Synthesis of major histocompatibility complex class I binding glycopeptides

Gemma Arsequell,[a] John S. Haurum,[b] Tim Elliott,[b] Raymond A. Dwek[a] and Annemarie C. Lellouch *,[a]

[a] *Glycobiology Institute, Department of Biochemistry, University of Oxford, South Parks Road, Oxford OX1 3QU, UK*

[b] *Institute of Molecular Medicine, John Radcliffe Hospital, Oxford OX3 9DU, UK*

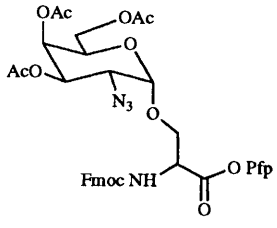
Four Major Histocompatibility Complex (MHC) Class I binding glycopeptides and two peptide analogues, from a cytotoxic T-lymphocyte (CTL) epitope of Sendai Virus Nucleoprotein, have been prepared using solid-phase peptide synthesis employing the following glycosyl amino acid building blocks: FmocSer(Ac_3-β-D-GlcNAc)OH **1**, FmocSer(Ac_3-α-D-GalN_3)OPfp **2**, FmocAsn(Ac_3-β-D-GlcNAc)OH **3** and FmocAsn(Ac_3-β-D-GalNAc)OH **4**. Previously, we examined the influence of glycosylation on peptide binding to the MHC Class I molecule and CTL recognition of these peptides. The synthesis and characterization of compounds **1–4** as well as the resulting glycopeptides is described. In addition, results of NMR investigations demonstrating that peptide K3, and glycopeptides K3-*O*-GlcNAc and K3-*O*-GalNAc, show two distinct conformations in solution as a result of *cis–trans* isomerization about a Tyr-Pro amide bond are reported.

Introduction

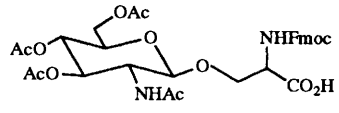
Major Histocompatibility Complex (MHC) Class I molecules[1] are cell-surface glycoproteins found on most types of mammalian cells. MHC Class I molecules present proteolytic peptide fragments of endogenous protein antigens for recognition by cytotoxic T lymphocytes (CTL). CTL activation[2] results in the lysis of the antigen-presenting cell. Peptides presented by MHC Class I are restricted in both amino acid composition and in length (8–10 residues).[3] All T-cell antigens identified to date consist of peptides with unmodified amino acid side chains. However, by using synthetic peptides heptenated with trinitrophenyl, it has been shown that chemically modified peptide antigens can be specifically recognized by T cells.[4] It is therefore possible that a glycosylated peptide antigen, mimicking a post-translation modification that plays a key role in many cellular processes, could also be recognized by T cells.

Protein glycosylation[5] takes a variety of forms. In *N*-linked glycosylation the oligosaccharide is attached *via* a core *N*-acetylglucosamine (GlcNAc) residue to Asn in a β-N-glycosidic linkage. In *O*-linked glycosylation, such as that found commonly on mucoproteins and mucin-like protein domains, the oligosaccharide chains are linked to serine or threonine through an α-anomeric linkage of a core *N*-acetylgalactosamine (GalNAc) residue. Both *N*- and *O*-glycosylation occur in the endoplasmic reticulum and the Golgi apparatus on proteins that are destined for secretion or incorporation into the plasma membrane. Recently a new form of glycosylation[6] has been identified in which a single residue of GlcNAc is attached *via* a β-*O*-glycosidic linkage to serines and threonines of cytoplasmic and nuclear proteins. Most of the MHC Class I presented peptide antigens are derived from proteins of a cytoplasmic or nuclear origin, and could therefore encompass an *O*-GlcNAc glycosylation site.

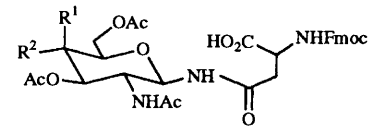
In order to determine whether peptides containing glycosyl amino acids such as Ser-β-*O*-GlcNAc could bind to the MHC Class I and be specifically recognized by T cells, a series of four glycopeptides was prepared. These glycopeptides are variants of the immunodominant CTL epitope (H-2K^{b}restricted) from Sendai virus nucleoprotein[7] 324-332 (SEV-9), containing modifications at either position 4 or 5. We have recently shown



FmocSer(Ac_3-α-D-GalN_3)Opfp **2**



FmocSer(Ac_3-β-D-GlcNAc)OH **1**



R^1 = H, R^2 = OAc FmocAsn(Ac_3-β-D-GlcNAc)OH **3**

R^1 = OAc, R^2 = H FmocAsn(Ac_3-β-D-GalNAc)OH **4**

that glycopeptides containing Ser(β-GlcNAc), Ser(α-GalNAc) or Asn(β-GlcNAc) bind to the MHC Class I, and elicit a glycopeptide-specific CTL response.[8]

Here we report the synthesis and characterization of the four glycopeptide analogues *via* the incorporation of FmocSer(Ac_3-α-D-GalN_3)OPfp **2**, FmocAsn(Ac_3-β-D-GlcNAc)OH **3**, FmocAsn(Ac_3-β-D-GalNAc)OH **4**, and, most importantly, the recently described FmocSer(Ac_3-β-D-GlcNAc)OH **1**, into a standard solid-phase peptide synthesis procedure. In addition, we report NMR data demonstrating that some of the SEV-9 analogues prepared display at least two distinct conformations in solution as a result of *cis–trans* proline isomerization.

Results and discussion

The crystal structure of SEV-9 (FAPGNYPAL)† peptide bound to a murine MHC Class I molecule (H-2K^b)[9] reveals

† Single-letter amino acid code.

that Tyr-6 and Leu-9 are involved in anchoring the peptide into the MHC binding cleft whereas the side chains of Gly-4 and Asn-5 point out of the groove and towards the T-lymphocyte antigen receptor (TCR). Two peptide-sequence analogues of the SEV-9 sequence were prepared (K1 and K3), each containing a single amino acid modification from the wild type (see below). In K1, proline at position 7 is replaced by serine, thus creating an NXS/T *N*-glycosylation motif. In K3, glycine at position 4 is replaced by serine to create an *O*-glycosylation site. Four glycopeptides whose amino acid sequences are based on these two SEV-9 analogues were prepared. The K1 glycopeptides, K1-*N*-GlcNAc and K1-*N*-GalNAc, were modified at Asn in position 5. The K3 glycopeptides, K3-*O*-GlcNAc and K3-*O*-GalNAc, were modified at Ser in position 4. Aside from mimicking naturally occurring glycosidic linkages (with the exception of K1-*N*-GalNAc), this series of four glycopeptides facilitated the exploration of the influence of both the position and nature (α *vs.* β anomers, *O*- *vs.* *N*-glycosidic linkages) of the glycan in the MHC–TCR interaction.

