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Multiple Column Synthesis of a Library of T-Cell Stimulating T_n-Antigenic Glycopeptide Analogues for the Molecular Characterization of T-Cell–Glycan Specificity

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Abstract: A series of peptides and glycopeptides derived by amino acid and glycosyl amino acid scans through the self peptide from CBA/J mouse haemoglobin Hb (67–76), VITAFNEGLK, was synthesized by multiple column peptide synthesis (MCPS). Investigation of glycopeptide binding to the mouse major histocompatibility class II molecule E^k showed that glycans in position 72 did not interfere with the binding to E^k. Immunization experiments revealed that glycopeptides with the glycan in position 72 were immunogenic. Therefore a series of *N*-linked and *O*-linked glycopeptides with the glycan attached in the position 72 either to serine, threonine or asparagine was synthesized by MCPS. The glycan structure was furthermore varied with respect to monosaccharide component, size of oligosaccharide, anomer configuration and stereochemistry of essential hydroxyl groups in order to investigate the specificity of the interaction with the T-cell receptor. Easy synthesis of ready to use Ser and Thr building blocks corresponding to mucin core 1, the T_n-antigen and its β-anomer were developed using trichloroacetimidates as glycosyl donors and reduction with *in situ* acetylation of the azide containing glycosylation products. Synthesis of an α-linked GlcNAc-Thr building block was achieved by glycosylation of Fmoc-Thr-OPfp with 2-azido-2-deoxy-3,4,6-tri-*O*-acetyl-D-glycopyranosyl trichloroacetimidate as a glycosyl donor. Other building blocks were obtained by previously described procedures.

Keywords: Keywords: mucin glycopeptides; tumour associated antigen; cancer; MHC Class II binding; glycopeptide synthesis

INTRODUCTION

Mucin glycoproteins are represented by the common α-GalNAc-serine or threonine core structure found in MUC 1 - MUC 4 and are the most important *O*-linked glycoproteins found on the surface of epithelial cells and in the mucous [1]. Thus the gastrointestinal, respiratory and genitourinary tracts are covered with mucin glycoproteins which serve as lubricants and protect against mechanical and chemical damage of the tissue [2]. The structure of the glycan shows a large tissue-specific variation even in the core oligosaccharide. This is due to the stepwise assembly of the glycan structures by a number of glycosyl-

Abbreviations: Dhbt-OH, 3,4-dihydro-3-hydroxy-4-oxo-1,2,3-benzotriazine; MCPS, multiple column peptide synthesis; MSNT, 2,4,6-mesitylene-sulfonyl-3-nitro-1,2,4-triazole; Pfp, pentafluorophenol; TBAF, tetrabutyl ammonium fluoride; TBDMS, *t*-butyl-dimethyl-silyl; TBTU, *O*-(1H-benzotriazol-1-yl)-*N,N,N',N'*-tetramethyluronium tetrafluoroborate; TMSOTf, trimethylsilyl trifluoromethanesulfonate.

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transferases present in different concentrations depending on the tissue [2]. Until now, only a few of the enzymes responsible for the biosynthesis of the different core structures have been identified and characterized [3]. The *O*-GalNAc moiety is first attached to a Ser- or a Thr-residue in the protein backbone by the *N*-acetylgalactosamine polypeptide transferase and then different mucin-type core structures are assembled by specific glycosyltransferases [1].

In the case of malignant tumour tissue the relative concentration of the enzymes responsible for the biosynthesis of the mucin glycans is changed and in particular the concentration of the GlcNAc transferases is downregulated [4]. Therefore aberrant glycosylation occurs in which the normal core 2 structure and further elongated and sialylated structures cannot be formed [4]. These aberrant glycosylations are known as tumour-associated antigens, among them the T_n-antigen (α-D-GalNAc-(1-O)Ser/Thr) and the core 1 structure or T-antigen (β-D-Gal-(1 → 3)-α-D-GalNAc-(1-O)Ser/Thr). Abnormally fucosylated and sialylated core 1 structures have also been identified in cancer tissues [4].

Proteins entering the endocytotic pathway are processed intracellularly and the resulting peptide fragments of 10–25 amino acids are bound to MHC class II molecules and transported to the cell surface. Here the peptide-MHC class II complex is able to be recognized by CD 4 positive T-cells which then trigger the immune response [5, 6]. It is not clear yet whether the oligosaccharides of the glycoprotein fragments remain attached during antigen processing or whether the glycan is directly involved in the interaction with the T-cell receptor. The immune response to glycoproteins and glycopeptides involves processing in the endocytic pathway before the putative glycopeptide epitope binds to the MHC class II molecule and is transported out on the cell surface. However, glycopeptides of the proper size possessing the required binding motif may conceivable bypass the endocytic pathway and bind directly to MHC class II molecules on the cell surface for presentation to helper T-cells.

A peptide fragment of CBA/J mouse hemoglobin Hb (67–76), VITAFNEGLK, has been selected to investigate the influence of glycosylation on T-cell response. This peptide is non-immunogenic in the CBA/J mouse, but able to bind to CBA/J mouse MHC class II molecule E^k [7]. It is therefore conceivable that this self peptide may readily be converted into a strong immunogen by small modifications and perhaps even by glycosylation. Synthetic glycopeptides are valuable tools when studying the influence of abnormal glycans in the immune regulation of tumour activity.

