Conformational analysis of sugar–peptide adducts in the solution state by NMR spectroscopy and molecular modelling

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A combined use of NMR spectroscopy and molecular modelling has enabled insight into conformational features of the novel sugar–peptide adducts **1**–**3**. Cyclic neoglycopeptide **1**, having the β-D-glucopyranose moiety which connects terminal parts of the Tyr-Pro-Phe sequence into a 14-membered ring, has been found in a rigid conformation with a sandwich-like arrangement of the proline residue flanked by the tyrosine and the phenylalanine side-chains. However, cyclic Tyr-Pro-Phe-Val-related Amadori compound **2**, with an 18-membered glycopeptide ring, has shown more flexibility in the peptide backbone and amino acid side-chains. Nevertheless, mutarotation was obstructed and the 1-deoxy-D-fructofuranose moiety was found in the β configuration exclusively. The analysis of the Amadori compound **3**, with an unsubstituted C-terminal of the Tyr-Pro-Phe-Val peptide, has revealed the presence of conformational isomers arising from *trans–cis* isomerism of the Tyr^1-Pro^2 peptide bond, while the 1-deoxy-D-fructose has been found in the β-pyranose form. The results presented here point towards peptide sequence-governed overall conformation of the studied neoglycopeptides.

Introduction

In spite of the biological importance of many peptides and proteins, their therapeutic use is limited due to unfavourable pharmacological properties. *In vitro* glycosylation of naturally unglycosylated peptides has led to a family of compounds named neoglycoconjugates,[1–4] which possess interesting biochemical and structural features. Modification of biologically active peptides and proteins with carbohydrate molecules has been shown to alter their activity profile affecting both the affinity and receptor selectivity, and to improve proteolytic stability and properties unavoidable for crossing the biological membranes.[5–8] Moreover, owing to their well-defined structures, chemical homogeneity and the possibility of obtaining a series of derivatives with specific structural differences, neoglycoconjugates are good models for studying the contribution of carbohydrate moieties to the functions mediated by glycoproteins.[9] Neoglycoconjugates are widely used in glycohistochemistry to study protein–carbohydrate interactions in tumor systems,[10] cell recognition processes[11] and inhibitory effects of histo-blood groups on H_2O_2 generation by human leukocytes.[12]

We have recently prepared neoglycopeptides with an ester type of linkage between the C-6 hydroxy group of the D-glucose and the C-terminal carboxy group of the peptide Tyr-Pro-Phe-Val and its smaller N-terminal fragments in order to examine the influence of peptide length on the Amadori rearrangement reaction.[13,14] It has been demonstrated that both peptide sequence and peptide length determine the products formed from 6-*O*-peptidyl-D-glucopyranoses under conditions suitable for the Amadori rearrangement. As the result of the intramolecular reactions in the studied carbohydrate esters, new sugar–peptide adducts **1**–**3** were obtained. The glucosylamine **1** and the Amadori compound **2** can be viewed as a new class of sugar-bridged cyclic peptides. It should be pointed out that, owing to the presence of the proline residue, compounds **1** and **2** are representatives of tricyclic systems. Cyclic peptides and their derivatives are an important group of compounds extensively used in protein folding studies and enzyme active site mimicry, as scaffolds for library syntheses[15] or molecular adapters for pore-forming proteins.[16] Peptide cyclization involving sugar moieties is a rather innovative, but promising approach in this field. An interesting example of cyclic peptide–nucleic acid hybrids with stability toward nuclease S1 cleavage has been reported recently.[17]

Incorporation of carbohydrate unit(s) can influence peptide conformation,[18,19] allowing insight into conformational preferences necessary for the interaction with a precise counterpart molecule. NMR spectroscopy and molecular modelling studies have been employed to determine the conformational features of new sugar–peptide adducts **1**–**3** and the impact of the sugar moiety on the peptide backbone and side-chain spatial arrangement. In the light of the latest constant demands for templates capable of reducing peptide flexibility and keeping them in a biologically relevant conformation, the results of our study should contribute to better understanding of the mutual carbohydrate–peptide influence on the overall conformation of sugar–peptide adducts.

Results and discussion

Compounds

In the cyclic neoglycopeptide **1** the D-glucopyranose moiety connects terminal parts of the Tyr-Pro-Phe sequence. The N-terminal tyrosine residue is linked through its amino group to the C-1 atom, while the C-terminal phenylalanine residue is linked through an ester bond to the C-6 hydroxy group of the sugar molecule. The cyclic Amadori compound **2** may be considered as the Tyr-Pro-Phe-Val peptide (human casein-derived peptide with a weak opioid activity[20]) bridged with the 1-deoxy-D-fructofuranose moiety in the same manner as

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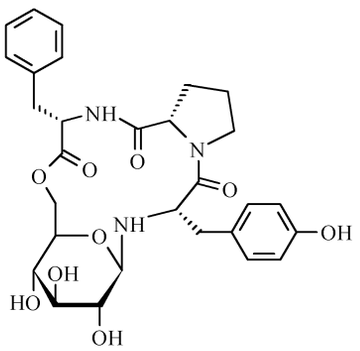

