarimeter and $[a]_D$-values are given in units of 10 cm^{-1} deg cm^2 g^{-1}. 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 1.1 cm^3 min^{-1} for semipreparative separations) under isocratic conditions using buffer A [57% methanol in 0.1% trifluoroacetic acid (TFA)] and buffer B (40% MeOH in 0.1% TFA) with UV detection at 280 nm. ^{13}C NMR spectra were recorded on a Varian Gemini 300 spectrometer operating at 75.5 MHz (^{13}C) and 300.1 MHz (^{1}H); δ-values are given in ppm relative to internal 1,4-dioxane standard. Elemental analyses were carried out at the Microanalytical Laboratory, Ruđer Bošković Institute.

The leucine-enkephalin acetate salt was purchased from Sigma (St. Louis, USA). 6-*O*-(L-Tyrosyl-glycylglycyl-L-phenylalanyl-L-leucyl)-D-glucopyranose **1**, 6-*O*-(L-tyrosyl-glycylglycyl-L-phenylalanyl-L-leucyl)-D-mannopyranose **2** and 6-*O*-(L-tyrosyl-glycylglycyl-L-phenylalanyl-L-leucyl)-D-galactopyranose **3**

Table 2 ^{13}C NMR spectral data for Amadori compounds **4**, **5** and **7**[a]

		Compound and tautomeric form						
		4		**5**		**7**[b]		
Residue	Carbon atom	α-*f*	β-*f*	α-*f*	β-*f*	α-*p*	β-*p*	α-*f*
Ketose[c]	C-1	50.9	52.1	51.5	50.1	52.9	52.9	51.3
	C-2	103.4	100.4	104.8	101.6	95.8	96.7	103.7
	C-3	83.3	77.7	78.6	73.5	73.3	66.3	79.4
	C-4	77.2	74.5	72.0	71.9	71.5	71.4	71.9
	C-5	80.5	79.3	78.6	78.0	66.4	69.7	80.5
	C-6	67.2	67.4	64.7	64.9	63.4	61.0	60.8
Tyr[1]	α	63.5	63.8	63.1	63.2	63.3	(62.7)	
	β	36.4	36.2	36.3	36.1	36.0	(35.9)	
	γ	130.2	130.2	127.6	127.6	126.0	(125.9)	
	δ	131.7	131.7	131.7	131.7	131.8	131.8	131.8
	ε	116.8	116.6	116.6	116.7	116.9	(116.8)	
	ζ	156.0	155.9	155.8	155.9	156.3	(156.2)	
Gly[2]	α	42.7	42.7	42.9	43.5	42.9	42.9	42.9
Gly[3]	α	44.0	44.1	43.6	43.7	43.2	43.2	43.2
Phe[4]	α	55.3	55.2	55.3	55.8	55.6	55.6	55.6
	β	38.4	38.4	37.3	37.9	37.8	37.8	37.8
	γ	137.6	137.5	137.4	137.5	137.1	137.1	137.1
	δ	130.3	130.3	130.1	130.0	130.2	130.2	130.2
	ε	129.7	129.7	129.7	129.7	129.7	129.7	129.7
	ζ	127.6	128.1	128.1	128.1	128.1	128.1	128.1
Leu[5]	α	52.9	53.0	52.5	52.9	52.2	52.2	52.2
	β	39.3	39.4	39.2	39.7	40.2	40.2	40.2
	γ	25.0	25.1	24.93	24.87	25.0	25.0	25.0
	δ	21.5	21.5	21.2	21.2	21.4	21.4	21.4
	δ′	22.8	22.8	22.9	22.8	22.9	22.9	22.9
CO		172.6	171.9	(172.0, 172.4, 173.8,		169.6	169.6	169.6
		173.9	172.3	173.9, 174.7, 176.1)[d]		171.6	171.6	171.6
		174.0	174.1			171.8	171.8	171.8
		174.9	175.4			173.8	173.8	173.8
						176.8	176.8	176.8

[a] Solvent: D_2O; δ [ppm] chemical shifts with 1,4-dioxane as internal standard (δ_C 66.6). [b] The values in parenthesis refer to the chemical shifts of the β-*p* and/or α-*f* tautomers. [c] Ketose: 1-deoxy-D-fructos-1-yl for **4**; 1-deoxy-D-tagatos-1-yl for **5** and **7**. [d] The values in parenthesis refer to the α- and β-*f* tautomers.

were obtained under the conditions described by Horvat *et al.*[5] *N*-(1-Deoxy-D-fructos-1-yl)-L-tyrosyl-glycylglycyl-L-phenylalanyl-L-leucine **6** was prepared by the procedure of Jakas and Horvat.[10]

cyclo-{*N*-[-6)-1-Deoxy-α,β-D-fructofuranos-1-yl]-L-tyrosyl-glycylglycyl-L-phenylalanyl-L-leucyl-(1 ⟶ *O*} 4

