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Synthesis, Conformation and T-Helper Cell Stimulation of an O-Linked Glycopeptide Epitope Containing Extended Carbohydrate Side-Chains

Mare Cudic, Hildegund C.J. Ertl and Laszlo Otvos, Jr.*

The Wistar Institute, 3601 Spruce Street, Philadelphia, PA 19104, USA

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Abstract—To answer the question whether or not T cells to immunodominant protein fragments recognize glycosylated antigens, we synthesized a series of glycopeptides corresponding to peptide 31D, a major T-helper cell epitope of the rabies virus nucleoprotein. Thr4 of the epitope is known to allow mono- or disaccharide side-chain substitutions in either α- or β-anomeric configuration without interfering with MHC-binding. To model naturally occurring glycoprotein fragments that carry extended sugar chains, we prepared Fmoc-Ser/Thr-OPfp building blocks containing α- and β-linked linear tri- and heptasaccharides. Peptide 31D was synthesized with the complex carbohydrates attached to Thr4, and the T-helper cell activity of the glycopeptides was determined. Addition of α-linked carbohydrates, that mimic most of the natural O-linked glycoproteins, resulted in a major drop in the T-cell stimulatory ability in a sugar length-dependent manner. In contrast, the cytosolic glycoprotein mimicking β-linked glycopeptides retained their T-cell stimulatory activity, with the trisaccharide-containing analogue being almost as potent as the unglycosylated peptide. When the peptides were preincubated with diluted human serum, all peptides lost their ability to stimulate the 9C5.D8-H hybridoma. These findings indicated that (i) in contrast to cytosolic glycosylation, incorporation of long O-linked carbohydrates into T-helper cell epitopes abrogates the antigenicity of these protein fragments, and (ii) glycosylation is not a viable alternative to improve the immunogenic properties of subunit peptide vaccines. Glycosylation with all four carbohydrate moieties similarly destroyed the inducible α-helical structure of peptide 31D as detected by CD, indicating that the differences in the T-cell activity were not due to different peptide conformations.

Introduction

The way the immune system presents antigens for T-cell stimulation is well understood by now. The major histocompatibility complex (MHC) class I and class II antigen-processing pathways play an essential role in the activation of pathogen-specific T lymphocytes by presenting peptide fragments derived from foreign proteins.[1] The basic unit of recognition is a trimolecular complex consisting of the T-cell receptor (TcR), the MHC molecule, and the MHC-bound peptide ligand.[2] In the first step, the processed peptide antigens bind to the MHC determinant followed by recognition by the TcR coming from the opposite direction.[3] Whereas no T-cell stimulation occurs without snug fit to the MHC proteins, it is not clear how tolerant the T-cell receptor is towards side-chain substituted peptide antigens. Proteins expressed in a diversity of intracellular sites, including the cell surface, endoplasmic reticulum and cytosol can gain access to the MHC molecule, albeit with different efficiencies.[4]

Glycosylation is a very common co-translational modification of proteins, and a basic immunological question is whether native glycopeptides can be included in the natural T-cell epitope repertoire.[5] For peptides to have antagonistic activity for T-cells (targeting, for example, autoimmune diseases), binding to the MHC complex is a prerequisite that is not necessarily fulfilled by synthetic glycopeptides.[6] While N-terminal glycosylation of an MHC class II-restricted hen egg lysozyme-originated epitope results in increased MHC-binding of synthetic peptides, internal glycosylation abolishes the ability of the same peptide to bind MHC.[7] Among the internally glycosylated analogues the study did not find any difference in binding when the sugar was positioned on an

*Corresponding author. Tel.: +1-215-898-3772; fax: +1-215-898-5821; e-mail: otvos@wistar.upenn.edu

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asparagine or on an adjacent serine. Haurum and coworkers demonstrated that a MHC class I binding peptide can be modified by both α- and β-linked O-glycosylation as well as by β-linked N-glycosylation without affecting its binding to MHC class I, provided the modifications do not involve amino acids located at MHC anchor positions.[8] Some of the glycopeptide-MHC complexes retain their ability to stimulate cytotoxic T-cell lymphocyte (CTL) lines, but another subset of the glycosylated analogues are not able to bind the TcR anymore.[8] In another experiment, recognition by T cells, directed to a non-glycosylated decapeptide, is lost when a single galactosyl moiety is attached N-terminally to the antigens.[9] This decapeptide is likely the shortest core sequence that binds to the corresponding MHC class II, and any further modification, such as glycosylation is not tolerated. T-cells directed to the N-terminally glycosylated version of this peptide recognize glycopeptides having significant variation in the saccharide structure. The authors proposed that these T cells recognize a peptide structure specific to glycopeptide-MHC complexes, and that the recognition does not involve specific interaction between the carbohydrate moiety and the T-cell receptor. Later their view was modified when they discovered a new, glycopeptide-specific set of T-cells. They demonstrated that the new T-cells are specific for the immunizing galabiose, and do not recognize the same peptide coupled to cellobiose.[10] Likewise, when neo-glycopeptides are used to generate CTL, the polyclonal cell population preferentially kills target cells treated with glycopeptide compared to those treated with the core peptide.[11] The resulting glycopeptide-specific CTL and unrestricted carbohydrate-specific CTL populations exhibit different TcR expression panels.

As outlined above, experiments using model T-cell epitopes have demonstrated that carbohydrate can modulate T-cell responses in a variety of ways. Significantly, short sugar-containing glycopeptide-specific T-cell responses have been detected to native glycoproteins.[12] While investigations using viral glycoproteins suggest that the large N-linked glycans influence the T cell response to glycoproteins but that this does not involve specific interactions between the carbohydrate and the TcR, the smaller O-linked carbohydrates found on collagen or mucins are able to elicit a carbohydrate specific T cell responses.[13] The ability of carbohydrate to influence T-cell recognition of antigen has important consequences for a broad range of immune responses.

Peptides presented by human class I MHC molecules in vivo encompass up to 0.1% glycopeptides, containing the cytosolic *O*-β-GlcNAc substitution, and synthetic peptides carrying this substitution are efficiently transported into the endoplasmic reticulum.[14] Studies on the immunogenicity of glycosylated type II collagen indicate that antigen presenting cells can indeed process glycoproteins to glycopeptides, which are presented to MHC class II proteins and the complex elicits T cell responses.[15] In addition to a pure scientific inquiry, if naturally glycosylated epitopes were excluded from the antigenic pool, this knowledge would significantly reduce the candidate protein fragments in the process of mapping the antigenic regions. Prediction methods for identifying epitopic peptides are ever-so-popular,[16–18] because a successful algorithm would reduce the number of peptides required to be synthesized and assayed, and thereby greatly facilitate the identification of T-cell epitopes of proteins.[19]

Earlier we found that mid-chain incorporation of β-linked mono- and disaccharides into asparagines greatly diminishes the T-cell stimulatory activity due to the reduced ability of the glycopeptides to bind to MHC class II.[20,21] This finding seems to support the idea about the lack of natural *N*-glycosylated epitopes. β-*O*-glycosylation with short sugars similarly decreases the T-cell stimulation, but the extent of the decrease is dependent upon the peptide sequence and the host amino acid.[21,22] Our data on the short sugar-containing glycopeptides indicate that in contrast to glycosylated asparagine, the T-cell epitope repertoire may contain both α- and β-linked *O*-glycopeptides. Since many natural *O*-glycosylated protein fragments (e.g., mucins, blood group antigens, etc.) contain longer carbohydrate side-chains,[23] the next question we addressed in the current report was how the carbohydrate length affected the peptides' ability to stimulate T-helper cells. Our model is peptide 31D, an immunodominant epitope delineated from the rabies virus nucleoprotein.[24] The sequence of peptide 31D contains a threonine residue in position 4, which was earlier shown not to be involved in binding to the I-E^k MHC II molecule, and could be decorated with O-linked short carbohydrates without interfering with MHC binding.[25] We also studied the conformation-modifying effect of incorporation of long sugars into T-cell epitopic peptides. To complete the immunological and structural studies first the synthetic procedures for the preparation of *O*-glycopeptides containing α- and β-linked long carbohydrates had to be worked out. Here we report the synthesis of not only the glycosylated threonine building blocks used for the immunological and structural studies, but of the analogous serine derivatives as well. These glycopeptide precursors will open new avenues in studying an array of biological, biochemical and pharmacological properties of model glycopeptides and fragments of native glycoproteins.