MATERIALS AND METHODS

General Procedures and Materials

All solvents were purchased from Labscan Ltd (Dublin, Ireland). Fmoc–amino acid–OPfp esters were purchased at Bachem (Switzerland) and NovaBiochem (Switzerland), Macrosorb SPR 250 resin from Sterling Organics (UK). TLC was performed on Merck Silica Gel 60 F_{254} alumina sheets with detection by charring with sulfuric acid, and by UV light when applicable. Flash chromatography was performed on Merck Silica Gel 60 (0.015–0.040 mm). Optical rotations were recorded on a Perkin-Elmer 241 polarimeter and are given in units of 10^{-1} deg cm^3 g^1. NMR spectroscopy was performed on a Bruker DRX 250. NMR experiments were performed at 300 K in $CDCl_3$. Chemical shifts are given in p.p.m. and referenced to residual $CHCl_3$ (δ_H 7.28) and $CDCl_3$ (δ_C 77.0). Coupling constants are given in hertz. For all compounds the assignment of the ^{1}H-NMR spectra was based on 2D proton–proton shift correlation spectra. The assignment of the ^{13}C-NMR spectra was based on carbon–proton shift correlation spectra. MALDI-TOF-MS was performed on a Finnigan MAT 2000 instrument with a matrix of α- cyano-4-hydroxy-cinnamic acid. ES-MS was performed on a VG-Quatro instrument from Fisons. HPLC was performed on a Waters HPLC system with delta pak C18 columns (200 × 25 mm, 15 μm, flow rate 10 ml/min for preparative separations) with buffer A (0.1% TFA in water) and buffer B (0.1% TFA in 90% aq. MeCN). Nomenclature is in agreement with IUPAC recommendations.

N^{α}-(Fluoren-9-ylmethoxycarbonyl)-O-(3,4,6-tri-O-acetyl-2-azido-2-deoxy-α-D-galactopyranosyl)-L-serine pentafluorophenyl ester (4). Compound **1** [8] (940 mg, 1.976 mmol) and compound **2** [9] (820 mg, 1.662 mmol) were dissolved in CH_2Cl_2 (40 ml). Molecular sieves (4 Å) were added and the solution cooled to −30 °C. Trimethylsilyl trifluoromethanesulfonate (70 μl) in CH_2Cl_2 (1400 μl) was added slowly. The reaction mixture was stirred for 90 min, quenched by addition of potassium hydrogen carbonate and washed with potassium hydrogen carbonate solution and water. The organic layer was

dried ($MgSO_4$) and the solvent removed *in vacuo* (NMR: α/β 6:1). Chromatography (PE/EA 3:1) on a column of dried silica gel yielded **4** (1.08 g α-anomer, 80%). NMR and MS data were in accord with published values [10]. The β-anomer (0.18 g, 13%) was also isolated.

N^{α}-(Fluoren-9-ylmethoxycarbonyl)-O-(3,4,6-tri-O-acetyl-2-azido-2-deoxy-α-D-galactopyranosyl)-L-threonine pentafluorophenyl ester (5). Compound **1** [8] (1280 mg, 2.691 mmol) and compound **3** [9] (1187 mg, 2.339 mmol) were dissolved in CH_2Cl_2 (60 ml). Molecular sieves (4 Å) were added and the solution cooled to -30 °C. Trimethylsilyl trifluoromethanesulfonate (90 µl) in CH_2Cl_2 (1800 µl) was added slowly. The reaction mixture was stirred for 90 min, quenched by addition of potassium hydrogen carbonate and washed with potassium hydrogen carbonate solution and water. The organic layer was dried ($MgSO_4$) and the solvent removed *in vacuo*. Chromatography (PE/EA 3:1) on a column of dried silica gel yielded **5** (1581 mg, only α, 82%). Data (NMR, MS) were in agreement with published values [10].

General Method for the Reduction of the Azido Group of the Building Blocks

The appropriate azide derivative (100 mg) was dissolved in THF/acetic acid/acetic anhyride (3:1:2) (5 ml). Zinc dust, activated for 2 min in 2% copper sulfate solution and twice washed with water, was added and the mixture stirred for 30 min. The solid was removed by filtration and washed with THF/acetic acid. The solution was evaporated under reduced pressure and the residue was redissolved in CH_2Cl_2. The solution was washed with water, dried ($MgSO_4$) and evaporated to give a nearly quantitative yield of a crude product. The product was essentially pure and was employed in SPPS without further purification.

N^{α}-(Fluoren-9-ylmethoxycarbonyl)-O-(3,4,6-tri-O-acetyl-2-acetamido-2-deoxy-α-D-galactopyranosyl)-L-serine pentafluorophenyl ester (6). Reduction of **4** by the general procedure for azide reduction yielded 95% of compound. Data (NMR, MS) are in agreement with published values [11].

N^{α}-(Fluoren-9-ylmethoxycarbonyl)-O-(3,4,6-tri-O-acetyl-2-acetamido-2-deoxy-α-D-galactopyranosyl)-L-threonine pentafluorophenyl ester (7). Reduction of **5** by the general procedure for azide reduction yielded 95% of compound. Data (NMR, MS) were in agreement with published values [11].

N^{α}-(Fluoren-9-ylmethoxycarbonyl)-O-(2,3,4,6-tetra-O-acetyl-β-D-galactopyranosyl)-L-threonine pentafluorophenyl ester (9). Compound **8** [12] (490 mg, 1.150 mmol) and compound **3** [9] (450 mg, 0.887 mmol) were dissolved in CH_2Cl_2 (10 ml). Molecular sieves (4 Å) were added and the solution cooled to -30 °C. Trimethylsilyl trifluoromethanesulfonate solution (500 µl, 800 µl in 20 ml CH_2Cl_2) was added slowly. The reaction mixture was stirred for 90 min, quenched by addition of potassium hydrogen carbonate and washed with potassium hydrogen carbonate solution and water. The organic layer was dried ($MgSO_4$) and the solvent removed *in vacuo*. **9** was obtained by chromatography (PE/EA 5:3) on a column of dried silica gel (406 mg, 55%). ES-MS: m/z 860.4 ($M+Na^+$) (calc. M: 836.7), $[\alpha]_D$ -24.1 ($c=1$, $CHCl_3$), ^1H and ^{13}C-NMR data are presented in Tables 1 and 2.

N^{α}-(Fluoren-9-ylmethoxycarbonyl)-O-(3,4,6-tri-O-acetyl-2-acetamido-2-deoxy-β-D-galactopyranosyl)-L-serine pentafluorophenyl ester (10). Reduction of the β-linked compound **4** by the general procedure for azide reduction yielded 90% of compound **10**. ES-MS: m/z 845.4 ($M+Na^+$) (calc. M: 822.2), $[\alpha]_D$ -12.1 ($c=0.5$, $CHCl_3$), ^1H and ^{13}C-NMR data are presented in Tables 1 and 2.