Sendai virus nucleoprotein
wt: FAPGNYPAL (SEV-9)

K1-*N*-GlcNAc FAPGNYSAL
|
β-GlcNAc

K1-*N*-GalNAc FAPGNYSAL
|
β-GalNAc

K3-*O*-GlcNAc FAPSNYPAL
|
β-GlcNAc

K3-*O*-GalNAc FAPSNYPAL
|
α-GalNAc

Amino acid sequence and saccharide composition of the SEV-9 analogues

Fmoc glycosyl amino acid building block and glycopeptide synthesis

A number of different strategies for the preparation of glycopeptides by either chemical[10] or enzymic[11] synthesis have been reported. The most versatile and general approach currently available for the chemical synthesis of a large variety of glycopeptides is solid-phase glycopeptide synthesis (SPGPS) incorporating glycosylated building blocks. Several groups have shown that the milder acid conditions[12,13] of the N^{α}-(fluoren-9-yl)methoxycarbonyl) (Fmoc) amino-protection scheme is compatible with the potentially labile glycosidic linkage, in particular with *O*-seryl and *O*-threonyl glycosides.

Syntheses of Fmoc-glycosyl amino acids derivatives have been reported in which the α-carboxy group of the amino acid is left as the free acid[14] or is protected with an activating group such as a pentafluorophenyl ester (OPfp).[15] Acetates are a common choice for the protection of the carbohydrate hydroxy groups. *O*-Acetyl groups stabilize the glycosidic bonds under the acidic conditions used for side-chain deprotection and cleavage from the resin in SPGPS and are easily removed later by treatment with sodium methoxide in methanol.[16]

Four glycosyl amino acids building blocks were synthesized. FmocSer(Ac_3-β-D-GlcNAc)OH **1** for K3-*O*-GlcNAc, FmocSer(Ac_3-α-D-$GalN_3$)OPfp **2** for K3-*O*-GalNAc, FmocAsn(Ac_3-β-D-GlcNAc)OH **3** for K1-*N*-GlcNAc and FmocAsn(Ac_3-β-D-GalNAc)OH **4** for K1-*N*-GalNAc.

FmocSer(Ac_3-β-D-GlcNAc)OH was prepared in a one-step reaction by glycosylation of FmocSerOH with peracetylated GlcNAc by an optimized method, recently described by us,[17] using boron trifluoride–diethyl ether as a Lewis acid catalyst.

The synthesis of FmocSer(Ac_3-α-D-$GalN_3$)OPfp was achieved by glycosylation of FmocSerOPfp with tri-*O*-acetyl-2-azido-2-deoxy-α-D-galactosyl bromide by using silver triflate as catalyst to afford a mixture of α and β glycosides of FmocSer(Ac_3-D-$GalN_3$)OPfp. The 2-azidogalactosyl bromide derivative was prepared from tri-*O*-acetyl-D-galactal by using the azidonitration and bromination methods described by Lemieux and Ratcliffe.[18] The pentafluorophenyl ester derivative of FmocSer was prepared by esterification of FmocSerOH with pentafluorophenol in the presence of *N*,*N*′-diisopropylcarbodiimide (DIC). The desired α-anomer, FmocSer(Ac_3-α-D-$GalN_3$)OPfp, was purified from the crude by column chromatography on silica gel. The synthesis of this glycosylated building block has been described using other catalysts[19] such as a mixture of silver perchlorate and silver carbonate. The transformation of the azido groups into acetamido functions was achieved after the completion of the peptide chain on the solid support by using multiply distilled thioacetic acid.[20]

FmocAsn(Ac_3-β-D-GlcNAc)OH **3** and FmocAsn(Ac_3-β-D-GalNAc)OH **4** were prepared by the coupling of commercially available FmocAspOBu^t and the free glycosylamines of GlcNAc and GalNAc, respectively. The glycosylamines were prepared[21] by reaction of the hydroxy-group-free GlcNAc and GalNAc with ammonium hydrogen carbonate at 30 °C for several days, with subsequent repeated lyophilization to a constant weight. The coupling was achieved in dimethylformamide (DMF) by using (benzotriazol-1-yloxy)tripyrrolidinophosphonium hexafluorophosphate (PyBOP) and 1-hydroxybenzotriazole (HOBt) activation.[22] After removal of the solvent, the free hydroxy groups of the sugar moiety were acetylated *in situ* with acetic anhydride in the presence of dry pyridine to afford the peracetylated building blocks. Although several groups have also reported the synthesis of N-linked glycosyl asparagine derivatives,[23] our procedure facilitates a simple column chromatographic purification of the final building blocks avoiding an otherwise time-consuming large-scale HPLC purification. The Bu^t ester group was removed by addition of trifluoroacetic acid (TFA) and the product was repurified by column chromatography.

The glycopeptides were synthesized manually using a standard Fmoc protocol. The Wang resin, preloaded with FmocLeu, was employed to generate a free acid terminus. The Fmoc group was removed in each cycle with 20% piperidine in DMF. The coupling of each (glycosyl) amino acid was achieved with PyBOP activation, using *N*,*N*-diisopropylethylamine (DIEA) as a catalyst. After addition of the last amino acid residue, the Fmoc group was removed and the peptide was deprotected and cleaved from the resin by treatment with TFA–water (95:5). In the case of the K3-*O*-GalNAc glycopeptide, containing the α-linked 2-azido-2-deoxy-D-galactose, the azido group was converted into an acetamido group by treatment of the resin with freshly distilled thioacetic acid. We found it necessary to leave the N-terminus of the peptide protected with Fmoc during this treatment to avoid unwanted *N*-acetylation. Each of the glycopeptides was purified once by reversed-phase HPLC, before deacetylation of the carbohydrate moiety with sodium methoxide in methanol. The glycopeptides were then subjected to an additional HPLC purification step before being characterized by amino acid analysis, mass spectrometry and NMR spectroscopy.

Structural analysis of glycopeptides

One- and two-dimensional NMR spectra were obtained for each of the peptides and glycopeptides in D_2O in order to verify

their identity and purity. The assignments and chemical shifts for the peptides and glycopeptides are reported in Tables 1 and 2. Upon analysis of the spectra from the compounds based on the K3 peptide sequence (K3-*O*-GlcNAc, K3-*O*-GalNAc and K3), many more peaks were detected than would be expected for the nine-amino-acid sequence. The three compounds in question were first re-analysed by HPLC and mass spectroscopy in order to double check their purity. The extra signals observed were subsequently identified as resulting from a significant fraction of the peptide containing a *cis*-amide bond between Tyr-6 and Pro-7.

