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Role of the Galactosyl Moiety of Collagen Glycopeptides for T-Cell Stimulation in a Model for Rheumatoid Arthritis

Björn Holm,[a] Syed M. Baquer,[a] Lotta Holm,[a] Rikard Holmdahl[b] and Jan Kihlberg[a,*]

[a]*Organic Chemistry, Department of Chemistry, Umeå University, SE-901 87 Umeå, Sweden*
[b]*Section of Medical Inflammation Research, Lund University, Sölvegatan 19, I11 BMC, SE-221 84 Lund, Sweden*

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Abstract—Two protected derivatives of β-D-galactopyranosyl-5-hydroxy-L-lysine, in which HO-4 of galactose has been *O*-methylated or replaced by fluorine, have been prepared. The building blocks were incorporated at position 264 of the peptide fragment CII259-273 from type II collagen by solid-phase synthesis. The ability of these two glycopeptides, and two CII259-273 glycopeptides in which HO-4 of galactose was either unmodified or deoxygenated, to elicit responses from T-cell hybridomas obtained in a mouse model for rheumatoid arthritis was then determined. The hybridomas were all highly sensitive towards modifications at C-4 of the β-D-galactosyl residue of CII259-273, highlighting the role of HO-4 as an important contact point for the T-cell receptor. Most likely, this glycopeptide hydroxyl group is involved in hydrogen bonding with the T-cell receptor.
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Introduction

The ability to distinguish self from non-self in order to mount an effective response against foreign antigens is vital to the immune system. Specialized cells, that is antigen-presenting cells, are able to process extracellular proteins into short peptides that become bound to class II major histocompatibility complex (MHC) molecules and displayed on the cell surface.[1] Recognition of complexes between class II MHC molecules and peptides from *foreign* antigens by $CD4^+$ helper T cells should result in release of immunomodulating cytokines, followed by elimination of the protein antigen from which the peptide was derived. During their early development T cells are subjected to several mechanisms of selection to ensure that they do not react against endogenous antigens, which are also presented by MHC molecules. Should this tolerance fail, the immune system may be directed against endogenous proteins leading to development of autoimmune disease.

Rheumatoid arthritis (RA) is a chronic disease, which affects about 1% of the worldwide population and is generally considered to be of autoimmune origin.[2] RA leads to destruction of joint cartilage which results in erythema and severe, painful swelling of periferal joints. The events that initiate RA are still largely unknown and although the symptoms can be treated no definite cure is available. A frequently used animal model for studies of rheumatiod arthritis is murine collagen-induced arthritis (CIA), in which mice are immunized with type II collagen from rat in combination with complete Freund's adjuvant (CFA).[3,4] This induces symptoms and histopathology that are very similar to those of patients suffering from rheumatoid arthritis. High incidence as well as development of severe forms of CIA is linked to presentation of an immunodominant peptide epitope, located between residues 256 and 273 of type II collagen, by the murine MHCII molecule H-$2A^q$ on the surface of antigen-presenting cells.[5] Previous studies in CIA have revealed the importance of post-translational modifications of the immunodominant epitope for stimulation of T cells.[6] The epitope contains two lysine residues that may be hydroxylated and subsequently glycosylated with a β-D-galactopyranosyl- or an α-D-glucopyranosyl-(1→2)-β-D-galactopyranosyl unit.[7,8] In fact, the majority of a panel of T-cell hybridomas generated in CIA responded specifically to glycopeptides carrying a β-D-galactopyranosyl moiety on hydroxylysine at position 264 (Hyl^{264}).[9,10] Alanine scanning, together with modelling of the MHCII–peptide complex, revealed that H2-A^q binds glycopeptides by placement of Ile^{260} and Phe^{263} in two hydrophobic pockets designated P1 and P4.[11] This positions the

*Corresponding author. Tel.: +46-90-7866890; fax: +46-90-138885; e-mail: jan.kihlberg@chem.umu.se

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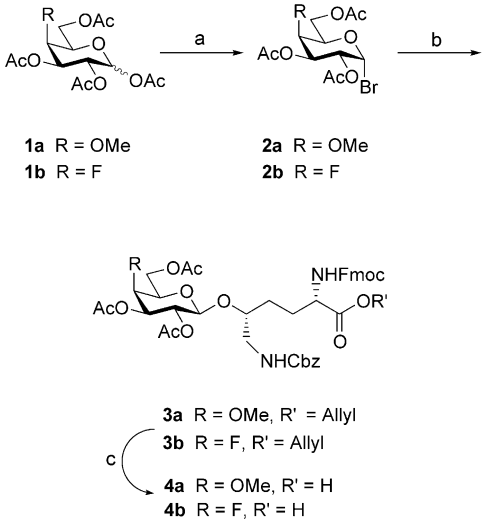
galactosylated Hyl[264] roughly in the middle of the binding cleft of the H2-A^q class II MHC molecule, pointing outwards in an ideal position for interactions with a T-cell receptor.

