Design and Synthesis of Glycosylated β^3-Peptides Capable of Folding into the 3_{14}-Helical Conformation in Water

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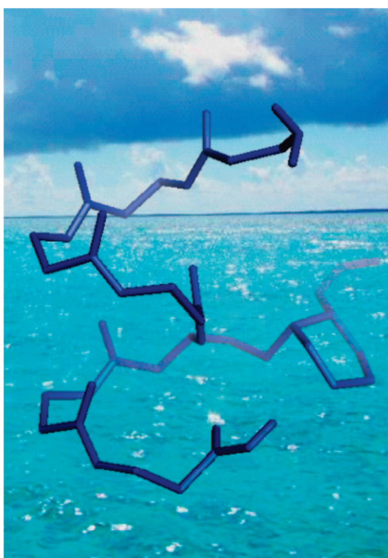

Herein, we describe the synthesis of seven glycosylated β^3-peptides, **1**−**7**, which were designed to adopt stable 3_{14}-helical conformations in aqueous solution. Such molecules are representative for a novel class of functionalized foldamers in which a natural post-translational modification is attached to an unnatural peptidomimetic backbone. Conformational studies by CD spectroscopic measurements were performed in methanol and in water (pH 7). Additionally, the influence of temperature, pH, and concentration on the ability of glycosylated β^3-peptides to adopt stable helical conformations were investigated. The first NMR-derived solution state structure of a glycosylated β^3-peptide in water is also presented.

Introduction

Folding of linear chain macromolecules into complex three-dimensional structures is one of the key strategies used by nature to generate molecules with widespread molecular function, i.e., molecular recognition, information storage, and catalysis, despite the use of only a small number of chemical building blocks. The ability to form well-ordered structures is also of central interest in the design of new homogeneous, sequence-specific artificial biomimetic macromolecules, a research field known as foldamer science.[1]

One of the most thoroughly investigated peptidomimetic foldamer to date is β-peptides, i.e., oligomers of β-amino acids.[2] These building blocks represent the smallest step away from α-amino acids in "backbone space", and when combined into oligomers they, like α-peptides, contain amide bond capable of forming structure stabilizing, intramolecular hydrogen bonds. Examples of secondary structures that β-peptides have shown to adopt are helices,[3] parallel and antiparallel pleated sheets,[4] and hairpins.[3] Surprisingly, even short β-peptides with as few as six residues can fold into defined secondary structures;

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moreover, the groups of Schepartz [5] and Gellman[6] recently demonstrated that β-peptide helices and mixed α/β-peptide helices can assemble into bundles, thus forming quaternary structures.

Throughout nature, a majority of all peptides are post-translationally modified. Such modifications introduce significant structural changes of the peptide/protein and thereby also change the biological properties and activities of the biomacromolecule. The most common post-translational modifications (PTMs) include glycosylation, phosphorylation, and lipidation of the peptide via the side chains of the amino acid residues. For instance, glycoproteins constitute more than 50% of the human proteins.

