Glycoconjugate Journal 15, 563–570 (1998)

Influence of glycation on *cis/trans* isomerization and tautomerization in novel morphiceptin-related Amadori compounds

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The influence of *N*-glycation of the N-terminus on amide bond stereochemistry and tautomeric distribution has been explored *via* the synthesis and NMR analysis of novel *N*-(1-deoxy-D-fructos-1-yl) derivatives (Amadori compounds) of the exogenous, milk derived, opioid tetrapeptide morphiceptin (H-Tyr-Pro-Phe-Pro-NH_2). NMR analysis of the protected Amadori compounds revealed the presence of four configurational isomers in DMSO solution arising from *cis/trans* isomerization about Tyr^1-Pro^2 and Phe^3-Pro^4 peptide bonds. Comparison of the data obtained for protected Amadori compounds with those obtained for morphiceptin showed that equilibrium fraction of all-*trans* isomers in *N*-glycated peptide derivatives was smaller than in the parent peptide compound. Spectroscopic investigation of unprotected morphiceptin-related Amadori compound revealed the presence of multiple conformers in solution due to *cis/trans* isomerization of the peptide backbone and tautomerization of the sugar moiety. The equilibrium composition in DMSO is markedly shifted towards furanose forms, amounting to two-thirds of the mixture. The estimated equilibrium of the tautomeric forms in water solution revealed the β-pyranose form as the major tautomer (66%).

Keywords*: Amadori, glycation, morphiceptin, *cis/trans

Introduction

Glycation, the nonenzymatic addition of reducing sugars to the amino groups of amino acids, peptides and proteins, collectively known as the Maillard reaction, is indicated in the development of pathophysiology in age-related diseases such as diabetes mellitus, arteriosclerosis, Alzheimer's disease, and in dialysis-related amyloidosis [1]. The initial phase of the reaction proceeds by the formation of an unstable glycosylamine, a cyclized Schiff base that under acidic conditions rearranges into the more stable 1-amino-1-deoxy-ketose derivative, a process known as Amadori rearrangement [2]. Over a period of time, the Amadori products lead, after intra- and intermolecular rearrangements, to a very reactive species called AGEs (advanced glycation end products) capable of cross-linking of proteins [3]. Particularly interesting are data showing that short-term administration of exogenous AGE-modified peptides into otherwise normal rats lead to a range of physiological dysfunctions, resembling those observed in diabetes [4].

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Changes in the monosaccharide fraction observed in some milk products [5], led us to assume that small exogenous opioid peptides, such as β-casomorphins, cleaved from β-casein during processing of milk or milk-based food products, can be also modified in the presence of glucose or other reducing sugars. β-Casomorphins may occur in milk as well as in milk products under normal conditions or may be formed *in vivo* after ingestion of milk-based food [6, 7]. Once formed, β-casomorphins are considered to be fairly resistant to enzymatic breakdown and can be transported from the mucosal to the serosal side in the small intestine [6]. It was reported that these peptides may disturb many physiological functions by exerting their effects through the activation of opioid receptors [8–10]. Concerning the recent controversy of whether exposure to cow milk in infancy is a triggering event for the development of diabetes [11–13], there was also a report that β-casein derived peptides precipitates diabetes in NOD mice and possibly in humans [14].

In an effort to better understand the reactivity of Amadori products and the mechanism of their further reactions, we have in the present study prepared novel morphiceptin-related Amadori compounds. Morphiceptin (H-Tyr-Pro-Phe-Pro-NH_2) is the most active opioid peptide from the bovine β-casomorphin group of peptides [15].

It is reasonable to suppose that glycation of the N-terminal amino group of the tyrosine residue in morphiceptin will affect the conformation and biological activity of the parent peptide compound. It was, therefore, of interest not only to prepare a novel class of Amadori compounds by unambiguous synthetic sequence, and thus provide substrate for bioactivity studies, but also to investigate the effect of glycation on the peptide backbone *cis*/*trans* isomerization by using NMR spectroscopy.

Materials and methods

Melting points were determined on a Tottoli (Büchi) apparatus and are uncorrected. Optical rotations were measured using an Optical Activity LTD automatic AA-10 Polarimeter. Column chromatography was performed on Silica Gel (Merck, 0.040–0.063) and TLC on glass plates (Merck silica gel 60 F_{254}). Spots were visualized by heating with ninhydrin, the chlorine-iodine reagent, or charring with H_2SO_4. The Amadori compounds were purified by semipreparative HPLC on Varian 9010 liquid chromatograph using Eurospher 100 reversed-phase C-18, 5 μm (250 × 8 mm) column (flow rate 1 ml min^{-1}) under isocratic conditions. HPLC column eluates were monitored by their UV absorbance at 280 nm. For analytical HPLC, an Eurospher 100 reversed-phase C-18, 5 μm (250 × 4 mm) column was used. Elemental analyses were performed in Microanalytical laboratory at Institute Ruđer Bošković, Zagreb. NMR spectra were recorded on a Varian Gemini spectrometer operating at 300.1 MHz and 600.14 MHz for ^1H and 75.5 MHz for ^{13}C nuclei. Experiments were performed at 25 °C with 50 mM solution in DMSO-d_6 or D_2O. Samples were equilibrated for 12 h in the deuterated solvents before recording the NMR spectra. Chemical shifts are reported in ppm relative to TMS as internal standard. Signals were assigned by the combined use of DQF-COSY, ROESY, TOCSY, HETCOR, HMQC and HSQC spectra. Homonuclear DQF-COSY, TOCSY and ROESY experiments were run in the phase-sensitive mode using quadrature detection in ω_1 by time-proportional phase incrementation of the initial pulse. Mixing times of 250 ms, and relaxation delays of 1.5 and 1.8 s, were employed for TOCSY and ROESY experiments, respectively. Delay times used in the ^{13}C NMR spectra were 1.5 s. FAB MS were measured with Jeol JMS SX 102 A spectrometer. Ions were produced by a beam of Xenon ions (6 keV) from a glycerole matrix.

