Glycopeptide Specificity of Helper T Cells Obtained in Mouse Models for Rheumatoid Arthritis

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Five protected analogues of β-D-galactosyl-(5R)-5-hydroxy-L-lysine were prepared, in which the galactosyl moiety was modified by monodeoxygenation or inversion of stereochemistry at C-4. The building blocks were used in the solid-phase synthesis of a set of glycopeptides related to the peptide fragment CII256–273 from type II collagen. Evaluation of the glycopeptides revealed that T-cell hybridomas obtained in collagen-induced arthritis (CIA), which is a common mouse model for rheumatoid arthritis, recognized the galactosyl moiety with high specificity for individual hydroxy groups. Moreover, T-cell hybridomas obtained in a humanized variant of CIA were also found to recognize the glycopeptides in an equally carbohydrate-specific manner. The results allowed the generation of models of the complexes formed between the appropriate class II major histocompatibility complex (MHC) molecule, glycopeptide, and the T-cell receptor, that is, of an interaction that is critical for the stimulation of T cells in the arthritis models. In the structural models, peptide side chains anchor the glycopeptide in pockets in the class II MHC molecule, whereas the galactosylated hydroxylysine residue forms the key contacts with the T-cell receptor. Importantly, the results also suggest that a T-cell response towards glycopeptide fragments from type II collagen could play an important role in the development of rheumatoid arthritis in humans.

KEYWORDS:

glycopeptides · immunology · molecular recognition · rheumatoid arthritis · solid-phase synthesis

Introduction

The last 10–20 years has led to a tremendous increase in our knowledge of how cell-mediated immune responses occur, and many key interactions are now understood at the molecular and structural level.[1] Proper functioning of the immune system of higher vertebrates requires the processing of protein antigens into shorter peptides by antigen-presenting cells.[2–4] Peptides resulting from this degradation are bound by major histocompatibility complex (MHC) molecules, and the complexes are then transported to the cell surface where they are displayed to T cells. Recognition of the complexes by receptors on circulating T cells triggers responses that depend on the origin of the protein antigen and also on the kind of antigen-presenting cell and T cell that are involved. Almost all cells can process foreign protein antigens produced intracellularly, for example, due to a viral infection or transformation of the cell during cancer. Recognition of the resulting peptide–class-I-MHC-molecule complexes by receptors on "cytotoxic" ($CD8^+$) T cells leads to destruction of the presenting cell, thereby eliminating the viral infection or preventing the development of cancer. In a similar manner, specialized antigen-presenting cells, such as macrophages, B cells, and dendritic cells, take up and process extracellular protein material, for example, bacterial proteins. Recognition of the complexes between peptides and class II MHC molecules on the surface of such antigen-presenting cells by "helper" ($CD4^+$) T cells leads to the release of immunomodulating cytokines, such as interleukines. These cytokines are essential for inducing production of high-affinity IgG antibodies in B cells as well as production of memory B cells and activation of phagocytic cells; these events are all essential for the elimination of the bacterial infection.

The interactions that occur in ternary complexes of MHC molecules, peptides, and T-cell receptors are thus critical for eliciting proper immune responses from both cytotoxic and helper T cells. Studies based on X-ray crystallography have recently revealed the structures of such complexes, which provide a platform for understanding how structural features relate to signal transduction.[5–9] In both class I and class II MHC molecules, two α helices form the sides of the elongated peptide-binding groove and the floor consists of an eight-stranded β sheet.[10, 11] The groove of class I MHC molecules is closed at both ends, which limits the length of the bound peptides to between eight and ten residues. Class II MHC molecules have peptide-binding grooves that are open at both ends, thereby allowing the binding of longer peptides that often contain more than 13 amino acids. Pockets in the binding

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groove require insertion of side chains from two or three residues in the bound peptide and thus provide specificity for the interaction. Since the peptide is deeply bound in the groove of the MHC molecule, only a few of its side chains (usually between one and three) are accessible for binding to the T-cell receptor.[5–9] It appears to be a common theme that the CDR3 loops of the T-cell receptor, which have the highest potential diversity, form specific interactions with a side chain that protrudes from the center of the bound peptide.

It is important to keep in mind that endogenous proteins are processed in antigen-presenting cells in the same manner as "foreign" proteins. Large numbers of self-peptides are therefore presented by MHC molecules on the surface of antigen-presenting cells. Normally, T cells that recognize complexes between self-peptides and MHC molecules are deleted during development in the thymus or inactivated in the periphery.[1] As a consequence the organism becomes tolerant to its endogenous proteins; however, if tolerance is broken then an autoimmune disease may develop. Rheumatoid arthritis (RA), which affects 0.5 – 1% of the human population, is generally considered to be an autoimmune inflammatory disease.[12] This conclusion is supported by the association of RA with certain human class II MHC molecules, that is, HLA-DR4 and HLA-DR1. A detailed insight into how RA develops requires studies that range from animal models to the molecular level. Nowadays, the use of transgenic animals may provide a more "human" setting for such investigations. Immunization of mice with type II collagen, which is the major glycoprotein found in joint cartilage, induces an autoimmune inflammatory response termed collagen-induced arthritis (CIA).[13, 14] In CIA, self-tolerance is broken by using type II collagen from rat as immunogen in Complete Freund's Adjuvant (CFA). CIA is accompanied by erythema and severe, painful swelling of peripheral joints, that is, symptoms and histopathology similar to those displayed by patients suffering from rheumatoid arthritis. Development of CIA in mice is associated with the presence of H-2A^q class II MHC molecules on antigen-presenting cells, which results in the display of an immunodominant peptide epitope located between residues 256 and 270 of type II collagen (CII256 – 270) to the T-cell receptor.[15] Recently, mice that were transgenic for both the human, RA-associated DR4 class II molecule and the human CD4 T cell co-receptor were generated.[16, 17] Interestingly, these "humanized" mice are also highly susceptible to the development of arthritis after immunization with type II collagen. Furthermore, the immunodominant T-cell epitope in these mice was found to be located within residues 259 – 273 of type II collagen (CII259 – 273), that is, in a peptide closely related to that found in CIA.

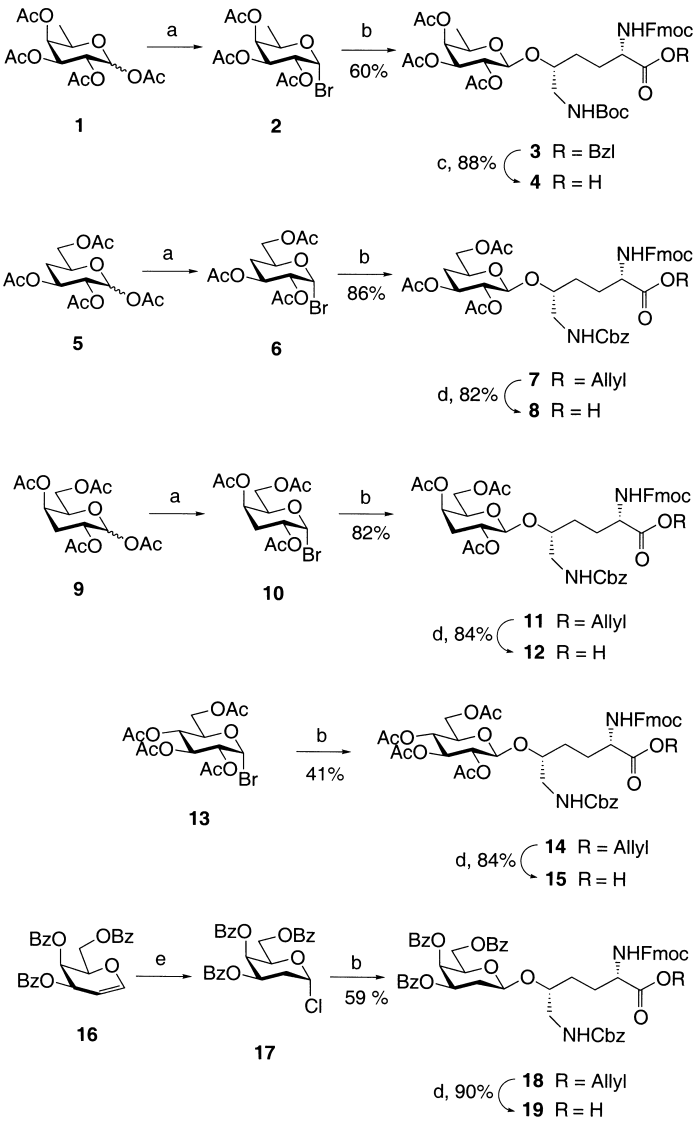
We have previously demonstrated that the majority of the members of a panel of T-cell hybridomas obtained in CIA[15, 18] actually recognize the immunodominant peptide epitope CII256 – 270 in a post-translationally modified form when it is presented by H-2A^q class II MHC molecules.[19, 20] Lysine residues in type II collagen can undergo hydroxylation followed by glycosylation with β-D-galactopyranosyl or α-D-glucopyranosyl-(1 $\rightarrow$2)-β-D-galactopyranosyl residues,[21, 22] and CII256 – 270 recognized by the hybridomas was found to carry a β-D-galactosyl residue attached to hydroxylysine at position 264. We now report the synthesis of five CII-derived glycopeptides, which have modified galactosyl residues attached to hydroxylysine 264. The glycopeptides were used to investigate the carbohydrate specificity of the T-cell hybridomas from CIA that recognize galactosylated CII256 – 270. Moreover, they were also used to study whether glycosylation of CII259 – 273 is important when arthritis is induced in mice that are transgenic for the human DR4 class II molecule. We believe that the results of these studies contribute to the development of a molecular understanding of how arthritis develops not only in mice, but also in humans.

Results and Discussion

Synthesis of glycosylated amino acids and glycopeptides

To investigate the fine specificity of the T-cell hybridomas obtained in CIA, five glycopeptides with modified D-galactosyl residues were selected as synthetic targets. The modifications consisted of the sequential monodeoxygenation of the four hydroxy groups on the galactose moiety and the inversion of the stereochemistry at C-4 (i.e., by using D-glucose). Published methods for the synthesis of glycosylated derivatives of hydroxylysine have often involved the use of acid-labile protecting groups on the carbohydrate moiety.[19, 23, 24] Glycosides of deoxygenated sugars are, however, substantially more susceptible to acid-catalyzed degradation than their nondeoxygenated counterparts.[25] As a consequence, protection with acetyl groups is necessary to prevent degradation during acid-catalyzed cleavage from the solid support of peptides glycosylated with the 6-deoxysugar L-fucose.[26, 27] Acetyl or benzoyl (Bz) groups were therefore chosen for the carbohydrate moieties in the present study.

Attempts to find conditions for the attachment of monodeoxygenated D-galactosyl residues to (5*R*)-5-hydroxy-L-lysine were first focused on the coupling of readily available, *O*-acetylated D-fucosyl donors to Fmoc-Hyl(Boc)-OBzl (Fmoc = *N*-(9-fluorenylmethyloxycarbonyl), Boc = *tert*-butoxycarbonyl, Bzl = benzyl). Unfortunately, the use of ethyl 1-thio-2,3,4-tri-*O*-acetyl-β-D-fucopyranoside in combination with *N*-iodosuccinimide (NIS) and either trifluoromethanesulfonic acid (TfOH)[28, 29] or silver trifluoromethanesulfonate (AgOTf) as promotors did not give the desired product. This was also the case when 2,3,4-tri-*O*-acetyl-α-D-fucopyranosyl bromide was used with promotion by silver trifluoromethanesulfonate. The thiofucoside/NIS/TfOH conditions were too acidic and led to cleavage of the Boc group from Fmoc-Hyl(Boc)-OBzl, whereas the other less acidic conditions (fucosyl bromide, AgOTf, and 2,6-di-*tert*-butylpyridine) resulted in formation of mixtures of α- and β-glycosides of hydroxylysine together with varying amounts of the corresponding orthoester. However, silver silicate promoted[30] glycosylation of Fmoc-Hyl(Boc)-OBzl with 2,3,4-tri-*O*-acetyl-α-D-fucopyranosyl bromide (**2**) in dichloromethane gave the β anomer **3** in 60% yield (Scheme 1). Hydrogenolysis of the benzyl ester[19, 31] of **3** over Pd/C in ethyl acetate at atmospheric pressure then gave the



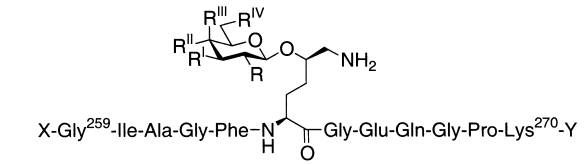
Scheme 1. *a) HBr, HOAc, Ac_2O; b) Fmoc-Hyl(Boc)-OBzl or Fmoc-Hyl(Cbz)-OAll, silver silicate, 3 Å MS, CH_2Cl_2; c) H_2, Pd/C, EtOAc; d) $(PPh_3)_4Pd(0)$,* N*-methylaniline, THF; e) HCl(g), toluene.*

glycosylated building block **4** (88% yield), which is ready for use in solid-phase glycopeptide synthesis.

