Synthesis of a *C*-Glycoside Analogue of β-D-Galactosyl Hydroxynorvaline and Its Use in Immunological Studies

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A C-linked isostere of β-D-galactosylated hydroxynorvaline has been prepared in eight steps from per-O-benzylated galactopyranolactone. Addition of a homoallylic Grignard reagent to the lactone, reduction of the resulting hemiacetal with triethylsilane, and a Wittig reaction with Garner's aldehyde were key steps in this synthesis. The C-linked building block was then incorporated at position 264 into the fragment CII(256 – 270) from type II collagen by solid-phase synthesis using a combination of the tert-butoxycarbonyl (Boc) and 9-fluorenylmethoxycarbonyl (Fmoc) protective group strategies. Deprotection of the benzylated C-linked galactosyl moiety was achieved simultaneously with cleavage of the glycopeptide from the solid phase by using triethylsilyl trifluoromethanesulfonate in TFA. Helper T-cell hybridomas obtained in a mouse model for rheumatoid arthritis responded to the C-linked glycopeptide when presented by class II MHC molecules. However, 10- to 20-fold higher concentrations were required as compared to when O-linked β-D-galactosylated hydroxynorvaline or hydroxylysine (Hyl) were present at position 264 of CII(256 – 270). Thus, replacement of a single oxygen atom by a methylene group in the carbohydrate moiety of a glycopeptide antigen had a substantial influence on the T-cell response. This reveals that T cells are able to recognize the carbohydrate moiety of glycopeptide antigens with high specificity. Finally, the results suggest that structural modifications of β-D-Gal-Hyl^{264} in CII(256 – 270) may give altered peptide ligands that can be used for induction of tolerance in autoimmune rheumatoid arthritis.

KEYWORDS:

glycopeptides · glycosides · immunochemistry · molecular recognition · rheumatoid arthritis

Introduction

Collagen is a fibrous protein and a major component of the extracellular matrix.[1] Its triple-helical structure is formed by three α-chains, which adopt a left-handed poly(proline-II)-like conformation. During synthesis of the α-chains in the endoplasmatic reticulum, proline and lysine residues that are predominantly located in Gly-Xxx-Pro and Gly-Xxx-Lys sequences may become posttranslationally hydroxylated to form *trans*-4-hydroxy-L-proline and (5*R*)-5-hydroxy-L-lysine, respectively.[1] In a subsequent step some of the hydroxylysine residues become glycosylated, either with a β-D-galactopyranosyl or an α-D-glucopyranosyl-(1 →2)-β-D-galactopyranosyl moiety.[1, 2]

Collagen-induced arthritis (CIA) is the most widely used animal model for rheumatoid arthritis (RA), which is regarded to be an inflammatory autoimmune disease.[3] In this model, immunization of mice with type II collagen (CII) induces similar effects on the peripheral joints as observed in patients suffering from RA, that is erythema, swelling, and erosion of bone and cartilage. Development of CIA in mice is closely associated with expression of H-2A^q class II major histocompatibility complex (MHC) molecules on antigen-presenting cells (APCs) such as macrophages and B cells. After immunization, CII is taken up and degraded in the APC, a process which has been found to lead to presentation of peptides containing the epitope CII(256 – 270) by H-2A^q molecules on the surface of the APC.[4] Recognition of these complexes by receptors on "helper" (CD4$^+$) T cells results in activation of the T cells, which then play an important role in eliciting autoimmune disease. It is possible that development of RA in humans may occur through a related sequence of events since susceptibility to RA is associated with expression of HLA DR4 and DR1 class II MHC molecules, and activated T cells infiltrate the arthritic joints.[3b]

We have recently shown that most of the members of a panel of helper T-cell hybridomas obtained from mice with CIA recognized a posttranslationally modified form of CII(256 – 270).[5] In this immunodominant epitope, Lys^{264} has

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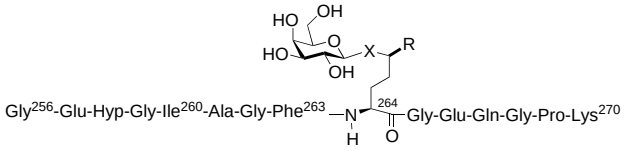
been hydroxylated (→Hyl[264]) and then glycosylated with a β-D-galactopyranosyl residue (see **1** in Figure 1). We could also show that the glycosylated hydroxylysine served as the primary T-cell contact point, that is, it dominated the contacts between the T-cell receptor (TCR) and the complex of **1** and H-2A^q.[5] In addition, the hydrophobic residues Ile[260] and Phe[263] were shown



1 X = O, R = CH_2NH_2
2 X = CH_2, R = H
3 X = O, R = H

Figure 1. *Glycopeptide **1** is derived from the peptide fragment CII(256 – 270) from type II collagen (CII), and has recently been shown to be the dominant epitope on CII which elicits autoimmune helper T cells in a mouse model for rheumatoid arthritis.[5] Some of the helper T-cell hybridomas which respond to **1** react equally well with **3**. C-Linked glycopeptide **2** has been prepared to allow further studies of the immune response to CII. Hyp=hydroxyproline.*

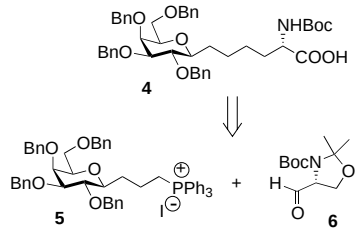
to anchor CII(256 – 270) in the P1 and P4 pockets of the H-2A^q class II MHC molecule.[6] Based on these anchor positions and on the crystal structure of a human class II MHC molecule,[7] the complex between H-2A^q and glycopeptide **1** was modelled.[6] This positioned galactosylated Hyl[264] facing toward the TCR in the center of the peptide – MHC complex, that is, in what appeared to be an ideal way for recognition by the TCR. Interestingly, the recently determined crystal structure of a ternary complex between a class II MHC molecule, a peptide, and a TCR revealed that a side chain in the center of the peptide formed critical contacts with the highly variable CD3 loops of the TCR.[8]

Studies in models for autoimmune disease such as experimental encephalomyelitis[9] have shown that slight structural modifications of MHC-restricted peptides may give altered peptide ligands[10] (APLs) which can induce tolerance, that is, break autoimmunity. It would be an advantage if transformation of an autoimmune peptide to an APL that induces tolerance could be achieved simultaneously with the introduction of increased stability toward degradation. *C*-Glycosylated amino acids are stable against both chemical and enzymatic degradation and have gained considerable attention in recent years,[11] but their use in biological investigations has been rare.[12] An important property of *C*-glycosides is that they adopt identical conformations as the corresponding *O*-linked carbohydrates at the glycosidic bond.[13] It is therefore possible that insertion of a *C*-glycosylated analogue of galactosylated hydroxylysine into the T-cell epitope **1** would result in an APL with ideal immunological and chemical properties.