N^{α}-(Fluoren-9-ylmethoxycarbonyl)-O-(3,4,6-tri-O-acetyl-2-azido-2-deoxy-α-D-glucopyranosyl)-L-threonine pentafluorophenyl ester (12). Compound **11** [8] (138 mg, 0.290 mmol) and compound **3** [9] (127 mg, 0.252 mmol) were dissolved in CH_2Cl_2 (7 ml). Molecular sieves were added and the solution cooled to -30 °C. Trimethylsilyl trifluoromethanesulfonate solution (320 µl, 150 µl in 4 ml CH_2Cl_2) was added slowly. The reaction mixture was stirred for 90 min, quenched by addition of potassium hydrogen carbonate and washed with potassium hydrogen carbonate solution and water. The organic layer was dried ($MgSO_4$) and the solvent removed *in vacuo*. Compound **12** was obtained by chromatography (PE/EA 2:1) on a column of dried silica gel to yield 122 mg (59 %). ES-MS: m/z 843.2 ($M+Na^+$) (calc. M: 820.2), $[\alpha]_D = +8.1$ ($c=0.5$, $CHCl_3$), ^1H and ^{13}C-NMR data are presented in Tables 1 and 2.

N^{α}-(Fluoren-9-ylmethoxycarbonyl)-O-(3,4,6-tri-O-acetyl-2-acetamido-2-deoxy-α-D-glucopyranosyl)-L-threonine pentafluorophenyl ester (13). Reduction of **12** by the general procedure for azide reduction

Table 1 ^{1}H-NMR Chemical Shift Assignment and Coupling Constants (Hz, in parentheses) for Compounds **9**, **10**, **12** and **13** Measured in $CDCl_3$ at 300 K

	Chemical shifts (δ)			
	9	**10**	**12**	**13**
1-H	4.54 (7.6)	4.89 (8.2)	5.17 (3.9)	5.01 (3.1)
2-H	5.19 (10.5)	3.94 (11.2)	3.51 (10.5)	4.39 (8.8)
3-H	5.06 (3.3)	5.26 (3.4)	5.47 (10.2)	5.19 (8.9)
4-H	5.40 (< 1)	5.38 (< 1)	5.09	5.11 (8.8)
5-h	3.92	3.94	4.09	4.05
6-Ha	4.07	4.14	4,28	4.20
6-Hb	4.04	4.12	4.14	4.15
NHAc		5.57 (8.2)		5.86 (9.5)
NH-AA	5.76 (9.0)	6.17 (8.6)	5.92 (9.3)	6.03 (9.2)
α-H	4.74 (2.5)	4.89	4.81 (2.1)	4.77
β-H	4.60 (6.2)	4.44 + 4.03	4.61 (6.4)	4.45 (6.1)
γ-H	1.32		1.47	1.43
FmocCH	4.28	4.27	4.28	4.29
$FmocCH_2$	4.50 + 4.45	4.56 + 4.46	4.50 + 4.48	4.59 + 4.56

Table 2 ^{13}C-NMR Chemical Shift Assignment for Compounds **9**, **10**, **12** and **13** Measured in $CDCl_3$ at 300 K

	Chemical shifts (δ)			
	9	**10**	**12**	**13**
C-1	98.6	100.7	99.8	99.5
C-2	68.9	51.6	61.4	51.5
C-3	70.5	69.6	76.5	71.0
C-4	66.7	66.6	68.2	68.3
C-5	70.5	71.0	67.8	68.5
C-6	60.8	61.3	61.9	62.0
C-α	58.6	54.4	58.4	58.4
C-β	73.7	68.1	72.0	77.1
C-γ	16.6		18.9	18.6
FmocCH	47.1	47.1	47.1	47.2
$FmocCH_2$	67.4	67.2	67.4	67.5

yielded 90% of compound **13**. ES-MS: m/z 859.3 ($M + Na^+$) (calc. M: 836.7), $[\alpha]_D = +30.4$ ($c = 1$, $CHCl_3$), ^{1}H and ^{13}C-NMR data are presented in Tables 1 and 2.

***tert*-Butyldimethylsilyl 2,3,4,6-tetra-O-acetyl-β-D-galactopyranosyl-(1 → 3)-2-azido-4,6-O-benzylidene-2-deoxy-β-D-galactopyranoside (15).** Compound **14** [8] (4.5 g, 11.04 mmol) and compound **8** [12] (6.5 g, 15.26 mmol) were dissolved in CH_2Cl_2 (60 ml) and cooled at − 30 °C. Trimethylsilyl trifluoromethanesulfonate solution solution (400 µl in 10 ml) was added slowly. The mixture was stirred for 60 min, triethylamine (1 ml) was added and washed with potassium hydrogen carbonate solution. The organic layer was dried ($MgSO_4$) and the solvent was removed under reduced pressure. The crude compound was purified by silica gel chromatography (PE/EA 6:4) yielding **15** (6.93 g, 85%). ES-MS: m/z 760.4 ($M + Na^+$) (calc. M: 737.8), $[\alpha]_D$ + 13.1 ($c = 1$, $CHCl_3$), ^{1}H and ^{13}C-NMR data are presented in Tables 3 and 4.

***2,3,4,6-Tetra-O-acetyl-β-D-galactopyranosyl-(1 → 3)-2-azido-4,6-O-benzylidene-2-deoxy-β-D-galactopyranose (16).** Compound **15** (4.81 g, 6.52 mmol) was dissolved in distilled THF (50 ml) and stirred for 30 min at 0 °C with molecular sieves. TBAF solution (14 ml, 1.4 M) was added slowly and the solution stirred for 30 min. The mixture was poured into ice water, extracted three times with diethyl ether and the combined organic layers dried ($MgSO_4$). The product **16** (3.38 g, 83%) was employed for the following reaction without further purification.