Table 1 ^{1}H NMR chemical shifts (δ, ppm) in D_2O for peptides of the K1 sequence FAPGNYSAL

Amino acid	Proton	K1	K1-*N*-GlcNAc	K1-*N*-GalNAc
Phe-1	CH$^\alpha$	4.26	4.20	4.10
	CH$^\beta$	3.18, 3.18	3.15	3.10, 3.10
	aromatics	7.39, 7.26	7.38, 7.27	7.39, 7.26
Ala-2	CH$^\alpha$	4.32	4.33	4.66
	CH$^\beta$	1.38	1.38	1.32
Pro-3	CH$^\alpha$	4.40	4.40	4.39
	CH$^\beta$	2.33, 1.95	2.32, 1.99	2.31, 1.95
	CH$^\gamma$	2.04	2.04	2.04
	CH$^\delta$	3.68	3.66	3.68, 3.63
Gly-4	CH$^\alpha$	not assigned	3.89	not assigned
Asn-5	CH$^\alpha$	4.69	4.71	4.73
	CH$^\beta$	2.76, 2.66	2.80, 2.66	2.80, 2.67
Tyr-6	CH$^\alpha$	4.53	4.54	4.53
	CH$^\beta$	3.06	3.04, 2.95	3.06
	aromatics	7.12, 6.83	7.10, 6.83	7.12, 6.83
Ser-7	CH$^\alpha$	4.36	4.39	4.39
	CH$^\beta$	3.78	3.78	3.79, 3.79
Ala-8	CH$^\alpha$	4.65	4.65	4.35
	CH$^\beta$	1.31	1.33	1.39
Leu-9	CH$^\alpha$	4.25	4.18	4.18
	CH$^\beta$	1.63	1.58	1.58
	CH$^\delta$	0.87	0.88	0.87
GlcNAc	1-H		5.02	
	2-H		3.79	
	3-H		3.60	
GalNAc	1-H			4.99
	2-H			3.99
	3-H			3.74

The chemical shifts of the proline ring signals in the *cis* conformation are typically shifted upfield from those resulting from the proline ring in the *trans* conformation[24] and, as such, are readily identified in a 2-D homonuclear chemical-shift correlation (COSY) spectrum. The COSY spectrum of K3-*O*-GlcNAc shows two sets of Pro αHβH crosspeaks as well as two sets of the Tyr-6 αHβH crosspeaks (see Fig. 1A). The resonances resulting from both the *cis* and the *trans* isomers of the Tyr-6–Pro-7 amide bond as well as the *trans* form of the Ala-2–Pro-3 amide bond could be positively identified in the K3-*O*-GlcNAc sequence from qualitative rotating-frame nuclear Overhauser enhancements (ROEs) of a ROESY experiment with a 200 ms mixing time. The Tyr-6–Pro-7 *cis* resonances were confirmed by the presence of a Tyr-6 CαH–Pro-7 CαH ROE, while the *trans* isomer resonances were identified by a Tyr-6 CαH–Pro-7 CδH ROE[25] (see Fig. 1B). Similarly, an ROE was observed between the Ala-2 CαH and Pro-3 CδH, thereby confirming the *trans* conformation of the Ala-2–Pro-3 amide bonds as well as allowing for the sequence specific assignment of the Ala-2 *vs.* Ala-8 resonances. Identical patterns of ROEs were observed in the spectra of compounds K3 and K3-*O*-GalNAc (data not shown). Of the two X-Pro amide bonds in the K3 sequence, Tyr-6–Pro-7 would be predicted to have the greater *cis*-forming propensity resulting from the bulky nature of the aromatic residue immediately preceding proline.[26] Indeed, for the K1 series of compounds (K1-*N*-GlcNAc, K1-*N*-GalNAc and K1), which contain only the Ala-2–Pro-3 sequence, the NMR spectra are extremely homogeneous, and show no evidence for the presence of *cis*-amide proline conformation.

Table 2 ^{1}H NMR chemical shifts (δ, ppm) in D_2O for peptides of the K3 sequence FAPSNYPAL

Amino acid	Proton	K3	K3-*O*-GlcNAc	K3-*O*-GalNAc
Phe-1	CH$^\alpha$	4.26	4.26	4.27
	CH$^\beta$	3.19	3.17, 3.17	3.21, 3.13
	aromatics	7.39, 7.29	7.85, 7.41	7.40, 7.30
Ala-2	CH$^\alpha$	4.65	4.65	4.69
	CH$^\beta$	1.33	1.33	1.35
Pro-3	CH$^\alpha$	4.42	4.41	4.42
	CH$^\beta$	2.33, 1.98	2.31, 1.97	2.27, 1.91
	CH$^\gamma$	2.02	2.00	1.99
	CH$^\delta$	3.66	3.69, 3.59	3.76, 3.54
Ser-4	CH$^\alpha$	4.43 (4.97)	4.45 (4.54)	4.60 (4.66)
	CH$^\beta$	3.85, 3.85 (3.80, 3.80)	4.00, 3.87 (4.05, 3.96)	3.80, 3.75 (3.98, 3.80)
Asn-5	CH$^\alpha$	4.70 (4.67)	4.65	4.73
	CH$^\beta$	2.77, 2.68 (2.66, 2.66)	2.67	2.74, 2.65
Tyr-6	CH$^\alpha$	4.80 *t*, 4.51 *c*	4.78 *t*, 4.51 *c*	4.80 *t*, 4.53 *c*
	CH$^\beta$	3.03, 2.83 *t*, 2.89 *c*	3.07. 2.84 *t*, 2.91 *c*	3.10, 2.86 *t*, 2.90 *c*
	aromatics	7.13, 6.86 (7.16, 6.87)	7.19, 6.87 *t*, 7.15, 6.89 *c*	7.20, 6.88, 7.15, 6.86
Pro-7	CH$^\alpha$	4.39 *t*, 3.78 *c*	4.89 *t*, 3.77 *c*	4.45 *t*, 3.78 *c*
	CH$^\beta$	2.24, 1.91 *t*, 1.86, 1.77 *c*	2.22, 1.90, 1.85, 1.77	2.34, 1.96 *t*, 1.86, 1.77 *c*
	CH$^\gamma$	1.96 *t*, 1.70 *c*	1.95 *t*, 1.68 *c*	2.03 *t*, 1.70 *c*
	CH$^\delta$	3.71, 3.50 *t*, 3.48, 3.34 *t*	3.69, 3.48 *t*, 3.48, 3.33 *c*	3.73, 3.64 *t*, 3.48, 3.34 *c*
Ala-8	CH$^\alpha$	4.27	4.25 (4.34)	4.33
	CH$^\beta$	1.31	1.37 (1.39)	1.40
Leu-9	CH$^\alpha$	4.29	4.35	4.21
	CH$^\beta$	1.63, 1.56	1.64	1.58
	CH$^\delta$	0.89, 0.89		0.88
GlcNAc	1-H		4.46	
	2-H		3.71	
	3-H			
GalNAc	1-H			4.92
	2-H			4.16
	3-H			3.83

