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Influence of saccharide size on the cellular immune response to glycopeptides

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Glycopeptides that bind to MHC molecules on antigen presenting cells may elicit carbohydrate selective T cells. In order to investigate how the cellular immune response depends on the size of the carbohydrate moiety, a trigalactosylated derivative of an immunogenic peptide from hen egg-white lysozyme (HEL52–61) was prepared. Synthesis was accomplished by assembly of an α-1,4-linked trigalactose peracetate which was coupled to Fmoc serine. After activation as a pentafluorophenyl ester the resulting building block was used in solid-phase synthesis. In contrast to the corresponding mono- and digalactosylated derivatives of HEL52–61, the trigalactosylated HEL52–61 was not immunogenic. Somewhat surprisingly, this was found to be because the trigalactosyl derivative bound approximately two orders of magnitude weaker to I-A^k MHC molecules than the mono- and digalactosyl peptides. Our observation suggests an explanation for previous findings, which show that glycopeptides isolated from MHC molecules in nature usually carry small saccharides.

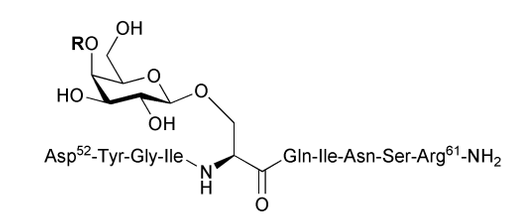
Introduction

Proper function of the immune system of higher vertebrates requires processing of protein antigens into shorter peptides in antigen presenting cells.[1–3] 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 immune responses that depend on the origin of protein antigen and also on the type of antigen presenting cell and T cell that is involved.

Most eucaryotic proteins and many viral proteins carry oligosaccharide chains; a fact which has generated interest in the role(s) of the saccharides during antigen processing, presentation and recognition by T cells.[4–7] Model studies performed with synthetic neoglycopeptides have revealed that mono- or small oligosaccharides can be attached to T cell immunogenic peptides which retain MHC binding if the position for the glycan is chosen carefully.[8–13] These studies also demonstrated that immunization of mice with neoglycopeptides can elicit T cells which specifically recognize the carbohydrate moiety if it is located in the center of the peptide. Recently, some reports have also described that glycoproteins found in nature can be degraded to glycopeptides in antigen presenting cells and presented to T cells as complexes with MHC molecules.[14–20] In most, but not all, of the cases that involve natural glycopeptides, the carbohydrate moities attached to the peptides have been found to be small, *i.e.* mono- or disaccharides. This may be considered as somewhat unexpected since both *N*- and *O*-linked carbohydrates found on proteins in general are substantially larger, usually consisting of at least 5–10 monosaccharide units.[21,22] Processing, *i.e.* degradation, of the carbohydrate moieties in the antigen presenting cell constitutes a possible explanation for the small size of oligosaccharides found on natural glycopeptides presented by MHC molecules. Alternatively, glycopeptides that carry large oligosaccharides may bind with low affinity to MHC molecules, or the T cell receptor may be unable to accommodate larger oligosaccharides while still maintaining contact with the surrounding MHC molecule.

The aim of the present study was to investigate how the cellular immune response elicited by glycopeptides depends on the size of the carbohydrate moiety. To avoid any structural differences between the saccharides, apart from size, affecting the immune responses the saccharides were chosen to have closely related structures, *i.e.* mono-, di- and trigalactosides. We decided to use a model system based on the peptide HEL52–61 (**1**, Fig. 1), which is presented to CD4 T cells by I-A^k class II MHC molecules on antigen presenting cells after immunization of mice with hen egg-white lysozyme (HEL).[23,24] In glycopeptides **2**–**4** the galactoside moieties are linked to a serine which has been inserted in place of Leu^{56} in the T cell immunogenic peptide HEL52–61 (**1**). This position was chosen since the crystal structure of the complex between I-A^k and **1** revealed that residue 56 is located in the center of the complex, with the side-chain pointing away from the binding site of I-A^k.[25] In this location the saccharide should thus be ideally located to allow binding to I-A^k as well as carbohydrate-specific interactions with the T cell receptor. In agreement with this expectation,

1 Asp^{52}-Tyr-Gly-Ile-Leu-Gln-Ile-Asn-Ser-Arg^{61}-NH_2 (HEL52-61)



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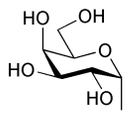
3 R =



4 R =

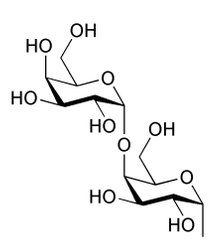


Fig. 1 Peptide **1** and neoglycopeptides **2**–**4** were used to study the influence of the size of the oligosaccharide residue for the cellular immune response elicited on immunization of mice.

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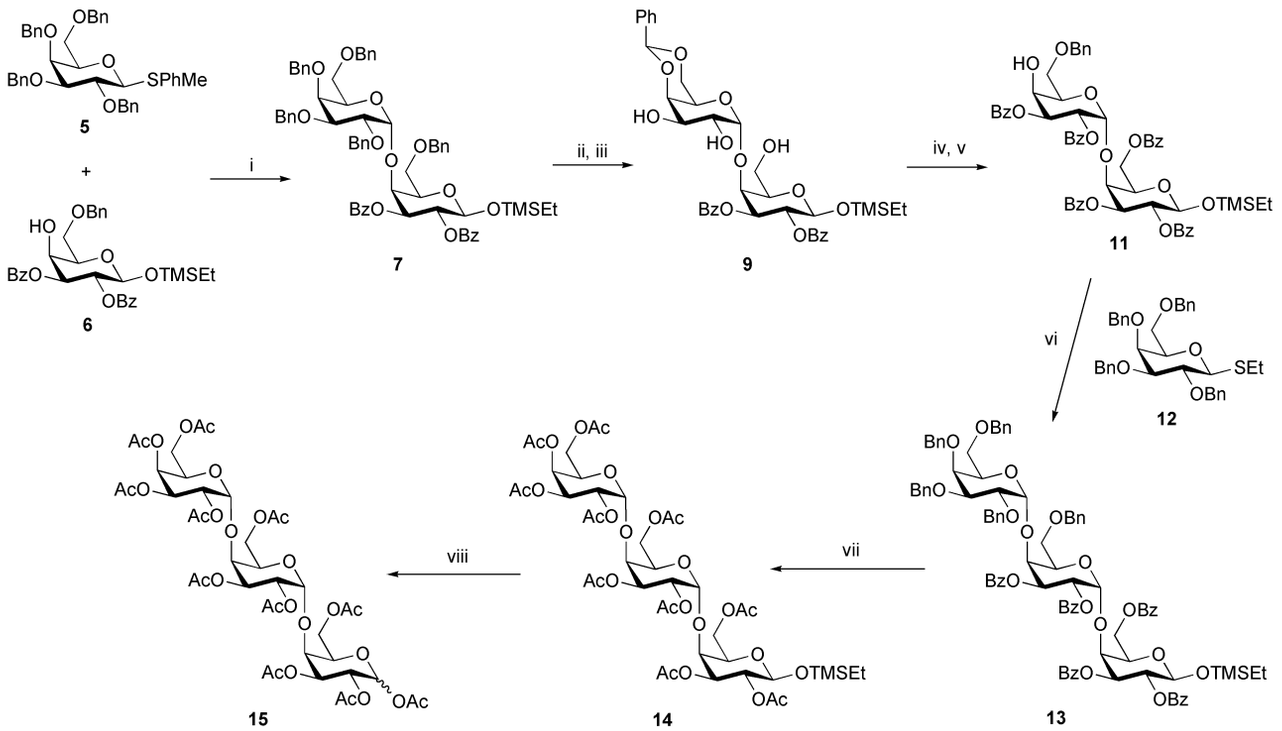


