Synthesis of T-antigen-containing glycopeptides as potential cancer vaccines

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In recent years, many tumour-associated carbohydrate antigens have been identified and some of them, including the Tn [GalNAcα(1→*O*)Ser/Thr] and T [Galβ(1→3)GalNAcα(1→*O*)Ser/Thr] antigens, are highly expressed in carcinoma-associated mucins. Two novel glycosyl building blocks (**3** and **4**) containing the T-antigen were synthesised by glycosylation of Fmoc-homoserine and glycolic acid having unprotected carboxy groups with 2,3,4,6-tetra-*O*-acetyl-β-D-galactopyranosyl-(1→3)-2-azido-4,6-*O*-benzylidene-2-deoxygalactopyranosyl trichloroacetimidate (**2**) as the glycosyl donor. These building blocks were used directly in the solid-phase synthesis of glycopeptides with varying distances between the peptide and glycan following two different methodologies. In the first strategy, the glycosylated amino acid building block was incorporated into the growing peptide chain using TBTU–NEM activation. The second strategy employed direct, selective, solid-phase acylation of an amino side chain of the relevant amino acid with a suitably protected glycosyl moiety, after the peptide had been completely synthesised. Reduction of the 2-azido group of the galactose moiety and acetylation to the corresponding acetamido function was performed on the solid phase.

Introduction

The carbohydrate moieties of glycoproteins are known to influence the properties of the parent protein in diverse ways.[1] For example, glycosylation provides protection against proteolysis, influences uptake of serum proteins by the liver, affects intracellular transport of enzymes to lysosomes, determines human blood groups, and regulates leukocyte trafficking to sites of inflammation. In addition, the glycosidic part of glycoproteins is responsible for many intercellular cell-surface-recognition phenomena.[2,3] Of fundamental biological and pharmaceutical importance are, for example, the aberrant glycosylation patterns of glycoproteins in cancer,[4] the carbohydrate-mediated cell adhesion involved in haematogeneous metastasis of cancer[5] and the inflammatory response mechanism.[6]

In recent years, many tumour-associated carbohydrate antigens have been identified and some of them, including the Tn [GalNAcα(1→*O*)Ser/Thr] and T [Galβ(1→3)GalNAcα(1→*O*)-Ser/Thr] antigens, are highly expressed in carcinoma-associated mucins, resulting from truncated carbohydrate chains during malignant transformation.[7] Synthetic glycopeptides have been shown to trigger humoral responses in murine and human immune systems against defined tumour antigens.[8,9] These data suggest the possibility of inducing immunity against cancer with fully synthetic carbohydrate vaccines. Simple carbohydrate conjugate vaccines have been synthesised, and their immunogenicity has been confirmed.[10–14]