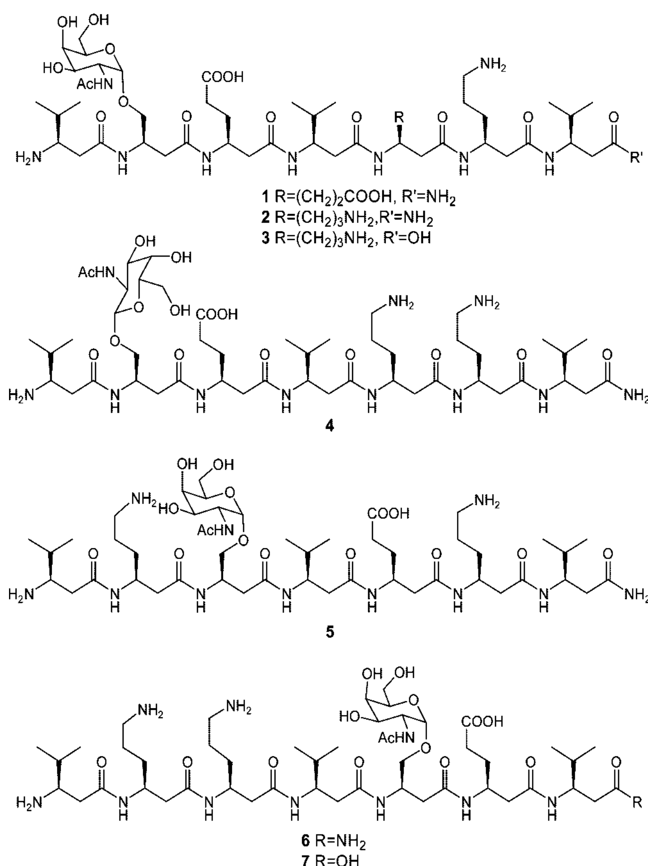
By introducing PTM on an artificial backbone, a functionalized foldamer hybrid is created. In the first demonstration of this concept,[7] we reported the synthesis and structural investigation of an *O*-glycosylated β-peptide. Subsequently, Gallagher[8] reported the use of similar constructs as a mean to study carbohydrate−carbohydrate interactions by placing them on the well-defined helical structure offered by cyclic β-amino acid oligomers, and our group reported the first biomolecular interaction study of glycosylated β-peptides and lectins.[9] It is easy to imagine a plethora of other interesting biomedical studies and applications of such conjugates, especially since β-peptides are known to be stable toward proteolytic and metabolic degradation.[10] Even more promising, the groups of Seeberger and Seebach recently concluded that *N*-linked glycosylated β-peptides are resistant toward degradation by glycoamidase A.[11]

In contrast to *N*-glycosylation, which in some cases can be similar between cell types, *O*-linked carbohydrates, such as the Tn epitope (αGalNAc-Ser/Thr), may offer greater potential for immune intervention, as these glycosylation patterns are only produced in cancer cells as a result of abnormal glycosylation.

Although the secondary structure is of less importance for such applications, we showed in a preliminary communication that an *O*-linked αGalNAc moiety introduced in a heptameric β^3-peptide destabilizes the 3_{14}-helical structure, especially in aqueous buffer.[7] Herein we present the design, synthesis, and conformational studies of seven GalNAc containing β^3-peptides (**1**−**7**) in an effort to enhance the helical stability of our glycosylated β^3-peptides.

Results and Discussion

Design. β-Peptides composed of acyclic proteinogenic β^3-amino acids derived from naturally occurring L-amino acids tend to fold into a left-handed 3_{14}-helical secondary structures, stabilized by hydrogen bonds between a backbone amide proton at position *i* and a main chain carbonyl oxygen at position $i + 2$, forming a series of intercatenated 14-membered rings.



In our earlier report,[7] glycosylated β^3-peptide **1** and an unglycosylated analogue were synthesized. The sequence was designed to fold into defined secondary 3_{14}-helical structures; the β^3hVal was introduced at positions 1, 4 and 7 for additional stabilization by hydrophobic interactions, whereas β^3hGlu and β^3hOrn were positioned as residues 3 and 6, respectively, forming electrostatic interactions.[3e]

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From the CD spectroscopic conformational studies of **1** and the unglycosylated analogue in methanol, characteristic features of the 3_{14}-helix was observed for both peptides. However, as seen from the intensity of the absorption, the incorporation of a GalNAc-carbohydrate residue on β^3hSer in position 2 lead to a slight destabilization of the 3_{14}-helical conformation. Further, the 3_{14}-helical conformation is known to be destabilized when changing solvent from methanol to water;[3e,12] however, the glycosylated peptide **1** lost more of its overall mean 3_{14}-helical population when recorded in water (pH 6.9) than its unglycosylated sequence analogue. NMR studies in methanol followed by a NOE-restrained Monte Carlo conformational sampling suggested that peptide **1** gave rise to two conformational families of comparable energies, whereas only one family was found for the peptide without a carbohydrate moiety. The two families of **1** arose as the β^3hOrn residue in position 6 could interact with both the β^3hGlu amino acid in position 3 and the GalNAc moiety at position 2, leading to an "opening" of the helix at the C-terminus. In order to address if the helical stability could be enhanced, a step-by-step design strategy was used to circumvent the interfering interaction between the β^3hOrn in position 6 and the GalNAc moiety at position 2 seen in the NMR investigations of **1**.[7] Consequently, glycosylated β^3-peptide **2** was synthesized. In this sequence, the β^3hGlu in position 5 of peptide **1** was exchanged to a positively charged β^3hOrn amino acid residue in an attempt to force the helical structure to "close". Furthermore, in order to extract the crucial factors for increased 3_{14}-helical stability of glycosylated β^3-peptides in aqueous solution, peptides **3**−**7** were designed and synthesized.