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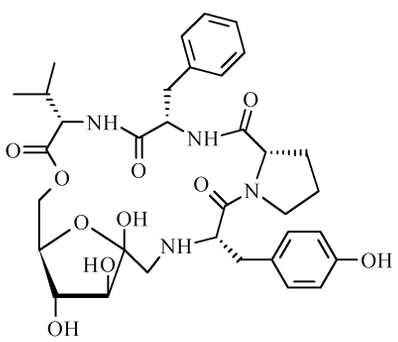

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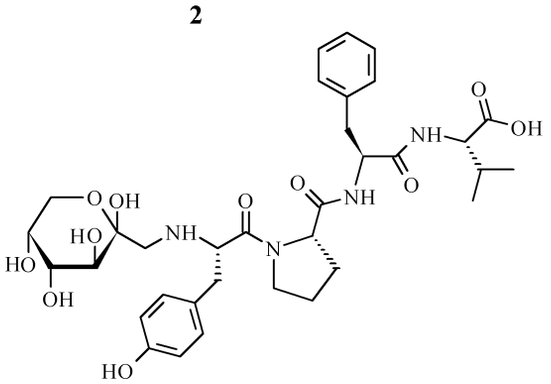


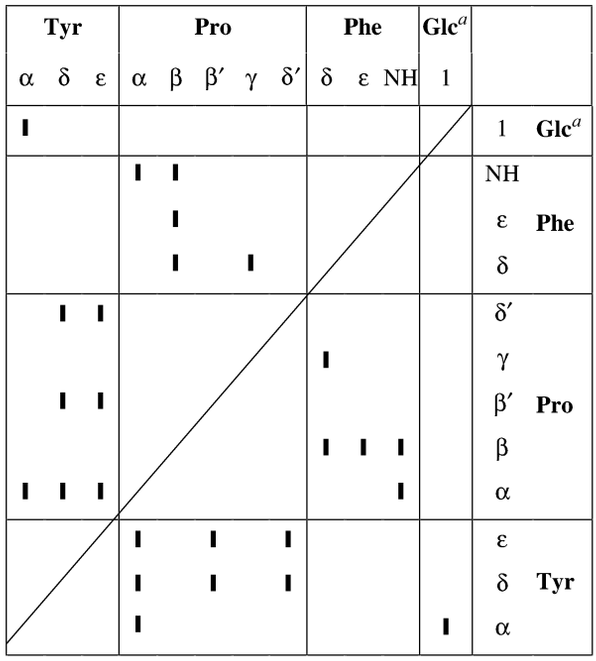
3

described for compound **1**. The Tyr-Pro-Phe-Val peptide in compound **3** bears the 1-deoxy-D-fructopyranose appendage at the N-terminal tyrosine residue.

Conformational analysis of compounds 1–3

^{1}H and ^{13}C resonances were assigned by using COSY and TOCSY experiments combined with ^{1}H–^{13}C correlation techniques through one bond (HSQC) and multiple bonds (HMBC). Chemical shifts of neoglycopeptides **1**–**3** are given in Table 1.

Tyr-Pro-Phe-related glucosylamine 1. A single set of resonances in the NMR spectra of Tyr-Pro-Phe-related glucosylamine **1** in DMSO-d_6 indicates one conformation of the pyranose ring. The chemical shift of the anomeric H-1 proton (Table 1) and the vicinal coupling constant $^{3}J_{1,2}$ = 7.70 Hz (Table 2) are in agreement with the β-anomeric configuration of the D-glucosylamine moiety.[21] Due to peak overlapping in the region 3.12–3.22 ppm, unambiguous coupling constant determination was not possible. However, additional data were



[a] β-D-Glucopyranosyl

Fig. 1 Inter-residual NOEs of **1** in DMSO-d_6.

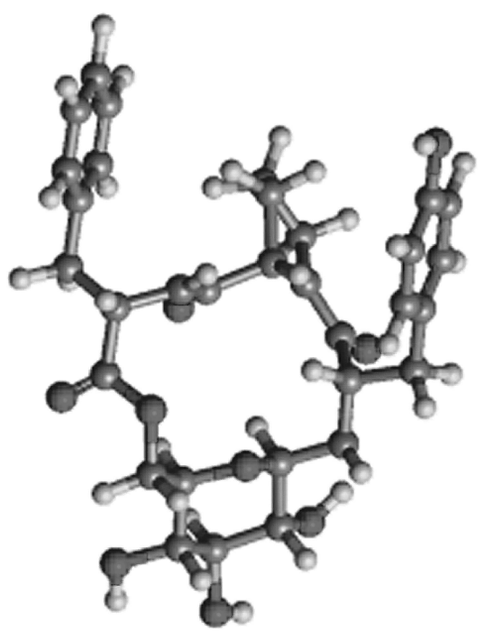


Fig. 2 The global minima conformation of **1**.

obtained from the NMR spectra of the glucosylamine **1** in CD_3CN. As seen in Table 1, a change in the solvent caused a separation of sugar proton resonances, thus allowing all coupling constants to be determined precisely (Table 2). Based on a similarity between the patterns in the ROESY spectra and the coupling constants in DMSO-d_6 and CD_3CN, it is assumed that **1** adopts a very similar conformation in both solvents.