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

Compound **2** was obtained from L-tyrosine (181 mg, 1 mmol) and 2,3:4,5-di-*O*-isopropylidene-*aldehydo*-*β*-D-*arabino*-hexos-2-ulo-2,6-pyranose (**1**) [16] (516 mg, 2 mmol) under conditions described in the literature [17] in 50% yield. The product was recrystallized from ethanol; mp 202–204 °C; $[\alpha]_D$–24.1° (c = 1.0, MeOH) [lit [17] mp 202–208 °C; $[\alpha]_D$–25° (c = 1.0, MeOH)].

N-tert-Butyloxycarbonyl-L-prolyl-L-phenylalanyl-L-proline amide (**3**)

To a cool (0 °C) solution of Boc-Pro-OH (110 mg, 0.5 mmol) in DMF (3 ml), NMM (55 μl, 0.5 mmol) and isobutyl chloroformate (65 μl, 0.5 mmol) were added. The reaction mixture was stirred for 2 min at the same temperature and a precooled solution of TFA salt of H-Phe-Pro-NH_2 [18] (190 mg, 0.5 mmol) in DMF (3 ml) containing NMM (55 μl, 0.5 mmol) was then added. The reaction mixture was stirred for 15 min at 0 °C and for 5 h at room temperature. The solvent was evaporated and the residue was purified by silica gel chromatography with EtOAc:HOAc:H_2O (70:2:2) as eluent to yield **3** which crystallized from MeOH/diisopropyl ether (180 mg, 80%); mp 80–85 °C; $[\alpha]_D$–85.5° (c = 1.0, MeOH). ^{13}C NMR (DMSO-d_6) [mixture of four conformers: *cis*/*trans* (*ct*), *trans*/*trans* (*tt*), *cis*/*cis* (*cc*) and *trans*/*cis* (*tc*)]; *ct*-**3**: δ 22.9 (Pro^1 γ-C), 24.4 (Pro^3 γ-C), 27.9 (Boc CH_3), 36.5 (Phe β-C), 51.9 (Phe α-C), 59.7 (Pro^1, Pro^3 α-C), 78.5 (Boc, C), 126.4 (Phe ζ-C), 128.2 (Phe ε-C), 138.2 (Phe γ-C), 153.6 (Boc CO), 170.2 (Phe CO), 172.6 (Pro^1 CO), 173.8 (Pro^3 CO); *tt*-**3**: δ 23.1 (Pro^1 γ-C), 24.4 (Pro^3 γ-C), 28.1 (Boc CH_3), 36.6 (Phe β-C), 51.8 (Phe α-C), 59.7 (Pro^1, Pro^3 α-C), 78.8 (Boc C), 126.4 (Phe ζ-C), 128.2 (Phe ε-C), 138.0 (Phe γ-C), 154.1 (Boc CO), 169.8 (Phe CO), 172.0 (Pro^1 CO), 173.8 (Pro^3 CO); *cc*-**3**: δ 21.7 (Pro^3 γ-C), 23.7 (Pro^1 γ-C), 28.0 (Boc CH_3), 38.2 (Phe β-C), 52.5 (Phe α-C), 59.7 (Pro^1, Pro^3 α-C), 78.7 (Boc C), 126.9 (Phe ζ-C), 128.5 (Phe ε-C), 136.9 (Phe γ-C), 153.6 (Boc CO), 169.6 (Phe CO), 172.9 (Pro^1 CO), 173.8 (Pro^3 CO); *tc*-**3**: δ 21.9 (Pro^3 γ-C), 22.9 (Pro^1 γ-C), 27.9 (Boc CH_3), 38.2 (Phe β-C), 52.3 (Phe α-C), 59.7 (Pro^1, Pro^3 α-C), 78.8 (Boc C), 126.9 (Phe ζ-C), 128.5 (Phe ε-C), 136.9 (Phe γ-C), 154.1 (Boc CO), 169.6 (Phe CO), 172.9 (Pro^1 CO), 173.8 (Pro^3 CO). Anal. Calcd. for $C_{24}H_{34}N_4O_5$: C, 62.86; H, 7.49; N, 12.22. Found: C, 62.99; H, 7.41; N, 12.37.