As part of a study toward development of APLs that can induce tolerance in rheumatoid arthritis, we now report the preparation of a *C*-linked isostere of β-D-galactosylated hydroxynorvaline (Hnv), its incorporation into CII(256 – 270) (→**2**, Figure 1), and immunological studies. This simplified analogue of galactosylated hydroxylysine was chosen as the first target since some of the T-cell hybridomas directed to the native glycopeptide **1** have been found to respond equally well to glycopeptide **3**, which has a β-D-galactosylated hydroxynorvaline at position 264 of CII(256 – 270).[5, 14]

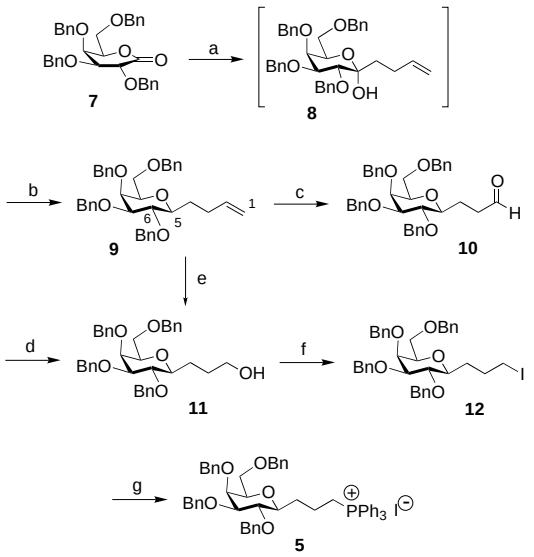
Results and Discussion

The protected *C*-glycoside analogue **4** of β-D-galactosyl hydroxynorvaline (β-D-Gal-CH_2-Hnv) was chosen as the key building block for synthesis of the *C*-linked glycopeptide **2** (Scheme 1). It was decided to attempt to prepare **4** from the phosphonium salt **5** and Garner aldehyde (**6**).[15] The latter can be conveniently prepared on a large scale from commercially available D-serine methyl ester in four steps.[16] This strategy is identical to that described recently for the preparation of β-D-galactosyl-CH_2-serine by Dondoni et al.[11h] The alternative approach, in which phosphonium salts derived from D-serine are coupled to an aldehyde prepared from D-galactose, was found to be unsatisfactory by these authors. Use of serine-derived phosphonium salts was therefore not explored by us in the synthesis of **4**.



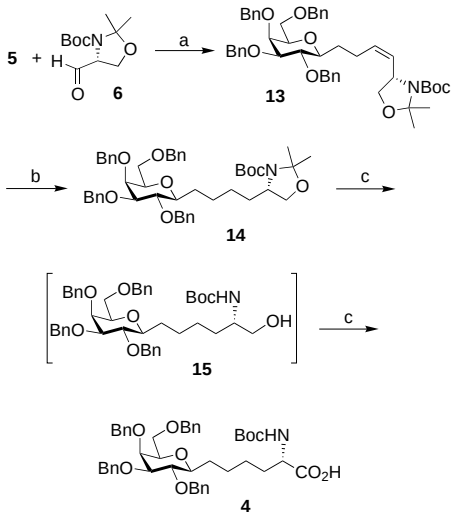
Scheme 1. *Retrosynthetic analysis of the protected β-D-Gal-CH_2-Hnv building block **4**. Bn=benzyl; Boc* = tert-*butoxycarbonyl.*

β-Linked *C*-glycosides have previously been prepared by addition of Grignard reagents to glycopyranolactones, followed by reduction of the resulting hemiacetals.[17] Thus, reaction of lactone **7**[18] with homoallylmagnesium bromide and subsequent reduction of hemiacetal **8** with triethylsilane and boron trifluoride etherate gave homoallylic *C*-galactoside **9** (81% yield from **7**; Scheme 2). As determined by ^{1}H NMR spectroscopy, only the β-D-*C*-galactoside **9** ($^3J_{5,6}$ = 9.1 Hz) was obtained by this sequence of reactions. The stereochemical outcome is most likely due to the Grignard reagent preferably attacking lactone **7** from the less sterically hindered, upper (*re*) face,[19] and also a result of the anomeric effect. Reduction of acetal **8** with triethylsilane[20] then occurs with retention of configuration. Ozonolysis of alkene **9** followed by treatment with triphenylphosphane provided aldehyde **10**, which was reduced with sodium borohydride to afford alcohol **11** (82% from **9**). Reductive workup of the ozonide obtained from **9** with sodium borohydride[21] constitutes a more straightforward approach that gave **11** in 91% yield. Finally, alcohol **11** was converted into the corresponding iodide **12** (88%) by reaction with iodine, triphenylphosphane, and imidazole,[22] after which **12** was transformed into phosphonium salt **5** (86%) by heating in a melt of triphenylphosphane at 120 °C.[23]



Scheme 2. *Synthesis of phosphonium salt **5**. a) Homoallylmagnesium bromide, THF, −78 →0 °C; b) Et_3SiH, $BF_3 \cdot Et_2O$, CH_3CN, −40 °C →r.t., 81% over steps a) and b); c) O_3, PPh_3, CH_2Cl_2, −78 °C →r.t., 88%; d) $NaBH_4$, Et_2O/MeOH (2:1), 0 °C, 93%; e) O_3, $NaBH_4$, CH_2Cl_2/MeOH (2:5), −78 °C →r.t., 91%; f) PPh_3, imidazole, I_2, toluene, 88%; g) PPh_3, 120 °C, 86%. r.t. = room temperature.*

In order to assemble the β-D-Gal-CH_2-Hnv building block, phosphonium salt **5** was deprotonated with potassium hexamethyldisilazane (KHMDS) to form the corresponding ylide. This was coupled with Garner aldehyde (**6**)[15, 16] in a Wittig reaction to give *Z*-alkene **13** together with a small amount of the corresponding *E*-isomer (71%; *Z*:*E* > 14:1 as determined by ^{1}H NMR spectroscopy), as expected under salt-free conditions (Scheme 3). The mixture of alkenes was directly submitted to hydrogenation without further separation. A first attempt to reduce the double bond with in situ generated diimide[11a, 24] did not result in any reaction and **13** was recovered. Hydrogenation



Scheme 3. *Synthesis of building block **4**. a) KHMDS, phosphonium salt **5**, THF, −45 °C →r.t., 71%; b) H_2 (1 bar), $Pd(OH)_2$/C, EtOAc/MeOH (4:1), 81%; c) Jones reagent, acetone, 0 °C →r.t., 66%.*

over Pd/C also proved to be difficult. Use of ethyl acetate as solvent and H_2 at atmospheric pressure gave only unreacted **13**, whereas higher pressure (5 bar) led to complete hydrogenolysis of the benzyl ether groups in addition to saturation of the double bond. Pearlman's catalyst ($Pd(OH)_2$/C) is known to possess a reduced catalytic activity for hydrogenolysis of benzyl ethers when employed under non-acidic conditions.[11e, 25] Indeed, when using Pearlman's catalyst, only reduction of the double bond in alkene **13** (and in the corresponding *E*-isomer) was observed, providing **14** in 81% yield. Treatment of **14** under Jones oxidation conditions resulted in both hydrolysis of the oxazolidine ring and oxidation of the resulting amino alcohol **15** to give the desired protected β-D-galactosyl-CH_2-hydroxynorvaline building block **4** (66%). Overall, the synthetic sequence described above provided building block **4** in eight steps and a total yield of 19% based on galactopyranolactone **7**.

In order to allow immunological studies, amino acid **4** was incorporated into the type II collagen fragment CII(256 – 270) to give *C*-linked glycopeptide **2** (see Figure 1) by using solid-phase glycopeptide synthesis.[26] A combination of the *tert*-butoxycarbonyl (Boc) and 9-fluorenylmethoxycarbonyl (Fmoc) protocols was used in this synthesis. In addition to avoiding protective-group manipulations of **4**, this allowed us to minimize trifluoroacetic acid(TFA)-induced partial cleavage from the solid phase during assembly, which may be problematic when employing the Boc protocol. The synthesis was performed on a Merrifield resin to which N^{α}-Boc-N^{ε}-(2-Cl-Cbz)-Lys (Cbz = benzyloxycarbonyl) was preloaded. After TFA-induced removal of the Boc group from Lys^{270} all amino acids, with the exception of Glu^{266} and β-D-Gal-CH_2-Hnv^{264}, were attached to the peptide resin carrying an N^{α}-Fmoc protective group. When assembly had been completed, peptide **2** was cleaved and fully deprotected in one step by treatment with triethylsilyl trifluoromethanesulfonate (TESOTf) in TFA containing thioanisole as cation scavenger.[27] The high stability of the linkage between the galactose residue and the peptide moiety permits such drastic conditions and demonstrates the advantage of *C*-glycopeptides over *O*- or *N*-linked glycopeptides. Purification of the crude product by reversed-phase HPLC gave **2** in 19% overall yield based on the resin capacity.