Recently, we have shown that T-cell hybridomas, reactive towards CII259-273 glycopeptides carrying a galactose moiety on Hyl[264], differed in their ability to recognize glycopeptides with modified galactosyl residues.[12] A panel of glycopeptides, carrying either 2-, 3-, 4- or 6-mono-deoxygenated galactose residues on Hyl[264], revealed that the 20 T-cell hybridomas in the study could be divided into four groups based on their response towards the modified CII glycopeptides. The same division into groups had been made independently based on the sequence of the T-cell receptors for some of the hybridomas.[9] Interestingly, all hybridomas were sensitive to deoxygenation at C-4 of the galactose moiety, which resulted in reduction or complete loss of the response. We now report the synthesis of two CII-derived glycopeptides carrying β-D-galactopyranosyl residues modified at C-4 by *O*-methylation and exchange of HO-4 for a fluorine atom. The glycopeptides were then used to further probe the role of HO-4 of the β-D-galactopyranosyl hydroxylysine moiety in interactions with T-cell receptors of CIA-derived hybridomas.

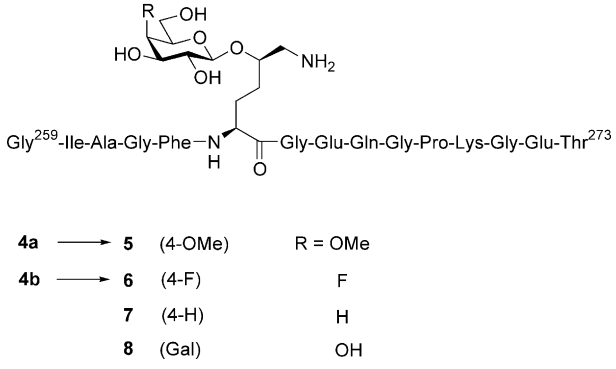
Results and Discussion

Synthesis of glycosylated hydroxylysine building blocks **4a** and **4b** was performed according to a procedure employed previously for preparation of a set of mono-deoxy-galactosylated hydroxylysines (Scheme 1).[12,13] Peracetylated 4-*O*-methyl- and 4-deoxy-4-fluoro-galactose (**1a** and **1b**)[14] were first converted into the corresponding glycosyl bromides (**2a**, **2b**) by treatment with hydrogen bromide in a mixture of acetic acid and acetic anhydride. The two bromides were then coupled to Fmoc-Hyl(Cbz)-OAllyl under promotion by silver-silicate to give **3a** and **3b** in 60 and 66% yields, respectively. The yields in the glycosylations are somewhat lower for these two less reactive glycosyl bromides, as compared to the mono-deoxygenated galactosyl bromides used in earlier studies.[12] When using glycosyl bromide **2b**, it was found that the purity of the glycosyl bromide was important for the outcome of the glycosylation. Although crude glycosyl bromides have been used earlier,[12,13] we have found that purification, by chromatography or crystallization, is advantageous in order to ensure that a successful and high-yielding glycosylation is achieved. Deallylation of **3a** and **3b** using $(PPh_3)_4Pd(0)$ and morpholine in THF gave glycosylated building blocks **4a** (76%) and **4b** (82%). These were then used in solid-phase glycopeptide synthesis on a Tentagel^{TM}-S-PHB-Thr-Fmoc resin according to the Fmoc protocol under conditions described previously.[12] Cleavage of the glycopeptides from the resin with TFA/H_2O/thioanisole/ethanedithiol (35:2:2:1) and subsequent deacetylation of the carbohydrate moieties using 20 mM methanolic sodium methoxide gave glycopeptides **5** and **6** (47 and 4.7% yields,[15] respectively, Scheme 2) after purification by reversed-phase HPLC. The two 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 (Tables 1 and 2).

To probe the sensitivity of T-cell hybridomas to modifications at C-4 of the galactose moiety, the modified glycopeptides **5**–**7** [henceforth also referred to as **5** (4-OMe), **6** (4-F), and **7** (4-H)] and reference glycopeptide **8** were incubated with antigen presenting spleen cells expressing H2-A^q class II MHC molecules and four of the 20 T-cell hybridomas obtained previously in CIA. The hybridomas were selected from each of the four groups that have different specificity for the galactose moiety of glycopeptide **8**, as revealed in our previous study with mono-deoxygenated glycopeptides.[12] The response of the hybridomas on incubation with increasing concentrations of glycopeptides was measured as interleukin-2 (IL-2) production detected by proliferation of IL-2 dependent CTLL T cells (Fig. 1).[16] The results, as expected, reveal that the four hybridomas are all sensitive towards modification at C-4 of the galactose moiety. However, the hybridomas do respond differ-



Scheme 1. (a) HBr/HOAc, Ac_2O; (b) Fmoc-Hyl(Cbz)-OAllyl, silver silicate, 3 Å molecular sieves, CH_2Cl_2 (or CH_2Cl_2/toluene); (c) $(PPh_3)_4Pd(0)$, morpholine, THF.