Although the yield and stereoselectivity of the glycosylation leading to **3** is adequate, the formation of a number of minor side-products was observed. Recent experience with acetobromogalactose donors in silver silicate promoted glycosylations of Fmoc-Hyl(Boc)-OBzl and Fmoc-Hyl(Cbz)-OAll (Cbz = carbobenzyloxy) led us to conclude that the latter acceptor is more suitable.[32] This conclusion was based on the increased stability of the Cbz group relative to the Boc group under acidic conditions such as those encountered during glycosylation. In spite of its higher stability towards acids, the Cbz group can still be removed by trifluoroacetic acid (TFA) under conditions commonly used for the cleavage of peptides and glycopeptides from the solid phase.[24, 33] The *O*-acetylated 4- and 3-deoxy-*xylo*-hexopyranosyl bromides (**6**) and (**10**) were therefore used in silver silicate promoted glycosylations of Fmoc-Hyl(Cbz)-OAll in dichloromethane, which gave **7** and **11** in 86 and 82% yields, respectively (Scheme 1). Use of the less reactive acetobromoglucose (**13**) under identical conditions gave the expected β-glycoside **14**; however, the corresponding othoester was formed as a side-product and proved difficult to remove. After repeated purification by chromatography, **14** was obtained in approximately 41% yield, although a small amount of the orthoester still remained (3% relative to **14**). Deallylation of **7**, **11**, and **14** was achieved by using $(PPh_3)_4Pd(0)$ and *N*-methylaniline in tetrahydrofuran (THF) and gave glycosylated building blocks **8**, **12**, and **15** (in 82, 84, and 84% yields, respectively). At this stage the last remnants of orthoester could be removed from **15**.

Activation of glycosyl halides by insoluble silver salts is an established method for creating β-glycosides in the absence of a participating group at C-2 of the glycosyl donor.[30, 34, 35] The 2-deoxyglycosyl chloride **17** was therefore generated from 3,4,6-tri-*O*-benzoyl-D-galactal (**16**) by treatment with gaseous HCl in toluene. Use of chloride donor **17** for silver silicate promoted glycosylation of Fmoc-Hyl(Cbz)-OAll in dichloromethane at 0 °C yielded glycoside **18** as an anomeric mixture ($\beta/\alpha = 2{:}1$). Lowering the temperature (−30 °C → 0 °C over 36 h) and also the polarity of the solvent (dichloromethane/toluene, 1:1) increased the β/α ratio to 10:1, and the β anomer **18** could be isolated in 59% yield. The increase in β/α ratio on lowering the polarity of the solvent may be explained by a preference for the direct substitution mechanism[30] of silver silicate over other pathways involving ionic intermediates. Deallylation of **18** (as described for **8**, **12**, and **15**) then gave the 2-deoxygalactosyl hydroxylysine building block **19** in 90% yield.

The five hydroxylysine building blocks with modified galactosyl residues were employed in the solid-phase synthesis of glycopeptides **20** – **24** (Scheme 2). Synthesis was performed under standard conditions according to the Fmoc protocol on a polystyrene resin grafted with poly(ethylene glycol) spacers



		R	R^I	R^{II}	R^{III}	R^{IV}	X	Y
19 →	**20** (2-deoxy)	H	OH	H	OH	OH	H	Gly-Glu-Thr
12 →	**21** (3-deoxy)	OH	H	H	OH	OH	H	Gly-Glu-Thr
8 →	**22** (4-deoxy)	OH	OH	H	H	OH	H	Gly-Glu-Thr
4 →	**23** (6-deoxy)	OH	OH	H	OH	H	Gly-Glu-Hyp	OH
15 →	**24** (Glc)	OH	OH	OH	H	OH	Gly-Glu-Hyp	OH
	25 (Gal)	OH	OH	H	OH	OH	Gly-Glu-Hyp	OH
	26 (Gal)	OH	OH	H	OH	OH	H	Gly-Glu-Thr

(Hyp = *trans*-4-hydroxy-L-proline)

Scheme 2. *Glycopeptides **20** – **26**, which correspond to residues 259 – 273 or 256 – 270 of type II collagen, were used to reveal the specificity of T-cell hybridomas obtained in mouse models for rheumatoid arthritis.*

(Tentagel) that were functionalized with a 4-alkoxybenzyl alcohol linker.[19, 24, 32] Acid-catalyzed cleavage from the solid phase, followed by deacylation of the carbohydrate moieties with methanolic sodium methoxide and purification by reversed-phase HPLC gave the target glycopeptides **20** – **24** in 13 – 43 % yields based on the resin capacity. The glycopeptides were homogeneous according to analytical reversed-phase HPLC and their structures were confirmed by mass spectrometry, amino acid analysis, and ^{1}H NMR spectroscopy. These analyses also revealed that the glycosidic bonds of the acid-labile, deoxygenated carbohydrate moieties had remained intact during purification with reversed-phase HPLC performed at low pH values (pH 1 – 2). Glycopeptides **23** and **24**, which carry a 6-deoxygalactose moiety and a glucose residue, respectively, were prepared first. The structures of these compounds were based on the peptide sequence of residues 256 – 270 in type II collagen from rat (Scheme 2), which is known to bind well to the murine H-2A^q class II MHC molecule.[15, 36] The two glycopeptides can therefore be used in studies of the specificity of H-2A^q-restricted T-cell hybridomas, that is, hybridomas obtained in collagen-induced arthritis. During the course of the synthetic work, T-cell hybridomas that recognize antigens bound by the human HLA-DR4 (DRB*0401) class II MHC molecule were produced (as discussed below). The DR4 class II MHC molecule binds a peptide epitope that is shifted four residues towards the C terminus of type II collagen as compared to peptides bound by the H-2A^q molecule.[16, 37] To allow studies of both H-2A^q- and HLA-DR4-restricted hybridomas, the peptide sequence was altered to that of CII259 – 273 for glycopeptides **20** – **22**, which have galactose moieties that are deoxygenated at C-2, C-3, and C-4, respectively. Glycopeptides **25** and **26**,[19] which have a galactosylated hydroxylysine residue at position 264 of either of the two peptide sequences were prepared to evaluate the influence of peptide structure on the hybridoma response and for use as controls.[38]

Table 1. *Response of H-2A^q-restricted T-cell hybridomas when incubated with antigen-presenting spleen cells and increasing concentrations of glycopeptides* ***20*** *–* ***25****.*[a]

	25 (Gal)	**20** (2-deoxy)	**21** (3-deoxy)	**22** (4-deoxy)	**23** (6-deoxy)	**24** (Glc)
Group 1						
HD13.1	++++	++++	++++	–	+++++	–
HD13.2	++++	++++	+++++	–	+++++	–
HD13.3	+++++	+++++	+++++	(+)	+++++	(++)
HD13.4	++++	++++	++++	–	+++++	–
HD13.5	+++	+++	+++	–	++++	–
HD13.6	+++++	+++++	+++++	–	++++++	–
HD13.7	++++	++++	++++	–	++++	–
HD13.9	++++	++++	+++(+)	–	++++	–
HD13.10	+++++	+++++	+++++	–	++++++	
HM2.1	++++	+++	++++	–	+++++	–
HNC.1	++++++	++++	++++	–	+++++	(++)
Group 2						
HCQ.10	++++++	+++++	–	+++	+++++	+++
HCQ.1	+++++	+++++	–	+++	+++++	++(+)
HCQ.9	+++++	+++++	–	+++	+++++	+++
Group 3						
HCQ.3	++++++	–	++++	–	+++	–
HCQ.2	+++++	–	+++	–	+++	–
HCQ.6	+++++	–	+++	–	+++	–
Group 4						
HM1R.1	+++++	–	–	–	++++++	–
HM1R.2	+++++	–	–	–	+++++	–
HDC.1	+++	–	–	–	+++	–

[a] The magnitude of the response was determined from the concentration of antigen required to induce a proliferation of the IL-2-dependent T-cell clone CTLL that was 10 times greater than the background (that is, than without antigen): – = no response or [Ag] > 50 μg mL^{-1}; + = 50 μg mL^{-1}; ++ = 10 μg mL^{-1}; +++ = 2 μg mL^{-1}; ++++ = 0.4 μg mL^{-1}; +++++ = 0.08 μg mL^{-1}; ++++++ = 0.016 μg mL^{-1}; absence of a symbol = not tested; (+) = just below the threshold.

Specificity of T-cell hybridomas obtained in collagen-induced arthritis

Recent investigations into the response of a panel of helper-T-cell hybridomas obtained in collagen-induced arthritis revealed that the majority of the hybridomas (20 out of 29) responded to glycopeptide **25**, but not to the corresponding nonglycosylated CII256 – 270 peptide.[19] To unravel the fine specificity of these H-2A^q-restricted T-cell hybridomas, increasing concentrations of the modified glycopeptides **20** – **24** and the unmodified control **25** were incubated together with each of the hybridomas and antigen-presenting spleen cells that express H-2A^q. The response of the hybridomas, that is, the secretion of interleukin-2 (IL-2) into the medium on recognition of glycopeptide – H-2A^q complexes on the surface of the spleen cells, was determined in a secondary assay based on proliferation of the IL-2-dependent CTLL T-cell clone.[20, 39] These studies revealed that the 20 hybridomas can be divided into four groups with different patterns of fine specificity for the galactosyl moiety of glycopeptide **25** (Table 1). The eleven hybridomas belonging to group 1 have a strong dependency on the hydroxy group at C-4 of the galactosyl moiety (compare data for hybridoma HD13.10 in Figure 1) as revealed by the loss of response towards glycopeptides **22** (4-deoxy) and **24** (Glc). Removal of any of the hydroxy groups at C-2, C-3, or C-6 (glycopeptides **20**, **21**, and **23**, respectively) had no effect on the response of the hybridomas in group 1. The three hybridomas of group 2 depended strongly on HO-3 and weakly on HO-4, whereas HO-2 and HO-6 lacked importance in eliciting a response from these hybridomas (see hybridoma HCQ.10 in Figure 1). Finally, the three hybridomas in group 3 depend strongly on both HO-2 and HO-4 and also weakly on HO-3 and HO-6 (see hybridoma HCQ.3 in Figure 1), whereas the three group 4 hybridomas have a strong dependency on HO-2, HO-3, and HO-4, but not on HO-6 (see hybridoma HM1R.2 in Figure 1). In addition, the hybridomas in groups 1 – 3, but not those in group 4, have previously been shown to require the ε-amino group in the side chain of the glycosylated hydroxylysine.[19]

X-ray crystallography reveals that T-cell receptors bury a surface area of approximately 900 – 1000 Å^2 when binding to complexes between peptides and class I or II MHC molecules.[5–9] Although not determined experimentally, it can be assumed that a similar surface area is involved in binding to complexes between glycopeptides and MHC molecules, such as the complex between glycopeptide **25** and H-2A^q. In view of the

