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Conformational study on glycosylated asparagine-oligopeptides by NMR spectroscopy and molecular dynamics calculations

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Abstract: The conformational properties of the homo oligomers of increasing chain length Boc-$(Asn)_n$-NHMe ($n = 2, 4, 5$), (GlcNAc-β-$Asn)_n$-NHMe ($n = 2, 4, 5, 8$) and Boc-[GlcNAc$(Ac)_3$-β-$Asn]_n$-NHMe ($n = 2, 4, 5$) were studied by using NOE experiments and molecular dynamic calculations (MD). Sequential NOEs and medium range NOEs, including (i,i+2) interactions, were detected by ROESY experiments and quantified. The calculated inter-proton distances are longer than those characteristic of β-turn secondary structures. Owing to the large conformational motions expected for linear peptides, MD simulations were performed without NMR constraints, with explicit water and by applying different treatments of the electrostatic interactions. In agreement with the NOE results, the simulations showed, for all peptides, the presence of both folded and unfolded structures. The existence of significant populations of β-turn structures can be excluded for all the examined compounds, but two families of structures were more often recognized. The first one with sinusoidal or S-shaped forms, and another family of large turns together with some more extended conformations. Only the glycosylated pentapeptide shows *in vacuo* a large amount of structures with helical shaped form. The results achieved in water and in DMSO are compared and discussed, together with the effect of the glycosylation.

Keywords: asparagine oligomers; conformation; glycosylated oligopeptides; molecular dynamics calculations; NMR spectroscopy

INTRODUCTION

Despite the fact that glycosylation of proteins represents one of the most significant post-translational events and is involved in important biological processes, such as cellular and molecular recognition, intercellular communication, cell control and growth [1–6], the consequences of glycosylation on the conformation and molecular properties of proteins are not yet fully understood [3–6]. Glycans intervene in protein folding during their biosynthesis, stabilize their biologically active conformation and display important functions in many other biological processes, but many questions on the role of glycoconjugate glycans are still open [4–6]. Small linear [7–13] and cyclic [14–16] glycosylated peptides have been used as model systems to study the effects of the sugar moieties on peptide conformation.

NMR spectroscopy, in particular through NOE experiments [17], can provide direct evidence for sugar–peptide local interaction and for peptide backbone conformational preference. The main difficulty in these studies is imputable to the high flexibility of small peptides, which generally adopt many conformations in solution. Furthermore, the detection of hydrogen bonds in the solvents used, H_2O or DMSO, is very difficult.

More detailed investigations have been carried out on *N*-glycoproteins in comparison with *O*-glycoproteins. NMR studies have shown that *N*-glycosylation drastically affects the peptide conformation in linear peptides [7], but no change in the backbone conformation of a cyclic hexapeptide was observed upon glycosylation [14,15]. Asparagine has a unique conformational property due to the possible side chain–backbone hydrogen bond formation, which allows the formation of a 10-member ring (Asn-turn) [18] similar to a β-turn structure [19]. A 7-member ring similar to a γ-turn can also occur [20]. Ten-member rings and type I or type II β-turns were more frequently found in proteins, mainly localized at the folding sites of the peptide backbone. The ability of asparagine to adopt positive values for Φ angles, analogous to D-proline [21]

Abbreviations: Standard abbreviations for amino acid derivatives and peptides are according to the suggestions of the IUPAC-IUB Commission on Biochemical Nomenclature (1984) *Eur. J. Biochem.* **138**: 9–37. Abbreviations listed in the guide published in *J. Peptide Sci.* 2003; **9**: 1–8 are used without explanation. Other abbreviations: GlcAc, 2-deoxy-2-acetamido-β-D-galactopyranosylamine; MD, molecular dynamic; RMS, root mean square; NOE, nuclear Overhauser effect; NOESY, nuclear Overhauser effect spectroscopy; ROESY, rotating frame Overhauser effect spectroscopy.

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results in a relative stabilization of the 'mirror image' β-turns (type I′ and type II′). The L-Asn-Gly segment was in fact identified as a potential β-sheet promoter, because it can be involved in a type I′ conformation and was then successfully used to nucleate β-hairpin formation [22–24]. The high propensity of asparagine to adopt turn-type structures has been suggested to explain its unique reactivity to the glycosylation reaction [8].

In order to study the conformational properties of natural glycosylated homopolymers, three series of asparagine-oligopeptides were synthesized [25]: the carbohydrate-free peptides (series **1**), the mono-acetylated glycopeptides (series **2**) and the fully acetylated glycopeptides (series **3**). The results of the IR and CD experiments [25] suggested the presence of turn-type structures for the carbohydrate-free peptides and for the fully acetylated glycopeptides, while ambiguous results were obtained for the series **2** peptides. A conformational study on selected compounds of the three series was performed by using NMR spectroscopy (NOE experiments) and molecular dynamics calculations.

RESULTS AND DISCUSSION

Assignment of Proton Resonances and H,H Coupling Constants

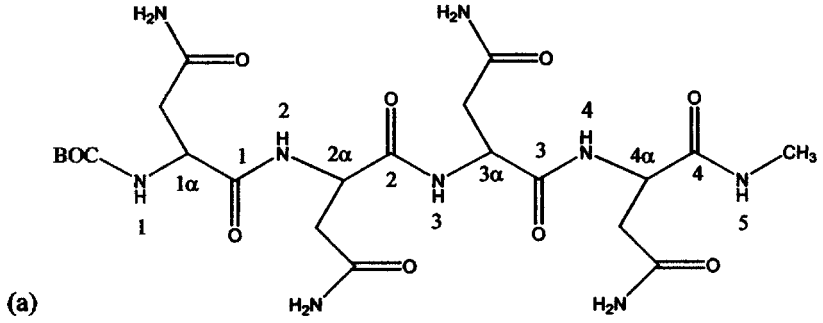
The sequential assignments in the peptide backbones were performed following the known strategy for protein structure [19]. The procedure was established using a series of TOCSY and NOESY experiments in DMSO and in deuterated and non-deuterated water; the ambiguities were resolved by acquiring spectra at different temperatures and different pH values. Starting from the NHMe signals of the *C*-terminal end, and following the NH-NH and αH-NH (i,i + 1)NOE interactions, all protons were assigned for the dipeptides and tetrapeptides. Some resonances of the inner residues of the other oligomers are overlapped. The data for the peptides of series **1** and **2** in water solution are reported in Table 1.

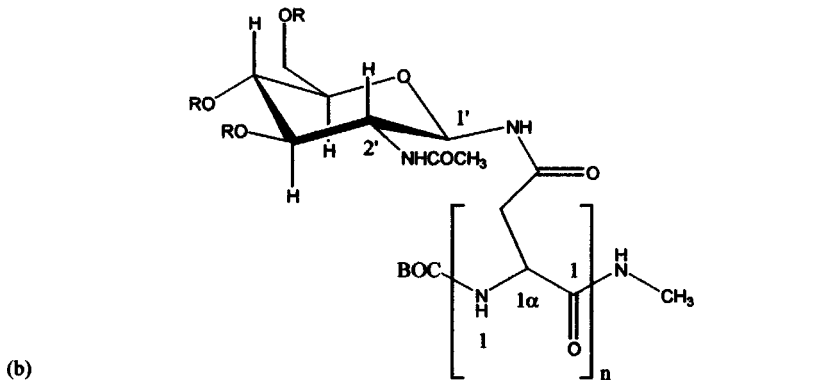
The resonances of the sugar moieties were completely assigned only for the dipeptide of series **2**. The proton signals are well separated for each sugar ring, except for 4′-H and 5′-H and all the coupling constants, except for $J(4',5')$, can be obtained by 1st order analysis. In

1 Boc-$(Asn)_n$ – NHMe ($n = 2,4,5$)

2 Boc-$(GlcNAc\text{-}\beta\text{-}Asn)_n$ – NHMe ($n = 2,4,5,8$)

3 Boc-$[GlcNAc(Ac)_3\text{-}\beta\text{-}Asn]_n$ – NHMe ($n = 2,4,5$)





Scheme 1 (a) Non-glycosylated tetrapeptide (series **1**) (b) Schematic representation of the peptides of series **2** (R = H) and **3** (R = COMe).