L-Prolyl-L-phenylalanyl-L-proline amide (**4**)

Compound **3** (100 mg, 0.22 mmol) was treated with TFA/H_2O (9:1, 1 ml) in the presence of anisole (0.4 ml) for 1 h at room temperature. After addition of cold diisopropyl ether, the precipitate was collected by centrifugation. Crystallization from MeOH/diisopropyl ether afforded pure **4** (90 mg, 90%); mp 110–114 °C; $[\alpha]_D$–50.0° (c = 1.0, H_2O). ^{13}C NMR (DMSO-d_6) (mixture of *cis* and *trans* isomers); *trans*-**4**: δ 23.3 (Pro^1 γ-C), 24.4 (Pro^3 γ-C), 29.1 (Pro^3 β-C), 29.4 (Pro^1 β-C), 36.2 (Phe β-C), 45.6 (Pro^1 δ-C), 46.7 (Pro^3 δ-C), 52.7 (Phe α-C), 58.6 (Pro^1 α-C), 59.6 (Pro^3 α-C), 126.4 (Phe ζ-C), 128.2 (Phe ε-C), 129.1 (Phe δ-C), 137.4 (Phe γ-C), 167.9 (Phe CO), 169.0 (Pro^1 CO), 173.2 (Pro^3 CO); *cis*-**4**: δ 21.7 (Pro^3 γ-C), 23.3 (Pro^1 γ-C), 29.5 (Pro^1 β-C),

31.2 (Pro^3 β-C), 38.4 (Phe β-C), 45.7 (Pro^1 δ-C), 46.2 (Pro^3 δ-C), 52.6 (Phe α-C), 58.6 (Pro^1 α-C), 59.4 (Pro^3 α-C), 126.6 (Phe ζ-C), 128.2 (Phe ε-C), 129.1 (Phe δ-C), 136.4 (Phe γ-C), 167.4 (Phe CO), 168.7 (Pro^1 CO), 172.6 (Pro^3 CO). Anal Calcd. for $C_{19}H_{26}N_4O_3 \cdot CF_3COOH$: C, 53.38; H, 5.77; N, 11.86. Found: C, 53.56; H, 5.88; N, 11.94.

N-(1-Deoxy-2,3:4,5-di-*O*-isopropylidene-β-D-fructopyranos-1-yl)-L-tyrosyl-L-prolyl-L-phenylalanyl-L-proline amide (**5**)

To a solution of the TFA salt of tripeptide **4** (100 mg, 0.2 mmol) in DMF (6 ml), compound **2** (90 mg, 0.2 mmol), BOP (98 mg, 0.2 mmol) and NMM (66 µl, 0.7 mmol) were added. After stirring overnight at room temperature, the solvent was evaporated and the residue was purified by silica gel chromatography with EtOAc:HOAc:H_2O (70:9:5). Crystallization from MeOH/diisopropyl ether gave crystals of **5** (75 mg, 50%); mp 105 °C (decomp.); $[\alpha]_D$–27.5° (c = 0.99, MeOH). For ^{13}C NMR data see Table 1. Mass spectrum (pos FAB) (relative intensity) *m/z* 764 ($M^+ + 1$, 95%). Anal Calcd. for $C_{40}H_{53}N_5O_{12} \cdot CH_3COOH$: C, 61.22; H, 6.99; N, 8.50. Found: C, 61.89; H, 6.54; N, 8.57.

N-(1-Deoxy-2,3-*O*-isopropylidene-β-D-fructopyranos-1-yl)-L-tyrosyl-L-prolyl-L-phenylalanyl-L-proline amide (**6**)

Fully protected Amadori compound **5** (100 mg, 0.13 mmol) was dissolved in cold TFA/H_2O (9:1, 1 ml) containing anisole (0.4 ml) and the solution was stirred at room temperature for 1 h. Addition of diethyl ether and subsequent centrifugation gave product which was purified by semi-preparative RP HPLC using 55.8% MeOH/0.1% TFA as eluent. Desalting on Dowex 1 × 2200 (Ac) column (5 × 1 cm) and lyophilization gave pure **6** (75 mg, 80%) which was crystallized from MeOH/diisopropyl ether; mp 145 °C (decomp.); $[\alpha]_D$–34.0° (c = 0.96, MeOH). For ^{13}C NMR data see Table 1. Mass spectrum (pos FAB) (relative intensity) *m/z* 724 ($M^+ + 1$, 45%). Anal Calcd. for $C_{37}H_{49}N_5O_{10}$:C, 61.39; H, 6.85; N, 9.68. Found: C, 61.13; H, 7.30; N 9.04.

N-(1-Deoxy-D-fructos-1-yl)-L-tyrosyl-L-prolyl-L-phenylalanyl-L-proline amide (**7**)