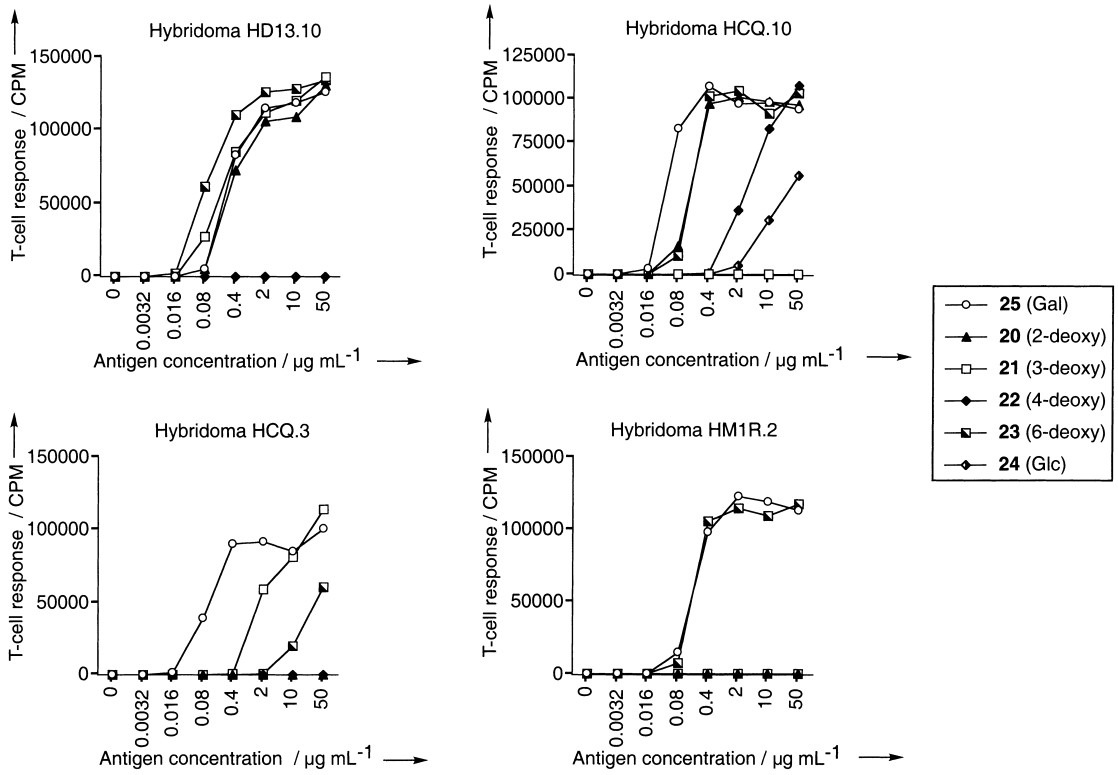


Figure 1. *Response of H-2A^q-restricted T-cell hybridomas on incubation with antigen-presenting spleen cells and increasing concentrations of glycopeptides **20** – **25**. The hybridomas were selected from each of the four groups that displayed different patterns of fine specificity for the galactosyl moiety in the glycopeptides. Recognition of complexes between glycopeptides and H-2A^q class II molecules on the surface of antigen-presenting cells by a T-cell hybridoma results in secretion of IL-2 in a dose-dependent manner. This T-cell response is subsequently determined in a radioassay based on proliferation of the IL-2-sensitive T-cell clone CTLL.[39]*

large size of the surface area buried, it is interesting to find that individual hydroxy groups of the galactose moiety in **25** are always critical for eliciting a proper response from each of the T-cell hybridomas. Similar observations have been made in model studies dealing with how T-cell hybridomas recognize neoglycopeptides.[40–42] However, in contrast to the present investigation, these studies employed less comprehensive sets of modified glycopeptides. Somewhat surprisingly, only three of the 20 hybridomas investigated here had a (weak) dependence on the less sterically hindered, primary hydroxy group at C-6 of the galactosyl residue. In contrast, all hybridomas require HO-4 of galactose to generate a full response. In a previous study, a similar importance of HO-4 of *N*-acetyl-D-galactosamine was found for class-II-MHC-restricted recognition of hemoglobin-derived neoglycopeptides by T-cell hybridomas.[40]

The complementarity-determining region-3 (CDR3) loops of T-cell receptors are of the utmost importance for specific recognition of peptide antigens.[5–8, 43] For helper T cells this is demonstrated in the crystal structure of a ternary complex between a T-cell receptor, a peptide, and a class II MHC molecule, in which the CDR3 loops contact the central parts of the bound peptide as well as the surrounding parts of the MHC molecule.[7] The high specificity with which the galactosylated hydroxylysine residue at position 264 of the type II collagen-derived glycopeptide **25** is recognized by all of the T-cell hybridomas reveals that this residue forms key contacts with the T-cell receptor, but it remains to be determined if it contacts the CDR3 loops. However, the CDR3 loops do appear to be involved in recognition of the galactosylated hydroxylysine, since the division of the hybridomas into groups based on carbohydrate fine specificity matches groups reported previously based on the nucleotide sequences of the T-cell receptor CDR3 loops.[20] In this context, it should be pointed out that CDR3-mediated recognition of centrally located carbohydrate moieties in glycopeptides was proposed in an early review[44] and experimentally supported in a study of a mouse hemoglobin-derived neoglycopeptide.[45, 46]

Recent studies of how peptides are bound by H-2A^q,[36] in combination with the present observations on the specific recognition of the galactose moiety of **25**, provide experimental support for a model of how the T-cell receptor recognizes the complex between H-2A^q and **25** (Figure 2). Single alanine substitutions revealed that Ile260 and Phe263 are required for binding of **25** into the groove of H-2A^q, and modeling placed these residues in the P1 and P4 binding pockets of H-2A^q.[36] As a consequence, the galactosylated Hyl264 is found in the P5 position, that is, facing up towards the T-cell receptor from the center of the glycopeptide – H-2A^q complex. Interestingly, the two reported structures of complexes between a T-cell receptor, a peptide, and a class II MHC molecule both reveal that the P5 peptide residue makes contacts with the variable CDR3 loops in the center of the T-cell receptor.[7, 9] In these complexes, the P5 residues are also known to be critical for recognition and T-cell signaling in the same way as the galactosylated Hyl264 in **25**.

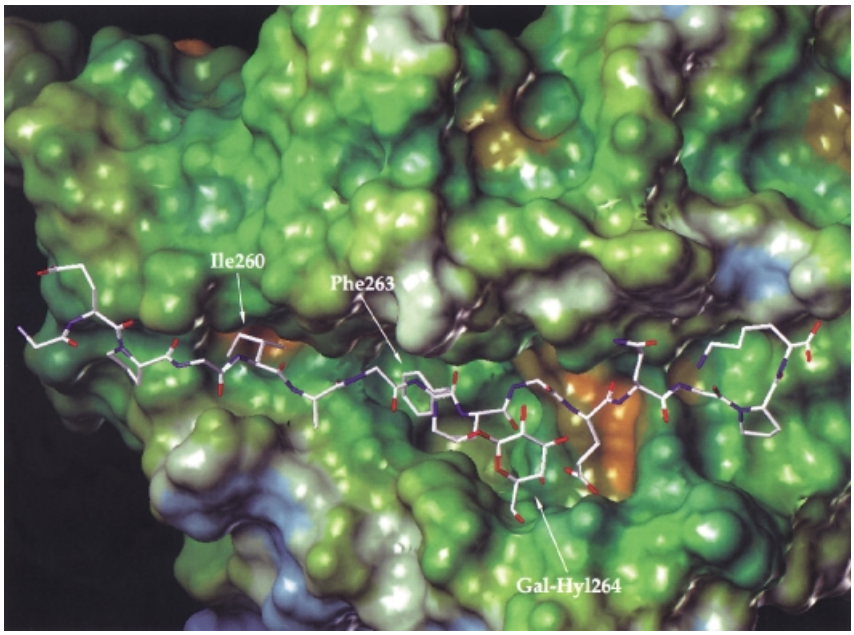
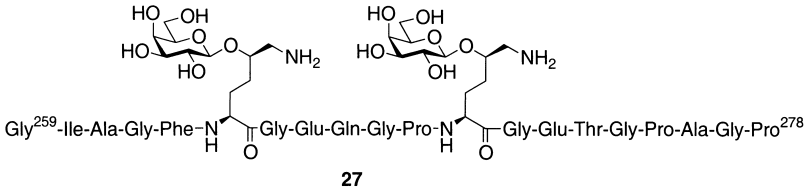


*Figure 2. Model of the complex between the class II MHC molecule H-2A^q and the immunodominant glycopeptide **25** from type II collagen, in which hydroxylysine 264 carries a β-D-galactopyranosyl moiety. The β-D-galactopyranosyl hydroxylysine residue is seen extending out of the peptide binding groove of H-2A^q in a way which allows specific interactions with the T-cell receptor. The side chains of isoleucine 260 and phenylalanine 263 anchor the glycopeptide in the P1 and P4 pockets of H-2A^q. Polar parts of the surface of H-2A^q are in blue, nonpolar parts are brown, while green represents intermediate polarity. The figure was generated with the program Sybyl.*

Specificity of T-cell hybridomas obtained in transgenic mice

The immune response towards type II collagen was recently investigated in a "humanized" mouse model, that is, in mice which are transgenic for the human DR4 class II MHC molecule and the human CD4 co-receptor.[47] It was found that the response of the polyclonal helper T cell population in these mice was dominated by reactivities towards the peptide CII259–273 with lysine residues at positions 264 and 270, and the corresponding monoglycosylated peptides with β-D-galactopyranosyl hydroxylysine residues at either position 264 or 270. To further investigate the glycopeptide-specific part of the T-cell response, the transgenic mice were immunized with bisgalactosylated peptide **27** (Figure 3),[19] which has a well-defined glycosylation pattern in contrast to type II collagen. Ten days later the mice were sacrificed, the lymph nodes were taken out, and helper-T-cell hybridomas were prepared by fusion with a T-cell-receptor-negative cell line. Altogether, this strategy gave ten helper-T-cell hybridomas that responded to glycopeptide **27** when used as immunogen without showing cross-reactivity to the non-glycosylated peptide, thereby confirming that glycosylation was required for the recognition. Eight out of the ten hybridomas also responded well to glycopeptide **26**, which has the galactosyl residue at Hyl264. The specificity of these hybridomas for the galactosyl residue was investigated in greater detail by incubation with monodeoxygenated glycopeptides **20**–**22** and antigen-presenting spleen cells, as described above for the H-2A^q-restricted hybridomas. This process allowed division of the eight hybridomas into two groups based on their fine specificity for



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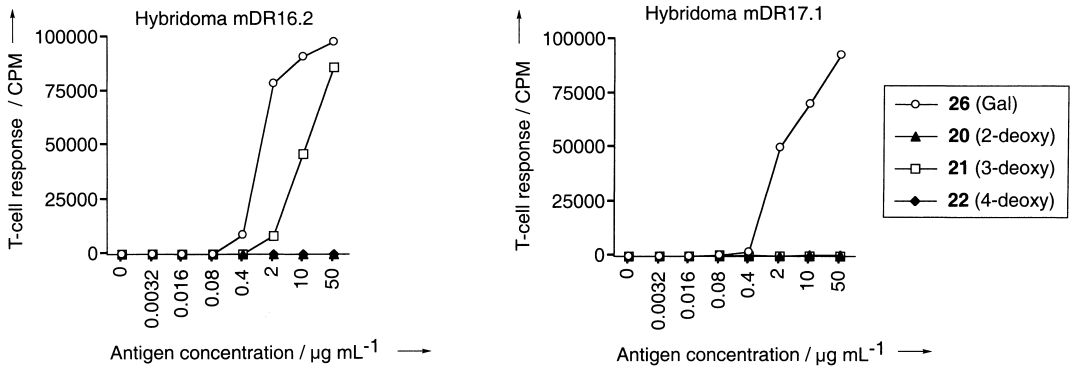

*Figure 3. Glycopeptide **27**, which corresponds to residues 259–278 from type II collagen, was used for the immunization of mice that were transgenic for the human DR4 class II MHC molecule. The graphs show the response of two T-cell hybridomas selected from each of the two groups obtained in the immunization with **27**. The hybridomas were incubated with antigen-presenting spleen cells and increasing concentrations of glycopeptides **20**–**22** and **26**. The assay is described in the legend of Figure 1.*

the galactosyl moiety (Table 2). The five hybridomas belonging to the first group did not respond to glycopeptides **20** (2-deoxy) and **22** (4-deoxy), thus revealing a strong dependence on the hydroxy groups at C-2 and C-4 (see hybridoma mDR16.2 in Figure 3). Removal of the hydroxy group at C-3 (glycopeptide **21**) led to a slight reduction of the response of these hybridomas. The three hybridomas in the second group were found to depend strongly on all three hydroxy groups, that is, HO-2, HO-3, and HO-4 (see hybridoma mDR17.1 in Figure 3).