Results and Discussion

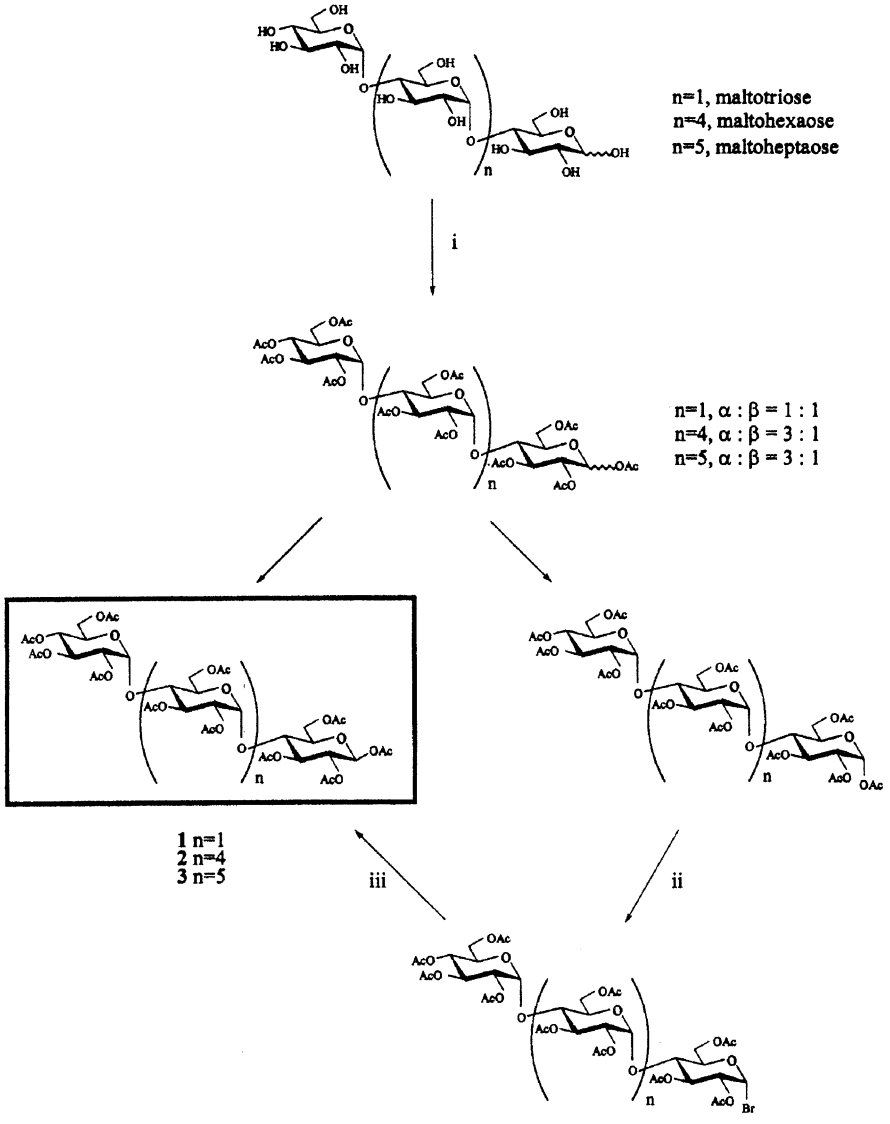
Synthesis of *O*-glycosylated amino acid building blocks

For modeling the extended carbohydrate chains attached to the fragments of natural O-glycoproteins we returned to oligomers of maltose. These were used successfully earlier for the immunological and conformational analysis of N-linked glycopeptides.[26,27] The starting compounds were maltotriose, maltohexaose and maltoheptaose. These oligosaccharides were acetylated with acetic acid anhydride/pyridine.[28] Mixtures of α- and β-anomers of peracetylated sugars (for maltotriose the α:β ratio was 1:1, and for maltohexaose and maltoheptose 3:1) were separated by silica gel

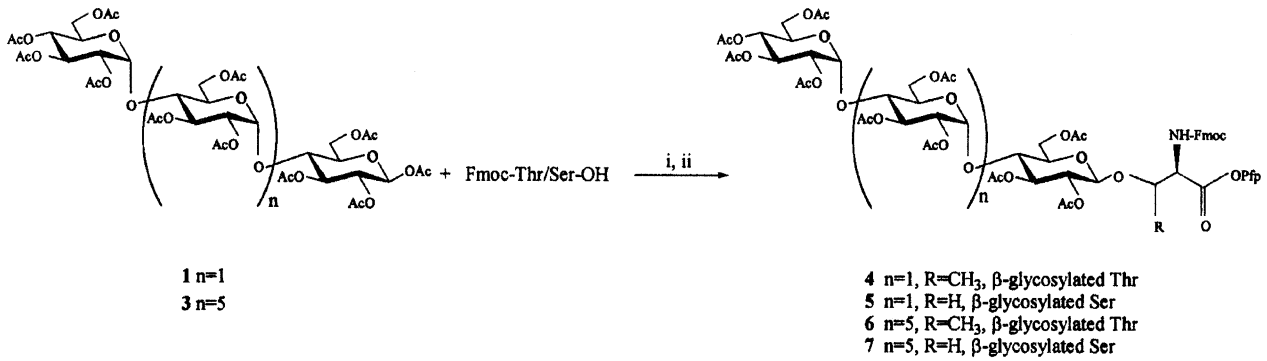
chromatography. The anomeric configuration was converted in order to obtain β-anomers of the peracetylated sugars, needed for the ensuing synthetic strategy. As a first step, the α-anomers were converted into acetylated glycosyl bromides with titanium tetrabromide.[29] This unusual protocol had to be employed, because the more conventional treatment with hydrogen bromide in acetic acid[28] failed to provide the target products due to intense decomposition, especially in case of maltohexaose and maltoheptaose. After bromination, the halogen was replaced by an acetate group using mercuric acetate in acetic acid, concomitant with simple inversion of the anomeric bonds (Scheme 1).

The next step was to prepare Fmoc-protected glycosyl serine and glycosyl threonine building blocks for the solid-phase synthesis of *O*-glycopeptides. The protocol introduced by Salvador et al.[30] gives an easy access to the target β-*O*-glycosylated building blocks, which carry the protective groups of choice (*O*-acetyl and *N*-α-Fmoc) for automated glycopeptide synthesis. We did not expect any major difficulty in applying this strategy for longer sugars because apparently an increase in the size of the carbohydrate is accompanied by an increase in the yield of the glycosylated Fmoc-Ser/Thr-OH derivatives.[31] Trisaccharide **1** and heptasaccharide **3** were coupled to Fmoc-Ser/Thr-OH using a Lewis acid ($BF_3 \cdot Et_2O$) as a promoter. After the crude products were purified by flash chromatography, the carboxyl group of the amino acids was activated with pentafluorophenol to afford Fmoc-protected and β-glycosylated Thr **4** and **6** as well as the corresponding Ser **5** and **7** derivatives in relatively high yields (60, 48, 55, and 44%, respectively) (Scheme 2). Clearly, our yields using long saccharides were comparable or better than those reported for the same derivatives carrying monosaccharide side-chains. The purity of the final *O*-glycosylated Ser/Thr building blocks was confirmed by HPLC chromatography and MALDI-MS (Table 1).

The formation of the α-glycosidic linkage can only be achieved if a nonparticipating substituent at C-2 of the glycosyl donor is used in a glycosylation reaction. The azido group was found to be efficient for such stereo-



Scheme 1. Reagents: (i) Ac_2O/pyridine; (ii) $TiBr_4$; (iii) $Hg(CH_3COO)_2$.