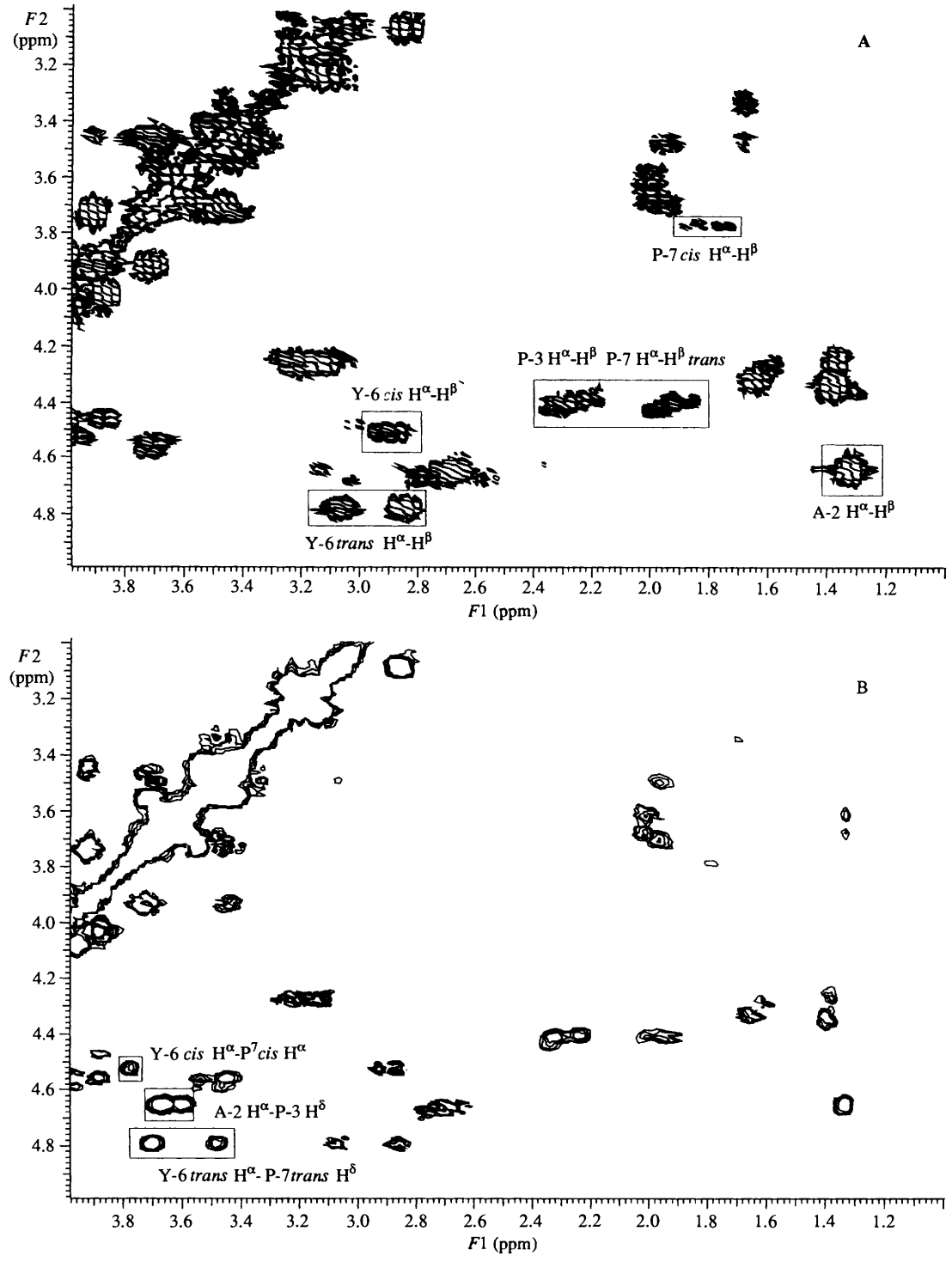


Fig. 1 A. COSY spectrum in which CH^{α}–CH^{β} crosspeaks have been highlighted for Pro-3 and Ala-2 as well as the *cis* and *trans* forms of Tyr-6 and Pro-7. **B.** 200 ms mixing time ROESY spectrum in which those intramolecular connectivities positively identifying the *cis* and *trans* forms of the Tyr-6–Pro-7 bond have been highlighted as have those identifying the *trans* conformation of the Ala-2–Pro-3 amide bond.

In comparing the glycopeptides with their unglycosylated peptide analogues, we found that there were virtually no differences in the chemical shifts of the amino acid resonances, with the exception that the glycosylated serine αH–βH crosspeaks were better resolved from one another in the K3-*O*-GalNAc and K3-*O*-GlcNAc structures than in the K3 peptide. Future investigations in H_2O–D_2O will allow for full assignment of the amide protons and therefore further analyses of the glycopeptide and peptide conformations.

In conclusion we have shown that Fmoc-glycosyl amino acids are an invaluable tool for the preparation of biologically important glycopeptides which have facilitated the study of the MHC Class I-TCR interaction. We report here as well the successful incorporation of the novel FmocSer(Ac_3-β-D-GlcNAc)OH building block into a solid-phase peptide synthesis protocol. Although we have prepared these glycopeptides manually, all the methods used here are compatible with automated and multi-peptide synthesizers.

The preliminary structural investigations reveal that the compounds based on the K3 sequence exist in at least two

conformations that are not exchanging on the NMR timescale. One of these two conformations contains a *cis*-amide bond at Tyr-6–Pro-7. Similar *cis*-amide bonds have been observed in other small peptides, containing in particular the Tyr–Pro sequence.[24,25] However, our findings are particularly salient given that native SEV-9 sequence, which also contains Tyr at position 6 and Pro at position 7, is found bound to the MHC complex in a highly extended structure which can only be accommodated by an all-*trans*-amide configuration of the peptide.[9]

Experimental

Materials and general methods

N-Acetylglucosamine, peracetylated *N*-acetylglucosamine, *N*-acetylgalactosamine and 2,3,6-tri-*O*-acetylgalactal were purchased from Sigma. All Fmoc amino acids, Fmoc-Leu Wang Resin and PyBOP were purchased from Novabiochem. HOBt, DIC, silver trifluoromethanesulfonate, DIEA, and pentafluorophenol were purchased from Fluka. Peptide-synthesis-grade DMF was purchased from Rathburn and used directly for synthesis. Immediately before use, dichloromethane (DCM), ethyl acetate and acetonitrile were dried by distillation from P_2O_5 and kept over molecular sieves (4 Å). Triethylamine was distilled from potassium hydroxide and kept over molecular sieves (4 Å). Thioacetic acid was freshly prepared by fractional distillation. Concentrations were performed under reduced pressure at temperatures < 40 °C. TLC was performed on Silica Gel 60 F_{254} (Merck) with detection by UV light and charring with H_2SO_4. Column chromatography was performed on a Merck Silica Gel 60 (230–400 mesh, 40–60 μm) column under dry conditions with distilled solvents.