A panel of 29 T-cell hybridomas has previously been obtained after immunization of DBA/1 and C3H.Q mice with native rat CII.[4] The hybridomas were selected solely according to their reactivity toward heat-denatured rat CII in culture; that is, without any CII fragment or peptide selection in vitro. Recent investigations have revealed that more than two thirds of the hybridomas (20 out of 29) obtained from mice immunized with CII responded to glycopeptide **1**,[5a] in which Hyl^{264} carries a β-D-galactopyranosyl residue.[5, 28] When the specificity of the 20 hybridomas was investigated further, it was found that three of them responded equally well to glycopeptide **3**,[29] revealing that the ε-amino group of Hyl^{264} in **1** is not part of the epitope recognized by this subset of hybridomas.[14]

The response and specificity of the three hybridomas, which responded to both **1** and **3**, was now investigated with *C*-linked glycopeptide **2**. This was done by incubating each hybridoma for 24 h with dilution series of glycopeptides **1** – **3** in flat-bottom

microtiter plate wells containing syngenic spleen cells as antigen-presenting cells. Recognition of glycopeptide – MHC complexes on the spleen cell surface results in secretion of interleukin-2 (IL-2) by the hybridomas in amounts that depend on the dose of glycopeptide added to each well and on the specificity of the recognition. It should be pointed out that IL-2 is the major autocrine growth factor for T cells, and that the amounts of IL-2 produced by activated helper T cells reflects the magnitude of the immune response. After incubating for 24 h, aliquots of the IL-2-containing supernatants were removed from the wells and frozen in order to kill any transferred T-cell hybridomas. Secreted IL-2 was then determined in a radioassay[30] in which the thawed supernatant was incubated with the T-cell clone CTLL. This results in an IL-2-dependent proliferation of CTLL, which is quantified by addition of [^{3}H]thymidine.

Two of the hybridomas (HM1R.1 and HM1R.2, see Figures 2a and b, respectively) responded by secreting IL-2 at lower concentrations of **1** and **3** than the third hybridoma (HDC.1, Figure 2c). Interestingly, these two strong responders also recognized *C*-linked glycopeptide **2**, but at 10- to 20-fold higher concentrations than required to reach the same levels of IL-2 secretion as with **1** and **3**.[31] Thus, even a minor structural change such as replacement of an oxygen atom by a methylene group in a T-cell epitope has a significant influence on the T-cell response. The weakly responding hybridoma HDC.1 did not show any stimulation on incubation with **2**, but it is possible that this could have been detected if higher concentrations of **2** had been evaluated. All three hybridomas responded well to CII, but not to peptide CII(256 – 270), which were included as positive and negative controls, respectively. As expected, *C*-linked glycopeptide **2** failed to stimulate a hybridoma (HCQ.10) which has previously[5] been shown to respond to glycopeptide **1** but not to **3** (data not shown).

T cells are known to be highly specific in their recognition of complexes between MHC molecules and peptide antigens. Indeed, 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, ranging from induction of selective stimulatory functions to completely turning off the functional capacity of the cell.[10, 32] Peptides in which TCR contact sites have been manipulated, but which retain the capactity to activate some TCR-mediated effector functions, have been termed altered peptide ligands (APLs, see Introduction).[10a] Importantly, such selective activation may result in induction of anergy, that is, a reduced ability of the T cell to respond to a subsequent exposure to the stimulatory antigen. It can also lead to T-cell antagonism, defined as down-modulation of agonist-induced T-cell proliferation when both agonist and APL are simultaneously presented to the T cell. These results suggest that APLs could be used in the immunotherapy of autoimmune diseases, and promising results have already been obtained in animal models of experimental autoimmune encephalomyelitis.[9] Recently, the nature of the effector functions induced by a peptide ligand have been found to correlate with the half-life of the short-lived ternary complexes formed between the TCR and the peptide-liganded MHC complex.[10b, 32]

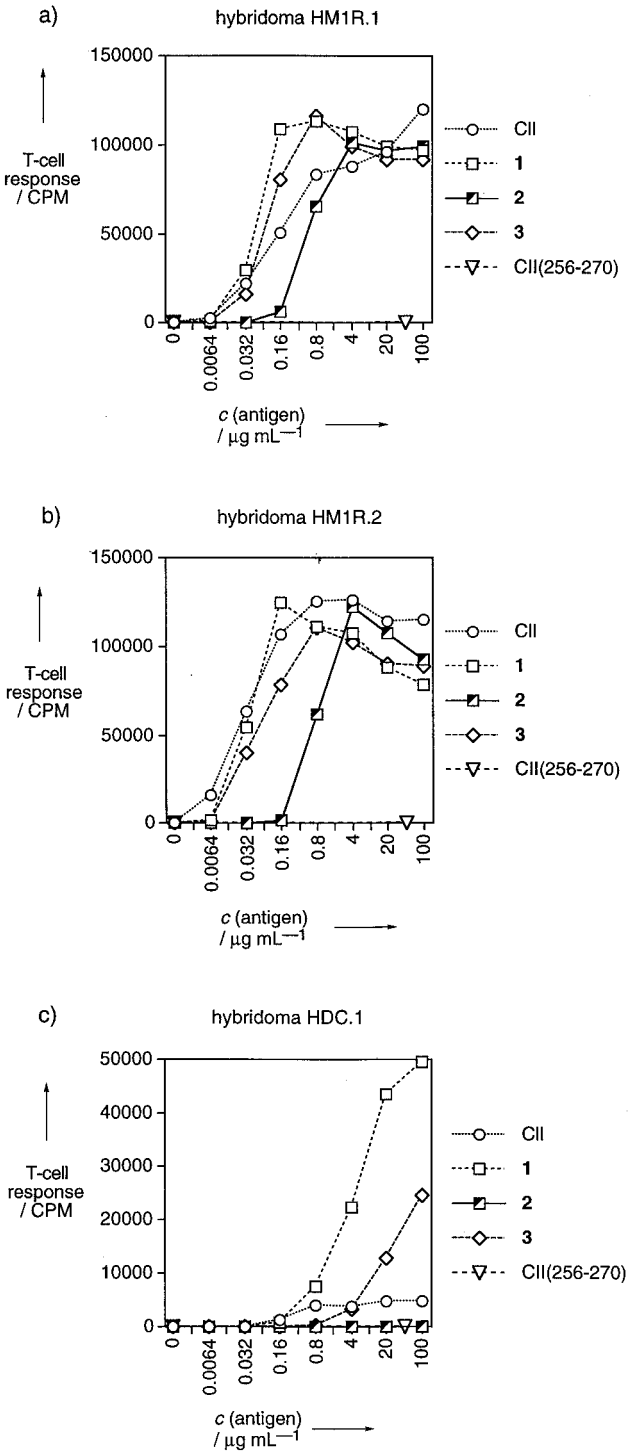


Figure 2. *The response of three T-cell hybridomas that have previously been shown to respond to glycopeptide **3**,[14] as well as to **1** and CII. The hybridomas were incubated with antigen-presenting spleen cells and increasing concentrations of type II collagen (CII) and glycopeptides **1** – **3**. Peptide CII(256 – 270), which had a lysine at position 264, was only evaluated at one concentration (50 μg mL^{-1}). Recognition of glycopeptide – MHC complexes on the surface of APCs by a hybridoma resulted in secretion of IL-2, which was subsequently determined in a radioassay.[30] a, b) Hybridomas HM1R.1 and HM1R.2 both show equal responses to glycopeptides **1** and **3**, which have β-D-galactosylated hydroxylysine and hydroxynorvaline, respectively, at position 264 of CII(256 – 270). Both hybridomas also show a weaker response to the C-linked analogue **2**. c) Hybridoma HDC.1 responds to **1** and **3** at higher concentrations than HM1R.1 and HM1R.2, but does not respond to **2**. CPM = counts per minute.*

