A molecular basis for glycosylation-induced conformational switching

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Background: Asparagine-linked glycosylation has the capacity to greatly influence the structure and function of glycoproteins. In most cases, however, it is unclear specifically how the carbohydrate moiety interacts with the protein to influence its conformation.

Results: A series of glycopeptides based on the critical A285 glycosylation site of the hemagglutinin glycoprotein from influenza virus was used as a model system to study the effects of asparagine-linked glycosylation. Derivatization of this peptide with a family of short carbohydrates reveals that subtle changes in the structure of the carbohydrate have a dramatic impact on peptide conformation. Modification of the hemagglutinin glycopeptide with a truncated version of the native carbohydrate induces a β-turn structure similar to the structure found in the native protein. Replacement of the C2 and C2′ *N*-acetyl groups of the carbohydrates with hydroxyl moieties results in a less well-ordered peptide conformation.

Conclusions: It is likely that the *N*-acetyl groups of the carbohydrates have a critical role in promoting the more compact β-turn conformation through steric interactions with the peptide. This study has demonstrated that relatively small changes in carbohydrate composition can have dramatic ramifications on glycopeptide conformation.

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Introduction

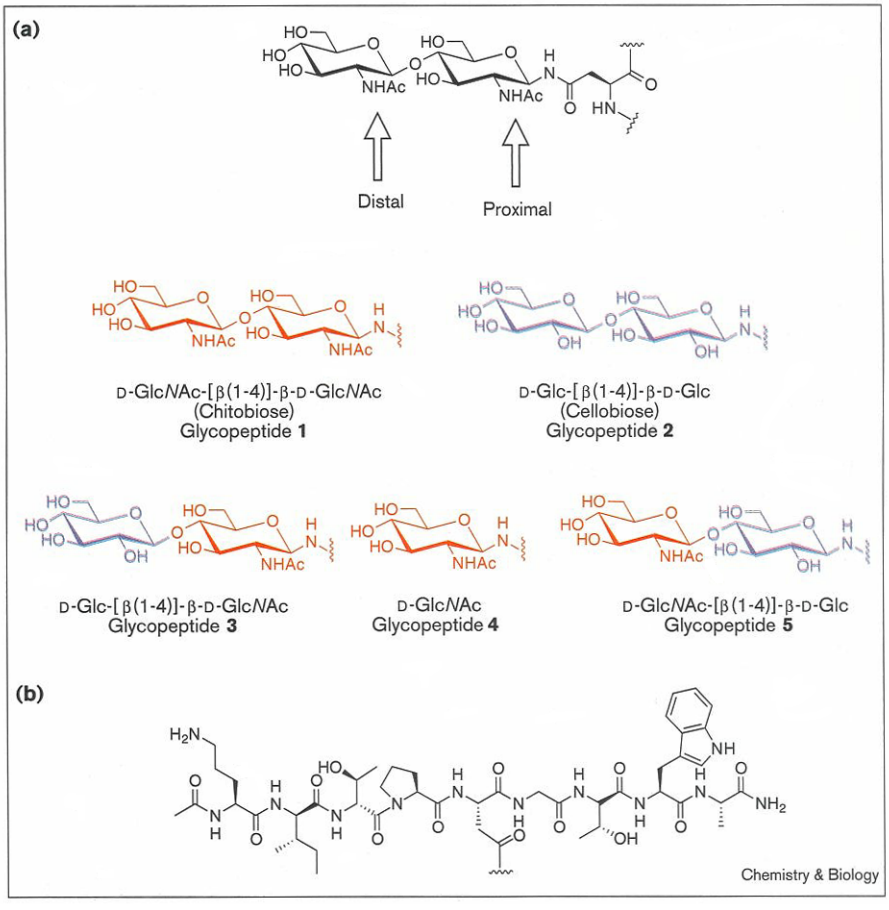
Glycoproteins play a critical role in a variety of biological processes including the immune response, cell–cell recognition and protein folding, stability and solubility [1,2]. Glycosylation frequently has a dramatic effect on protein structure, impacting either overall structure or local conformation [3]. Because *N*-linked glycosylation occurs co-translationally, as the protein is being biosynthesized, glycosylation-induced conformational changes could affect protein folding. Indeed, numerous examples exist in which glycoproteins fail either to fold correctly or to be secreted when certain *N*-linked glycosylation sites are removed by site-directed mutagenesis [3–11]. Furthermore, a number of short glycopeptides that mimic flexible, nascent polypeptides undergo well-defined conformational changes after glycan derivatization [12–16].

In a few cases, the carbohydrate moiety has been shown to stabilize the structure of the fully folded protein by making specific, stabilizing contacts with the protein [17,18]. In systems in which these types of interactions do not prevail, however, understanding the mechanisms by which carbohydrates can influence protein structure is difficult. In the absence of observable, specific carbohydrate–protein contacts, it has been proposed that oligosaccharides might influence the local structure of the glycosylation site [1,2] either by altering the solvation properties of the peptide or by participating in hydrogen-bonding interactions with the polypeptide [19]. Notably, evidence for specific hydrogen bonds between peptide and carbohydrate functionality has only been observed in non-aqueous solvents. The carbohydrate might also have a steric role, in which the bulk of the carbohydrate limits the conformational space accessible to the peptide [16,20]. Definitive evidence to support any of these proposals in aqueous media has not yet been presented, however.

As the importance of *N*-linked glycosylation for protein stability and function continues to emerge, it is essential to gain a full understanding of how carbohydrates influence peptide structure and function. Recent advances in the chemical synthesis of glycopeptides are making access to this class of compounds easier [21–25]. Thus, the opportunity now arises to answer specific questions concerning the molecular basis of glycosylation-induced conformational modulation.

In our ongoing efforts to unravel the relationship between the saccharide moiety and its effect on peptidyl structure, we synthesized a family of short glycopeptides in which key molecular elements of the sugar, specifically the *N*-acetyl groups, were modulated (Figure 1a). The short peptide sequence AcNH-Orn–Ile–Thr–Pro–Asn–Gly–Thr–Trp–Ala-$CONH_2$, based on the essential glycosylation

Figure 1



Chemical structures of all glycopeptides examined in this study. In each figure, *N*-acetylglucosamine is red, and glucose is blue.

