Journal of Peptide Science
J. Peptide Sci. **6**: 585–593 (2000)

Synthesis of Tumor Associated Sialyl-T-Glycopeptides and their Immunogenicity

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Received 24 March 2000
Accepted 31 March 2000

Abstract: Sialyl-T-glycopeptides were synthesized by solid-phase techniques, using a PEGA resin as the solid support. An appropriately protected building block containing α-Neu5Ac-(2→3)-β-Gal-(1→3)-α-GalN$_3$-(1→) attached to Fmoc-Thr/Ser-OPfp was employed in a solid-phase glycopeptide assembly of a 10-mer glycopeptide, using a general Fmoc/OPfp-ester strategy. Reduction of the azido group of the GalN$_3$ residue was effected on solid-phase, using DTT and DBU. After acidolytic cleavage from the resin, the methyl ester of the sialic acid residue and acetyl groups were removed with 30% NaOMe/MeOH in MeOH and water pH 14, at −30°C for 2 h. At this low temperature, the highly basic conditions did not result in any detectable β-elimination. However, one *O*-acetyl group, located at the 2-position of the Gal was resistant to hydrolysis. To remove this remaining acetyl group, reaction with hydrazine hydrate in $CHCl_3$ and MeOH at room temperature for 2.5 h was successful. The two target sequences of sialyl-T-glycopeptides were obtained in good yield. In contrast to the the analogs carrying the T-antigen, the Sial-T-glycopeptides were non-immunogenic, supporting the idea that the sialylation is a method of circumventing the recognition by the immune system. Copyright © 2000 John Wiley & Sons, Ltd.

Keywords: sialyl-T-glycopeptide; sialyl-T-antigen; glycopeptide synthesis; MHC class II

INTRODUCTION

Aberrant glycosylation is one of the most constant traits of the malignant cell phenotype [1], and a significant part of the humoral antitumor response of a cancer patient is directed against the epithelial cell membrane carbohydrates. MUC1 is a major surface molecule in the gastrointestinal tract. In mammalian tissue, the MUC1 glycoprotein is glycosylated with β-D-Gal-(1→3)-α-D-GalNAc, also named the T antigen on Thr and Ser, frequently terminated with sialic acid in cancerous tissue. In normal tissue, this disaccharide is further elongated with lactosamine units. However, in malignant epithelial tumors, there is a down regulation of GlcNAc transferase, and instead of elongation, the T antigen is directly terminated by sialylation of the 6-position of the GalNAc residue to give (β-D-Gal-(1→3)-[α-Neu5Ac-(2→6)]-α-D-GalNAc), or, particularly, the 3-position of the Gal residue, resulting in (α-Neu5Ac-(2→3)-β-D-Gal-(1→3)-α-D-GalNAc) [2]. The T-antigen may also be fucosylated at the 2-position of the Gal residue to give (α-L-Fuc-(1→2)-β-D-Gal-(1→3)-α-D-GalNAc) [1,3–6]. These structures, which are absent in normal tissue, could be expected to be immunogenic [7–9] as a result of lack of immunological tolerance, and they may,

Abbreviations: Dhbt-OH, 3,4-dihydro-3-hydroxy-4-oxo-1,2,3-benzotriazine; DIPEA, *N*,*N*-diisopropylethylamine; DTT, dithiothreitol; MeIm, 1-methylimidazole; MSNT, 1-(2-mesitylenesulfonyl)-3-nitro-1,2,4-triazole; NEM, *N*-ethylmorpholine; PEGA, *bis*-aminopropyl ethylene glycol polyacrylamide copolymer; Pfp, pentafluorophenyl; RPMI, Roswell Parks Memorial Institute tissue culture medium; Three or one letter codes are used for the amino acids, according to IUPAC.

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therefore, together with the T and Tn antigens, be involved in host rejection of malignant tissue. The negatively charged sialic acids have been considered to shield against host recognition [10–12] and, therefore, an antigenic response against these abundant antigens elicited by a challenge with a glycopeptide vaccine could be an effective way of cancer treatment.

In order to understand in more detail the physiochemical and biological properties of the MUC1 glycoprotein of malignant tissue, substantial efforts have been dedicated to the chemical synthesis of glycopeptides carrying these antigens. Recently, the synthesis method of Thr and Ser carrying the (2→3)-linked sialyl-T-antigen has been developed [13]. This method comprises a short, convenient route consisting of sialyl-T-antigen tri-saccharide building blocks. Furthermore, in a recent study of glycopeptide recognition by T-cells, it was demonstrated that substitution of N^{72} in the self peptide sequence VITAFNEGLK, derived from CBA/J mouse hemoglobin Hb(67-76) with T(α-GalNAc) (Tn-antigen), resulted in highly carbohydrate specific T-cell immunogenicity [14]. To study the effect of sialylation on glycopeptide immunogenicity, the same peptide Hb (67-76), carrying the Thr/Ser(sialyl-T) at position 72, was prepared. The two glycopeptides were subsequently used in immunization studies, in order to clarify the role of sialylation in the carbohydrate specific T-cell activation by cancer mucins.