Compound **5** (100 mg, 0.13 mmol) was treated with TFA/H_2O (9:1, 1 ml) for 24 h at room temperature. Purification of the product in the same way as described for **6**, afforded 58 mg (65%) of **7**; mp 136 °C (decomp.); $[\alpha]_D$–65.5° (c = 0.96, MeOH). ^{13}C NMR (D_2O) [mixture of *ct*, *tt*, *tc* and *cc* conformers and β-pyranose (β-*p*), β-furanose (β-*f*) and α-furanose (α-*f*) tautomers]: δ 22.4, 22.5, 25.1, 25.3, 25.4 (Pro^2, Pro^4 γ-C), 29.5, 29.7, 30.2, 30.3, 32.1, 32.2, 32.4 (Pro^2, Pro^4 β-C), 36.9, 37.0, 37.2, 38.4, 38.6, 38.8, 39.4 (Tyr, Phe β-C), 47.8, 48.0, 48.2, 48.5, 48.8 (Pro^2, Pro^4 δ-C), 52.7, 53.1, 53.2, 53.3 (dFru C-1), 53.7, 53.8, 54.0 (Phe α-C), 60.2, 60.4, 61.0, 61.3, 61.5 (Pro^2, Pro^4 α-C), 62.2 (Tyr α-C), 62.9, 63.1, 64.4 (dFru C-6), 69.3, 69.9, 70.4 (dFru β-*p* C-3, 4, 5), 75.0, 75.4 (dFru β-*f* C-4), 76.9, 77.1 (dFru α-*f* C-4), 78.3, 78.7 (dFru β-*f* C-3), 81.8 (dFru β-*f* C-5), 82.5, 82.9 (dFru α-*f* C-5), 83.4, 83.6 (dFru α-*f* C-3), 97.3, 97.5 (dFru β-*p* C-2), 101.3, 101.4, 102.0 (dFru β-*f* C-2), 103.2, 103.5 (dFru α-*f* C-2), 116.5, 116.7 (Tyr ε-C), 127.5, 128.0, 128.3, 128.5 (Phe ζ-C), 129.8, 129.9 (Phe ε-C), 130.3 (Phe δ-C), 131.6, 132.0 (Tyr δ-C), 136.5, 137.1, 137.7 (Phe γ-C), 155.7, 155.9, 156.0 (Tyr ζ-C), 172.3, 172.5, 172.7, 172.9, 173.3, 173.5, 173.9, 177.6, 177.9 (Tyr, Pro^2, Phe, Pro^4 CO). Mass spectrum (pos FAB) (relative intensity) *m/z* 684 ($M^+ + 1$, 95%). Anal Calcd. for $C_{34}H_{45}N_5O_{10} \cdot CH_3COOH$: C, 58.13; H, 6.65; N, 9.42. Found: C, 58.27; H, 7.94; N, 9.12.

Results and discussion

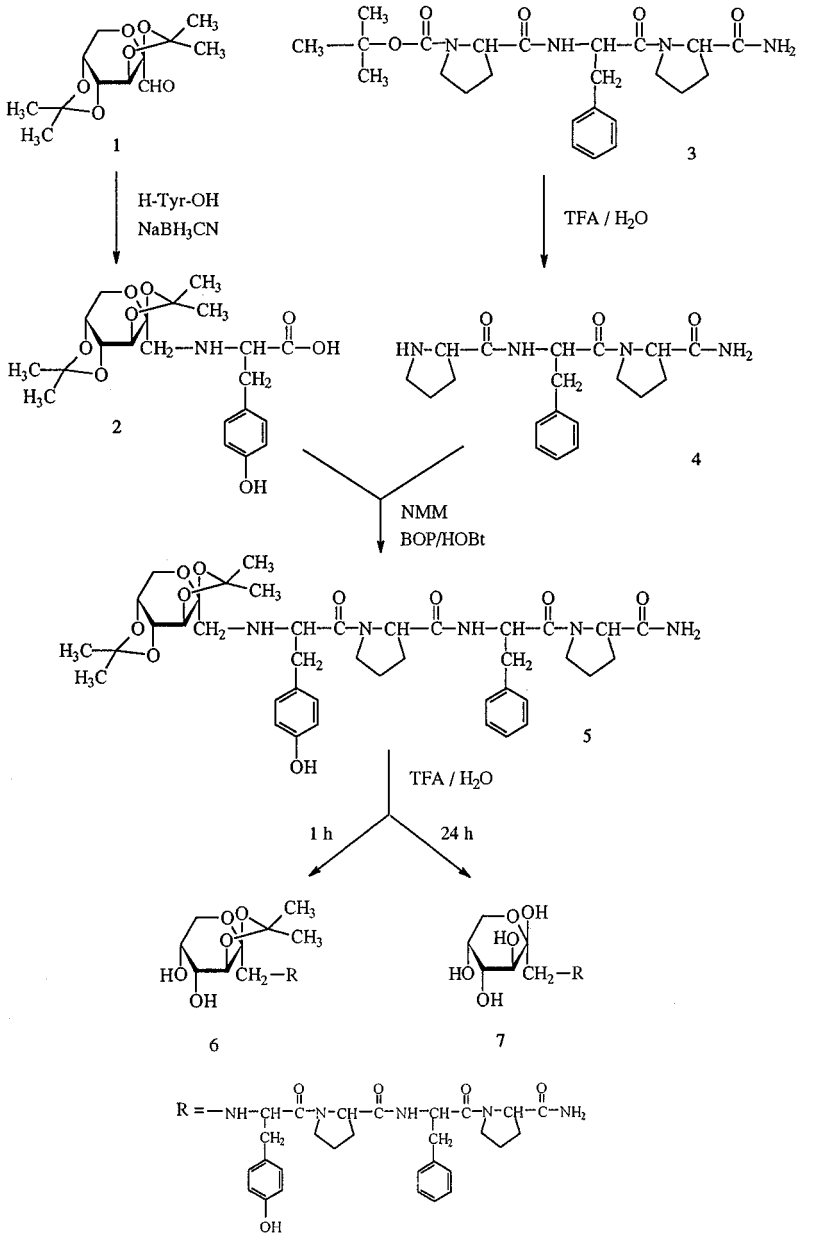
Synthesis of Amadori compounds

The synthetic approach to Amadori compounds related to the milk-derived peptide morphiceptin, involved synthesis of the protected Amadori compound **2** and its incorporation into a solution-phase synthesis, thus avoiding the possibility of side reactions and non-selectivity, usually observed in Amadori rearrangement of glycosylamines.