Peptide **3** has the same sequence as glycosylated β-peptide **2**, except for the inclusion of a free C-terminus. Further, during the synthesis of the αGalNAcβ^3hSer building block, there was always a certain amount β-product being formed (65:35 α/β-ratio, determined by ^{1}H NMR). In the natural system, the GalNAc moiety α-linked to either Ser or Thr represents the tumor-associated Tn antigen found in mucins, proteins secreted from epithelial cells. However, by incorporating a βGalNAcβ^3hSer residue into the sequence of peptide **2**, forming peptide **4**, the influence of the carbohydrate moiety's stereochemistry could be further investigated.

Additionally, the inclusion of β^3-amino acid residues not involved in any stabilizing interactions, such as β^3hSer, have been reported by Schepartz to influence the secondary structure stability depending on where in the sequence the incorporation was made.[13] Because of this, we synthesized glyco-β^3-peptides **5** and **6**, which had the GalNAc-linked β^3hSer derivative at position 3 and 5, respectively. It should be noted that even though the backbone sequences of **5** and **6** differ slightly, the unglycosylated analogues do not differ significantly in helical population at pH 7,[3e,7] and the results from the studies herein can therefore be seen as arising as a result of the position of the carbohydrate moiety rather than the difference in backbone sequence.

The Schepartz group has also made extensive studies to enhance the helical stability of the β^3-peptide 3_{14}-helical structure in aqueous buffer, taking into account the effect of the peptide sequence and macrodipole moment[14] as well as the salt-bridge identity,[15] before proceeding to study quaternary structures.[5] β-Peptides have a unique hydrogen bonding pattern that causes the helical macrodipole moment to orient in the opposite direction relative to the α-helix, with a partial positive charge at the C-terminus and a partial negative charge at the N-terminus. Therefore, the 3_{14}-helical structure can be stabilized by introducing a positively charged side chain close to the N-terminus and a negatively charged side chain close to the C-terminus and by preserving the charge associated with free N- and C-termini. Consequently, since peptide **6** was sequenced such that β^3hOrn was located close to the N-terminus and β^3hGlu close to the C-terminus, all in agreement with the design principles of Schepartz except for a free C-terminus, glycosylated β^3-peptide **7** was synthesized in order to study the effect of a free C-terminus on the folding propensity in buffer solution.

Synthesis. Glycosylated β^3-peptides **1**−**7** were synthesized by standard Fmoc solid-phase peptide synthesis on either Rink (**1**, **2**, **4**−**6**) or Wang solid support (**3** and **7**) as described in

TABLE 1. Analytical Reverse-Phase HPLC Retention Times and Observed MALDI Masses for Glycosylated β-Peptides 1−7

peptide	retention time (min)[a]	calcd exact mass	obsd mass[b]
1	6.0	1074.612	1075.639
2	3.6	1059.654	1060.708
3	3.1	1060.638	1062.992
4	4.5	1059.654	1060.746
5	3.2	1059.654	1060.681
6	3.5	1059.654	1060.746
7	3.7	1060.638	1061.893

[a] Analytical HPLC: gradient: 5 → 40% MeCN over 15 min on a C-18 column. [b] MALDI run using α-cyano-4-hydroxycinnamic acid matrix.

