Synthesis and characterization of mannose-related imidazolidinones formed by the intramolecular rearrangement of the mannopyranose ester of leucine-enkephalin

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Detailed scrutiny of the intramolecular reactivity of mannopyranose ester **1**, a neoglycopeptide in which D-mannose is linked to leucine-enkephalin through an ester bond involving the carboxylic function of the C-terminal leucine residue and the hydroxylic function at C-6 in the D-mannopyranose moiety, demonstrates for the first time that, in addition to intramolecular Amadori rearrangement (in Py–HOAc), an alternative pathway is possible. Thus, incubation of **1** in methanol or water as solvent gives the previously unknown bicyclic mannose-related imidazolidinone **2**. Its formation is studied as a function of solvent and temperature. Hydrolysis of the ester linkage in bicyclic compound **2** gives the novel mannose-related imidazolidinone **3**, in almost quantitative yield.

Introduction

It is well known that monosaccharides, under physiological conditions, can react non-enzymically, as well as enzymically, with the N-terminal amino group of proteins. The process is known as the Maillard reaction[1] in which the initial step is the formation of a Schiff base, followed by its rearrangement to a more stable keto amine or Amadori product.[2]

In our ongoing efforts to unravel the reactivity of individual sugar components in the Maillard reaction, we have studied the chemical behaviour of different monosaccharide esters in which leucine-enkephalin (H-Tyr-Gly-Gly-Phe-Leu-OH), an endogenous opioid pentapeptide exhibiting a broad spectrum of biological activities,[3] is linked, through its C-terminal carboxy group, to the C-6 hydroxy group of D-glucose, D-mannose and D-galactose.[4] We found that these monosaccharide esters under certain reaction conditions readily undergo intramolecular rearrangement. Thus, in pyridine–acetic acid solution a novel type of bicyclic Amadori compound was obtained,[5] while in methanol the aforementioned monosaccharide esters of leucine-enkephalin gave the corresponding carbohydrate-related imidazolidinones, demonstrating for the first time that, in addition to Amadori rearrangement, an alternative pathway for the carbohydrate-induced modification of peptides is possible.

In this paper we substantiate our previous[6] work by investigating the intramolecular rearrangement reactions of the D-mannopyranose ester of leucine-enkephalin, compound **1** (Scheme 1). We study the effects of solvent and temperature on the formation of carbohydrate–peptide adducts from ester **1**. For comparison we also determine the products formed in the reaction of free D-mannose with leucine-enkephalin under the same conditions. Convenient procedures for the synthesis of the mannose-related imidazolidinones **2** and **3** from the parent ester **1** are described, their stabilities are discussed, and their structural properties are deduced from NMR and MS spectra.