***2,3,4,6-Tetra-O-acetyl-β-D-galactopyranosyl-(1 → 3)-O-2-azido-4,6-O-benzylidene-2-deoxy-β-D-galactopyranosyl trichloroacetimidate (17).** A solution of **16** (1000 mg, 1.60 mmol) in CH_2Cl_2 (15 ml) was stirred with dry potassium carbonate (1.60 g). Trichloroace-

Table 3 ^{1}H-NMR Chemical Shift Assignment and Coupling Constants (Hz, in parentheses) for Compounds **15**, **17**, **18** and **20** Measured in $CDCl_3$ at 300 K

	Chemical shifts (δ)			
	15	**17**	**18**	**20**
1-H	4.57 (7.6)	5.66 (8.5)	5.09 (3.2)	5.17 (3.2)
2-H	3.74 (10.6)	4.12 (10.5)	3.95 (10.6)	4.67
3-H	3.45 (3.4)	3.64 (3.4)	4.04	3.95
4-H	4.21 (< 1)	4.32 (< 1)	4.41 (< 1)	4.32
5-H	3.37 (< 1)	3.59 (< 1)	3.73 (< 1)	3.67
6-Ha	4.28 (12.9)	4.38 (12.9)	4.19	4.22
6-Hb	4.06 (12.9)	4.08 (12.9)	4.15	3.97
NH	-			5.65 (8.1)
1′-H	4.82 (7.9)	4.87 (7.7)	4.74 (7.9)	4.74 (8.2)
2′-H	5.25 (10.4)	5.32 (10.4)	5.31 (10.2)	5.22 (10.2)
3′-H	5.05 (3.5)	5.06 (3.4)	5.04 (3.4)	5.01 (3.3)
4H	5.41 (< 1)	5.42 (< 1)	5.41 (<1)	5.40 (<1)
5′-H	3.91 (6.6;6.6)	3.94 (6.2;6.2)	3.89	3.90
6′-Ha	4.20	4.20	4.23	4.16
6′-Hb	4.14	4.16	4.00	4.13
NH-Imidate		8.73		
NH-AA			6.08 (8.3)	6.11 (8.0)
α-H			4.96	4.93
β-H			4.43 + 4.11	4.27 + 4.07
FmocCH			4.28	4.28
$FmocCH_2$			4.55 + 4.48	4.53 + 4.50
Bzl-H	5.56	5.60	5.57	5.55

Table 4 ^{13}C-NMR Chemical Shift Assignment for Compounds **15**, **17**, **18** and **20** Measured in $CDCl_3$ at 300 K

	Chemical shifts (δ)			
	15	**17**	**18**	**20**
C-1	97.5	97.1	100.6	99.9
C-2	64.7	61.9	58.7	48.2
C-3	78.3	78.4	75.5	73.6
C-4	75.0	74.7	75.5	75.1
C-5	66.6	67.4	63.9	63.8
C-6	69.1	68.7	68.9	68.9
C-1′	102.9	102.2	102.4	100.9
C-2′	68.7	68.6	68.2	68.9
C-3′	70.9	71.0	70.9	70.7
C-4′	67.0	67.0	66.9	66.9
C-5′	70.8	71.0	70.9	71.2
C-6′	61.3	61.4	61.3	61.4
C-α			54.5	54.5
C-β			70.1	68.9
FmocCH			47.0	47.0
$FmocCH_2$			67.4	67.4
Bzl	100.8	100.7	100.8	100.9

tonitrile (1.2 ml) was added. After 3 h the mixture was filtered and the solvent removed under reduced pressure. Chromatography on a column of silica gel (PE/EA 1:2) yielded **17** (654 mg, 56%). ES-MS: m/z 790.5 ($M + Na^+$) (calc. M: 767.9), $[\alpha]_D$ -12.1 (c = 1, $CHCl_3$), ^{1}H and ^{13}C NMR data are presented in Tables 3 and 4.

***N*$^{\alpha}$*-(Fluoren-9-ylmethoxycarbonyl)-O-(2,3,4,6-tetra-O-acetyl-β-D-galactopyranosyl-(1 → 3)-O-2-azido-4,6-O-benzylidene-2-deoxy-α-D-galactopyranosyl)-L-serine pentafluorophenyl ester (18).** Compound **17** (254 mg, 0.330 mmol) and compound **2** [9] (155 mg, 0.314 mmol) were dissolved in CH_2Cl_2 (9 ml). Molecular sieves (4 Å) were added and the solution cooled to − 30 °C. Trimethylsilyl trifluoromethanesulfonate solution (150 µl, 800 µl in 20 ml CH_2Cl_2) was added slowly. The reaction mixture was stirred for 90 min, quenched by addition of potassium hydrogen carbonate and washed with potassium hydrogen carbonate solution and water. The organic layer was dried ($MgSO_4$) and the solvent removed *in vacuo* (NMR: α/β 12:1). Chromatography (PE/EA 1:1) on a column of dried silica gel yielded **18**

(168 mg α-anomer, 50%). ES-MS: *m/z* 1121.3 ($M+Na^+$) (calc. M: 1098.3), $[\alpha]_D = +63.1$ (c = 1, $CHCl_3$), ^1H and ^{13}C-NMR data are presented in Tables 3 and 4.

N^{α}-(Fluoren-9-ylmethoxycarbonyl)-O-(2,3,4,6-tetra-O-acetyl-β-D-galactopyranosyl-(1 → 3)-2-azido-4,6-O-benzylidene-2-deoxy-α-D-galactopyranosyl)-L-threonine pentafluorophenyl ester (19). Compound **17** (850 mg, 1.110 mmol) and compound **3** [9] (540 mg, 1.064 mmol) were dissolved in CH_2Cl_2 (20 ml). Molecular sieves (4 Å) were added and the solution cooled to − 30 °C. Trimethylsilyl trifluoromethanesulfonate solution (600 µl, 150 µl in 4 ml CH_2Cl_2) was added slowly. The reaction mixture was stirred for 90 min, quenched by addition of potassium hydrogen carbonate and washed with potassium hydrogen carbonate solution and water. The organic layer was dried ($MgSO_4$) and the solvent removed *in vacuo* (NMR: α/β 15:1). Chromatography (PE/EA 1:1) on a column of dried silica gel yielded **19** (687 mg α-anomer, 58%). Data (NMR, MS) are in agreement with published values [13].