Table 1 Assignments of the Proton Resonance for the Peptide Fragment of Asn-oligopeptides of Series **1** and **2**[a]

	1 ($n = 2$)			**2** ($n = 2$)		
	NH	αH	$\beta\beta'$H	NH	αH	$\beta\beta'$H
Asn1	7.33	4.50	2.87, 2.82	7.29	4.45	2.72, 2.80
Asn2	8.68	4.74	2.87, 2.82	8.58	4.66	2.72, 2.80
NHMe	8.03			7.94		
	1 ($n = 4$)			**2** ($n = 4$)		
Asn1	7.23	4.45	2.95, 2.75	7.17	4.42	2.85, 2.70
Asn2	8.67	4.70	2.85, 2.75	8.56	4.67	[b]
Asn3	8.65	4.70	2.85, 2.75	8.53	4.67	2.75, 2.75
Asn4	8.47	4.62	2.95, 2.93	8.41	4.59	2.80, 2.80
NHMe	7.95			7.83		

	1 ($n = 5$)		**2** ($n = 5$)		**2** ($n = 8$)		
	NH	αH[c]	NH	αH[d]		NH	αH[e]
Asn1	7.22	4.53	7.15	4.44	Asn1	7.20	4.48
Asn2	8.73	4.78	8.60	4.72	Asn2	8.60	4.69
Asn3	8.61	4.78	8.51	4.72	Asn3,4,5	8.55	4.69
Asn4	8.64	4.78	8.51	4.72	Asn6	8.50	4.69
Asn5	8.50	4.72	8.40	4.62	Asn7	8.72	4.69
NHMe	7.95		7.85		Asn8	8.45	4.62
					NHMe	7.88	

[a] Spectra measured in ppm (δ) from external DSS reference. Solvent $H_2O:D_2O$ (9 : 1), pH 6.0–6.7, temperature 5 °C unless otherwise specified. β and β' stand for low and up-field, respectively. Boc methyl protons lie at 1.43–1.46 ppm and the terminal NMe protons lie at 2.72–2.76 ppm for all oligomers. The geminal protons of γ-NH_2 groups lie at 7.03–7.06 and at 7.75–7.79 ppm for all oligomers.
[b] Not detected.
[c] β and β' protons lie at 3.00 for Asn1, 2.85, 2.78 for Asn5 and 2.80, 2.79 for the others.
[d] β and β' protons at 2.81, 2.70 for Asn1, 2.81, 2.77 for Asn5 and 2.77 for the others.
[e] β and β' protons at 2.85, 2.68 for Asn1, 2.85, 2.79 for Asn8 and 2.79 for the others.

the case of the other oligomers, the proton resonances on each glucose ring are very close and only the protons of the terminal residues can be distinguished. The 1′-H and 1′-NH resonances were detected for all residues of the tetrapeptide of series **2**. Although not assigned, they are enough separated to allow the measurement of the most significant coupling constants, i.e. $J(1',2')$ and $J(1',NH1')$, which were used to obtain information about the geometry at the glycosidic bond. The coupling constants values for the di- and the tetrapeptide of series **2** are reported in Table 2. The sugar protons of the penta and octapeptide of the same series are overlapped except for the 1′-H resonances, as occurs for the 4-mer. This allowed $J(1',2')$ to be measured, which for all units was in the range 9.8–10.0 Hz. The coupling constant $J(1',NH1')$ (8.8 Hz) was measured for the pentapeptide Asn 5 unit only.

Table 2 Chemical Shift and Coupling Constant Values for the Sugar Moieties of the Dipeptide and the Tetrapeptide of Series **2**[a]

2 ($n = 2$)

Shift[a]	Asn1	Asn2	J(H, H)[b]	
1′-H	5.03[c]	5.07[c]	J(1′,2′)	9.8
2′-H	3.81	3.81	J(2′,3′)	10.0
3′-H	3.60[d]	3.61[d]	J(3′,4′)	8.5
4′-H, 5′-H	3.48–3.58	3.48–3.58	J(5′,6′)	2.0
6′-H	3.87	3.87	J(5′,6″)	4.9
6″-H	3.75	3.75	J(6′,6″)	12.0
1′-NH	8.82[e]	8.80[e]	J(1′,NH1′)	9.0
2′-NH	8.32	8.35	J(2′,NH2′)	9.2
NCOMe	2.02	2.02		

2 ($n = 4$)

Shift[a]		J(1′,2′)[d]
1′-H	5.09, 5.07, 5.06, 5.05[d]	9.8
2′-H	3.84–3.79	9.6
3′-H	3.78–3.73	9.8
4′-H, 5′-H	3.54–3.46	10.0
6′-H	3.86–3.90	
1′-NH	8.72[f], 8.79[g], 8.77[h]	J(1′,NH1′)[d]
2′-NH	8.29–8.32	9.0[b]

[a] Measured in ppm (δ) from external DSS reference; solvent D_2O, 22 °C, pH 8.9. NH data were obtained in $H_2O:D_2O$ (9 : 1), 5 °C, pH 6.7. *J*s were measured in Hz by 1D experiments, estimated accuracy ±0.1 Hz.
[b] The values are the same for all the sugar units.
[c] Assigned by the NOE interaction with the Boc methyl protons. The scalar interaction with 1′-NH could not be used because 1′-H protons lie under the water peak and in D_2O the NH signals are not visible. 1′-H protons were thus related through TOCSY to the other protons, for each sugar ring.
[d] The separation is not sufficient for the assignment, but allowed to measure the coupling constants.
[e] Assigned by NOE interactions with αH of the same residue, as the vicinal βH of all units are coincident.
[f] Asn1.
[g] Asn4.
[h] Asn2 and Asn3.

The proton coupling constants of the peptide backbone, $J(H\alpha,NH)$ are good indicators for the dimension of the dihedral angles CO–N–Cα–CO = Φ [19]. The *J* values obtained (Table 3) were in the range 7.2–8.5 Hz, as normally found in linear peptides. In the case of the glycopeptides of series **2** ($n = 4$, 5, 8), the inner residues showed values (7.2–7.6 Hz) slightly lower than the terminal ones (8.0 Hz) which were similar to those found for the dipeptide of the same series. However, the *J*s did not show the alternating values, typical of β-turn structure [19], but rather indicate a conformational averaging due to the high flexibility of the molecules. The values for the

Table 3 Coupling Constant Values for the Backbone of the Peptides **1** and **2**[a]

J(αH, NH)	**2**-mer			**4**-mer				**5**-mer		**8**-mer
	DMSO		H_2O	H_2O		DMSO		DMSO	H_2O	H_2O
	1	**2**	**2**	**1**	**2**	**1**	**2**	**1**	**2**	**2**
Asn1	7.4	8.0	8.0	7.8	8.0	7.2	8.0	8.2	8.4	8.0
Asn2	8.2	9.0	8.3	7.4	7.4	7.2	[b]	7.5	7.4	7.5
Asn3				7.4	7.6	7.8	7.5	7.3	7.2	[b]
Asn4				7.8	7.8	8.0	8.0	7.5	7.2	[b]

[a] Measured in Hz by 1D experiments and without presaturation of water signal. Estimated accuracy ±0.1 Hz. In DMSO at 22 °C, in H_2O at 5 °C and pH 6.0–6.1.
[b] Not detected for partial overlapping with other signals.

carbohydrate-free compounds (series **1,** $n = 2, 4, 5$) were similar, although their spectra were broader.