As presented in Scheme 1, reductive amination of isopropylidene-protected *aldehydo*-hexulopyranose **1** [16] by tyrosine in the presence of sodium cyanoborohydride gave Amadori compound **2** in 50% yield [17]. The protected Amadori tetrapeptide **5**, comprising the sequence of morphiceptin (H-Tyr-Pro-Phe-Pro-NH_2), was obtained in 50% yield by coupling **2** with tripeptide H-Pro-Phe-Pro-NH_2 **4** in the presence of BOP reagent. Treatment of fully protected Amadori compound **5** with aqueous trifluoroacetic acid, to remove the isopropylidene protection from the fructose moiety, afforded two products which were purified by RP HPLC. A short deprotection time (1 h) for **5** provided partially protected compound **6** as a major product (80%). This result is consistent with the observation that the acetal rings attached to the anomeric carbon atoms are less easily hydrolyzed than those attached at other positions, in part because of the special polar environment at the anomeric carbon atom [19]. A longer deprotection of **5** under acidic conditions (24 h) gave the desired morphiceptin-related Amadori compound **7** as the major product in 65% yield.

NMR studies

The assignments of various resonances in ^{13}C NMR spectra of both the fully protected Amadori compound **5** and the partially protected **6** were based on experiments mentioned in Materials and methods, chemical shift values for morphiceptin [20] as well as on assignments made for the related tripeptides **3** and **4**. A summary of the ^{13}C chemical shifts determined for **5** and **6** is presented in Table 1. Inspection of these data revealed the presence of four discernible



Scheme 1

isomers in DMSO solution of each Amadori compound, arising from *cis*/*trans* isomerization about Tyr[1]-Pro[2] and Phe[3]-Pro[4] peptide bonds. The presence of multiple *cis*/*trans* rotamers made the ^{1}H NMR spectra of **5** and **6** very complex, the signals for the sugar ring protons as well as for Pro[2] and Pro[4] protons of the various isomers appearing within a narrow range, making the assignments ambiguous because of peak overlap. However, the Phe NH and α-CH signals in the ^{1}H NMR spectra of compounds **5** and **6**, being relatively isolated from other proton signals, provided the most compelling evidence of multiple isomers present in DMSO solution (Figure 1). To assign the observed Phe amide and α-CH resonances (listed in Table 2) to the particular conformer, we used the following rationale based on the hypothesis that amide resonances in proline-containing peptides reflect proximal and distal proline isomerization [21]. Since the α-CH of the Phe residue is closer than the amide proton to the Phe[3]-Pro[4] peptide bond by one covalent bond, this α-proton will experience a greater change in chemical shift ($\Delta\delta$) resulting from Pro[4] isomerization than the NH proton of the Phe residue. Similarly, the amide proton of the Phe residue would show a greater $\Delta\delta$ than the α-proton due to Pro[2] isomerization. As presented in Figure 1a, in the amide region of the ^{1}H NMR spectra of

Table 1. ^{13}C Chemical Shifts (δ, ppm) of Carbon Atoms in Amadori compounds **5**, **6** and **7**.[a]