MATERIALS AND METHODS

N^{α}-Fmoc amino acids and Pfp esters protected in the side-chains with TFA labile protecting groups were purchased from MilliGen (Taastrup, Denmark) or Bachem (Bubendorf, Switzerland). MALDI-TOF MS was performed in the positive mode on a Finnigan MAT 2000, using a matrix of α-cyano-4-hydroxycinnamic acid, and a renin substrate (M.W. = 1759.0) as a standard. ^{1}H-NMR spectra were recorded at neutral pH on a Bruker 600 MHz or Bruker DRX 250 MHz spectrometer. Chemical shifts are given in ppm and referenced to MeOH (δ 3.35) or H_2O (δ 4.75). Preparative reverse phase HPLC separations were performed on a Waters HPLC system, using a Delta PAK C-18 column (15 μm, 300 Å, 25 × 200 mm), with a flow rate of 10 ml min^{-1} and detection at 215 nm with a photodiode array detector (Waters 991). The solvent system was A: TFA/water (1/1000); B: TFA/acetonitril/water (1/900/100). Analytical reverse phase HPLC separations were performed on a Waters HPLC system, using RCM 25 × 10 module containing a 25 NV C_{18} column, with a flow rate of 1 ml min^{-1} with detection at 215 nm, with a programmable multi wavelength detector (Waters 490E). Buffers were as above.

Sialyl-T-Glycopeptide Synthesis by the Syringe Method, General Procedure

Synthesis of glycopeptides was performed in DMF using the PL-PEGA 1900 [poly(ethylene glycol dimethylacrylamide copolymer]. The resin **1** (100 mg) was packed into a 5 ml disposable syringe (Discardit II, Beckton Dickinson) fitted with a teflon filter. The syringe was connected to a suction flask through a teflon tube with a manual two-way teflon valve; excess reagent, DMF etc., was removed by applying vacuum. The resin was derivatized with the HMPA-linker: HMPA (15 mg, 80 μmol), TBTU (24 mg, 76 μmol) and NEM (20 μl, 160 μmol) were dissolved in DMF, and after 5 min, the solution was added to the resin. After 2 h, the solution was removed, and the resin was washed with DMF (3 × 3 ml), and a solution (3 ml) of acetic anhydride/DMF (1/7) was added. After 20 min, the resin was washed thoroughly with DMF (15 × 3 ml), and the solvent removed from the resin. The washing procedure was repeated after each coupling step. The first amino acid, Fmoc-Lys-OH (56 mg, 120 μmol), MeIm (7 μl, 90 μmol) and MSNT (36mg, 120 μmol) were dissolved in CH_2Cl_2 (1 ml), and, after 5 min, added to the resin. After 1 h, the resin was quickly washed with DMF (3 × 3 ml), and CH_2Cl_2 (3 × 3ml). The coupling procedure was repeated once more, and the resin was washed with DMF (2 × 3ml), CH_2Cl_2 (2 × 3ml) and DMF (10 × 3ml), and dried. The Fmoc group was cleaved from the Lys residue, and the loading of PEGA resin was determined from UV-absorbance of the eluate to be 0.33 mmol/g. N^{α}-Fmoc deprotection was effected by 22 min treatment of resin with 20% piperidine in DMF (10 ml).

The Fmoc amino acid Pfp esters (3.0 equivalent) and Dhbt-OH (1.0 equivalent) were dissolved in DMF (3 ml), and the solution was added to the resin. The suspension was agitated several times and then left for 24 h. After washing, the N^{α}-Fmoc group was removed, and the resin was washed as described above. The synthesis cycle was repeated with the remaining amino acids, except **3** (Thr) and **4** (Ser), containing the sialyl-T building block. The glycosylated building block was coupled with the

peptide **2**, using 1.0 equivalent of building block and 1.0 equivalent Dhbt-OH. These reactions were left for 3 days.

At the end of peptide assembly, the resin was washed successively with DMF (15×3 ml) and CH_2Cl_2 (10×3ml) and dried.

Azide Reduction. General Procedure

The azido groups of $GalN_3$ residues **5** or **6** were reduced with dithiothreitol (DTT). The resin was swelled in a solution of 0.25 M DTT/DMF (2.5 ml), and DBU (8 μl, 53 μmol) was added. Nitrogen gas evolved during the reaction. After 3 h, the resin was washed with DMF (6×3 ml). It was treated with 20% acetic acid in DMF (3 ml) for 30 min for *N*-acetylation, and the resin was washed with DMF (10×3 ml), CH_2Cl_2 (5×3ml) and dried.