Scheme 1 *Reagents, conditions and yields*: i) NIS, TfOH, 4 Å molecular sieves, CH_2Cl_2–Et_2O (1 : 1, v/v), −45 °C (84%); ii) H_2, 10% Pd/C, HOAc (⟶**8**, quant.); iii) α,α-dimethoxytoluene, *p*-TsOH, MeCN (63%); iv) BzCl, pyridine, 0 °C ⟶ room temp. (⟶**10**, 96%); v) $NaCNBH_3$, HCl (g) in Et_2O, 4 Å molecular sieves, THF (85%); vi) NIS, TfOH, 4 Å molecular sieves, CH_2Cl_2–Et_2O (1 : 1, v/v), −45 °C (77%); vii) NaOMe, MeOH; then H_2, 10% Pd/C, HOAc; then Ac_2O, pyridine (72%); viii) $BF_3{\cdot}Et_2O$, Ac_2O–toluene (1 : 1, v/v), 0 °C (86%, α–β, 1 : 1).

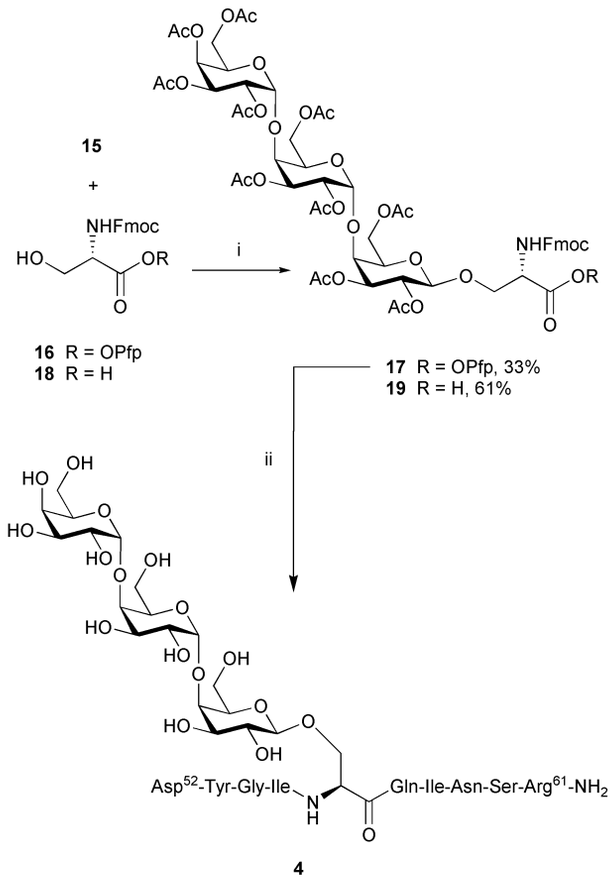
synthetic neoglycopeptides[26] **2** and **3** have previously been shown to bind to I-A^k class II MHC molecules with an affinity close to that of peptide **1**. Moreover, immunization of mice with **3** elicited T cells that recognized the digalactosyl moiety with high specificity.[10,27]

Results and discussion

Synthesis of the trigalactoside moiety of glycopeptide **4** was begun by glycosylation of acceptor **6**[28] with donor **5**[29] under promotion[30,31] by *N*-iodosuccinimide and trifluoromethanesulfonic acid (Scheme 1). Several portions of the promoters had to be added to achieve complete conversion of **6**; this was assumed to be because the molecular sieves adsorbed the promoters. When the glycosylation was performed in a mixture of dichloromethane and diethyl ether (1 : 1) the α-linked disaccharide **7** was obtained in 84% yield. Hydrogenation of **7** over Pd/C followed by protection of HO-4′ and HO-6′ with a benzylidene acetal gave **9** in a modest yield (63% over two steps). Benzoylation of **9** (⟶**10**, 96%) and subsequent reductive opening of the benzylidene acetal by treatment with sodium cyanoborohydride and ethereal hydrochloric acid liberated HO-4′ to give acceptor **11** (85%). Attempts to glycosylate **11** with thiocresyl galactoside **5**, using *N*-iodosuccinimide and trifluoromethanesulfonic acid as promoters, were unsuccessful under a variety of conditions. Fortunately, replacement of **5** with the more reactive thioethyl galactoside **12**[32] furnished the desired α-1,4-linked trigalactoside **13** as the only isolated product in an excellent yield (77%). Trisaccharide **13** was then converted into a glycosyl donor in four steps. Debenzoylation with methanolic sodium methoxide, followed by hydrogenolysis of the benzyl groups and acetylation with acetic anhydride in pyridine gave **14** in good overall yield (72%). Treatment of 2-trimethylsilylethyl glycoside **14** with acetic anhydride in toluene under activation with boron trifluoride etherate gave the anomeric mixture of acetates **15** (86%, α–β, 1 : 1).

The first attempt to convert acetates **15** into a glycosylated amino acids involved reaction with N^{α}-Fmoc-serine pentafluorophenyl ester **16** (Scheme 2). Boron trifluoride etherate promoted glycosylation[33,34] of **16** with **15** in acetonitrile provided building block **17** in a rather poor yield (33%). The low yield was explained by the fact that the crude product had to be purified three times with radial chromatography to remove the unreacted α-anomer of **15**. This lead to substantial loss of material because the pentafluorophenyl ester in **17** was hydrolysed slowly on the silica gel matrix. The purification problem could be circumvented by glycosylation of N^{α}-Fmoc serine (**18**) instead of pentafluorophenyl ester **16**. When the glycosylation was performed under the same conditions as in the synthesis of **17**, glycosylated serine **19** was obtained in 61% yield after purification with reversed phase HPLC.

Synthesis of the glycosylated HEL-peptide **4** was performed manually on a polystyrene resin grafted with polyethylene glycol spacers (TentaGel S NH_2). The resin was functionalized with the Rink amide linker[35,36] and DMF was used as solvent throughout the synthesis. The N^{α}-Fmoc amino acids (4 equiv.) were coupled to the resin activated as the corresponding benzotriazolyl esters.[37] Surprisingly, only building block **17** (1.3 equiv.) could be used in the synthesis of **4**. Attempts to use **19**, after preactivation with 1-hydroxy-7-azabenzotriazole[38] (HOAt) and *N*,*N*′-diisopropyl carbodiimide in DMF, resulted in migration of one of the actyl protective groups to the *N*-terminus of the peptide-resin thus terminating peptide growth. However, pentafluorophenyl esters, such as **17**, may be prepared *in situ* from the corresponding carboxylic acids and used directly in solid-phase peptide synthesis thereby circumventing this problem.[39] All couplings were monitored using bromophenol blue as indicator of free amino groups and removal of N^{α}-Fmoc protecting groups was accomplished with piperidine (20% in DMF). After completion of the solid-phase synthesis the glycopeptide was cleaved from the solid support and the amino acid side chains were simultaneously deprotected by treatment with trifluoroacetic acid containing water (5%), thioanisole (5%) and ethanedithiol (2.5%).[40] In order to obtain complete cleavage this was performed at 40 °C for 2 h instead of at room temperature.[41] Finally, deacetylation with methanolic sodium methoxide and purification with reversed



Scheme 2 *Reagents, conditions and yields*: i) $BF_3{\cdot}Et_2O$, MeCN; ii) solid-phase peptide synthesis; then NaOMe, MeOH (28%, based on resin capacity).

phase HPLC furnished the target glycopeptide **4** (Table 1) in 28% overall yield based on the resin capacity.