Table 2. *Response of DR4-restricted T-cell hybridomas when incubated with antigen-presenting spleen cells and increasing concentrations of glycopeptides **20** – **22** and **26**.*[a]

	26 (Gal)	**20** (2-deoxy)	**21** (3-deoxy)	**22** (4-deoxy)
Group 1				
mDR-1.1	+++++	–	++++	–
mDR-2.2	++++	–	+++	–
mDR-8.4	++++	–	++++	–
mDR-14.1	+++++	–	++++	–
mDR-16.2	++++	–	+++	–
Group 2				
mDR-6.3	+++	–	–	–
mDR-15.2	++	–	–	–
mDR-17.1	+++++	–	–	–

[a] The magnitude of the response was determined from the concentration of antigen required to induce a proliferation of the IL-2-dependent T-cell clone CTLL that was 10 times greater than the background that is, than without antigen): – = no response or [Ag] $>50\ \mu g\,mL^{-1}$; + = $50\ \mu g\,mL^{-1}$; ++ = $10\ \mu g\,mL^{-1}$; +++ = $2\ \mu g\,mL^{-1}$; ++++ = $0.4\ \mu g\,mL^{-1}$; +++++ = $0.08\ \mu g\,mL^{-1}$; ++++++ = $0.016\ \mu g\,mL^{-1}$.

Single alanine substitutions revealed that Phe263 serves as the only crucial anchor residue when the CII259 – 273 peptide binds into the groove of DR4 class II MHC molecules.[16, 48] In addition, Lys264 and Glu266 were found to make less important contributions in binding to DR4. Our findings with deoxygenated glycopeptides **20** – **22** reveal that the T-cell receptor recognizes the galactosyl moiety attached to Hyl264 in glycopeptide **26** in a highly specific manner. Altogether, these observations support a model in which Phe263 is located in the P1 pocket of DR4, whereas Hyl264 extends towards the T-cell receptor and allows specific recognition of the galactosyl moiety (Figure 4). This model is closely related to one of the crystal structures of a complex between a T-cell receptor, a peptide, and a class II MHC molecule,[7] in which the P2 residue of the complexed peptide is specifically recognized by the CDR1 and CDR3 loops of the T-cell receptor.

The finding that glycopeptides from type II collagen are recognized in a highly specific manner by T-cell hybridomas obtained in DR4 transgenic mice has implications for our understanding of how rheumatoid arthritis may develop in humans. Studies in rheumatoid arthritis patients have in general failed to identify DR4-restricted helper T cells specific for the peptide epitope CII259 – 273.[16, 17, 37] One potential explanation for this problem is that these studies did not address the possibility of post-translational hydroxylation and glycosylation of the two lysine residues found at positions 264 and 270 in

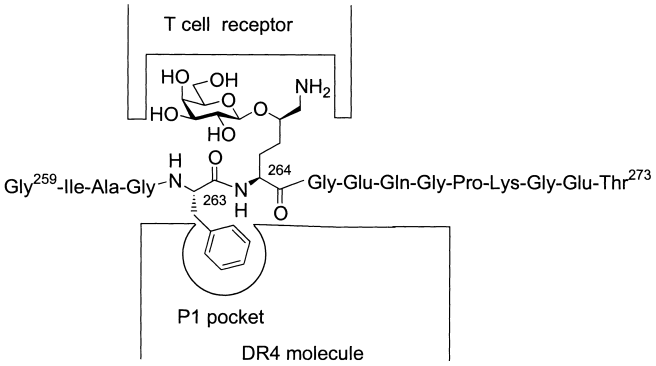

Figure 4. *A schematic model of the ternary complex of glycopeptide **26**, the DR4 class II MHC molecule, and a T-cell receptor. The side chain of phenylalanine 263 anchors **26** in the P1 pocket of DR4. Galactosylated hydroxylysine 264 is bound by the T-cell receptor in agreement with the finding that the hydroxy groups at C-2, C-3, and C-4 are required for proper stimulation of the T-cell hybridomas obtained in DR4 transgenic mice.*

CII259 – 273. The results presented in this work suggest the possibility that a T-cell-mediated autoimmune response towards glycopeptide fragments from type II collagen from joints could play an important role in disease development in humans. If correct, this could open possibilities for the development of treatments for rheumatoid arthritis patients based on glycopeptides or their analogues. It is known that structural modifications of amino acid side chains that contact the T-cell receptor can have a dramatic influence on the response of the T cell, which ranges from induction of selective stimulatory functions to completely turning off the functional capacity of the cell.[49–51] Peptides in which T-cell receptor contact sites have been manipulated, but which retain the capacity to activate some functions of the T cell have been termed altered peptide ligands (APLs).[49] Promising results in using APLs for immunotherapy in autoimmune disease have already been obtained in animal models of experimental autoimmune encephalomyelitis.[52, 53] Since all glycopeptide-specific T-cell hybridomas investigated in this study show recognition of HO-4 of the galactose moiety, modification of this position, for instance by replacement of the hydroxy group by fluorine, appears interesting for attempts to generate APLs.

Conclusions

Five protected analogues of β-D-galactosyl-(5*R*)-5-hydroxy-L-lysine were prepared in which the galactosyl moiety has been modified by sequential monodeoxygenation or inversion of the stereochemistry at C-4. The five glycosylated building blocks were then employed in solid-phase synthesis of derivatives of the peptide fragments CII256 – 270 and CII259 – 273 from type II collagen (CII). The resulting glycopeptides were used to evaluate the specificity of panels of T-cell hybridomas that occur in two variants of collagen-induced arthritis, that is, in mouse models for rheumatoid arthritis in which disease is induced by immunization with the glycoprotein type II collagen. The first panel of hybridomas were obtained in mice in which peptide epitopes were presented to T cells by H-2A^q class II MHC

molecules on the surface of antigen-presenting immune cells. These hybridomas, which have previously been shown to recognize the glycopeptide CII256 – 270 in which Hyl264 carries a β-D-galactosyl residue, were now found to recognize the galactosyl moiety with high specificity for the individual hydroxy groups. The second group of hybridomas was obtained in a "humanized" mouse model, in which the mice were transgenic for the human, arthritis-associated DR4 class II MHC molecule and the human CD4 T-cell co-receptor. T cells from this transgenic mouse recognized the glycopeptide CII259 – 273 with a β-D-galactosyl moiety attached to Hyl264, but not the corresponding nonglycosylated peptide; this again reveals the importance of post-translational glycosylation for the cellular immune response. Moreover, the DR4-restricted T-cell hybridomas were also found to recognize the galactosyl moiety with high fine specificity. The results presented in this work allowed construction of models of how the T-cell receptor interacts with complexes between glycopeptides and class II MHC molecules—a key interaction in the pathway to rheumatoid arthritis. In addition, the results suggest the possibility that a T-cell-mediated autoimmune response towards glycopeptide fragments from type II collagen found in joints could also play an important role in disease development in humans.

Experimental Section

General: TLC was performed on silica gel 60 F_{254} (Merck) with detection by UV light, charring with 10% aqueous sulfuric acid, or by treatment with phosphomolybdic acid and ceric sulfate in 6% aqueous sulfuric acid followed by heating. Flash column chromatography was performed on silica gel (Matrex, 60 Å, 35 – 70 μm, Grace Amicon) with solvents of HPLC grade, analytical grade, or distilled technical grade. Dimethyl formamide (DMF) was distilled and then dried over 3-Å molecular sieves. Dry THF and CH_2Cl_2 were obtained by distilling from potassium and CaH_2, respectively.

The ^{1}H and ^{13}C NMR spectra of compounds **3**, **4**, **7**, **8**, **11**, **12**, **14**, **15**, **18**, and **19** were recorded on a Bruker DRX-400 spectrometer at 400 MHz and 100 MHz, respectively. Chemical shifts are referenced to residual $CHCl_3$ ($\delta_H = 7.27$ ppm) and $CDCl_3$ ($\delta_C = 77.0$ ppm) for solutions in $CDCl_3$ or to CD_2HOD ($\delta_H = 3.31$ ppm) and CD_3OD ($\delta_C = 49.0$ ppm) for solutions in a 1:1 mixture of $CDCl_3$ and CD_3OD. For these compounds, resonances for aromatic protons and protons that could not be assigned are omitted. The ^{1}H NMR spectra of compounds **3**, **4**, **7**, **8**, **11**, **12**, **14**, **15**, **18**, and **19** all contained broad, minor peaks. These have previously been shown to be caused by the existence of rotamers about the amide bond in the Fmoc urethane.[54, 55] Spectra for glycopeptides **20** – **22** and **24** were recorded on a Bruker AMX-500 spectrometer in H_2O/D_2O (9:1) at 278 K and pH 5.4. Spectra for glycopeptide **23** were recorded on a Bruker DRX-600 spectrometer at 600 MHz in phosphate buffer (40 mM) containing NaN_3 (1.5 mM) and D_2O (10%) at 278 K at pH 6.2. H_2O ($\delta_H = 4.98$ ppm) was used as internal standard for **20** – **24**. First-order chemical shifts and coupling constants were determined from one-dimensional spectra and resonances were assigned from appropriate combinations of COSY, TOCSY, ^{1}H – ^{13}C-HSQC, ^{1}H – ^{15}N-HSQC, ^{1}H – ^{13}C-HSQC-TOCSY, DEPT, and NOESY experiments. Optical rotations were recorded on a Perkin – Elmer 343 polarimeter.

Analytical reversed-phase HPLC was performed on a Kromasil C-8 column (25 × 4.6 mm, 5 μm, 100 Å), eluted with a linear gradient of MeCN (0 → 100% over 60 min) in H_2O, both of which contained TFA (0.1%). A flow rate of 1.5 mL min^{-1} was used and detection was at 214 nm. Preparative reversed-phase HPLC was performed on a Kromasil C-8 column (250 × 20 mm, 5μm, 100 Å), with the same eluent, a flow rate of 11 mL min^{-1}, and detection at 214 nm. Analytical normal-phase HPLC was performed on a Kromasil silica column (25 × 4.6 mm, 5μm, 100 Å), eluted with a linear gradient of MeCN (0 → 30% over 60 min) in CH_2Cl_2, a flow rate of 2 mL min^{-1}, and detection at 254 nm.

Silver silicate was prepared as previously reported.[32] Fmoc-Hyl(Boc)-OBzl and Fmoc-Hyl(Cbz)-OAll were prepared as described elsewhere.[19, 24] 1,2,3,4-Tetra-*O*-acetyl-D-fucopyranose (**1**),[56] 1,2,3,6-tetra-*O*-acetyl-4-deoxy-D-*xylo*-hexopyranose (**5**),[57] 1,2,4,6-tetra-*O*-acetyl-3-deoxy-D-*xylo*-hexopyranose (**9**),[58] and 2,3,4,6-tetra-*O*-acetyl-α-D-glucopyranosyl bromide (**13**)[59] were prepared essentially as described in the cited references. 3,4,6-Tri-*O*-benzoyl-D-galactal (**16**) is commercially available.

Bromosugars **2**, **6**, and **10** were prepared by treatment of the corresponding 1-*O*-acyl sugars with HBr (15%) in HOAc/Ac_2O (6:1) for 2 – 3 h. The solutions were diluted with CH_2Cl_2, washed with water, saturated aqueous $NaHCO_3$, and water, dried (Na_2SO_4), filtered, and concentrated. The crude products were analyzed with ^{1}H NMR spectroscopy and were then used without further purification.