Scheme 2. Glycopeptides **5**–**8**, which correspond to residues 259–273 of type II collagen, were used to probe the role of HO-4 in the interactions between glycopeptide–MHCII complexes and T-cell receptors of hybridomas obtained in a mouse model of rheumatoid arthritis.

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

Residue	NH	Hα	Hβ	Hγ	Others
Thr^{273}	7.98	4.28	4.21	1.23	
Glu^{272}	8.50	4.44	2.18, 1.99	2.35[b]	
Gly^{271}	8.52	4.06, 3.98			
Lys^{270}	8.67	4.37	1.90, 1.84	1.51	1.74 (Hδ), 3.04 (Hε)
Pro^{269}		4.47	2.33, 1.98	2.07[b]	3.65[b] (Hδ)
Gly^{268}	8.46	4.19, 4.04			
Gln^{267}	8.67	4.42	2.19, 2.03	2.44[b]	7.65, 6.96 ($CONH_2$)
Glu^{266}	8.50	4.35	2.12, 1.98	2.35[b]	
Gly^{265}	7.94	3.98, 3.91			
Hyl^{264c}	8.57	4.34	2.05, 1.78	1.64[b]	4.05 (Hδ), 322, 3.02 (Hε)
Phe^{263}	8.23	4.66	3.13[b]		7.31,7.40 (H–arom.)
Gly^{262}	8.50	3.95[b]			
Ala^{261}	8.68	4.36	1.49		
Ile^{260}	8.62	4.26	1.89	0.98 (CH_3), 1.51, 1.21	0.91 (H-δ)
Gly^{259}	3.89				

[a]Measured at 500 MHz, pH 4.7 and 286 K.
[b]Degeneracy has been assumed.
[c]Chemical shifts for the 4-*O*-methyl-β-D-galactopyranosyl moiety of **5**: 4.45 (H1), 3.75 (H3), 3.69 (H4), 3.57 (OMe), 3.52 (H2). Chemical shifts not reported could not be unambigously assigned from the available spectra due to overlap.

Table 2. ^{1}H NMR chemical shifts for glycopeptide **6** recorded in water containing 10% D_2O[a,d]

Residue	NH	Hα	Hβ	Hγ	Others
Thr^{273}	7.91	4.16	4.23	1.15	
Glu^{272}	8.37	4.39	2.12, 1.93	2.32[b]	
Gly^{271}	8.42	3.98, 3.90			
Lys^{270}	8.55	4.30	1.84, 1.77	1.45[b]	1.67 (Hδ), 2.98 (Hε)
Pro^{269}	4.40	2.26	1.90	1.98	3.59[b] (Hδ)
Gly^{268}	8.36	4.13, 3.98			
Gln^{267}	8.58	4.36	2.12, 1.97	2.36[b]	7.57, 6.88 ($CONH_2$)
Glu^{266}	8.41	4.29	2.05, 1.92	2.29[b]	
Gly^{265}	7.85	3.88[b]			
Hyl^{264c}	8.47	4.28	1.97, 1.72	1.60[b]	4.03 (Hδ), 3.01, 3.17 (Hε)
Phe^{263}	8.14	4.60	3.06[b]		7.33, 7.25 (H–arom)
Gly^{262}	8.41	3.88[b]			
Ala^{261}	8.58	4.30	1.37		
Ile^{260}	8.53	4.19	1.83	0.91 (CH_3), 1.45, 1.17	0.84 (H-δ)

[a]Measured at 500 MHz, pH 4.7 and 286 K.
[b]Degeneracy has been assumed.
[c]Chemical shifts for the 4-deoxy-4-fluoro-β-D-galactopyranosyl moiety of **6**: 4.50 (d, $J = 7.7$ Hz, H1), 3.77 ($^3J_{F4-H3} \approx 30$ Hz[d], H3), 3.58 (H2). Chemical shifts not reported could not be unambigously assigned from the available spectra due to overlap and complicated crosspeak patterns resulting from the F–H couplings.
[d]Approximate value measured from 2D-TOCSY spectrum.

ently to the modifications and none of the modified glycopeptides gave a full response, that is a response identical to that of the galactose-containing reference glycopeptide **8**.

Interpretation of the responses from the T-cell hybridomas is based on that glycopeptides **5**–**7** do not retain the full ability of HO-4 in the galactose moiety of **8** to be involved in hydrogen bonding to the T-cell receptor recognition site. *O*-Methylation in **5** introduces a steric hindrance, but the oxygen atom can still function as a hydrogen bond acceptor. The fluorine atom in glycopeptide **6** mimics the galactose HO-4 in size and in polarization of the bond between C-4 and the heteroatom. The use of organic fluoro analogues as hydrogen bond acceptors to elucidate hydrogen bonding patterns is, however, hazardous since several reports indicate that fluorine atoms in organofluoro compounds seldom act as hydrogen bond acceptors.[17,18] We have therefore chosen not to view the fluorine atom of **6** as a hydrogen bond acceptor. Hybridomas HCQ.3 and HM1R.2 respond well to the reference glycopeptide **8**, but not to any of the modified glycopeptides **5**–**7**. The lack of response against these glycopeptides displayed by hybridomas HCQ.3 and HM1R.2 implies that HO-4 both donates and accepts hydrogen bonds from the recognition sites of the T-cell receptors in these hybridomas. Hybridoma HNC.1 recognizes glycopeptide **6** (4-F) with a slightly less than full response but it does not respond to **7** (4-H) and **5** (4-OMe). A possible interpretation could be that the recognition site of the T-cell receptor is too small to accommodate the 4-*O*-methyl group. Furthermore, removal of the electronegative hydroxy group at C-4 [→**7** (4-H)] may disrupt important polar interactions with the T-cell receptor, whereas the fluorine atom might be similar enough, in size and electonegativity, to enable a somewhat weaker response. Hybridoma HCQ.10 shows weaker but significant responses for all the modified glycopeptides with the responses for **7** (4-H) and **5** (4-OMe) being