The ^1H NMR and ^{13}C NMR spectra were recorded with a Varian Unity 500 spectrometer. Chemical shifts are given in ppm and referenced to internal standards, for solutions in $CDCl_3$ [residual $CHCl_3$ (δ_H 7.25) and $CDCl_3$ (δ_C 77.9) as internal standards], $(CD_3)_2SO$ ([$^2H_6$]DMSO) [residual [$^2H_5$]DMSO (δ_H 2.49; δ_C 39.5)], and D_2O {0.75% 3-(trimethylsilyl)[2,2,3,3-$^2H_4$]propionic acid sodium salt as internal standard}. Coupling constants (J) are given in Hz. First-order chemical shifts and coupling constants were obtained from one-dimensional spectra. Proton resonances were assigned from COSY,[27] phase-sensitive 2-dimensional total correlation spectroscopy (TOCSY)[28] and ROESY[29] experiments. Glycopeptide samples were prepared for ^1H NMR spectroscopy by lyophilization several times from D_2O. Samples used were typically glycopeptide (5 mg) in unbuffered D_2O (800 mm^3) with an internal standard [20 mm^3; 0.75% 3-(trimethylsilyl)-[2,2,3,3-$^2H_4$]propionic acid sodium salt]. Two-dimensional COSY, TOCSY and ROESY spectra were obtained of each sample at 30 °C. Spectra were processed with sine bell and cosine bell functions as necessary. Optical rotations were measured in a Perkin-Elmer 141 polarimeter, and $[\alpha]_D$ values are given in units of 10^{-1} deg cm^2 g^{-1}. Matrix-assisted laser desorption/ionization mass spectrometry (MALDI) was performed on either a Fisons VG Autospec QFPD with 2,5-dihydroxybenzoic acid as a matrix or on a Finnigan Lasermat™ time-of-flight mass spectrometer (Finnigan MAT), with α-cyano-4-hydroxycinnamic acid and renin [m/z 1760 (M + 1)] as internal standard.

All analytical and preparative reversed-phase HPLC was performed on a Waters HPLC system equipped with two Waters 510 Pumps, a Valco U6 manual injector, and a Waters M-490 multi-wavelength detector. Analytical HPLC was performed using a Vydac C4 column (214TP54; 5 μm; 250 mm × 4.6 mm) run at 1.0 cm^3 min^{-1}. HPLC purification of all compounds was performed on a Vydac C4 column (214TP54; 5 μm; 250 mm × 10 mm) run at 4.0 cm^3 min^{-1}. Solvent system used was A: 0.1% TFA in water and B: 0.1% TFA in MeCN.

For hexosamine and amino acid analysis, glycopeptide samples were hydrolysed in vapour phase HCl (6 mol dm^{-3} HCl) for either 6 h at 100 °C (hexosamine analysis) or 24 h at 110 °C (amino acid composition). (±)-2-Aminobutyric acid (Aldrich 16,266-3) was used as an internal standard in both cases. The hydrolysates were derivatized with phenyl isothiocyanate according to the Waters Picotag manufacturer instructions. The phenylthiocarbamoyl derivatives were separated by reversed-phase HPLC on a 5 μm Spherisorb ODS2 column (0.46 × 25 cm) at 50 °C by using modifications of the Picotag solvent and gradient system.

General procedure. Solid-phase glycopeptide synthesis

Synthesis of glycopeptides was carried out manually by using the Wang resin prederivatized with FmocLeu. The side chains of Ser and Tyr were protected with *tert*-butyl groups. Amino acids were coupled as their free acids (3 mol equiv.) by addition of PyBOP (3 mol equiv.) and DIEA (1 mol equiv.). The couplings were performed in DMF for 2 h. After coupling, the resin was rinsed successively with DMF, methanol, and DCM, before monitoring of the success of the coupling with Kaiser ninhydrin assay.[30] The glycosylated building blocks (2 mol equiv.) were coupled in the same way. The resin was acetylated between couplings by treatment (30 min) with acetic anhydride and DIEA in DCM. Deprotection of the Fmoc group was achieved by treatment (10 min) with 20% piperidine in DMF. After the final removal of the Fmoc group the resin was washed successively with DMF, MeOH and DCM, and dried *in vacuo*.

In the case of K3-*O*-GlcNAc, containing the α-glycosidically linked 2-azido-2-deoxy-D-galactose, the transformation of the azido group into an acetamido function was performed on the resin-bound glycopeptide once the synthesis was complete. The Fmoc group was not removed after coupling of the final amino acid in order to prevent unwanted *N*-acetylation. The resin-bound peptide was treated with multiply distilled thioacetic acid for 2 days. The thioacetic acid was replaced several times during this time. Completion of the reaction was monitored by the disappearance of the azide band at ~2110 cm^{-1} in the FTIR spectrum of a KBr pellet containing resin. The resin was subsequently washed several times with MeOH, the terminal Fmoc was removed, and the resin was washed as described above.

The resin-bound glycopeptides were removed from the resin and amino acid side chains were deprotected by treatment (2 h) with TFA–water (95:5). The crude cleavage product was precipitated with cold diethyl ether and dissolved in DMF to be used directly for RP-HPLC purification. Pure glycopeptides were deacetylated by using a catalytic amount of sodium methoxide in absolute methanol, followed by repurification by RP-HPLC. The deacetylation reaction was monitored by HPLC and was usually complete after 1 h, the methanolic solutions were neutralized with acetic acid, the solvent was removed by evaporation, and the resulting residues were purified by semi-preparative RP-HPLC.

Synthesis of *O*-(2-acetamido-3,4,6-tri-*O*-acetyl-2-deoxy-β-D-glucopyranosyl)-*N*$^{\alpha}$-(fluoren-9-ylmethoxycarbonyl)-L-serine 1

Commercially available peracetylated GlcNAc (240 mg, 0.616 mmol) were placed under argon in a flask containing 4 Å molecular sieves and DCM (4 cm^3) was added. After cooling of this mixture to 0 °C freshly distilled BF_3·Et_2O (200 mm^3, 1.91 mmol, 3.1 mol equiv.) was added dropwise to the suspension. After being stirred overnight at room temp. the formation of the oxazoline was complete as monitored by TLC [$CHCl_3$–

MeOH (10:1)]. Then Et_3N (80 mm^3, 0.574 mmol) was added dropwise at 0 °C. The reaction mixture was stirred for 10 min and a solution of FmocSerOH (208 mg, 0.635 mmol) in a mixture of DCM–acetonitrile was added. The reaction mixture was left at room temp. and was periodically monitored by TLC [$CHCl_3$–MeOH–AcOH (80:10:1)] and RP-HPLC [70:30 to 20:80 (A:B) in 30 min]. To enhance the yield further a second batch of oxazoline (240 mg, 0.616 mmol) prepared in the same way as described above may be added after 24 h. When the reaction showed no more progress (from 48 h to 150 h), the crude was neutralized at 0 °C with Et_3N, diluted with DCM, and filtered through Celite. The filtrate was concentrated and the crude was purified by column chromatography [silica gel; $CHCl_3$–MeOH (from 30:1 to 10:1)]. After purification, FmocSer(Ac_3-β-D-GlcNAc)OH **1** was obtained in 55% yield, HPLC t_R 15.5 min [Vydac C4 analytical column; gradient 95:5 (A:B) for 5 min, then to 30:70 (A:B) in 25 min]; $[\alpha]_D^{22}$ 27.8 (*c* 1, MeOH); δ_H(500 MHz; [$^2H_6$]DMSO) 1.74 (3 H, s, NH*Ac*), 1.92, 1.98 and 2.02 (9 H, s, Ac), 3.69 (1 H, t, *J* 9.5, 2-H), 3.78–3.84 (2 H, m, 5-H, Ser β-CH), 3.96–4.04 (3 H, m, 6-H, Ser α-CH, Ser β-CH), 4.19–4.31 (4 H, m, 6-H, C*H*Ar_2, $NHCO_2CH_2$), 4.73 (1 H, d, *J* 8.4, 1-H), 4.84 (1 H, t, *J* 9.6, 4-H), 5.12 (1 H, t, *J* 9.6, 3-H), 6.15 (1 H, d, *J* 9.3, $NHCO_2$), 7.32–7.94 (8 H, m, ArH) and 7.97 (1 H, d, *J* 8.5, N*H*Ac); δ_C(125 MHz; [$^2H_6$]DMSO) 20.32, 20.36 and 20.45 (*Me*CO), 22.53 (NHCO*Me*), 46.69 (*C*HAr_2), 53.17 (Ser C-α), 61.82–70.74 (C-2, -3, -4, -5, -6 and $NHCO_2CH_2$), 72.71 (Ser C-β), 100.36 (C-1), 120.09–143.96 (ArC), 155.63 ($NHCO_2$) and 169.30–170.08 (MeCO_2, Me*C*ONH, CO_2H). MALDI: *m/z* 680 (M + 23).