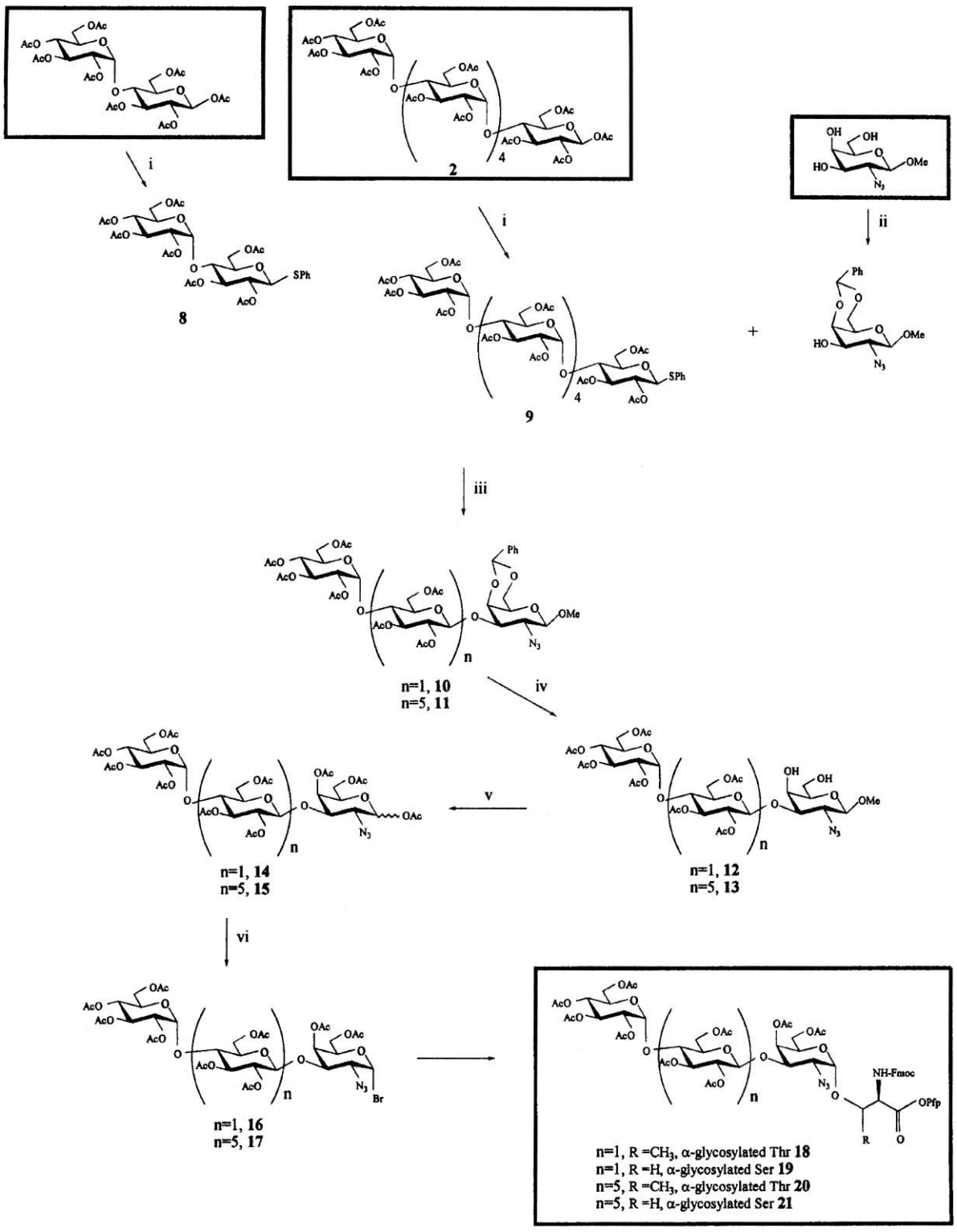


Scheme 2. Reagents: (i) BF_3–OEt_2; (ii) Pfp–OH, DIC.

Table 1. Synthetic *O*-glycosylated amino acid building blocks and their characterization

Compound	RP-HPLC retention time (min)	MALDI mass spectrometry	
		Calculated (Da)	Observed $(M+Na)^+$ (m/z)
Fmoc-Thr(maltotriose β1→*O*)-OPfp **4**	33.3	1413	1437
Fmoc-Ser(maltotriose β1→*O*)-OPfp **5**	31.7	1399	1423
Fmoc-Thr(maltoheptose β1→*O*)-OPfp **6**	37.7	2566	2589
Fmoc-Ser(maltoheptose β1→*O*)-OPfp **7**	36.5	2552	2575
Fmoc-Thr(maltose-$GalN_3$ α1→*O*)-OPfp **18**	33.1	1396	1419
Fmoc-Ser(maltose-$GalN_3$ α1→*O*)-OPfp **19**	31.8	1382	1406
Fmoc-Thr(maltohexaose-$GalN_3$ α1→*O*)-OPfp **20**	38.0	2549	2572
Fmoc-Ser(maltohexaose-$GalN_3$ α1→*O*)-OPfp **21**	36.9	2535	2558

selective α-glycosylation and for the subsequent conversion into an acetamido function, which is predominantly present at C-2 in natural *O*-glycoproteins.[29] The formation of α-linked glycosides of serine and threonine can be further promoted by the presence of fused cyclic acetal at the C-4 and C-6 hydroxyls of *N*-acetylgalactosamine.[32] Accordingly, methyl 2-azido-4,6-di-*O*-benzylidene-2-deoxy-β-D-galactopyranoside,[29,33–35] was used for the preparation of the α-*O*-glycosylated building blocks. Our strategy towards the synthesis of α-anomers **18**–**21** with extended carbohydrate chains involved the preparation of fully acetylated trisaccharide/heptasaccharide bromides **16, 17** as glycosyl donors which were then coupled to Fmoc-Ser/Thr pentafluorophenyl esters to yield Fmoc - Ser/Thr(Ac_7 - maltose - Ac_2 - Gal-N_3/Ac_{19}-maltohexaose-Ac_2-$GalN_3$-α1→*O*)-OPfp **18**–**21** (Scheme 3). Peracetylated β-sugars, β-D-maltose octaacetate and the peracetylated maltohexaose **2**, were treated with thiophenol in the presence of boron trifluoride etherate to give the corresponding phenyl-β-thioglycosides **8** and **9**.[36] The trisaccharide/heptasaccharide glycosyl donors were then prepared from peracetylated maltose/maltohexaose phenyl β-1-thioglycosides **8** and **9**. These carbohydrates underwent a condensation reaction with methyl 2-azido-4,6-di-*O*-benzylidene-2-deoxy-β-D-galactopyranoside promoted by *N*-iodosuccinimide-triflic acid to exclusively afford the β-anomers **10** and **11** in good yields. The thioglycoside donors were used on the basis of their superiority compared with trichloroacetimidate donors because of the ease of their preparation and the high yields they are reported to provide.[35] The benzylidine protective group was removed in aqueous acetic acid at 80 °C resulting in the formation of partially acetylated glycosyl azides **12** and **13**. Acetylation of the hydroxyl groups and acetolysis of the anomeric methyl glycoside were carried out in a single step using a mixture of acetic acid and sulfuric acid (50:1, v:v), affording anomeric mixtures of the peracetylated trisaccharides or heptasaccharides **14** or **15**. These peracetylated carbohydrates were converted to the corresponding glycosyl bromides **16** and **17**, now in α-anomeric configuration, using titanium tetrabromide. Finally, a modified Koenigs-Knorr glycosylation reaction was employed for the sugar incorporation into Fmoc-protected pentafluorophenyl esters of serine and threonine. This reaction is based on an observation that when the glycosylation is carried out with $AgClO_4$ at −40 °C, the α-isomer is preferably formed.[33] Accordingly, we coupled the Fmoc-Ser/Thr-OPfp derivatives with glycosyl bromide donors **16** and **17** in the presence of silver perchlorate at −40 °C to obtain the stereochemically almost clean α-anomers of Thr **18** and **20** as well as the Ser derivatives **19** and **21** in excellent yields (72, 63, 65, and 60%, respectively). The synthesis of the α-linked glycoamino acids is outlined in Scheme 3. All synthesized glycoamino acid derivatives were purified by silica gel chromatography. The purity of the final *O*-glycosylated Ser/Thr building blocks was confirmed by HPLC chromatography and MALDI-MS (Table 1). MHC class II epitopic peptide 31D as well as its α- and β-glycosylated analogues were then prepared by stepwise incorporation of unglycosylated threonine or the corresponding glycoamino acids **4**, **6**, **18**, and **20** during conventional solid-phase peptide synthesis. Similar to the glycoamino acid building blocks, the final glycopeptides were characterized by



Scheme 3. Reagents: (i) PhSH, BF_3–OEt_2; (ii) $PhCH(OMe)_2$, TsOH; (iii) NIS, TfOH, −45 °C; (iv) 80% AcOH, 80 °C; (v) Ac_2O–H_2SO_4, −25 °C; (vi) $TiBr_4$. The final coupling to Fmoc-Thr/Ser-OPfp utilized $AgClO_4$ at −45 °C.

RP-HPLC and MALDI-MS (Table 2). Figure 1 shows the RP-HPLC profile and the mass spectrum of the most complex glycopeptide, carrying an α-linked heptasaccharide side-chain attached to Thr4. One dimensional NMR spectra of the 31D glycopeptides supported the expected structures. The same glycoamino acid derivatives were used to synthesize a series of glycopeptides corresponding to the human epithelial mucin 1 protein. These mucin glycopeptides were characterized by homonuclear two-dimensional NMR experiments. Identity of the products and their anomeric configuration was confirmed by comparison of the protein shifts with those of model compounds in the sugar database and by analysis of the TOCSY and NOESY spectra.