The yields for glycopeptides **20** – **24** have been corrected with regard to the peptide content as determined by quantitative amino acid analysis. Glycopeptides **25** – **27** were prepared as described previously.[19]

(5*R*)-*N*$^{\alpha}$-(Fluoren-9-ylmethoxycarbonyl)-*N*$^{\varepsilon}$-(*tert*-butoxycarbonyl)-5-*O*-(2,3,4-tri-*O*-acetyl-β-D-fucopyranosyl)-5-hydroxy-L-lysine benzyl ester (3): A solution of **2** (228 mg, 0.6 mmol) in CH_2Cl_2 (4 mL) was added dropwise to a stirred mixture of silver silicate (600 mg), crushed 3-Å molecular sieves (600 mg), and Fmoc-Hyl(Boc)-OBzl (230 mg, 0.40 mmol) in dry CH_2Cl_2 (7 mL) at $-10\,^{\circ}C$ under N_2 in the absence of light. After stirring for 50 min, the temperature was raised to $0\,^{\circ}C$. After stirring for an additional 5 h, Et_3N (150 μL, 1.1 mmol) was added, and the mixture was allowed to warm to room temperature. The solid material was filtered off, washed with CH_2Cl_2 (50 mL), and the combined filtrates were concentrated. Flash column chromatography of the residue (toluene/MeCN 5:1) gave **3** (199 mg, 60%). $[\alpha]_D^{20} = -10$ ($c = 1.0$, $CHCl_3$); ^{1}H NMR ($CDCl_3$): $\delta = 5.55$ (m, 1H; NH-ε), 5.42 (d, 1H, $J = 8.0$ Hz; NH-α), 5.24 (d, 1H, $J = 3.1$ Hz; H-4), 5.19 (s, 2H; PhCH_2O), 5.15 (dd, 1H, $J = 10.5$, 8.0 Hz; H-2), 5.00 (dd, 1H, $J = 10.5$, 3.5 Hz; H-3), 4.48 – 4.30 (m, 4H; H-1, H-α, OCOCH_2CH), 4.21 (t, 1H, $J = 6.8$ Hz; OCOCH_2C*H*), 3.81 (q, 1H, $J = 6.4$ Hz; H-5), 3.59 – 3.50 (m, 1H; H-δ), 3.39 – 3.27 (m, 1H; H-ε), 3.07 – 2.97 (m, 1H; H-ε), 2.19, 1.99, 1.96 (3s, 3 × 3H; 3CH_3CO), 2.01 – 1.90 (m, 1H; H-β), 1.76 – 1.38 (m, 3H; H-β, H-γ,γ), 1.44 (s, 9H; C$(CH_3)_3$), 1.25 (d, 3H, $J = 6.4$ Hz; H-6) ppm; ^{13}C NMR ($CDCl_3$): $\delta = 171.9$, 170.7, 170.6, 169.5, 156.1, 155.9, 143.8, 143.7, 141.3, 135.0, 128.7, 128.6, 128.4, 127.7, 127.1, 127.1, 125.0, 120.0, 101.3, 81.4, 79.1, 71.2, 70.1, 69.3, 69.0, 67.4, 67.1, 53.7, 47.1, 44.2, 28.7, 28.4, 20.6, 20.6, 20.6, 15.9 ppm; HRMS (FAB): *m/z* calcd for $C_{45}H_{54}N_2NaO_{14}$: 869.3473 [*M*+Na]$^+$; found: 869.3465.

(5*R*)-*N*$^{\alpha}$-(Fluoren-9-ylmethoxycarbonyl)-*N*$^{\varepsilon}$-(*tert*-butoxycarbonyl)-5-*O*-(2,3,4-tri-*O*-acetyl-β-D-fucopyranosyl)-5-hydroxy-L-lysine (4): A solution of **3** (115 mg, 0.14 mmol) in EtOAc (10 mL) was hydrogenated at atmospheric pressure over 10% Pd/C (115 mg) for 8.5 h. The mixture was filtered (Hyflo-Supercel), the solid material was washed with EtOAc, and the combined filtrates were concentrated. Flash column chromatography of the residue (toluene/EtOH 10:1 → 3:1) gave **4** (91 mg, 88%) as a white amorphous solid. $[\alpha]_D^{20} = -2.2$ ($c = 0.6$, $CHCl_3$); ^{1}H NMR ($CDCl_3$): $\delta = 5.80 - 5.70$ (m, 2H; NH-α, NH-ε), 5.22 (d, 1H, $J = 3.1$ Hz; H-4), 5.15 (dd, 1H, $J = 10.5$,

7.9 Hz; H-2), 4.99 (dd, 1H, $J = 10.5$, 3.4 Hz; H-3), 4.45 – 4.30 (m, 4H; H-1, H-α, OCOCH_2CH), 4.18 (t, 1H, $J = 7.1$ Hz; OCOCH$_2$CH), 3.83 – 3.75 (m, 1H; H-5), 3.62 – 3.54 (m, 1H; H-δ), 3.47 – 3.35 (m, 1H; H-ε), 3.15 – 2.96 (m, 1H; H-ε), 2.17, 2.01, 1.97 (3s, 3 × 3H; CH_3CO), 2.01 – 1.95 (m, 1H; H-β), 1.78 – 1.40 (m, 3H; H-β, H-γ,γ), 1.43 (s, 9H, C(CH_3)$_3$), 1.22 (d, 3H, $J = 6.3$ Hz, H-6) ppm; ^{13}C NMR ($CDCl_3$): $\delta = 174.5$, 170.6, 170.2, 169.8, 156.6, 156.3, 143.8, 143.6, 141.2, 127.7, 127.1, 127.1, 125.1, 119.9, 101.4, 81.6, 79.8, 71.2, 70.1, 69.2, 69.1, 67.1, 53.4, 47.0, 44.3, 28.7, 28.4, 28.1, 20.7, 20.6, 20.6, 15.9 ppm; HRMS (FAB): m/z calcd for $C_{38}H_{47}N_2Na_2O_{14}$: 801.2816 $[M - H+2Na]^+$; found: 801.2816.

(5*R*)-*N*$^{\alpha}$-(Fluoren-9-ylmethoxycarbonyl)-*N*$^{\varepsilon}$-benzyloxycarbonyl-5-*O*-(2,3,6-tri-*O*-acetyl-4-deoxy-β-D-*xylo*-hexopyranosyl)-5-hydroxy-L-lysine allyl ester (7): Bromosugar **6** (211 mg, 0.6 mmol) was reacted with Fmoc-Hyl(Cbz)-OAll (223 mg, 0.4 mmol) as described for **3** at 0 °C for 2 h to give **7** (285 mg, 86%) as a white amorphous solid, after purification by flash column chromatography (toluene/MeCN 5:1). $[\alpha]_D^{20} = -2.9$ ($c = 1.0$, $CHCl_3$); ^{1}H NMR ($CDCl_3$): $\delta = 5.98 – 5.84$ (m, 1H; CH=CH$_2$), 5.71 (br t, 1H, $J = 5.4$ Hz; NH-ε), 5.44 (d, 1H, $J = 8.5$ Hz; NH-α), 5.34 (d, 1H, $J = 17.2$ Hz; OCH$_2$CH=CH_{2trans}), 5.27 (d, 1H, $J = 10.4$ Hz; OCH$_2$CH=CH_{2cis}), 5.10 (s, 2H; PhCH_2O), 4.96 (dt, 1H, $J = 10.5$, 5.3 Hz; H-3), 4.86 (dd, 1H, $J = 9.6$, 7.8 Hz; H-2), 4.70 – 4.60 (m, 2H; OCH_2CH=CH$_2$), 4.49 – 4.29 (m, 4H; OCOCH_2CH, H-1, H-α), 4.23 (t, 1H, $J = 6.9$ Hz; OCOCH$_2$CH), 4.04 (dd, 1H, $J = 11.7$, 3.7 Hz; H-6), 4.04 (dd, 1H, $J = 11.0$, 6.4 Hz; H-6), 3.86 – 3.78 (m, 1H; H-5), 3.72 – 3.62 (m, 1H; H-δ), 3.48 – 3.37 (m, 1H; H-ε), 3.25 – 3.13 (m, 1H; H-ε), 2.14 – 2.06 (m, 1H; H-4$_{eq}$), 2.04 – 1.94 (m, 1H; H-β), 2.03, 2.01, 1.97 (3s, 3 × 3H; CH_3CO), 1.72 – 1.52 (m, 4H; H-β, H-γ,γ, H-4$_{ax}$) ppm; ^{13}C NMR ($CDCl_3$): $\delta = 171.8$, 170.5, 170.2, 169.6, 156.7, 155.9, 143.8, 143.7, 141.2, 136.5, 131.3, 128.5, 128.1, 128.1, 127.7, 127.1, 127.0, 125.0, 101.1, 80.7, 72.2, 70.4, 69.5, 67.0, 66.6, 66.1, 65.1, 53.7, 47.0, 44.9, 32.3, 28.8, 28.3, 20.9, 20.7, 20.5 ppm; HRMS (FAB): m/z calcd for $C_{44}H_{50}N_2NaO_{14}$: 853.3160 $[M+Na]^+$; found: 853.3177.

(5*R*)-*N*$^{\alpha}$-(Fluoren-9-ylmethoxycarbonyl)-*N*$^{\varepsilon}$-benzyloxycarbonyl-5-*O*-(2,3,6-tri-*O*-acetyl-4-deoxy-β-D-*xylo*-hexopyranosyl)-5-hydroxy-L-lysine (8): $(Ph_3P)_4Pd$ (33 mg, 30 μmol) was added to a stirred solution of **7** (236 mg, 0.28 mmol) and *N*-methyl aniline (93 μL, 0.88 mmol) in dry THF (6 mL) under N_2 in the absence of light. After stirring for 1.5 h at room temperature, the solution was diluted with EtOAc and washed with saturated aqueous NH_4Cl, dried (Na_2SO_4), filtered, and concentrated. Flash column chromatography of the residue (toluene/EtOH 50:1 →5:1) gave **8** (183 mg, 82%) as a white amorphous solid. $[\alpha]_D^{20} = -4.3$ ($c = 1.0$, $CHCl_3$); ^{1}H NMR ($CDCl_3$/CD_3OD 1:1): $\delta = 5.04$ (s, 2H; PhCH_2O), 4.99 – 4.91 (m, 1H; H-3), 4.78 (dd, 1H, $J = 9.4$, 8.0 Hz; H-2), 4.48 (d, 1H, $J = 7.9$ Hz; H-1), 4.33 (d, 2H, $J = 7.1$ Hz; OCOCH_2CH), 4.18 (t, 1H, $J = 6.9$ Hz; OCOCH$_2$CH), 4.15 – 4.06 (m, 2H; H-6, H-α), 4.02 (dd, 1H, $J = 11.8$, 6.3 Hz; H-6), 3.77 – 3.67 (m, 1H; H-5), 3.66 – 3.56 (m, 1H; H-δ), 3.35 – 3.25 (m, 1H; H-ε), 3.17 – 3.06 (m, 1H; H-ε), 2.09 – 1.96 (m, 2H; H-4$_{eq}$, H-β), 2.00, 1.98, 1.93 (3s, 3 × 3H; 3CH_3CO), 1.71 – 1.46 (m, 4H; H-γ,γ, H-β, H-4$_{ax}$) ppm; ^{13}C NMR ($CDCl_3$/CD_3OD 1:1): $\delta = 175.8$, 171.8, 171.3, 171.1, 157.9, 157.7, 144.6, 144.4, 141.9, 137.2, 137.0, 129.4, 129.0, 128.9, 128.7, 128.6, 128.3, 127.7, 127.5, 125.7, 125.4, 120.5, 101.8, 81.3, 73.2, 71.3, 70.0, 67.4, 67.2, 65.9, 54.8, 45.7, 32.8, 29.9, 28.6, 21.0, 20.9, 20.7 ppm; HRMS (FAB): m/z calcd for $C_{41}H_{46}N_2NaO_{14}$: 813.2847 $[M+Na]^+$; found: 813.2827.