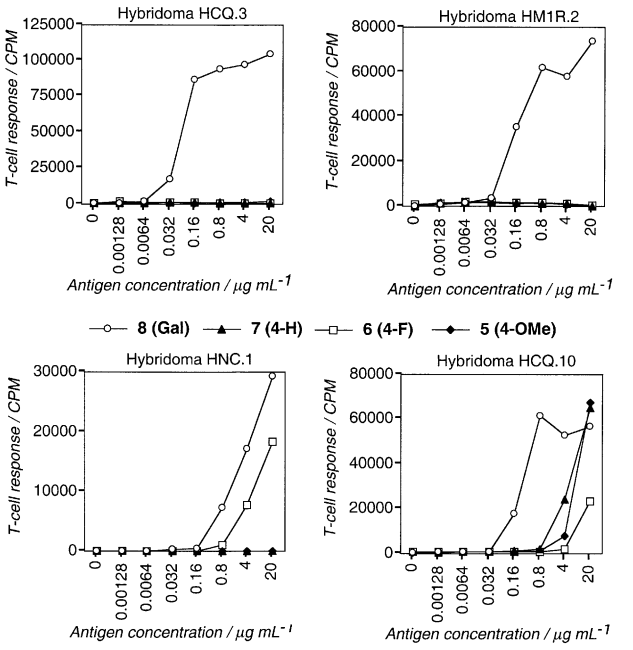


Figure 1. Response of H-2A^q restricted T-cell hybridomas on incubation with antigen-presenting spleen cells and increasing concentrations of glycopeptides **5**–**8**. The hybridomas were selected from each of the four groups that displayed different patterns of fine specificity for the galactosyl moiety 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 interleukin 2 (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.

slightly stronger as compared to **6** (4-F). The specificity pattern of HCQ.10 is not as readily explained as those of the other three hybridomas and may be the result of more complex interactions.

The results of the studies with the four hybridomas emphasize the importance of HO-4 in the galactose moiety of glycopeptide **8** in contacting the T-cell receptor, and demonstrates that very minor modifications of the galactose residue leads to large changes in the T-cell response. Activation of the various T-cell effector functions upon binding to MHC–peptide complexes is believed to be influenced by on and off rates as well as by affinity, and occurs through a series of complex intracellular events.[19] It is known that a minor modification of a peptide ligand at a position which contacts the T cell receptor can drastically modify the response of a T cell, including sending it into an anergic state.[20,21] Moreover, immunotherapeutic use of altered peptide ligands (APLs) have shown promise in animal models for experimental autoimmune encephalomyelitis.[22,23] Increased knowledge about the interactions between T cell receptors and glycopeptides could be utilized in the generation of APL's, based on analogues of glycopeptides from collagen. In glycopeptide **8**, HO-4 of the galactose moiety and its immediate surroundings, provide one possible point of modification for generation of altered peptide ligands that could be investigated for therapeutic applications in rheumatoid arthritis.

Conclusion

A set of glycopeptides derived from type II collagen carrying modified β-D-galactosyl moieties (4-deoxy,[12] 4-deoxy-4-fluoro and 4-*O*-methyl) at hydroxylysine 264 has been prepared. The ability of the glycopeptides to elicit responses from T-cell hybridomas obtained in a mouse model for rheumatoid arthritis was evaluated in a cell-based assay. The four hybridomas that were evaluated were all sensitive towards modifications at C-4 of the galactose residue. This highlights the role of HO-4 of the β-D-galactosyl moiety of the type II collagen glycopeptide CII259-273 as an important contact point for the T-cell receptor.