Table 2. Characterization of the synthetic peptide (31D) and its glycosylated analogues

Peptide	Sequence	Side-chain modification (X)	RP-HPLC (min)	$(M+H)^+$ (m/z)	(Calculated, Da)
31D	AVYTRIMMNGGRLKR	—	29.9	1766	1765
31D-Tβ-$(Glc)_3$	AVYT(X)RIMMNGGRLKR	Glc(α1–4)Glc(α1–4)Glc(β1-O)	24.5	2252	2251
31D-Tα-$(Glc)_2$GalNAc	AVYT(X)RIMMNGGRLKR	Glc(α1–4)-Glc(β1–4)-GalNAc(α1-O)	26.7	2293	2292
31D-Tβ-$(Glc)_7$	AVYT(X)RIMMNGGRLKR	$[Glc(\alpha1–4)\text{-}Glc]_3$-Glc(β1-O)	23.8	2901	2900
31D-Tα-$(Glc)_6$GalNAc	AVYT(X)RIMMNGGRLKR	$[Glc(\alpha1–4)\text{-}Glc]_3$-GalNAc(α1-O)	26.2	2941	2940

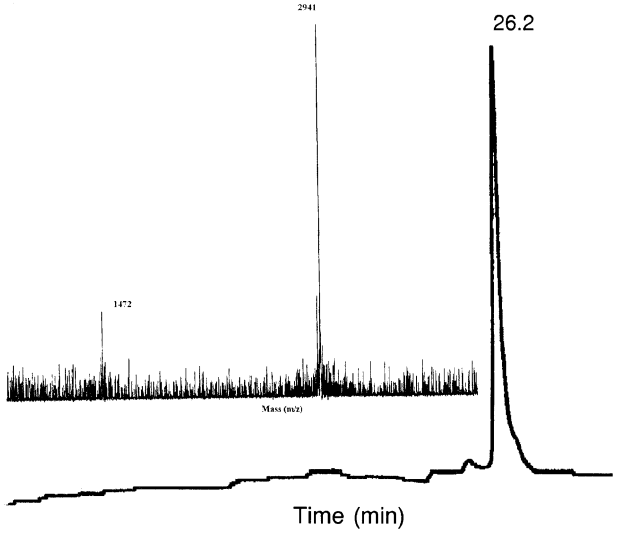


Figure 1. Reversed-phase high performance liquid chromatogram and matrix-assisted laser ionization/desorption mass spectrum (inset) of peptide 31D-Tα-$(Glc)_6$GalNAc. The glycopeptide eluted at 26.2 min as a single peak and exhibited the expected molecular ion of 2941 Da. The peak at 1472 m/z unit is the double charged mass spectrometry product.

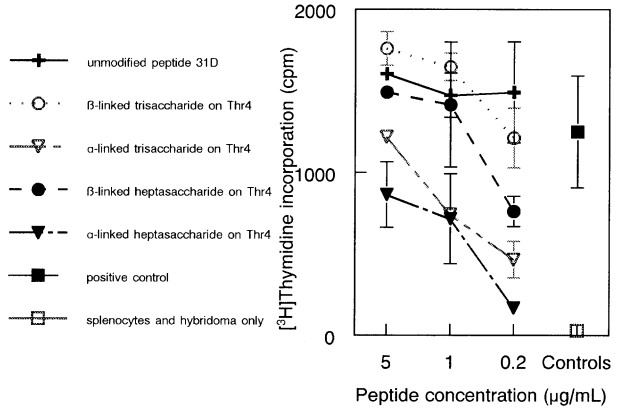


Figure 2. Stimulation of the 9C5.D8.H T-cell hybridoma with synthetic peptides. The solid line (crosses) represents the unmodified native peptide epitope 31D. The other four analogues carried carbohydrate side chains. The solid symbols represent trisaccharide substitutions, and the open symbols correspond to heptasaccharide substitutions. The two curves with circles represent α-linked sugars, and the triangles correspond to the β-derivatives. The glycopeptides are: 31D-Tβ-$(Glc)_3$ (dots), 31D-Tβ$(Glc)_7$ (dashes); 31D-Tα-$(Glc)_2$GalNAc (double dots and dashes), and 31D-Tα-$(Glc)_6$GalNAc (dots and dashes). The positive control was a rat concanavalin A solution (RCAS), regularly used to propagate the lymphokine-dependent HT-2 cell line.

High resolution conformational analysis of the products will be published elsewhere.

T-cell stimulation

To answer the question how *O*-linked extended carbohydrates modulate T cell responses, the 31D glycopeptides were tested for their ability to stimulate T helper cells. Peptide 31D, in context with the $I\text{-}E^k$ MHC determinant, vigorously stimulates cytokine secretion by the 9C5.D8-H T-cell hybridoma. From our earlier studies we know that Thr4 can carry mono or disaccharide side-chains in either α- or β-anomeric configuration without interfering with MHC-binding. In the current study, addition of α-linked carbohydrates, that mimic the sugar structures of natural *O*-linked glycoproteins, resulted in a major drop in the T-cell stimulatory ability in a sugar length-dependent manner (Fig. 2). In contrast, the β-linked glycopeptides retained most of their T-cell stimulatory activity, at least at higher antigen concentrations, with the trisaccharide-containing analogue being as potent as the non-glycosylated peptide (Fig. 2). These experiments demonstrated that the stimulation of T-helper cells was diminished if an extended α-linked sugar moiety was attached to a Thr residue, not involved in MHC-binding. Either the TcR was unable to accommodate the bulky carbohydrate, or, less likely, the sugar interfered with the formation of the trimolecular complex needed to elicit $CD4^+$ T-cell responses. The same glycopeptide remained somewhat stimulatory, when the heptasaccharide was attached in the non-natural β-anomeric configuration. This further supported the idea, that the MHC groove could accommodate the long carbohydrate side-chain attached to Thr4.

When the glycopeptides were preincubated in 50% human serum for 3 h, all peptides lost their T-cell stimulatory ability, probably due to cleavage by serum proteases. Although some peptides were probably cleaved by the 10% fetal bovine serum (FBS) used as a medium for the T-cell stimulation assay, this proteolytic strength has been shown to be inadequate to cleave significant amounts of peptide 31D or its analogues.[37] These findings indicated that (i) long and short sugars coupled to hydroxyamino acids in native T cell epitopes are non-equivalent in terms of antigenicity, and (ii) glycosylation of peptide epitopes is not an efficient alternative to improve the half-live, and consequently the immunogenic properties of subunit peptide vaccines.

These results are in accord with earlier studies on the antigenicity and immunogenicity of MHC class II restricted O-linked glycoproteins or synthetic glycopeptides that carry shorter sugar side-chains. When T-cells were generated to various *O*-glycosylated fragments of type II collagen, most of the resulting hybridomas specifically recognized the collagen peptide glycosylated with a monosaccharide.[38] Likewise, while glycopeptides carrying mono- and disaccharides attached to the mouse hemoglobin-derived T helper cell epitope Hb(67–76) elicit measurable immune responses, the same peptide with a natural mucin core 2 trisaccharide structure remains without any immunogenic properties.[39] In the third example the disaccharide present in the T antigen is extended with sialylation to yield a trisaccharide-coupled peptide epitope. Regardless of whether the carbohydrate is attached to a serine or a threonine residue, the peptide carrying trisaccharide is always less immunogenic than the analogous peptide coupled to the disaccharide moiety.[40] Although the authors explain these findings with the shielding effects of sialic acid in cellular recognition processes, a simple sugar-length dependent inhibition of interaction with the T-cell receptor can not be excluded either. To the best of our knowledge, *O*-glycopeptides containing longer than trisaccharides have not been studied for their T-cell stimulatory properties.

It is worth speculating on the T cell antigenicity of native glycopeptides as regulated by the differences in the anomeric configuration and the length of the carbohydrate side-chains. While the less frequent cytosolic glycosylation involves a single GlcNAc residue coupled to serines or threonines via a β-anomeric linkage,[41] most other glycoproteins contain α-linked extended carbohydrate side-chains. In accord with earlier studies on MHC class I epitopes, our β-linked trisaccharide-containing 31D variant retained most of the antigenic properties of the native peptide epitope. Apparently this type of glycosylation, native or artificial, does not interfere with binding to the MHC determinants or the recognition by the TcR. These and our earlier results also indicate that our particular 9C5.D8-H T cell hybridoma does not directly bind to the hydroxyl group of Thr4. For all other α-linked glycoprotein-mimicking glycopeptides, if our single example can be generalized, the interaction with the TcR is abrogated upon sugar incorporation. Whether this reflects the lack of native *O*-glycosylated epitopes for the α-linked glycoproteins can be later investigated by using the various glycosylated 31D fragments as immunogens.

Conformational analysis

Peptide 31D is a prototype of protein fragments assuming an inducible α-helical structure.[20] The conformational analysis of the 31D glycopeptides carrying the extended sugar chains served two purposes. First, we wanted to see whether the observed differences in the T-cell stimulatory ability of the α- and β-linked carbohydrates could be rationalized by different secondary structures of the glycopeptides. Second, the current state of knowledge concerning the conformation-modifying effect of long carbohydrates attached to the peptide backbone is very limited. Our data provided some insight into the natural conformation of glycoproteins and their fragments.