Results and discussion

Preparation of mannose-related imidazolidinones 2 and 3

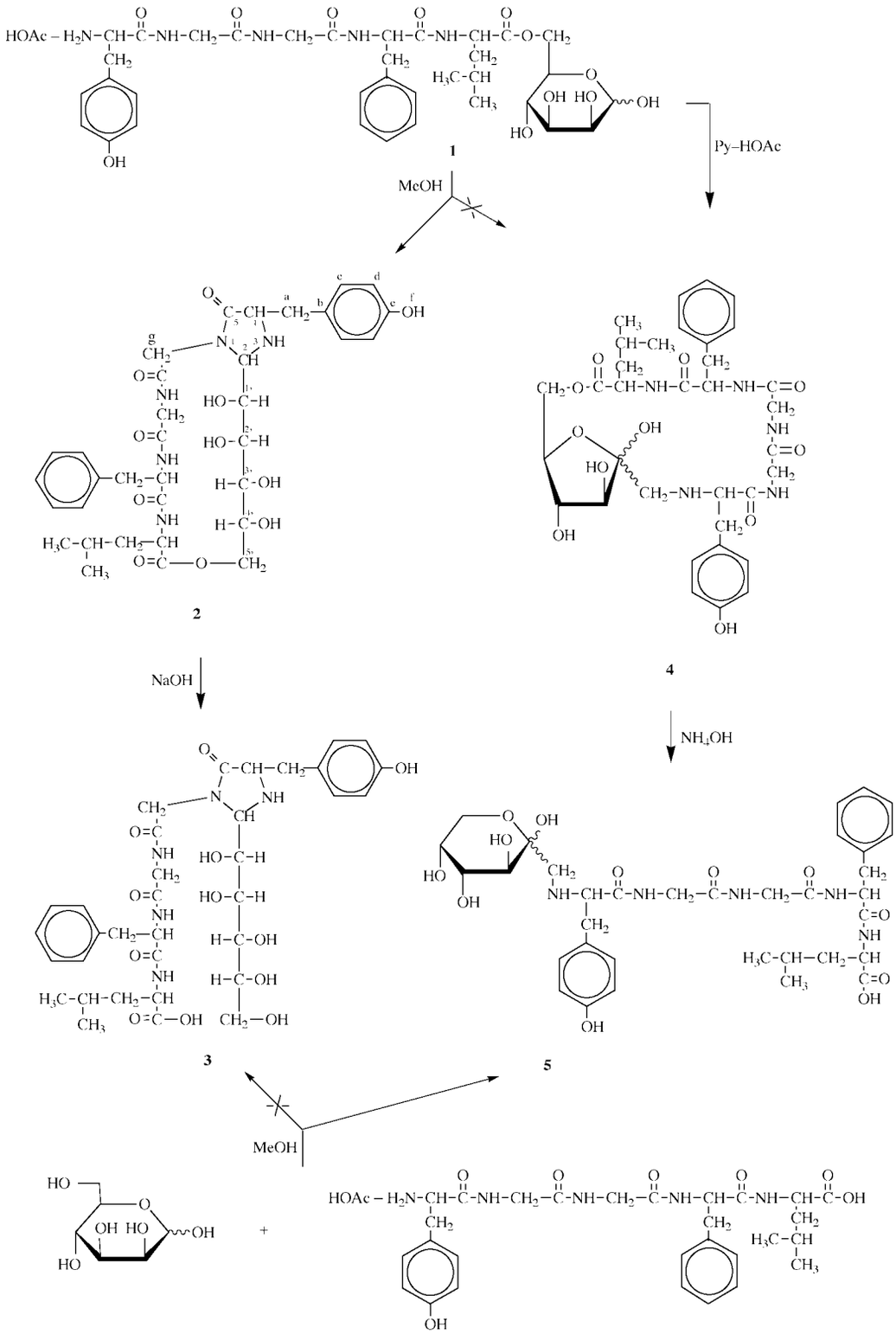
Imidazolidinone formation by rearrangement of mannopyranose ester **1** was studied as a function of temperature and solvent. The results are given in Table 1. Incubation of **1** in water affords imidazolidinone **2** in low yield. Simultaneously, mannopyranose ester **1** was hydrolysed, resulting in leucine-enkephalin liberation. In contrast the incubation of **1** in methanol gave mannose-related imidazolidinone **2** as the main product. Increasing the temperature accelerated the imidazolidinone-ring formation. Thus, at 50 °C the reaction equilibrium was attained after 24 h, while at 37 °C the maximum yield was obtained after 5 days. We recently showed[5] that the mannopyranose ester **1** (in pyridine–acetic acid) gave, as the main product, the bicyclic Amadori compound **4**, due to intramolecular rearrangement. In contrast, the incubation of **1** in methanol gave no trace of **4** at all.

Table 1 Time course for the formation of bicyclic mannose-related imidazolidinone **2** and leucine-enkephalin (Leu-E) liberation during the intramolecular rearrangement of mannopyranose ester **1** in water and methanol

		Compound (%)[a]					
		Water			Methanol		
T/°C	Incubation time/days	**1**	**2**	**Leu-E**	**1**	**2**	**Leu-E**
37	1	83	8	9	56	38	
	2	78	8	17	46	47	1
	5	39	7	37	19	67	3
50	1	55		29	26	60	2
	2	45		39	24	61	2
	5	12		66	15	64	3

[a] Quantitation was done by measuring the peak area in RP-HPLC chromatograms.