The large number of observed NOE cross peaks (Fig. 1) suggested a well-ordered structure of **1** in DMSO-d_6 solution. Therefore, a conformational space search of **1** was performed by using Tripos and MM3 force fields and simulated annealing protocols. Data so obtained were compared with NMR results. The conformational search found a single low energy conformation of the central 14-membered glycopeptide ring and the global minima conformation shown in Fig. 2. However, alternative conformations of Tyr and Phe side-chains were energetically accessible at room temperature, *i.e.* they fell within

Table 1 ^{1}H NMR chemical shifts (ppm) of neoglycopeptides **1**–**3**[a]

Residue	Proton(s)	**1**		**2**	**3**[c]	
		DMSO-d_6	CD_3CN	DMSO-d_6	D_2O	
					cis	*trans*
Tyr	α	3.85	4.09	3.98		(4.53)
	β/β′	2.95	2.94; 3.18	2.90; 3.13		(2.83; 3.00; 3.07)
	δ	6.91	7.03	7.00	6.92	7.04
	ε	6.67	6.74	6.70	6.79	6.75
Pro	α	3.39	3.25	3.30	2.92	4.38
	β/β′	1.04; 1.17	1.25	1.30; 1.35	1.55; 1.65	1.74; 2.02
	γ/γ′	1.48; 1.59	1.58; 1.67	1.10; 1.49		(1.40; 1.55)
	δ/δ′	3.14; 3.43	3.25; 3.56	3.13; 3.51	3.21; 3.37	2.88; 3.47
Phe	α	4.03	4.00	4.59		(4.54; 4.58)
	β/β′	2.86; 3.12	2.94; 3.20	2.90; 3.36		(2.80; 2.82; 3.00; 3.10)
	δ	7.12	7.12	7.25		(7.18; 7.25)
	ε	7.27	7.28	7.25		(7.25; 7.41)
	ζ	7.20	7.22	7.17		(7.18; 7.34)
	NH	8.65	7.02	7.86		[d]
Val	α			3.91	4.11	4.06
	β			2.06	2.03	2.00
	γ			0.95; 1.01	0.78; 0.79	0.80; 0.81
	NH			8.40		[d]
Sugar[b]	H-1	3.62	3.85	3.28; 3.51		(2.65; 2.83; 3.10)
	H-2	3.19	3.56			
	H-3	3.19	3.45	4.08	3.59	3.55
	H-4	2.95	3.20	3.99	3.78	3.72
	H-5	3.19	3.45	3.93		(3.87; 3.91)
	H-6/H-6′	3.55; 5.10	3.62; 5.13	3.78; 4.85		(3.60; 3.68; 3.86; 3.90)

[a] At 25 °C. [b] β-D-Glucopyranosyl for **1**; 1-deoxy-β-D-fructofuranos-1-yl for **2**; 1-deoxy-β-D-fructopyranos-1-yl for **3**. [c] The values in parentheses could not be assigned to *cis* or *trans* isomer unambiguously. [d] Not observed.

Table 2 Observed ^3J coupling constants (Hz) in neoglycopeptides **1**–**3** and calculated ^3J coupling constants (Hz) and dihedral angles (°) based on the global minima conformation in compound **1**

Residue	Type	**1**				**2**	**3**	
		DMSO-d_6	CD_3CN	φ_{model}	$^3J_{calc}$[b]	DMSO-d_6	D_2O	
							cis	*trans*
Tyr	$^3J_{\alpha\beta}$	5.50	5.00	69.57	1.72			
	$^3J_{\alpha\beta'}$	10.70	10.62	−174.40	12.07	6.60		
Pro	$^3J_{\alpha\beta}$	0		−84.28	0.70	1.80	3.15	4.35
	$^3J_{\alpha\beta'}$	8.15		36.25	7.08	8.30	8.05	8.55
Phe	$^3J_{\alpha\beta}$	11.20		−168.01	11.81	3.84		
	$^3J_{\alpha\beta'}$	4.30		−52.71	4.20			
	$^3J_{\alpha NH}$	6.65	6.30			9.14		
Val	$^3J_{\alpha\beta}$					2.40	6.10	6.65
	$^3J_{\beta\gamma}$					6.75		
	$^3J_{\alpha NH}$					5.40		
Sugar[a]	$^3J_{1,2}$	7.70	8.74	174.97	8.22			
	$^3J_{2,3}$		9.17	−172.64	8.92			
	$^3J_{3,4}$		9.17	174.51	8.98	6.73	9.75	9.96
	$^3J_{4,5}$		9.17	−179.36	9.37		3.36	3.28
	$^3J_{5,6}$	11.50	10.00	−170.52	9.10	1.60		
	$^3J_{5,6'}$		1.43	71.30	1.57	1.60		