The *cis-trans* stereoisomers at the terminal amide bonds of the dipeptides of the series **1** and **2** can be easily quantified by simple integration of the well-separated NH peaks. The inter-conversion process is slow with respect to the NMR time scale, and the resonances can be correlated by NOE-exchange peaks. The most abundant isomer (85%–90%) must have the most stable *trans* configuration [26]. For the inner amide bond, a small amount of the *cis* isomer was present, but it could not be quantified. In the case of tetra- and pentapeptides, the ratio of *cis-trans* stereoisomers at the amide bonds can be measured only for Asn1 (*trans* isomer 80%). Owing to their low solubility in water the fully acetylated peptides (series **3)** were measured in TFE–H_2O mixture and gave similar results (data not reported).

NOE Experiments

Dipeptides and tetrapeptides of series 1, 2 and 3. The conformational study of the dimeric species was a basis for the analysis of the other homo-oligomers. Two solvents, DMSO and water, were used for the NOE experiments. Intra-residue and sequential NOE interactions involving αH, βH and NH protons were detected, in both solvents, in the glycosylated and in the non-glycosylated peptides. The strong sequential N,N and α,N interactions suggest [19] the presence of folded structures, which seems to be strengthened by the finding of an (i,i + 2)α,N NOE between αH and NH protons of the two last units at the *C*-terminal ends. The same results and specifically the same (i,i + 2) NOE interactions were found in TFE–H_2O mixture for the fully acetylated peptides (series **3**), whereas for the non-glycosylated compounds (series **1**), this interaction was detected in DMSO and not in water. An example of ROESY spectrum is reported in Figure 1.

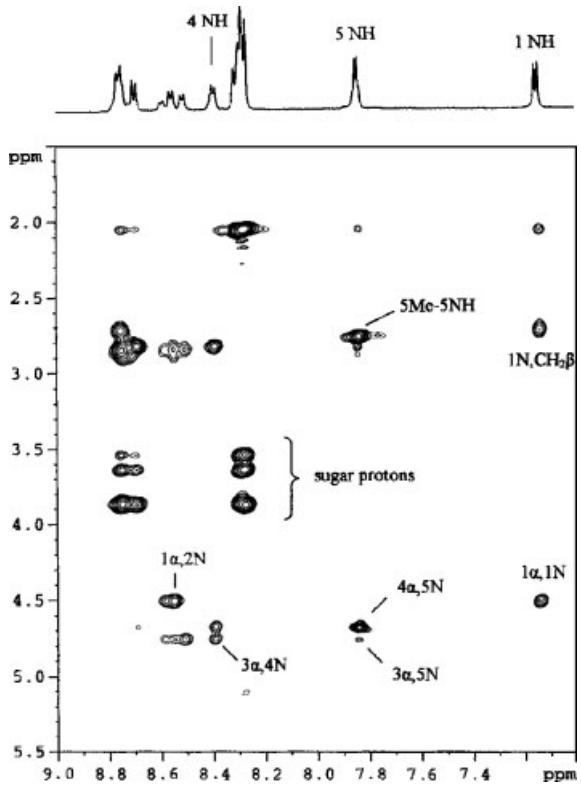


Figure 1 2D ROESY spectrum of the glycosylated tetrapeptide **2** ($n = 4$) in $H_2O : D_2O$ (9 : 1) at 5 °C pH 6.2.

For the tetrapeptides of the three series no interactions (zero NOE) were found between protons of Asn1 and Asn3, whereas NOE contacts of Boc-methyl protons with NH and αH of Asn1 and Asn2 were always detected. Other weaker (i,i + 2) interactions, found only by NOESY experiments for the glycosylated 4-mer, i.e. 2N,4N and 3N,5N, were affected by spin diffusion, as shown by experiments with different mixing times, from 50 to 300 ms, and confirmed by MD calculations. Only three of the 22 structures derived from MD calculations, presented corresponding distances shorter than 5 Å.

The quantification of the NOE results is a necessary step for conformational analysis; consequently the inter-proton distances were derived from the experimental volumes. The data for the tetrapeptides of series **1** and **2** are given in Table 4. The distances for the compounds of series **1** are not reported; although their solubility in water is low, the values for the intra-residue and the sequential interactions were similar to those obtained in DMSO, but the (i,i + 2) interactions were not detected. The data for the dipeptides are not reported, but they are available upon request.

The results showed that the (i,i + 1) $d_{N,N}$ and the (i,i + 2) $d_{\alpha N}$ distances were longer than those typical of β-turns [19]. In particular the (i,i + 2) α,N were 4.3–4.5 Å instead of 3.6 Å, and for the tetrapeptides the alternating intensities of the sequential interactions, generally required for β turn structures [19], were not

Table 4 Inter-proton Distances (Å) for the Tetrapeptides **1** and **2** ($n = 4$), obtained from the Experimental NOEs[a]

Peptide segment						
Intra-residue distance (Å)	**1**[b]	**2**[b]	**2**[c]	Sequential distance (Å)	**1**[b]	**2**[b]	**2**[c]
Asn1αH...NH	3.1	3.1	3.5	1NH...2NH	3.2	3.2	3.2
Asn2αH...NH	2.6	3.0	3.0	2NH...3NH	w	w	w
Asn3αH...NH	2.3	2.2	2.7	3NH...4NH	3.2	w	w
Asn4αH...NH	2.6	2.4	2.7	4NH...5NH	3.0	3.2	3.5
Asn1βH...NH	2.6	2.6	m[d]	1αH...2NH	3.2	3.6	3.6
Asn1β'H...NH	3.7	3.7	m[d]	2αH...3NH	m[e]	s[e]	s[e]
Asn4βH...NH	2.6	2.7	s[d]	3αH...4NH	m[e]	s[e]	s[e]
Asn4β'H...NH	3.7	3.6	s[d]	4αH...5NH	3.1	2.8	3.0
Medium range				Sugar-peptide			
Distance (Å)	**1**[b]	**2**[b]	**2**[c]	Distance (Å)	**2**[b]		**2**[c]
3αH...5NH	4.7	m[g]	4.5	Asn1NH1′...NH	w		4.5
2NH...5NH[f]	[h]	ww	[h]	Asn1NH1′...αH	w		4.7
1αH....3NH	zero NOE			Asn4NH1′...NH	w		w
BocMe...1NH	3.4	3.6	3.7	Asn4NH1′....αH	w		4.5
BocMe...2NH	4.0	3.8	4.0	Boc-Me...1H-1′	3.5[i]		3.5[i]
BocMe...1αH	3.6	3.4	3.4	Boc-Me...1NH1′	ww[i]		4.8[i]
BocMe...2αH	w	w	w	Boc-Me...1NH2′	w[i]		4.2[i]
BocMe...3NH	w	w	[h]	Boc-Me...1NH2′	w[j]		w[j]

[a] The NOEs were measured by ROESY experiments and converted in distances by using Felix software and referenced to the known distance (2.5 Å) between NH and CH_3 protons of the *C*-terminal end. For the volumes that could not be measured the distances were estimated as follows: s = 2.5–3.0 Å, m = 3.0–4.0 Å, w = 4.0–5.0 Å, ww = 5.0–5.5 Å. The α, β interactions were detected but not reported, β and β' stand for low and up-field, respectively. Sugar-free peptide **1**, although poorly soluble in water showed intra-residue and sequential NOEs as in DMSO but the 3αH...5NH was not detected.