In 50% aqueous trifluoroethanol, unglycosylated peptide 31D exhibited a loosened α-helical structure (Fig. 3). While the general shape of the CD curve resembled those of classical α-helices,[42] the blueshift of the bands representing the ππ* transition, especially that of the negative band to 205 nm, clearly documented a destabilized helical conformation. The calculated α-helix content based on the ellipticity value at 208 nm[43] reached 28%. Glycosylation with all four carbohydrate moieties similarly destroyed the inducible α-helical structure of peptide 31D (Fig. 3). This conformation-modifying effect could be seen from the blueshift of the ππ* bands which was accompanied by the significant decrease of the intensity of the positive band, as well as from the considerable drop of the intensity ratio of the 222:201–205 nm bands. Actually all four glycopeptides exhibited very similar CD spectra, characterized by a positive band at 186 nm, a negative band at 201 nm and a negative shoulder at 222 nm. These spectral features resembled those of type I (III) β-turns or mixtures of type I and type II turns.[44] These data indicated that the differences in the T-cell activity, as regulated by the anomeric configuration of the carbohydrate side-chains, were not due to different peptide conformations. Significantly, the local secondary structure around the glycosylation sites of *O*-glycopeptides appeared to be a reverse-turn, regardless of the length or the anomeric configuration of the carbohydrate moiety.

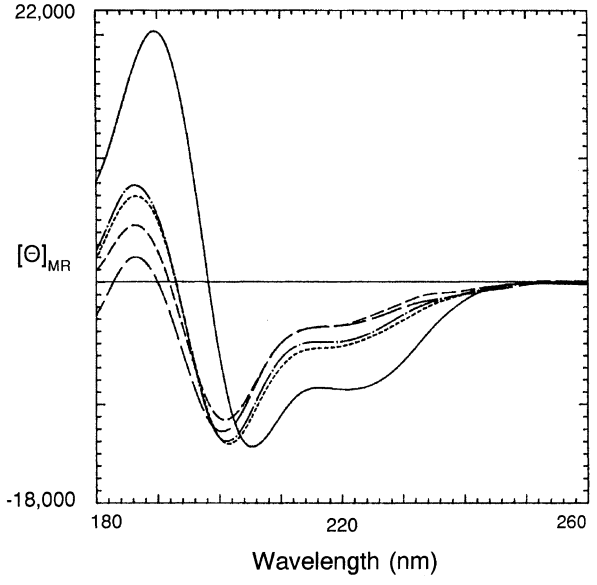


Figure 3. Circular dichroism spectra of peptide 31D and its glycopeptide analogues. The spectra were acquired in 50% aqueous trifluoroethanol at a peptide concentration of 0.2 mg/mL. The solid line represents unmodified peptide 31D, the rest are the glycopeptides. The substitutions are as follows: α-linked trisaccharide, peptide 31D-Tα-$(Glc)_2$GalNAc—short dashes; β-linked trisaccharide, peptide 31D-Tβ-$(Glc)_3$—dots; α-linked heptasaccharide, peptide 31D-Tα-$(Glc)_6$GalNAc—long dashes; and β-linked heptasaccharide, peptide 31D-Tβ$(Glc)_7$—dots and dashes.

Incorporation of O-linked carbohydrates into synthetic peptides are reported to result in small, but measurable conformational changes on the peptide backbone. In accordance with our data, the 204 nm negative CD band is blueshifted to 200 nm upon multiple glycosylation of a glycophorin fragment, when alternating serines and threonines are decorated with a GalNAc monosaccharide moiety.[45] Another stabilization of a β-turn structure was documented by addition of two GalNAc units at the two ends of the V3 loop of the human immunodeficiency virus glycoprotein.[46] *O*-Glycosylation also appears to modulate the conformation of synthetic mucin peptides,[47,48] although the similarity of the CD curves representing polyproline II helices and unordered conformations as well as the lack of intramolecular hydrogen bonding, sources of NOE cross-peaks in two-dimensional NMR, renders the polyproline II conformation of free peptides in solution indistinguishable from an irregular backbone structure.[49] The literature generally lacks conformational studies on O-linked glycopeptides with extended sugar chains. In one of the rare reports, only marginal changes in the backbone conformation was detected when a hexapeptide was decorated with two linear tetrasaccharide moieties.[50]

In conclusion, we prepared glycoamino acid building blocks, ready for incorporation into synthetic glycopeptides, carrying α- and β-linked trisaccharide and heptasaccharide side-chains attached to serines and threonines. These glycoamino acid derivatives will be invaluable tools to study the biological and biochemical effects the presence of carbohydrates make in natural and artificial glycoprotein fragments. In our particular example, an analogue of a native T-helper cell epitopic peptide, carrying an α-linked extended sugar chain lost its ability to stimulate the corresponding T-cell hybridoma. In contrast, the cytosolic-glycosylation mimicking β-linked short trisaccharide did not interfere with recognition by the TcR. Threonine-bound long sugars appear to break helices and stabilize reverse-turns.

Experimental

General procedures

All air- and moisture-sensitive reactions were performed under argon atmosphere. The solvents, purchased from Fluka, were devoid of any trace of water and kept over molecular sieves. Acid-washed molecular sieves (AW-300), activated powdered molecular sieves, and Celite (Celite 521) were purchased from Aldrich. The progress of the reactions was monitored by thin-layer chromatography (TLC) on Merck Silica Gel 60 F_{254} plates, visualized by charring with 10% aqueous sulfuric acid and/or by UV light when applicable. Flash-column chromatography was performed on Silica Gel columns (Merck, 230–400 mesh).

For the reversed-phase HPLC a Beckman HPLC system (two 110B solvent delivery modules, a System Gold analogue interface module 406, and a Beckman System Organizer) equipped with a Dynamax UV–vis-detector Model UV-C (Rainin Technologies) and an CR501-integrator (Shimadzu Corporation) was used. A Phenomenex 5 μm C18 300 Å (4×250 mm) Jupiter column was used at a flow rate 1 mL min^{-1} for analytical runs, and a Phenomenex 10 μm C18 300 Å (21.2×250 mm) Jupiter column was used at a flow rate 10 mL min^{-1} for preparative separations. Solvent A was 0.1% aqueous trifluoroacetic acid and solvent B was 0.1% trifluoroacetic acid in acetonitrile. The gradient is given as percentage of solvent B in the solvent A/solvent B mixture.

Matrix-assisted laser desorption/ionization mass spectrometry (MALDI-MS) was carried out at the Wistar Institute Protein Microchemistry Laboratory on a Voyager Biospectrometry Workstation by standard methods. Fmoc-Thr(O^{t}Bu)-OPfp, Fmoc-Ser(O^{t}Bu)-OPfp, Fmoc-Thr-OH and Fmoc-Ser-OH were purchased from Bachem. Maltotriose, maltoheptaose, and β-D-maltose octaacetate were purchased from Sigma, methyl-2-azido-2-deoxy-β-D-galactopyranoside from Senn Chemicals, and silver perchlorate from Aldrich. Thioacetic acid was purchased from Fluka.

Synthesis of *O*-glycosylated amino acid building blocks

β-Anomers of peracetylated sugars (maltotriose, maltohexaose, and maltoheptaose) (**1**–**3**) were prepared according to standard methods by acetylation of unprotected D-carbohydrates, and an α→β anomeric conversion of the fully acetylated sugars.[28]

Synthesis of Fmoc-Ser/Thr(Ac_{10}-maltotriose/Ac_{22}-maltoheptaose β1→O)-OPfp

The Lewis acid, $BF_3 \cdot Et_2O$ (39 μL, 0.3 mmol) was added to the β-peracetylated tri-/heptasaccharide (0.1 mmol) and the N-α-Fmoc-Ser/Thr-OH (0.12 mmol) in dry CH_2Cl_2 (10 mL) under argon atmosphere at room temperature. The progress of the glycosylation reaction was monitored by TLC and analytical HPLC (isocratic elution of 50% solvent B for 5 min followed by a linear gradient from 50 to 100% solvent B for 45 min). When the reaction was found not to progress further, the reaction mixture was diluted with CH_2Cl_2 (15 mL), washed with 1 M aqueous HCl (5 mL) and water (15 mL), dried over sodium sulfate (Na_2SO_4) and concentrated in vacuum. The residue was purified by flash chromatography (EtOAc/toluene/HOAc = 10:5:1, v/v). Pentafluorophenol (37 mg, 0.2 mmol) and 1,3-diisopropylcarbodiimide (31 μL, 0.2 mmol) were added to the purified Fmoc-Ser/Thr(Ac_{10}-maltotriose/Ac_{22}-maltoheptaose β1→O)-OH (0.1 mmol), dissolved in dry CH_2Cl_2 (10 mL), at room temperature. After the active ester formation was completed (approximately 2–3 h), the mixture was washed with water (10 mL), dried and concentrated by rotary evaporation. The crude products were purified by RP-HPLC (preparative runs using an isocratic elution of 50% solvent B for 5 min followed by a linear gradient from 50 to 100% solvent B for 45 min). The purity of the final β1→*O*-glycosylated Ser/Thr building blocks was confirmed by RP-HPLC chromatography and MALDI-MS.