Residue	*Carbon Atom*	*Amadori Compound*		
		5[b]	**6**[b]	**7**[c]
dFru	C-1	(52.0, 52.2, 52.3, 52.4, 52.5, 52.6)[d]	49.0, 50.2	52.0[e]
	C-2	103.37, 103.4, 103.5	103.17, 103.25	96.8 (*α-p*), 97.6 (*β-p*), 97.8 (*β-p*), 101.8 (*β-f*), 103.5 (*α-f*), 103.7 (*α-f*), 103.8 (*α-f*)
	C-3,4,5	69.7, 70.0, 70.2, 70.3, 70.5, 70.8	65.4, 65.8, 66.2	69.1 (*β-p*), 69.2 (*β-p*), 69.7 (*β-p*),69.9 (*β-p*), 70.1 (*β-p*), 75.3 (C-4 *β-f*), 75.5 (C-4 *α-f*), 75.6 (C-4 *α-f*) 77.1 (C-3 *β-f*), 77.4 (C-3 *β-f*), 81.0 (C-5 *β-f*), 81.3 (C-5 *β-f*), 82.3 (C-5 *α-f*), 82.5 (C-5 *α-f*), 84.3 (C-3 *α-f*), 84.7 (C-3 *α-f*)
	C-6	61.1, 61.4, 61.6	62.7, 63.6	61.7, 61.8, 63.2
Tyr	*α*-C	60.3, 60.4	61.3	60.6, 60.7, 60.9
	β-C	37.8, 38.2[e]	37.9[e]	(36.4, 36.8, 37.9, 38.0, 38.2, 38.4)[f]
	γ-C	(137.6, 137.8)[g]	(136.9, 137.8, 138.0)[g]	(137.1, 137.6, 137.9)[g]
	δ-C	130.2, 130.4, 130.5, 130.6	130.4, 130.7	130.4, 130.5
	ε-C	114.9, 115.0	115.0, 115.2	115.1, 115.2
	ζ-C	155.8, 156.0	155.9, 156.2	155.8 156.0
Pro^2, Pro^4	*α*-C	58.5, 59.0, 59.4, 59.6, 59.7, 59.8	58.7, 59.4, 59.7, 59.8	58.7, 59.0, 59.4, 59.8
	β-C	28.7, 29.2, 29.3, 31.0, 31.6, 31.8	28.7, 29.3, 31.1, 31.6	28.6, 29.2, 30.9, 31.3, 31.4, 31.6
	γ-C	21.7, 21.8, 24.4, 24.5	21.7, 21.8, 24.3, 24.5	21.7, 24.3, 24.5
	δ-C	46.2, 46.3, 46.5, 46.7	46.5, 46.7	46.3, 46.5, 46.7, 46.9
Phe	*α*-C	(52.0, 52.2, 52.3, 52.4, 52.5, 52.6)[d]	52.1, 52.3, 52.4	52.4[e]
	β-C	36.5, 36.9[e]	36.1, 36.8[e]	(36.4, 36.8, 37.9, 38.0, 38.2, 38.4)[f]
	γ-C	(137.6, 137.8)[g]	(136.9, 137.8, 138.0)[g]	(137.1, 137.6, 137.9)[g]
	δ-C	129.2, 129.3, 129.5	130.4, 130.5, 130.7, 130.8	129.4, 129.6
	ε-C	128.2, 128.4	128.3, 128.6	128.3, 128.5, 128.9
	ζ-C	126.4, 126.5, 126.8, 127.6	126.5, 126.7, 126.9	126.5, 126.9, 127.7
Tyr, Pro^2, Pro^4 Phe	CO	169.4, 169.6, 171.0, 171.3, 171.4, 172.5, 172.6, 172.7, 173.5, 173.6	169.8, 171.3, 171.5, 171.6, 172.4, 172.7, 173.1, 173.8, 173.9,	169.5, 169.6, 169.8, 171.1, 171.2, 171.3, 171.5, 172.3, 172.5, 172.6, 172.7, 173.1, 173.6, 173.8
Isopropylidene	CH_3	24.1, 25.4, 25.6, 25.8, 26.5	25.8, 26.0, 27.3, 27.7	
	C	107.0, 107.4, 107.9, 108.0	108.4, 108.8	

[a]In DMSO-d_6 at 25 °C.
[b]Mixture of four conformers (*ct, tt, tc, cc*).
[c]Mixture of four conformers (*ct, tt, tc, cc*) and four tautomers (*α-p*, *β-p*, *α-f*, *β-f*).
[d]The values in parentheses correspond to those of dFru C-1 and Phe *α*-C.
[e]Assignments of signals can be interchangeable.
[f]The values in parentheses correspond to those of Tyr and Phe *β*-C.
[g]The values in parentheses correspond to those of Tyr and Phe *γ*-C.

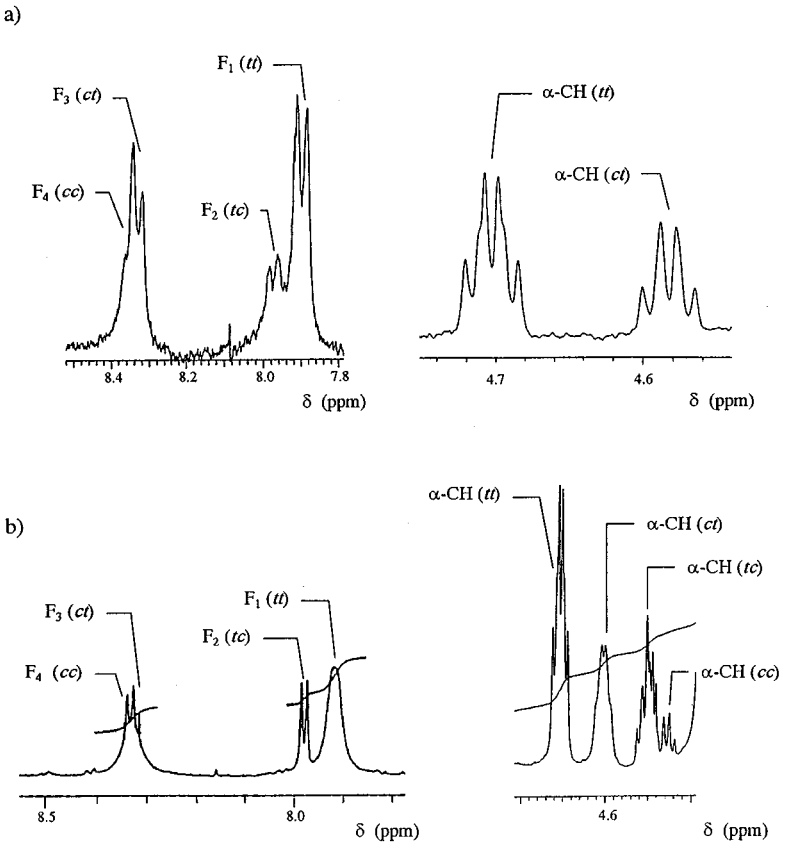


Figure 1. Expanded NH and *α*-CH regions of the Phe residue in ^{1}H NMR spectra of: a) fully protected Amadori compound **5** (*α*-CH signals of *tc* and *cc* conformers are not presented because of the spectral overlap with H-4 of the fructose moiety), and b) partially protected Amadori compound **6** in DMSO-d_6