CBA/J mice were then immunized, in parallel and under identical conditions, with each of glycopeptides **3** and **4** in complete Freund's adjuvant. Seven days later T cells were harvested from the draining lymph nodes and then cultured in the presence of **1**, **3** or **4** (Fig. 2). Since antigen presenting cells are found among the T cells isolated from the lymph nodes this allows proliferation of those T cells that recognize a specific antigen. In agreement with previous studies,[10] immunization with digalactosylated **3** gave a T cell population that proliferated well, and in a dose-dependent manner, when incubated with **3** (Fig. 2a). These T cells did not show any proliferative response towards trigalactosylated peptide **4**, or peptide **1**, thus revealing a high degree of specificity for glycopeptide **3** which was used as immunogen. In addition, T cell hybridomas produced from this population were found to be highly specific for **3**, in agreement with previous reports.[10,27] In sharp contrast, T cells harvested from mice immunized with trigalactosylated **4** did not react with either of **1**, **3** or **4** (Fig. 2b). Moreover, T cell hybridomas were not obtained from these mice, further confirming the unexpected finding that **4** is not immunogenic.

The lack of immunogenicity displayed by glycopeptide **4** could, for instance, result either from poor binding to the I-A^k class II MHC molecule or an inability of the T cell receptor to accommodate the trisaccharide moiety while still maintaining contact with the surrounding MHC molecule. In order to shed light on these alternatives the binding of **1**–**4** to purified I-A^k molecules was investigated. This was achieved by employing **1**–**4** as inhibitors of the binding of I-A^k to a ^{125}I-radiolabelled high-affinity peptide ligand (YEDYGILQINSR).[27] It was found that the mono- and diglycosylated peptides **2** and **3** bound almost as well as peptide **1** (Table 2). In contrast, trigalactosylated peptide **4** bound more than 200 times weaker than peptide **1**. This finding was unexpected since the side-chain of residue 56, which carries the oligosaccharide moieties, points away from the binding site of I-A^k in the co-crystal with **1**.[25] Since peptides which bind to I-A^k with an affinity equal to that of **4** are non-immunogenic,[42] we conclude that the lack of immunogenicity displayed by **4** is explained by the low affinity it displays for I-A^k.

Our results may also provide further understanding of previous investigations dealing with how the cellular immune system responds to neoglycopeptides.[11,43] These studies involved immunization of mice with neoglycopeptides that carried

Table 1 ^{1}H NMR chemical shifts (δ, ppm) for the peptide part of glycopeptide **4**.[a]

Residue	Proton	**4**
Asp^{52}	α	4.05
	β	3.49, 3.45
Tyr^{53}	NH	8.61
	α	3.33
	β	2.96, 2.68
	Arom.	7.04, 6.64
	OH	9.20
Gly^{54}	NH	8.33
	α	3.79
Ile^{55}	NH	7.86
	α	4.28
	β	1.70
	γ	1.44, 1.07
	β,γ-CH_3	0.82
Ser^{56}	NH	8.39
	α	4.49
	β	3.83, 3.58
Gln^{57}	NH	8.12
	α	4.28
	β	1.91, 1.71
	γ	2.09
	γ-$CONH_2$	7.54, 7.03
Ile^{58}	NH	7.91
	α	4.15
	β	1.71
	γ	1.41, 1.06
	β,γ-CH_3	0.80
Asn^{59}	NH	8.28
	α	4.59
	β	2.57, 2.47
	β-$CONH_2$	7.52, 6.98
Ser^{60}	NH	8.05
	α	4.15
	β	3.66, 3.53
Arg^{61}	NH	8.10
	α	4.11
	β	1.79, 1.53
	γ	1.49
	δ	3.07
	δ-NH_2	7.46
	δ-$CONH_2$	7.09

[a] Spectra were recorded at 500 MHz for a solution of **4** in [D_6]DMSO at 298 K using [D_5]DMSO (δ_H = 2.50) as internal standard.

Table 2 Binding of peptide **1** and glycopeptides **2**–**4** to I-A^k molecules.[a]

Peptide	IC_{50}/μM[b]		
1	0.12	0.08	0.08
2	n.d.[c]	n.d.[c]	0.27
3	0.55	0.75	0.43
4	n.d.[c]	30	24

[a] Peptide **1** and glycopeptides **2**–**4** were used as inhibitors of the binding of an ^{125}I-radiolabelled peptide (YEDYGILQINSR) to purified I-A^k molecules. [b] The concentrations of free (glyco)peptide that inhibits 50% of the binding of the radiolabelled peptide to I-A^k in three different experiments are shown. [c] Not determined.

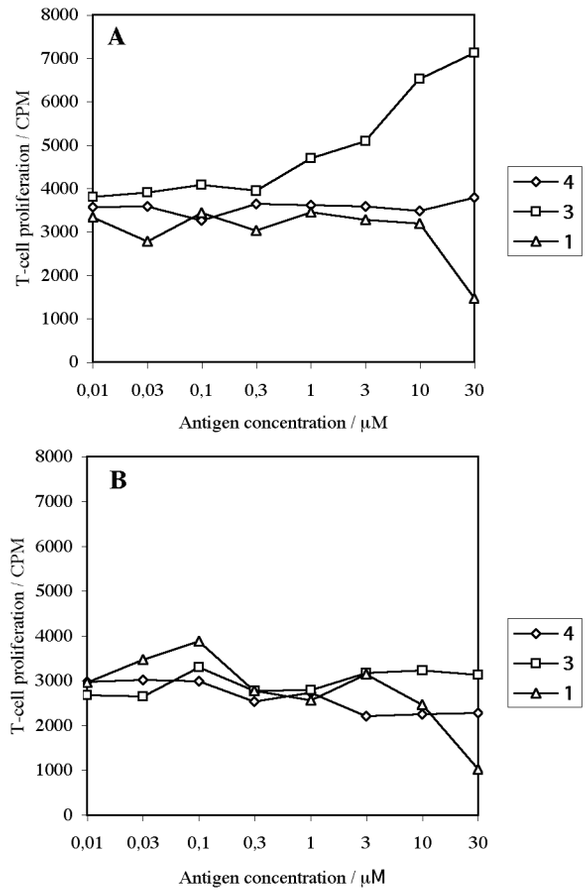


Fig. 2 Proliferation of T cells obtained after immunization of mice with (A) digalactosylated peptide **3** or (B) trigalactosylated peptide **4**. T cells obtained from each of the immunizations were incubated with peptide **1**, or glycopeptides **3** or **4** for three days, after which the amount of ^{3}H-thymidine incorporated in the DNA of the proliferating cells was determined.