Synthesis of *O*-(3,4,6-tri-*O*-acetyl-2-azido-2-deoxy-α-D-galactopyranosyl)-N^{α}-(fluoren-9-ylmethoxycarbonyl)-L-serine 2

Tri-*O*-acetyl-2-azido-2-deoxy-α-D-galactosyl bromide (890 mg, 2.26 mmol) was dissolved in DCM (8 cm^3) and added to a mixture of FmocSerOPfp (1.18 g, 2.26 mmol), silver triflate (580 mg, 2.26 mmol), and 4 Å molecular sieves in dry DCM (15 cm^3) under argon at −40 °C. The reaction mixture was stirred while its temperature was maintained between −25 and −40 °C. The reaction was followed by TLC [hexane–ethyl acetate (33:20)]. After 8 h, triethylamine (315 mm^3, 2.26 mmol) was added and the mixture was stirred at room temp. overnight. Dilution wth dry DCM, filtration through Celite, and concentration were followed by purification on column chromatography on dried silica gel with dry solvents [hexane–ethyl acetate (35:20)]. After purification the α-anomer of the building block was obtained in 52% yield, HPLC t_R 30.7 min [C4 Vydac analytical column; gradient 95:5 (A:B) for 10 min, then to 0:100 (A:B) in 30 min]; δ_H(500 MHz; $CDCl_3$) 1.98, 2.07 and 2.16 (9 H, s, Ac), 3.72 (1 H, dd, *J* 11, 2-H), 4.02–4.16 (3 H, m, 6-H_2, Ser β-CH^b), 4.18 (1 H, t, 5-H), 4.26 (1 H, t, C*H*Ar_2), 4.34 (1 H, dd, *J* 11.2 and 3.4, Ser β-CH^a), 4.5 (2 H, m, Fmoc CH_2), 4.96 (1 H, m, Ser α-CH), 5.01 (1 H, d, *J* 3.6, 1-H), 5.32 (1 H, m, 3-H), 5.46 (1 H, m, 4-H), 6.04 (1 H, d, *J* 8.4, Ser-NH) and 7.29–7.78 (8 H, 4 m, ArH). Data in accord with published values;[19b] (M + 1); MALDI: *m/z* 830 (M + 23).

Synthesis of *N*-(2-acetamido-3,4,6-tri-*O*-acetyl-2-deoxy-β-D-glucopyranosyl)-N^{α}-(fluoren-9-ylmethoxycarbonyl)-L-asparagine 3

In a typical synthesis, commercially available FmocAspOBu^t (220 mg, 0.5 mmol) in DMF (~500 mm^3) were treated with glycosamine (117 mg, 0.5 mmol), PyBOP (780 mg, 1.5 mmol), and HOBt (68 mg, 0.5 mmol), each dissolved in a minimal amount of DMF. After initial stirring at room temp., DIEA (100 mm^3, 0.5 mmol) was added to the reaction mixture. The coupling reaction was monitored by RP-HPLC. After being stirred overnight, the crude mixture was concentrated under reduced pressure and then was stirred overnight with an excess of Ac_2O in pyridine (1:5). The crude mixture was again concentrated under reduced pressure and purified by column chromatography on silica gel with $CHCl_3$–MeOH (8:1) as solvent. The purified α-*tert*-butyl ester was deprotected by treatment for 2 h with neat TFA. TFA was removed under a stream of N_2. Residual TFA was removed by co-evaporation from tetrahydrofuran (THF). The product was purified by silica gel column chromatography with $CHCl_3$–MeOH–AcOH (80:10:1) as solvent. HPLC t_R 24.4 min [C4 Vydac analytical column; gradient 95:5 (A:B) for 10 min, then to 0:100 (A:B) in 30 min]; δ_H(500 MHz; [$^2H_6$]DMSO) 1.89 (3 H, s, NH*Ac*), 1.95, 1.98 and 2.00 (9 H, 3 s, Ac), 2.41 (1 H, dd, *J* 7.0, β-CH^b), 2.61 (1 H, dd, *J* 16.1 and 7.0, β-CH^a), 3.80–4.00 (3 H, m, 2-, 5- and 6-H), 4.20 (4 H, m, 6-H, Fmoc CH_2, C*H*Ar_2), 4.36 (1 H, m, α-CH), 4.81 (1 H, dd, *J* 9.9, 4-H), 5.08 (1 H, dd, *J* 9.7, 3-H), 5.16 (1 H, dd, *J* 9.6, 1-H), 7.30–7.45 (4 H, m, ArH), 7.53 (1 H, d, *J* 8.0, α-NH), 7.69–7.91 (5 H, m, N*H*Ac and 4 × ArH) and 8.61 (1 H, d, *J* 9.0, γ-NH). MALDI: *m/z* 705 (M + 23) (Calc. M: 682.5). Data in accord with published values.[31]

Synthesis of *N*-(2-acetamido-3,4,6-tri-*O*-acetyl-2-deoxy-β-D-galactopyranosyl)-N^{α}-(fluoren-9-ylmethoxycarbonyl)-L-asparagine 4

Synthesis and purification as described above for **3**, except that galactosamine replaced glucosamine. Compound **4** showed HPLC t_R 14.5 min [C4 Vydac analytical; gradient 95:5 (A:B) for 5 min, then to 30:70 (A:B) in 25 min]; δ_H(500 MHz; [$^2H_6$]DMSO) 1.76 (3 H, s, NH*Ac*), 1.88, 1.97 and 2.07 (9 H, 3 s, Ac), 2.48 (1 H, dd, *J* 7, β-CH^b), 2.61 (1 H, dd, *J* 16.1 and 4.9, β-CH^a), 3.94–4.21 (3 H, m, 2-, 5- and 6-H), 4.20 (4 H, m, 6-H, Fmoc CH_2 and C*H*Ar_2), 4.25 (1 H, m, CH^a), 5.00 (1 H, dd, *J* 10.8 and 3.2, 3-H), 5.09 (1 H, dd, *J* 9.4, 1-H), 5.26 (1 H, dd, *J* 3.2, 4-H), 7.19 (1 H, br s, α-NH), 7.31–7.88 (8 H, m, ArH), 7.93 (1 H, d, N*H*Ac) and 8.54 (1 H, d, *J* 9.4, γ-NH); MALDI *m/z* 705.1 (M + 23) (Calc. M: 682.5).