(5*R*)-*N*$^{\alpha}$-(Fluoren-9-ylmethoxycarbonyl)-*N*$^{\varepsilon}$-benzyloxycarbonyl-5-*O*-(2,4,6-tri-*O*-acetyl-3-deoxy-β-D-*xylo*-hexopyranosyl)-5-hydroxy-L-lysine allyl ester (11): Bromosugar **10** (136 mg, 0.4 mmol) was reacted with Fmoc-Hyl(Cbz)-OAll (145 mg, 0.26 mmol) at 0 °C for 2 h as described for **3** to give **11** (176 mg, 82%) as a white amorphous solid, after purification by flash column chromatography (toluene/MeCN 5:1). $[\alpha]_D^{20} = -12$ ($c = 1.0$, $CHCl_3$); ^{1}H NMR ($CDCl_3$): $\delta = 5.99 – 5.85$ (m, 1H; CH=CH$_2$), 5.71 – 5.61 (m, 1H; NH-ε), 5.43 (d, 1H, $J = 6.7$ Hz; NH-α), 5.35 (d, 1H, $J = 16.9$ Hz; OCH$_2$CH=CH_{2trans}), 5.28 (dd, 1H, $J = 10.4$, 0.7 Hz; OCH$_2$CH=CH_{2cis}), 5.11, 5.10 (ABd, 2 × 1H, $J = 13.8$ Hz; PhCH_2O), 5.05 – 5.01 (m, 1H; H-4), 4.88 (ddd, 1H, $J = 11.8$, 8.0, 5.2 Hz; H-2), 4.69 – 4.61 (m, 2H; OCH_2CH=CH$_2$), 4.51 – 4.31 (m, 4H; OCOCH_2CH, H-1, H-α), 4.22 (t, 1H, $J = 6.7$ Hz; OCOCH$_2$CH), 4.16 (dd, 1H, $J = 11.5$, 5.4 Hz; H-6), 4.06 (dd, 1H, $J = 11.4$, 7.5 Hz; H-6), 3.86 – 3.78 (m, 1H; H-5), 3.72 – 3.62 (m, 1H; H-δ), 3.48 – 3.37 (m, 1H; H-ε), 3.25 – 3.13 (m, 1H; H-ε), 2.35 (ddd, 1H, $J = 14.2$, 5.2, 3.1 Hz; H-3$_{eq}$), 2.11 (s, 3H; CH_3CO), 2.05 – 1.95 (m, 1H; H-β), 1.97 (s, 6H; 2CH_3CO), 1.83 – 1.40 (m, 4H; H-β, H-γ,γ, H-3$_{ax}$) ppm; ^{13}C NMR ($CDCl_3$): $\delta = 171.8$, 170.4, 170.1, 169.5, 156.7, 155.9, 143.8, 143.7, 141.3, 136.5, 131.3, 128.5, 128.2, 128.1, 127.7, 127.0, 124.9, 120.0, 119.2, 102.6, 80.4, 74.2, 67.6, 66.9, 66.8, 66.7, 66.1, 62.4, 53.8, 47.1, 45.0, 32.8, 28.9, 28.2, 20.9, 20.8, 20.5 ppm; HRMS(FAB): m/z calcd for $C_{44}H_{50}N_2NaO_{14}$: 853.3160 $[M+Na]^+$; found: 853.3171.

(5*R*)-*N*$^{\alpha}$-(Fluoren-9-ylmethoxycarbonyl)-*N*$^{\varepsilon}$-benzyloxycarbonyl-5-*O*-(2,4,6-tri-*O*-acetyl-3-deoxy-β-D-*xylo*-hexopyranosyl)-5-hydroxy-L-lysine (12): Compound **11** (124 mg, 0.15 mmol) was reacted as described for **8** for 1.25 h. The crude product was purified by flash column chromatography (toluene/EtOH 50:1 →10:1 →5:1) to give **12** (99 mg, 84%) as a white amorphous solid. $[\alpha]_D^{20} = 0$ ($c = 1$, $CHCl_3$); ^{1}H NMR ($CDCl_3$/CD_3OD 1:1): $\delta = 5.05$ (s, 2H; PhCH_2O), 5.02 – 4.98 (m, 1H; H-4), 4.81 (ddd, $J = 12.0$, 8.0, 5.2 Hz, 1H; H-2), 4.51 (d, 1H, $J = 8.0$ Hz; H-1), 4.35 (d, 2H, $J = 7.0$ Hz; OCOCH_2CH), 4.18 (t, 1H, $J = 7.0$ Hz; OCOCH$_2$CH), 4.15 – 4.07 (m, 2H; H-6, H-α), 4.00 (dd, 1H, $J = 11.3$, 7.0 Hz; H-6), 3.89 (t, 1H, $J = 6.3$ Hz; H-5), 3.71 – 3.61 (m, 1H; H-δ), 3.34 – 3.18 (m, 1H; H-ε), 3.20 – 3.10 (m, 1H; H-ε), 2.28 (ddd, 1H, $J = 14.0$, 5.0, 3.1 Hz; H-3$_{eq}$), 2.06, 1.97, 1.93 (3s, 3 × 3H; 3CH_3CO), 1.99 – 1.92 (m, 1H; H-β), 1.74 – 1.52 (m, 4H; H-γ,γ, H-β, H-3$_{ax}$) ppm; ^{13}C NMR ($CDCl_3$/CD_3OD 1:1): $\delta = 176.5$, 171.7, 171.3, 171.2, 157.9, 157.6, 129.1, 128.8, 128.7, 128.6, 128.3, 127.7, 125.6, 120.5, 103.3, 81.2, 74.7, 68.4, 68.0, 67.4, 67.3, 63.0, 55.1, 47.8, 45.7, 33.2, 29.9, 28.7, 21.0, 20.7 ppm; HRMS (FAB): m/z calcd for $C_{41}H_{45}N_2Na_2O_{14}$: 835.2666 $[M - H+2Na]^+$; found: 835.2667.

(5*R*)-*N*$^{\alpha}$-(Fluoren-9-ylmethoxycarbonyl)-*N*$^{\varepsilon}$-benzyloxycarbonyl-5-*O*-(2,3,4,6-tetra-*O*-acetyl-β-D-glucopyranosyl)-5-hydroxy-L-lysine allyl ester (14): Acetobromoglucose **13** (141 mg, 0.34 mmol) was treated with Fmoc-Hyl(Cbz)-OAll (107 mg, 0.19 mmol) at 0 °C for 1 h and then at 10 °C for 8 h as described for **3**. The crude product was purified by flash column chromatography (toluene/MeCN 6:1, toluene/MeCN 8:1, and toluene/EtOH 20:1) to give **14** (70 mg, 41%, > 97% pure by ^{1}H NMR spectroscopy) as a white amorphous solid, which was used in the next step without further purification. ^{1}H NMR ($CDCl_3$): $\delta = 5.96 – 5.84$ (m, 1H; CH=CH$_2$), 5.62 (m, 1H; NH-ε), 5.42 (d, 1H, $J = 7.9$ Hz; NH-α), 5.34 (d, 1H, $J = 17.2$ Hz; OCH$_2$CH=CH_{2trans}), 5.34 (d, 1H, $J = 10.3$ Hz; OCH$_2$CH=CH_{2cis}), 5.16 (t, 1H, $J = 9.5$ Hz; H-3), 5.08 (s, 2H; PhCH_2O), 5.03 (t, 1H, $J = 9.7$ Hz; H-4), 4.95 (dd, 1H, $J = 9.6$, 8.2 Hz; H-2), 4.66 – 4.60 (m, 2H; OCH_2CH=CH$_2$), 4.50 (d, 1H, $J = 7.9$ Hz; H-1), 4.47 – 4.29 (m, 3H; OCOCH_2CH, H-α), 4.22 (t, 1H, $J = 6.7$ Hz; OCOCH$_2$CH), 4.18 – 4.05 (m, 2H; H-6), 3.69 – 3.58 (m, 2H; H-5, H-δ), 3.46 – 3.11 (m, 2H; H-ε,ε), 2.03 – 1.95 (m, 1H; H-β), 2.02 (s, 3H; CH_3CO), 1.99 (s, 6H; 2CH_3CO), 1.96 (s, 3H; CH_3CO), 1.80 – 1.76 (m, 3H; H-β, H-γ,γ) ppm; ^{13}C NMR ($CDCl_3$): $\delta = 171.7$, 170.5, 170.1, 169.3, 169.2, 156.7, 155.9, 143.7, 143.6, 141.3, 136.5, 131.3, 128.5, 128.1, 127.7, 127.0, 125.0, 120.0, 119.3, 100.8, 81.0, 72.6, 71.7, 71.3, 68.1, 67.0, 66.7, 61.7, 47.1, 44.8, 28.7, 28.4, 20.5 ppm.

(5*R*)-*N*$^{\alpha}$-(Fluoren-9-ylmethoxycarbonyl)-*N*$^{\varepsilon}$-benzyloxycarbonyl-5-*O*-(2,3,4,6-tetra-*O*-acetyl-β-D-glucopyranosyl)-5-hydroxy-L-lysine (15): Allyl ester **14** (55 mg, 64 μmol) was treated as described for **8**. The crude product was purified twice by flash column chromatography (toluene/EtOH 10:1 →4:1) to give **15** (42 mg, 84%) as a white amorphous solid. $[\alpha]_D^{20} = -11.7$ ($c = 1$,$CHCl_3$); ^{1}H NMR ($CDCl_3$/CD_3OD 1:1): $\delta = 5.12$ (t, 1H, $J = 9.6$ Hz; H-3), 5.01 (d, 2H; PhCH_2O), 4.96 (t, 1H, $J = 9.8$ Hz; H-4), 4.86 (t, 1H, $J = 8.7$ Hz; H-2), 4.52 (d, 1H, $J = 8.0$ Hz;

H-1), 4.37 – 4.23 (m, 2H; OCOCH_2CH), 4.19 – 3.97 (m, 4H; H-6,6, H-α, OCOCH$_2$CH), 3.68 – 3.56 (m, 2H; H-5, H-δ), 3.35 – 3.24 (m, 1H; H-ε), 3.13 – 3.01 (m, 1H; H-ε), 2.00 – 1.85 (m, 1H; H-β), 1.98, 1.95, 1.94, 1.90 (4s, 4 × 3H; 4CH_3CO), 1.68 – 1.45 (m, 3H; H-β, H-γ,γ) ppm; ^{13}C NMR ($CDCl_3$): δ = 178.9, 171.7, 171.0, 170.7, 170.5, 157.8, 157.5, 144.5, 144.4, 141.8, 137.0, 129.0, 128.8, 128.7, 128.5, 128.3, 127.6, 125.5, 120.5, 101.3, 81.5, 73.4, 72.1, 72.0, 67.3, 67.3, 62.3, 55.8, 47.7, 45.4, 29.5, 28.9, 20.8, 28.7 ppm; HRMS(FAB): *m/z* calcd for $C_{43}H_{48}N_2NaO_{16}$: 871.2902 [M+Na]$^+$; found: 871.2872.

(5*R*)-N^{α}-(Fluoren-9-ylmethoxycarbonyl)-N^{ε}-benzyloxycarbonyl-5-*O*-(3,4,6-tri-*O*-benzoyl-2-deoxy-β-D-*lyxo*-hexopyranosyl)-5-hydroxy-L-lysine allyl ester (18): HCl gas was bubbled through an ice-cold solution of 3,4,6-tri-*O*-benzoyl-D-galactal (**16**; 140 mg, 0.30 mmol) in dry toluene for 1 h. The solution was concentrated and the remaining traces of solvent and HCl were removed under high vacuum. The resulting crude **17** was dissolved in dry toluene (3 mL) and added dropwise to a stirred mixture of silver silicate (300 mg), crushed 3-Å molecular sieves (100 mg), and Fmoc-Hyl(Cbz)-OAll (112 mg, 0.20 mmol) in dry CH_2Cl_2 (1 mL) at $-30\,^{\circ}C$ under N_2 in the absence of light. After stirring overnight, dry CH_2Cl_2 (3 mL) was added and the temperature was raised slowly to 0 °C over 36 h. Et_3N (55 μL, 0.4 mmol) was added and the mixture was allowed to warm to room temperature. The solid material was filtered off, washed with CH_2Cl_2 (50 mL), and the combined filtrates were concentrated to give crude **18** (α/β ratio 1:10 as judged by normal-phase HPLC). Repeated flash chromatography of the residue (toluene/MeCN 6:1) gave **18** (120 mg, 59%) as a white amorphous solid. $[\alpha]_D^{20} = -7$ (c = 1, $CHCl_3$); ^{1}H NMR ($CDCl_3$): δ = 5.97 – 5.86 (m, 1H; CH=CH$_2$), 5.85 – 5.79 (m, 1H; NH-ε), 5.76 (d, 1H, J = 2.8 Hz; H-4), 5.56 (d, 1H, J = 7.9 Hz; NH-α), 5.39 – 5.31 (m, 2H; H-3, OCH$_2$CH=CH_{2trans}), 5.27 (d, 1H, J = 10.4 Hz; OCH$_2$CH=CH_{2cis}), 5.05 (s, 2H; PhCH_2O), 4.77 (br d, 1H, J = 8.4 Hz; H-1), 4.66 (d, 2H, J = 5.3 Hz; OCH_2CH=CH$_2$), 4.55 (dd, 1H, J = 11.3, 6.9 Hz; H-6), 4.48 – 4.35 (m, 4H; H-6, OCOCH_2CH, H-α), 4.23 (t, 1H, J = 7.0 Hz; OCOCH$_2$CH), 4.11 (t, 1H, J = 6.3 Hz; H-5), 3.82 – 3.74 (m, 1H; H-δ), 3.51 – 3.42 (m, 1H; H-ε), 3.32 – 3.23 (m, 1H; H-ε), 2.32 – 2.16 (m, 2H; H-2$_{ax}$, H-2$_{eq}$), 2.07 – 1.95 (m, 1H; H-β), 1.87 – 1.60 (m, 3H; H-β, H-γ,γ) ppm; ^{13}C NMR ($CDCl_3$): δ = 171.7, 166.0, 165.7, 165.3, 156.8, 155.9, 143.8, 143.7, 141.2, 136.5, 133.4, 133.2, 131.4, 129.9, 129.7, 129.7, 129.4, 129.4, 129.3, 128.6, 128.4, 128.4, 128.3, 128.1, 128.0, 127.7, 125.0, 119.9, 119.2, 100.5, 80.4, 71.7, 69.2, 67.1, 66.6, 66.1, 66.1, 62.5, 53.6, 47.1, 44.6, 32.6, 28.6, 28.5 ppm; HRMS(FAB): *m/z* calcd for $C_{59}H_{56}N_2NaO_{14}$: 1039.3629 [M+Na]$^+$; found: 1039.3640.