N^{α}-(Fluoren-9-ylmethoxycarbonyl)-3-*O*-[2,3,4,6-tetra-*O*-acetyl-α-D-glucopyranosyl-(1→4)-2,3,6-tri-*O*-acetyl-α-D-glucopyranosyl-(1→4)-2,3,6-tri-*O*-acetyl-β-D-glucopyranosyl]-L-threonine pentafluorophenyl ester (4). The protected and activated glycoamino acid derivative **4** was prepared by the condensation of peracetylated maltotriose **1** (100 mg, 0.1 mmol) with N-α-Fmoc-Thr-OH (42 mg, 0.12 mmol) according to the general procedure outlined in the previous paragraph. Purification of the pentafluorophenyl ester by flash chromatography followed by RP-HPLC afforded the final trisaccharide-containing activated amino acid derivative **4** (85 mg, 60%). MALDI-MS; calcd for $C_{63}H_{68}F_5N_1O_{30}$ 1413.37, found $(M+Na)^+$ 1436.50.

N^{α}-(Fluoren-9-ylmethoxycarbonyl)-3-*O*-[2,3,4,6-tetra-*O*-acetyl-α-D-glucopyranosyl-(1→4)-2,3,6-tri-*O*-acetyl-α-D-glucopyranosyl-(1→4)-2,3,6-tri-*O*-acetyl-β-D-glucopyranosyl]-L-serine pentafluorophenyl ester (5). Compound **5** was prepared by reaction of **1** (100 mg, 0.1 mmol) with N-α-Fmoc-Ser-OH (39 mg, 0.12 mmol). Purification of the crude product by flash chromatography, and RP-HPLC of the corresponding pentafluorophenyl ester afforded the protected activated ester of serine **5** (77 mg, 55%). MALDI-MS; calcd for $C_{62}H_{66}F_5N_1O_{30}$ 1399.36, found $(M+Na)^+$ 1422.59.

N^{α}-(Fluoren-9-ylmethoxycarbonyl)-3-*O*-[2,3,4,6-tetra-*O*-acetyl-α-D-glucopyranosyl-(1→4)-2,3,6-tri-*O*-acetyl-α-D-glucopyranosyl-(1→4)-2,3,6-tri-*O*-acetyl-α-D-glucopyranosyl-(1→4)-2,3,6-tri-*O*-acetyl-α-D-glucopyranosyl-(1→4)-2,3,6-tri-*O*-acetyl-α-D-glucopyranosyl-(1→4)-2,3,6-tri-*O*-acetyl-α-D-glucopyranosyl-(1→4)-2,3,6-tri-*O*-acetyl-β-D-glucopyranosyl]-L-threonine pentafluorophenyl ester (6). The protected and activated glycoamino acid derivative **6** was prepared by the condensation of the peracetylated heptasaccharide **3** (213 mg, 0.1 mmol) with N-α-Fmoc-Thr-OH (42 mg, 0.12 mmol) according to the general procedure described above. Purification of the crude product by flash chromatography followed by RP-HPLC afforded the final threonine derivative **6** (125 mg, 48%). MALDI-MS; calcd for $C_{111}H_{132}F_5N_1O_{62}$ 2565.71, found $(M+Na)^+$ 2588.69.

N^{α}-(Fluoren-9-ylmethoxycarbonyl)-3-*O*-[2,3,4,6-tetra-*O*-acetyl-α-D-glucopyranosyl-(1→4)-2,3,6-tri-*O*-acetyl-α-D-glucopyranosyl-(1→4)-2,3,6-tri-*O*-acetyl-α-D-glucopyranosyl-(1→4)-2,3,6-tri-*O*-acetyl-α-D-glucopyranosyl-(1→4)-2,3,6-tri-*O*-acetyl-α-D-glucopyranosyl-(1→4)-2,3,6-tri-*O*-acetyl-α-D-glucopyranosyl-(1→4)-2,3,6-tri-*O*-acetyl-β-D-glucopyranosyl]-L-serine pentafluorophenyl ester (7). Compound **7** was prepared by the reaction of the heptasaccharide **3** (213 mg, 0.1 mmol) with N-α-Fmoc-Ser-OH (39 mg, 0.12 mmol). Purification of the crude product by flash chromatography, and RP-HPLC of the corresponding pentafluorophenyl ester afforded the protected activated ester of serine **7** (115 mg, 44%). MALDI-MS; calcd for $C_{110}H_{130}F_5N_1O_{62}$ 2551.70, found $(M+Na)^+$ 2575.30.

Synthesis of Fmoc-Ser/Thr(Ac_7-maltose-Ac_2-GalN_3/Ac_{19}-maltohexaose-Ac_2-GalN_3 α1→*O*)-OPfp. Methyl 2-azido-4,6-*O*-benzylidene-2-deoxy-β-D-galactopyranoside was prepared according to Paulsen et al.[17]

Phenyl 2,3,4,6-tetra-*O*-acetyl-α-D-glucopyranosyl-(1→4)-2,3,6-tri-*O*-acetyl-1-thio-β-D-glucopyranoside (8). The Lewis acid, $BF_3{\cdot}Et_2O$ (280 μL, 2.1 mmol) was added to β-D-maltose octaacetate (500 mg, 0.7 mmol) and thiophenol (111 μL, 1.05 mmol) in dry CH_2Cl_2 (20 mL) under argon atmosphere. After 12 h at room temperature, the reaction mixture was washed two times with saturated aqueous sodium bicarbonate ($NaHCO_3$), and then with water. The separated organic layer was dried over sodium sulfate (Na_2SO_4) and concentrated under vacuum. The residue was purified by flash chromatography (EtOAc:toluene = 1:1, v/v) to obtain the thioglycoside **8** (320 mg, 58%). MALDI-MS; calcd for $C_{32}H_{40}O_{17}S_1$ 728.20, found $(M+Na)^+$ 751.20.

Phenyl 2,3,4,6-tetra-*O*-acetyl-α-D-glucopyranosyl-(1→4)-2,3,6-tri-*O*-acetyl-α-D-glucopyranosyl-(1→4)-2,3,6-tri-*O*-acetyl-α-D-glucopyranosyl-(1→4)-2,3,6-tri-*O*-acetyl-α-D-glucopyranosyl-(1→4)-2,3,6-tri-*O*-acetyl-α-D-glucopyranosyl-(1→4)-2,3,6-tri-*O*-acetyl-1-thio-β-D-glucopyranoside (9). In a similar manner, compound **9** was prepared starting from peracetylated β-D-maltohexaose **2** (1500 mg, 0.82 mmol) and thiophenol (130 μL, 1.23 mmol) in dry CH_2Cl_2 (20 mL) under $BF_3{\cdot}Et_2O$ (328 μL, 2.46 mmol) promotion. Purification of the crude product by flash chromatography (EtOAc:toluene = 3:1, v/v) afforded the hexasaccharide thioglycoside **9** (1150 mg, 75%). MALDI-MS; calcd for $C_{80}H_{10}4O_{49}S_1$ 1880.54, found $(M+Na)^+$ 1903.78.

Methyl 2,3,4,6-tetra-*O*-acetyl-α-D-glucopyranosyl-(1→4)-2,3,6-tri-*O*-acetyl-β-D-glucopyranosyl-(1→3)-2-azido-4,6-*O*-benzylidene-2-deoxy-β-D-galactopyranoside (10). Phenyl β-thioglycoside **8** (720 mg, 0.96 mmol) and methyl 2-azido-4,6-*O*-benzylidene-2-deoxy-β-D-galactopyranoside (255 mg, 0.81 mmol) were dissolved in dry CH_2Cl_2 (20 mL). Acid-washed molecular sieves (AW-300, 1.8 g) were added and the reaction mixture was stirred at room temperature for 1 h under argon atmosphere. The flask was cooled down to −45 °C and N-iodosuccinimide (234 mg, 1.02 mmol) was added. After a period of 10 min, trifluoromethane sulfonic acid (84 μL, 0.96 mmol) in acetonitrile (1 mL) was added slowly and the reaction mixture was vigorously stirred for another 2 h. Triethylamine (0.1 mL) was added at the same temperature to neutralize the acid and the mixture was filtered through Celite. The organic filtrate was then washed with saturated aqueous sodium thiosulfate ($Na_2S_2O_3$) and sodium bicarbonate ($NaHCO_3$). The separated organic layer was dried over Na_2SO_4 and was concentrated. The residue was purified by flash chromatography (EtOAc/toluene = 2:1, v/v) to obtain the pure methyl glycoside **10** (495 mg, 66%). MALDI-MS; calcd for $C_{40}H_{51}N_3O_{22}$ 925.30, found $(M+Na)^+$ 948.31.