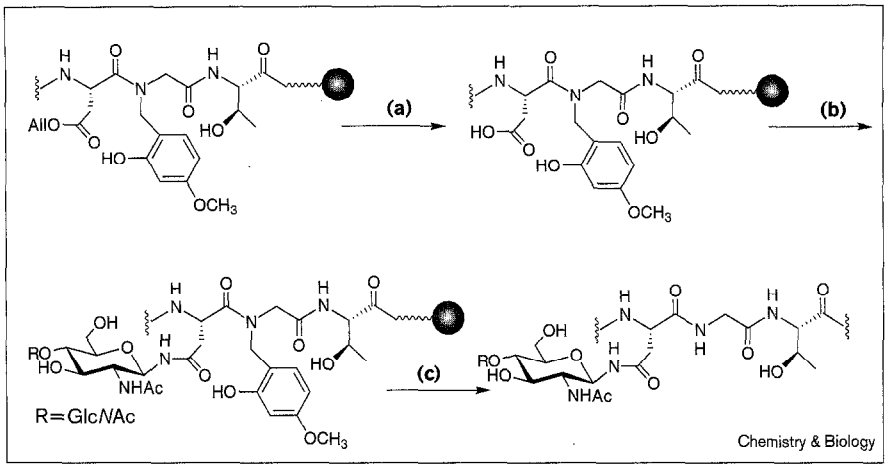
site at residue A285 of the hemagglutinin protein of influenza virus, was synthesized and derivatized with five different carbohydrates (Figure 1b). The various conformations of the glycopeptides were then assessed using two-dimensional nuclear magnetic resonance (NMR) methods.

Previously, two-dimensional NMR studies of glycopeptide **1** provided evidence that glycosylation with chitobiose, a disaccharide representing the first two β(1-4) linked *N*-acetylglucosamine residues of the native tetradecasaccharide, induced the formation of a compact β-turn remarkably similar to the structure found in the fully folded glycoprotein [13,26]. Despite the dramatic change in peptide conformation that was observed upon protein glycosylation, no obvious specific interactions between the peptide and the chitobiose moiety were observed in the NMR analysis. Because ~30% of all glycosylation sites occur at sites that fold ultimately to β-turns, this model peptide provides a relatively 'general' context for studying the conformational implications of *N*-linked glycosylation [27]. Moreover, β-turn formation is believed to be critical for the protein-folding process, as folding can be promoted by the long range contacts between two β strands [28]. For these reasons, this model peptide was chosen for further conformational analysis.

Derivatization with a variety of saccharides (Figure 1) revealed that chitobiose plays a unique role in modulating peptide conformation; no other saccharide appears to definitively induce the native β-turn structure. Because no discreet peptide–carbohydrate interactions were discovered, it is likely that the conformational behavior of the saccharides is a key factor that influences peptide conformation. The average structure of each glycopeptide in aqueous media was determined by examining nuclear Overhauser effect (NOE) data and $^{3}J_{HN\alpha}$ values. To complement these structural studies, the conformational stability of the glycopeptides was assessed using ^{13}C T_1 relaxation values. The results indicate that the addition of any carbohydrate to the asparagine sidechain perturbs the aspartate/asparagine (Asx)-turn structure of the nonglycosylated peptide [13], but the bias towards β-turn structure is greatest when the truncated native carbohydrate (chitobiose) is in place. Additionally, these studies have implicated the *N*-acetyl group of the sugar proximal to the peptide as a critical component required

Figure 2

Typical synthesis of an *N*-linked glycopeptide. The 2-hydroxy-4-methoxy group on the glycine amide nitrogen is utilized to prevent succinimide formation (see the Materials and methods section). **(a)** $Pd(PPh_3)_4$, $CHCl_3$/AcOH/*N*-methyl morpholine (37/2/1, v/v), 18 h. **(b)** BOP, DIPEA, glycosylamine, DMF, 8h. **(c)** TFA-PhOH-Ethanedithiol-triisopropylsilane (92/3/3/2 v/w/v/v).



for the induction of β-turn formation, probably as a result of steric interactions with the peptide backbone. Surprisingly, the addition of an *N*-acetyl group to the distal sugar also appears to play a key role in rigidifying both the saccharide and the peptide.

Results and discussion

Glycopeptide synthesis

Glycopeptide synthesis was effected using the strategy developed by Cohen-Anisfied and Lansbury [29] and Albericio and coworkers [30]. Specifically, glycopeptides were prepared by coupling glycosylamines to the sidechain carboxylate of an aspartic acid residue to form an *N*-glycosidic linkage. In order to ensure regioselective coupling, an allyl-protected aspartic acid was substituted for asparagine in the peptide synthesis. While on the solid support, the allyl ester was selectively removed using tetrakis(triphenylphosphine) palladium, coupled to the desired amino sugar, and then cleaved from the resin (Figure 2). The disaccharides cellobiose and chitobiose octaacetate are commercially available. The disaccharide D-Glc-[β(1-4)]-β-D-Glc*N*Ac-β-NH_2 (for derivatization of glycopeptide **3**) and D-Glc*N*Ac-[β(1-4)]-β-D-Glc-β-NH_2 (for derivatization of glycopeptide **5**) were synthesized as illustrated in Figures 3 and 4.

Glycopeptide structure

It should be noted that these short peptides are expected to be somewhat flexible, and that NMR-derived structures represent a conformational average. Previous fluorescence resonance energy transfer studies [26], along with several long-range NOEs (most notably, between the Ile2 and Trp8 sidechains in two-dimensional ROESY experiments), suggest that these glycopeptides exhibit some prevelance of secondary structure. A structural analysis of the chitobiose derivative glycopeptide **1** has been described previously (Figure 5a) [13]. In addition to the distance restraints provided by NOESY spectra (109 individual NOEs were observed and 92 distance restraints resulted after pseudoatom correction), amide proton variable temperature (VT) coefficients were also examined. Lowered amide VT coefficients suggest that the amide proton is protected from exchange with water, most likely by the presence of an intramolecular hydrogen bond. Consistent with the *i* to *i*+3 [CO→NH] hydrogen bond characteristic of a β-turn, a slightly lowered

Figure 3

Synthesis of D-Glc-[β(1-4)]-β-D-Glc*N*Ac for glycopeptide **3**. **(a)** AgOTf, CH_2Cl_2/toluene, −40°C (40%); **(b)** H_2, Pd/C, MeOH (95%); **(c)** NaOMe, MeOH (95%); **(d)** $(NH_4)_2CO_3$, H_2O.