N^{α}-(Fluoren-9-ylmethoxycarbonyl)-O-(2,3,4,6-tetra-O-acetyl-β-D-galactopyranosyl-(1 → 3)-O-2-acetamido-4,6-O-benzylidene-2-deoxy-α-D-galactopyranosyl)-L-serine pentafluorophenyl ester (20). Reduction of **18** by the general procedure for azide reduction yielded 92% of compound **20**. ES-MS: *m/z* 1137.4 ($M+Na^+$) (calc. M: 1114.3), $[\alpha]_D = +65.2$ (c = 1, $CHCl_3$), ^1H and ^{13}C-NMR data are presented in Tables 3 and 4.

N^{α}-(Fluoren-9-ylmethoxycarbonyl)-O-(2,3,4,6-tetra-O-acetyl-β-D-galactopyranosyl-(1 → 3)-O-2-acetamido-4,6-O-benzylidene-2-deoxy-α-D-galactopyranosyl)-L-threonine pentafluorophenyl ester (21). Reduction of **19** by the general procedure for azide reduction yielded 89% of compound **21**. Data (NMR, MS) were in agreement published values [11].

Building blocks 22-27 were obtained from previous work [14–17].

Synthesis of Peptides 28–47 and Glycopeptides 48–67 (Table 5)

Macrosorb SPR 250 resin (10 g) was placed in a disposable syringe with a Teflon filter connected via a two-way valve to a vacuum waste container and the resin was swelled in DMF (40 ml, 20 min). Hydroxymethyl phenoxy acidic acid (0.91 g, 5 mmol) and

Table 5 MS data of Substituted Peptide Hb (67–76): VITAFNEGLK[a]

No.	Sequence	M + H	M calc.
Serine scan, X = Ser (MALDI-TOF-MS data)			
28	*X*ITAFNEGLK	1080.3	(1079.2)
29	V*X*TAFNEGLK	1065.9	(1065.2)
30	VI*X*AFNEGLK	1078.1	(1077.3)
31	VIT*X*FNEGLK	1108.4	(1107.3)
32	VITA*X*NEGLK	1032.1	(1031.2)
33	VITAF*X*EGLK	1065.0	(1064.3)
34	VITAFN*X*GLK	1049.9	(1048.6)
35	VITAFNE*X*LK	1122.1	(1121.3)
36	VITAFNEG*X*K	1066.1	(1065.2)
37	VITAFNEGL*X*	1251.6	(1250.2)
Threonine scan, X = Thr (MALDI-TOF-MS data)			
38	*X*ITAFNEGLK	1094.6	(1092.3)
39	V*X*TAFNEGLK	1080.0	(1079.2)
40	VI*X*AFNEGLK	1092.3	(1091.3)[b]
41	VIT*X*FNEGLK	1122.7	(1121.3)
42	VITA*X*NEGLK	1046.1	(1045.2)
43	VITAF*X*EGLK	1079.6	(1078.3)
44	VITAFN*X*GLK	1062.6	(1063.7)
45	VITAFNE*X*LK	1136.6	(1135.3)
46	VITAFNEG*X*K	1080.6	(1079.2)
47	VITAFNEGL*X*	1065.2	(1064.2)
T_n-Ser scan, X = Ser(α-D-GalNAc-1-) (ES-MS data)			
48	*X*ITAFNEGLK	1282.6	(1281.6)
49	V*X*TAFNEGLK	1268.7	(1267.6)
50	VI*X*AFNEGLK	1280.8	(1279.6)
51	VIT*X*FNEGLK	1310.8	(1309.6)
52	VITA*X*NEGLK	1234.7	(1233.6)
53	VITAF*X*EGLK	1267.7	(1266.6)
54	VITAFN*X*GLK	1252.8	(1251.6)
55	VITAFNE*X*LK	1324.8	(1323.6)
56	VITAFNEG*X*K	1268.6	(1267.6)
57	VITAFNEGL*X*-NH_2	1251.6	(1252.4)
T_n-Thr scan, X = Thr(α-D-GalNAc-1-) (ES-MS data)			
58	*X*ITAFNEGLK	1296.8	(1295.6)
59	V*X*TAFNEGLK	1282.7	(1281.6)
60	VI*X*AFNEGLK	1294.9	(1293.6)
61	VIT*X*FNEGLK	1324.9	(1323.6)
62	VITA*X*NEGLK	1248.8	(1247.6)
63	VITAF*X*EGLK	1281.8	(1280.6)
64	VITAFN*X*GLK	1266.7	(1265.6)
65	VITAFNE*X*LK	1338.8	(1337.7)
66	VITAFNEG*X*K	1282.8	(1281.6)
67	VITAFNEGL*X*-NH_2	1265.6	(1266.4)

[a] All peptide and glycopeptides eluted as single compounds on analytical HPLC (buffer A–buffer B 100:0 → 0P100 (60 min)).
[b] Native peptide.

TBTU (1.53 g, 4.75 mmol) were dissolved in DMF (40 ml). 4-Ethyl-morpholine (575 mg, 630 µl, 5 mmol) was added and after 5 min the solution was added to the resin. After 1.5 h the resin was washed carefully with DMF (six times). Fmoc-Lys(Boc)-OH (3.51 g, 7.5 mmol) and *N*-methyl imidazol (461.8 mg, 447 µl, 5.62 mmol) were dissolved in CH_2Cl_2 (35 ml). MSNT (2.22 g, 7.42 mmol) was added and the solution was added to the resin after 3 min. After 2.5 h the resin was washed with CH_2Cl_2 (three times) and DMF (four times). Unreactive amino groups of the resin were acetylated with DMF/acetic acid (7:1) (2 × 2 min) and the resin was washed with DMF (eight times), diethyl ether (four times) and dried *in vacuo*. In case of peptides **37** and **47** the macrosorb resin was derivatized with FmocSer-(Bu^t)-OH and Fmoc-Thr(Bu^t)-OH respectively. For the synthesis of glycopeptides **57** and **67** a PEGA resin with Rink linker was employed [18].

The derivatized resin was packed in a 20 column manual peptide synthesizer (100 mg/column). The Fmoc deprotection was performed by treatment with 20% piperidine in DMF (1 × 2 min, 2 × 20 min). The resin was washed with DMF (five times). Each Fmoc amino acid OPfp ester (3 mol equiv.) and Dhbt-OH (3 mol equiv.) was dissolved in DMF (0.6 ml). In the case of glycosylated building blocks 1.6 equiv. of both reagents were employed. The solution of the reagents were transferred to the column and after 5 h removed by washing with DMF (5×).