(5*R*)-N^{α}-(Fluoren-9-ylmethoxycarbonyl)-N^{ε}-benzyloxycarbonyl-5-*O*-(3,4,6-tri-*O*-benzoyl-2-deoxy-β-D-*lyxo*-hexopyranosyl)-5-hydroxy-L-lysine (19): Allyl ester **18** (91 mg, 89 μmol) was treated as described for **8**. The crude product was purified twice by flash chromatography (toluene/EtOH/HOAc 50:2:1 followed by CH_2Cl_2/EtOH/HOAc 200:2:1 → CH_2Cl_2/EtOH 10:1) to give **19** (79 mg, 90%) as a white amorphous solid after lyophilization. $[\alpha]_D^{20} = 0$ (c = 1, $CHCl_3$); ^{1}H NMR ($CDCl_3$/CD_3OD 1:1): δ = 6.36 (t, 1H, J = 5.3 Hz; NH-ε), 5.71 (d, 1H, J = 2.7 Hz; H-4), 5.39 – 5.31 (m, 1H; H-3), 4.98 (d, 1H, J = 12.3 Hz; PhCH_2O), 4.93 (d, 1H, J = 12.4 Hz; PhCH_2O), 4.82 (br d, 1H, J = 9.3 Hz; H-1), 4.48 (dd, 1H, J = 11.7, 7.0 Hz; H-6), 4.41 – 4.31 (m, 3H, H-6; OCOCH_2CH), 4.27 – 4.21 (m, 1H; H-α), 4.20 – 4.12 (m, 2H, H-5; OCOCH$_2$CH), 3.85 – 3.75 (m, 1H; H-δ), 3.41 – 3.32 (m, 1H; H-ε), 3.27 – 3.17 (m, 1H; H-ε), 2.28 – 2.11 (m, 2H; H-2$_{ax}$, H-2$_{eq}$), 2.05 – 1.93 (m, 1H; H-β), 1.83 – 1.72 (m, 1H; H-β), 1.70 – 1.58 (m, 2H; H-γ,γ) ppm; ^{13}C NMR ($CDCl_3$): δ = 174.8, 166.9, 166.7, 166.2, 157.8, 157.5, 144.5, 144.3, 141.8, 137.1, 134.2, 133.9, 133.1, 133.1, 132.5, 132.4, 132.3, 131.3, 130.3, 130.2, 130.1, 129.9, 129.9, 129.4, 129.4, 129.3, 129.2, 129.0, 128.9, 128.7, 128.5, 128.4, 128.2, 127.6, 125.6, 120.4, 100.9, 80.6, 72.0, 70.1, 67.5, 67.2, 67.1, 63.3, 54.3, 47.7, 33.1, 29.4, 28.5 ppm; HRMS(FAB): *m/z* calcd for $C_{56}H_{52}N_2NaO_{14}$: 999.3316 [M+Na]$^+$; found: 999.3310.

General procedure for solid-phase glycopeptide synthesis: Glycopeptides **20** – **24** were synthesized on Tentagel-S-PHB-Lys-Fmoc or Tentagel-S-PHB-Thr-Fmoc resins essentially as described elsewhere.[19] N^{α}-Fmoc amino acids with standard side-chain protecting groups were coupled by using either *O*-(benzotriazol-1-yl)-*N*,*N*,*N*′,*N*′-tetramethyluronium hexafluorophosphate (HBTU; 6 equiv) and diisopropylethylamine (DIPEA; 6 equiv), diisopropyl carbodiimide (DIC; 4 equiv), and 1-hydroxybenzotriazole (HOBt; 6 equiv), or DIC (4 equiv) and 1-hydroxy-7-azabenzotriazole (HOAt, 6 equiv) as coupling reagents. Glycosylated building blocks **4**, **8**, **12**, **15**, and **19** (1.2 – 1.5 equiv) were coupled to the peptide resins in manually agitated reactors by using DIC (1.2 – 1.5 equiv) and HOAt (3.6 – 4.5 equiv) as coupling reagents in dry DMF for 29 – 38 h. After coupling of the glycosylated building blocks, unreacted amino groups were acetylated with Ac_2O/DMF (1:1) for 1 h. Cleavage of the glycopeptides from the resin with trifluoroacetic acid/water/thioanisole/ethanedithiol (35:2:2:1) and subsequent workup were performed essentially as described elsewhere.[19]

Glycyl-L-isoleucyl-L-alanylglycyl-L-phenylalanyl-5-*O*-(2-deoxy-β-D-*lyxo*-hexopyranosyl)-5-hydroxy-L-lysylglycyl-L-glutam-1-yl-L-glutaminylglycyl-L-prolyl-L-lysylglycyl-L-glutam-1-yl-L-threonine (20): Synthesis was performed with building block **19** on resin (50 μmol) according to the general procedure. Cleavage and then purification by reversed-phase HPLC gave the 3,4,6-*O*-benzoylated glycopeptide (42.5 mg). A portion (14 mg) of this glycopeptide was treated with NaOMe in MeOH (0.2 M) for 3 h at room temperature (monitored by analytical reversed-phase HPLC). The solution was then neutralized (HOAc) and concentrated, and the residue was purified by preparative reversed-phase HPLC to give **20** (9 mg, 74% peptide content, 24% yield based on the amount of resin used). MS (ES): *m/z* calcd: 819.4 [M+2H]$^{2+}$; found: 819.3; amino acid analysis: Ala 0.99 (1), Glu 3.00 (3), Gly 4.93 (5), Hyl 1.03 (1), Ile 0.99 (1), Lys 1.03(1), Phe 1.02 (1), Pro 1.01 (1), Thr 1.00 (1). ^{1}H NMR data are given in Table 3.

Glycyl-L-isoleucyl-L-alanylglycyl-L-phenylalanyl-5-*O*-(3-deoxy-β-D-*xylo*-hexopyranosyl)-5-hydroxy-L-lysylglycyl-L-glutam-1-yl-L-glutaminylglycyl-L-prolyl-L-lysylglycyl-L-glutam-1-yl-L-threonine (21): Synthesis was performed with building block **12** on resin (50 μmol) according to the general procedure. Cleavage and then purification by reversed-phase HPLC gave the 2,4,6-*O*-acetylated glycopeptide (43.7 mg). A portion (25 mg) of this glycopeptide was treated with NaOMe in MeOH (0.02 M) for 3 h at room temperature (monitored by analytical reversed-phase HPLC). The solution was then neutralized (HOAc) and concentrated, and the residue was purified by preparative reversed-phase HPLC to give **21** (13 mg, 75% peptide content, 20% yield based on the amount of resin used). MS (ES): *m/z* calcd: 819.4 [M+2H]$^{2+}$; found: 819.3; amino acid analysis: Ala 1.01 (1), Glu 2.98 (3), Gly 4.94 (5), Hyl 1.01 (1), Ile 1.00 (1), Lys 1.00(1), Phe 1.02 (1), Pro 1.04 (1), Thr 1.01 (1). ^{1}H NMR data are given in Table 4.

Glycyl-L-isoleucyl-L-alanylglycyl-L-phenylalanyl-5-*O*-(4-deoxy-β-D-*xylo*-hexopyranosyl)-5-hydroxy-L-lysylglycyl-L-glutam-1-yl-L-glutaminylglycyl-L-prolyl-L-lysylglycyl-L-glutam-1-yl-L-threonine (22): Synthesis was performed with building block **8** on resin (50 μmol) according to the general procedure. Cleavage and then purification by preparative reversed-phase HPLC gave the 2,3,6-*O*-acetylated glycopeptide (48.2 mg). A portion of this glycopeptide (30 mg) was treated with NaOMe in MeOH (0.02 M) for 2 h 40 min at room temperature (monitored by analytical reversed-phase HPLC). The solution was then neutralized (HOAc), concentrated, and the residue was purified by preparative reversed-phase HPLC to give **22** (21.9 mg, 79% peptide content, 34% yield based on the amount of

Table 3. ^{1}H NMR chemical shifts for glycopeptide **20** in water containing 10% D_2O.[a]

Residue	NH	Hα	Hβ	Hγ	Others
Gly259		3.82[b]			
Ile260	8.59	4.18	1.82	1.43, 1.14, 0.91 (CH_3)	0.82 (Hδ)
Ala261	8.65	4.28	1.36		
Gly262	8.48	3.88[b]			
Phe263	8.21	4.57	3.05[b]		7.31 (H3,H5), 7.30 (H4), 7.23 (H2,H6)
Hyl264[c]	8.54	4.25	1.88, 1.67	1.50[b]	4.01 (Hδ), 3.14, 2.89 (Hε)
Gly265	7.89	3.84, 3.89			
Glu266	8.47	4.26	2.03, 1.89	2.24[b]	
Gln267	8.62	4.34	2.11, 1.95	2.36[b]	6.61, 6.92 ($CONH_2$)
Gly268	8.41	4.12, 3.95			
Pro269		4.38	2.25, 1.90	1.98[b]	3.58[b] (Hδ)
Lys270	8.63	4.30	1.83, 1.75	1.44[b]	1.66[b] (Hδ), 2.97[b] (Hε)
Gly271	8.48	3.99, 3.84			
Glu272	8.46	4.35	1.99, 1.90	2.25[b]	
Thr273	7.93	4.20	4.12	1.14	

[a] Spectra were recorded at 500 MHz, 278 K, and pH 5.4 with H_2O ($\delta_H = 4.98$ ppm) as internal standard. [b] Degeneracy has been assumed. [c] Chemical shifts for the 2-deoxy-β-D-*lyxo*-hexopyranosyl moiety in **20**: $\delta = 4.65$ (H-1), 3.84 (H-3), 3.76 (H-6,6), 3.75 (H-4), 3.56 (H-5), 2.03 (H-2_{eq}), 1.62 (H-2_{ax}) ppm.