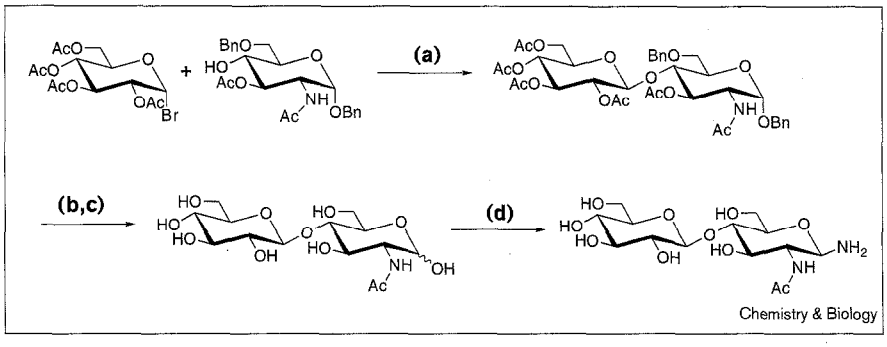
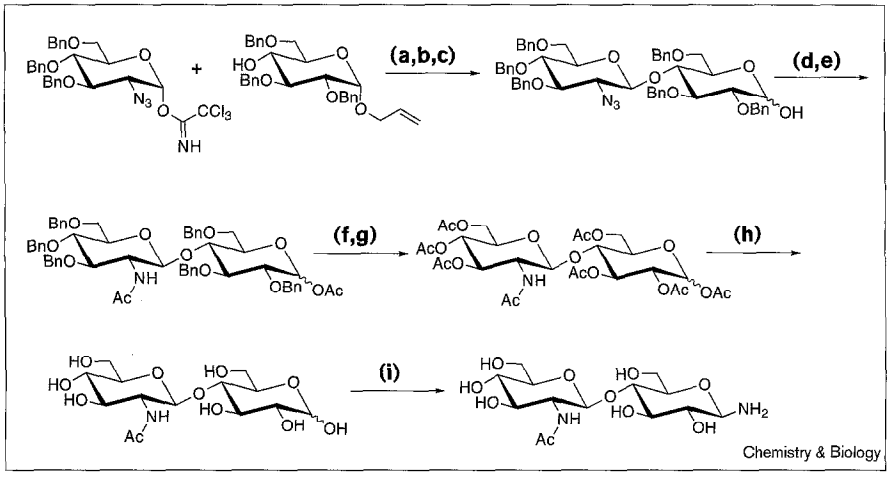


Figure 4

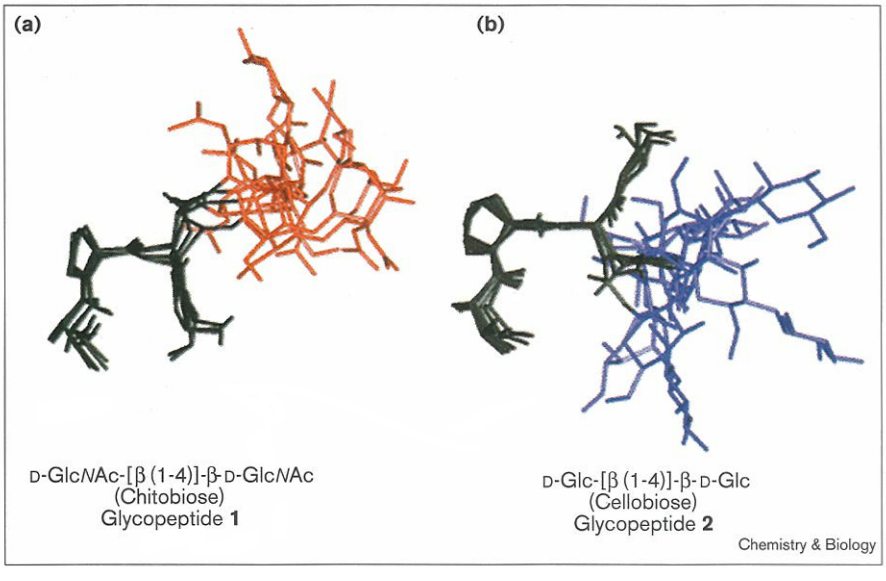


Synthesis of D-Glc*N*Ac-[β(1-4)]-β-D-Glc for glycopeptide **5**. **(a)** BF_3 Et_2O, 4 Å ms, CH_2Cl_2, −70 to −40°C (48%); **(b)** $[PMe_2Ph)_2IrCOD]PF_6$, THF; **(c)** $HgO/HgCl_2$ acetone/water (82% for b and c); **(d)** Raney-Ni (cat.), H_2, Ac_2O-EtOH; **(e)** Ac_2O, pyridine, DMAP, CH_2Cl_2 (100% for d and e); **(f)** H_2, Pd/C, EtOH/MeOH; **(g)** Ac_2O, pyridine, DMAP (cat.) (97% for f and g); **(h)** NaOMe, MeOH (95%); **(i)** $(NH_4)_2CO_3$, H_2O.

amide VT coefficient (–6.8 ppb/K) for the Gly6 residue was observed. Additionally, the distinctively high $^3J_{HN\alpha}$ value (9.6 Hz) observed for the Asn5 residue is indicative of a type I β-turn [31]. A simulated annealing protocol, using distance restraints derived from the NMR data, produced five out of ten structures that converged to a well-ordered type I β-turn at the glycosylation site (Figure 5a).

In the present study, similar structural analyses were performed for glycopeptides **2**–**5**, which have been derivatized with the various monosaccharides and disaccharides illustrated in Figure 1. Glycopeptide **2**, modified with the disaccharide D-Glc-[β(1-4)]-β-D-Glc (cellobiose), exhibited a half-turn structure with an extended backbone as illustrated by the structures resulting from the simulated annealing protocol (Figure 5b is based on 83 distance restraints that were derived from 100 individual NOEs). Fewer intra-residue carbohydrate NOEs were observed (8/100 intraresidue carbohydrate NOEs compared to 19/109 observed for glycopeptide **1**), suggesting that cellobiose, and consequently, the cellobiose-derivatized glycopeptide **2**, are more mobile than chitobiose and glycopeptide **1**. Also, fewer NOEs between the Asn5 sidechain amide and carbohydrate were seen. A low VT coefficient for Thr7 (–5.6 ppb/K) was noted and the $^3J_{HN\alpha}$ value for Ile3 (9.7 Hz) instead of Asn5 was significantly elevated. Together, these results signify a large deviation from the conformation of glycopeptide **1** at the glycosylation site, suggesting an important role for both *N*-acetyl groups in modulating local peptide conformation.