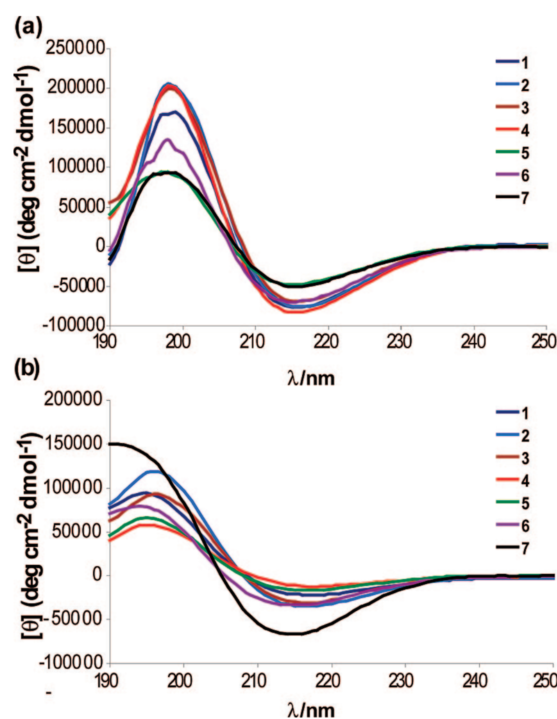

FIGURE 1. CD spectra of glycosylated β^3-peptides **1**−**7** in methanol (a) and in PBC buffer (pH 7) (b). All spectra were acquired at room temperature, $c = 0.1$ mM. The spectra have not been normalized as all peptides contain the same number of residues.

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TABLE 2. Summary of Helical Stability Represented by CD Spectra Minimum at ~215 nm; Relative Intensities between Peptides and Solvents Also Given for Comparison

	methanol		PBC (pH 7)		
peptide	minimum intensity of the 3_{14}-helix (deg cm^2 dmol^{-1})	change in helical population, sequence effect (%)[a]	minimum intensity of the 3_{14}-helix (deg cm^2 dmol^{-1})	change in helical population, sequence effect (%)[a]	change in helical population, solvent effect (%)[b]
1	−76695	+1	−22258	−35	−71
2	−75587		−34407		−54
3	−69076	−9	−30696	−11	−55
4	−83159	+10	−12636	−63	−85
5	−47940	−36	−16912	−51	−65
6	−70032	−7	−32855	−5	−53
7	−50418	−33	−66985	+95	+33

[a] Relative peptide. [b] Methanol → water.

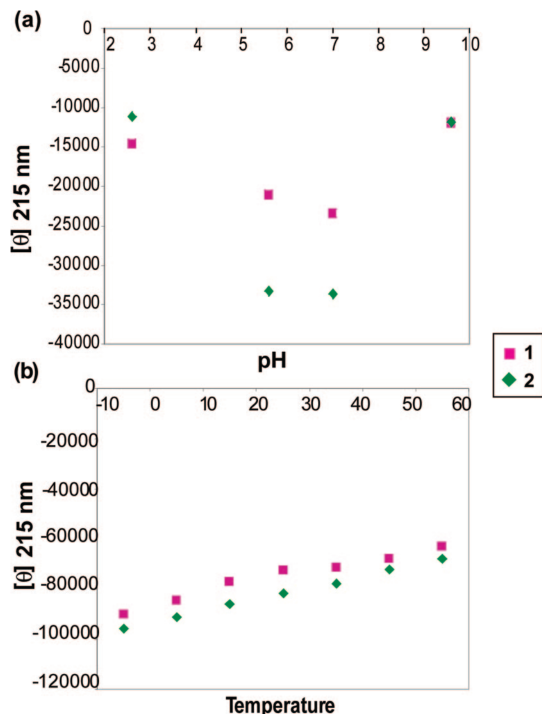

FIGURE 2. (a) Results from pH dependence measurements, comparing the absolute minima of peptides **1** and **2** arising at different pH conditions: 2.6, 5.6, 7, and 9.6. (b) Results from temperature dependence measurements, comparing the absolute minima of peptide **1** and **2** arising at different temperatures between 5 and 55 °C in intervals of 10 °C.