Scheme 2 gives the proposed mechanism for the formation of bicyclic mannose-related imidazolidinone **2** from mannopyranose ester **1**. Similarly to the formation of bicyclic Amadori compound **4**,[5] the process is initiated by nucleophilic attack of the amino terminus of the peptide moiety on the acyclic sugar form **1a**. Dehydration of the tetrahedral intermediate **1b** yields Schiff base **1c**. In pyridine–acetic acid, under well balanced acid-base catalysis, Schiff base **1c** gave, through keto–enol tautomerism, cyclic Amadori rearrangement product **4**.[5] In methanol the catalytic effect of the solvent is missing, and



Scheme 1

instead Schiff base **1c** is trapped intramolecularly by the amide moiety, resulting in the formation of bicyclic mannose-related imidazolidinone **2**.

To determine the reactivities of the parent peptide and carbohydrate moiety in terms of imidazolidinone ring formation, leucine-enkephalin and D-mannose were incubated in methanol at 50 °C for 24 h. Although we expected to obtain mannose-related imidazolidinone **3**, only traces of this compound were detected, while the main product (25%) was the previously prepared Amadori compound.[5] One could speculate that the free peptide COOH group effectively catalyzes the rate-determining step in the Amadori rearrangement to compound **5** due to intramolecular protonation. A similar catalytic effect of acids (especially carboxylic acids) on the Amadori rearrangement has been established previously in different model systems.[2,7]

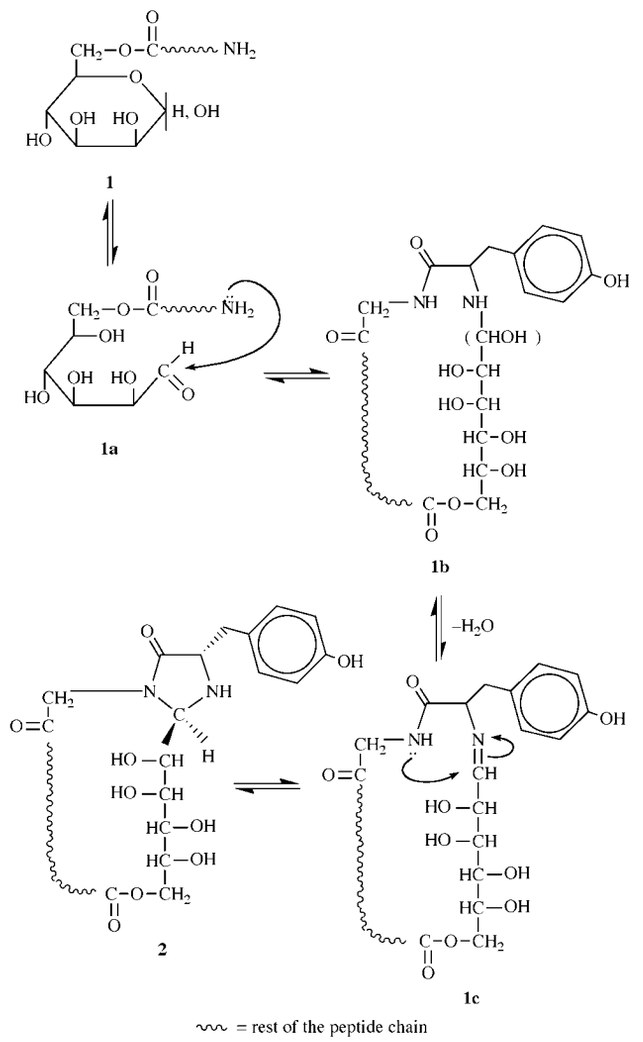
It is interesting to note that compound **2** gave bicyclic Amadori product **4** during incubations in acetic acid or in methanol containing different amounts of acetic acid (Table 2). Since, in all solvent mixtures, in addition to the unchanged starting compound **2**, a certain amount of mannopyranose ester **1** was detected, rearrangement of **2** to **4** is likely to proceed through Schiff base **1c**.