Cleavage of the Sialyl-T-Glycopeptide from the Resin. General Procedure

Cleavage of the sialyl-T-glycopeptides **7** or **8** from the resin was performed by treatment with 95% TFA (3 ml) for 2.5 h, followed by filtration and washing of the resin with 95% TFA, water and CH_2Cl_2. The filtrate was concentrated, and the sialyl-T-glycopeptide was purified by preparative HPLC, first using 85% solvent A and 15% solvent B for 10 min, then the linear gradient 15–100% solvent B for 180 min.

Saponification and *O*-deacetylation of the Carbohydrate Moiety. General Procedure

The purified sialyl-T-glycopeptides **9** or **10** (e.g. 6.19 mg, 3.0 μmol) were dissolved in MeOH (800 μl) and the solution cooled. When the temperature reached −30°C, the 30% NaOMe/MeOH (150 μl) was added with stirring. After 2.5 h, water (400 μl) was added to the mixture, and stirring continued for 2 h at −30°C. The reaction mixture was neutralized with AcOH and the mixture allowed to warm. The product was isolated by preparative HPLC, using a gradient of 0–100% B, 120 min.

Complete *O*-deacetylation of the Carbohydrate Moiety. General Procedure

The purified sialyl-T-glycopeptides **11** or **12** (e.g. 9.41 mg, 5.2 μmol) was dissolved in $CHCl_3$/MeOH (1/5, 1.3 ml). The hydrazine hydrate (250 μl) was added to the solution with stirring. After 2.5 h, the reaction mixture was directly injected to a preparative HPLC system, and the product purified using a gradient of 0–100% B, 120 min.

Val-Ile-Thr-Ala-Phe-Thr{-*O*-(methyl 5-acetamido-4,7,8,9-tetra-*O*-acetyl-3,5-dideoxy-D-*glycero*-α-D-*galacto*-2-nonulopyranosylonate-(2→3)-*O*-2,4,6-tri-*O*-acetyl-β-D-galacto-pyranosyl-(1→3)-*O*-2-acetamido-2-deoxy-α-D-galactopyranosyl)}-Glu-Gly-Leu-Lys (9)

Solid-phase synthesis, azide reduction, cleavage from resin and purification (rf 50.75 min), as described in the general procedures, gave compound **9** (23.2 mg, 33%). Selected ^{1}H-NMR data for amino acid residues are presented in Table 1. Additional ^{1}H-NMR data (250 MHz, CD_3OD): $\delta = 1.86$, 2.01, 2.06, 2.06, 2.08, 2.11, 2.12, 2.20 and 2.32 (9s, 27H, 7Ac and 2NAc); sialic acid: $J_{3eq.,4}$ 4.4, J_{gem} 12.3, $J_{6,7}$ 2.5, $J_{7,8}$ 9.8 Hz, $\delta = 3.90$, CO_2CH_3; $GalN_3$: $J_{1,2}$ 3.4 Hz. ES-MS$^+$ 2044.3 $[M+H]^+$; $C_{90}H_{139}N_{13}O_{40}$ requires *M*, 2043.12.

Val-Ile-Thr-Ala-Phe-Ser{-*O*-(methyl 5-acetamido-4,7,8,9-tetra-*O*-acetyl-3,5-dideoxy-D-*glycero*-α-D-*galacto*-2-nonulopyranosylonate-(2→3)-*O*-2,4,6-tri-*O*-acetyl-β-D-galactopyranosyl-(1→3)-*O*-2-acetamido-2-deoxy-α-D-galactopyranosyl)}-Glu-Gly-Leu-Lys (10)

Solid-phase synthesis, cyclization, azide reduction, cleavage from resin and purification (rf 51.75 min), as described in the general procedures, gave compound **10** (27.8 mg, 40%). Selected ^{1}H-NMR data for amino acid residues are presented in Table 1. Additional ^{1}H-NMR data (250 MHz, CD_3OD): $\delta = 1.86$, 2.01, 2.06, 2.07, 2.09, 2.10, 2.11, 2.22 and 2.32 (9s, 27H, 7Ac and 2NAc); sialic acid: $J_{3eq.,4}$ 4.7, J_{gem} 12.5, $J_{6,7}$ 2.5, $J_{7,8}$ 9.8 Hz, $\delta = 3.90$ CO_2CH_3; $GalN_3$: $J_{1,2}$ 3.3 Hz. ES-MS$^+$ 2029.6 $[M+H]^+$; $C_{89}H_{137}N_{13}O_{40}$ requires *M*, 2029.12.