[b] In DMSO at 22 °C.

[c] In H_2O-D_2O (9 : 1) at 5 °C, pH 6.2.

[d] β and β' protons are overlapped.

[e] Estimated value, because the signal is partially overlapped by the intra α,N cross-peak.

[f] Detected only for **2** at −20 °C in DMSO : $CDCl_3$ (1 : 1) by NOESY.

[g] Overlapped by the sequential 4α,5N, but detected (w) at −20 °C as in (*f*).

[h] Not detected.

[i] The signals for all the residues lie together: a single cross-peak was detected, which is assumed to belong to Asn1.

[j] Presumed intra-residue interaction, as the signal for all the residues lie together.

found. Actually the NOE data must be considered as an average of the interactions occurring for many conformations, and the amount of unfolded conformations might be relevant in the case of linear peptides.

Most of the sugar–sugar and sugar–peptide interactions, such as those between 1′-NH and $\beta\beta'$H within the same residue, are obvious but other NOE contacts gave information about the glycosydic bonds and the orientation of the sugar moieties with respect to the peptide backbone. As expected, both glucose rings adopt the most stable chair $^4C_1$ conformation, with all protons in axial orientation. This was easily deduced from the high value (9–10 Hz) of the coupling constants involving the protons on the ring, reported in Table 2. $J(4', 5')$ was missing, but the orientation of 5′-H followed from the *S* configuration of C-5′ in the glucose moiety. The geometry at the glycosidic bonds was deduced from the 9.8 Hz value of $J(1',2')$, which confirms the β configuration at C-1′. The orientation of the glucose moieties was obtained from the value of $J(1',NH1')$ and from the NOE interaction between 1′-NH and 2′-NH. The value of the coupling constants (9.0 Hz) indicated values of ± (150°–180°) for the dihedral angles H(1′)–C(1′)–N(1′)–H = θ, which correspond to O–C(1′)–N(1′)–CO angles in the range from −80° to −140°. Small θ values of 20°–30°, also consistent with the coupling constants, are not allowed because of the steric hindrance.

Pentapeptides of series 1 and 2 and octapeptide of series 2. For these peptides, the resonances of some inner residues are either overlapped or have too close chemical shifts. However, four N,N and four α,N sequential interactions, together with the (i,i + 2) 4α,6N, were found for the pentapeptides (Table 5). NOEs between Boc-methyl protons and the two proximal Asn residues were also detected. The intensities of the 4α,6N cross-peaks are very low in the case of the glycosylated peptide, in both DMSO and water and correspond to distance values of 5.0–5.5 Å. The non-glycosylated peptide is almost insoluble in water, but in DMSO the 4α,6N interaction was stronger than that of the glycosylated partner, corresponding to distance values of 4.0–5.0 Å. As occurs for tetrapeptides, zero NOEs between protons of Asn1 and Asn3 at the *N*-terminal end were also found. Owing to the NMR signals being too broad, the fully acetylated pentapeptide of series **3** was not analysed.

Six sequential interactions were found for the glycosylated octapeptide, together with a very weak cross-peak between 7NH and 9NH, corresponding to distance values of 5.0–5.5 Å. Zero NOEs were also found between protons of Asn1 and Asn3, as well as the expected NOEs of Boc protons with Asn1. For this peptide, good spectra were obtained in water, but not in DMSO, and only with NOESY experiments. The data are not reported.

Chemical Shift Variation with Temperature

The chemical shift values show a general shielding effect in DMSO in comparison with water; $\Delta\delta$: peptide

Table 5 NOE Interactions for Pentapeptides **1** and **2** ($n = 5$) Obtained from Experimental NOE Interactions[a]

Peptide segment

Intra-residue NOE	**1**[b]	**2**[b]	**2**[c]	Sequential NOE	**1**[b]	**2**[b]	**2**[c]
Asn1αH...NH	2.5–3.0	2.5–3.0		1NH...2NH	3.0–4.0		4.0–5.0
Asn2αH...NH	3.0–4.0[f]	3.0–4.0		2NH...3NH	[d]	[d]	4.0–5.0[e]
Asn3αH...NH	3.0–4.0[f]	3.0–4.0[g]		3NH...4NH	4.0–5.0	[d]	[d]
Asn4αH...NH	3.0–4.0	3.0–4.0[g]		4NH...5NH	[d]		4.0–5.0
Asn5αH...NH	2.5–3.0	3.0–4.0		5NH...6NH	3.0–4.0		3.0–4.0
Asn1βH...NH	3.0–4.0	3.0–4.0		1αH...2NH	2.5–3.0		3.0–4.0
Asn1β′H...NH	4.0–5.0	3.0–4.0		2αH...3NH	3.0–4.0[h]		3.0–4.0
Asn5βH....NH	3.0–4.0	3.0–4.0		4αH...5NH	3.0–4.0		3.0–4.0
Asn5β′H...NH	3.0–4.0	3.0–4.0		5αH...6NH	3.0–4.0		3.0–4.0
Medium range NOE	**1**[b]	**2**[b]	**2**[c]	Sugar-peptide		**2**[c]	
4αH....6NH	4.0–5.0	5.0–5.5		BocMe...1NH1′		4.0–5.0[i]	
1αH....3NH		zero NOE		BocMe...1NH2′		4.0–5.0[i]	
Boc-Me...1NH	4.0–5.0	4.0–5.0	3.0–4.0	Me[j,k]...NH		5.0–5.5	
Boc-Me...1Hα	4.0–5.0	4.0–5.0		5Me[j]...6NH		4.0–5.0	
Boc-Me...2NH	5.0–5.5	5.0–5.5		Me[j,k]...NH1′		4.0–5.0	
Boc-Me...2αH	4.0–5.0	5.0–5.5	4.0–5.0	5NH1′...6NH		5.0–5.5	

[a] Measured by ROESY experiments. The volumes could not be measured by Felix program, thus the distances were estimated as in note (a) of Table 3. The α,β interactions were detected but not reported. β and β′ stand for low and up-field, respectively.
[b] In DMSO at 22 °C.
[c] In $H_2O:D_2O$ (9 : 1), at 5 °C, pH 6.7.
[d] Not detected.
[e] Very close to the diagonal.
[f] 2αH and 3αH are partially overlapped.
[g] The cross-peaks are overlapped.
[h] Partially overlapped by 3αH...3NH cross-peak.
[i] Presumed with the vicinal Asn1, as the signals of NH1′ and respectively NH2′ lie together for all the residues.
[j] Methyl protons of NHCOMe group at C-2′.
[k] Intra-residue interaction.