The present investigation, together with two previous studies,[33] reveals that T cells are able to recognize the carbohydrate moiety of glycopeptide antigens with an equally exquisite specificity as shown for the side chains of peptides. In model systems, it has been found that deoxygenation[33a] or inversion of a specific hydroxy group[33b] in the carbohydrate moiety of a glycopeptide resulted in complete loss of response by T-cell hybridomas. In contrast, as described above, the less drastic replacement of a glycosidic oxygen atom by a methylene group to give a *C*-glycoside reduces the T-cell response, but does not extinguish it. It thus appears that introduction of structural modifications in the galactosylated hydroxylysine at position 264 of CII(256 – 270) constitutes a useful route to altered peptide ligands. Since Gal-Hyl[264] serves as the primary T-cell contact point in CII(256 – 270), such APLs may prove to be useful in attempts to induce tolerance in models for autoimmune rheumatoid arthritis. Moreover, *C*-linked glycopeptides may be ideal in this respect since they combine the property of an APL with increased stability toward chemical and enzymatic degradation.

Conclusion

A *C*-linked isostere of β-D-galactosylated hydroxynorvaline has been prepared in eight steps from perbenzylated galactopyranolactone. The *C*-linked building block was then incorporated at position 264 into the fragment CII(256 – 270) from type II collagen by solid-phase synthesis using a combination of the Boc and Fmoc protective-group strategies. Helper T-cell hybridomas obtained in a mouse model for rheumatoid arthritis responded to the *C*-linked glycopeptide when presented by class II MHC molecules. However, significantly higher concentrations were required as compared to when *O*-linked β-D-galactosylated hydroxynorvaline or hydroxylysine were found at position 264 of CII(256 – 270). This reveals that T cells are able to recognize the carbohydrate moiety of glycopeptide antigens with high specificity. Moreover, structural modifications of β-D-Gal-Hyl[264] in CII(256 – 270) may result in altered peptide ligands that can be used for the induction of tolerance in autoimmune rheumatoid arthritis.

Experimental Section

General: All reactions were carried out in an inert gas atmosphere with dried solvents under anhydrous conditions, unless otherwise stated. Acetonitrile and dichloromethane were distilled over calcium hydride, whereas THF and toluene were distilled over potassium benzophenone and sodium, respectively. DMF was distilled and then dried over 3 Å molecular sieves. Thin-layer chromatography (TLC) was performed on Silica Gel 60 F_{254} (Merck) with detection by UV light and charring with aqueous sulfuric acid or phosphomolybdic acid/ceric sulfate/aqueous sulfuric acid. Flash column chromatography was performed on silica gel (60 Å, 35 – 70 μm). Organic solutions were dried over Na_2SO_4 before being concentrated.

^{1}H and ^{13}C NMR spectra were recorded at 400 and 100 MHz, respectively, in $CDCl_3$ (residual $CHCl_3$ ($\delta_H = 7.27$) or $CDCl_3$ ($\delta_C = 77.0$) as internal standards) at 300 K, unless otherwise specified. The ^{1}H NMR spectrum of *C*-glycopeptide **1** was recorded at 600 MHz in a 9:1 mixture of H_2O and D_2O (H_2O ($\delta_H = 4.98$) as internal standard) at 278 K. For compounds **4** – **14**, first-order chemical shifts and coupling constants were obtained from one-dimensional spectra; carbon and proton signals were assigned from COSY, TOCSY, NOESY, and HETCOR experiments. Signals for aromatic protons and signals that could not be assigned are not reported. For *C*-glycopeptide **2**, COSY,[34a] TOCSY,[34b] and NOESY[34c] experiments were used for assignment of signals and determination of chemical shifts. Ions for positive-mode fast atom bombardment (FAB) mass spectra were produced by a beam of xenon atoms (6 keV) from a matrix of glycerol and thioglycerol. In the amino acid analysis, glutamine was determined as glutamic acid. Optical rotations were measured at 20 °C in $CHCl_3$.

5,9-Anhydro-6,7,8,10-tetra-*O*-benzyl-1,2,3,4-tetradeoxy-D-*glycero*-L-*manno*-dec-1-enitol (9): A solution of lactone **7** (2.72 g, 5.05 mmol) in THF (15 mL) was cooled to −78 °C and treated with a solution of homoallylmagnesium bromide in THF (1 M, 8 mL, 8.0 mmol). After stirring for 1 h, the solution was allowed to reach 0 °C. After stirring for an additional 30 min at 0 °C, NH_4Cl (1.5 g) was added and the solution was stirred a further 5 min. The solids were removed by filtration, and the filter cake was washed with several small portions of diethyl ether. Concentration of the solution gave a yellow solid, to which CH_2Cl_2 (25 mL) and water (25 mL) were added. Aqueous $KHSO_4$ (1 M) was added dropwise until the precipitate disappeared. The organic layer was separated, the aqueous layer was extracted with CH_2Cl_2 (2 × 30 mL), and the combined organic layers were concentrated to give crude **8** as an oil. The oil was dissolved in dry acetonitrile (25 mL) and cooled to −40 °C. $BF_3 \cdot Et_2O$ (1.43 g, 1.28 mL, 10.1 mmol) and Et_3SiH (1.17 g, 1.61 mL, 10.1 mmol) were added, the mixture was stirred for 30 min and then allowed to warm to room temperature. The reaction was quenched with saturated aqueous $NaHCO_3$ and extracted with EtOAc (3 × 30 mL). The organic solution was washed with brine and water and finally concentrated. Flash column chromatography of the residue (*n*-heptane/EtOAc, 3:1) gave **9** (2.38 g, 81 %) as a white amorphous solid. $[\alpha]_D = -2.0$ ($c = 1$); ^{1}H NMR ($CDCl_3$): $\delta = 5.82$ (m, 1H, H-2), 4.99 and 4.66 (2d, 2H, $J = 10.4$ Hz, PhCH_2), 4.99 and 4.65 (2d, 2H, $J = 11.7$ Hz, PhCH_2), 4.97 (m, 2H, H-1), 4.76 and 4.68 (2d, 2H, $J = 11.8$ Hz, PhCH_2), 4.49 and 4.43 (2d, 2H, $J = 11.8$ Hz, PhCH_2), 4.00 (d, 1H, $J_{7,8} = 2.3$ Hz, H-8), 3.69 (dd, 1H, $J_{5,6} = J_{6,7} = 9.1$ Hz, H-6), 3.61 (m, 1H, H-7), 3.57 (m, 1H, H-10b), 3.51 (m, 1H, H-9), 3.50 (m, 1H, H-10a), 3.24 (ddd, 1H, $J_{5,6} = J_{5,4a} = 9.1$, $J_{5,4b} = 2.1$ Hz, H-5), 2.35 – 2.23 (m, 1H, H-3), 2.18 – 2.05 (m, 1H, H-3), 2.01 – 1.90 (m, 1H, H-4), 1.66 – 1.53 (m, 1H, H-4); ^{13}C NMR ($CDCl_3$): $\delta = 138.9$, 138.6 (C-2), 138.5, 138.5, 138.1, 128.5, 128.5, 128.5, 128.3, 128.3, 128.2, 128.0, 127.8, 127.8, 127.7, 127.6, 114.6 (C-1), 85.1 (C-7), 79.3 (C-6), 79.1 (C-5), 77.2 (C-9), 75.6 (OCH_2Ph), 74.5 (OCH_2Ph), 73.8 (C-8), 73.6 (OCH_2Ph), 72.4 (OCH_2Ph), 69.2 (C-10), 31.1 (C-4), 29.9 (C-3); HR-MS (FAB): calcd for $C_{38}H_{42}NaO_5$ 601.2930 $[M+Na]^+$, found 601.2944.