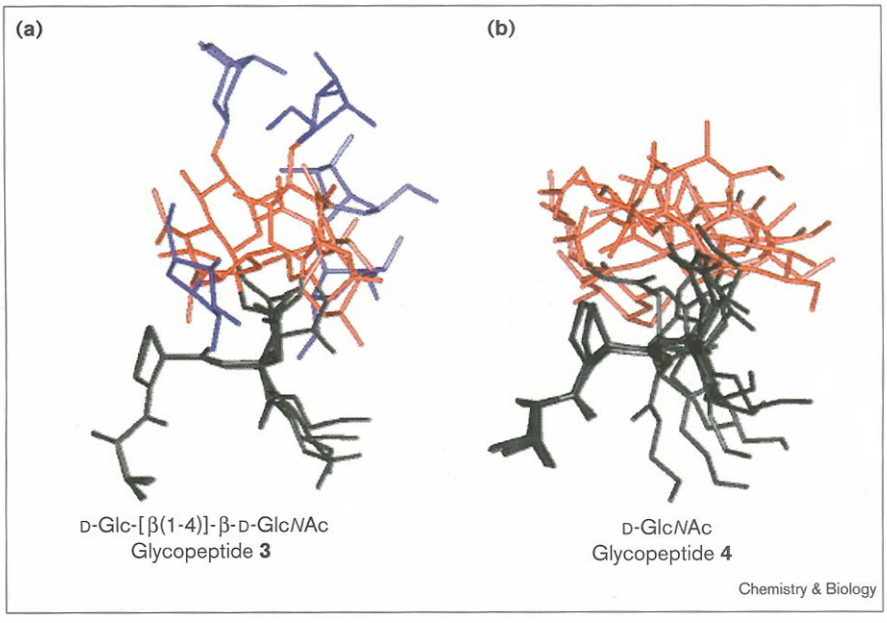
Figure 5



Minimized structures resulting from a simulated annealing protocol that incorporated NOE-derived distance and $^3J_{HN\alpha}$-derived dihedral restraints. **(a)** Five of ten NMR-derived structures of glycopeptide **1** that converge to a type I β-turn. Residues Thr3–Gly6 are shown. Root mean square deviation (rmsd) values were calculated from all atoms. In all cases, the large rmsd value for asparagine reflects the highly disordered carbohydrate that is covalently linked to this residue. Thr3, 0.76 ± 0.07 Å; Pro4, 1 ± 0.09 Å; Asn5, 6.5 ± 2.6 Å; Gly6, 2.3 ± 0.7 Å. **(b)** Seven of ten NMR-derived structures of glycopeptide **2**, illustrating the more extended backbone. Residues Thr3–Gly6 are shown. Thr3, 1.2 ± 0.4 Å, Pro4, 0.9 ± 0.2 Å; Asn5 7, ± 1.6 Å; Gly6, 1.8 ± 0.5 Å.

Figure 6

Minimized structures resulting from a simulated annealing protocol that incorporated NOE derived distance and $^3J_{HN\alpha}$-derived dihedral restraints. **(a)** Five of ten NMR-derived structures of glycopeptide **3** that converge to a type I β-turn. Residues Thr3–Gly6 are shown. Thr3, 0.82 ± 0.06 Å; Pro4, 0.88 ± 0.3 Å; Asn5, 7.9 ± 3.1 Å; Gly6, 1.2 ± 0.3 Å. **(b)** Seven of ten NMR-derived structures of glycopeptide **4** that conform to a disordered β-turn. Residues Thr3–Gly6 are shown. Thr3, 0.86 ± 0.05 Å; Pro4, 0.76 ± 0.3 Å; Asn5, 4.1 ± 1.5 Å; Gly6, 2.4 ± 0.2 Å.

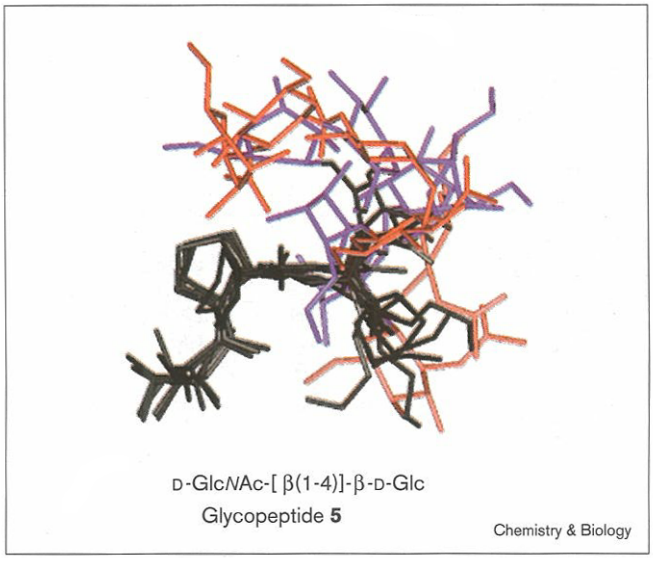


To further elucidate the role of the *N*-acetyl moieties, glycopeptide derivatives that have a single *N*-acetyl group on the proximal sugar (as in glycopeptides **3** and **4**) or on the distal sugar (as in glycopeptide **5**) were prepared. Addition of the *N*-acetyl group to the proximal sugar in glycopeptide **3** promoted β-turn formation analogous to that observed for glycopeptide **1**. Five of ten structures generated from a simulated annealing protocol based on the NOE data (64 distance restraints were generated from 75 individual NOEs) converged to a highly ordered type I β-turn (Figure 6a). An elevated $^3J_{HN\alpha}$ value at Asn5 that would characterize a type I β-turn was not observed, however. Additionally, the VT coefficient for Gly6 (–7.1 ppb/K) was slightly higher than that observed in peptide **2**. Notably, the NMR-derived structures indicate a turn conformation that is less compact than that of peptide **1**. Taken together, these data suggest that although the *N*-acetyl group of the proximal sugar is critical for compact β-turn formation, the *N*-acetyl of the distal sugar also plays a role in modulating peptide conformation. It should be noted that the glycopeptide derivatized with D-Gal-[β(1-4)]-β-D-Glc*N*Ac failed to produce a β-turn conformation, and instead adopted an extended structure that resembled that of glycopeptide **2** (S.E.O. and B.I., unpublished observations). This suggests that a highly specific carbohydrate conformation generated by the D-Glc-[β(1–4)]-β-D-Glc*N*Ac linkage might be necessary to create the more compact β-turn.