Methyl 2,3,4,6-tetra-*O*-acetyl-α-D-glucopyranosyl-(1→4)-2,3,6-tri-*O*-acetyl-α-D-glucopyranosyl-(1→4)-2,3,6-tri-*O*-acetyl-α-D-glucopyranosyl-(1→4)-2,3,6-tri-*O*-acetyl-α-D-glucopyranosyl-(1→4)-2,3,6-tri-*O*-acetyl-α-D-glucopyranosyl-(1→4)-2,3,6-tri-*O*-acetyl-α-D-glucopyranosyl-(1→3)-2-azido-4,6-*O*-benzylidene-2-deoxy-β-D-galactopyranoside (11). In a similar manner, the methyl hexa-

saccharide glycoside **11** was prepared from phenyl β-thioglycoside **9** (1431 mg, 0.76 mmol) and methyl 2-azido-4,6-*O*-benzylidene-2-deoxy-β-D-galactopyranoside (200 mg, 0.64 mmol). Purification of the crude product by flash chromatography (EtOAc/toluene = 3:1,v/v) afforded product **11** (900 mg, 68%). MALDI-MS; calcd for $C_{88}H_{115}N_3O_{54}$ 2077.64, found $(M+Na)^+$ 2100.97.

Methyl 2,3,4,6-tetra-*O*-acetyl-α-D-glucopyranosyl-(1→4)-2,3,6-tri-*O*-acetyl-β-D-glucopyranosyl-(1→3)-2-azido-2-deoxy-β-D-galactopyranoside (12). To remove the benzylidene protection, compound **10** (450 mg, 0.49 mmol) was heated with 80% aqueous acetic acid (5 mL) for 2–3 h at 80 °C. The resulting mixture was diluted with toluene and evaporated to dryness. The crude product was purified by flash chromatography (EtOAc/toluene/HOAc = 10:5:1, v/v/v) to obtain the pure deprotected methyl trisaccharide **12** (250 mg, 61%). MALDI-MS; calcd for $C_{33}H_{47}N_3O_{22}$ 837.27, found $(M+Na)^+$ 859.88.

Methyl 2,3,4,6-tetra-*O*-acetyl-α-D-glucopyranosyl-(1→4)-2,3,6-tri-*O*-acetyl-α-D-glucopyranosyl-(1→4)-2,3,6-tri-*O*-acetyl-β-D-glucopyranosyl-(1→4)-2,3,6-tri-*O*-acetyl-α-D-glucopyranosyl-(1→4)-2,3,6-tri-*O*-acetyl-α-D-glucopyranosyl-(1→4)-2,3,6-tri-*O*-acetyl-α-D-glucopyranosyl-(1→3)-2-azido-2-deoxy-β-D-galactopyranoside (13). In a similar manner, compound **13** was prepared by acidic hydrolysis of the benzylidene derivative **11** (700 mg, 0.34 mmol). After flash chromatography (EtOAc/toluene/HOAc = 10:5:1, v/v/v) the methyl heptasaccharide **13** was obtained as a white solid (550 mg, 81%). MALDI-MS; calcd for $C_{81}H_{111}N_3O_{54}$ 1989.61, found $(M+Na)^+$ 2012.86.

Acetyl 2,3,4,6-tetra-*O*-acetyl-α-D-glucopyranosyl-(1→4)-2,3,6-tri-*O*-acetyl-β-D-glucopyranosyl-(1→3)-2-azido-4,6-di-*O*-acetyl-2-deoxy-1-*O*-D-galactopyranoside (14). The solution of the methyl glycoside **12** (250 mg, 0.3 mmol) in acetic anhydride (3 mL), was stirred at −20 °C, and a precooled (0 °C) mixture of acetic anhydride and sulfuric acid [Ac_2O/H_2SO_4 (50:1, v/v, 2 mL)] was added to it during a time period of 5 min. The acetylation reaction was allowed to proceed for 5 days at-20 °C. The mixture was diluted with cold CH_2Cl_2 (50 mL) and was carefully washed with aqueous sodium bicarbonate solution ($NaHCO_3$) and water. The organic layer was dried over Na_2SO_4 and was evaporated to complete dryness after adding and removing toluene. Product **14** was obtained in practically quantitative yields. MALDI-MS; calcd for $C_{38}H_{51}N_3O_{25}$ 949.28, found $(M+Na)^+$ 972.32.

Acetyl 2,3,4,6-tetra-*O*-acetyl-α-D-glucopyranosyl-(1→4)-2,3,6-tri-*O*-acetyl-α-D-glucopyranosyl-(1→4)-2,3,6-tri-*O*-acetyl-α-D-glucopyranosyl-(1→4)-2,3,6-tri-*O*-acetyl-α-D-glucopyranosyl-(1→4)-2,3,6-tri-*O*-acetyl-α-D-glucopyranosyl-(1→4)-2,3,6-tri-*O*-acetyl-α-D-glucopyranosyl-(1→3)-2-azido-4,6-di-*O*-acetyl-2-deoxy-1-*O*-D-galactopyranoside (15). In a similar manner, the acetyl heptasaccharide glycoside **15** was prepared starting from the methyl derivative **13** (600 mg, 0.3 mmol). The reaction proceeded smoothly and the product was isolated in quantitative yields. MALDI-MS; calcd for $C_{86}H_{115}N_3O_{57}$ 2101.62, found $(M+Na)^+$ 2124.95.

2,3,4,6-Tetra-*O*-acetyl-α-D-glucopyranosyl-(1→4)-2,3,6-tri-*O*-acetyl-β-D-glucopyranosyl-(1→3)-2-azido-4,6-di-*O*-acetyl-2-deoxy-α-D-galactopyranosyl bromide (16). The acetyl trisaccharide glycoside **14** (250 mg, 0.26 mmol) and titanium tetrabromide (195 mg, 0.53 mmol) were stirred in dry CH_2Cl_2 and EtOAc mixture (10:1, v/v, 11 mL) for 12 h at room temperature. The solution was diluted with dry toluene (50 mL), and anhydrous sodium acetate was added until the mixture became colorless. The reaction mixture was filtered through Celite, and the filtrate was dried over Na_2SO_4 and concentrated in vacuum. The crude product was purified by flash chromatography (EtOAc/toluene = 2:1, v/v) to obtain the pure glycosyl bromide **16** (100 mg, 40%). MALDI-MS; calculated for $C_{36}H_{48}Br_1N_3O_{23}$ 969.19, found $(M+Na)^+$ 992.08.

2,3,4,6-Tetra-*O*-acetyl-α-D-glucopyranosyl-(1→4)-2,3,6-tri-*O*-acetyl-α-D-glucopyranosyl-(1→4)-2,3,6-tri-*O*-acetyl-α-D-glucopyranosyl-(1→4)-2,3,6-tri-*O*-acetyl-α-D-glucopyranosyl-(1→4)-2,3,6-tri-*O*-acetyl-α-D-glucopyranosyl-(1→4)-2,3,6-tri-*O*-acetyl-α-D-glucopyranosyl-(1→3)-2-azido-4,6-di-*O*-acetyl-2-deoxy-α-D-galactopyranosyl bromide (17). In a similar manner, the acetyl heptasaccharide glycoside **15** (600 mg, 0.29 mmol) was used to prepare the corresponding bromide **17**. The reaction was run in the presence of titanium tetrabromide (210 mg, 0.57 mmol). Purification of the crude heptasaccharide bromide by flash chromatography (EtOAc/toluene = 3:1, v/v) afforded the pure product **17** (300 mg, 50%). MALDI-MS; calcd for $C_{84}H_{112}Br_1N_3O_{55}$ 2121.53, found $(M+Na)^+$ 2146.72.