Experimental

General methods and materials

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 solvent of HPLC grade or analytical grade. Dimethylformamide (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 ^1H and ^{13}C NMR spectra of compounds **2**–**4** were recorded on a Bruker DRX-400 spectrometer at 400 and 100 MHz, respectively. Chemical shifts are referenced to residual $CHCl_3$ ($\delta_H = 7.27$ ppm) and $CDCl_3$ ($\delta_H = 77.0$ ppm) for solutions in $CDCl_3$ or to CD_2HOD ($\delta_H = 3.31$ ppm) and CD_3OD ($\delta_H = 49.0$ ppm) for solutions in a 1:1 mixture of $CDCl_3$ and CD_3OD. For compounds **2**–**4**, resonances for aromatic protons are not reported. The ^1H NMR spectra of compounds **3**–**4** contain broad minor peaks. These have previously been shown to be caused by the existence of rotamers about the amide bond in the Fmoc urethane.[24,25] Spectra for glycopeptides **5** and **6** in H_2O/D_2O 9:1 (pH 4.7) were recorded at 283 K on a Bruker AMX-500 spectrometer operating at 500 MHz. First order chemical shifts and coupling constants were determined from one-dimensional spectra and resonances were assigned from appropriate combinations of two-dimensional COSY, TOCSY, NOESY, and ^1H–^{13}C-HSQC spectra. Optical rotations were recorded with a Perkin-Elmer 343 polarimeter. Analytical reversed-phase HPLC was performed on a Kromasil C-8 column (250×4.6 mm, 5 μm, 100 Å), eluted with a linear gradient of MeCN (0→100% over 60 min) in H_2O; both eluents containing 0.1% TFA. A flowrate of 1.5 mL/min was used and detection was at 214 nm. Preparative reversed-phase HPLC was performed on a Kromasil C-8 column (250×20 mm, 5 μm, 100 Å), with the same eluents, a flowrate of 11 mL/min and detection at 214 nm. Fmoc-Hyl(Cbz)-OAllyl[26] and silver silicate[13] were prepared as described in the cited references. Glycopeptides **7**[12] and **8**[10] have been prepared earlier.

2,3,6-tri-*O*-Acetyl-4-*O*-methyl-α-D-galactopyranosyl bromide (2a) and 2,3,6-tri-*O*-acetyl-4-deoxy-4-fluoro-α-D-galactopyranosyl bromide (2b). Bromosugars **2a** and **2b** were prepared by treatment of the corresponding 1-*O*-acetyl sugars **1a** and **1b** with 15% HBr in $HOAc/Ac_2O$ (6:1) for 2–3 h at room temperature. The solutions were diluted with CH_2Cl_2 and washed with water, saturated aqueous $NaHCO_3$ and water, dried (Na_2SO_4), filtered and concentrated. Bromosugar **2a** was used without further purification whereas **2b** was purified by flash column chromatography (heptane/ethyl acetate 19:1→4:1). ^{1}H NMR ($CDCl_3$) **2a**: δ 6.68 (d, 1H, J = 3.9 Hz, H-1), 5.32 (dd, 1H, J = 10.6, 2.9 Hz, H-3), 5.14 (dd, 1H, J = 10.6, 3.8 Hz, H-2), 4.31–4.13 (m, 3H, H-5, H-6,6′), 3.81 (d, 1H, J = 2.9 Hz, H-4), 3.50 (s, 3H, OCH_3), 2.12, 2.09, and 2.09 (3s, each 3H, OAc); **2b**: δ 6.70 (d, 1H, J = 4.0 Hz, H-1), 5.34 (ddd, 1H, J = 26.7, 10.8, 2.5 Hz, H-3), 5.12 (dd, 1H, J = 10.4, 3.9 Hz, H-2), 5.00 (dd, 1H, J = 50.1, 2.9 Hz, H-4), 4.44–4.26 (m, 3H, H-5, H-6,6′), 2.16, 2.12, and 2.11 (3s, each 3H, OAc).