Table 4. ^{1}H NMR chemical shifts for glycopeptide **21** in water containing 10% D_2O.[a]

Residue	NH	Hα	Hβ	Hγ	Others
Gly259[b]					
Ile260	8.60	4.17	1.82	1.44, 1.14, 0.92 (CH_3)	0.83 (Hδ)
Ala261	8.67	4.28	1.36		
Gly262	8.44	3.84[c]			
Phe263	8.22	4.58	3.05[c]		7.32 (H3,H5), 7.23 (H2,H6)
Hyl264[d]	8.55	4.24	1.98, 1.71	1.58[c]	4.01 (Hδ), 3.15, 2.96 (Hε)
Gly265	7.90	3.89, 3.84			
Glu266	8.48	4.27	2.04, 1.90	2.27[c]	
Gln267	8.61	4.32	2.11, 1.90	2.27[c]	
Gly268	8.44	4.12, 3.96			
Pro269		4.39	2.26, 1.91	1.99[c]	3.59[c] (Hδ)
Lys270	8.62	4.29	1.84, 1.76	1.45[c]	1.67[c] (Hδ), 2.94[c] (Hε)
Gly271	8.50	3.99, 3.90			
Glu272	8.47	4.35	2.11, 1.97	2.28[c]	
Thr273	7.96	4.21	4.15	1.15	

[a] Spectra were recorded at 500 MHz, 278 K, and pH 5.4 with H_2O ($\delta_H = 4.98$ ppm) as internal standard. [b] Not assigned due to spectral overlap. [c] Degeneracy has been assumed. [d] Chemical shifts for the 3-deoxy-β-D-*xylo*-hexopyranosyl moiety in **21**: $\delta = 4.43$ (H-1), 3.97 (H-4), 3.70 (H-2), 2.17 (H-3_{eq}), 1.68 (H-3_{ax}) ppm.

resin used). MS (ES): *m/z* calcd: 546.6 $[M+3H]^{3+}$; found: 546.6; Amino acid analysis: Ala 1.02 (1), Glu 3.01 (3), Gly 4.93 (5), Hyl 1.02 (1), Ile 1.01 (1), Lys 1.03(1), Phe 1.04 (1), Pro 0.94 (1), Thr 1.02 (1). ^{1}H NMR data are given in Table 5.

Glycyl-L-glutamin-1-yl-*trans*-4-hydroxy-L-prolylglycyl-L-isoleucyl-L-alanylglycyl-L-phenylalanyl-5-*O*-(β-D-fucopyranosyl)-5-hydroxy-L-lysylglycyl-L-glutam-1-yl-L-glutaminylglycyl-L-prolyl-L-lysine (23): Synthesis was performed with building block **4** on resin (60 μmol) according to the general procedure to give glycopeptide-containing resin (352 mg). Cleavage and workup of a portion of the resin (125 mg, ≈21 μmol) according to the general procedure, followed by purification by preparative reversed-phase HPLC gave the 2,3,4-*O*-acetylated glycopeptide (26 mg). A portion of this glycopeptide (25.5 mg) was treated with NaOMe in MeOH (9 mM) for 6 h at room temperature (monitored by analytical reversed-phase HPLC). The solution was then neutralized (HOAc) and concentrated, and the residue was purified by preparative reversed-phase HPLC to give **23** (20.6 mg, 74% peptide content, 43% yield based on the amount of resin used). MS (FAB): *m/z* calcd: 1649 $[M+H]^+$; found: 1649; amino acid analysis: Ala 1.03 (1), Glu 2.87 (3), Gly 4.89 (5), Hyl 1.04 (1), Ile 1.02 (1), Lys 1.05(1), Phe 1.04 (1), Pro 1.03 (1), Hyp 1.03 (1). ^{1}H NMR data are given in Table 6.

Glycyl-L-glutamin-1-yl-*trans*-4-hydroxy-L-prolylglycyl-L-isoleucyl-L-alanylglycyl-L-phenylalanyl-5-*O*-(β-D-glucopyranosyl)-5-hydroxy-L-lysylglycyl-L-glutam-1-yl-L-glutaminylglycyl-L-prolyl-L-lysine (24): Synthesis was performed with building block **15** on resin (35 μmol) according to the general procedure. Cleavage and then purification by preparative reversed-phase HPLC gave 2,3,4,6-*O*-acetylated glycopeptide (22 mg). A portion of this glycopeptide (20 mg) was treated with NaOMe in MeOH (5 mM) for 3.5 h at room temperature (monitored by analytical reversed-phase HPLC). The solution was then neutralized (HOAc) and concentrated, and the residue was purified by preparative reversed-phase HPLC to give **24** (9.4 mg, 70% peptide content, 13% yield based on the amount of resin used). MS (FAB): *m/z* calcd: 1665 $[M+H]^+$; found: 1665; amino acid analysis: Ala 1.01 (1), Glu 2.98 (3), Gly 4.99 (5), Hyl 0.99 (1), Ile 1.00 (1), Lys 1.01(1), Phe 1.01 (1), Pro 1.01 (1), Hyp 1.01 (1). ^{1}H NMR data are given in Table 7.

Generation of DR4-restricted T-cell hybridomas: Three mice that were transgenic for the human DR4 class II MHC molecule and the

Table 5. ^{1}H NMR chemical shifts for glycopeptide **22** in water containing 10% D_2O.[a]

Residue	NH	Hα	Hβ	Hγ	Others
Gly259		3.82[b]			
Ile260	8.60	4.18	1.82	1.44, 1.15, 0.91 (CH_3)	0.84 (Hδ)
Ala261	8.67	4.29	1.39		
Gly262	8.49	3.88[b]			
Phe263	8.21	4.59	3.06[b]		7.33 (H3,H5), 7.30 (H4), 7.23 (H2,H6)
Hyl264[c]	8.55	4.26	1.99, 1.71	1.57[b]	3.97 (Hδ), 3.15, 2.96 (Hε)
Gly265	7.89	3.90, 3.84			
Glu266	8.48	4.27	2.05, 1.91	2.28[b]	
Gln267	8.65	4.35	2.14, 1.98	2.37[b]	7.62, 6.93 ($CONH_2$)
Gly268	8.44	4.12, 3.96			
Pro269		4.40	2.27, 1.91	2.00[b]	3.60[b] (Hδ)
Lys270	8.65	4.31	1.84, 1.78	1.46[b]	1.68[b] (Hδ), 2.97[b] (Hε), 7.59 (ε-NH_2)
Gly271	8.48	3.98, 3.89			
Glu272	8.47	4.37	2.10, 1.91	2.28[b]	
Thr273	7.96	4.21	4.14	1.15	

[a] Spectra were recorded at 500 MHz, 278 K, and pH 5.4 with H_2O ($\delta_H = 4.98$ ppm) as internal standard. [b] Degeneracy has been assumed. [c] Chemical shifts for the 4-deoxy-β-D-*xylo*-hexopyranosyl moiety in **22**: $\delta = 4.39$ (H-1), 3.74 (H-3), 3.71 (H-5), 3.71, 3.59 (H-6), 3.19 (H-2), 1.93 (H-4_{eq}), 1.43 (H-4_{ax}) ppm.

Table 6. ^{1}H NMR chemical shifts for glycopeptide **23** in 40 mM phosphate buffer containing 10% D_2O and 1.5 mM NaN_3.[a]

Residue	NH	Hα	Hβ	Hγ	Others
Gly256		3.78, 3.63			
Glu257	8.74	4.62	2.00, 1.79	2.24[b]	
Hyp258		4.49	2.31, 2.04	4.60	3.82[b] (Hδ)
Gly259	8.71	3.90[b]			
Ile260	8.09	4.15	1.85	1.14[b], 0.88 (CH_3)	0.79 (Hδ)
Ala261	8.66	4.24	1.34		
Gly262	8.46	3.84[b]			
Phe263	8.18	4.53	3.05[b]		7.30 (H3,H5), 7.27 (H2,H6), 7.2 (H4)
Hyl264[c]	8.50	4.24	1.95, 1.66	1.53[b]	3.95 (Hδ), 3.11, 2.91 (Hε)
Gly265	7.79	3.87, 3.80			
Glu266	8.48	4.24	2.02, 1.88	2.24[b]	
Gln267	8.66	4.32	2.10, 1.95	2.24[b]	7.62, 6.90 ($CONH_2$)
Gly268	8.43	4.12, 3.93			
Pro269		4.38	2.23, 1.96	1.98[b]	3.66[b] (Hδ)
Lys270	8.17	4.10	1.78, 1.68	1.39[b]	1.65[b] (Hδ), 2.95[b] (Hε), 7.54 (ε-NH_2)

[a] Spectra were recorded at 600 MHz, 278 K, and pH 5.4 with H_2O ($\delta_H = 4.98$ ppm) as internal standard. [b] Degeneracy has been assumed. [c] Chemical shifts for the β-D-fucopyranosyl moiety in **23**: $\delta = 4.34$ (H-1), 3.72 (H-5), 3.70 (H-4), 3.60 (H-3), 3.42 (H-2), 1.19 (H-6) ppm.

Table 7. ^{1}H NMR chemical shifts for glycopeptide **24** in water containing 10% D_2O.[a]

Residue	NH	Hα	Hβ	Hγ	Others
Gly256		3.80, 3.67			
Glu257	8.74	4.65	2.02, 1.82	2.27[b]	
Hyp258		4.50	2.32, 2.06	4.62	3.87[b] (Hδ)
Gly259	8.70	3.87[b]			
Ile260	8.08	4.17	1.87	1.41, 1.17, 0.90 (CH_3)	0.80 (Hδ)
Ala261	8.65	4.26	1.37		
Gly262	8.45	3.87[b]			
Phe263	8.18	4.56	3.06[b]		7.32 (H3,H5, H4), 7.22 (H2,H6)
Hyl264[c]	8.49	4.28	1.98, 1.69	1.58[b]	3.97 (Hδ), 3.13, 2.94 (Hε)
Gly265	7.80	3.86[b]			
Glu266	8.49	4.27	2.04, 1.91	2.26[b]	
Gln267	8.65	4.34	2.12, 1.97	2.37[b]	7.62, 6.92 ($CONH_2$)
Gly268	8.42	4.13, 3.97			
Pro269		4.39	2.25, 1.98	1.98[b]	3.59[b] (Hδ)
Lys270	8.16	4.13	1.79, 1.65	1.40[b]	1.64[b] (Hδ), 2.96[b] (Hε), 7.54 (ε-NH_2)

[a] Spectra were recorded at 500 MHz, 278 K, and pH 5.3 with H_2O ($\delta_H = 4.98$ ppm) as internal standard. [b] Degeneracy has been assumed. [c] Chemical shifts for the β-D-glucopyranosyl moiety in **24**: $\delta = 4.45$ (H-1), 3.78, 3.72 (H-6), 3.45 (H-3), 3.43 (H-5), 3.39 (H-4), 3.27 (H-2) ppm.

human CD4 co-receptor were used in the study. The mice were immunized subcutaneously with $3 \times 50\,\mu g$ of glycopeptide **27** (CII259 – 278 in which hydroxylysine at positions 264 and 270 carried β-D-galactopyranosyl residues) emulsified in CFA in the footpads and at the base of the tail. Ten days later the mice were sacrificed and the draining popliteal and inguinal lymph nodes were pooled and single-cell suspensions prepared. Cells were restimulated in vitro with **27**, that is, the same peptide used for immunization, at a concentration of $20\,\mu g\,mL^{-1}$ for 7 days. The last three days' cells were expanded by the addition of IL-2-containing medium to the cultures. The cells were then fused with α/β T-cell receptor-negative thymoma cell line BW5147.[60] After selection, positive cells were tested for IL-2 production in the presence of **27** as described below. Positive clones were further subcloned by limited dilution.

Determination of T-cell hybridoma response: The response of each T-cell hybridoma, that is, IL-2 secreted on incubation of the hybridoma with antigen-presenting spleen cells and increasing concentrations of glycopeptides **20** – **26**, was determined in a standard assay by using the T cell clone CTLL.[39] In brief, 5×10^4 T-cell hybridomas were cocultured with 5×10^5 syngeneic, irradiated (1500 rad) spleen cells and antigen in a volume of 200 μL in flat-bottomed microtiter plate wells. After 24 h, 100-μL aliquots of the supernatants were removed and frozen to kill any transferred T-cell hybridomas. Then, 10^4 IL-2-sensitive CTLL T cells were added to the thawed supernatant. The CTLL cultures were incubated for 24 h, after which they were pulsed with 1 μCi of ^{3}H-TdR for an additional 15 – 18 h. The cells were harvested on glass fiber sheets in a Filtermate cell harvester (Packard Instruments, Meriden, CT) and the amount of radioactivity was determined in a matrix 96 Direct Beta Counter (Packard). All experiments were performed in duplicate.

Molecular modeling: The model of glycopeptide **25** bound to H-2A^q was generated from a previous model of the corresponding peptide CII256 – 270 bound to H-2A^q.[36] The β-D-galactopyranosyl moiety was added to hydroxylysine 264 by using the program Sybyl and was oriented manually to avoid steric interactions with H-2A^q.

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