[a] β-D-Glucopyranosyl for **1**; 1-deoxy-β-D-fructofuranos-1-yl for **2**; 1-deoxy-β-D-fructopyranos-1-yl for **3**. [b] Calculated according to ref. 24.

a 3 kcal mol^{-1} margin above the global minima. Calculated vicinal coupling constants and inter-proton distances based on the global minima conformation are listed in Tables 2 and 3, respectively. A comparison of calculated data with the results deduced from NMR spectra proved our model to be a good representative of the solution state conformation of glucosylamine **1**. The discrepancies between the inter-proton distances calculated from the MM3 model and NOE data are a consequence either of an exchange effect (*e.g.* decrease of the NOE signal for Phe-α–Phe-NH contact) or peak overlap (*e.g.* contribution of neighboring peaks to the NOE signal for Glc-1–Glc-5 contact) or limited amino acid side-chain rotations (*e.g.* increased NOE signal for Tyr-δ–Pro-α contact due to a small rotation–oscillation of the Tyr C-β–C-γ bond).

The *cis* conformation of the Tyr^1–Pro^2 peptide bond is confirmed by the large upfield shift of the proline α proton (Table 1), as well as by the strong NOE cross peak between the Tyr H-α and the Pro H-α (Fig. 1). No trace of the Tyr^1–Pro^2 *trans* conformer was found in the NMR spectra. The strong NOE contact found between the Pro H-α and the Phe NH is indicative of the *trans* conformation of the Pro^2–Phe^3 peptide bond.

Coupling constants in the pyranose ring reflect the diaxial orientation of vicinal protons and the $^4C_1$ conformation of the

β-D-glucopyranosylamine moiety.[21] The diastereotopic H-6/H-6′ protons in **1** exhibit a remarkable chemical shift difference; one being found at 3.55 ppm and the other at 5.10 ppm (Table 1). Since the C-6 glucose atom is linked to the C-terminal phenylalanine residue through an ester bond, the anisotropic effect of the nearby π electrons can contribute significantly to the observed shift difference. The large difference between $^3J_{5,6}$ and $^3J_{5,6'}$ (Table 2) reflects a considerable rigidity in this part of the molecule.

The appearance of the Pro H-α only as a doublet with the vicinal coupling constant $^3J_{\alpha\beta'}$ of 8.15 Hz corresponds to a single puckered state of the pyrrolidine ring. Calculated coupling constants of the proline residue, as well as the geometry of the model, are in agreement with the C-β-*endo*/C-γ-*exo* conformation of the pyrrolidine ring.[22]

The side-chain conformation of the aromatic amino acids, tyrosine and phenylalanine, can be derived from the $^3J_{\alpha\beta}$ and $^3J_{\alpha\beta'}$ coupling constants.[23] Averaged values of ^3J constants correspond to equal populations of three rotamers (*trans*, *gauche*$^+$ and *gauche*$^-$) arising from rotation about the C-α–C-β bond, while the difference in ^3J values suggests the preponderance of a single rotamer. The observed coupling constants (Table 2) indicate a high population of a single rotameric form of the tyrosine and the phenylalanine side-chains. MM3 force field results ruled out the presence of the g^+ (*gauche*) conformation of the tyrosine side-chain, because it is 3.63 kcal mol^{-1} less stable than the global minima conformation (*trans, t*). The g^- conformation is found to be 0.79 kcal mol^{-1} less stable than the *trans* conformation and should be, according to the Boltzmann distribution, populated in the solution state in the ratio 0.27 : 1 with respect to the global minima conformation. Calculated values of Tyr $^3J_{\alpha\beta}$ and $^3J_{\alpha\beta'}$ for the *trans* conformation, based on the Altona modification of a Karplus type equation,[24] are 1.72 and 12.07 Hz, respectively (Table 4). Calculated values of $^3J_{\alpha\beta}$ and $^3J_{\alpha\beta'}$ for the g^- conformation are 12.15 and 2.78 Hz, respectively. However, the measured values of Tyr $^3J_{\alpha\beta}$ and $^3J_{\alpha\beta'}$ in DMSO-d_6 solution are 5.50 and 10.70 Hz, respectively (Table 2). Therefore, the linear combination of $^3J_{calc}$ values for the *t* and g^- conformations (Table 4) gave observed ^3J values for 37% or 15% of the g^- conformation population, based on $^3J_{\alpha\beta}$ or $^3J_{\alpha\beta'}$ values, respectively.