L-Phenylalanyl-L-alanyl-L-prolyl-L-glycyl-L-asparagyl-L-tyrosyl-L-seryl-L-alanyl-L-leucine K1

Peptide was synthesized (30 μmol scale) and cleaved from the resin as described previously. HPLC [Vydac C4 semi-preparative column; gradient 90:10 to 40:60 (A:B) in 30 min; t_R 19.35 min] gave K1 (12 mg, 45%); MALDI *m/z* 961 (M + 23) (Calc. *m/z* 939.02). Amino acid analysis results were as expected. ^1H NMR data are reported in Table 1.

L-Phenylalanyl-L-alanyl-L-prolyl-L-seryl-L-asparagyl-L-tyrosyl-L-prolyl-L-alanyl-L-leucine K3

Peptide was synthesized (32 μmol scale) and cleaved from the resin as described previously. Purification by HPLC [Vydac C4 semi-preparative column; gradient 90:10 to 40:60 (A:B) in 30 min; t_R 20.2 min], gave K3 (14 mg, 46% overall); MALDI *m/z* 999 (M + 23) (Calc. M + 3, 979.08). Amino acid analysis results were as expected. For ^1H NMR data see Table 2.

L-Phenylalanyl-L-alanyl-L-prolyl-L-glycyl-*N*-(2-*N*-acetyl-β-D-glucopyranosylamino)-L-asparagyl-L-tyrosyl-L-seryl-L-alanyl-L-leucine K1-*N*-GlcNAc

The glycopeptide was synthesized (29 μmol scale) and cleaved from the resin as described previously. The crude cleavage product was purified by HPLC [Vydac C4 semi-preparative column; gradient 90:10 to 40:60 (A:B) in 30 min; t_R 19.0 min], to give the *O*-acetylated glycopeptide [MALDI *m/z* 1289 (M + 23)]. Deacetylation and purification by HPLC [Vydac C4 semi-preparative column; gradient 90:10 to 60:40 (A:B) in 30 min; t_R 18.5 min] gave K1-*N*-GlcNAc (14 mg, 41% overall); MALDI *m/z* 1164.6 (M + 23) and 1180.9 (M + 39) [Calc. M: 1142.02].

Amino acid and hexosamine analysis results as expected. For ^{1}H NMR spectra see Table 1.

L-Phenylalanyl-L-alanyl-L-prolyl-L-glycyl-*N*-(2-*N*-acetyl-β-D-galactopyranosylamino)-L-asparagyl-L-tyrosyl-L-seryl-L-alanyl-L-leucine K1-*N*-GalNAc

The glycopeptide was synthesized (28 μmol scale) and cleaved from the resin as described previously. The crude cleavage product was purified by HPLC [Vydac C4 semi-preparative column; gradient 90:10 to 60:40 (A:B) in 30 min; t_R 23.3 min] to give *O*-acetylated peptide, MALDI *m/z* 1289 (M + 23)]. Deacetylation and purification by HPLC [Vydac C4 semi-preparative column; gradient 90:10 to 60:40 (A:B) in 30 min; t_R 18.5 min] gave K1-*N*-GalNAc (14 mg, 43% overall); MALDI *m/z* 1142.6 (M + H), 1164.1 (M + 23) and 1181.4 (M + 39) (renin 1760) [Calc. *m/z* 1165.02 (M + 23) and 1181.02 (M + 39)]. Amino acid and hexosamine analysis results were as expected. For ^{1}H NMR data see Table 1.

L-Phenylalanyl-L-alanyl-L-prolyl-*O*-(2-*N*-acetyl-β-D-glucopyranosylamino)-L-seryl-L-asparagyl-L-tyrosyl-L-prolyl-L-alanyl-L-leucine K3-*O*-GlcNAc

The glycopeptide was synthesized (26 μmol scale) and cleaved from the resin as described previously. The crude cleavage product was purified by HPLC [Vydac C4 semi-preparative column; gradient 90:10 to 60:40 (A:B) in 30 min; t_R 25.2 min], to give the *O*-acetylated peptide [MALDI *m/z* 1332 (M + 23)]. Deacetylation and purification by HPLC [Vydac C4 semi-preparative column; gradient 90:10 to 60:40 (A:B) in 30 min; t_R 18.5 min] gave K3-*O*-GlcNAc (12 mg, 39% overall), LD-MS *m/z* 1184.9 (M + H), 1204.03 (M + 23) and 1221.44 (M + 39) [Calc. *m/z* 1182.10 (M), 1205.10 (M + 23) and 1221.10 (M + 39)]. Amino acid and hexosamine analysis results were as expected. For ^{1}H NMR data, see Table 2.

L-Phenylalanyl-L-alanyl-L-prolyl-*O*-(2-*N*-acetyl-α-D-galactopyranosylamino)-L-seryl-L-asparagyl-L-tyrosyl-L-prolyl-L-alanyl-L-leucine K3-*O*-GalNAc

The glycopeptide was synthesized (25 μmol scale), treated with thioacetic acid, and cleaved from the resin as described previously. The crude cleavage product was purified by HPLC [Vydac C4 semi-preparative column; gradient 90:10 to 60:40 (A:B) in 30 min; t_R 23.3 min], to give the *O*-acetylated glycopeptide [MALDI *m/z* 1333 (M + 23)]. Deacetylation and purification by HPLC [Vydac C4 semi-preparative column; gradient 90:10 to 60:40 (A:B) in 30 min; t_R 18.5 min] gave K3-*O*-GalNAc (9 mg, 30% overall); MALDI *m/z* 1182.5 (M), 1205.4 (M + 23) and 1222.0 (M + 39) (1760) [Calc. *m/z* 1182.10 (M), 1205.10 (M + 23) and 1221.10 (M + 39)]. Amino acid and hexosamine analysis results were as expected. For ^{1}H NMR data, see Table 2.

Acknowledgements

We thank Dr M. R. Wormald for technical assistance with the NMR spectroscopy, and Dr D. J. Harvey for mass spectroscopy. G. Arsequell is an E. C. fellow, J. S. Haurum is a Carlsberg-Wellcome Travelling Research Fellow, T. Elliott is a Wellcome Senior Research Fellow and A. C. Lellouch is a National Institute of Health Postdoctoral Research Fellow.