4,8-Anhydro-5,6,7,9-tetra-*O*-benzyl-2,3-dideoxy-D-*glycero*-L-*manno*-nononal (10): A solution of **9** (1.79 g, 3.09 mmol) in CH_2Cl_2 (20 mL) was cooled to −78 °C and O_3 was bubbled through the solution until the colour became slightly blue. The excess of O_3 was purged by bubbling O_2 through the solution until it became colourless. Triphenylphosphane (816 mg, 3.11 mmol) was added and the reaction mixture was allowed to reach room temperature overnight. The solvent was evaporated under reduced pressure and the resulting oil was submitted to flash column chromatography (*n*-heptane/EtOAc, 3:1 → 2:1) to give **10** (1.54 g, 88 %) as a viscous liquid. $[\alpha]_D = -13.7$ ($c = 0.6$); ^{1}H NMR ($CDCl_3$): $\delta = 9.70$ (t, 1H, $J_{1,2} = 1.7$ Hz, H-1), 4.96 and 4.66 (2d, 2H, $J = 10.8$ Hz, PhCH_2), 4.93 and 4.62 (2d, 2H, $J = 11.5$ Hz, PhCH_2), 4.75 and 4.67 (2d, 2H, $J = 11.6$ Hz, PhCH_2), 4.45 and 4.40 (2d, 2H, $J = 11.8$ Hz, PhCH_2), 3.98 (d, 1H, $J_{6,7} = 2.8$ Hz,

H-7), 3.68 (t, 1H, $J_{5,6} = J_{4,5} = 9.2$ Hz, H-5), 3.58 (dd, 1H, $J_{5,6} = 9.2$, $J_{6,7} = 2.8$ Hz, H-6), 3.50 (m, 3H, H-8, H-9a, b), 3.22 (ddd, 1H, $J_{4,3a} = 2.7$, $J_{4,3b} = 9.2$ Hz, H-4), 2.56 – 2.41 (m, 2H, H-2), 2.20 (m, 1H, H-3), 1.79 (m, 1H, H-3); ^{13}C NMR ($CDCl_3$): $\delta = 202.7$ (C-1), 138.8, 138.3, 138.3, 138.0, 128.5, 128.5, 128.5, 128.4, 128.3, 128.3, 128.2, 128.0, 127.9, 127.8, 127.7, 127.6, 84.8 (C-6), 78.7 (C-4), 78.6 (C-5), 77.1 (C-8), 75.5 (OCH_2Ph), 74.6 (OCH_2Ph), 73.7 (C-7), 73.6 (OCH_2Ph), 72.3 (OCH_2Ph), 68.9 (C-9), 40.4 (C-2), 24.8 (C-3); HR-MS (FAB): calcd for $C_{37}H_{40}NaO_6$ 603.2723 $[M+Na]^+$, found 603.2723.

4,8-Anhydro-5,6,7,9-tetra-*O*-benzyl-2,3-dideoxy-D-*glycero*-L-*manno*-nonitol (11): *Method A*: A solution of **10** (1.37 g, 2.36 mmol) in diethyl ether (20 mL) and MeOH (10 mL) was cooled to 0 °C and $NaBH_4$ (90 mg, 2.38 mmol) was added. After 30 min a second portion of $NaBH_4$ (36 mg, 0.95 mmol) was added. Stirring was continued for an additional 30 min at 0 °C, then the reaction was quenched with acetone (5 mL) and the solvents were evaporated under reduced pressure. Flash column chromatography (*n*-heptane/EtOAc, 4:1 →1:1) gave **11** (1.28 g, 93%) as a viscous liquid. *Method B*: A solution of **9** (173 mg, 0.30 mmol) in MeOH (5 mL) and CH_2Cl_2 (2 mL) was cooled to −78 °C, and O_3 was bubbled through the solution until it became blue. The excess of O_3 was purged by a stream of O_2, and the ozonide was reduced by addition of $NaBH_4$ (33 mg, 0.87 mmol). After stirring for 10 min at −78 °C the solution was allowed to reach room temperature, and after an additional 1 h aqueous HCl (5%, 10 mL) was added. The aqueous layer was extracted with diethyl ether (3 × 10 mL), and the combined organic phases were concentrated. Flash column chromatography (*n*-heptane/EtOAc, 4:1 →1:1) gave **11** (159 mg, 91%) as a viscous liquid. $[\alpha]_D = +7.2$ ($c = 1$); ^{1}H NMR ($CDCl_3$): $\delta = 4.99$ and 4.67 (2d, 2H, $J = 10.9$ Hz, PhC*H*$_2$), 4.96 and 4.65 (2d, 2H, $J = 10.9$ Hz, PhC*H*$_2$), 4.78 and 4.70 (2d, 2H, $J = 11.7$ Hz, PhC*H*$_2$), 4.49 and 4.40 (2d, 2H, $J = 11.8$ Hz, PhC*H*$_2$), 3.96 (d, 1H, $J_{6,7} = 1.7$ Hz, H-7), 3.72 (t, 1H, $J_{6,5} = J_{5,4} = 9.5$ Hz, H-5), 3.63 (m, 3H, H-6, H-1a, b), 3.56 (m, 2H, H-8, 9), 3.43 (m, 1H, H-9), 2.51 (br. s, 1H, O*H*), 2.05 (m, 1H, H-3), 1.72 (m, 2H, H-2), 1.61 (m, 1H, H-3); ^{13}C NMR ($CDCl_3$): $\delta = 138.3$, 138.1, 138.1, 137.6, 128.4, 128.3, 128.2, 128.2, 128.1, 128.0, 127.8, 127.7, 127.6, 127.6, 127.5, 84.6 (C-6), 79.9 (C-4), 78.6 (C-5), 77.0 (C-8), 75.5 (OCH_2Ph), 74.3 (OCH_2Ph), 73.4 (OCH_2Ph), 73.2 (C-7), 72.1 (OCH_2Ph), 69.0 (C-9), 62.7 (C-1), 29.5 (C-2), 28.6 (C-3); HR-MS (FAB): calcd for $C_{37}H_{42}NaO_6$ 605.2879 $[M+Na]^+$, found 605.2886.