According to MM3 force field calculations the g^+ conformation of the phenylalanine side-chain is highly unstable (5.22 kcal mol^{-1}) to influence NMR parameters, while the *t* conformation is 1.17 kcal mol^{-1} less stable than g^-. Therefore, the *t* conformation is expected to contribute in solution with the ratio 0.14 : 1. Calculated values of Phe $^3J_{\alpha\beta}$ and $^3J_{\alpha\beta'}$ for the g^- conformation are 11.81 and 4.20 Hz, respectively, and for the *t* conformation 1.56 and 12.15 Hz, respectively (Table 4). However, observed values of Phe $^3J_{\alpha\beta}$ and $^3J_{\alpha\beta'}$ in DMSO-d_6 solution are 11.20 and 4.30 Hz, respectively (Table 2). Therefore, the estimated population of the *t* conformer, based on $^3J_{\alpha\beta}$ and $^3J_{\alpha\beta'}$ values, is 6% and 1%, respectively (Table 4).

Force field calculations and analysis of the NMR data of **1** have shown that only three conformations are present in solution with populations that may influence the observed NMR spectra. These conformations differ in Tyr and Phe side-chain orientation: Tyr_*t*–Phe_g^-, Tyr_g^-–Phe_g^- and Tyr_*t*–Phe_*t*. According to MM3 force field calculations their population ratio should be 1 : 0.27 : 0.14 or 71% : 19% : 10% which is close to the population estimated from NMR ^3J values (Table 4).

The most striking characteristic of the solution state conformation of **1** (Fig. 2) is stacking of Tyr, Pro and Phe side-chains. Such an arrangement of aromatic rings and the Pro side-chain is strongly supported by findings in the ROESY spectrum (Fig. 1). The Tyr H-δ and H-ε show correlations with Pro H-α, Pro H-β′ and H-δ′ as a consequence of their spatial proximity. Orientation of the Phe aromatic ring with respect to the pyrrolidine ring is derived from Phe H-δ–Pro H-β, Phe H-δ–Pro H-γ and Phe H-ε–Pro H-β NOE connectivities. A similar disposition of the aromatic side-chains flanking the proline residue has been found in small peptides of the type VI β-turn.[25] In a number of five- and six-residue peptides of general

Table 3 Inter-proton distances (Å) in the compound **1** calculated from the model and estimated from the integrated NOE cross peak volumes (DMSO-d_6)

H1	H2	d_{HH}(MM3)	d_{HH}(NOE)
Glc-6[a]	Glc-6′	1.77	1.57
Pro-δ′	Pro-δ	1.80	1.78
Tyr-α	Pro-α	2.12	2.12
Phe-α	Phe-NH	2.30	2.62
Phe-β	Phe-δ	2.31	2.32
Pro-α	Phe-NH	2.32	2.35
Glc-1	Glc-5	2.36	2.09
Pro-α	Pro-β	2.37	2.79
Tyr-β	Tyr-δ	2.41	2.48
Phe-α	Phe-β′	2.42	2.65
Tyr-β′	Tyr-δ	2.45	2.61
Phe-α	Phe-δ	2.54	2.93
Glc-5	Glc-6′	2.61	2.23
Phe-β′	Phe-δ	2.63	2.52
Glc-4	Glc-6	2.66	2.49
Pro-α	Pro-β	2.71	2.99
Tyr-α	Glc-1	2.77	3.19
Pro-β	Phe-NH	2.88	3.16
Tyr-α	Tyr-δ	2.95	2.74
Pro-γ′	Pro-δ	3.03	2.79
Glc-4	Glc-6′	3.06	2.92
Pro-β	Phe-δ	3.07	3.37
Tyr-α	Tyr-β′	3.12	2.74
Tyr-δ	Pro-δ	3.21	2.92
Tyr-δ	Pro-α	3.22	2.54
Phe-NH	Phe-δ	3.85	4.06
Pro-γ	Phe-δ	3.99	3.90

[a] Glc = β-D-Glucopyranosyl.

Table 4 Estimated population of the tyrosine and the phenylalanine side-chain conformations of the compound **1** based on NMR data and MM3 calculations

Residue	Type	$^3J_{calc}(t)$[a]	$^3J_{calc}(g^-)$[b]	$^3J_{exp}$[c]	*t* : g^- ratio NMR[d]	*t* : g^- ratio MM3	% of *t* NMR (MM3)	% of g^- NMR (MM3)[j]
Tyr	$^3J_{\alpha\beta}$	1.72	12.15	5.50	1 : 0.58	1 : 0.27[e]	63–85[g] (81)[h]	15–37 (19)
	$^3J_{\alpha\beta'}$	12.07	2.78	10.70	1 : 0.17			
Phe	$^3J_{\alpha\beta}$	1.56	11.81	11.20	0.06 : 1	0.14 : 1[f]	1–6 (10)[i]	94–99 (90)
	$^3J_{\alpha\beta'}$	12.15	4.20	4.30	0.01 : 1			