Glycopeptide **4**, which is derivatized with the monosaccharide Glc*N*Ac, was also examined (Figure 6b is based on 75 distance restraints derived from 88 observed NOEs). The NMR-derived structures of this peptide also support the assertion that although the first *N*-acetyl group is the most critical, the second saccharide appears to enhance β-turn formation. Although the NOE data indicate that β-turn character is present in this peptide, the carboxy-terminal region of the turn is highly disordered. Moreover, the high $^3J_{HN\alpha}$ value of Asn5 and the low amide VT coefficient of Gly6 characteristic of a β-turn were not observed. β-Turn structure appears to be more prevalent in glycopeptide **3** than in glycopeptide **4**. These data again suggest that the distal saccharide plays a role in β-turn formation.

The NMR analysis of glycopeptide **5**, in which only the distal sugar is derivatized with an *N*-acetyl moiety, supports the notion that the proximal *N*-acetyl group is most critical for β-turn formation. NOE studies suggest that peptide **5** adopts a conformation in which the carboxy-terminal residues of the β-turn are disordered (Figure 7 is based on 75 distance restraints derived from 90 observed NOEs). The distinctively high $^3J_{HN\alpha}$ value of Asn5 observed in peptide **1** (9.6 Hz) was not observed in this derivative, although a slight elevation was noted (7.5 Hz). The VT coefficients were similar to those observed in peptide **3**. Despite the importance of the proximal *N*-acetyl group, glycopeptide **5** still displays β-turn-like character, in contrast to glycopeptide **2**, which is derivatized with the cellobiose disaccharide that lacks both *N*-acetyl groups. This observation, along with the imperfect formation of β-turn structure in glycopeptides **3** and **4**, implies that the distal *N*-acetyl group also contributes to the formation of β-turn structure.

Figure 7



Six of ten NMR-derived structures of glycopeptide **5** that converge to a disordered β-turn. Residues Thr3–Gly6 are shown. Thr3, 2.9 ± 1.6 Å; Pro4, 1.2 ± 0.1 Å; Asn5, 12.5 ± 3.7 Å; Gly6, 4.2 ± 0.7 Å.

Assessment of the mobility of asparagine

It is likely that the carbohydrate has an impact on the dynamic nature of the peptide, as well as its conformation; numerous reports have suggested that, even when the carbohydrate does not appear to dramatically affect protein conformation, it does rigidify the protein, particularly at the glycosylation site [32,33]. Thus, in addition to promoting different peptide conformations, the carbohydrate moiety might also be imparting varying amounts of rigidity to the peptide by limiting available conformational space. Furthermore, the saccharides themselves could have varying mobilities.

Measurement of heteronuclear ^{13}C T_1 values for glycopeptides **1–5** were made to compare the mobilities of these derivatives. T_1 values provide information regarding motional properties in the frequency range of 10^8–10^{12} s^{-1}. Heteronuclear T_1 values can be interpreted in a relatively straightforward manner; differences in the relaxation times of a given amino acid in different environments, for example in different glycopeptides, can be directly related to the different effective rotational correlation times [34]. This type of comparison has also been performed, for example, in substrate-binding studies, in which the increased rigidity observed after substrate binding is reflected by longer T_1 values [35].

Measurements of the T_1 values of the α carbons of proline and asparagine residues indicated certain differences in the rigidity of glycopeptides **1–5**. The low T_1 value (238 ms) measured for the proline residue of glycopeptide **1** suggested that this derivative is relatively rigid, whereas the slightly higher T_1 value (279 ms) displayed by glycopeptide **2** suggested that there is more mobility in glycopeptide **2**. Glycopeptides **3** and **5** each displayed intermediate T_1 values for the proline residue (313 and 304 ms, respectively), suggesting a larger degree of mobility. Glycopeptide **4**, which is derivatized with a monosaccharide, displayed the highest T_1 value (377 ms). Glycopeptides **2** and **3** exhibited high T_1 values for the asparagine residues (377 and 371 ms, respectively). The remaining glycopeptides exhibited similar T_1 values for asparagine, indicative of intermediate rigidity (glycopeptide **1**, 299 ms; glycopeptide **5**, 284 ms; glycopeptide **4**, 296 ms). For comparison, the nonglycosylated peptide displayed intermediate T_1 values for both the proline (300 ms) and asparagine (308 ms) residues. Although the role that the individual *N*-acetyl groups have in enhancing the rigidity of each residue at the glycosylation site did not emerge from this study, the data nevertheless strongly suggest that glycopeptide **1**, derivatized with the native saccharide, is the most rigid of the glycopeptides at the glycosylation site.

Role of the proximal *N*-acetyl group

It is possible that the *N*-acetyl of the proximal sugar might interact with the peptide backbone to assist β-turn formation. The NMR data suggest that the proximal *N*-acetyl group is not rigid in water as has been reported in other cases [36]; NOEs from the amide proton of the *N*-acetyl group to the protons of the sugar were of similar strength, suggesting that no defined conformation exists. Obviously, if the *N*-acetyl group is flexible then the likelihood of its involvement in a hydrogen bond or some other specific interaction is greatly reduced. The VT coefficient of the amide proton of the glycosidic linkage is not significantly reduced (~ –7.5 ppb/K for all glycopeptides). The VT coefficients for the amide protons of the *N*-acetyl groups are also not significantly lowered (~ –7 ppb/K), which would be the case if either of these protons were to serve as the donor in a hydrogen bond. Overall, these data suggest that the influence of the *N*-acetyl group on the peptide backbone conformation is steric.