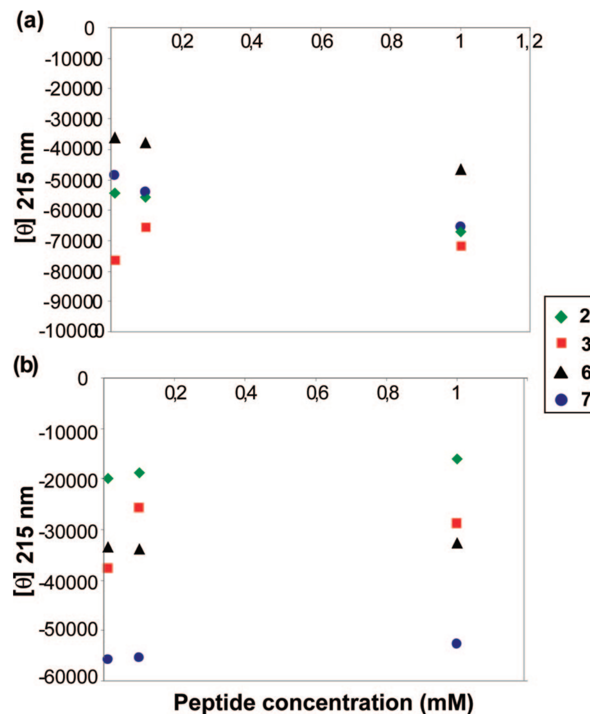

FIGURE 3. Concentration dependence study performed on peptides **2**, **3**, **6**, and **7** in methanol (a) and water (b), comparing the absolute helical minima arising at different concentrations: 0.01, 0.1 and 1 mM.

Experimental Section. Pure glycosylated β^3-peptides **1**−**7** were obtained after purification by reverse-phase HPLC (C-18 column); see Table 1.

Conformational Studies. The conformational preferences for glycosylated β^3-peptides **1**−**7** were initially studied by circular dichroism (CD) spectroscopy at a concentration of 0.1 mM at 25 °C. The CD spectrum of a left-handed 3_{14}-helix of a β^3-peptide in methanol is well-established and gives rise to a minimum, zero crossing, and maximum at 215, 208, and 198 nm, respectively.[16] Because CD spectroscopy is very dependent on the concentration and therefore intensity-related errors can arise from the sample preparation, this risk was minimized by running at least two independent measurements, in combination with repeated lyophilization of the peptide.

The CD spectra of the glycosylated β-peptides **1**−**7** in methanol and pH 7 PBC buffer are shown in Figure 1a and b, respectively, and are all indicative of 3_{14}-helical structures. It is important to remember that the CD data of β^3-peptides always should be interpreted with care;[17] however, the CD spectra of 3_{14}-helices have been extensively studied over the past 10 years,[16] and it is therefore rational to conclude that changes in intensity at 215 nm correlate to relative changes in overall mean 3_{14}-helical population.[2b,18]

Comparing the CD spectra of glycosylated β-peptides **1** and **2** recorded in methanol, no difference in intensity could be observed. However, analyzing the spectra recorded in a pH 7 PBC buffer (1 mM Na phosphate, 1 mM Na borate, 1 mM Na citrate), an increase in helical stability could be observed for peptide **2** relative to peptide **1**. Indeed, whereas β-peptide **1** only

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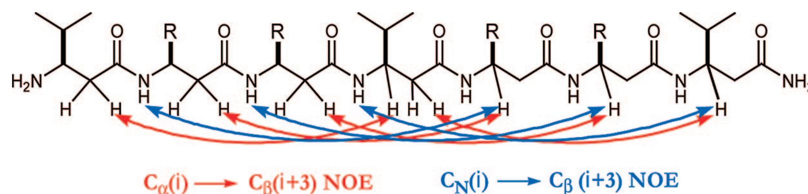

FIGURE 4. Representative backbone NOEs extracted from the ROESY spectra of the glycosylated peptides **1**−**7** recorded in methanol: $C_\alpha H(i) \rightarrow C_\beta H(i+3)$ NOEs are in red, and $C_N H(i) \rightarrow C_\beta H(i+3)$ NOEs are in blue.

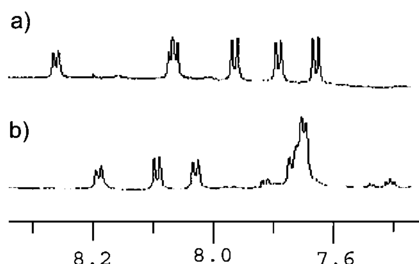

FIGURE 5. Expansion of the amide regions from the ^{1}H NMR spectra of peptide **7** recorded in water (a) and methanol (b).

retained 29% of its 3_{14}-helical conformation observed in methanol, peptide **2** retained as much as 46% of the intensity upon change of solvent (see Figure 1 and Table 2), indicating that the 3_{14}-helix could be stabilized by replacing the β^3hGlu at position 5 (**1**) with β^3hOrn (**2**). In the following discussion, the percent changes in 3_{14}-helicity of the glycosylated β^3-peptides will be calculated on the basis of the helical minima arising from the CD data of peptide **2** if nothing else is mentioned; it should, however, be noted that this percent change represent a relative change rather than a comparison with a completely (100%) folded helix.