N^{α}-(Fluoren-9-ylmethoxycarbonyl)-3-*O*-[2,3,4,6-tetra-*O*-acetyl-α-D-glucopyranosyl-(1→4)-2,3,6-tri-*O*-acetyl-β-D-glucopyranosyl-(1→3)-2-azido-4,6-di-*O*-acetyl-2-deoxy-α-D-galactopyranosyl]-L-threonine pentafluorophenyl ester (18). Bromide **16** (100 mg, 0.1 mmol) and Fmoc-Thr-OPfp (102 mg, 0.2 mmol) were dissolved in a mixture of dry CH_2Cl_2 and toluene (1:1, v/v, 5 mL). Activated powdered molecular sieves (Aldrich, 150 mg) were added and the reaction mixture was stirred at room temperature for 1 h under argon atmosphere. Then the reaction flask was cooled down to −40 °C and silver perchlorate ($AgClO_4$) (21 mg, 0.1 mmol) was added. The progress of the glycosylation reaction was monitored by TLC (EtOAc/toluene = 2:1, v/v). After 2 h, a complete disappearance of the starting glycosyl bromide **16** was observed. The reaction mixture was allowed to warm up to room temperature, was diluted with chloroform (10 mL) and was filtered through Celite. The organic filtrate was washed with water, separated, dried over Na_2SO_4 and concentrated. The residue was purified by flash chromatography (EtOAc/toluene = 2:1, v/v) to obtain the pure activated glycoamino acid **18** (102 mg, 72%). MALDI-MS; calcd for $C_{61}H_{65}F_5N_4O_{28}$ 1396.37, found $(M+Na)^+$ 1419.26.

N^{α}-(Fluoren-9-ylmethoxycarbonyl)-3-*O*-[2,3,4,6-tetra-*O*-acetyl-α-D-glucopyranosyl-(1→4)-2,3,6-tri-*O*-acetyl-β-D-glucopyranosyl-(1→3)-2-azido-4,6-di-*O*-acetyl-2-deoxy-

α-D-galactopyranosyl]-L-serine pentafluorophenyl ester (19). In a similar manner, the serine derivative **19** was prepared from glycosyl bromide **16** (100 mg, 0.1 mmol) and Fmoc-Ser-OPfp (99 mg, 0.2 mmol) in the presence of silver perchlorate ($AgClO_4$) (21 mg, 0.1 mmol). The crude product was purified by flash chromatography (EtOAc/toluene = 2:1, v/v) to obtain the pure activated glycoamino acid **19** (90 mg, 65%). MALDI-MS; calcd for $C_{60}H_{63}F_5N_4O_{28}$ 1382.35, found $(M+Na)^+$ 1405.67.

N^{α}-(Fluoren-9-ylmethoxycarbonyl)-3-*O*-[2,3,4,6-tetra-*O*-acetyl-α-D-glucopyranosyl-(1→4)-2,3,6-tri-*O*-acetyl-α-D-glucopyranosyl-2,3,6-tri-*O*-acetyl-α-D-glucopyranosyl-2,3,6-tri-*O*-acetyl-α-D-glucopyranosyl-2,3,6-tri-*O*-acetyl-α-D-glucopyranosyl-2,3,6-tri-*O*-acetyl-α-D-glucopyranosyl-(1→3)-2-azido-4,6-di-*O*-acetyl-2-deoxy-α-D-galactopyranosyl]-L-threonine pentafluorophenyl ester (20). In a similar manner, the activated threonine derivative carrying a long carbohydrate side-chain **20** was prepared from the corresponding glycosyl bromide **17** (214 mg, 0.1 mmol) and Fmoc-Thr-OPfp (102 mg, 0.2 mmol) in the presence of silver perchlorate ($AgClO_4$) (21 mg, 0.1 mmol). The crude product was purified by flash chromatography (EtOAc/toluene = 3:1, v/v) to obtain the pure glycoamino acid **20** (160 mg, 63%). MALDI-MS; calcd for $C_{109}H_{129}F_5N_4O_{60}$ 2548.71, found $(M+Na)^+$ 2571.90.

N^{α}-(Fluoren-9-ylmethoxycarbonyl)-3-*O*-[2,3,4,6-tetra-*O*-acetyl-α-D-glucopyranosyl-(1→4)-2,3,6-tri-*O*-acetyl-α-D-glucopyranosyl-2,3,6-tri-*O*-acetyl-α-D-glucopyranosyl-2,3,6-tri-*O*-acetyl-α-D-glucopyranosyl-2,3,6-tri-*O*-acetyl-α-D-glucopyranosyl (1→3)-2-azido-4,6-di-*O*-acetyl-2-deoxy-α-D-galactopyranosyl]-L-serine pentafluorophenyl ester (21). In a similar manner, the serine derivative **21** was prepared from the glycosyl bromide **17** (214 mg, 0.1 mmol) and Fmoc-Ser-OPfp (99 mg, 0.2 mmol) in the presence of silver perchlorate ($AgClO_4$) (21 mg, 0.1 mmol). The crude glycoamino acid was purified by flash chromatography (EtOAc/toluene = 3:1, v/v) to obtain the pure serine building block **21** (153 mg, 60%). MALDI-MS; calcd for $C_{108}H_{127}F_5N_4O_{60}$ 2534.70, found $(M+Na)^+$ 2558.27.

Solid-phase (glyco)peptide synthesis

Unmodified peptide 31D and the corresponding glycosylated analogues were synthesized by conventional solid-phase methods. The peptide chain assembly proceeded on a Rainin PS3 automated synthesizer and using TentaGel S-Ram-Fmoc resin with an initial load of 0.3 mmol/g (Advanced ChemTech, Louisville, KY). Standard Fmoc-chemistry was used throughout[51] with a 4-molar excess of the acylating amino acids and HATU (1-hydroxy-7-azabenzotriazole uronium salt) activation, recommended for the synthesis of complex peptides.[52] The glycoamino acids were incorporated in the same manner as the unmodified amino acids, except that they were coupled in a 1.5 molar excess (to reduce glycoamino acid usage) in the presence of di-isopropyl-ethylamine (DIPEA). On completion of the assembly of the peptide chain, the N-terminal Fmoc-protection was retained. Still polymer-bound, the azido group on the sugar ring was transformed into an acetamido group using thioacetic acid for 4 days with 4 consecutive changes of the reagent. After reducing the azido group, the Fmoc protection on the N-terminal amino acid was removed using 20% piperidine in dimethyl formamide (DMF). The resulting glycopeptides as well unmodified peptide 31D were cleaved from the solid support with trifluoroacetic acid (TFA) in the presence of thioanisole (5%), and water (5%) as scavengers for 2 h. Deacetylation of the sugar hydroxyl groups was accomplished by a treatment with 0.1 M NaOH.[20] A reaction time of 10 min was sufficient for the peptides containing trisaccharides, and 20 min was needed for those carrying heptasaccharide side-chains. After cleavage, the peptides were purified by RP-HPLC, (preparative runs: isocratic elution of 5% solvent B for 5 min followed by a linear gradient from 5 to 65% solvent B for 120 min). The final products were characterized by RP-HPLC (during the analytical runs the same gradient was developed over a 45 min period) and MALDI-MS.

Conformational analysis

Circular dichroism (CD) spectra were performed on a Jasco J720 instrument at room temperature in a 0.2 mm pathlength cell. Double-distilled water and spectroscopy grade trifluoroethanol were used as solvents. Peptide concentration was 0.2 mg/mL, as determined by RP-HPLC. Curves were smoothed by algorithm provided by Jasco. Mean residue ellipticity $[\theta]_{MR}$ is expressed in deg cm^2 $dmol^{-1}$ using mean residue weights calculated from the molecular weight of the glycopeptides divided by the number of amide bonds in the sequences.

Cytokine release assay

The 9C5.D8.H cell line, a fusion product between a thymoma cell line and splenocytes from C3H/He mice immunized with inactivated rabies virus of the Evelyn–Rokitnicki–Abelseth (ERA) strain, was used for the studies. These T-cell hybridomas secrete IL-2 in response to peptide 31D presented by the $I\text{-}E^k$ MHC determinant as it was detailed earlier.[24] The 9C5.D8.H cells were grown in Dulbecco's Modified Eagles medium (DMEM) supplemented with 10% FBS, glutamine and antibiotics. Low passage numbers were used for the experiments. The IL-2 and IL-4 sensitive HT-2 cell line was grown in DMEM supplemented with 10% FBS, 10% rat concanavalin A supernatant and 1 μM 2-mercaptoethanol.

Splenocytes from young naive C3H/He mice were prepared and irradiated with 1800 rads. Cells were plated at a density of 3×10^6 cell/wells onto 48-well Costar plates in 300 μL of DMEM supplemented with 10% FBS and 1 μM 2-mercaptoethanol. Peptides were added followed by the addition of 1×10^5 9C5.D8.H to each well and the medium was adjusted to a final volume of 1 mL. Cell-free supernatants were harvested 24 h later; 75 μL of concentrated supernatant was added in triplicate to round bottom microtiter plate wells that contained 2×10^3 HT-2 cells. The final volume was 150 μL. Plates

were pulsed 3 days later for 6 h with 0.5 μCi of ^{3}[H]-thymidine. Cells were then harvested onto filtermates and incorporation of radioactivity was determined in a β-counter. The specificity of this assay for this particular peptide epitope and T-helper cell line was documented earlier when under identical assay conditions peptide 31D failed to stimulate any other rabies virus-related T cell clone.[53]

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