The pure compound **2** was prepared by heating 6-*O*-(H-Tyr-

Table 2 Distribution of products formed from bicyclic mannose-related imidazolidinone **2** under different reaction conditions

Solvent	*T*/°C	Incubation time/h	Compound (%)[a]		
			1	**2**	**4**
MeOH	37	24	2	96	
MeOH	50	24	4	92	
MeOH–HOAc (9 : 1)	50	24	23	44	17
HOAc	50	24	4	46	26
MeOH	reflux	4	10	83	
MeOH–HOAc (32 : 1)	reflux	4	9	63	17
MeOH–HOAc (9 : 1)	reflux	4	9	34	28
MeOH–HOAc (4 : 1)	reflux	4	14	32	24

[a] As in Table 1.



Scheme 2

Gly-Gly-Phe-Leu-)-D-mannopyranose **1**[4] in dry methanol at 50 °C over a period of 24 h. Removing the solvent and crystallizing from methanol–water gave **2** in 49% yield. This reaction is completely stereospecific, only the *trans*-diastereomer being obtained (see Structural analysis). In this paper the *cis* and *trans* descriptors refer to the relative geometry of the carbon substituents on the imidazolidinone moiety. In contrast, the enkephalin-related imidazolidinones so far described in the literature,[8] formed by condensation of enkephalins with various aldehydes and ketones, were obtained as mixtures of their *cis* and *trans* isomers.[8a] Since incubations of D-*gluco* and D-*galacto* 6-*O*-peptidyl esters gave imidazolidinone derivatives as mixtures of *cis* and *trans* isomers,[6] stereoselectivity in the imidazolidinone-ring formation from the mannopyranose ester **1** quite possibly is a consequence of the steric limits imposed by the C-2 configuration of the sugar moiety.

Table 3 Half-lives of mannose-related imidazolidinone **3** in various media[a]

Media	*T*/°C	Half-life
0.1 M NaOH	37	30 min
	50	15 min
0.1 M HCl	37	18 days
	50	6 days
0.05 M phosphate buffer, pH 7.4	37	8 days

[a] As in Table 1.

Cleavage of the ester linkage in compound **2** was carried out in 0.1 M sodium hydroxide, at room temperature, for 5 min. Purification by reversed-phase high-performance liquid chromatography (RP-HPLC) gave chiral mannose-related imidazolidinone **3** in 90% yield.

The stability of compound **3** was investigated by incubation under acidic, basic and neutral conditions (Table 3). The results show that **3** is considerably less stable in basic than in acidic or neutral media. In neutral medium the mannose-related imidazolidinone of leucine-enkephalin, compound **3**, is more stable than leucine-enkephalin-related imidazolidinones derived from acetaldehyde and propionaldehyde.[8c] In phosphate buffer solution degradation of **3** gave leucine-enkephalin as the main product, whereas under acidic and basic conditions a number of degradation products were detected.

Structural analysis of mannose-related imidazolidinones 2 and 3

The NMR chemical shifts, in DMSO-d_6 solution, of imidazolidinones **2** and **3** are summarized in Table 4. Designations of particular atoms are given in Scheme 1. For compound **2** only one set of resonances was observed in ^{1}H and ^{13}C NMR spectra, indicating that the intramolecular rearrangement **1**→**2** proceeded stereospecifically. The assignment was performed by different one- and two-dimensional techniques (see Experimental section). Thus, the OH-2′ of the carbohydrate moiety was assigned on the basis of three-bond C–H coupling with C-2 of the imidazolidinone ring in HMBC spectra. The remaining sugar OH protons were determined from 1D TOCSY experiments performed with different contact times and selective excitation at H-2, and from cross-peaks in T-ROESY spectra. Comparison of ^{1}H chemical shifts of the mannopyranose ester **1** (data are presented in the Experimental section) with those of bicyclic mannose-related imidazolidinone **2** (Table 4) revealed a few characteristic features. Most striking is the disappearance of the Gly^2 amide proton in the spectrum of **2**. Furthermore, the signal of the Tyr^1 amino proton is shifted from δ 8.08 in **1** to δ 3.49 in **2**. This significant shielding effect was caused by the imidazolidinone formation involving Tyr^1 and the sugar moiety, as corroborated by spin–spin interactions between NH-3 and H-2 as well as between NH-3 and H-4, observed in DQFCOSY spectra. The signals of the anomeric proton at δ 4.57 and 4.87 for the sugar moiety of **1** are replaced by a single new signal at δ 4.55 (H-2) in **2**, which corresponds to the CH proton of the imidazolidinone ring.