NH 0.4–0.5 ppm, αH and βH 0.2–0.3 ppm, 1′-NH and 2′-NH 0.6 ppm. Actually, solvents which can act as H-donors, like water, might shift the NMR signals of the neighbouring protons to a lower field *via* protonation of the carbonyl oxygen. IR absorption measurements in the solid state suggested [25] the presence of weak intra-molecular hydrogen bonds. The identification of hydrogen bonds in DMSO or H_2O solution is very difficult. Although variable temperature NMR measurements in DMSO are extensively used to study peptides and proteins [10,11,18,27] the experimental errors are always high and the obtained results must be taken with caution. These experiments allowed the determination of the temperature coefficients for the exchangeable NH protons of peptides of series **1** and **2** ($n = 2$, 4, 5) (Table 6). According to the literature data, values of $-\Delta\delta/\Delta T \times 10^3$ ppb/K below 3 are consistent with intra-molecular hydrogen-bonding, while values of 5, or greater, are typical of hydrogen-bonding to the solvent. For instance in the case of the tripeptide Bz-Asn-Leu-Thr, a coefficient value, $-\Delta\delta/\Delta T$ of 3.2 ppb/K is reported for Thr NH as consistent with the Asx-turn structure [10]. Therefore, the values of 2.5–3.2 ppb/K, found for the terminal NHMe of the dipeptide **2**, for the NH protons of Asp4 and for the terminal NHMe of the tetrapeptides of both series **1** and **2**,

Table 6 Temperature Dependence of the NH Protons Chemical Shift (ppb/K) for Peptides **1** and **2** ($n = 2, 4, 5$)[a]

	$-\Delta\delta/\Delta T$ (DMSO)				
	2-mer		4-mer		5-mer
	1	**2**	**1**	**2**	**2**
Asn1NH	4.4	5.0	5.0	3.5	5.0
Asn2NH	3.2	3.6	4.7	3.2	[b]
Asn3NH	[b]	3.0	4.4	4.6	[b]
Asn4NH			3.1	3.0	[b]
Asn5NH			2.5	3.2	4.0
Asn6NH					3.5
NH-1′		5.0, 5.3		4.8	[b]

[a] Measured in 5° increments over the temperature range 283 to 320 K.
[b] Not detected.

should suggest that these NH protons are more solvent-protected than the others, and thus a number of folded conformations with intra-molecular hydrogen-bonding might be in equilibrium with the non-hydrogen-bonded forms. If compared with these values, those obtained for the NH protons of the two units at the *N*-terminal end of the glycosylated pentapeptide, 3.5 and 4.0 ppb/K, suggest more open conformations.

MD Simulations

Because of the large conformational motions expected for these peptides, MD simulations were performed without experimental constraints [28]. To investigate the behaviour of these molecules in solution, different treatments of the electrostatic interactions were applied by using a dielectric constant ε of 1, a distant-dependent dielectric constant of 4, which should mimic the solution in DMSO, and simulations with explicit water. A set of 22 minimized structures of comparable energies was obtained for each run.

In order to analyse the results, for each structure, the distances $d_{N,C}$ between the nitrogen of Asn1 and the carbonyl carbon atom of the last Asn unit were considered first, and these values were compared with those of the all-extended conformation. The average values of $d_{N,C}$ obtained from the simulations are reported in Table 7. In order to assess the reliability of the MD results, the sets of the calculated conformations must be compared with the NMR data, and the most important NMR parameters to do it are the (i,i + 2) NOE interactions. The NOE data, converted in distances, were thus compared with the corresponding average distances $d_{1\alpha,3N}$, $d_{3\alpha,5N}$, $d_{4\alpha,6N}$ and $d_{7N,9N}$, obtained from the simulations for the 2-mer, 4-mer, 5-mer and 8-mer, respectively.

The dipeptides of series **1** and **2** gave similar results (Table 7). Two families can be recognized in the ensembles of structures obtained with $\varepsilon = 1$ and 4; one is formed by large turns, the other displays S-shaped forms, but some extended structures are also

Table 7 Data Obtained by MD Simulations Performed *in vacuo* ($\varepsilon = 1$), with a Distance-dependent Relative Permittivity ($\varepsilon = 4$) and with Explicit Water

	$\varepsilon = 1$	$\varepsilon = 4$	H_2O
Dipeptide **1**			
Average $d_{N,C}$ (Å)[a]	5.1	5.1	4.8
a.a. deviation[b]	0.24	0.29	0.13
Average $d_{1\alpha,3N}$ (Å)[c]	4.1	4.4	5.3
RMS[d]	1.3	0.5	0.5
Dipeptide **2**			
Average $d_{N,C}$ (Å)	5.0	5.2	4.7
a.a. deviation	0.31	0.36	0.20
Average $d_{1\alpha,3N}$ (Å)	4.3	4.3	5.9
RMS	1.1	0.9	0.7
Tetrapeptide **1**			
Average $d_{N,C}$ (Å)	9.0	7.1	7.9
a.a. deviation	1.39	1.34	0.45
Average $d_{3\alpha,5N}$ (Å)	5.0	4.9	5.2
RMS	1.4	1.3	0.4
Tetrapeptide **2**			
Average $d_{N,C}$ (Å)	9.8	9.1	8.7
a.a. deviation	0.93	1.45	0.86
Average $d_{3\alpha,5N}$ (Å)	4.5	4.4	4.3
RMS	1.1	1.0	0.4
Pentapeptide **1**			
Average $d_{N,C}$ (Å)	9.2	8.5	10.8
a.a. deviation	1.76	1.89	1.17
Average $d_{4\alpha,6N}$ (Å)	4.5	5.4	4.7
RMS	1.0	1.1	0.7
Pentapeptide **2**			
Average $d_{N,C}$ (Å)	8.5	10.5	13.0
a.a. deviation	1.23	1.42	0.55
Average $d_{4\alpha,6N}$ (Å)	4.6	5.0	5.6
RMS	1.2	1.2	0.3
Octapeptide **2**			
Average $d_{N,C}$ (Å)	10.1	[e]	14.2
a.a. deviation	3.40		1.18
Average $d_{7N,9N}$ (Å)	4.8		5.0
RMS	1.1		0.4

[a] Arithmetic average of the distances between the nitrogen of Asn1 and the carbonyl carbon atom of the last Asn unit. In the all-extended conformation this distance is 6.1 Å for the dipeptides, 13.0 Å for the tetrapeptides, 17.4 Å for the pentapeptides and 27.9 Å for the octapeptide.
[b] Absolute average deviation, i.e. average of the absolute deviations from the average $d_{N,C}$.
[c] Arithmetic average of the (i,i + 2) distances between αH and NH protons.
[d] The RMS deviation was calculated from an average structure derived from those obtained by MD simulations.
[e] Not performed.