mono-, di- or trisaccharides. Interestingly, immune responses were only obtained towards glycopeptides having a covalently bound mono- or disaccharide. No clear explanation was provided for this observation, but in one case it was proposed that the lack of immune response to a trisaccharide was due to the presence of a terminal, non-immunogenic α2,3-linked sialic acid residue in the trisaccharide.[43] As shown in the present study, the lack of immunogenicity of glycopeptides that carry large saccharides may instead be explained by a weak binding to the appropriate MHC molecule. Alternatively, the immune system may be unable to produce T cells with receptors that recognize glycopeptides that carry tri- or larger saccharides.[4,7] Only a few glycopeptides originating from natural glycoproteins have been isolated from MHC molecules, or found to be able to stimulate T cells. These glycopeptides usually carry smaller saccharides, such as β-D-galactose or α-D-glucopyranosyl-(1⟶2)-β-D-galactopyranose linked to hydroxylysine,[17,18,44] or β-D-*N*-acetylglucosamine linked to serine or threonine.[20] It is interesting to speculate on why glycopeptides, which carry small saccharides, appear to be produced predominantly by the cellular immune system. This could be a consequence of poor binding to MHC molecules, as suggested by the model study presented herein. Another possibility is that nature has evolved enzymatic pathways for removal of larger oligosaccharides, such as *N*-linked glycosides and *O*-linked saccharides of the mucin type, during processing in the antigen presenting cell. For *N*-linked glycoproteins, studies have shown that the large *N*-linked carbohydrate may be processed so that only a single *N*-acetylglucosamine residue remains attached to asparagine,[14] or to give complete removal of the *N*-linked glycan.[45] Other studies dealing with *O*-linked glycopeptides have, however, shown that the carbohydrate moieties do not undergo processing in antigen presenting cells.[46,47] The extent of processing may therefore depend on the individual glycoprotein and whether the carbohydrates are *N*- or *O*-linked.

Experimental

General

All reactions were carried out at room temperature under an inert nitrogen atmosphere using dry, freshly distilled solvents under anhydrous conditions, unless otherwise stated. MeCN and CH_2Cl_2 were distilled from calcium hydride. Diethyl ether and THF were distilled from sodium benzophenone. MeOH and pyridine were dried over 3 Å and 4 Å molecular sieves, respectively. Organic solutions were dried over Na_2SO_4 before being concentrated. TLC was performed on Silica Gel F_{254} (Merck) and detection was carried out by examination under UV light and by charring with 10% sulfuric acid. Flash column chromatography was performed on Silica Gel (Matrix, 60 Å, 35–70 μm, Grace Amicon). Preparative HPLC separations were performed on a Beckman System Gold HPLC, using a Kromasil C-8 column (250 × 20 mm, 5 μm, 100 Å) with a flow rate of 11 mL min^{-1}, detection at 214 nm, and the following eluent systems: *A*, aq. 0.1% CF_3CO_2H; and *B*, 0.1% CF_3CO_2H in MeCN. Analytical HPLC was performed on a Beckman System Gold HPLC, using a Kromasil C-8 column (250 × 4.6 mm, 5 μm, 100 Å) with a flow rate of 1.5 mL min $^{-1}$, detection at 214 nm, and the following eluent systems: *A*, aq. 0.1% CF_3CO_2H; and *B*, 0.1% CF_3CO_2H in MeCN. ^{1}H and ^{13}C NMR spectra were recorded with a Bruker DRX-400 or ARX-500 spectrometer for solutions in $CDCl_3$ [residual $CHCl_3$ (δ_H 7.26 ppm), $CDCl_3$ (δ_C 77.0 ppm) as internal standard], [D_6]DMSO [residual [D_5]DMSO (δ_H 2.50 ppm), [D_6]DMSO (δ_C 39.51 ppm) as internal standard] or CD_3OD [residual CD_2HOD (δ_H 3.35 ppm), CD_3OD (δ_C 49.0 ppm) as internal standard] at 298 K. First order chemical shifts and coupling constants were determined from one-dimensional spectra and proton resonances were assigned from COSY, TOCSY and HETCOR experiments. Proton resonances that could not be assigned are not reported. Specific optical rotations were measured in units of 10^{-1} deg cm^2 g^{-1} using a Perkin Elmer 343 polarimeter.

2-(Trimethylsilyl)ethyl 2,3-di-*O*-benzoyl-6-*O*-benzyl-4-*O*-(2,3,4,6-tetra-*O*-benzyl-α-D-galactopyranosyl)-β-galactopyranoside (7)

Compound **5** (1.90 g, 2.94 mmol), **6** (1.42 g, 2.45 mmol) and molecular sieves (1.0 g, 4 Å powder, activated) were suspended in CH_2Cl_2–Et_2O (35 mL, 1 : 1, v/v) in the absence of light. The mixture was cooled to −45 °C (CO_2–MeCN) and a red–brown coloured solution of *N*-iodosuccinimide (0.75 g, 3.33 mmol) and TfOH (56 μL, 0.63 mmol) in CH_2Cl_2 (25 mL) was added in several portions over 4 h. After being stirred for 4.5 h the mixture was diluted with CH_2Cl_2 (100 mL) and filtered (Hyflo – Supercel). The filtrate was washed with sat. $Na_2S_2O_3$–sat. $NaHCO_3$–H_2O (100 mL, 1 : 1 : 2), water (50 mL). The organic phase was concentrated and chromatographed (SiO_2, heptane–EtOAc 10 : 1) to give **7** (2.26 g, 84%); $[\alpha]_D^{25}$ +70 (*c* 1.6, $CHCl_3$); ^{1}H NMR (400 MHz, $CDCl_3$) δ 7.27–8.04 (m, 35 H; Ar*H*), 5.81 (dd, *J* 10.6, 7.8 Hz, 1 H; H-2), 5.27 (dd, *J* 10.7, 2.8 Hz, 1 H; H-3), 4.99 (d, *J* 3.4 Hz, 1 H; H-1′), 4.92 (dd, *J* 11.4, 5.4 Hz, 2 H; PhC*H*$_2$O), 4.83 (s, 2 H; PhC*H*$_2$O), 4.76 (d, *J* 7.8 Hz, 1 H; H-1), 4.70 (d, *J* 10.8 Hz, 1 H; PhC*H*$_2$O), 4.54 (d, *J* 11.1 Hz, 1 H; PhC*H*$_2$O), 4.45 (d, *J* 2.9 Hz, 1 H; H-4), 4.42 (m, 1 H; PhC*H*$_2$O), 4.34 (s, 2 H; PhC*H*$_2$O), 4.22 (dd, *J* 10.3, 2.5 Hz, 1 H; H-3′), 4.16 (br s, 1 H; H-4′), 4.11 (br s, 3 H; OC*H*$_2$, PhC*H*$_2$O), 4.08 (m, 1 H; H-2′), 4.03 (dd, *J* 9.8, 6.2 Hz, 1 H; H-6 or H-6′), 3.94 (t, *J* 6.2 Hz, 1 H; H-5 or H-5′), 3.76 (dd, *J* 9.8, 6.2 Hz, 1 H; H-6 or H-6′), 3.69 (dt, *J* 10.0, 6.6 Hz, 1 H; OC*H*$_2$), 3.46 (t, *J* 9.0 Hz,

1 H; H-5 or H-5′), 3.04 (dd, *J* 8.3, 5.0 Hz, 1 H; H-6 or H-6′), 0.98 (m, 2 H; CH_2Si), −0.01 (s, 9 H; SiMe_3); ^{13}C NMR (100 MHz, $CDCl_3$): δ 166.5, 165.5, 138.9, 138.8, 138.7, 138.4, 138.1, 133.1, 132.9, 129.9, 129.9, 129.6, 129.2, 128.4, 128.3, 128.2, 128.2, 128.0, 128.0, 127.9, 127.6, 127.5, 127.5, 127.4, 127.4, 127.3, 127.3, 100.9, 100.5, 78.9, 77.21, 76.4, 75.6, 74.9, 74.8, 74.5, 74.3, 73.7, 73.0, 72.8, 72.4, 69.9, 69.4, 68.4, 67.5, 67.3, 18.0, −1.5; MS (FAB) calcd for $C_{66}H_{72}O_{13}SiNa$ 1123 *m*/*z* $(M + Na)^+$, observed 1123; anal. calcd for $C_{66}H_{72}O_{13}Si$: C, 72.0; H, 6.6. Found: C, 72.0; H, 6.3%.