Val-Ile-Thr-Ala-Phe-Thr(-*O*-5-acetamido-3,5-dideoxy-D-*glycero*-α-D-*galacto*-2-nonulopyrano-sylonic acid-(2→3)-*O*-2-*O*-acetyl-β-D-galactopyranosyl-(1→3)-*O*-2-acetamido-2-deoxy-α-D-galactopyranosyl)-Glu-Gly-Leu-Lys (11)

Saponification, *O*-deacetylation and purification (rf 35.25 min) of the compound **9** (6.19 mg, 3.0 μmol), as described in the general procedure, gave compound **11** (3.3 mg, 61%). Selected ^{1}H-NMR data (250 MHz, CD_3OD): $\delta = 2.05$, 2.10 and 2.26 (3s, 9H, OAc and 2NAc), 2.77 (dd, 1H, $J_{3eq.,4}$ 3.7, J_{gem} 13.3 Hz, H-3eq. sialic acid), 5.11 (dd, 1H, $J_{1,2}$ 7.9, $J_{2,3}$ 9.9 Hz, H-2 Gal). ES-MS$^+$ 1777.12 $[M+H]^+$; $C_{77}H_{125}N_{13}O_{34}$ requires *M*, 1776.87.

Table 1 ^{1}H-NMR Chemical Shift Assignments (δ: ppm, 500 MHz, CD_3OD, Reference $CD_3OD = 3.35$ ppm) for Protected Sialyl-T-Glycopeptides **9** and **10**

Comp. **9**	α-NH	α-H	β-H	Others			
Lys 10	8.37	4.46	1.93, 1.83	1.52	1.75	3.03	7.80
Leu 9	8.12	4.44	1.66	1.71	0.99	0.95	–
Gly 8	8.32	4.00	–	–	–	–	–
Glu 7	8.40	4.44	2.19, 2.03	2.46	–	–	–
Thr 6	8.22	4.55	4.38	1.32	–	–	–
Phe 5	8.10	4.69	3.26, 3.05	7.25–7.32	–	–	–
Ala 4	8.15	4.36	1.32		–	–	–
Thr 3	8.06	4.40	4.16	1.18	–	–	–
Ile 2	8.63	4.32	1.91	1.62	1.26	0.96	0.94
Val 1		3.86	2.23	1.07	1.04	–	–
	H1	H2	H3	H4	H5	H6/H6′	NHAc
GalNAc	4.90	4.36	4.02	4.14	–	3.75	7.57
Gal	4.90	4.97	4.59	5.05	5.40	3.52/3.72	–
	H3a/H3e	H3e	H4/NHAc	H5	H6	H7	H9/H9′
Neu5Ac	2.60/1.48	3.59	3.80/7.86	3.68	5.41	5.53	4.24/4.05
Comp. **10**	α-NH	α-H	β-H	Others			
Lys 10	8.36	4.44	1.96, 1.82	1.50	1.72	3.00	7.79
Leu 9	8.05	4.42	1.66	1.69	0.98	0.93	–
Gly 8	8.30	4.00	–	–	–	–	–
Glu 7	8.31	4.39	2.20, 2.05	2.53	–	–	–
Thr 6	8.28	4.59	4.02,3.87	–	–	–	–
Phe 5	8.03	4.65	3.24, 3.03	7.28–7.33	–	–	–
Ala 4	8.23	4.30	1.32	–	–	–	–
Thr 3	8.06	4.38	4.20	1.21	–	–	–
Ile 2	8.64	4.34	1.90	1.61	1.26	0.95	0.93
Val 1		3.85	2.23	1.07	1.05	–	–
	H1	H2	H3	H4	H5	H6/H6′	NHAc
GalNAc	4.90	4.42	4.04	4.13	–	–	7.97
Gal	4.90	4.97	4.58	5.06	5.41	3.51/3.71	–
	H3a/H3e	H3e	H4/NHAc	H5	H6	H7	H9/H9'
Neu5Ac	2.56/1.48	3.59	3.80/7.86	3.68	5.41	5.53	4.25/4.05

Val-Ile-Thr-Ala-Phe-Ser(-*O*-5-acetamido-3,5-dideoxy-D-*glycero*-α-D-*galacto*-2-nonulopyranosylonic acid-(2→3)-*O*-2-*O*-acetyl-β-D-galacto-pyranosyl-(1→3)-*O*-2-acetamido-2-deoxy-α-D-galactopyranosyl)-Glu-Gly-Leu-Lys (12)

Saponification, *O*-deacetylation and purification (rf 35.0 min) of the compound **10** (10.9 mg, 5.4 μmol), as described in the general procedure, gave compound **12** (6.23 mg, 66%). Selected ^{1}H-NMR data (250 MHz, CD_3OD): $\delta = 2.05$, 2.07 and 2.25 (3s, 9H, OAc and 2NAc), 2.76 (dd, 1H, $J_{3eq.,4}$ 4.5, J_{gem} 11.5 Hz, H-3eq. sialic acid), 5.10 (dd, 1H, $J_{1,2}$ 8.2, $J_{2,3}$ 9.6 Hz, H-2 Gal). ES-MS$^+$ 1763.42 $[M+H]^+$; $C_{76}H_{123}N_{13}O_{34}$ requires *M*, 1762.87.