To establish exactly how the *N*-acetyl group could effect a steric influence, the favored position of the *N*-acetyl group relative to the peptide was established. All peptides in this study are likely to have a trans *N*-glycosidic linkage in the *Z* conformation. This conformation has been previously observed for the *N*-glycosidic linkages of glycopeptides [33,36]. The structure of this linkage is determined by the high $^3J_{HN\alpha}$ value (approximately 9 Hz) and the strong dipolar interaction of the amide proton of the linkage with the H2 proton of the proximal sugar. Additionally, observation of a strong $\delta\gamma_{NH\beta}$ (Asn5) NOE and the lack of a $\delta_{C1\beta}$ (Glc, Asn5) NOE support this assertion. The β protons of the asparagine sidechain are not resolved; this precludes χ

Figure 8

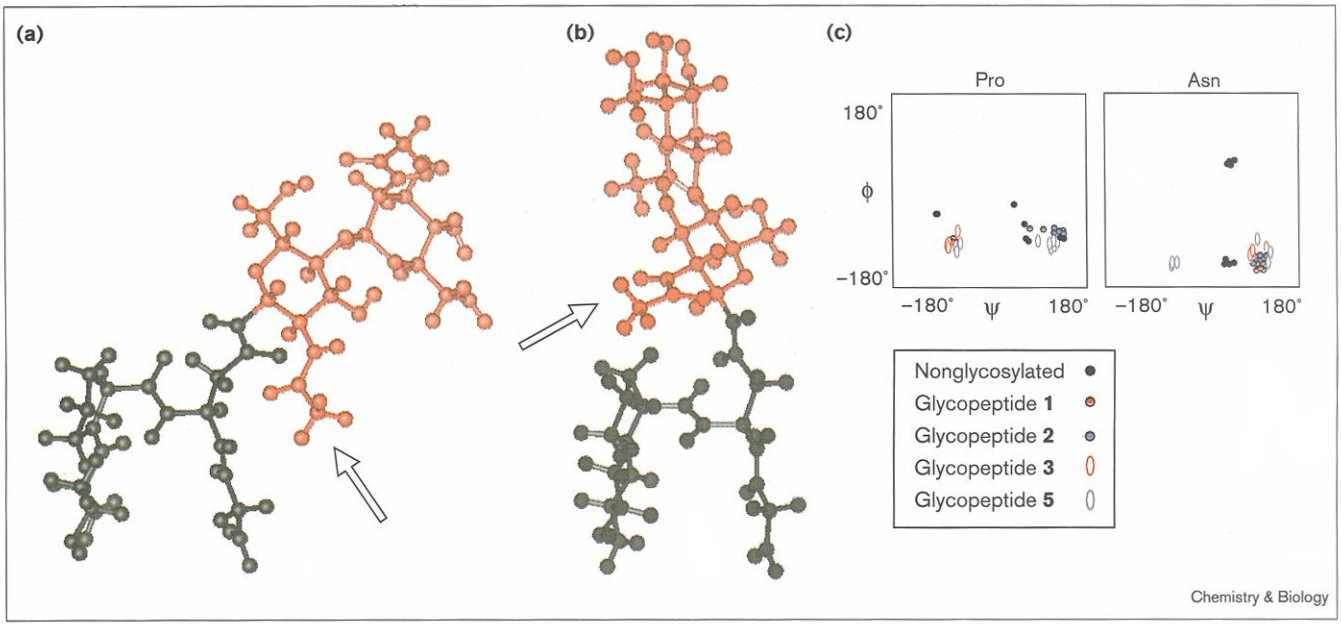


Illustration of how the *N*-acetyl group of the proximal sugar can interact sterically with the **(a)** carboxy-terminal and **(b)** amino-terminal regions of the peptide. **(c)** Ramachandran plots illustrating the ϕ/ψ dihedral space for the Pro4 and Asn5 residues of the glycopeptide derivatives. The nonglycosylated peptide [13] is also included for comparison.

dihedral determinations and also suggests that the asparagine sidechain is highly mobile. A mobile asparagine sidechain would enable the proximal *N*-acetyl group to interact with both the carboxy- and amino-terminal regions of the turn as illustrated in Figure 8 a,b. By sterically interacting with both sides of the glycosylation site, the *N*-acetyl moiety could be expected to affect the Pro4 and Asn5 dihedral angles. A Ramachandran plot of the Pro4 and Asn5 residues (Figure 8c) constructed from the results of the NMR-derived structures suggests that the proline dihedral angles are more affected by the switch of carbohydrates then are the asparagine dihedrals. Notably, motion of the asparagine–GlcNAc linkage has been proposed to increase the shielding of the glycoprotein by the carbohydrate [1].

Role of the distal *N*-acetyl group

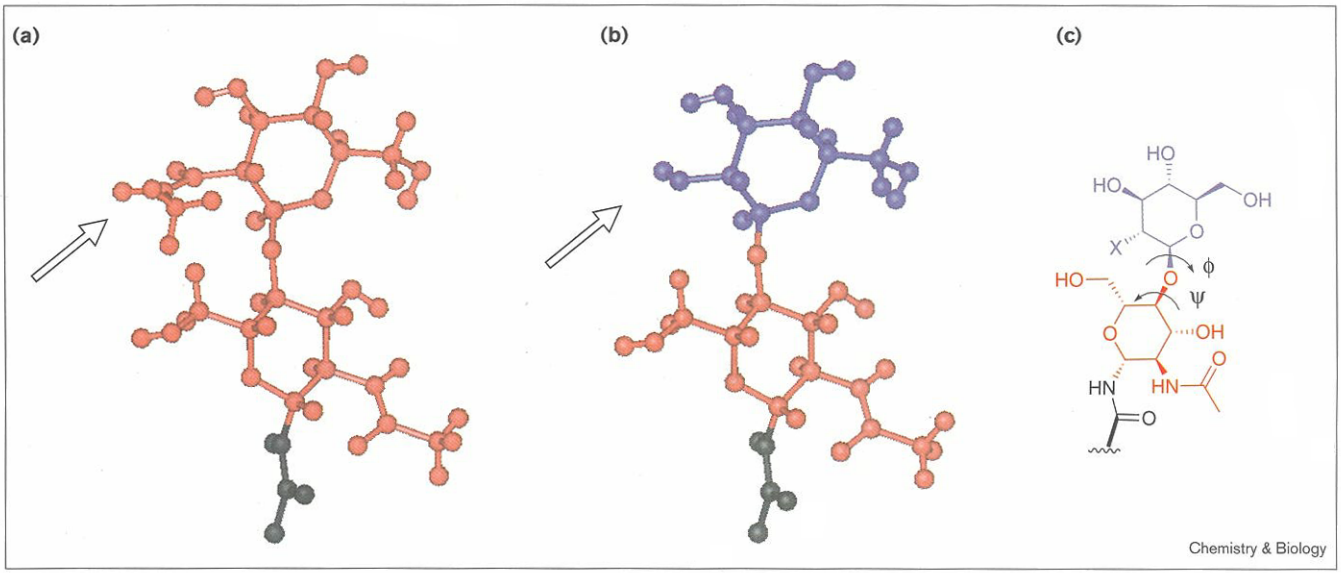
Although carbohydrate residues were difficult to resolve in the one-dimensional proton-detected spectra used to measure the ^{13}C T_1 values, ^{13}C T_1 values have previously been measured for the RNAse B glycan using both two-dimensional [37] and direct-detection methods [38]. Similar studies have also been performed on chorionic gonadotropin [39] and a glycopeptide from human serum transferrin [40]. In all cases, the motion of the interior monosaccharides was found to be relatively restricted compared to the residues of the outer branches, indicating that a relatively constrained carbohydrate conformation is often proximal to the glycoprotein.