The sequence of peptides **2** and **3** had a positively charged side chain close to the C-terminus and the GalNAc moiety close to the N-terminus. Surprisingly, a weak destabilization of the 3_{14}-helical structure was observed upon inclusion of a free C-terminus **3** compared to an amide modified terminus **2**, and a 9% (methanol) and 11% (water) decrease was seen for β^3-peptide **3**, see Figure 1 and Table 2).

The 3_{14}-helical content of the βGalNAc-β-peptide **4** in methanol was in the same range as its αGalNAc-containing sequence analogue **2**, but with slightly increased intensity. Interestingly, upon change of solvent to water, the overall helical content of peptide **4** were almost completely lost, retaining only 15% of its helical stability observed in methanol (see Figure 1 and Table 2)! The reason for this is unclear but could be related to more fully developed charges in water, as compared to methanol, which explains why structural effects is observed to higher extent when water is used as solvent.

The positional effect on the conformational stability was investigated by comparing the CD spectra arising from peptide **2**, **5**, and **6**, having the αGalNAc linked β^3hSer in position **2**, **3**, and **5**, respectively. Peptide **5** showed a significant decrease in mean 3_{14}-helical population in both methanol and water, concluding that the inclusion of a glycosylated β^3hSer derivative at position 3 was directly destabilizing. However, there was no significant change in helical intensity of peptide **6** compared to peptide **2**.

By replacing the amidated C-terminus of peptide **6** with a free carboxyl group, i.e., peptide **7**, a 28% drop of intensity (relative **6**) was observed in methanol. However, when analyzing the folding propensities of **7** in water at neutral pH, a dramatic increase of intensity (+100% as compared to **6**) was observed; see Figure 1 and Table 2. Although anticipated, as peptide **7** incorporated all helix-enhancing features described by Schepartz, such a large difference is truly amazing.

Next, the effects of pH and temperature on glycosylated β^3-peptides **1** and **2** were investigated. The results from the pH studies of β^3-peptides **1** and **2** were similar to each other; see Figure 2a. Again, the risk of misleading results due to errors in the sample preparation was minimized by running two independent measurements of two different lyophilized samples at each pH. From the studies of **1**, it could be observed that the 3_{14}-helical intensity lies within the same range at pH 7 and pH 5.6, showing only slightly increased overall mean helical elipticity at pH 7 as compared with pH 5.6, while a decrease in overall mean helical elipticity was observed at pH 2.6 and pH 9.6. For peptide **2**, the helical intensity at pH 2.6 and pH 9.6 were in the same range as for peptide **1**; again, a decrease in helical intensity was observed at pH 2.6 and pH 9.6, as compared to pH 5.6 and pH 7. The increased aqueous helical stability of peptide **2**, as compared to **1**, as observed above, is seen at both pH 5.6 and pH 7.

For the temperature studies, CD spectroscopic studies were performed in methanol at temperatures between −5 and 55 °C in intervals of 10 °C; see Figure 2b. Both peptides showed marked temperature dependence, with a proportional decrease in helical intensity with increasing temperature.

The concentration dependence of peptides **2**, **3**, **6**, and **7** were investigated in both methanol and water (pH 7) in the range of 0.01 to 1 mM. According to Figure 3a, a possible tendency for aggregation is observed at the highest concentration for peptides **2**, **6**, and **7** in methanol. However, no such tendencies were observed in water at neutral pH; see Figure 3b. The concentration dependence of peptide **3** did not follow the same trend, neither in methanol nor in water. In order to fully evaluate the concentration dependence, analytical centrifugation studies would be needed. However, the results given from the CD spectroscopic studies do not affect the conclusion made from the NMR experiments below.

Detailed ^{1}H and 2D NMR (TOCSY, P.E.-COSY, ROESY) studies were performed on all glycosylated β^3-peptides **1**−**7** in CH_3OH/CD_3OD (9:1). All peptides showed good dispersion of the amide region, indicating the presence of secondary structures. Furthermore, ROESY spectra were recorded using mixing times of 150 and 300 ms. The 3_{14}-helical conformation is characterized by NOEs between $H_N(i)$ and $H_\beta(i+3)$ and between $H_\alpha(i)$ and $H_\beta(i+3)$; such NOEs were observed for *all* glycosylated β^3-peptides in methanol; see Figure 4.