Val-Ile-Thr-Ala-Phe-Thr(-*O*-5-acetamido-3,5-dideoxy-D-*glycero*-α-D-*galacto*-2-nonulopyranosylonic acid-(2→3)-*O*-β-D-galactopyranosyl-(1→3)-*O*-2-acetamido-2-deoxy-α-D-galactopyranosyl)-Glu-Gly-Leu-Lys (13)

Complete *O*-deacetylation and purification (rf 33.0 min) of the compound **11** (9.41 mg, 5.3 μmol), as described in the general procedure, gave target compound **13** (6.57 mg, 71%). Selected ^{1}H-NMR data (250 MHz, CD_3OD): $\delta = 2.05$ and 2.08 (2s, 6H, 2NAc), 2.45 (m, 2H, H_γ Glu), 2.20 (m, 1H, H_β Glu), 2.05 (m, 1H, H_β Glu), 2.23 (m, 1H, H_β Val), 3.00 (m, 1H, H_ε Lys), 3.05 (m, 1H, H_β Phe), 3.25 (dd, 1H, H_β Phe), 2.78 (dd, 1H, H-3eq. sialic acid). ES-MS$^+$ 1735.57 $[M+H]^+$; $C_{75}H_{123}N_{13}O_{33}$ requires *M*, 1734.86.

Val-Ile-Thr-Ala-Phe-Ser(-*O*-5-acetamido-3,5-dideoxy-D-*glycero*-α-D-*galacto*-2-nonulopyranosylonic acid-(2→3)-*O*-β-D-galactopyranosyl-(1→3)-*O*-2-acetamido-2-deoxy-α-D-galactopyranosyl)-Glu-Gly-Leu-Lys (14)

Complete *O*-deacetylation and purification (rf 33.5 min) of the compound **12** (11.74 mg, 6.7 μmol), as described in the general procedure, gave target compound **14** (7.48 mg, 65%). Selected ^{1}H-NMR data (250 MHz, CD_3OD): $\delta = 2.05$ (s, 6H, 2NAc), 2.48 (m, 2H, H_γ Glu), 2.20 (m, 2H, H_β Glu), 2.23 (m, 1H, H_β Val), 3.00 (m, 2H, H_ε Lys), 3.05 (m, 1H, H_β Phe), 3.25 (dd, 1H, H_β Phe), 2.90 (dd, 1H, H-3eq. sialic acid). ES-MS$^+$ 1721.55 $[M+H]^+$; $C_{74}H_{121}N_{13}O_{33}$ requires *M*, 1720.84.

Immunization with Glycopeptide

7–8 week female CBA/J mice (H-2^k) were immunized in the hind foot pads and at the base of the tail with 0.1 ml of a water in oil emulsion, containing equal amounts of PBS and Complete Freunds Adjuvant with Mycobacterium butyricum (Difco) and peptide or glycopeptide antigen. A total of 100 μg of the antigen was injected into each mouse.

T-cell Proliferation Assay

Lymph node cells (LNC) from the popliteal and inguinal lymph nodes (LN) of the immunized mice were prepared on day 10 after immunization. Peptide was added to *in vitro* cultures of 4×10^5 LNC/well. The culture was performed in quadruplicate in RPMI 1640, containing 100 U/ml penicillin, 100 μg/ml streptomycin, 5×10^{-5} M 2-mercaptoethanol, 4 mM glutamine, 1 mM sodium pyruvate, 1% non-essential amino acids, and 1% syngeneic mouse serum. Purified protein derivative (PPD) of tuberculin was used as positive control. Background proliferation was measured by PBS. The final volume per well was 200 μl. [^{3}H]thymidine (1 μCi) was added per well after 72 h. Cells were harvested on a Filtermate 196 (Packard Instruments) after 90 h in culture, and the [^{3}H]-incorporation was determined by liquid scintillation counting (Topcount, Packard Instruments).