(5*R*)-*N*$^{\alpha}$-(Fluoren-9-yl-methoxycarbonyl)-*N*$^{\varepsilon}$-benzyloxycarbonyl-5-*O*-(2,3,6-tri-*O*-acetyl-4-*O*-methyl-β-D-galactopyranosyl)-5-hydroxy-L-lysine allyl ester (3a). A solution of *O*-methylated galactosyl bromide **2a** (141 mg, 0.38 mmol) in dry CH_2Cl_2 (2 mL) was added dropwise to an ice-cold stirred mixture of Fmoc-Hyl(Cbz)-OAllyl (142 mg, 0.26 mmol), silver silicate (384 mg) and crushed 3 Å molecular sieves (142 mg) in dry CH_2Cl_2 (4 mL) under N_2 in the absence of light. After stirring for 3 h additional **2a** (50 mg) in CH_2Cl_2 (0.5 mL) was added, the mixture was stirred for a further 2 h and was then allowed to attain room temperature. The solid material was filtered off (Hyflo supercel) and washed with CH_2Cl_2 and the combined filtrates were concentrated. Flash column chromatography of the residue (toluene/MeCN 5:1) gave **3a** (137 mg, 0.159 mmol, 60%): $[\alpha]_D^{20}$ 0° (*c* 1, $CHCl_3$); ^{1}H NMR ($CDCl_3$) δ 5.97–5.80 (m, 1H, $OCH_2C\underline{H}=CH_2$), 5.64 (t, 1H, J = 5.2 Hz, NH-ε), 5.45 (d, 1H, J = 8.1 Hz, NH-α), 5.33 (d, 1H, J = 17.1 Hz, $OCH_2CH=C\underline{H}_{2trans}$), 5.28–5.20 (m, 2H, H-2, $OCH_2CH=C\underline{H}_{2cis}$), 5.11 (ABd, 1H, J = 14.0 Hz, $OC\underline{H}_2Ph$), 5.07 (ABd, 1H, J = 14.0 Hz, $OC\underline{H}_2Ph$), 4.89 (dd, 1H, J = 10.5, 3.0 Hz, H-3), 4.63 (d, 2H, J = 5.0 Hz, $OC\underline{H}_2CH=CH$), 4.48–4.28 (m, 4H, H-1, $NH(CO)OC\underline{H}_2CH$, H-α), 4.28–4.17 (m, 3H, $NH(CO)OCH_2C\underline{H}$, H-6,6), 3.67 (t, 1H, J = 6.3 Hz, H-5), 3.71–3.58 (m, 2H, H-4, H-δ), 3.49 (s, 3H, OCH_3), 3.45–3.36 (m, 1H, H-ε), 3.21–3.11 (m, 1H, H-ε), 2.08, 1.99, and 1.99 (3s, each 3H, OAc), 2.02–1.90 (m, 1H, H-β), 1.75–1.36 (m, 3H, H-β, H-γ,γ); ^{13}C NMR ($CDCl_3$) δ 171.7, 170.4, 170.3, 169.2, 156.7, 156.9, 143.9, 143.8, 141.2, 136.5, 131.3, 128.4, 128.2, 128.0, 127.9, 127.7, 127.0, 127.0, 125.0, 119.9, 119.2, 101.3, 80.8, 76.2, 73.7, 72.2, 69.4, 66.9, 66.6, 66.1, 62.3, 61.4, 53.7, 47.1, 44.8, 28.7, 28.3, 20.7, 20.6, 20.6; HRMS (FAB): calcd for $C_{45}H_{52}N_2NaO_{15}$ 883.3265 (M + Na), found 883.3268.

(5*R*)-*N*$^{\alpha}$-(Fluoren-9-yl-methoxycarbonyl)-*N*$^{\varepsilon}$-benzyloxycarbonyl-5-*O*-(2,3,6-tri-*O*-acetyl-4-deoxy-4-fluoro-β-D-galactopyranosyl)-5-hydroxy-L-lysine allyl ester (3b). A solution of fluorinated galactosyl bromide **2b** (50 mg, 0.135 mmol) in dry toluene/CH_2Cl_2 (1:1, 1.5 mL) was added dropwise to an ice-cold stirred mixture of Fmoc-Hyl(Cbz)-OAllyl (50 mg, 0.09 mmol), silver silicate (135 mg) and crushed 3 Å molecular sieves (100 mg) in dry toluene/CH_2Cl_2 (1:3, 4 mL) under N_2 in the absence of light. After stirring for 5.5 h the solid material was filtered off (Hyflo supercel) and washed with CH_2Cl_2 (5 mL) and the combined filtrates were concentrated. Flash column chromatography of the residue (toluene/MeCN 100:1→6:1) gave **3b** (50 mg, 0.059 mmol, 66%): $[\alpha]_D^{20}$ 5° (*c* 0.44, $CHCl_3$); ^{1}H NMR ($CDCl_3$) δ 5.88–5.79 (m, 1H, $OCH_2C\underline{H}=CH_2$), 5.47–5.42 (m, 1H, NH-ε), 5.32 (d, 1H, J = 7.2 Hz, NH-α), 5.27 (d, 1H, J = 17.1 Hz, $OCH_2CH=C\underline{H}_{2trans}$), 5.20 (d, 1H, J = 10.4 Hz, $OCH_2CH=C\underline{H}_{2cis}$), 5.15 (dd, 1H, J = 10.4, 8.1 Hz), 5.05, and 5.01 (2 ABd, each 1H, J = 12.3 Hz, $OC\underline{H}_2Ph$), 4.83 (ddd, 1H, J = 14.3, 8.0, 2.5 Hz, H-3), 4.72 (dd, 1H, J = 50.2, 2.6 Hz, H-4), 4.58 (d, 2H, J = 4.6 Hz, $OC\underline{H}_2CH=CH_2$), 4.45–4.24 (m, 4H, $NH(CO)OC\underline{H}_2CH$, H-1, H-α), 4.22–4.12 (m, 3H, $NH(CO)OCH_2C\underline{H}$, H-6,6), 3.72–3.55 (m, 2H, H-δ, H-5), 3.38–3.30 (m, 1H, H-ε), 3.15–3.07 (m, 1H, H-ε), 2.03 (s, 3H, OAc), 1.94 (s, 6H, 2 OAc), 1.70–1.40 (m, 3H, H-β, H-γ,γ); ^{13}C NMR ($CDCl_3$) δ 170.2, 169.1, 156.7, 143.8, 143.6, 141.3, 136.5, 131.3, 128.5, 127.0, 125.0, 120.0, 119.3, 101.0, 86.6, 84.8, 80.7, 71.3, 71.1, 70.9, 68.7, 67.0, 66.7, 66.2, 61.5, 53.6, 47.1, 44.8, 29.6, 28.6, 28.4, 20.6; HRMS (FAB): calcd for $C_{44}H_{49}FN_2NaO_{14}$ 871.3066 (M + Na), found 871.3086.