2-(Trimethylsilyl)ethyl 2,3-di-*O*-benzoyl-4-*O*-(α-D-galacto-pyranosyl)-β-galactopyranoside (8)

A solution of compound **7** (4.40 g, 4.00 mmol) in HOAc (50 mL) was treated with 10% Pd/C (2.0 g) under hydrogen (55 psi) for 46 h. The catalyst was removed by filtration (Hyflo – Supercel) and co-concentrated several times with toluene to afford **8** (2.83 g, quant.); $[\alpha]_D^{25}$ +100 (*c* 1.0, MeOH); ^{1}H NMR (400 MHz, MeOD) δ 7.26–7.89 (m, 10 H; Ar*H*), 5.56 (dd, *J* 10.7, 2.8 Hz, 1 H; H-2), 5.34 (dd, *J* 10.7, 2.8 Hz, 1 H; H-3), 4.92 (d, *J* 3.7 Hz, 1H; H-1′), 4.81 (d, *J* 7.8 Hz, 1 H; H-1), 4.39 (d, *J* 2.8 Hz, 1 H; H-4), 4.08 (t, *J* 5.7 Hz, 1 H; H-5 or H-5′), 4.00 (dt, *J* 9.8, 5.1 Hz, 1 H; OCH_2), 3.92 (d, *J* 2.5 Hz, 1 H; H-4′), 3.85 (m, 1 H; H-3′), 3.57 (m, 1 H; H-2′), 0.80 (m, 2 H; CH_2Si), −0.17 (s, 9 H; SiMe_3); ^{13}C NMR (100 MHz, MeOD): 167.4, 167.1, 134.6, 134.5, 130.9, 130.6, 130.6, 129.6, 129.5, 102.5, 102.1, 76.2, 75.8, 75.4, 71.9, 71.7, 71.4, 71.2, 70.7, 68.7, 62.3, 60.5, 18.9, −1.4; MS (ESI) calcd for $C_{31}H_{42}O_{13}Si$ 650 *m*/*z* $(M)^+$, observed 650.

2-(Trimethylsilyl)ethyl 2,3-di-*O*-benzoyl-4-*O*-(4,6-*O*-benzyl-idene-α-D-galactopyranosyl)-β-D-galactopyranoside (9)

TsOH (110 mg, 0.58 mmol) was added to a solution of **8** (2.70 g, 4.33 mmol) and α,α-dimethoxytoluene (0.82 mL, 5.40 mmol) in MeCN (20 mL). After being stirred for two days the reaction was neutralized with Et_3N (0.2 mL) and concentrated. The residue was chromatographed (SiO_2, heptane–EtOAc 1 : 3) to give **9** (1.94 g, 63%); $[\alpha]_D^{25}$ +92 (*c* 1.4, $CHCl_3$); ^{1}H NMR (400 MHz, $CDCl_3$) δ 7.27–8.00 (m, 15 H; Ar*H*), 5.64 (dd, *J* 10.7, 7.9 Hz, 1 H; H-2), 5.41 (s, 1 H; PhC*H*O_2), 5.20 (dd, *J* 10.8, 2.9 Hz, 1 H; H-3), 5.02 (d, *J* 3.1 Hz, 1 H; H-1′), 4.74 (d, *J* 7.9 Hz, 1 H; H-1), 4.50 (d, *J* 2.5 Hz, 1 H; H-4), 4.24 (d, *J* 3.5 Hz, 1 H; H-4′), 4.13 (m, 1 H; H-3′), 4.07 (s, 1 H; H-5 or H-5′), 4.03 (m, 1 H; OCH_2), 3.96 (m, 3 H; H-6 or H-6′, H-2′), 3.86 (t, *J* 6.7 Hz, 1 H; H-5 or H-5′), 3.65 (br s, 1 H; OH), 3.58 (m, 2 H; H-6 or H-6′, OCH_2), 3.43 (d, *J* 12.5 Hz, 1 H; H-6 or H-6′), 2.84 (br s, 1 H; OH), 1.85 (br s, 1 H; OH), 0.89 (m, 2 H; CH_2Si), −0.11 (s, 9 H; SiMe_3); ^{13}C NMR (100 MHz, $CDCl_3$): 166.1, 165.7, 137.7, 133.5, 133.2, 129.8, 129.7, 129.6, 129.2, 129.0, 128.6, 128.3, 128.1, 126.3, 102.1, 100.9, 100.9, 77.2, 76.5, 75.9, 74.3, 70.1, 69.9, 69.0, 68.9, 67.9, 63.7, 60.0, 18.0, −1.5; MS (ESI) calcd for $C_{38}H_{46}O_{13}Si$ 739 *m*/*z* $(M)^+$, observed 739. MS (ESI) calcd for $C_{38}H_{46}O_{13}Si$ 739 *m*/*z* $(M)^+$, observed 739.

2-(Trimethylsilyl)ethyl 2,3,6-tri-*O*-benzoyl-4-*O*-(2,3-di-*O*-benzoyl-4,6-*O*-benzylidene-α-D-galactopyranosyl)-β-D-galacto-pyranoside (10)

Benzoylchloride (1.2 mL, 10.3 mmol) was added to a solution of compound 9 (1.91 g, 2.58 mmol) in pyridine (15 mL) at 0 °C. After stirring at room temperature for 2 h the mixture was diluted with CH_2Cl_2 (100 mL), washed with sat. $NaHCO_3$ (2 × 30 mL) and water (40 mL). The organic phase was concentrated and chromatographed (SiO_2, heptane–EtOAc 5 : 1) to give 10 (2.59 g, 96%); $[\alpha]_D^{25}$ +177 (*c* 0.4, $CHCl_3$); ^{1}H NMR (400 MHz, $CDCl_3$) δ 7.27–8.04 (m, 30 H; Ar*H*), 5.95 (dd, *J* 11.0, 3.2 Hz, 1 H; H-3′), 5.90 (dd, *J* 11.0, 3.1 Hz, 1 H; H-2′), 5.78 (dd, *J* 10.7, 7.8 Hz, 1 H; H-2), 5.48 (d, *J* 3.2 Hz, 1 H; H-1′), 5.41 (s, 1 H; PhC*H*O_2), 5.18 (dd, *J* 10.7, 2.9 Hz, 1 H; H-3), 4.76 (d, *J* 7.8 Hz, 1 H; H-1), 4.72 (d, *J* 2.4 Hz, 1 H; H-4′), 4.47 (d, *J* 2.6 Hz, 1 H; H-4), 4.06 (m, 1 H; OCH_2), 3.65 (dt, *J* 9.9, 6.6 Hz, 1 H; OCH_2), 0.97 (m, 2 H; CH_2Si), −0.03 (s, 9 H; SiMe_3); ^{13}C NMR (100 MHz, $CDCl_3$) δ 166.2, 165.9, 165.7, 165.6, 137.5, 133.7, 133.3, 133.3, 133.2, 133.1, 129.9, 129.8, 129.8, 129.7, 129.7, 129.6, 129.6, 129.2, 129.1, 129.0, 128.8, 128.7, 128.5, 128.4, 128.3, 128.3, 128.0, 126.1, 100.7, 100.4, 100.2, 77.2, 76.4, 74.3, 74.1, 71.6, 69.6, 69.1, 68.9, 68.7, 67.4, 63.1, 60.6, 18.0, −1.5; MS (ESI) calcd for $C_{59}H_{58}O_{16}Si$ 1050 *m*/*z* $(M)^+$, observed 1050.