As the NOEs extracted from the ROESY spectra of peptides **2**−**7** recorded in methanol resembled the NOEs of the already published glycopeptide **1**[7] to such large extent, no detailed computational calculations of the solution state conformation of these peptides were performed. Further, the difference between α- and β-glycosylation seen from the CD studies

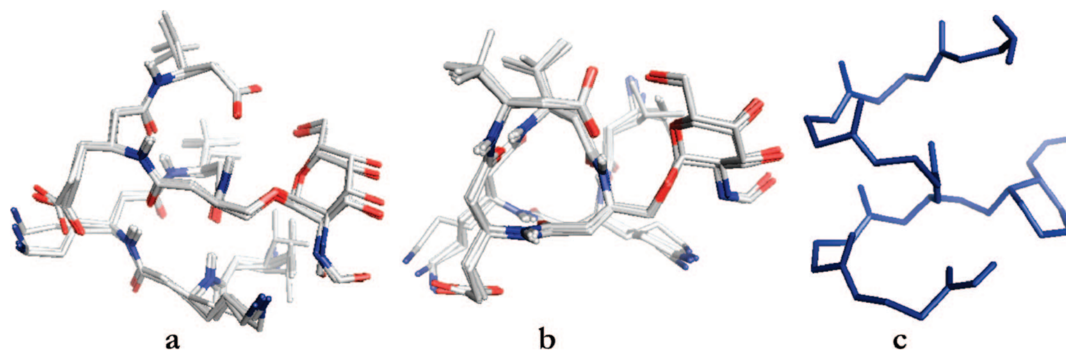

FIGURE 6. NMR-derived structures of glycosylated β^3-peptide **7** in water (pH 7.2) as a bundle of six low-energy structures calculated from a restrained Monte Carlo conformational search shown from the side (a) and from the top (b). All hydrogens, except amide hydrogens, have been omitted for clarity. Carbons are shown in gray, nitrogens in blue, and oxygens in red. (c) Shows a structure (blue) where all side chains, except the carbohydrate moiety, and hydrogens, except the amide hydrogens, have been omitted for clarity.

(glycosylated β^3-peptides **2** and **4**) were not further investigated by NMR and calculations since the difference was only observed in aqueous buffer and not in methanol, which was the solvent of choice for the NMR experiments of these peptides.

A detailed computational NOE restrained conformational search was, however, done for the glycosylated β^3-peptide **7** in water, as the CD spectra of the glycosylated β^3-peptide **7** suggested that this peptide adopts a more stable 3_{14}-helical structure in water at neutral pH as compared to in methanol. Additional NMR experiments on this peptide were therefore acquired in H_2O/D_2O (9:1) at pH = 7.2. A simple inspection of the amide region in the ^{1}H NMR spectra originating from peptide **7** in methanol and water at neutral pH (7.2), respectively, suggest the presence of only one major conformer in both solvents, Figure 5. Moreover, it is seen that the amide proton resonances have larger dispersion and display sharper signals in the spectra recorded in aqueous solution, as compared to methanol, thus confirming the observations made by CD spectroscopy, i.e., that **7** forms a more stable secondary structure in water than in methanol. It was also observed that the amide region of peptide **7** in MeOH displayed less well defined signals than any of the other glycosylated β^3-peptides did when recorded in MeOH.

Hydrogen-bond patterns defining the helical structures of **7** in water were provided from the ROESY spectra (mixing time 150 and 300 ms). Thirteen out of 14 characteristic long-range NOEs of a 3_{14}-helical conformation was observed. A computational conformational search was made for peptide **7** in water, using parameters extracted from the NMR experiments (49 NOEs, see Supporting Information). From the calculations, one conformational family was found, in agreement with the result from the CD measurements; see Figure 6. A positively charged side chain close to the N-terminus and a negatively charged side chain close to the C-terminus in combination with preserved free termini is known to stabilize a 3_{14}-helical structure, which already have been stated by the CD analysis. Stabilization also arose from hydrophobic interactions between the β^3hVal residues positioned on one side of the helical triangle and a salt-bridge interaction between β^3hOrn in position 2 and β^3hGlu in position 5. On the third rim of the helical structure, a stabilizing interaction arose between the *N*-acetyl group on the GalNAc moiety in position 5 and the β^3hOrn in position 2. The N-terminus is somewhat "opened" as the valine side chains of residue 1 is positioned more over the carbohydrate moiety than over the other β^3hVal residues.