Monosaccharide ester **1** or **2** (71 mg, 0.1 mmol) was dissolved in dry pyridine–acetic acid (1:1; 35 cm^3) and the solution was stirred for 24 h at room temp. The solvent was evaporated off, and the residue was dissolved in 25% MeOH (1 cm^3), applied to a short (10 × 0.8 cm) Dowex 1X2 200 (Ac) column, and eluted with water. Fractions containing product were pooled, evaporated and purified by semipreparative RP-HPLC using buffer A. Fractions containing compound **4** were combined, desalted on an anion-exchange resin (Dowex 1X2 200, Ac) and crystallized from MeOH–dry diethyl ether to give the pure *title compound* **4** (**1** ⟶ **4**, 41 mg, 58%; **2** ⟶ **4**, 35 mg, 50%), mp 142–146 °C (decomp.); $[\alpha]_D$ +54 (*c* 1, MeOH); for ^{13}C NMR data see Table 2 (Found: C, 58.5; H, 6.6; N, 10.0. $C_{34}H_{45}N_5O_{11}$ requires C, 58.4; H, 6.5; N, 10.0%).

cyclo-{*N*-[-6)-1-Deoxy-α,β-D-tagatofuranos-1-yl]-L-tyrosyl-glycylglycyl-L-phenylalanyl-L-leucyl-(1 ⟶ *O*} 5

Ester **3** (50 mg, 0.07 mmol) was dissolved in a chilled solution (0 °C) of dry pyridine–acetic acid (1:1; 25 cm^3) and kept at 4 °C for 20 h. After evaporation off of the solvent the residue was treated in the same way as described for compound **4** to give pure *compound* **5** (10 mg, 20%), mp 138–142 °C (decomp.); $[\alpha]_D$ −14 (*c* 1, MeOH); for ^{13}C NMR data see Table 2 (Found: C, 54.9; H, 6.8; N, 9.1. $C_{34}H_{45}N_5O_{11}\cdot CH_3CO_2H\cdot 1.5H_2O$ requires C, 54.9; H, 6.7; N, 8.9%).

Hydrolysis of compounds 4 and 5

Bicyclic Amadori compounds **4** and **5** (70 mg, 0.1 mmol) were dissolved in 0.1 M NH_4OH (14 cm^3) and the solution was stirred at room temp. for 1 h. The solvent was removed and the residue was purified by semipreparative RP-HPLC using buffer B as the eluent to furnish the corresponding Amadori compounds **6** and **7** (**4** ⟶ **6**, 39 mg, 55%; **5** ⟶ **7**, 28 mg, 39%) which were found to be identical with authentic samples of *N*-(1-deoxy-D-fructos-1-yl)-L-tyrosyl-glycylglycyl-L-phenylalanyl-L-leucine **6** and *N*-(1-deoxy-D-tagatos-1-yl)-L-tyrosyl-glycylglycyl-L-phenylalanyl-L-leucine **7** by comparison of their mp, optical rotation, RP-HPLC retention times and NMR data.

***N*-(1-Deoxy-D-tagatos-1-yl)-L-tyrosyl-glycylglycyl-L-phenylalanyl-L-leucine 7**

D-Galactose (490 mg, 2.72 mmol) was suspended in dry MeOH (200 cm^3), the suspension was ultrasonicated for 10 min, and then the leucine-enkephalin acetate salt (AcOH·H-Tyr-Gly-Gly-Phe-Leu-OH) (100 mg, 0.16 mmol) was added. The solution was kept in a well-closed, round-bottom flask for 3 days at 50 °C. After removal of the solvent, the residue was purified by semipreparative RP-HPLC using buffer A as the eluent to provide *compound* **7**, which was crystallized from MeOH–dry diethyl ether (38 mg, 32%), mp 115–120 °C (decomp.); $[\alpha]_D$ +15 (*c* 1, MeOH); for ^{13}C NMR data see Table 2 (Found: C, 57.1; H, 6.7; N, 9.9. $C_{34}H_{47}N_5O_{12}$ requires C, 57.1; H, 6.7; N, 9.9%).

RP-HPLC Analysis of the Amadori rearrangement

Monosaccharide esters **1**, **2** and **3** (2 mg, 2.8 μmol) were each incubated in pyridine–acetic acid (1:1; 1 cm^3) at 22 °C. Free sugars (D-glucose, D-mannose, D-galactose) (1 mg, 5.6 μmol) dissolved in pyridine–acetic acid (1:1; 2 cm^3) were each

incubated with leucine-enkephalin acetate salt (3.4 mg, 5.6 μmol) at 22 °C. In parallel experiments, in order to monitor decomposition of the formed products, Amadori compounds **4**–**7** (2 mg, 2.8 μmol) were also incubated in pyridine–acetic acid (1 : 1; 1 cm^3), at the same temperature. Samples were withdrawn from the incubation mixtures after 3 h and 24 h intervals, immediately frozen, and lyophilized. The respective samples were directly analysed by RP-HPLC using buffer A as the eluent.

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