In the ^{13}C NMR spectrum of compound **2**, the signal at δ 75.2 is typical for C-2 of the imidazolidinone moiety. The signals corresponding to the anomeric C-atom in ester **1** (at δ 94.6 and 95.0) disappeared in **2**. The ^{13}C chemical shifts of C-4 and C-g in **2** are considerably greater than those of the corresponding C-atoms in **1** (C-α atoms in Tyr^1 and Gly^2). The other carbons have more or less the same chemical shifts in both **1** and **2**. This confirms that the cyclization takes place at Tyr^1 and Gly^2 in **1**, hence giving rise to deshielding at C-4 and C-g in the product **2**.

Table 4 NMR Chemical shift data (δ, ppm) of compounds **2** and **3**[a]

		2		**3**	
Residue[b]	Atom	δ_H	δ_C	δ_H	δ_C
Imidazolidinone ring	CH-2	4.55	75.2	4.50	75.4
	NH-3	3.49		3.40	
	CH-4	3.68	59.5	3.57	59.2
	a/a′	2.50/2.91	36.6	2.50/2.90	36.6
	b		128.9		128.9
	c	7.06	129.8	7.05	130.2
	d	6.66	115.1	6.66	115.3
	e		156.0		156.1
	f	9.14			
	g	3.92/3.98	46.8	4.09	45.5
Carbohydrate moiety	1′	3.54	72.0	3.53	71.9
	2′	3.60	68.8	3.61	69.8
	3′	3.61	66.8	3.55	71.2
	4′	3.58	67.8	3.56	70.4
	5′/5″	3.98/4.40	66.0	3.59/3.80	64.0
	OH-1′	5.25			
	OH-2′	5.00			
	OH-3′	4.13			
	OH-4′	4.48			
Gly[3]	NH	8.35		8.21	
	α/α′	3.38/3.98	42.4	3.59	42.0
Phe[4]	NH	8.07		8.11	
	α	4.46	54.0	4.55	53.9
	β/β′	2.77/3.14	37.6	2.76/3.05	37.7
	γ	7.19–7.29	138.4	7.18–7.27	138.1
	δ		129.4		129.5
	ε		128.3		128.3
	ξ		126.5		126.6
Leu[5]	NH	8.28		8.29	
	α	4.21	51.2	4.22	50.5
	β	1.61	39.6	1.58	39.2
	γ	1.61	24.2	1.55	24.4
	δ_1	0.86	21.3	0.86	21.2
	δ_2	0.91	23.0	0.91	23.0

[a] In DMSO-d_6. [b] Desginations of particular atoms are given in Scheme 1.

The relative configuration of the C-atom substituents on the imidazolidinone ring in compound **2** was determined to be *trans*. This means that the 2- and 4-substituents in **2**, *i.e.* the D-*manno*-pentitolyl and hydroxybenzyl groups, point in opposite directions with respect to the plane defined by the heterocyclic moiety. Such a conformation was derived from the NOE interactions observed between H-2 on the imidazolidinone ring and the H-c,c′ protons of the hydroxybenzyl substituent at C-4 of that ring, and from molecular modelling,[9] which showed that these protons are spatially closer in the *trans* than in the *cis* arrangement. If free rotation along the C-4–C-a bond is allowed, then the H-2 and H-c,c′ protons may spatially approach each other to a distance of 3.84 Å in the *cis* form, while they may be within 1.37 Å in the *trans* form. On the other hand, the spatial distances between the H-4 and H-2 protons and between the H-4 and H-c,c′ protons are very similar in both the *cis* and the *trans* isomers (≈3.85 Å for H-4–H-2 and ≈2.70 and 4.30 Å for H-4–H-c,c′).