RESULTS AND DISCUSSION

The building blocks, N^α-(fluoren-9-ylmethoxycarbonyl)-*O*-[(methyl 5-acetamido-4,7,8,9-tetra-*O*-acetyl-3,5-dideoxy-D-glycero-α-D-galacto-2-nonulopyranosylonate)-(2→3)-2,4,6-tri-*O*-acetyl-β-D-galactopyranosyl-(1→3)-2-azido-4,6-*O*-benzylidene-2-deoxy-α-D-galactopyranosyl]-L-threonine pentafluorophenyl ester (Fmoc-Thr(sialyl-T)-OPfp), and N^α-(fluoren-9-ylmethoxycarbonyl)-*O*-[(methyl 5-acetamido-4,7,8,9-tetra-*O*-acetyl-3,5-dideoxy-D-glycero-α-D-galacto-2-nonulopyranosylonate)-(2→3)-2,4,6-tri-*O*-acetyl-β-D-galactopyranosyl-(1→3)-2-azido-4,6-*O*-benzylidene-2-deoxy-α-D-galactopyranosyl]-L-serine pentafluorophenyl ester (Fmoc-Ser(sialyl-T)-OPfp), recently reported [13], were used in solid-phase glycopeptide assembly. Synthesis of the sialyl-T-glycopeptide were performed in DMF by the syringe method [15], using the PL-PEGA 1900 resin [16,17] derivatized with the HMPA-linker (Figure 1). The first amino acid, Fmoc-Lys-OH, was attached to the HMPA-linker by activation with 1-methylimidazole (MeIm) and 1-(2-mesitylenesulfonyl)-3-nitro-1,2,4-triazole (MSNT) [18]. The glycopeptide assembly was performed by the Fmoc/OPfp-ester strategy [19], using 3,4-dihydro-3-hydroxy-4-oxo-1,2,3-benzotriazine (Dhbt-OH) as a catalyst. The use of Dhbt-OH as an auxiliary nucleophile allowed the progress of the amide bond formation to be followed visually.

After assembly of the linear peptide chains, the N_3 group was reduced to NHAc by 0.25 M DTT/DMF and 1.5 eq. DBU/DMF at room temperature for 3 h (Figure 2). The reagents were removed and *N*-acylation was effected with acetic anhydride.

The sequence of reaction which would be most advantageous was considered, the saponification of the sialic acid methyl ester on solid-phase with simultaneous deacetylation of hydroxy groups or cleavage of the protected glycopeptide from the resin, followed by deprotection. When the

saponification and de-acylation was carried out first, optimal reaction condition was difficult to establish, as removal of *O*-acetates and the free carboxylic acid on the 1-position of the sialic acid rendered the glycoside linkage between sialic acid and Gal more susceptible toward acidic condition. Therefore, the glycopeptides were first cleaved off from the resin with 95% aqueous TFA, and purified by HPLC. The purified glycopeptides **9** and **10** still protected with *O*-acetyl groups were completely characterized at this stage by 1-D and 2-D ^{1}H-NMR spectroscopy, because the *O*-acetylation increased the dispersion of the carbohydrate protons, thus, facilitating structural assignment.

Cleavage of the methyl ester of the sialic acid was attempted using several different basic conditions (Figure 1). To avoid β-elimination in the glycopeptide synthesis, the pH is usually kept below 10. After treatment of the glycopeptide with 0.1 N NaOH in H_2O and EtOH at pH 10 and 0°C for 3 h, HPLC of the mixture still showed starting compound as the main peak. No saponification product was observed. The same result was observed by using both 0.2 N KOH, pH 10 at 0°C for 3 h, or by addition of 30% NaOMe/MeOH to the glycopeptide in MeOH/H_2O, pH 10 at 0°C for 3 h. This indicated that, in order to cleave the methyl ester, more basic conditions were required. However, as has often been demonstrated, β-elimination can easily occur at such high pH conditions. In order to prevent β-elimination, the reaction was, therefore, carried out at -30°C, using conditions with 30% NaOMe/MeOH in MeOH and H_2O (pH 14), previously used at higher temperatures for gangliosides. Despite the high pH, no β-elimination product was formed, and only one product, the deprotected glycopeptide, was obtained. However, analysis of the compound by MALDI-TOF MS showed an additional 42 mass units corresponding to a resistant acetate remaining on the target molecule. Careful inspection of the mass spectrum revealed some loss of of glycan resulting from fragmentation in the ion source, yielding peaks at 291 and 495 mass units less than the molecular ion. These correspond to loss of sialic acid and the mono acetylated disaccharide α-Neu5Ac-(1-3)-*O*-Ac-Gal, indicating the resistant acetyl group to be located at the Gal residue. Analysis of the ^{1}H-NMR spectrum of **11** and **12** showed