The peptides were cleaved with 95% aq. TFA (1 ml) for 2 h at room temperature. The resin was washed with 95% aq. TFA and four times with acetic acid. After evaporation the peptides were freeze dried with water. The removal of the acetyl groups from the glycopeptides was performed with sodium methoxide at a pH of 8.5 in methanol. After 5 h the solution was acidified with acetic acid, evaporated and lyophilized from water. The peptides and glycopeptides were purified by RP-HPLC (buffer A–buffer B 100:0 → 50:50 (60 min)). The pure compounds were lyophilized and characterized by ES-MS (glycopeptides) and MALDI-TOF-MS (peptides) (Table 5).

RESULTS AND DISCUSSION

The present paper describes the synthesis of a large variety of peptide and glycopeptide analogues of the Hb (67–76) peptide. First a series of peptides was synthesized where all positions of the peptide were subsequently substituted by serine, threonine, T_n-serine and T_n-threonine (Figure 1). One of the most efficient methods for the synthesis of such series of peptides is by multiple column peptide synthesis (MCPS) performed in a Teflon block of columns organized in a matrix. Synthesis was carried out on Macrosorp resin using a two-step reaction cycle with acylation reactions using preformed solutions of preactivated Fmoc–amino acid–OPfp esters with Dhbt–OH catalysis and Fmoc cleavage with 20% piperidine in DMF. The glycosylated Fmoc–amino acid-OPfp esters were coupled as the usual amino acids in MCPS using extented reaction times and only a small excess of active ester. The use of Dhbt–OH allowed the acylation reaction to be monitored visually because of the colour change observed when the yellow ion pair between amino groups and Dhbt–OH disappears and the resin turns white with progress of acylation. Peptides were synthesized on the hydroxymethyl phenoxyacetic acid linker or the Rink-amide linker.

Synthesis of the T_n-antigen building blocks

The trichloroacetimidate derivative **1** was employed in the synthesis of the α-D-GalNAc-(1-*O*)Ser/Thr (T_n-antigen) building blocks [19]. Glycosylation of partially protected hydroxyamino acids Fmoc–Ser/Thr–OPfp ester [9] with **1** [8] was carried out with high stereoselectivity and with good yield in the presence of TMSOTf as catalyst. The threonine building block **5** [10] was obtained in 82% yield as pure α-anomer, the serine derivative **4** [10] as a mixture of α/β anomers 6:1 and 80% isolated α-anomer. The azido derivative of the building blocks were previously used during the MCPS [20]. Then the final conversion of the azido group to the acetamide had been achieved after the synthesis of the peptide by the reduction with thioacetic acid on the solid phase. The inconvenience of the long reaction time required, of the side products of *N*-thioacetates inherently formed and of the difficulties performing and analysing these reactions lead us to use a different strategy. The azido functions of the building blocks **4** and **5** were converted into acetamido groups using activated zinc dust in the presence of acetic anhydride for reduction with simultaneous acylation of the amino group formed. The acetylation of the amine was performed under acidic conditions with excess of acetic anhydride and the acylation was fast enough to avoid the intramolecular attack of the amino group on the Pfp ester which would result in the formation of a lactam. The building blocks **6** and **7** were used directly in MCPS and no manipulation of the acetamido function was required after peptide synthesis (Figure 1).

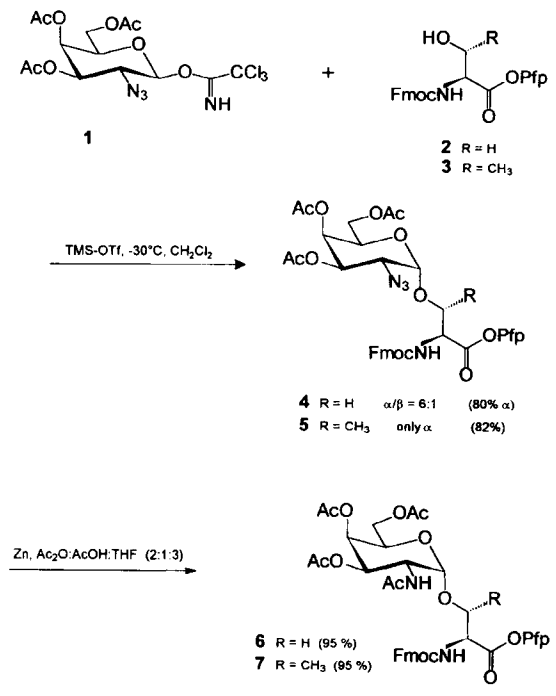

Figure 1 Synthesis of T_n antigen building blocks.

After distribution of the resin in the columns of the synthesizer the amino acids were coupled in DMF by use of 3 equiv. of the Fmoc–amino acid–OPfp ester's side chain protected with Bu^t-(Ser, Thr and Glu) and Boc-groups (Lys) and the reactions were catalyzed by the addition of 3 equiv. of Dhbt–OH. The acylation reactions were allowed to proceed for 4 h. The Fmoc cleavage was carried out with 20% piperidine in DMF. After the peptide synthesis was complete, the last Fmoc group was removed and the peptides were cleaved simultaneously by reaction with 95% aq. TFA for 2 h. During the cleavage from the resin all protecting groups of the amino acid side chains were removed, whereas the acetyl groups of the carbohydrates were completely stable. The *O*-acetyl protecting groups of the carbohydrate moiety of the glycopeptides were removed by mild transesterification with sodium methoxide in methanol at pH 8.5 to obtain the fully deprotected glycopeptides. The peptides **28–47** and glycopeptides **48–67** were purified by preparative reversed-phase HPLC (buffer A-buffer B 100:0 → 50:50 (60 min)) and yielded 50–75%. MALDI-TOF-MS data are presented in Table 6.

Peptide and Glycopeptide synthesis

The MCPS was performed in a 20 column manual peptide synthesizer. Macrosorp (SPR 250) resin was first derivatized in a syringe synthesizer with the acid labile hydroxymethyl phenoxy acetic acid using TBTU and *N*-ethyl morpholine. The first amino acid was esterified to the hydroxymethyl group using MSNT and *N*-methyl-imidazol as a coupling reagent.