To better understand the dynamic nature of the neoglycoconjugates used in this study, Monte Carlo conformational searches were performed in a continuum solvation model on the four disaccharides that derivatize peptides **1**, **2**, **3** and **5**. An *N*-acetyl group was substituted for the hydroxyl group at the anomeric carbon to represent more accurately the amide linkage that characterizes *N*-linked glycosylation. The results of the molecular modeling suggest that the ϕ dihedral of the glycosidic bond for chitobiose and D-Glc*N*Ac-[β(1-4)]-D-Glc is less flexible than those observed for cellobiose and D-Glc-[β(1-4)]-D-Glc*N*Ac (Figure 9). The C-2′ *N*-acetyl group of the distal sugar appears to greatly hinder rotation around the glycosidic bond, thereby playing a critical role in maintaining the rigidity of the disaccharide (Figure 9a). Additionally, molecular dynamics studies performed with the inclusion of explicit water molecules also indicate that the glycosidic bond of cellobiose is more mobile than that of chitobiose [41,42]. A less flexible carbohydrate derivative could more rigorously exclude conformational space, thereby disfavoring excessive motion of the peptide.

Impact of glycosylation on asparagine: destabilization of the Asx turn

The nonglycosylated analog of glycopeptide **1** is found to be in a predominantly Asx-turn conformation [13]. This turn involves interactions between the asparagine sidechain carbonyl and peptide backbone (Figure 10). The Asx and β-turns are topologically similar and are believed to have

Figure 9



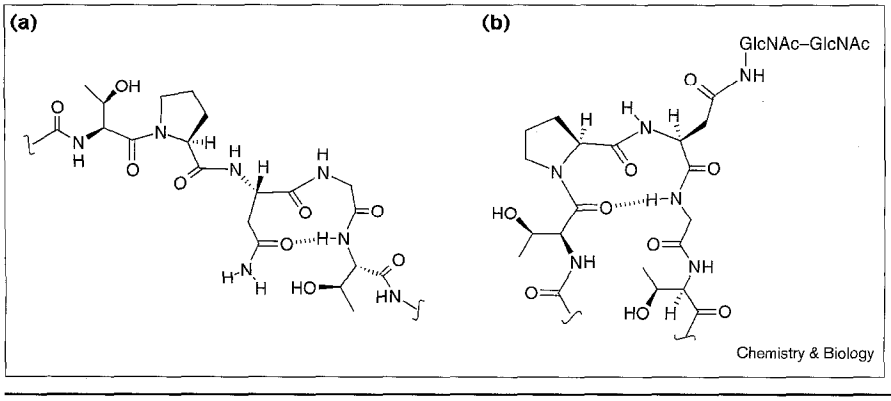
(a,b) The distal *N*-acetyl group can hinder rotation around the glycosidic bond. **(c)** Rotation around the glycosidic ϕ and ψ dihedral angles.

similar energies [43]. Moreover, the backbone dihedrals of asparagine do not change substantially between the two turn types; a type I β-turn has ϕ and ψ dihedrals of approximately –130° and 75°, whereas a standard Asx turn (type I_t) has ϕ and ψ values of –120° and 80° [43]. It is entirely possible, therefore, that the addition of a bulky substituent to this key asparagine residue could result in the predominance of the β-turn over the Asx turn. The Asx turn could become disfavored in the glycosylated state for steric reasons; the bulky glycosylated asparagine sidechain might no longer be in close proximity with the backbone. Additionally, the flexible sugar that now adorns the asparagine sidechain might disrupt the hydrogen bond between the backbone and asparagine sidechain that characterizes an Asx turn.

Significance

Work in numerous laboratories has established that glycosylation can have a dramatic effect on peptide and protein conformation. The work presented here explores the effect of systematic changes in carbohydrate composition on glycopeptide conformation. Notably, relatively minor changes in the carbohydrate appear to produce dramatic structural effects in the model peptide system described here. It is perhaps worthwhile to speculate that the pentameric core $GlcNAc_2Man_3$ that is found in all *N*-linked glycans [44] might be conserved in part for structural reasons. Without the innermost *N*-acetylglucosamine residues, glycoproteins might lose their required rigidity or local conformation. A common modification that could

Figure 10



Comparison of the Asx turn and β-turn. **(a)** Asx turn. **(b)** Type I β-turn.

further enforce the rigidity of the peptide is the attachment of a fucose to the proximal *N*-acetyl glucosamine. Although the influence of this derivation on the conformation of the polypeptide backbone has not been studied, it is clear that addition of this carbohydrate stabilizes the conformation of the saccharide [45].

The results of this study have led us to propose that the steric bulk of chitobiose causes glycopeptide **1** to adopt the more compact β-turn over the more extended Asx-turn conformation. The structures of the various glycopeptides described in this study reveal that the *N*-acetyl groups of the native sugar play distinct, but equally critical roles. Glycopeptide **2**, derivatized with cellobiose, adopts a much more extended structure than glycopeptide **1**; moreover, analysis of ^{13}C T_1 values indicate that glycopeptide **1** is more rigid than other derivatives at the glycosylation site. Addition of an *N*-acetyl group to the proximal sugar in glycopeptide **3** promotes β-turn formation, as revealed by the nuclear overhauser effect (NOE) data. This *N*-acetyl group appears to be located in the appropriate position to engage in steric interactions that can affect both the amino- and carboxy-terminal regions of the turn. These studies, performed in aqueous media, do not reveal any specific hydrogen bond between the carbohydrate and the peptide. The nuclear magnetic resonance (NMR) data for glycopeptide **4** indicates that although glycosylation-induced β-turn formation is not nearly as dramatic, this peptide is more rigid than the cellobiose derivative; this result suggests that the *N*-acetyl group of the distal sugar plays a role in rigidifying the peptide. Molecular modeling studies suggest that the stabilization might occur because the distal *N*-acetyl group prohibits rotation around the glycosidic bond. The more rigid saccharide conformation can serve to exclude conformational space more rigorously, thereby promoting a more rigid, compact peptide conformation.