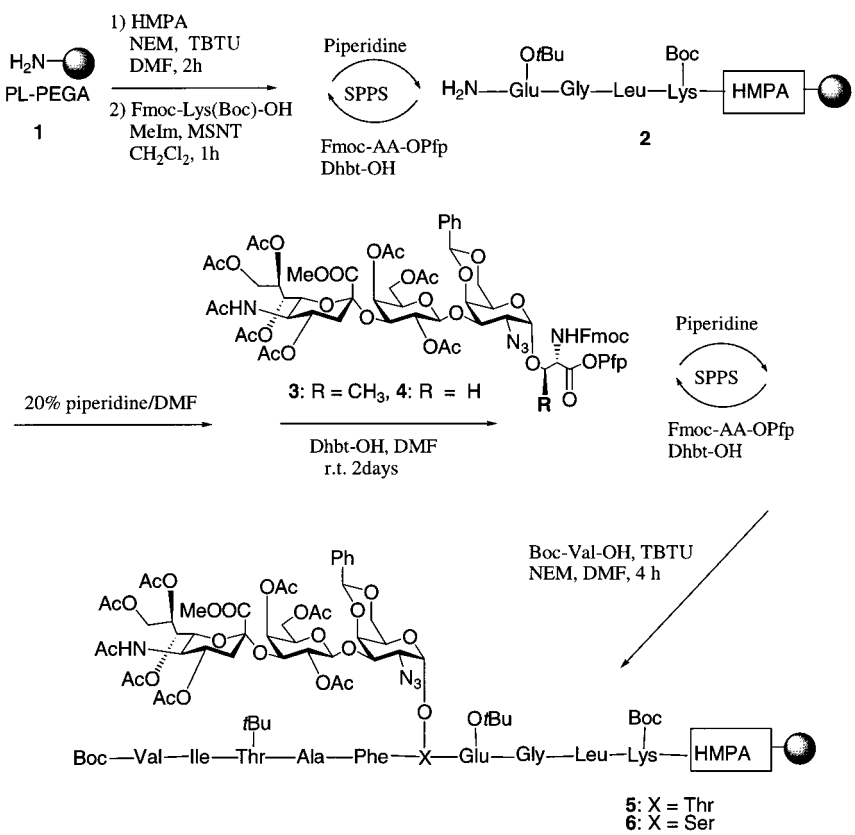

Figure 1 Solid-phase glycopeptide synthesis.

significant down field shifts of the 2-position of Gal to δ 5.11 (dd, 1H, $J_{1,2} = 7.9$, $J_{2,3} = 9.9$Hz) and 5.10 (dd, 1H, $J_{1,2} = 8.2$, $J_{2,3} = 9.6$Hz), respectively. Furthermore, three acetyl signals were observed at δ 2.05, 2.10 and 2.26 (3s, 9H) and 2.05, 2.07 and 2.25 (3s, 9H), respectively, indicating that two *N*-acetyl groups and one *O*-acetyl group located at the 2-position of Gal still remained. To remove the remaining *O*-acetyl group, the glycopeptide was treated with hydrazine hydrate in $CHCl_3$ and MeOH at room temperature for 2.5 h. After purification by HPLC, the compounds **13** and **14** were obtained in 71% and 65%, respectively. The target sialyl-T-glycopeptide **13** and **14** were analyzed in the positive ion mode by MALDI-TOF MS and gave the expected molecular masses at 1735.57 and 1721.55 μ, respectively.

This new two step method of deprotection (Figure 2) of the methyl ester protected sialyl-T-antigen gives a high yield, and is a valuable alternative to previously used methods employing more elaborate protection schemes to facilitate the ester deprotection of sialic acid. Although β-elimination does not occur at mildly basic conditions, it is not generally recommended to use pH 14 for reactions with glycopeptides. However, the use of high concentration of NaOH at low temperature, followed by treatment with NH_2-NH_2, resulted in a clean reaction, leading to a single deprotected compound obtained from a glycopeptide assembled with simple synthetic precursors.