The ^{1}H and ^{13}C NMR spectral data of mannose-related compound **3** also confirmed the presence of an imidazolidinone ring. The shift of mannose signals for H-5′/5″ from δ 3.98/4.40 to δ 3.59/3.80 and for C-5′ from δ 66.0 to 64.0, on proceeding from **2** to **3**, revealed that the ester bond between the C-terminal carboxy group of the peptide and the C-5′ hydroxy group of the sugar is broken in compound **3**. This was confirmed by two-bond C–H coupling between C-5′ and OH in the HMBC spectrum of **3**. The cleavage of the ester bond is also reflected in the chemical shift of C-4′, which is more deshielded (by ≈2.60 ppm) in **3** than in **2**.

The structures of mannose-related imidazolidinones **2** and **3** were confirmed by electron-impact mass spectrometry (EI-MS) in negative-ion mode. It is interesting to note that the observed fragmentation of mannose-related imidazolidinone **3** is quite different from the fragmentation behaviour of Amadori compounds under EI conditions, which results in two well stabilized fragments, the oxonium and the imonium ions.[2b]

Conclusions

6-*O*-(Leucine-enkephalyl)-D-mannopyranose **1** in methanol as the solvent was found to undergo intramolecular rearrangement to yield the previously unknown bicyclic, mannose-related imidazolidinone **2**. The reaction proceeded with a high degree of stereocontrol, *i.e.* only the *trans*-diastereomer was obtained. Comparison of the extent of mannose-related imidazolidinone formation during incubation, in methanol, of free D-mannose and leucine-enkephalin, with those obtained from incubations of mannopyranose ester **1** in the same solvent, demonstrates that esterification of the primary hydroxy group of the sugar moiety greatly accelerates the imidazolidinone-ring formation. It was also observed that the nature of the product formed depends upon the reactants. Thus, while incubation of D-mannose with leucine-enkephalin in methanol gave Amadori compound **5** as the main product, and only traces of mannose-related imidazolidinone **3**, incubation of mannopyranose ester **1** in the same solvent gave only the bicyclic mannose-related imidazolidinone **2**. Hydrolysis of compound **2** gave the novel mannose-related imidazolidinone **3** in almost quantitative yield. Imidazolidinone **3** is much more stable in acidic than in basic or neutral conditions. In neutral medium (phosphate buffer) compound **3** is more stable than those imidazolidinones of leucine-enkephalin which are derived from simple aldehydes. It is worth emphasizing that this type of derivatization is bioreversible with the parent peptide being

formed by spontaneous hydrolysis at physiological pH and temperature.

Experimental

Materials and methods

The leucine-enkephalin acetate salt (RP-HPCL retention time t_R = 15.99 min) was purchased from Sigma (St. Louis, USA), and D-mannose from Aldrich (Steinheim, Germany). All 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 and $[\alpha]_D$-values are given in units of 10^{-1} deg cm^2 g^{-1}. Reactions were monitored by TLC on Silica Gel 60 F_{254} plates (Merck; Darmstadt, Germany) using detection with ninhydrin, the chlorine–iodine reagent,[5] or heating with H_2SO_4. RP-HPLC analyses were performed on a Varian 9010 HPLC system with a Eurospher 100 reversed-phase C-18 semipreparative column (Knauer, Bad Homburg, Germany) (250 × 8 mm ID; 5 μm) under isocratic conditions (36% methanol in 0.1% aq. trifluoroacetic acid), flow rate 1.1 cm^3 min^{-1}. UV detection (Varian Model 9050 variable-wavelength UV-Vis detector) was performed at 280 nm. The chromatograms were obtained directly from lyophilized samples taken from the incubation mixtures at appropriate time intervals. Quantitation of the compounds was done by measuring peak areas (%).

The ^{1}H and ^{13}C one- and two-dimensional NMR spectra were recorded with Varian Gemini 300 and UNITY Inova 500 spectrometers, operating at 75.5 MHz and 125.7 MHz for the ^{13}C nucleus, respectively. All samples were measured in DMSO-d_6 solution at 20 °C in 5 mm NMR tubes. Chemical shifts, in ppm, are referred to TMS. The digital resolution in ^{1}H NMR spectra was 0.20 Hz (Gemini 300) and 0.15 Hz (Inova 500), while in ^{13}C NMR spectra it was 0.63 Hz per point. For complete signal assignments two-dimensional ^{1}H–^{1}H, ^{1}H–^{13}C and ^{1}H–^{15}N chemical-shift-correlation spectroscopy was used. The following spectra were recorded on a Gemini 300 spectrometer: broadband proton decoupling, gated proton decoupling, APT, COSY-45, long-range (delayed) COSY-45, NOESY and HETCOR. The COSY-45 and delayed COSY-45 spectra were measured in magnitude mode, while NOESY spectra were measured in phase-sensitive mode.