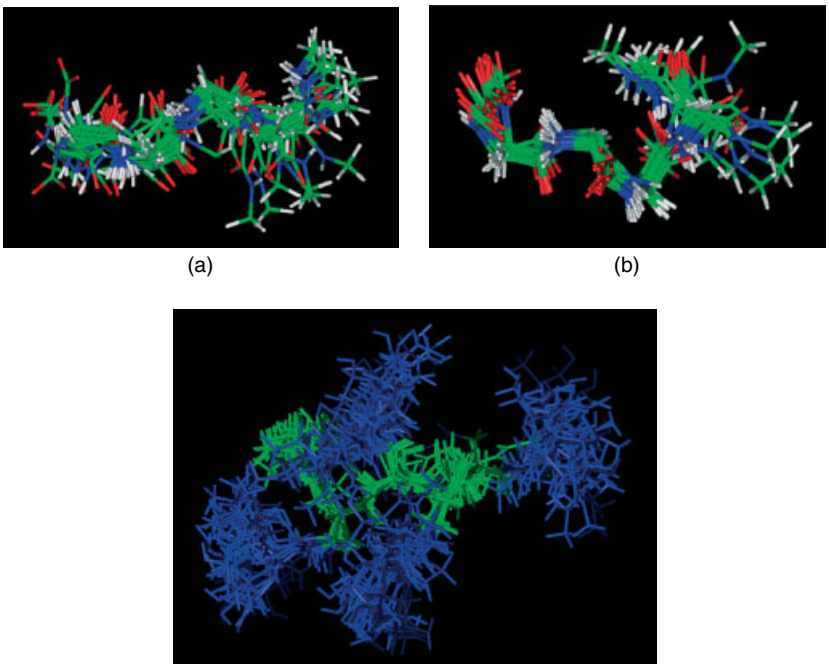


Figure 2 Ensembles of structures from the MD simulations of the glycosylated tetrapeptide **2** ($n = 4$); (a) (b) and (c) backbone profile, with (a) $\varepsilon = 4$, (b) with explicit water; (c) the same ensemble as in (b) with the Boc groups and the sugar moieties.

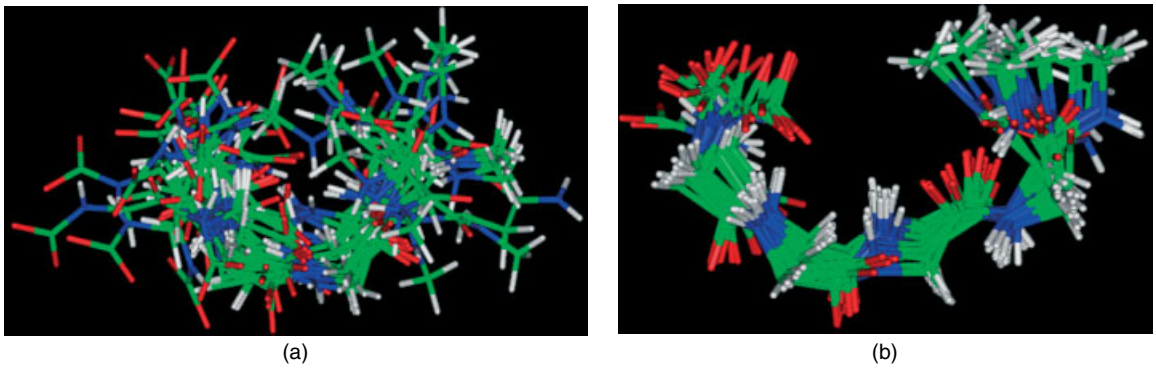


Figure 3 Ensembles of structures (backbone profile only) from the MD simulations of the tetrapeptide **1** ($n = 4$); (a) with $\varepsilon = 4$; (b) with explicit water.

present. The average $d_{1\alpha,3N}$ values are 4.1–4.4 Å, in agreement with the experimental values of 4.3 Å found in DMSO. The structures derived from the simulations with explicit water can instead be grouped in a single family of large turns, with $d_{1\alpha,3N}$ of 5.3 and 5.9 Å, values in line with the NOE results, i.e. zero NOE for the dipeptide of series **1** and 5.0–5.5 Å for the glycosylated partner **2**. The figures for the simulations of the dipeptides are not reported, but are available.

Tetrapeptides. The structures of the tetrapeptides of series **1** and **2** derived from the simulation with explicit water appear slightly more closed than those obtained *in vacuo*, as follows from the average $d_{N,C}$ values reported in Table 7. The ensembles are always more ordered in water, with the backbones well superimposed, as appears from the RMS = 0.4. The glycosylated tetramer presents sinusoidal shape conformations, while the non-glycosylated partner shows predominantly large turns (Figures 2 and 3).

The comparison with the NOE data shows that almost all the structures derived from the MD simulations display distances between 3Hα and 5NH protons within 4.5 Å (average $d_{3\alpha,5N}$ between 4.3 and 5.0 Å), in agreement with the (i,i + 2) NOE interaction. For the non-glycosylated peptide in water this distance is slightly longer (5.2 Å), but in line with the experimental results. The distances between protons of Asn1 and Asn3 are in agreement with the zero NOEs found for both tetramers, and the NOE interactions between Boc and backbone protons are compatible with the distances obtained from the large amount of the simulated structures.

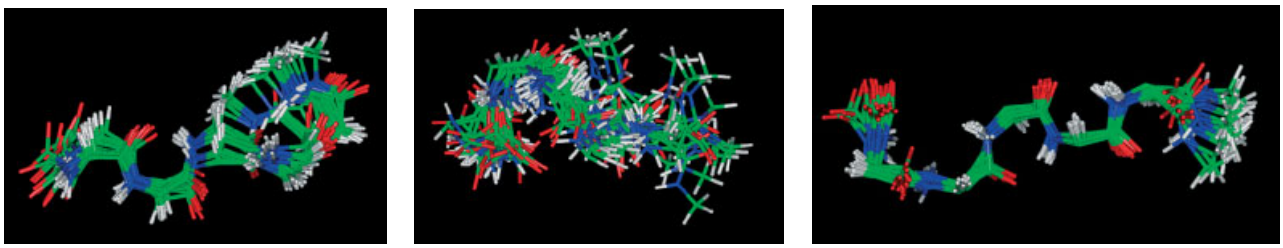


Figure 4 Ensemble of structures (backbone profile only) from the MD simulations of (a) the pentapeptide **1** ($n = 5$) with explicit water; (b,c) the glycosylated pentapeptide **2** ($n = 5$) with (b) $\varepsilon = 1$ and (c) with explicit water.

The 22 conformations of the glycosylated tetrapeptide, obtained from MD calculations with explicit water (Figure 2c), show the mobility of the sugar moieties. The glucose rings can rotate around the C(1′)–N(1′) bonds. The values of the coupling constants J(1′,NH1′) found for all the units allow a range of values for the angle O–C(1′)–N(1′)–CO, from −80° to −140°, which are in line with the simulation results.

Pentapeptides and octapeptide. The simulations of both pentapeptides of series **1** and **2**, with $\varepsilon = 1$ and 4 are quite disordered (RMS = 1.1 and 1.2). However, two families of structures can be recognized in the ensemble of the glycosylated peptide obtained with $\varepsilon = 1$ (Figure 4b). The most abundant family surprisingly shows helical shape conformations, the less abundant one, S-shaped forms. With the other simulations no helical structures were recognized, as well as with those performed for the sugar-free peptide. With $\varepsilon = 4$, for both peptides the structures appear as large turns or with S-shaped forms and the ensembles, though disordered, gave average $d_{4\alpha,6N}$ values (5.4 and 5.0 Å) and $d_{1\alpha,3N}$ in agreement with the experimental NOE data in DMSO (5.0–5.5 Å and zero NOE, respectively).

The simulations with water (Figure 4a and 4c) show some interesting results: the ensembles are very ordered, as was observed for dimers and tetramers, but the conformations are more extended than *in vacuo*, and also with respect to $\varepsilon = 4$, especially in the case of the glycosylated peptide, which has an average $d_{N,C}$ of 13 Å (for all-extended conformation it is 17.4 Å). This is also reflected in the average distance $d_{4\alpha,6N} = 5.6$ Å, which is in agreement with the value (5.0–5.5 Å) obtained from NOE experiments. For the sugar-free peptide this NOE interaction was not detected in water. The behaviour of the glycosylated pentapeptide in water is in contrast with the trend observed for the other peptides. Table 7 and Figure 4 show that the RMS values are low in water and the backbones very well superimposed, while the fluttering of the *C*-terminal end is more pronounced for the carbohydrate-free pentapeptide. The zero NOEs found between protons of Asn1 and Asn3 are in line with the models.