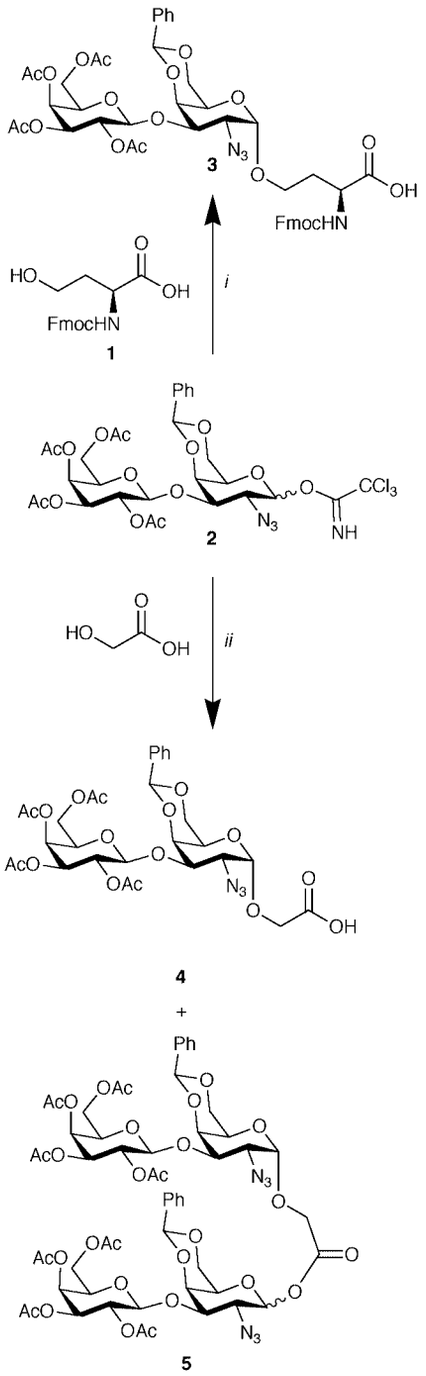
In previous work directed towards the development of anticancer carbohydrate-based vaccines, it was shown that both the nature of the peptide and the glycan portion of the glycopeptide H-VITAFXEGLK-OH, where X represents several different mono-, di- and trisaccharides linked to Ser or Thr, were crucial in eliciting a particular immune response.[15] To generate *in vivo* a carbohydrate-specific and peptide-independent cytolitic T-cell response we designed two polyalanine peptide backbones with high binding affinity for the murine class I MHC alleles K^b and K^k to be used in the *in vivo* studies. The glycan is in a central position for T cell receptor (TCR) recognition. We aim to examine the *in vivo* effects of varying the distance between the glycan and the peptide scaffold. To this end, two novel glycosyl building blocks (**3** and **4**), containing the T-antigen, were synthesised and incorporated into glycopeptides using two different strategies: the building block approach and glycosylation by acylation of a preformed peptide. We describe herein the synthesis of the glycosylated building blocks as well as the four different glycopeptides (**6**–**9**) containing variations in both the peptide amino acids and the distance between the T-antigen and the peptide scaffold.

Results and discussion

Preparation of building blocks

Fmoc-homoserine (Fmoc-*h*Ser) **1** was prepared according to the method of Paquet described for the N^{α} protection of Ser, Thr and Tyr.[16] The yield obtained (49%) was lower than that normally obtained for the protection of serine and threonine, in part because of the formation of the Fmoc γ-lactone upon acidification. The glycosyl donor, 2,3,4,6-tetra-*O*-acetyl-β-D-galactopyranosyl(1→3)-2-azido-4,6-*O*-benzylidene-2-deoxy-D-galactopyranosyl trichloroacetimidate **2**,[17] was used for the glycosylation of both N^{α}-Fmoc-protected homoserine **1** and glycolic acid (Scheme 1), affording, respectively, the two building blocks **3** and **4**. The use of *h*Ser increases the distance of the glycan from the peptide chain by one carbon atom compared with its serine analogue. The azido group in the glycosyl donor was employed as a masked amine functionality as well as a non-participating neighbouring group that facilitates α-glycosylation. In both cases the carboxylic acid group of the acceptors was unprotected.

Glycosylation of *h*Ser **1** with **2** upon activation with catalytic trimethylsilyl triflate (TMSOTf) was carried out at −50 °C in a



Scheme 1 *Reagents, conditions and yields*: *i*, 0.05 M TMSOTf, CH_2Cl_2–THF (3:1), −50 °C, 92%; *ii*, 0.05 M TMSOTf, CH_2Cl_2–THF (2:1), −50 °C, 77% for **4**.

mixture of CH_2Cl_2–THF (3:1) in order to solubilise the glycosyl acceptor and afforded derivative **3** in an α:β mixture (20:1) of anomers in 92% yield. Separation of the two anomers by flash chromatography was somewhat difficult; however, the α-anomer could be obtained in sufficiently pure quantities (912 mg, 66%) after one purification column. This yield is much greater than that obtained from the similar glycosylation of amino acids containing unprotected carboxylic acids using peracetylated mono- and disaccharides as the glycosyl donor.[18] It is also noteworthy that no traces of by-products due to the glycosylation of both the hydroxy and the free carboxy group of homoserine could be detected.

Glycosylation of glycolic acid was carried out using an analogous procedure (Scheme 1). In this case, a larger quantity of THF was required to allow solubility of the glycolic acid [CH_2Cl_2–THF (2:1)]. The reaction was carried out at −50 °C, using a two-fold excess of the glycosyl acceptor and 0.02 equivalents of catalyst (TMSOTf), and afforded the desired compound **4** in 77% yield. No trace of the β-anomer was detected. Instead, a by-product **5** (12%) formed by the glycosylation of both the hydroxy and carboxy groups of the acid was obtained. The by-product was identified using ^{1}H NMR and MALDI-TOF-MS after purification of the crude reaction mixture.