The pulsed field gradient techniques used on a UNITY Inova 500 MHz spectrometer were the following: HSQC ^{1}H–^{13}C and ^{1}H–^{15}N, HMBC, DQFCOSY, TOCSY and selectively excited 1D TOCSY, NOESY and T-ROESY. In all experiments standard pulse sequences were used, while proton decoupling was performed by Waltz-16 modulation.

Elemental analyses were carried out at the Microanalytical Laboratory, Ruđer Bošković Institute. Mass spectra were recorded on a FT MS 2001 DD Fourier transform mass spectrometer (FTMS, Madison, WI, USA) equipped with a 3T superconducting magnet.

6-*O*-(L-Tyrosylglycylglycyl-L-phenylalanyl-L-leucyl)-D-mannopyranose 1

Compound **1** (RP-HPLC t_R = 12.78 min) was obtained under the conditions described by Horvat *et al.*[4]

δ_H (DMSO-d_6) 0.85 (Leu^5 δ_1), 0.91 (Leu^5 δ_2), 1.57 (Leu^5 γ), 1.60 (Leu^5 β), 2.58 (Tyr^1 β), 2.83 (Phe^4 β), 2.86 (Tyr^1 β′), 3.43–3.63 (Man H-2–5), 3.54 (Tyr^1 α), 3.65 (Phe^4 β′), 3.66 (Gly^3 α/α′), 3.71 (Gly^2 α/α′), 3.75/4.05 (Man H-6/6′), 4.35 (Leu^5 α), 4.53 (βMan H-1), 4.57 (Phe^4 α), 4.87 (αMan H-1), 6.67 (Tyr^1 ε), 6.95 (Tyr^1 δ), 7.18–7.26 (Phe^4 ArH), 8.08 (Tyr^1 NH_2), 8.10 (Phe^4 NH), 8.23 (Gly^2, Gly^3 NH), 8.35 (Leu^5 NH); δ_C (DMSO-d_6) 21.2 (Leu^5 δ_1), 22.8 (Leu^5 δ_2), 24.3 (Leu^5 γ), 37.7 (Tyr^1 β), 38.0 (Phe^4 β), 39.4 (Leu^5 β), 41.8 (Gly^2 α), 42.1 (Gly^3 α), 50.5 (Leu^5 α), 53.7 (Phe^4 α), 55.5 (Tyr^1 α), 65.3 (Man C-6), 67.5–75.0 (Man C-2–5), 94.6 (βMan C-1), 95.0 (αMan C-1), 115.3 (Tyr^1 ε), 126.6 (Phe^4 ξ), 128.3 (Phe^4 ε), 128.5 (Tyr^1 γ), 129.5 (Phe^4 δ), 130.5 (Tyr^1 δ), 138.0 (Phe^4 γ), 156.2 (Tyr^1 ξ).

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

Compound **4** (RP-HPLC t_R = 22.43 min) was obtained by intramolecular rearrangement of **1** under the conditions described by Horvat *et al.*[5]

N-(1-Deoxy-D-fructos-1-yl)-L-tyrosylglycylglycyl-L-phenylalanyl-L-leucine 5

Compound **5** (RP-HPLC t_R = 15.32 min) was obtained from **4** under the conditions described by Horvat *et al.*[5]

cyclo-[*N*-({2-[-5)-D-*manno*-Pentitol-1-yl]-4-(4-hydroxybenzyl)-5-oxoimidazolidin-1-yl-(1 ⟶ *O*}acetyl)glycyl-L-phenylalanyl-L-leucyl-] 2