4,8-Anhydro-5,6,7,9-tetra-*O*-benzyl-1,2,3-trideoxy-1-iodo-D-*glycero*-L-*manno*-nonitol (12): Triphenylphosphane (1.06 g, 4.05 mmol), imidazole (310 mg, 4.55 mmol), and iodine (814 mg, 3.21 mmol) were added to a stirred solution of **11** (988 mg, 1.70 mmol) in toluene (20 mL). The reaction mixture was stirred for 1 h at room temperature, diluted with diethyl ether (20 mL), and washed with aqueous $Na_2S_2O_3$ (5%, 10 mL). The aqueous layer was extracted with diethyl ether (20 mL), and the combined organic layers were concentrated. The residue was purified by flash column chromatography (*n*-heptane/EtOAc, 40:1 →1:1) to provide **12** (1.04 g, 88%) as a viscous liquid. $[\alpha]_D = -5.4$ ($c = 1$); ^{1}H NMR ($CDCl_3$): $\delta = 4.96$ and 4.68 (2d, 2H, $J = 10.8$ Hz, PhC*H*$_2$), 4.96 and 4.65 (2d, 2H, $J = 11.9$ Hz, PhC*H*$_2$), 4.78 and 4.69 (2d, 2H, $J = 11.7$ Hz, PhC*H*$_2$), 4.48 and 4.43 (2d, 2H, $J = 11.9$ Hz, PhC*H*$_2$), 3.99 (d, 1H, $J_{6,7} = 2.6$ Hz, H-7), 3.68 (t, 1H, $J_{5,6} = J_{4,5} = 9.4$ Hz, H-5), 3.60 (dd, $J_{6,7} = 2.6$, $J_{5,6} = 9.4$ Hz, 1H, H-6), 3.57 (m, 1H, H-9), 3.53 (m, 1H, H-8), 3.52 (m, 1H, H-9), 3.21 (m, 1H, H-4), 3.20 and 3.14 (2m, 2H, H-1a, b), 2.07 (m, 1H, H-3), 2.04 and 1.98 (2m, 2H, H-2a, b), 1.51 – 1.60 (m, 1H, H-3); ^{13}C NMR ($CDCl_3$): $\delta = 138.8$, 138.4, 138.4, 138.1, 128.5, 128.4, 128.3, 128.3, 128.0, 127.9, 127.8, 127.7, 127.7, 85.0 (C-6), 78.9 (C-5), 78.9 (C-4), 77.2 (C-8), 75.6 (OCH_2Ph), 74.6 (OCH_2Ph), 73.8 (C-7), 73.6 (OCH_2Ph), 72.4 (OCH_2Ph), 69.2 (C-9), 32.9 (C-3), 30.1 (C-2), 7.3 (C-1); HR-MS (FAB): calcd for $C_{37}H_{41}INaO_5$ 715.1896 $[M+Na]^+$, found 715.1897.

(4,8-Anhydro-5,6,7,9-tetra-*O*-benzyl-1,2,3-trideoxy-D-*glycero*-L-*manno*-nonitol-1-yl)triphenylphosphonium iodide (5): The iodide **12** (660 mg, 0.95 mmol) was treated with triphenylphosphane (1.25 g, 4.76 mmol) for 2 h at 120 °C, then cooled to room temperature and submitted to flash column chromatography (CH_2Cl_2/MeOH, 1:0 →0:1) to give **5** (777 mg, 86%) as a white amorphous solid. $[\alpha]_D = -29.4$ ($c = 1$); ^{1}H NMR ($CDCl_3$): $\delta = 4.91$ and 4.59 (2d, 2H, $J = 10.8$ Hz, PhC*H*$_2$), 4.88 and 4.58 (2d, 2H, $J = 11.4$ Hz, PhC*H*$_2$), 4.75 and 4.68 (2d, 2H, $J = 12.0$ Hz, PhC*H*$_2$), 4.37 – 4.28 (m, 2H, PhC*H*$_2$), 3.93 (br. s, 1H, H-7), 3.64 (m, 1H, H-8), 3.62 (m, 2H, H-5, H-6), 3.56 (m, 1H, H-1), 3.49 (m, 2H, H-1, H-9), 3.36 (m, 2H, H-4, H-9), 2.16 (m, 1H, H-3b), 1.78 – 1.66 (m, 3H, H-3a, H-2a, b); ^{13}C NMR ($CDCl_3$): $\delta = 138.6$, 138.3, 138.3, 137.9, 135.2 (d, $^4J_{P,C} = 3.0$ Hz, *p*-C in Ph_3P), 133.7 (d, $J_{P,C} = 10.1$ Hz, *o*-C or *m*-C in Ph_3P), 130.6 (d, $J_{P,C} = 12.8$ Hz, *m*-C or *o*-C in Ph_3P), 128.6, 128.5, 128.4, 128.4, 128.3, 127.9, 127.9, 127.8, 127.7, 127.7, 118.0 (d, $^1J_{P,C} = 85.9$ Hz, *i*-C in Ph_3P), 84.6 (C-6), 79.1 (C-4), 78.2 (C-5), 77.0 (C-8), 75.3 (OCH_2Ph), 74.7 (OCH_2Ph), 73.9 (C-7), 73.4 (OCH_2Ph), 72.4 (OCH_2Ph), 69.4 (C-9), 31.4 (d, $J_{P,C-3} = 15.7$ Hz, C-3), 22.7 (d, $J_{P,C-1} = 50.9$ Hz, C-1), 19.3 (d, $J_{P,C-2} = 3.4$ Hz, C-2); HR-MS (FAB): calcd for $C_{55}H_{56}O_5P$ 827.3865 $[M]^+$, found 827.3869.

(*Z*)-7,11-Anhydro-8,9,10,12-tetra-*O*-benzyl-2,3,4,5,6-pentadeoxy-1,2-*N*,*O*-isopropylidene-2-(*tert*-butoxycarbonylamino)-D-*threo*-L-*galacto*-dodec-3-enitol (13): A solution of **5** (900 mg, 0.94 mmol) in THF (15 mL) was cooled to −45 °C and potassium hexamethyldisilazane (3.1 mL of a 0.33 M solution in toluene, 1.02 mmol) was added. The mixture was stirred for 10 min and aldehyde **6** (237 mg, 1.03 mmol) in THF (2 mL) was then slowly added to the yellow solution. The mixture was allowed to reach −25 °C in 1.5 h and then room temperature after removal of the cooling bath. The mixture was diluted with diethyl ether (50 mL) and quenched with phosphate buffer (20 mL, pH 7). Concentration and flash column chromatography (*n*-heptane/EtOAc, 10:1 →1:1) gave **13** as a viscous liquid (519 mg, 71%), which contained a small amount of the corresponding *E*-alkene (< 5%); the following data refer to the *Z*-isomer only. ^{1}H NMR ([D$_6$]DMSO, 110 °C): $\delta = 5.39$ (dd, 1H, $J_{3,4} = 10.5$, $J_{4,5} = 7.2$ Hz, H-4), 5.30 (dd, 1H, $J_{3,4} = 10.5$, $J_{2,3} = 9.4$ Hz, H-3), 4.84 and 4.60 (2d, 2H, $J = 11.6$ Hz, PhC*H*$_2$), 4.82 and 4.54 (2d, 2H, $J = 11.4$ Hz, PhC*H*$_2$), 4.77 and 4.64 (2d, 2H, $J = 12.0$ Hz, PhC*H*$_2$), 4.51 and 4.46 (2d, 2H, $J = 12$ Hz, PhC*H*$_2$), 4.57 – 4.46 (m, 1H, H-2), 4.00 (br. s, 1H, H-10), 3.94 (dd, 1H, $J_{1a,1b} = 8.6$, $J_{1a,2} = 6.6$ Hz, H-1), 3.68 (m, 1H, H-9), 3.64 (m, 1H, H-11), 3.59 and 3.54 (2m, 2H, H-12a, b), 3.52 (m, 1H, H-8), 3.24 (ddd, 1H, $J_{6a,7} = J_{7,8} = 8.7$, $J_{6b,7} = 2.4$ Hz, H-7), 2.06 – 2.29 (m, 2H, H-5a, b), 1.87 – 1.76 (m, 1H, H-6), 1.49 – 1.37 (m, 1H, H-6), 1.48 (s, 3H, CH_3), 1.41 (s, 3H, CH_3), 1.35 (s, 9H, *t*Bu); ^{13}C NMR ([D$_6$]DMSO, 110 °C): $\delta = 151.8$ (C=O), 139.7, 139.4, 139.3, 139.1, 131.9 (C-3), 130.8 (C-4), 128.7, 128.6, 128.5, 128.1, 128.0, 128.0, 127.9, 127.8, 127.8, 127.7, 93.6 (N*C*Me$_2$O), 84.7 (C-9), 79.5 (Me$_3$*C*O), 79.3 (C-8), 78.5 (C-7), 77.1 (C-11), 75.4 (C-10), 74.6 (OCH_2Ph), 74.5 (OCH_2Ph), 73.1 (OCH_2Ph), 72.0 (OCH_2Ph), 70.0 (C-12), 69.0 (C-1), 54.8 (C-2), 32.2 and 23.6 (C-5 and C-6), 28.7 (C(*C*H$_3$)$_3$), 27.1 and 25.0 (2*C*H$_3$); HR-MS (FAB): calcd for $C_{48}H_{59}NNaO_8$ 800.4138 $[M+Na]^+$, found 800.4152.