(5*R*)-*N*$^{\alpha}$-(Fluoren-9-yl-methoxycarbonyl)-*N*$^{\varepsilon}$-benzyloxycarbonyl-5-*O*-(2,3,6-tri-*O*-acetyl-4-*O*-methyl-β-D-galactopyranosyl)-5-hydroxy-L-lysine (4a). $(PPh_3)_4Pd(0)$ (18 mg, 15 μmol) was added to a stirred solution of **3a** (124 mg, 0.144 mmol) and morpholine (38 μL, 0.43 mmol) in dry THF under N_2 with protection from light. After stirring at room temperature for 35 min the solution was diluted with EtOAc (50 mL), washed with 1 M aqueous $KHSO_4$ and brine, dried (Na_2SO_4), filtered and concentrated. Flash column chromatography of the residue (toluene/EtOH 50:1→10:1→5:1) gave **4a** (90 mg, 0.11 mmol, 76%): $[\alpha]_D^{20}$ 3° (*c* 1, $CHCl_3$); ^{1}H NMR ($CDCl_3$/MeOD 1:1) δ 6.27 (t, 1H, J = 4.9 Hz, NH-ε), 5.16 (dd, 1H, J = 10.4, 8.0 Hz, H-2), 5.05 (s, 2H, $OC\underline{H}_2Ph$), 4.96 (dd, 1H, J = 10.4, 2.8 Hz, H-3), 4.48 (d, 1H, J = 7.9 Hz, H-1), 4.39–4.30 (m, 2H, $NH(CO)OC\underline{H}_2CH$), 4.22–4.11 (m, 4H, $NH(CO)OCH_2C\underline{H}$, H-6, H-6, H-α), 3.71 (t, 1H, J = 6.1 Hz, H-5), 3.67–3.59 (m, 2H, H-4, H-δ), 3.48 (s, 3H, OCH_3), 3.36–3.25 (m, 1H, H-ε), 3.18–3.09 (m, 1H, H-ε), 2.05, 1.99 and 1.98 (3s, each 3H, OAc), 2.01–1.85 (m, 1H, H-β), 1.70–1.49 (m, 3H, H-β, H-γ,γ); ^{13}C NMR ($CDCl_3$/MeOD 1:1) δ 174.7, 171.6, 171.2, 170.8, 157.9, 157.6, 144.5, 144.4, 141.9, 137.2, 129.0, 128.6, 128.5, 128.2, 127.6, 125.6, 120.4, 101.8, 81.3, 77.0, 74.4, 72.7, 70.4, 67.4, 67.2, 63.0, 61.8, 54.3, 47.7, 45.5, 29.7, 28.4, 20.9, 20.8; HRMS (FAB): calcd for $C_{42}H_{48}N_2NaO_{15}$ 843.2905 (M + Na), found 843.2946.

(5*R*)-*N*$^{\alpha}$-(Fluoren-9-yl-methoxycarbonyl)-*N*$^{\varepsilon}$-benzyloxycarbonyl-5-*O*-(2,3,6-tri-*O*-acetyl-4-deoxy-4-fluoro-β-D-galactopyranosyl)-5-hydroxy-L-lysine (4b). Deallylation and workup of **3b** (50 mg) as described for the preparation of **4a** followed by flash column chromatography ($CHCl_3$/MeOH 100:1→20:1) gave **4b** (39 mg, 82%): $[\alpha]_D^{20}$ 9° (*c* 1.2, $CHCl_3$); ^{1}H NMR ($CDCl_3$) δ 5.77 (d, 1H,

$J = 7.0$ Hz, NH-α), 5.62 (t, $J = 5.4$ Hz, NH-ε), 5.20 (dd, 1H, $J = 10.1$, 8.5 Hz, H-2), 5.10 and 5.06 (2 ABd, $J = 12.3$ Hz), 4.88 (dd, 1H, $J = 27.4$, 10.3 Hz, H-1), 4.77 (d, 1H, $J = 52.3$ Hz, H-4), 4.46 (d, 1H, $J = 7.6$ Hz, H-1), 4.42–4.28 (m, 3H, NH(CO)OC$\underline{H}_2$CH, H-α), 4.25–4.13 (m, 3H, NH(CO)OCH$_2$C$\underline{H}$, H-6,6), 3.71 (dt, $J = 26.6$, 6.1 Hz, H-5), 3.67–3.60 (m, 1H, H-δ), 3.46–3.33 (m, 1H, H-ε), 3.23–3.10 (m, 1H, H-ε), 2.06, 1.98, and 1.97 (3s, each 3H, 3 OAc), 2.01–1.94 (m, 1H, H-β), 1.77–1.42 (m, 3H, H-β, H-γ,γ); ^{13}C NMR ($CDCl_3$) δ 170.3, 160.5, 143.7, 143.6, 141.2, 128.9, 127.0, 125.2, 125.0, 119.9, 101.0, 86.6, 84.8, 71.2, 71.1, 70.9, 70.8, 68.7, 67.1, 66.9, 61.5, 53.3, 47.0, 27.9, 20.6, 20.5; HR-MS (FAB): calc for $C_{42}H_{48}N_2NaO_{15}$ 831.2753 (M + Na), found 831.2756.