2-(Trimethylsilyl)ethyl 2,3,6-tri-*O*-benzoyl-4-*O*-(2,3,di-*O*-benzoyl-6-*O*-benzyl-α-D-galactopyranosyl)-β-galactopyranoside (11)

Compound **10** (0.70 g, 0.67 mmol), $NaCNBH_3$ (0.30 g, 4.77 mmol) and molecular sieves (0.5 g, 4 Å, activated) were suspended in dry THF (15 mL). The mixture was stirred for 1 h and saturated etheral HCl was added until the mixture became acidic (pH 2–3), and gas evolution ceased. After 2 h the reaction was neutralized with $NaHCO_3$ (s). The mixture was diluted with CH_2Cl_2 (100 mL), filtrated (Hyflo – Supercel), washed with sat. $NaHCO_3$ (40 mL) and water (50 mL). The organic phase was concentrated and chromatographed (SiO_2, heptane–EtOAc 3 : 1) to give **11** (0.60 g, 85%); $[\alpha]_D^{25}$ +80 (*c* 2.7, $CDCl_3$); ^{1}H NMR (400 MHz, $CHCl_3$) δ 7.18–8.03 (m, 30 H; Ar*H*), 5.89 (dd, *J* 10.8, 3.5 Hz, 1 H; H-2′), 5.81 (dd, *J* 10.8, 2.7 Hz, 1 H; H-3′), 5.72 (dd, *J* 10.6, 7.7 Hz, 1 H; H-2), 5.49 (d, *J* 3.6 Hz, 1 H; H-1′), 5.27 (dd, *J* 10.6, 2.7 Hz, 1 H; H-3), 4.74 (d, *J* 7.7 Hz, 1 H; H-1), 4.70 (m, 1 H; H-5), 4.62 (br s, 1 H; H-4′), 4.51 (br s, 1 H; PhCH_2O), 4.43 (d, *J* 2.3 Hz, 1 H; H-4), 4.37 (m, 2 H, H-6, H-6′), 4.19 (d, *J* 11.8 Hz, 1 H; H-6′), 4.02 (m, 2 H; H-6, OCH_2), 3.77 (d, *J* 1.0 Hz, 1 H; H-5′), 3.61 (dt, *J* 10.0, 6.5 Hz, 1 H; OCH_2), 3.55 (d, *J* 3.7 Hz, 2 H; PhCH_2O), 0.95 (m, 2 H; CH_2Si), −0.04 (s, 9 H; SiMe_3); ^{13}C NMR (100 MHz, $CDCl_3$) δ 166.3, 166.1, 165.6, 165.5, 165.2, 137.2, 133.4, 133.2, 133.1, 133.1, 133.0, 130.0, 129.9, 129.9, 129.8, 129.7, 129.7, 129.3, 128.8, 128.5, 128.4, 128.3, 128.3, 127.8, 127.6, 100.8, 99.3, 77.2, 76.1, 74.0, 73.8, 72.0, 70.9, 69.9, 69.8, 69.3, 68.7, 67.3, 61.4, 18.0, −1.5; MS (FAB) calcd for $C_{59}H_{60}O_{16}SiNa$ 1075 *m*/*z* $(M + Na)^+$, observed 1075; anal. calcd for $C_{59}H_{60}O_{16}Si$: C, 67.3; H, 5.7. Found: C, 66.9; H, 5.9%.

2-(Trimethylsilyl)ethyl 2,3,6-tri-*O*-benzoyl-4-*O*-[2,3-di-*O*-benzoyl-6-*O*-benzyl-4-*O*-(2,3,4,6-tetra-*O*-benzyl-α-D-galacto-pyranosyl)-α-D-galactopyranosyl]-β-galactopyranoside (13)

Compound **11** (0.73 g, 0.69 mmol), **12** (0.61 g, 1.04 mmol) and molecular sieves (0.6 g, 4 Å powder, activated) were suspended in CH_2Cl_2–Et_2O (10 mL, 1 : 1, v/v) in the absence of light. The mixture was cooled to −45 °C (CO_2–MeCN) and a red–brown coloured solution of *N*-iodosuccinimide (0.47 g, 2.09 mmol) and TfOH (120 μL, 1.35 mmol) in CH_2Cl_2 (4 mL) was added in several portions over 2 h. After being stirred 6 h the mixture was diluted with CH_2Cl_2 (300 mL) and filtrated (Hyflo – Supercel). The filtrate was washed with sat. $Na_2S_2O_3$–sat. $NaHCO_3$–H_2O (120 mL, 1 : 1 : 2). The organic phase was concentrated and chromatographed (SiO_2, heptane–CH_2Cl_2–methyl *t*-butyl ether 25 : 20 : 1) to give **13** (0.84 g, 77%); $[\alpha]_D^{25}$ +92 (*c* 1.1, $CHCl_3$); ^{1}H NMR ($CDCl_3$) δ 7.05–8.00 (m, 50 H; Ar*H*), 5.83 (m, 2 H; H-2′, H-3′), 5.79 (dd, *J* 10.2, 7.4 Hz, 1 H; H-2), 5.46 (d, *J* 3.0 Hz, 1 H; H-1′), 5.32 (dd, *J* 10.3, 2.7 Hz, 1 H; H-3), 5.08 (d, *J* 3.5 Hz, 1 H; H-1″), 4.78 (m, 2 H; PhCH_2O), 4.75 (d, *J* 7.4 Hz, 1 H; H-1), 4.73 (s, 1 H; H-6, H-6′ or H-6″), 4.64 (m, 3 H; H-4′, H-6, H-6′ or H-6″, PhCH_2O), 4.47 (d, *J* 3.3 Hz, 1 H; H-4), 4.42 (t, *J* 5.4 Hz, 1 H; H-5, H-5′ or H-5″), 4.39 (s, 1 H; PhCH_2O), 4.29 (m, 1 H; H-5, H-5′ or H-5″), 4.16 (s, 2 H; PhCH_2O), 4.13 (dd, *J* 10.4, 2.6 Hz, 1 H; H-3″), 4.03 (m, 4 H; H-2″, H-4″, H-6, H-6′ or H-6″, OCH_2), 3.94 (t, *J* 9.3 Hz, 1 H; H-5, H-5′ or H-5″), 3.75 (dd, *J* 24.6, 12.9 Hz, 2 H; PhCH_2O), 3.60 (dt, *J* 10.0, 6.3 Hz, 1 H; OCH_2), 3.30 (m, 2 H; H-6, H-6′, or H-6″), 2.73 (dd,

J 8.1, 5.1 Hz, 1 H; H-6, H-6′, or H-6″), 0.93 (m, 2 H; CH_2Si), −0.07 (s, 9 H; SiMe_3); δ ^{13}C NMR (100 MHz, $CDCl_3$) 166.5, 166.3, 166.2, 166.7, 165.0, 138.9, 138.8, 138.6, 138.1, 133.3, 133.1, 133.1, 132.9, 130.0, 129.8, 129.8, 129.7, 129.4, 129.0, 129.0, 128.4, 128.4, 128.3, 128.2, 128.2, 128.2, 128.1, 128.1, 128.1, 128.0, 127.8, 127.4, 127.4, 127.3, 127.3, 127.2, 127.2, 127.1, 127.1, 100.7, 99.6, 98.9, 78.8, 77.2, 76.3, 75.5, 75.1, 74.8, 74.8, 74.0, 73.7, 72.7, 72.5, 72.3, 72.2, 71.1, 69.8, 69.6, 69.2, 67.9, 67.5, 67.1, 66.5, 61.8, 25.6, 17.9, −1.5; MS (FAB) calcd for $C_{93}H_{94}O_{21}SiNa$ 1598 *m/z* (M + Na)$^+$, observed 1598; anal. calcd for $C_{93}H_{94}O_{21}Si$: C, 70.9; H, 6.0. Found: C, 70.5; H, 6.3%.