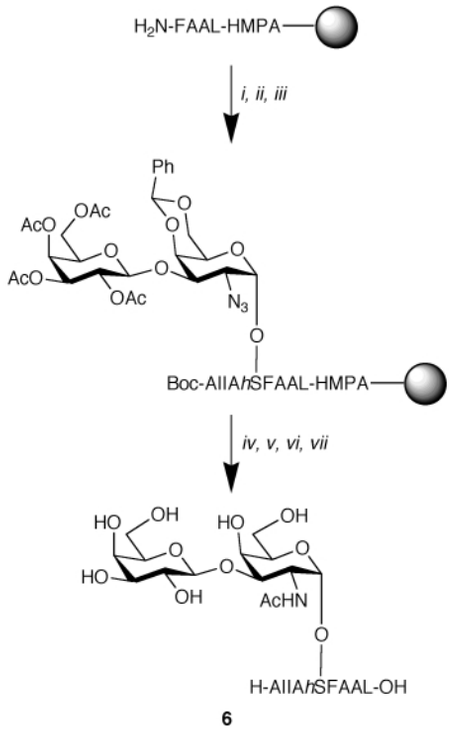
The two building blocks (**3** and **4**), having the 2-azido group instead of the desired 2-acetamido function present in the structure of the T-antigen, were used in the solid-phase synthesis of four different T-antigen-containing glycopeptides (Schemes 2 and 3).

Solid-phase synthesis of glycopeptides

The solid-phase synthesis of the glycopeptides exploited two different methodologies. In the first strategy, the building block containing the protected glycan attached to an amino acid side chain was used directly in solid-phase synthesis of the glycopeptide.[19–21] The second strategy employed direct solid-phase acylation of an amine side chain with a suitably protected glycosyl moiety, after the peptide had been assembled. In both cases, reduction of the azido group on the sugar moiety and subsequent acetylation to the acetamido function present in the T-antigen was performed on a solid support.