Conclusion

In this account we reported the synthesis of seven glycosylated β^3-peptides. Such molecules are representative for a novel class of functionalized foldamers, in which a natural post-translational modification is attached to an unnatural peptidomimetic backbone. Considering the vast importance of glycopeptides in biology, such molecules are expected to find broad utility in biomedical applications. Although the need for secondary structure formation depends upon the application of interest, we set out to study the factors affecting the 3_{14}-helix stability of these glycosylated β-peptides.

It was concluded that, in methanol, the highest degree of helical content was observed for the β-peptides having the C-terminus amidated and the GalNAc-linked β^3hSer derivative in position 2. Further, the GalNAc moiety preferred inteactions with a positively charged side chain instead of a negatively charged side chain, cf. peptide **1** and **2**.

The important difference between methanol and water was demonstrated by peptide **7**. It could be seen that the more fully developed charges present in water, as compared to methanol (where the carboxylic acids in the peptide are expected to be protonated because of the acidic solvent used in purification), enhanced the helical stability of this β-peptide in water. In fact, β^3-peptide **7**, which incorporated all the design principles stated by Schepartz[12b,13–15] for 3_{14}-helical stabilty of unglycosylated β^3-peptides in water, was even more stable in aqueous buffer than in methanol!

Furthermore, a detailed NMR structure of peptide **7** in water proved this peptide to adopt the 3_{14}-helical conformation. This represents the first solution-state structure of a glycosylated β^3-peptide in water and one of few β-peptidic structures determined in water.

Experimental Section

Amino Acid Synthesis. β^3-Amino acids used in the peptide synthesis were synthesized according to a procedure developed by Seebach, utilizing Arndt–Eistert homologation of α-amino acid derivatives.[19] The synthesis of glycosylated β^3hSer have been described elsewhere.[20]

Solid-Phase Peptide Synthesis. All glycosylated β^3-peptides were synthesized according to the following procedure. The first amino acid (β^3hVal) was linked to the resin by preactivation of

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the amino acid using either a HBTU/HOBT/DIPEA mixture (Rink) or *N*,*N*-diisopropylcarbodiimide and DMAP (Wang). The coupling reaction were in general left to react over 2 h but were in some cases left overnight as β^3-amino acids do not racemize. Unreacted free amines were capped after coupling of the first amino acid to the resin by treatment of Ac_2O in CH_2Cl_2 for 3 h. Capping was performed once again before linkage of the GalNAcβ^3hSer.After inclusion of the GalNAc-bearing β^3hSer residue, Fmoc deprotection was performed using 20% piperidine in DMF, instead of the more harsch cleavage cocktail 2% DBU and 2% piperidine in DMF. Upon completion of the peptide synthesis, final Fmoc deprotection was made before cleavage and side chain deprotection utilizing a standard TFA/H_2O/TIPS mixture (95:2.5:2.5). Partial deprotection of the *O*-acetyl groups occurred during solid-phase synthesis, probably due to basic conditions in combination with insufficiently dry solvents. Removal of the remaining *O*-acetyl protecting groups was made using NH_3/MeOH. The glycosylated β^3-peptides **1**−**7** were obtained after purification by reverse-phase HPLC (C-18 column); see Table 1 and Supporting Information for details.

NMR Solution-State Structure Calculations. The studies were performed using Monte Carlo simulation followed by PR Conjugate Gradient minimization in MacroModel 7.0.[21] As distance restraints, parameters extracted from 2D NOESY and ROESY spectra were used and classified into three categories with upper bond distance limits: strong 2.5 ± 1 Å, medium 3.0 ± 1 Å, and weak 4.0 ± 1 Å. The NH−C(β)−H dihedral angle restraints,[22] derived from the coupling constants, was used in the conformational search.

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Supporting Information Available: General experimental procedures and details about the 2D NMR spectroscopy measurements, ^{1}H NMR spectra, and HPLC traces for all synthesized peptides. This material is available free of charge via the Internet http://pubs.acs.org.

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