2-(Trimethylsilyl)ethyl 2,3,6-tri-*O*-acetyl-4-*O*-[2,3,6-tri-*O*-acetyl-4-*O*-(2,3,4,6-tetra-*O*-acetyl-α-D-galactopyranosyl)-α-D-galactopyranosyl]-β-galactopyranoside (14)

Sodium methoxide in methanol (6.6 mL, 2.0 M) was added to a solution of **13** (1.04 g, 0.66 mmol) in MeOH (20 mL). The mixture was stirred at room temperature for 3 h, then neutralized with Amberlite IR-120 resin, filtered and concentrated. Residual methanol was removed by co-concentration with toluene. A solution of the crude product in HOAc (30 mL) was treated with 10% Pd/C (0.5 g) under hydrogen (55 psi). After 48 h, the catalyst was removed by filtration (Hyflo – Supercel) and by co-concentration several times with toluene. The residue was dissolved in pyridine (30 mL) and acetic anhydride (30 mL). After 6 h of stirring the solution was concentrated and residual pyridine was removed by co-concentration with toluene. The residue was chromatographed (SiO_2, heptane–EtOAc 1 : 1) to give **14** (0.30 g, 72%); $[\alpha]_D^{25}$ +94 (*c* 1.5, $CHCl_3$); ^{1}H NMR (400 MHz, $CDCl_3$) δ 5.53 (d, *J* 2.0 Hz, 1 H; H-4″), 5.36 (dd, *J* 11.1, 3.2 Hz, 1 H; H-3″), 5.29–5.16 (m, 3 H; H-2″, H-2′, H-3′), 5.12 (dd, *J* 10.6, 7.8 Hz, 1 H; H-2), 4.96 (d, *J* 3.5 Hz, 1 H; H-1″), 4.93 (d, *J* 3.3 Hz, 1 H; H-1′), 4.80 (dd, *J* 10.7, 2.0 Hz, 1 H; H-3), 4.44 (m, 2 H, H-1; H-6, H-6′ or H-6″), 4.29 (d, *J* 2.0 Hz, 1 H; H-4′), 4.17 (dd, *J* 11.1, 6.5 Hz, 1 H H-6, H-6′ or H-6″), 4.05 (d, *J* 1.5 Hz, 1 H; H-4), 3.97 (m, 1 H; OCH_2), 3.76 (t, *J* 6.5 Hz, 1 H; H-5, H-5′ or H-5″), 3.52 (dt, J 10.0, 6.7 Hz, 1 H; OCH_2), 2.11–1.96 (m, 30 H; O*Ac*), 0.91 (m, 2 H; CH_2Si), −0.02 (s, 9 H; SiMe_3); ^{13}C NMR (100 MHz, $CDCl_3$) δ 170.8, 170.5, 170.3, 170.3, 170.2, 170.2, 170.1, 170.0, 169.0, 100.6, 99.4, 99.2, 77.7, 77.2, 76.8, 72.7, 71.9, 69.4, 68.9, 68.8, 68.3, 68.1, 67.8, 67.4, 67.3, 66.9, 62.2, 61.0, 60.4, 21.0, 21.0, 20.8, 20.7, 20.7, 20.6, 17.9, −1.3; MS (FAB) calcd for $C_{43}H_{64}O_{26}SiNa$ 1047 *m/z* (M + Na)$^+$, observed 1047; anal. calcd for $C_{43}H_{64}O_{26}Si$: C, 50.4; H, 6.3. Found: C, 50.8; H, 6.7%.

1,2,3,6-Tetra-*O*-acetyl-4-*O*-[2,3,6-tri-*O*-acetyl-4-*O*-(2,3,4,6-tetra-*O*-acetyl-α-D-galactopyranosyl)-α-D-galactopyranosyl]-galactopyranoside (15)

Boron trifluoride etherate (103 μL, 0.82 mmol) was added to a solution of **13** (0.27 mg, 0.26 mmol) in toluene (4 mL) and acetic anhydride (4 mL) at 0 °C. The solution was stirred for 3 h, then diluted with CH_2Cl_2 (100 mL), washed with sat. $NaHCO_3$ (40 mL) and water (40 mL). The organic phase was concentrated and chromatographed (SiO_2, heptane–EtOAc 2 : 3) to give a 1 : 1 α–β mixture of **15** (0.22 g, 86%); MS (FAB) calcd for $C_{40}H_{54}O_{27}Na$ 989 *m/z* (M + Na)$^+$, observed 989.

N$^{\alpha}$-(Fluoren-9-ylmethoxycarbonyl)-3-*O*-(2,3,6-tri-*O*-acetyl-4-*O*-[2,3,6-tri-*O*-acetyl-4-*O*-(2,3,4,6-tetra-*O*-acetyl-α-D-galactopyranosyl)-α-D-galactopyranosyl]-β-D-galactopyranosyl)-L-serine pentafluorophenyl ester (17)

$BF_3{\cdot}Et_2O$ (70 μL, 0.55 mmol) was added to a solution of compound **15** (0.21 g, 0.22 mmol) and **16** (0.14 g, 0.29 mmol) in MeCN (7 mL) at room temperature. After 2 h, the solution was diluted with CH_2Cl_2 (100 mL), washed with sat. $NaHCO_3$ (60 mL) and water (60 mL). The aqeous phase was re-extracted with CH_2Cl_2 (2 × 40 mL). The combined organic phases were concentrated and purified with radical chromatography (1 mm, heptane–EtOAc 1 : 2) to give **17** (0.10 g, 33%); $[\alpha]_D^{25}$ +69 (*c* 1.5, $CHCl_3$); ^{1}H NMR (400 MHz, $CDCl_3$) δ 7.79–7.29 (m, 8 H; Ar*H*), 6.13 (d, *J* 8.8 Hz, 1 H; Ser-N*H*), 5.55 (d, *J* 2.2 Hz, 1 H; H-4″), 5.40 (dd, *J* 11.1, 3.3 Hz, 1 H; H-3″), 5.32–5.15 (m, 4 H; H-2′, H-2″, H-3′, H-2), 5.00 (d, *J* 3.5 Hz, 1 H; H-1″), 4.96 (d, *J* 3.3 Hz, 1 H; H-1′), 4.87 (d, *J* 8.9, 1 H; Ser-*H*α), 4.81 (dd, *J* 10.8, 2.4 Hz, 1 H; H-3), 4.62–4.52 (m, 3H; H-5, H-5′ or H-5 H-6, H-6′ or H-6″), 4.43 (dd, *J* 10.5, 6.0 Hz, 1 H; H-6, H-6′ or H-6″), 4.34 (d, *J* 7.6 Hz, 1 H; H-1), 4.31–4.23 (m, 3 H; H-4, Ser-*H*β), 4.17–4.00 (m, 6 H; Ser-*H*β, H-6, H-6′ or H-6″, H-4), 3.50 (t, *J* 6.2 Hz, 1 H; H-5, H-5′ or H-5″), 2.13–1.90 (m, 30 H; O*Ac*); ^{13}C NMR (100 MHz, $CDCl_3$) δ 170.8, 170.4, 170.4, 170.3, 170.3, 170.2, 170.1, 170.0, 169.2, 166.0, 155.9, 144.8, 143.7, 141.4, 141.3, 127.8, 127.2, 127.1, 125.0, 124.9, 120.0, 120.0, 101.5, 99.4, 99.3, 77.7, 77.2, 72.4, 69.5, 69.2, 69.0, 68.4, 68.3, 68.2, 67.8, 67.4, 66.9, 62.6, 61.1, 60.4, 54.4, 47.2, 20.9, 20.7, 20.6, 20.6, 20.4, 14.2; MS (FAB) calcd for $C_{66}H_{72}F_5NO_{30}Na$ 1422 *m/z* (M + Na)$^+$, observed 1422; anal. calcd for $C_{66}H_{72}F_5NO_{30}$: C, 53.2; H, 4.8. Found: C, 53.2; H, 5.1.