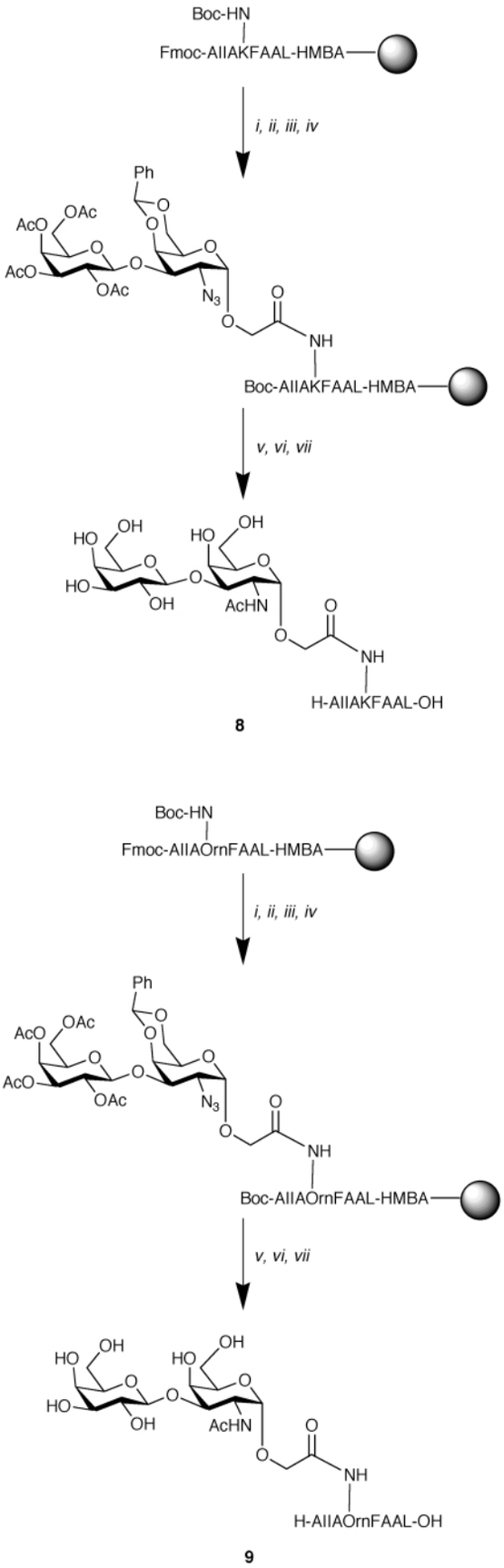
The first methodology (Scheme 2) was used for the incorporation of building block **3** into peptides H-AIIA*h*S(T-Ag)FAAL-OH **6** and H-AEA*h*S(T-Ag)AAAI-OH **7**, on $PEGA_{800}$ resin. The peptides were attached to the resin *via* the acid-labile hydroxymethylphenoxyacetic acid (HMPA) linker. Normal amino acids were incorporated using three equivalents of the Fmoc–pentafluorophenyl (Pfp) derivative and one equivalent of 3,4-dihydro-3-hydroxy-4-oxo-1,2,3-benzotriazine (Dhbt-OH) as an acylation catalyst and a simultaneous indicator of reaction completeness. The glycosylated building block **3** (two reaction cycles of 3 h, 2.0 equiv. cycle^{-1}) was coupled to the growing peptide chain upon preactivation with *O*-(benzotriazol-1-yl)-*N*,*N*,*N*′,*N*′-tetramethyluronium tetrafluoroborate (TBTU) and *N*-ethylmorpholine (NEM). The N-terminal amino acid was protected with a Boc group instead of the usual Fmoc group, in order to avoid cleavage of the Fmoc protecting group during the reduction of the azido function with dithiothreitol (DTT) and DBU under basic conditions. The glycopeptides were cleaved from the resin and the side-chain-protecting group was simultaneously deprotected with 95% TFA in water, and the *O*-acetyl protecting groups removed under Zèmplen conditions. The crude glycopeptides were purified by reversed-phase HPLC yielding the products **6** and **7** in 83 and 30% overall yield, respectively. The highly hydrophobic nature of glycopeptide **6** rendered its purification by HPLC extremely difficult. Its solubility was eventually achieved by first completely solubilising it in neat TFA and then slowly adding water up to 50%.

Two additional glycopeptides, H-AIIAK(T-Ag)FAAL-OH **8** and H-AIIAOrn(T-Ag)FAAL-OH **9** (Scheme 3), were synthesised by direct acylation of the corresponding Fmoc-protected peptide (Fmoc-AIIAXFAAL-HMBA-resin where X is an unprotected lysine (Lys) or ornithine (Orn) residue) with compound **4** upon TBTU–NEM preactivation. In these peptides, the glycan was attached to the peptide as an amide (N-linked) in contrast to the others where the peptide was attached as the O-linked peptide. This is a more versatile strategy since only one building block is required to synthesise several different glycopeptides with varying amino acid sequence and tether length. In the two glycopeptides synthesised, the distance between the glycan and the peptide scaffold was roughly 6 and 7 atoms for glycopeptides **8** and **9** respectively. The peptides were synthesised on $PEGA_{800}$ resin as described for glycopeptides **6** and **7** with the exception that the peptides were attached to the solid support using the base-labile hydroxymethylbenzoic acid



Scheme 2 *Reagents and conditions*: *i*, **3**, TBTU–NEM, DMF; *ii*, Fmoc-Aa-OPfp, Dhbt-OH, DMF; *iii*, Boc-Ala-OH, TBTU–NEM, DMF; *iv*, DTT–DIPEA–DBU, DMF; *v*, 20% Ac_2O in DMF; *vi*, 50% TFA in CH_2Cl_2; *vii*, 0.05 M NaOMe.