The simulations of the glycosylated octapeptide of series **2** were performed *in vacuo* and with explicit water. Only one of the five families of structures recognized *in vacuo* is enough extended. The others are folded but not helical shaped. The ensemble of structures obtained with water (Figure 5) is quite ordered and presents a slight folding at the *C*-terminal end. Although only few experimental data are available, the weak (i,i + 2) NOE interaction found between 7NH and 9NH confirms the folding of the *C*-terminal end toward the inner of the curve (average $d_{7N,9N}$ value from the simulations 5.0 Å).

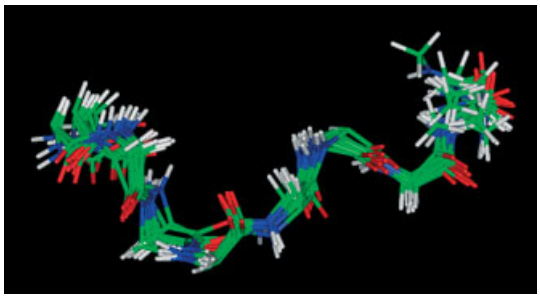


Figure 5 Ensemble of structures (backbone profile only) from the MD simulations of the octapeptide **2** ($n = 8$) with explicit water.

CONCLUSIONS

The results of the NOE experiments and MD simulations without constraints show that, beside the β-turn secondary structures, many conformations are compatible with the presence of sequential NOE interactions and with the medium range (i,i + 2) α,N. The ROESY cross-peak intensities depend on the distance between the two involved protons, as well as by the molecule mobility, and it was important to compare the NOE experimental data and the MD simulations without NOE constraints.

The NOE interactions showed that the inter-proton distances obtained from the experimental data are longer than those characteristic of type I and type II β-turn forms [19]. For instance, the (i,i + 2) $d_{\alpha,N}$ were found in the range 4.3–5.5 Å, compared with 3.6 Å, a value typical for β-turns. Consequently, a significant population of β-turns structures can be excluded for

all compounds which can be realistically depicted by ensembles of folded and unfolded conformations, especially in DMSO solution. This is confirmed by the simulations with dielectric constants, $\varepsilon = 1$ and $\varepsilon = 4$, which mimic the situation *in vacuo* and in DMSO solution, respectively. The ensembles of structures obtained with explicit water, especially those of the glycosylated peptides, are generally more ordered. The dimers' and tetramers' structures are more closed in water than *in vacuo*, whereas the opposite is true for the penta and octapeptides, also with respect to $\varepsilon = 4$.

The two conformation families more often recognized were either one with sinusoidal or S-shaped forms, or another one of large turns. Only the glycosylated pentapeptide shows *in vacuo* a large amount of helical structures (>60%). No helical shaped structures were found in water, not even with other simulations, including those of the octapeptide.

The calculated (i,i + 2) $d_{\alpha,N}$, average distances, involving the protons of the *C*-terminal units are in agreement with the values obtained from the corresponding NOE data. The fluttering of the *C*-terminal end is common to all compounds, as well as the folding toward the inner of the turn, which is more evident for the dipeptides and tetrapeptides, and is in part due to the presence of the methyl group. The temperature coefficient values calculated for the terminal NH protons of 2-mers and 4-mers, suggest that they are more solvent protected, and are in line with the above finding. The *N*-terminal end is in general more extended, in line with the zero NOEs found between protons of Asn1 and Asn3.

The experimental data indicate that, in the case of dipeptides and tetrapeptides, the sugar moieties do not induce relevant conformational changes, especially in DMSO solution. For the glycosylated pentapeptide, the intensities of the (i,i + 2)α,N NOE interactions are significantly lower than those of the tetrapeptides and dipeptides. This is more marked in water, indicating that the population of more extended structures in water increases from the glycosylated tetra-oligomer to the penta- and octa-oligomer.

The interactions with water of the polar glucose moieties appear to stabilize the conformations of the backbones, by acting as a protection from the solvent. The mobility of the sugar rings, which can rotate around the C(1′)–N(1′) bonds (the angle O–C(1′)–N(1′)–CO range from −80° to −140°) creates a sort of an hydrophilic cloud around the backbone of these peptides.

EXPERIMENTAL

NMR Experiments

The NMR spectra were recorded on a Bruker AMX 600 spectrometer operating at a frequency of 600.13 MHz for ^{1}H nucleus. The chemical shifts (δ) were measured in ppm and referenced to external DSS signal for DMSO solutions; for the other solvents, the residual water signal set at 4.78 ppm was used at a temperature of 22 °C and 5.02 ppm for 5 °C; estimated accuracy ±0.01 ppm. D_2O, H_2O–D_2O (90 : 10 v/v), DMSO-d_6 and TFE–H_2O (75 : 25 v/v) were used as solvents. Owing to the low solubility of the glycosylated peptides, the TFE–H_2O mixture was only occasionally used. The experiments in water were generally performed at 5 °C and pH 6.0–6.7 otherwise as specified in the Tables. The spectra in the other solvents were measured at 22 °C. Some NOE experiments were performed at −20 °C in DMSO–$CDCl_3$ (1 : 1) (peptide concentration 5–6 mM).

NOESY and ROESY spectra were acquired in the phase sensitive TPPI mode, with 1K × 512 complex FIDs, spectral width of 6024.096 Hz for water solvent, 9090.910 Hz for DMSO, recycling delay of 1.3 s, 64 scans, mixing times from 50 ms to 300 ms for NOESY and to 400 for ROESY. TOCSY and ROESY spectra were recorded with the use of MLEV-17 spin-lock pulse [29] (field strength 11 360 Hz and 6250 Hz, respectively, 60 ms total duration). All spectra were transformed and weighted with a 90° shifted sine-bell squared function to 1K × 1K real data points. The RF carrier frequency for the ROESY experiments in DMSO was placed at 6.0 ppm in order to avoid problems from Hartmann–Hahn correlations, but also in water such signals were not observed. For the ROESY spectra in water, the solvent suppression was achieved by pre-saturation technique, placing the carrier frequency on the H_2O resonance, whereas the NOESY spectra were measured by using gradient-based pulse programs, capable of suppressing the water signal and minimizing the magnetization loss due to saturation transfer. The volumes of the NOE peaks were integrated and transformed in inter-proton distances by FELIX software included in the Insight II & Discover programs, using as reference the known distance (2.5 Å) between NH and CH_3 protons of the *C*-terminal. The effect of the methyl group has been considered. The distance between the vicinal protons Hα, NH of Asn1 with measured coupling constant, was also used, finding a good agreement.

The NH temperature coefficients were obtained in DMSO, by monitoring the amide NH chemical shifts over a temperature range of 283 to 320 K in 5° increments. All changes in NH chemical shifts ($-\Delta\delta/\Delta$T) were linear over the above temperature range. The experiments were performed with the maximum care, but the error was estimated within ±0.38 ppb/K, by considering that the digital resolution is ±0.18 ppb and the accuracy in the temperature measurement by the Bruker system is within 0.1 °C, which corresponds to ±0.2 ppb/K.