6-*O*-(L-Tyrosylglycylglycyl-L-phenylalanyl-L-leucyl)-D-mannopyranose **1** (220 mg, 0.31 mmol) was dissolved in dry methanol (20 cm^3) and the solution was stirred for 24 h at 50 °C. The solvent was evaporated off and the residue was crystallized from methanol–water to give the pure title compound **2** (106 mg, 49%). RP-HPLC t_R = 31.50 min. Mp 190–195 °C (decomp.); $[\alpha]_D^{22}$ −19 (*c* 1 in *N*,*N*-dimethylformamide) (Found: C, 58.5; H, 6.7; N, 10.0. $C_{34}H_{45}N_5O_{11}$ requires C, 58.4; H, 6.5; N, 10.0%). For ^{1}H and ^{13}C NMR data see Table 4. EI-MS: *m*/*z* 699 (53%, $[M - H]^-$. $C_{34}H_{45}N_5O_{11}$ requires *M*, 699.7529), 597 (100, $[M - H]^- - C_4H_6O_3$), 555 (61, $[597 - C_2H_2O]$), 376 (83, $[555 - C_9H_{10}N_2O_2]$), 344 (60), 278 (57) and 186 (52).

N-{[2-(D-*manno*-Pentitol-1-yl)-4-(4-hydroxybenzyl)-5-oxoimidazolidin-1-yl]acety}glycyl-L-phenylalanyl-L-leucine 3

Compound **2** (70 mg, 0.1 mmol) was dissolved in 0.1 M sodium hydroxide (1 cm^3). The solution was stirred at room temperature and, after 5 min, the pH was adjusted to 5 by addition of 0.1 M hydrochloric acid. The solution was applied to a Sephadex G-15 column (30 × 1.5 cm) and eluted with 1% aq. acetic acid. Fractions containing compound **3** were combined. After removal of the solvent, the residue was purified by semi-preparative RP-HPLC (for details see Materials and methods subsection) to provide compound **3**, which was crystallized from dry methanol–diethyl ether (65 mg, 90%). RP-HPLC t_R = 18.17 min. Mp 130–135 °C (decomp.); $[\alpha]_D^{22}$ −28 (*c* 1 in methanol) (Found: C, 56.8; H, 6.8; N, 9.9. $C_{34}H_{47}N_5O_{12}$ requires C, 56.9; H, 6.6; N, 9.8%). For ^{1}H and ^{13}C NMR data see Table 4. EI-MS: *m*/*z* 717 (15%, $[M - H]^-$. $C_{34}H_{47}N_5O_{12}$ requires *M*, 717.7679), 650 (32) and 596 (100, $[M - H]^- - C_4H_9O_4$).

RP-HPLC analysis

In order to obtain bicyclic mannose-related imidazolidinone **2**, mannopyranose ester **1** (2 mg, 2.8 μmol) was incubated in water (1 cm^3) and in dry methanol (1 cm^3) at both 37 °C and 50 °C (Table 1).

The reactivity of the free peptide and carbohydrate in terms of imidazolidinone-ring formation was examined by incubation of the leucine-enkephalin acetate salt (3.4 mg, 5.6 μmol) and D-mannose (1 mg, 5.6 μmol) in dry methanol (2 cm^3) at 50 °C.

Compound **2** (2 mg, 2.8 μmol) was incubated in dry methanol, glacial acetic acid and in different volume ratios of methanol–acetic acid (1 cm^3) at 37 and 50 °C during 24 h, and under reflux during 4 h (Table 2).

Samples from all experiments were withdrawn from the incubation mixtures, immediately frozen, and lyophilized. The respective samples were directly analysed by RP-HPLC (for details see Materials and methods subsection).

Compound **3** (1 mg, 1.4 μmol) was incubated in 0.1 M hydro-

chloric acid (1 cm^3) and in 0.1 M sodium hydroxide (1 cm^3) at 37 °C and 50 °C, as well as in 0.05 M phosphate buffer–0.1 M sodium chloride (pH 7.4) (1 cm^3) at 37 °C. The progress of hydrolysis was monitored by RP-HPLC (for details see Materials and methods subsection) on frozen and lyophilized aliquots removed at suitable intervals from the incubation solutions. The results are presented in Table 3.

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