[a] Calculated for *trans* conformation of the C-α–C-β bond of corresponding amino acid side-chain. [b] Calculated for g^- conformation of the C-α–C-β bond of corresponding amino acid side-chain. [c] DMSO-d_6 solution. [d] 1 : ((1 − *x*)/*x*), where $x = (^3J_{calc}(g^-) - {}^3J_{calc}(t))/(^3J_{calc}(g^-) - {}^3J_{exp})$. [e] 1 : $e^{(E_{MM3}(Tyr\_t\text{-}Phe\_g^-) - E_{MM3}(Tyr\_g^-\text{-}Phe\_g^-))/RT}$. [f] $e^{(E_{MM3}(Tyr\_t\text{-}Phe\_g^-) - E_{MM3}(Tyr\_t\text{-}Phe\_t))/RT}$: 1. [g] 100% × *x*, where *x* is as described in note *d*. [h] 100% × (1 + 0.14)/(1 + 0.27 + 0.14), because the ratio of stable conformers Tyr_*t*-Phe-g^- : Tyr_g^--Phe_g^- : Tyr_*t*-Phe_*t* is 1 : 0.27 : 0.14 (see notes *e* and *f*). [i] 100% × (0.14)/(1 + 0.27 + 0.14) (see notes *e*, *f* and *h*). [j] 100% − (% of *t*).

sequence AA-Ar-*cis*-Pro-Ar-Asp-AA, where AA is any amino acid and Ar is an aromatic residue, it was established that the main source of stabilization arose from the close stacking interaction involving the proline and neighboring aromatic rings, while hydrogen bonds were not found to participate in conformation stabilization. It is worth noting that in our model only hydrogen bonds between hydroxy groups of the glucosylamine moiety are present. Such an arrangement brings hydrophobic groups of the molecule into close proximity, while the hydrophilic sugar moiety is located on the opposite side. Also all carbonyl groups are orientated in the same direction with respect to the central 14-membered ring (Fig. 2).

As previously reported, glycosylamines related to amino acids and peptides are usually unstable and readily undergo an Amadori rearrangement into the corresponding 1-amino-1-deoxy-2-keto sugar derivatives.[26] However, attempts to rearrange Tyr-Pro-Phe-related glucosylamine **1** into the corresponding keto-sugar derivative (Amadori product) were unsuccessful.[14] On the basis of the above presented results, it is concluded that the main reason for it is a particularly stable and rigid conformation of the 14-membered ring additionally stabilized by favorable stacking interactions between the aromatic side-chains and the pyrrolidine ring.

Tyr-Pro-Phe-Val-related Amadori compound 2. Solutions of Amadori compounds usually contain a mixture of various tautomeric forms arising from mutarotation.[27] However a single set of resonances in the ^{1}H NMR spectrum of cyclic Amadori compound **2** in DMSO-d_6 implies the presence of a single anomeric form of the D-fructofuranose moiety (Table 1). The observed strong NOE H-1–H-3 correlation, a characteristic of the β-anomeric configuration, and the absence of the H-1–H-4 connectivity, a characteristic of the α-anomer, have confirmed the β configuration at the anomeric C-2 atom. Diastereotopic H-1/H-1′ protons are found at 3.28 and 3.51 ppm, whereas H-6/H-6′ resonate at 3.78 and 4.85 ppm. Fructose H-1/H-1′–Tyr H-α and fructose H-1/H-1′–Tyr H-β/H-β′ NOE correlations, as well as fructose H-6–Val H-β and fructose H-6–Val H-γ/H-γ′, are in accordance with the structure in which the 1-deoxy-D-fructofuranose moiety connects N- and C-terminal parts of the Tyr-Pro-Phe-Val peptide into the cyclic Amadori compound.

An attempt to calculate the solution state conformation of **2** in a fashion similar to that for compound **1** was unsuccessful. Even with eight constrictions based on unambiguous inter-residue observed NOE cross peaks, simulations gave a large number of low energy conformers, all satisfying NOE criteria. Therefore, it is concluded that glycopeptide **2** is too flexible a molecule for an explicit description of its geometry in the solution state.

The upfield shift of the proline H-α (Table 1) and the observed NOE correlation between the Tyr H-α and the Phe H-α (Fig. 3) are in agreement with the *cis* conformation of the Tyr^1–Pro^2 peptide bond. A severe peak overlapping prevented a complete determination of the ^3J coupling constants and stereospecific assignment of the β protons in the tetrapeptide part of the neoglycopeptide **2**. However, $^3J_{\alpha\beta}$ and $^3J_{\alpha\beta'}$ coupling constants were determined for the proline residue based on the α-proton splitting (Table 2). A single conformation of the pyrrolidine ring is consistent with the two different couplings (8.30 and 1.80 Hz).[22]

The vicinal coupling constants $^3J_{5,6}$ and $^3J_{5,6'}$ are equal and amount to 1.60 Hz as the result of similar dihedral angles and somewhat limited rotation about the C-5–C-6 bond. Inter-residual interactions Pro H-α–Phe H-α, Pro H-α–Phe NH and Phe H-α–Val H-α in the ROESY spectrum (Fig. 3) are consistent with the folded form of the peptide part in **2**.[28] The medium-range Tyr H-α–Phe NH as well as fructose H-1–Phe NH NOE correlations are in agreement with the presence of turn in the compound **2**.