General procedure for solid-phase glycopeptide synthesis

Glycopeptides **5** and **6** were synthesized on a Tentagel-S-PHB-Thr(*t*Bu)-Fmoc resin essentially as described.[12] N^{α}-Fmoc amino acids carrying standard side-chain protective groups (4 equiv) were coupled to the resin in DMF using either *O*-(benzotriazol-1-yl)-*N*,*N*,*N*′,*N*′-tetramethyluronium hexafluorophosphate (HBTU, 4 equiv) in the presence of diisopropyl ethyl amine (8 equiv) or diisopropyl carbodiimide (DIC, 3.9 equiv) in the presence of 1-hydroxy-benzotriazole (HOBt, 6 equiv). Glycosylated amino acids **4a** and **4b** (1.35 and 1.2 equiv, respectively) were coupled in DMF using DIC (1.35 or 1.2 equiv) and 1-hydroxy-7-aza-benzotriazole (2.20 or 1.8 equiv). Removal of Fmoc protective groups was performed using 20% piperidine in DMF. Cleavage from the solid phase was performed with TFA/H_2O/thioanisole/ethanedithiol (35:2:2:1).

Glycyl-L-isoleucyl-L-alanylglycyl-L-phenylalanyl-5-*O*-(4-*O*-methyl-β-D-galactopyranosyl)-5-hydroxy-L-lysylglycyl-L-glutam-1-yl-L-glutaminylglycyl-L-prolyl-L-lysylglycyl-L-glutam-1-yl-L-threonine (5). Synthesis was performed with building block **4a** (67 mg, 81 μmol) on Tentagel-S-PHB-Thr(*t*Bu)-Fmoc resin (60 μmol) according to the general procedure. Cleavage and workup gave 117 mg of crude *O*-acetylated peptide. A portion (60 mg) of this peptide was treated with NaOMe (30 mL, 20 mM in MeOH) for 2 h (monitored by analytical reversed-phase HPLC). The solution was neutralized (HOAc), concentrated and the residue was purified by reversed-phase HPLC to give **5** (31.5 mg, 77% peptide content, 47% yield based on the amount of resin used): MS (FAB): *m*/*z* calcd: 1668 [M + H$^+$]; found 1667; amino acid analysis: Ala 0.99 (1), Glu 2.99 (3), Gly 5.06 (5), Hyl 0.99 (1), Ile 0.97 (1), Lys 1.00 (1), Phe 1.01 (1), Pro 1.00 (1), Thr 0.99 (1). ^{1}H NMR data are given in Table 1.

Glycyl-L-isoleucyl-L-alanylglycyl-L-phenylalanyl-5-*O*-(4-deoxy-4-fluoro-β-D-galactopyranosyl)-5-hydroxy-L-lysylglycyl-L-glutam-1-yl-L-glutaminylglycyl-L-prolyl-L-lysylglycyl-L-glutam-1-yl-L-threonine (6). Synthesis was performed with building block **4b** (49 mg, 60 μmol) on resin (50 μmol) according to the general procedure. Cleavage, workup and deacetylation using NaOMe (35 mL, 20 mM in MeOH) for 0.5 h (monitored by analytical reversed-phase HPLC) followed by neutralization (HOAc), concentration and purification by reversed-phase HPLC gave **6** (7.2 mg, 54% peptide content, 4.7% yield based on the amount of resin used): MS (FAB): *m*/*z* calcd: 1655 [M + H$^+$]; found 1655; Ala 0.94 (1), Glu 3.10 (3), Gly 5.08 (5), Hyl 1.01 (1), Ile 0.92 (1), Lys 1.05 (1), Phe 0.98 (1), Pro 0.97 (1), Thr 0.95 (1). ^{1}H NMR data are given in Table 2.

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 **5**–**8**, was determined in a standard assay using the T-cell clone CTLL.[16] In brief, 5×10^4 T cell hybridomas were co-cultured with 5×10^5 syngeneic, spleen cells and antigen in a volume of 200 μL in flat-bottom microtiter plate wells. After 24 h, 100-μL aliquots of the supernatants were removed and frozen to kill any transferred T-cell hybridomas. To the thawed supernatant, 10^4 IL-2 sensitive CTLL T cells were added. The CTLL cultures were incubated for 24 h, after which they were pulsed with 1 μCi of ^{3}H-dThd for an additional 15–18 h. The cells were harvested on glassfiber sheets in a Filtermate TM cell harvester (Packard Instruments, Meriden, CT, USA) and the amount of radioactivity was determined in a matrix 96^{TM} Direct Beta Counter (Packard). All experiments were performed in triplicate.

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