N$^{\alpha}$-(Fluoren-9-ylmethoxycarbonyl)-3-*O*-(2,3,6-tri-*O*-acetyl-4-*O*-[2,3,6-tri-*O*-acetyl-4-*O*-(2,3,4,6-tetra-*O*-acetyl-α-D-galacto-pyranosyl)-α-D-galactopyranosyl]-β-D-galactopyranosyl)-L-serine (19)

$BF_3{\cdot}Et_2O$ (49 μL, 0.39 mmol) was added to a solution of **15** (0.13 g, 0.13 mmol) and **18** (64 mg, 0.19 mmol) in MeCN (4 mL) at room temperature. After stirring for 4 h the solution was diluted with CH_2Cl_2 (120 mL), washed with 1 M HCl (30 mL) and water (40 mL). The organic phase was concentrated and purified with reversed phase HPLC (gradient: 0 ⟶ 80% *B* in *A* during 60 min) to give **19** (97 mg, 61%); $[\alpha]_D^{25}$ +84 (*c* 1.7, $CHCl_3$); ^{1}H NMR (400 MHz, $CDCl_3$) δ 7.88–7.30 (m, 8 H; Ar*H*), 6.39 (d, *J* 8.4 Hz, 1 H; Ser-N*H*), 5.52 (dd, *J* 2.9, 1.0 Hz, 1 H; H-4″), 5.24–5.16 (m, 3 H; H-2″, H-2′, H-2), 5.15 (br s, 1 H; H-1″), 5.11 (d, *J* 3.5 Hz, 1 H; H-1′), 5.06 (dd, *J* 10.6, 2.9 Hz, 1 H; H-3), 4.73 (d, *J* 7.7 Hz, 1 H; H-1), 4.65 (t, *J* 6.9 Hz, 1 H; H-5, H-5′ or H-5″), 4.29 (d, *J* 2.3 Hz, 1 H; H-4), 4.14 (d, *J* 6.9 Hz, 2 H; H-6, H-6′ or H-6″), 4.05 (t, *J* 6.5 Hz, 1 H; H-5, H-5′ or H-5″), 3.96 (dd, *J* 10.6, 4.0 Hz, 1 H; Ser-*H*β), 2.15–1.90 (m, 30 H; O*Ac*); ^{13}C NMR (400 MHz, $CDCl_3$) δ 171.6, 171.0, 170.9, 170.7, 170.4, 170.3, 170.2, 170.2, 170.0, 169.6, 156.2, 143.9, 143.7, 141.3, 127.7, 127.1, 125.3, 125.2, 120.0, 102.5, 99.2, 99.2, 77.7, 77.2, 76.5, 73.4, 72.0, 71.2, 70.0, 69.5, 68.6, 68.5, 68.3, 67.7, 67.4, 67.1, 66.9, 63.4, 60.8, 60.4, 47.1, 21.2, 20.9, 20.9, 20.8, 20.7, 20.7, 20.6; MS (ESI) calcd for $C_{56}H_{67}NO_{30}$ 1233 *m/z* (M)$^+$, observed 1233.

L-α-Aspartyl-L-tyrosylglycyl-L-isoleucyl-*O*-{4-*O*-[4-*O*-(α-D-galactopyranosyl)-α-D-galactopyranosyl]-β-D-galactopyranosyl}-L-seryl-L-glutaminyl-L-isoleucyl-L-asparaginyl-L-seryl-L-arginine amide (4)

Synthesis was performed manually in a mechanically agitated reactor on a TentaGel S NH_2 resin (14.8 μmol), functionalized with the the Rink amide linker *p*-[α-(fluoren-9-ylmethoxyform-amido)-2,4-dimethoxybenzyl]-phenoxyacetic acid,[35,36] according to procedures that have been reported previously.[48] After cleavage from the solid phase the crude product was treated with sodium methoxide in methanol (10 mL, 6.0 mM) during 2.5 h at room temperature. The solution was neutralized with Amberlite IR-120 resin and filtered. The organic phase was concentrated and the residue was purified by preparative reversed-phase HPLC (gradient: 0 ⟶ 80% *B* in *A* during 60 min) to give **4** (6.7 mg, 28% based on resin capacity); MS (ESI) calcd for $C_{66}H_{107}N_{15}O_{33}$ 1637 *m/z* (M)$^+$, observed 1637; amino acid analysis: Arg 1.00 (1), Asp 1.99 (2), Glu 1.02 (1), Gly 1.01 (1), Ile 1.99 (2), Ser 1.96 (2), Tyr 1.00 (1).

Immunization with glycopeptides 3 and 4

Five CBA/J mice were immunized with 10 nmol of each of glycopeptides **3** and **4** emulsified in 100 μL of complete Freund's adjuvant. The mice were females of 10 weeks of age purchased from Jackson Laboratories (Bar Harbor, Maine) and the adjuvant was obtained from Difco Chemical Co. Each mouse received approximately 50 μL of glycopeptide emulsion in each of two footpads. Seven days later the popliteal lymph nodes were dissected, a cell suspension was made and a proliferative assay was carried out. In this assay 5×10^5 cells in 200 μL of Dulbecco's minimal essential media containing 10% fetal calf serum were added to each of the wells in 96 well plates. Glycopeptides **3** and **4**, or the parent peptide HEL52–61 (**1**), were added to the wells to give concentrations ranging from 30 μM to 10 nmol (*cf.* Fig. 2). The cells were incubated for three days, the last 24 h in the presence of ^{3}H-thymidine. Then the cells were harvested and the amount of ^{3}H-thymidine incorporated in the DNA was determined. The data presented in Fig. 2 is the average of three proliferative assays, having a variation of ~10% in the cpm.

Binding of glycopeptides to I-A^k class II MHC molecules

Class II I-A^k molecules were produced in baculovirus and purified as described elsewhere.[49] Peptide **1** and glycopeptides **2**–**4** were then tested for binding to the I-A^k molecules as described previously.[27] In brief, the assay relied on incubation of purified I-A^k molecules with an ^{125}I-radiolabelled peptide known to bind to I-A^k with high affinity (YEDYGILQINSR) in the presence of increasing concentrations of **1**–**4**.

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