Substitution at Position 72

The biological investigation of the peptides and glycopeptides indicated that the Hb (67–76) analogues were immunogenic when position 72 of the peptide was T_n-glycosylated [21]. To further investigate this glycosylation influence, a set of peptides substituted with different glycan structures at this particular position was synthesized.

Table 6 MALDI-TOF-MS Data of Peptide Hb (67–76): VITAFNEGLK Substituted on Position 72[a]

No.	Sequence	M + H	M calc.
68	VITAFS(β-D-Gal(1-3)-α-D-GalNAc-1-)EGLK	1430.4	(1429.6)
69	VITAFS(β-D-GalNAc-1-)EGLK	1268.7	(1267.5)
70	VITAFS(α-L-Fuc-1-)EGLK	1211.4	(1210.4)
71	VITAFS(β-L-Fuc-1-)EGLK	1211.3	(1210.4)
72	VITAFN(β-D-GlcNAc-1-)ETLK	1339.6	(1338.5)
73	VITAFT(β-D-Gal(1-3)-α-D-GalNAc-1-)EGLK	1444.4	(1443.6)
74	VITAFT(α-D-Man-1-)EGLK	1241.5	(1240.4)
75	VITAFT(β-D-Gal-1-)EGLK	1241.5	(1240.4)
76	VITAFN(β-D-GlcNAc-1-)EGLK	1295.8	(1294.5)
77	VITAFN(α-D-Glc(1-4)β-D-Glc-1-)EGLK	1416.4	(1415.6)
78	VITAFN(α-D-Glc(1-4)α-D-Glc(1-4)-β-D-Glc-1-)EGLK	1579.3	(1577.7)
79	VITAFT(α-D-GlcNAc-1-)EGLK	1281.8	(1280.7)

[a] All peptides and glycopeptides eluted as single compounds on analytical HPLC (buffer A–buffer B 100:0 → 0:100 (60 min)).

Building block **9** was synthesized by reaction of trichloroacetimidate **8** [12] with Fmoc–Thr–OPfp ester. The reaction resulted in the exclusive formation of the β-anomer in 55% yield. The minor β-linked **4**, obtained during the glycosylation of serine derivative **3** with **1**, was converted to the acetamido derivative **10** using the zinc/acetic anhydride method. Building blocks **6** and **10** differ only in the linkage of the GalNAc to the amino acid (Figure 2). The α-linked GlcNAc building block **13** which differs from **7** by being an epimer at the 4 position of the glycan was also synthesized. The α/β mixture of the imidate **11** [8] was used for the glycosyation of Fmoc–Thr–OPfp ester **3** yielding exclusively the α-product **12** which in high yield was converted to the building block **13** by reduction of the azido group and *in situ* acetylation with a mixture of zinc, acetic anhydride, acetic acid and THF (Figure 3). In order to investigate the influence of the size of the glycans, building blocks **20** and **21** which contained the T-antigen (β-Gal-(1 → 3)-α-GalNAc-(1-*O*)Ser/Thr) were synthesized. Glycosylation of the partially protected derivative **14** [13] with **8** [12] afforded a 85% yield of the disaccharide **15**. Only the formation of the β-linkage was observed. Two steps were required to obtain the glycosyl donor **17**. The TBDMS group was removed with TBAF to afford **16** and imidate **17** was then formed by reaction of **16** with trichloroacetonitrile and potassium carbonate, yielding only the β-product (Figure 4). Glycosylation of serine derivative **2** with **17** resulted in the formation of **18** in 50% yield of α-product (α/β 12:1). The reaction with threonine derivative **3** gave 58% of the α-product **19** (α/β 15:1). Both building blocks were successfully converted to the acetamido derivatives **20** and **21** in good yields using the zinc/acetic anhydride method for azide conversion (Figure 5). In addition to the synthesized building blocks, a set of previously described building blocks **22** + **23** [14], **24** [15], **25** [16] and **26** + **27** [17] was employed (Figure 6).

The glycopeptides were synthesized as described above for the peptides. Only 1.2 equiv. of the glycosylated building block was used and any remaining amino function capped with acetic anhydride. The fucosyl building blocks **22** and **23** were preactivated with TBTU before coupling. Removal of the carbohydrate *O*-acetyl protecting groups of the glycopeptides was performed with sodium methoxide in methanol at pH 8.5 after cleavage from the resin. Only the fucosyl glycopeptides **70** and **71** were treated with hydrazine hydrate as a milder method to avoid cleavage of the more labile fucose to serine bond. In those cases where silyl protection of the

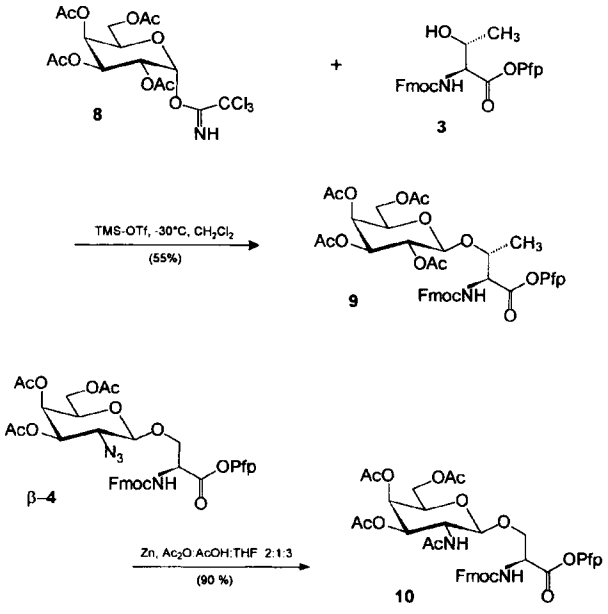

Figure 2 Synthesis of building blocks **9** and **10**.