References

1 T. Elliott, M. Smith, P. Driscoll and A. McMichael, *Curr. Biol.*, 1993, **3**, 854; R. N. Germain, *Cell*, 1994, **76**, 287.
2 A. R. Townsend and H. Bodmer, *Annu. Rev. Immunol.*, 1989, **7**, 601.
3 G. M. Van Bleek and S. G. Nathenson, *Nature*, 1990, **348**, 213; K. Falk, O. Roetzschke, S. Stevanovic, G. Jung and H. G. Rammensee, *Nature*, 1991, **351**, 290; J. W. Yewdell and J. R. Bennink, *Adv. Immunol.*, 1992, **52**, 1; R. N. Germain and D. H. Margulies, *Annu. Rev. Immunol.*, 1993, **11**, 403; S. Joyce, K. Kuzushima, G. Kepecs, R. H. Angeletti and S. G. Nathenson, *Proc. Natl. Acad. Sci. USA*, 1994, **9**, 4145.
4 A. Bonin, B. Ortmann, S. Martin and H. U. Weltzien, *Int. Immunol.*, 1992, **4**, 869.
5 J. Montreuil, *Carbohydr. Chem. Biochem.*, 1980, **37**, 157; T. W. Rademacher, R. B. Parekh and R. A. Dwek, *Annu. Rev. Biochem.*, 1988, **57**, 785; A. Varki, *Glycobiology*, 1993, **3**, 97.
6 G. W. Hart, G. D. Holt and R. S. Haltiwanger, *Trends Biochem. Sci.*, 1988, 380; G. W. Hart, R. S. Haltiwanger, G. D. Holt and W. G. Kelly, *Annu. Rev. Biochem.*, 1989, **58**, 841; R. S. Haltiwanger, M. A. Blomberg and G. W. Hart, *J. Biol. Chem.*, 1992, **267**, 9005; R. S. Haltiwanger, W. G. Kelly, E. P. Roquemore, M. A. Blomberg, L. Y. Dennis Dong, L. Kreppel, T. Y. Chou and G. W. Hart, *Biochem. Soc. Trans.*, 1992, **20**, 264; D. L. Y. Dong, Z. S. Xu, M. R. Chevrier, R. J. Cotter, D. W. Cleveland and G. W. Hart, *J. Biol. Chem.*, 1993, **268**, 16679.
7 W. M. Kast, L. Roux, J. Curren, H. J. Blom, A. C. Voordouw, R. H. Meloen, D. Kolafofsky and C. J. Meleief, *Proc. Natl. Acad. Sci. USA*, 1991, **88**, 2283.
8 J. S. Haurum, G. Arsequell, A. C. Lellouch, S. Y. C. Wong, R. A. Dwek, A. J. McMichael and T. Elliott, *J. Exp. Med.*, 1994, **180**, 739.
9 D. H. Fremont, M. Matsumurs, E. A. Stura and P. A. Peterson, *Science*, 1992, **257**, 919.
10 For reviews see: H. Paulsen, *Angew. Chem., Int. Ed. Engl.*, 1982, **21**, 155; H. Kunz, *Angew. Chem., Int. Ed. Engl.*, 1987, **26**, 294; H. Paulsen, *Angew. Chem., Int. Ed. Engl.*, 1990, **29**, 823; K. H. Jung and R. R. Schmidt, *Curr. Opin. Struct. Biol.*, 1991, **1**, 721; H. Kunz, *Pure Appl. Chem.*, 1993, **65**, 1223; O. Kanie and O. Hindsgaul, *Curr. Opin. Struct. Biol.*, 1992, **2**, 674; M. Meldal, *Curr. Opin. Struct. Biol.*, 1994, **4**, 710.
11 C. H. Wong, M. Schuster, P. Wang and P. Sears, *J Am. Chem. Soc.*, 1993, **115**, 5893.
12 H. Paulsen and J. P. Hölck, *Carbohydr. Res.*, 1982, **109**, 89.
13 J. Kihlberg and T. Vuljanic, *Tetrahedron Lett.*, 1993, **34**, 6135.
14 M. Eloffson, B. Walse and J. Kihlberg, *Tetrahedron Lett.*, 1991, **32**, 7613.
15 M. Meldal and K. J. Jensen, *J. Chem. Soc., Chem. Commun.*, 1990, 483; M. Meldal and K. Bock, *Tetrahedron Lett.*, 1990, **31**, 6987; L. Kifslaudy and I. Schön, *Synthesis*, 1983, 325; I. Schön and L. Kifslaudy, *Synthesis*, 1986, 303.
16 B. Lüning, T. Norberg and J. Tejbrant, *J. Chem. Soc., Chem. Commun.*, 1989, 1267.
17 G. Arsequell, L. A. Krippner, R. A. Dwek and S. Y. C. Wong, *J. Chem. Soc., Chem. Commun.*, 1994, 2383.
18 R. U. Lemieux and R. M. Ratcliffe, *Can. J. Chem.*, 1979, **57**, 1244.
19 (*a*) H. Paulsen, A. Richter, V. Sinnwell and W. Stenzel, *Carbohydr. Res.*, 1978, **64**, 339; (*b*) H. Paulsen, T. Bieldfeldt, S. Peters, M. Meldal and K. Bock, *Liebigs Ann. Chem.*, **4**, 1994, **4**, 369.
20 T. Bieldfeldt, S. Peters, M. Meldal, K. Bock and H. Paulsen, *Angew. Chem., Int. Ed. Engl.*, 1992, **31**, 857; H. Paulsen, T. Bieldfeldt, S. Peters, M. Meldal and K. Bock, *Liebigs Ann. Chem.*, 1994, **4**, 381.
21 L. M. Likhosherstov, O. S. Novikova, V. A. Derevitskaja and N. K. Kotchetkov, *Carbohydr. Res.*, 1986, **146**, C1.
22 S. T. Anisfeld and P. T. Lansbury, *J. Org. Chem.*, 1990, **55**, 5560; S. T. Cohen-Anisfeld amd P. T. Lansbury, Jr., *J. Am. Chem. Soc.*, 1993, 115, 10531.
23 L. Urge, L. Otvos, Jr., E. Lang, K. Wroblewski, I. Laczko and M. Hollosi, *Carbohydr. Res.*, 1992, **235**, 83; L. Urge, E. Kollat, M. Hollosi, I. Laczko, K. Wroblewski, J. Thurin and L. Otvos, Jr., *Tetrahedron Lett.*, 1991, **32**, 3445; L. Otvos, Jr., K. Wroblewski, E. Kollat, A. Perczel, M. Hollosi, G. D. Fasman, H. C. J. Ertl and J. Thurin, *Pept. Res.*, 1989, **2**, 362.
24 H. J. Dyson, M. Rance, R. A. Houghten, R. A. Lerner and P. E. Wright, *J. Mol. Biol.*, 1988, **201**, 161.
25 K. Wüthrich, M. Billeter and W. Braun, *J. Mol. Biol.*, 1984, **180**, 715.
26 C. Grathwohl and K. Wüthrich, *Biopolymers*, 1981, **20**, 2623.
27 D. J. States, R. A. Haberkorn and D. J. Ruben, *J. Magn. Reson.*, 1982, **48**, 286.
28 L. Braunschweiler and R. R. Ernst, *J. Magn. Reson.*, 1983, **53**, 521.
29 A. Bax and D. G. Davies, *J. Magn. Reson.*, 1985, **63**, 207.
30 E. Kaiser, R. L. Colescott, C. D. Bossinger and P. A. Cook, *Anal. Biochem.*, 1970, **54**, 595.
31 L. Biondi, F. Filira, M. Gobbo, B. Scolaro, R. Rocchi and F. Cavaggion, *Int. J. Pept. Protein Res.*, 1991, **37**, 112.

Paper 4/07642G
Received 15*th December* 1994
Accepted 17*th February* 1995