7,11-Anhydro-8,9,10,12-tetra-*O*-benzyl-2,3,4,5,6-pentadeoxy-1,2-*N*,*O*-isopropylidene-2-(*tert*-butoxycarbonylamino)-D-*threo*-L-*galacto*-dodecitol (14): A solution of **13** containing the corresponding *E*-isomer (see above; 444 mg, 0.57 mmol) in EtOAc (8 mL) and MeOH (2 mL) was treated with $Pd(OH)_2/C$[35] (130 mg) under hydrogen (1 bar) at room temperature for 3 h. The catalyst was removed by filtration (Hyflo-Supercel), and the filter cake was washed several times with small portions of MeOH. The combined filtrates were concentrated, and flash column chromatography (*n*-heptane/EtOAc, 6:1 →5:1) of the residue gave **14** (360 mg, 81%). $[\alpha]_D = +4.3$ ($c = 1$); ^{1}H NMR ([D$_6$]DMSO, 110 °C): $\delta = 4.82$ and 4.58 (2d, 2H, $J = 11.2$ Hz, PhC*H*$_2$), 4.81 and 4.53 (2d, 2H, $J = 11.3$ Hz, PhC*H*$_2$), 4.77 and 4.64 (2d,

2H, $J = 11.9$ Hz, PhCH_2), 4.50 and 4.45 (2d, 2H, $J = 12.3$ Hz, PhCH_2), 4.02 (d, 1H, $J_{9,10} = 2.7$ Hz, H-10), 3.84 (dd, 1H, $J_{1a,1b} = 8.6$, $J_{1a,2} = 5.9$ Hz, H-1a), 3.70 (m, 1H, H-2), 3.66 (m, 1H, H-9), 3.61 (m, 1H, H-1b), 3.59 (m, 2H, H-12, H-11), 3.49 (m, 1H, H-12), 3.48 (m, 1H, H-8), 3.17 (ddd, 1H, $J_{7,8} = J_{7,6a} = 9.5$, $J_{7,6b} = 2.7$ Hz, H-7), 1.75 – 1.68 (m, 1H, H-6), 1.53 – 1.64 (m, 1H, H-3), 1.45 (s, 3H, CH_3), 1.39 (m, 1H, H-3), 1.38 (s, 12H, CH_3 and $C(CH_3)_3$), 1.35 (m, 1H, H-6), 1.25 (m, 2H, H-4), 1.16 (m, 2H, H-5); ^{13}C NMR ([D$_6$]DMSO, 110 °C): $\delta = 151.8$ (N(*C*=O)O), 139.7, 139.3, 139.3, 139.0, 128.7, 128.7, 128.6, 128.2, 128.0, 128.0, 127.9, 127.8, 127.8, 127.8, 93.2 (N*C*Me$_2$O), 84.7, (C-9), 79.3 (Me$_3$*C*O), 79.2 (C-8), 79.0 (C-7), 77.1 (C-11), 75.2 (C-10), 74.6 (O*C*H$_2$Ph), 74.5 (O*C*H$_2$Ph), 73.0 (O*C*H$_2$Ph), 71.9 (O*C*H$_2$Ph), 69.9 (C-12), 67.2 (C-1), 57.5 (C-2), 33.5, 32.0, 28.7 ((*C*H$_3$)$_3$C), 27.4 (CH_3), 26.0, 25.5, 24.5 (CH_3); HR-MS (FAB): calcd for $C_{48}H_{61}NNaO_8$ 802.4295 $[M+Na]^+$, found 802.4293.

7,11-Anhydro-8,9,10,12-tetra-*O*-benzyl-2,3,4,5,6-pentadeoxy-2-(*tert*-butoxycarbonylamino)-D-*threo*-L-*galacto*-dodecanoic acid (4): A solution of **14** (340 mg, 0.44 mmol) in acetone (10 mL) was cooled to 0 °C. Freshly prepared Jones reagent[36] (1 M, 1.33 mL, 1.33 mmol) was slowly added, the mixture stirred for 10 min and then allowed to reach room temperature. After 2 h at room temperature the reaction was quenched with isopropyl alcohol (0.5 mL), the pH was adjusted to 7 with saturated aqueous $NaHCO_3$, diethyl ether (100 mL) was added, and the phases were separated. The organic layer was washed with brine (2 × 20 mL) and concentrated. Flash column chromatography (*n*-heptane/EtOAc, 5:1 → 2:1, containing 0.1% HOAc) followed by freeze-drying from benzene gave **4** (219 mg, 66%) as a white fluffy powder. $[\alpha]_D = -1.4$ ($c = 0.54$); ^{1}H NMR ($CDCl_3$): $\delta = 10.55$ (br. s, 1H, COOH), 4.93 and 4.63 (2d, $J = 11.0$ Hz, PhCH_2), 4.92 and 4.62 (2d, $J = 11.8$ Hz, PhCH_2), 4.74 and 4.67 (2d, $J = 11.7$ Hz, PhCH_2), 4.51 and 4.42 (2d, $J = 11.8$ Hz, PhCH_2), 4.97 (br. s, 1H, N*H*), 4.22 (m, 1H, H-2), 3.93 (d, 1H, $J_{10,9} = 2.1$ Hz, H-10), 3.64 (dd, 1H, $J_{8,9} = J_{7,8} = 9.2$ Hz, H-8), 3.58 (m, 1H, H-12), 3.57 (m, 1H, H-9), 3.48 (m, 1H, H-11), 3.46 (m, 1H, H-12), 3.18 (m, 1H, H-7), 1.69 (m, 1H, H-3), 1.46 (m, 1H, H-6), 1.44 (s, 9H, C(CH_3)$_3$), 1.86 – 1.30 (m, 6H, H-6, H-5a, b, H-4a, b, H-3); ^{13}C NMR ($CDCl_3$): $\delta = 175.6$ (C-1), 155.8 (N(*C*=O)O), 138.7, 138.5, 138.4, 137.8, 128.5, 128.5, 128.3, 128.3, 128.2, 128.1, 127.9, 127.8, 127.7, 127.7, 127.6, 85.0 (C-9), 80.3 (Me$_3$*C*O), 79.3 (C-7), 79.2 (C-8), 77.1 (C-11), 75.6 (O*C*H$_2$Ph), 74.4 (O*C*H$_2$Ph), 73.8 (C-10), 73.5 (O*C*H$_2$Ph), 72.4 (O*C*H$_2$Ph), 69.5 (C-12), 53.4 (C-2), 32.2 (C-6 or C-3), 31.5 (C-3 or C-6), 28.4 (C(*C*H$_3$)$_3$), 25.2 (C-5 or C-4), 25.1 (C-4 or C-5); HR-MS (FAB): calcd for $C_{45}H_{55}NNaO_9$ 776.3775 $[M+Na]^+$, found 776.3790.