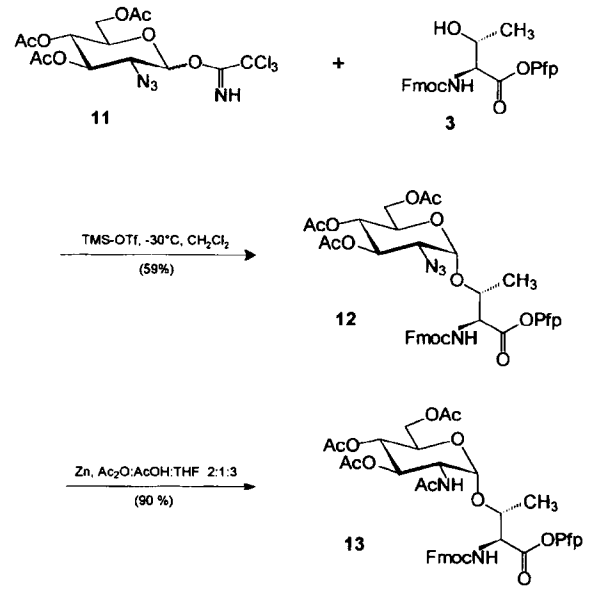

Figure 3 Synthesis of building block **13**.

glycan was employed (glycopeptides **77** and **78**) the deprotection of the carbohydrate occurred simultaneously with the cleavage from the resin [17]. The glycopeptides were eluted from HPLC as essentially a single compound and after preparative RP-HPLC they were obtained in yields of 50–65% based on the initial resin loading. MALDI-TOF-MS data are presented in Table 6.

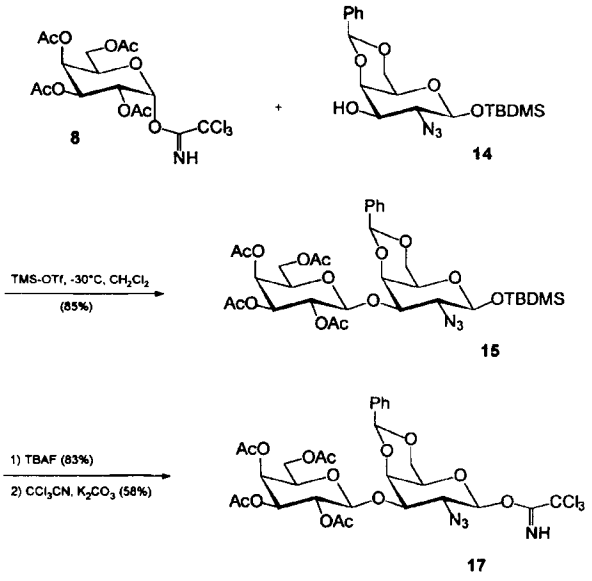

Figure 4 Synthesis of glycosyl donor **17**.

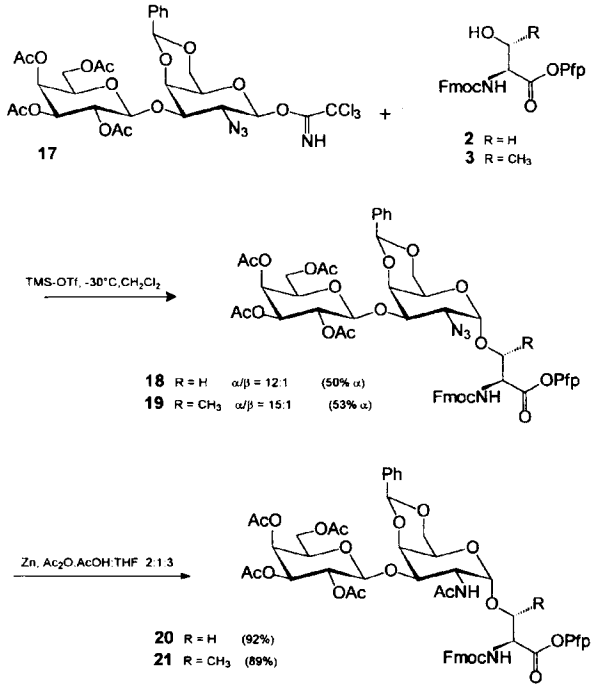

Figure 5 Synthesis of the T antigen building blocks.

CONCLUSIONS

Series of 20 peptides and 32 glycopeptides for the characterization of the interaction of the T-cell receptor with MHC-bound glycopeptides were successfully synthesized using MCPS. High yielding and

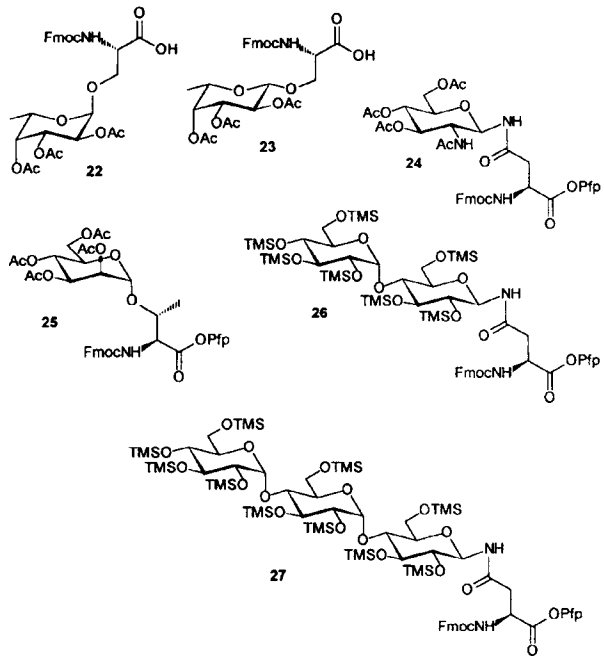

Figure 6 Building blocks **22–27**.

stereospecific glycosylations using trichloroacetimidates were developed for the convenient preparation of glycosyl amino acid building blocks with the T and T_n antigens as well as for α-linked GlcNAc residues. An efficient protocol with reduction and *in situ* acetylation was developed for the transformation of azido sugars into *N*-acetates on the level of the building block, eliminating the need for post-assembly conversion. High yield and purity could be obtained for a large range of glycan structures. Glycoproteins substituted at position 72 showed a large increase in T-cell stimulation index and glycanspecific T-cell clones could be produced, as will be reported elsewhere [21].

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