Table 2. ^{1}H Chemical Shifts (δ, ppm) of *α*-CH and NH proton of the Phe residue and relative content (%) of *trans* (*t*) and *cis* (*c*) isomers in tripeptides **3** and **4** and Amadori compounds **5** and **6**.[a]

Compound	*Isomeric form (%)*		*Phe*	
			α-CH	*NH*
Boc-Pro-Phe-Pro-NH_2 (**3**)	*tt*	24	4.73	7.94
	ct	55	4.73	8.04
	tc	9	4.48	7.94
	cc	12	4.48	7.94
H-Pro-Phe-Pro-NH_2 (**4**)	*t*	75	4.70	8.89
	c	25	4.54	8.77
5	*tt*	45	4.70	7.90
	ct	30	4.58	8.33
	tc	15	4.50	7.97
	cc	10	4.46	8.35
6	*tt*	47	4.71	7.92
	ct	24	4.60	8.33
	tc	18	4.50	7.98
	cc	11	4.46	8.35

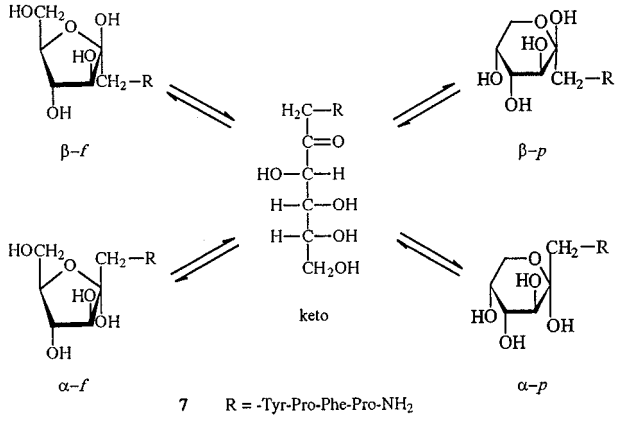
[a]In DMSO-d_6 at 25 °C.

Amadori compound **5** four doublets (F_{1-4}) for Phe NH were observed. Analysis of the cross-peaks in the COSY spectra of **5** revealed larger differences in the associated *α*-CH chemical shifts for F_1/F_2 ($\Delta\delta$ 0.20 ppm) and F_3/F_4 ($\Delta\delta$ 0.12 ppm) and smaller differences in the chemical shifts for NH ($\Delta\delta$ 0.07 and 0.02 ppm, respectively). Comparison of F_1/F_3 and F_2/F_4 doublets yielded greater $\Delta\delta$ for NH ($\sim$ 0.4 ppm) than for *α*-CH ($\Delta\delta$ 0.12 and 0.04 ppm, respectively). These results suggest that F_1/F_2 as well as F_3/F_4 are related by Phe^3-Pro^4 *cis-trans* isomerization and the F_1 is related to F_3 and F_2 to F_4 by distal Tyr^1-Pro^2 isomerization. Based on this assumption, the four Phe amide resonances arise from Pro^2 and Pro^4 isomerizations in the following conformations, respectively: F_1 (*trans*, *trans*), F_2 (*trans*, *cis*), F_3 (*cis*, *trans*) and F_4 (*cis*, *cis*). The ratio of configurational isomers for compound **5**, determined from the integration of signals in amide and *α*-CH region, was *tt*:*ct*:*tc*:*cc* (45:30:15:10) (Table 2). The *cis*:*trans* ratio for Pro^2 and Pro^4 in DMSO solution of **5**, calculated from integrated intensities of the appropriate resonances, were Tyr^1-Pro^2 (40:60) and Phe^3-Pro^4 (25:75). In the partially protected Amadori compound **6**, analysis of the four Phe amide doublets and associated *α*-CH signals (Figure 1b,

Table 2) by using the same considerations as above, revealed the following ratio of configurational isomers present in DMSO solution, *tt*:*ct*:*tc*:*cc* (47:24:18:11). The *cis*/*trans* ratio for each proline were Tyr[1]-Pro[2] (35:65) and Phe[3]-Pro[4] (29:71). The data obtained for compounds **5** and **6** showed that *cis* ⇄ *trans* equilibrium about Tyr[1]-Pro[2] and Phe[3]-Pro[4] peptide bonds is shifted towards the *trans* form (60–75%) in both compounds. Removal of one isopropylidene group in **5** did not influence the population of major *tt* isomers in DMSO solution. However, the slight decrease of the second configurational isomer with a *cis* conformation about Tyr[1]-Pro[2] was observed for partially deprotected compound, amounting to 30% for **5** and 24% for **6**.