Materials and methods

Glycopeptide synthesis

Peptides were synthesized using standard Fmoc based chemistry on a PAL–PEG polystyrene resin (Millipore, 0.2 mmol/g substitution). The amino terminus of the completed peptide was acetylated on the resin by shaking with 20-fold excess of acetic anhydride and fivefold excess of triethyl amine for 2 h in dimethylformamide (DMF). The resin was rinsed with DMF, CH_2Cl_2 and then MeOH, followed by drying under vacuum overnight. Glycopeptides were chemically synthesized from a peptide in which allyl-protected aspartic acid was substituted for asparagine. While on the solid support, the allyl ester was removed with tetrakis(triphenylphosphine) palladium by shaking with a three-fold excess of the reagent in CH_3Cl/AcOH/*N*-methylmorpholine (37:2:1 v/v/v) for 12–16 h. The resin was washed extensively with chloroform, 0.5% (w/v) diethyldithiocarbamate/DMF, 0.5% (v/v) diisopropylethylamine (DIPEA)/DMF and DMF to ensure complete removal of the palladium reagent. Glycosylamines were coupled to the aspartyl sidechain with benzotriazole-1-yl-oxy-tris-(dimethylamino)-phosphoniumhexafluorophosphate (BOP) activation prior to cleavage from the solid support. A 1.5–5-fold excess of the glycosylamine was used along with DIPEA (three equivalents). In order to prevent succinimide formation during the peptide synthesis, Hmb (2-Hydroxy-4-methoxy benzyl) amide-protected glycine (Novabiochem) was incorporated as the residue carboxy-terminal to the aspartic acid [46]. The 2-hydroxy group of Hmb is acetylated during peptide synthesis; to ensure efficient Hmb cleavage after glycan coupling, the resin-bound peptide was treated with hydrazine hydrate/DMF (5% v/v) to deacetylate the 2-hydroxy group [46]. Glycopeptides were cleaved by shaking with 92:3:3:2 trifluoroacetic acid (TFA)/phenol/ethanedithiol/triisopropylsilane. Glycopeptides were triturated with diethyl ether and purified using reverse phase high-performance liquid chromatography (HPLC). Predicted masses of glycopeptides **1–5** were confirmed using electrospray or matrix-assisted laser desorption/ionization (MALDI) mass spectroscopy (Table 1).

Table 1

Analysis of mass of glycopeptides 1–5.

Glycopeptide	Formula	Calc'd	Observed [M+H]+
1	$C_{62}H_{97}N_{15}O_{23}$	1420.5	1421
2	$C_{58}H_{91}N_{13}O_{23}$	1338.4	1339
3	$C_{60}H_{94}N_{14}O_{23}$	1379.4	1380
4	$C_{54}H_{84}N_{14}O_{18}$	1217.3	1218
5	$C_{60}H_{94}N_{14}O_{23}$	1379.4	1379

Carbohydrate synthesis

Disaccharides were either purchased (Aldrich, Toronto Research Chemicals) or synthesized as outlined in Figures 3 and 4. Monosaccharide building blocks were synthesized using standard methods. Glycosylamines were prepared by reaction of the free sugars with a saturated solution of ammonium bicarbonate [47]. The reaction took several days to complete, at which point the reaction mixture was successively lyophilized until all excess ammonium carbonate was removed.

NMR studies

NMR samples were prepared at 1–4 mM glycopeptide in 90:10 H_2O:D_2O or 100% D_2O. All samples were adjusted to a pH between 4.45 and 4.50 using minute amounts of HCl or NaOH. Uncorrected pH values for D_2O samples were adjusted with minute amounts of DCl or NaOD. All rotating frame overhauser effect spectroscopy (ROESY) spectra were acquired on a 600 MHz Varian Unity Plus spectrometer with a mixing time of 400 ms using presaturation to suppress the water signal. Amide proton VT data were calculated from total correlated spectroscopy (TOCSY) spectra acquired on an AMX 500 MHz Bruker spectrometer at temperatures ranging from 7°–32°C, in 5°C increments. The water signal was suppressed by a WATERGATE gradient suppression. Phi dihedral angle restraints were calculated from $^3J_{HN\alpha}$ values obtained from a double quantum filtered correlated overhauser spectroscopy (DQF–COSY) also acquired on a 600 MHz Varian Unity Plus at 7°C. Twice the number of points (4096) were acquired in the F1 dimension to enhance resolution. Presaturation during the relaxation delay was used to suppress the water signal. Data was processed using Felix95 (Biosym, San Diego) software on a Personal Iris SGI workstation.

^{13}C T_1 Values were acquired from a heteronuclear multiple quantum correlation (HSQC) derived pulse program designed to provide selective T_1 values [48]. Only the first increment of this two-dimensional pulse sequence was taken. T_1 values were obtained from line heights (measured in Varian software) and fit to an exponential curve in Kaleidagraph. All fits were shown to display a regression coefficient R of 0.98 or better.

Structure determination

All structure determination was carried out in the NMRefine module of Insight (MSI, San Diego) using the cvff forcefield. ROESY peaks were visually inspected and sorted into two bins of distance restraints, strong (1–3 Å) and weak (2–4 Å). Approximately 70–100 distance

restraints were tabulated for each peptide. Any $^{3}J_{HN\alpha}$ values greater than 9 Hz were included in the restraint file as dihedral restraints (−150° to −80° degrees). Amide proton VT data were not included in any restraint file. Peptides were then subjected to a multi-step simulated annealing protocol incorporating ROESY-derived distance restraints. In each case, ten structures were determined.

Molecular modeling

Monte Carlo searches of disaccharides were performed using Macromodel 2.0, using a continuum-solvation water solvent. A total of 1000 structures were generated, and the ten lowest energy conformers were compared.

Supplementary material available

Additional data on the distance restraints for glycopeptides **1–5** are available as Supplementary material published with this article on the internet.

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