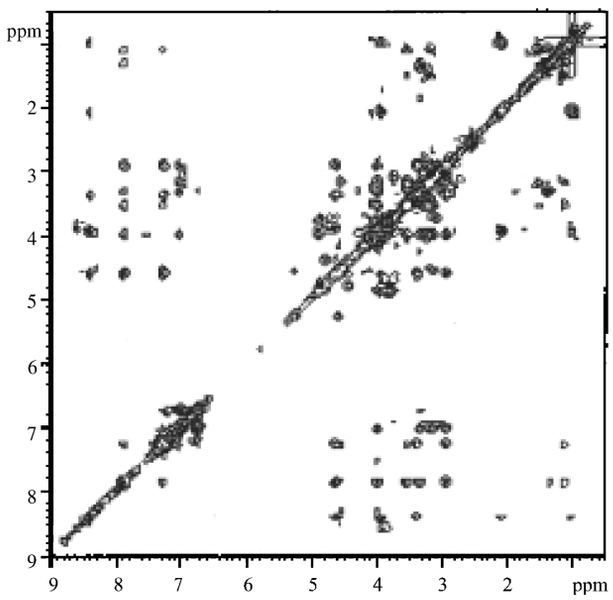


Fig. 3 ROESY spectrum of compound **2** in DMSO-d_6.

Since Amadori compound **2** was obtained from the 6-*O*-(Tyr-Pro-Phe-Val)-D-glucopyranose under the same conditions as the glucosylamine **1**,[14] it is apparent that elongation of the peptide chain causes disruption of the close-stacking interactions within the Tyr-Pro-Phe sequence and provides enough conformational space for the Amadori rearrangement into cyclic keto-derivative **2**. However, the presence of the 18-membered ring hinders mutarotation, since only the β configuration of the D-fructofuranose moiety is found. For comparison, a solution of a cyclic Amadori compound related to the leucine-enkephalin (Tyr-Gly-Gly-Phe-Leu) with a 21-membered glycopeptide ring is found as a mixture of α- and β-furanose forms.[29]

Tyr-Pro-Phe-Val-related Amadori compound 3. Both ^{13}C and ^{1}H NMR spectra of the Amadori compound **3** have shown two sets of resonances in D_2O solution. A close inspection of the spectra has revealed the presence of two conformers in solution arising from *trans–cis* isomerization of the Tyr^1–Pro^2 peptide bond as evidenced from chemical shifts for the proline C-β and C-γ atoms. Furthermore, chemical shifts of the sugar carbons have demonstrated the presence of the 1-deoxy-D-fructose moiety in the β-pyranose form exclusively.[14] The ratio of the two conformers, calculated from the integrated peak intensities of the well-resolved signals in the ^{1}H NMR spectrum, was *cis : trans* 60 : 40.

Chemical shifts of the protons in the 1-deoxy-D-fructose (Table 1) are in good agreement with those of the closely related Amadori compounds.[27] The vicinal coupling constants within the fructopyranose ring were determined from the well resolved H-4 signal for both isomers, indicating the $^2C_5$ conformation of the pyranose ring (Table 2).

In spite of the severe peak overlapping, all amino acid proton resonances were unambiguously assigned (Table 1). Complete assignment of the resonances corresponding to the *trans* and *cis* conformer of the proline and valine side-chain was performed following the spin systems in the TOCSY spectrum. An absence of a distinct set of inter-residual connectivities in the ROESY spectrum suggested free rotation about the single bonds and a random conformation of the peptide backbone in the Amadori compound **3**.

Amadori compound **3** was obtained after the base hydrolysis of an ester linkage, from the analogous cyclic derivative **2**.[14] In spite of increased conformational freedom resulting from the loss of the main 18-membered ring, a single tautomeric form is found in D_2O solution of the Tyr-Pro-Phe-Val-related Amadori compound **3**. Also, the equilibrium of the *cis*⟶*trans*

isomerization of the Tyr[1]–Pro[2] peptide bond was shifted, with significant preponderance toward the *cis* isomer. A similar *cis* : *trans* ratio was established for the highly μ-receptor selective Tyr-Pro-Phe-Val-NH_2 peptide,[30] suggesting a *trans–cis* equilibrium undisturbed by the presence of the sugar moiety. These findings suggest the peptide sequence-dependent mutarotation of the Amadori compound, as well as the *trans–cis* isomerism of the peptidyl–prolyl amide bond.