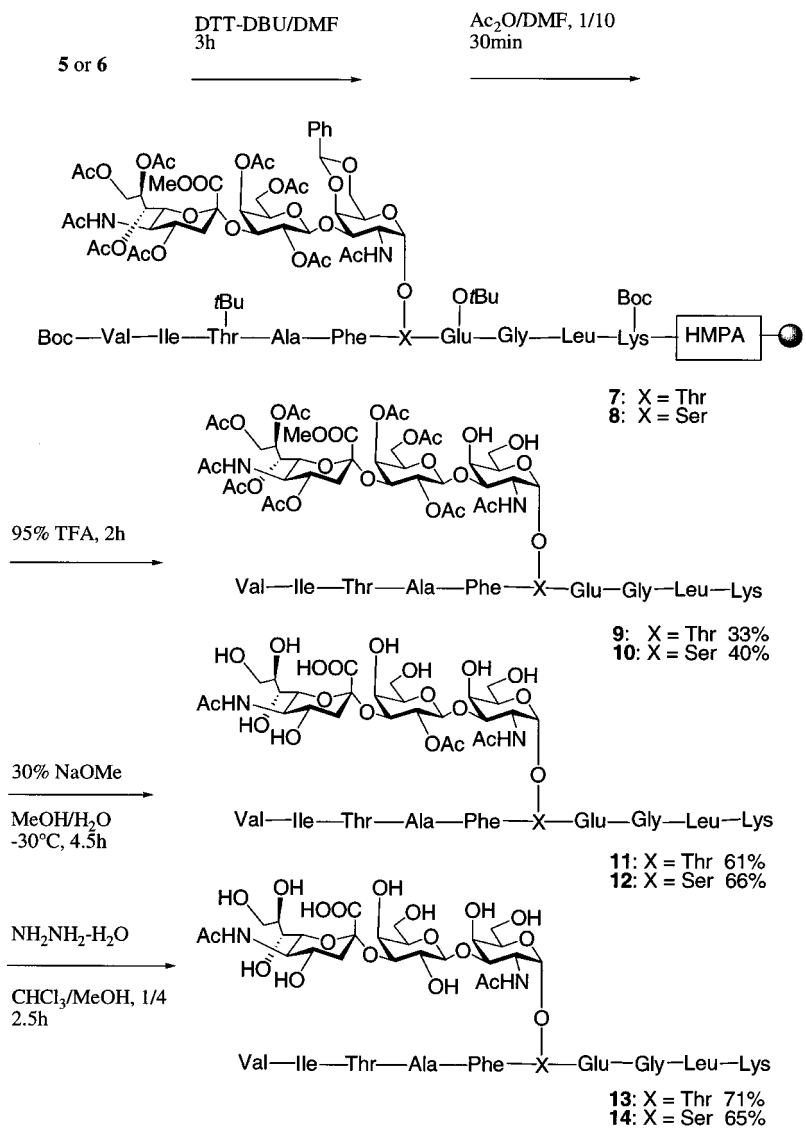

Figure 2 Cleavage and deprotection.

Table 2 T-cell Proliferation in Response to Immunization with Sialyl-T-Glycopeptides Measured by Incorporation of Radiolabeled Thymidine into T-cells

Mean cpm [^{3}H]Thymidine incorporation/culture ± S.D. in response to:						
Experiment no.	HB 67-76 glycopeptide used for immunization	PBS	Glycopeptide			PPD[a]
			180 μg/ml	60 μg/ml	20 μg/ml	30 μg/ml
1	S^{72}(SiGGN)[b]	641 ± 47	631 ± 85	839 ± 283	600 ± 85	42552 ± 1842
2	T^{72}(SiGGN)	1233 ± 215	1167 ± 147	1291 ± 67	1301 ± 186	39872 ± 1142
3	S^{72}(GGN)[c]	1389 ± 341	10292 ± 4042	8572 ± 1308	5445 ± 213	58936 ± 8509
4	T^{72}(GGN)[c]	3834 ± 215	8098 ± 554	7687 ± 516	5770 ± 675	42952 ± 5023

[a] Highest response obtained with 30 μg PPD/ml.
[b] SiGGN: α-Neu5Ac-(2→3)-β-D-Gal-(1→3)-α-D-GalNAc.
[c] Taken from reference [14].

Biological Results

The glycopeptides were incorporated into an emulsion of PBS and Freunds complete adjuvant and Balb/c mice were immunized. After 10 days the antigen presenting cells and T-cells were harvested from the draining lymph nodes, and tested in a T-cell proliferation assay. In microtiter plates, the cells were stimulated with the glycopeptides in the presence of radio-labeled [^{3}H]thymidine. Thymidine incorporation into cells was measured and evaluated against a negative (PBS) and a positive (PPD, purified protein derivative of tuberculin) control culture with no glycopeptide added (Table 2). The sialylated glycopeptides **13** and **14** were unable to stimulate T-cells in these immunization studies, in contrast to the immunogenic analogeous glycopeptides carrying the T-antigen (particularly S^{72}-T, having the core 1 structure linked to Ser) [14], suggesting a shielding effect of this type of sialylation. These shielding effects of sialic acid have also been reported in cellular recognition phenomenon, controlling cell morphology, and in the inhibition of entry of virus particles into the host cells [20,21]. A method for tumor suppression based on tumor targeted inhibition of 2-3-sialyl transferase may, therefore, be suggested as a valuable remedy for the treatment of cancer.

Acknowledgements

We thank Bent Ole Petersen for expert assistance with the NMR spectrometric analysis. This work was supported by the Danish Cancer Society.

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