Molecular Modelling

Molecular models were built using a Silicon Graphics 4D35GT workstation running the Insight II & Discover

software. Molecular mechanics (MM) and molecular dynamics (MD) were carried out using AMBER force-fields; a scaling factor of 0.5 was used for 1–4 interactions. The starting geometry of the peptides was generated using standard bond lengths and angles. The simulations were performed *in vacuo* with relative permittivity $\varepsilon = 1.0$, and with a distance-dependent relative permittivity ($\varepsilon = 4.0 \times r$) to simulate the solvent effect. For the aqueous environment, the system was surrounded by a sphere of water molecules with radius ranging from 15 to 25 Å. At the first step a minimization by Discover was performed with steepest-descendent algorithm followed by conjugate gradient minimization. Then MD simulations were performed from 20 to 40 ps, at a constant temperature of 1000 K, for $\varepsilon = 1.0$ and $\varepsilon = 4.0 \times r$, and at 300 K for the simulations with explicit water. The results from 20 and 40 ps MD simulations were similar. Every structure obtained from MD was further minimized. The energies of the minimized structures were within ±21 KJ/mol for $\varepsilon = 1$ and 4, and ±314 KJ/mol for the simulations with explicit water. An average structure was created for each MD simulation, in order to calculate the RMS deviation. This structure was used to superimpose the backbone of each single frame of the MD calculations.

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REFERENCES

1. Kassenbrock CK, Garcia PD, Walter P, Kelly RB. Heavy-chain binding protein recognizes aberrant polypeptides translocated *in vitro*. *Nature* 1988; **333**: 90–93.
2. Kozutsumi Y, Segal M, Normington K, Gething M, Sambrook MJ. The presence of unfolded proteins in the endoplasmic reticulum signals the induction of glucose-regulated proteins. *Nature* 1988; **332**: 462–464.
3. Dwek RA. Glycobiology: toward understanding the function of sugars. *Chem. Rev.* 1996; **96**: 683–720.
4. Imperiali B, O'Connor SE. Effect of N-linked glycosylation on glycopeptide and glycoprotein structure. *Curr. Opin. Chem. Biol.* 1999; **3**: 643–649.
5. Helenius A, Aebi M. Intracellular functions of N-linked glycans. *Science* 2001; **291**: 2364–2369.
6. Wyss DF, Wagner G. The structure of sugar in glycoproteins. *Curr. Opin. Biotechnol.* 1996; **7**: 409–416.
7. Hamilton Andreotti A, Kahne D. Effects of glycosylation on the peptide backbone conformation. *J. Am. Chem. Soc.* 1993; **115**: 3352–3353.
8. Imperiali B, Shannon KL, Rickert KW. Role of peptide conformation of asparagine-linked glycosylation. *J. Am. Chem. Soc.* 1992; **114**: 7942–7944.
9. Imperiali B, Spenser JR, Struthers MD. Structural and functional characterization of a constrained Asx-turn motif. *J. Am. Chem. Soc.* 1994; **116**: 8424–8425.
10. Lee K-C, Falcone ML, Davis JT. Sequence-specific peptide-carbohydrate interactions in an asparagines-linked glycopeptide. *J. Org. Chem.* 1996; **61**: 4198–4199.
11. Xu T, Marshall Werner RM, Lee K-C, Fettinger JC, Davis JT, Coward JK. Synthesis and evaluation of tripeptides containing asparagine analogs as potential substrates or inhibitors of oligosaccharyltransferase. *J. Org. Chem.* 1998; **63**: 4767–4778.
12. Live DH, Kumar RA, Beebe X, Danishefsky SJ. Conformational influence of glycosylation of a peptide. *Proc. Natl Acad. Sci. USA* 1996; **93**: 12 759–12 761.
13. Bailey D, Renouf DV, Large DG, Warren CD, Hounsell EF. Conformational studies of the glycopeptide Ac-Tyr-[Man_5GlyNAc-β-(1 → 4)GlyNAc-β-(1 → N^{δ})]-Asn-Leu-Thr-Ser-Obz and the constituent peptide and oligosaccharide. *Carbohydr. Res.* 2000; **324**: 242–254.
14. Kessler H, Matter H, Gemmecker G, Kottenbahn M, Bats JW. Structure and dynamics of a synthetic *O*-glycosylated cyclopeptide in solution determined by NMR spectroscopy and MD calculations. *J. Am. Chem. Soc.* 1992; **114**: 4805–4818.
15. Hoffmann M, Burkhart F, Hessler G, Kessler H. *C*-glycoside analogues of N^4-(2-acetamido-2-deoxy-β-D-glucopyranosyl)-L-asparagine: synthesis and conformational analysis of a cyclic *C*-glycopeptide. *Helv. Chim. Acta* 1996; **76**: 1519–1532.
16. Vass E, Lang E, Samu J, Majer Zs, Kajtar-Peredy M, Mak M, Radics L, Hollosi M. Vibrational spectroscopic detection of H-bonded β- and γ-turns in cyclic peptides and glycopeptides. *J. Mol. Structure* 1998; **440**: 59–71.
17. Neuhaus D, Williamson M. *The Nuclear Overhauser Effect in Structural and Conformational Analysis*. VCH: New York, 1998.
18. Abbadi M, Mcharfi M, Aubry A, Premilat S, Boussard G, Marraud M. Involvement of side functions in peptide structures: the Asx turn. Occurrence and conformational aspects. *J. Am. Chem. Soc.* 1991; **113**: 2729–2735.
19. Wüthrich K. *NMR of Proteins and Nucleic Acids*. John Wiley & Sons: New York, 1986.
20. Rose GD, Gierasch LM, Smidth JA. Turns in peptides and proteins. *Adv. Prot. Chem.* 1985; **37**: 1–109.
21. Venkatraman J, Shankaramma C, Balaram P. Design of folded peptides. *Chem. Rev.* 2001; **101**: 3131–3152.
22. de Alba E, Jimenez MA, Rico M. Turn residue sequence determines β-hairpin conformation in designed peptides. *J. Am. Chem. Soc.* 1997; **119**: 175–183.
23. Maynard AJ, Searle MS. NMR structural analysis of a β-hairpin peptide designed for DNA binding. *J. Chem. Soc. Chem. Commun.* 1997; 1298.
24. Syud FA, Espinosa JF, Gellman SH. NMR-based quantification of β-sheet populations in aqueous solution through use of reference peptides for the folded and unfolded states. *J. Am. Chem. Soc.* 1999; **121**: 11 577–11 578.
25. Biondi L, Filira F, Gobbo M, Rocchi R. Conformational investigations on glycosylated asparagine-oligopeptides of increasing chain length. *J. Pept. Sci.* 2002; **7**: 80–92.
26. Kessler H, Anders U, Schudok M. An unexpected *cis* peptide bond in the minor conformation of a cyclic hexapeptide containing only secondary amide bonds. *J. Am. Chem. Soc.* 1990; **112**: 5908–5916.
27. Gellman SH, Dado GP, Liang G-B, Adams BR. Conformation-directing effects of a single intramolecular amide-amide hydrogen bond: variable temperature NMR and IR studies on a homologous diamide series. *J. Am. Chem. Soc.* 1991; **113**: 1164–1173.
28. Bartels C, Stote RH, Karplus M. Characterization of flexible molecules in solution: the RGDW peptide. *J. Mol. Biol.* 1998; **284**: 1641–1660.
29. Bax A, Davis DJ. MLEV-17 based two-dimensional homonuclear magnetization transfer spectroscopy. *J. Magn. Reson.* 1985; **65**: 355–360.