Conclusions

Cyclic Tyr-Pro-Phe-related glucosylamine **1** was found to be resistant toward Amadori rearrangement.[14] NMR spectroscopy and molecular modelling imply that the main reason for this is a rigid conformation of the central 14-membered ring with the hydrophobic tyrosine and the phenylalanine side-chains stacking against the pyrrolidine ring and the hydrophilic glucose moiety occupying the opposite side of the molecule. In contrast, the Tyr-Pro-Phe-Val-related cyclic Amadori compound **2** is a more flexible molecule with a large number of low energy conformations of the central glycopeptide ring. Extension of the main ring from 14-membered in **1** to 18-membered in **2** increased the conformational freedom of the peptide backbone and amino acid side-chains. However, mutarotation is effectively hindered, resulting in the β configuration of the 1-deoxy-D-fructofuranose moiety. Amadori compound **3**, with a free carboxy group at the C-terminal of the Tyr-Pro-Phe-Val peptide, was found to exist as a mixture of *trans* and *cis* isomers in D_2O solution. Although the presence of the 1-deoxy-D-fructose appendage at the N-terminal has no notable influence on the *trans–cis* equilibrium, the peptide sequence was responsible for the single β-pyranose form of the sugar moiety, most probably due to favorable intramolecular hydrogen bonds.

Experimental

Compounds

The cyclic glucosylamine **1** is obtained by the incubation of the 6-*O*-(Tyr-Pro-Phe)-D-glucopyranose in a pyridine–acetic acid mixture (1 : 1) at 37 °C for 24 h. The Amadori rearrangement of the 6-*O*-(Tyr-Pro-Phe-Val)-D-glucopyranose in the same solvent system at ambient temperature for 24 h afforded the cyclic ketofuranose or the Amadori compound **2**. Hydrolysis of an ester linkage in **2** (0.1 M NH_4OH) resulted in the Amadori derivative **3**. Synthesis of the above mentioned compounds, proposed mechanisms of formation and their chemical characterization are published elsewhere.[14]

NMR measurements

All NMR spectra were recorded on a Bruker Avance DRX500 spectrometer operating at 500.13 MHz for ^1H, equipped with a 5 mm diameter inverse detection probe and *z*-gradient accessory.

^1H NMR experiments were executed with a spectral width of 6600 Hz, 65 K data points and 64 scans. TMS was used as the internal standard. The sample concentration was 10–15 mg ml^{-1} in DMSO-d_6, acetonitrile-d_3 and D_2O. A presaturation technique was used for water signal suppression. The digital resolution was 0.1 Hz per point. In ^{13}C NMR spectra the spectral width was 31000 Hz, the number of data points 65 K and the number of scans 2500–7000 per spectrum. The digital resolution was 0.5 Hz per point. ^3J coupling constants were obtained from the high-resolution ^1H spectra.

Two-dimensional DQF COSY and TOCSY spectra were recorded under the following conditions. The spectral width was 6600 Hz in both domains, 2K data points were applied in the time domain and 512 increments were collected for each data set with linear prediction and zero filling to 2K. The relaxation delay was 1.5 s. DQF COSY and TOCSY spectra were processed with sine and squared sine functions, respectively. The digital resolution was 3.3 Hz per point in both $f1$ and $f2$ domains. The number of scans was 8 and 16 for DQF COSY and TOCSY, respectively.

HSQC and HMBC spectra were recorded with a relaxation delay of 1.5 s and 32 scans per increment. The spectral width was 31000 Hz in acquisition domain $f2$ and 6600 Hz in time domain $f1$. Data were collected into a 2048 × 256 acquisition matrix and processed using a 2K × 1K transformed matrix with zero filling in the $f1$ domain. Sine multiplication was performed prior to Fourier transformations. In HMBC spectra the delay for long range couplings was set at 60 ms.

TPPI ROESY spectra were obtained with a mixing time of 250 ms and processed with the sine squared function shifted by π/2 in both domains. The spectral width was 5300 Hz in both domains and the relaxation delay was 1.5 s. The number of scans was 32. Spectra were zero filled and the digital resolution was 1.3 and 2.6 Hz per point in the $f2$ and $f1$ domains, respectively.

Molecular modelling

All computations were performed on SGI Octane and SGI Indigo2 computers. Conformational analysis was performed using Sybyl 6.6 software.[31] Vicinal coupling constants were calculated from the model using an in house program based on the Altona modification of the Karplus equation.[24]

Compound **1** was subjected to 10 cycles of simulated annealing protocol with a 0.5 fs simulation step, Tripos force field and relative permittivity $\varepsilon = 48$ corresponding to DMSO-d_6 solvent. For each cycle the molecule was heated to 2000 K, equilibrated for 1000 fs and exponentially cooled to 0 K for 10000 fs. Resulting geometries were optimized by MM3 force field and sorted according to calculated MM3 energy. The most stable conformation of the 14-membered ring appeared in the global minima conformation and the Tyr and Phe side-chain rotamers.

Compound **2** was subjected to a number of simulations similar to those for compound **1** using different simulated annealing protocols and constrictions based on observed NOE signals. However, the number of stable conformers was high, growing with each round of simulations and making it difficult to draw an unequivocal conclusion about the completeness of the simulation and the relevance of the observed results.

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