Glycyl-L-glutam-1-yl-*trans*-4-hydroxy-L-prolylglycyl-L-isoleucyl-L-alanylglycyl-L-phenylalanyl-(2*S*)-6-(*β*-D-galactopyranosyl)-2-aminohexanoylglycyl-L-glutam-1-yl-L-glutaminylglycyl-L-prolyl-L-lysine (2): *Synthesis*: A Merrifield resin (Novabiochem, Läufelfingen, Switzerland), which carried Boc-Lys(2-Cl-Cbz) was used in the synthesis. *N*^{a}-Fmoc-protected amino acids had the following protective groups for their side chains: triphenylmethyl (Trt) for glutamine; *tert*-butyl for glutamic acid and hydroxyproline. *N*^{a}-Boc-glutamic acid was benzyl-protected. DMF was distilled before use. In the synthesis of **2**, 50 μmol of resin was used in a mechanically agitated reactor. The *N*^{a}-Fmoc amino acids and *N*^{a}-Boc-Glu(Bzl)-OH were activated as 1-benzotriazolyl esters.[37] These were prepared in situ by reaction of the amino acid (0.20 mmol), 1-hydroxybenzotriazole (HOBt, 41 mg, 0.30 mmol), and 1,3-diisopropylcarbodiimide (DIC, 30 μL of a 6.5 M solution in DMF, 0.195 mmol) in dry DMF (0.8 mL). After 1 h the activated amino acid was added to the resin together with bromophenol blue (125 nmol, 62.5 mL of a 2 M solution in DMF). The acylation was monitored by the change of colour from blue to pale yellow. When monitoring revealed the coupling to be complete, the resin was washed with DMF. *N*^{a}-Fmoc deprotection of the peptide resin was performed by washing the resin with 20% piperidine in DMF (4 × 2 mL) over a period of 10 – 12 min. After completion of the *N*^{a}-Fmoc deprotection, the peptide resin was again washed with DMF. In case of the *N*^{a}-Boc-protected amino acids Lys^{270}, Glu^{266}, and building block **4**, the resin was washed with CH_2Cl_2 and the Boc group was then cleaved by addition of 25% TFA in CH_2Cl_2. The resin was gently swirled for 20 min, washed with CH_2Cl_2 (5 × 2 mL) over 10 min, swirled with triethylamine in CH_2Cl_2 (1:10) for 20 min, washed with CH_2Cl_2 (3 × 2 mL) over 6 min, and DMF (2 × 2 mL) during 4 min. *C*-Galactosylated amino acid **4** (60 mmol) was activated in DMF (0.8 mL) at room temperature during 40 min by addition of DIC (60 μL of a 1 M solution in DMF, 60 mmol) and 1-hydroxy-7-azabenzotriazole (HOAt, 25 mg, 180 mmol). After completion of the synthesis, the resin carrying protected peptide **1** was washed with CH_2Cl_2 and dried under vacuum. *Cleavage*: The resin-bound peptide (40 mg, 11.4 mmol) was suspended in 1 M thioanisole in TFA (5 mL) and cooled to 0 °C. Triethylsilyl triflate (0.91 mL) was carefully added. After stirring for 1 h at 0 °C, the resin was removed by filtration and washed with TFA (2 × 5 mL). The combined filtrates were concentrated at 15 °C, then 80% TFA in water (12 mL) and HOAc (5 mL) were added and the solution was stirred for 10 min. After concentration, TFA/water/HOAc (1:1:1, 5 mL) was added, the solution was stirred for 5 min and then concentrated. Two portions of HOAc (each 5 mL) were then added, followed by concentration after each addition. Addition of diethyl ether (5 mL) led to precipitation of crude glycopeptide which was dissolved in a mixture of HOAc and water (9:1, 10 mL) and then freeze-dried. *Purification*: Glycopeptide **2** was analysed on a Kromasil C-8 column (100 Å, 5 μm, 4.6 × 250 mm) by using a linear gradient of 0 → 100% of eluant B in eluant A over 80 min with a flow rate of 1.5 mL min^{-1} and detection at 214 nm (eluants: A, 0.1% aqueous TFA; B, 0.1% TFA in MeCN). Purification of crude **2** was performed on Kromasil C-8 column (100 Å, 5 μm, 20 × 250 mm) by using the same eluants and a flow rate of 9 mL min^{-1} to give **2** (5 mg, 19%). The peptide content of purified **2** (72%) was determined by amino acid analysis and has been taken into account when calculating the final overall yield. ^{1}H NMR data for **2** are given in Table 1; MS (FAB): calcd 1635 $[M+H]^+$, found 1634; amino acid analysis: Ala 1.01 (1), Glu 3.07 (3), Gly 5.04 (5), *β*-D-Gal-CH_2-Hnv 0.97 (1), Hyp 0.97 (1), Ile 1.00 (1), Lys 1.02 (1), Phe 1.04 (1), Pro 1.06 (1).

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 antigen [type II collagen, glycopeptides **1** – **3**, peptide CII(256 – 270)], was determined in a standard assay by using the T-cell clone CTLL.[30] 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 tritiated thymidine ([^{3}H]TdR) for an additional 15 – 18 h. The cells were harvested on glassfiber sheets in a Filtermate TM 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.

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Table 1. ^1H NMR data (δ) for glycopeptide **2** in water containing 10% D_2O.[a]

Residue	NH	H-α	H-β	H-γ	H-δ	Others
Gly^{256}		3.86, 3.80				
Glu^{257}	8.70	4.61	1.97, 1.76	2.23[b]		
Hyp^{258}		4.46	2.26, 2.01	4.56		
Gly^{259}	8.67	4.00, 3.87				
Ile^{260}	8.03	4.13	1.81	1.35, 1.11	0.74	0.84 (β-CH_3)
Ala^{261}	8.61	4.22	1.31			
Gly^{262}	8.40	3.81[b]				
Phe^{263}	8.10	4.53	3.04, 2.98			7.24, 7.16 (arom.)
C-Hnv^{264}	8.37	4.17	1.69, 1.60	1.33	1.25	1.44, 1.33 (H-ε), Gal[c]
Gly^{265}	7.85	3.85, 3.78				
Glu^{266}	8.34	4.21	1.98, 1.84	2.22[b]		
Gln^{267}	8.58	4.28	2.08, 1.91	2.30[b]		7.57, 6.88 (CONH_2)
Gly^{268}	8.35	4.09, 3.90				
Pro^{269}		4.34	2.17, 1.89	1.93[b]	3.54[b]	
Lys^{270}	8.15	4.10	1.75	1.35[b]	1.59[b]	2.91[b] (H-ε), 7.49 (ε-NH_2)

[a] Obtained at 600 MHz, 278 K, and pH 5.4 with H_2O as internal standard ($\delta_H = 4.98$). [b] Degeneracy has been assumed. [c] Chemical shifts (δ) for the Gal monosaccharide moiety: 3.85 (H-4) 3.73 (H-6), 3.63 (H-5, 6), 3.50 (H-3), 3.33 (H-2), 3.11 (H-1).

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