The spectroscopic investigation of morphiceptin in DMSO solution [20] showed the presence of four isomers with an approximate ratio of 55:25:15:5. The most populated isomer (55%) was found to be all-*trans* one, whereas the second most abundant isomer (25%) was shown to contain a *cis* Tyr[1]-Pro[2] bond. The other two conformers were not assigned. Comparison of the data obtained for Amadori compounds **5** and **6** with those obtained for morphiceptin under similar conditions showed that equilibrium fraction of all-*trans* isomers in *N*-glycated morphiceptin derivatives was smaller than in the parent peptide compound. This observation suggests the stabilization of *cis*-amide bonds in **5** and **6** by hydrophobic interaction between the protected sugar moiety and peptide chain.

The NMR spectra of the unprotected morphiceptin-related Amadori compound **7**, studied in DMSO and D_2O, indicated the presence of multiple conformers in solution due to the *cis-trans* isomerism of Tyr[1]-Pro[2] and Phe[3]-Pro[4] peptide bonds as well as tautomerism of 1-deoxy-D-fructose moiety (Scheme 2). A summary of the ^{13}C chemical shifts determined for the mixture of conformers of compound **7** in DMSO is presented in Table 1. The equilibrium composition of the tautomeric forms was estimated from the



Scheme 2.

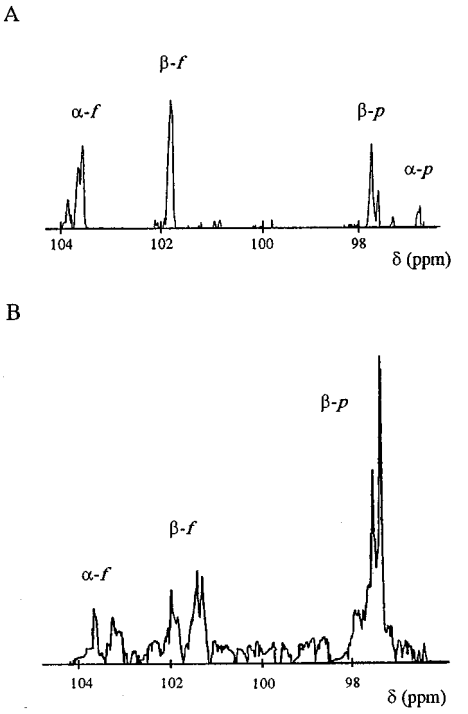


Figure 2. Anomeric (C-2) regions of the ^{13}C NMR spectra of Amadori compound **7**: (A) in DMSO-d_6; (B) in D_2O.

relative peak intensities in the anomeric region (Figure 2). According to NMR analysis the following equilibrium composition was found for **7** in DMSO solution: α-furanose 40%, *β*-furanose 28%, *β*-pyranose 27% and α-pyranose 5%. No signals were observed which could be attributed to C=O of the open chain form. Comparison of these data with published results for D-fructose [22] gave the same (~ 2:1) furanose:pyranose ratio for **7** in DMSO solution. However, it is worth noting that in contrast to D-fructose there was a great preponderance of the α- over the *β*-furanose form in solution. This spectral feature of **7** is similar to those observed for Amadori compounds related to endogenous opioid pentapeptide, Leu-enkephalin [17] confirming that peptide chain attached to the anomeric carbon atom in ketoses affects the proportion of one furanose form at equilibrium in DMSO solution.

The ^{13}C NMR spectrum of **7** in D_2O showed the presence of α- and *β*-furanose and *β*-pyranose forms (Figure 2). The estimated equilibrium composition of the tautomeric forms in water solution revealed that the *β*-pyranose form is by far the major tautomer (66%) whereas the second largest tautomer present was *β*-furanose form of **7** (27%). A predominance of the *β*-pyranose form is 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 [22, 23]. It is interesting to note that in water

enkephalin-related Amadori compounds adopt equilibria with higher content of the α-furanose tautomer (12–18%) [17] whereas compound **7** adopts equilibria with a lower proportion of this form (7%). Thus by comparing ^{13}C NMR data of Amadori compound **7** related to exogenous, milk derived, opioid peptide morphiceptin with that reported for *N*-glycated endogenous opioid peptide Leu-enkephalin [17], we assume that structure of the peptide backbone influence the tautomeric distribution in studied Amadori compounds.

Conclusions

A new class of Amadori compounds related to μ-opioid receptor selective ligand, morphiceptin, were synthesized and their equilibrium compositions have been studied by NMR spectroscopy in DMSO and D_2O solutions. We have examined consequences of the introduction of the ketose sugar moiety into *N*-terminal of a peptide backbone and have compared the obtained results with those concerning parent peptide compound and previously studied Amadori compound models. The information reported here on the behavior of these compounds is of importance for a better understanding of the chemical and biochemical reactivities of Amadori compounds formed *in vitro*, in particular compounds formed during processing of milk or milk-related products.

Acknowledgments

This work was supported by grant No. 00980704 from the Ministry of Science and Technology of Croatia. Part of the NMR experiments presented in this work were performed at the National Center for High Resolution NMR Spectroscopy at the National Institute of Chemistry, Ljubljana, Slovenia. The authors thank Dr. Primož Pristovšek for recording NMR spectra at 600.14 MHz.

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Received 22 July 1997, revised 19 September 1997, accepted 22 September 1997