(HMBA) linker which allowed simultaneous cleavage of the peptides and *O*-deacetylation of the final product. The side-chain amino function of Lys and Orn was temporarily protected with a Boc group. Removal of the Boc group by treatment with 50% TFA in CH_2Cl_2 was followed by acylation using 1.5–2.0 equivalents of the building block in one cycle. Acylation was complete within 1 h based on monitoring by the Kaiser test. The N-terminal Fmoc protecting group was cleaved by treatment with 20% piperidine–DMF, replaced with a Boc group by reaction with excess Boc anhydride, and the azido



Scheme 3 *Reagents and conditions*: *i*, 50% TFA in CH_2Cl_2; *ii*, **4**, TBTU–NEM, DMF; *iii*, 20% piperidine in DMF; *iv*, 10 equiv. Boc_2O, DMF; *v*, DTT–DBU, DMF; *vi*, 20% Ac_2O in DMF; *vii*, 0.1 M NaOH.

functionality reduced by treatment with DTT and DBU in dry DMF over 20 min. It was necessary to exchange the N-terminal protecting group because cleavage of the Fmoc group occurs

upon exposure to low concentrations (0.15%) of DBU at room temperature. It is also shown that, in contrast to previous reports,[22] it is not necessary to have diisopropylethylamine (DIPEA) in addition to DBU during the reduction of the azide. The Boc and benzylidene protecting groups were removed by treatment with 50% TFA in CH_2Cl_2 for 30 min. The peptides were cleaved from the resin and simultaneously deacetylated using 0.1 M NaOH over 2 h, resulting in the desired glycopeptides **8** and **9** in 58% and 55% yield, respectively. While the yields from both strategies are comparable, the second strategy is preferred because of its versatility. The structural dependence of the biological activity of glycopeptides **6**–**9** is currently being investigated and the results will be published elsewhere.

Experimental

General procedures

All solvents were purchased from Labscan Ltd. (Dublin, Ireland). Dichloromethane was distilled from P_2O_5 and was stored over 3 Å molecular sieves under argon in sealed vessels. Light petroleum refers to the fraction in the distillation range 60–80 °C. Fmoc amino acids and their pentafluorophenyl (Pfp) ester derivatives were purchased from Bachem (Bubendorf) and NovaBiochem (Laufelingen). The substitution of the resins was determined by spectrophotometric analysis at 290 nm of the dibenzofulvene-piperidine adduct formed upon deprotection of the amino terminal, using a Perkin-Elmer Lambda 7 UV–VIS spectrophotometer. TLC was performed on Merck Silica Gel 60 F_{254} with detection by charring with sulfuric acid, and by UV light when applicable. Flash chromatography was performed on Merck Silica Gel 60 (40–63 μm). Optical rotations were recorded on a Perkin-Elmer 241 polarimeter and $[\alpha]_D$-values are given in units of 10^{-1} deg cm^2 g^{-1}. NMR spectroscopy was performed on a Bruker AMX-250 or a Bruker DRX-600 MHz spectrometer. All the NMR experiments were performed at 300 K in $CDCl_3$ or $(CD_3)_2CO$. Chemical shifts are given in ppm and referenced to internal $SiMe_4$ (0 ppm). Coupling constants (J) are given in Hz. For all compounds the assignments of the ^{1}H NMR spectra were based on 2D proton–proton shift-correlation spectra. The assignments of ^{13}C NMR spectra were based on carbon–proton shift-correlation spectra. MALDI-TOF MS was performed on a Finnigan MAT 2000 instrument with a matrix of 2,5-dihydroxybenzoic acid. ES mass spectra were recorded with a VG-Quatro instrument from Fisons. Purification of glycopeptides was performed by preparative reversed-phase HPLC on a Waters HPLC system equipped with a delta pak C_{18} column (200 × 25 mm; 15 μm) and eluting at a flow rate of 20 cm^3 min^{-1} with Buffer A (0.1% TFA in water) and Buffer B (0.1% TFA in 90% aq. MeCN). Analytical HPLC purity checks were performed using an RCM (8 × 200 mm) C_{18} column eluting at a flow rate of 1 cm^3 min^{-1} and with the same buffer system. Detection was at 215 and 280 nm with a photodiode array detector. Amino acid sequencing was performed using an Applied Biosystems Sequencer (models 477A or 470A) equipped with an on-line phenylthiohydantoin analyser (Model 120A) according to the protocol of the manufacturer.

N$^{\alpha}$-(Fluoren-9-ylmethoxycarbonyl)-L-homoserine 1

Fmoc-succinimide (13.3 g, 39.5 mmol, 1.1 equiv.) was added to a solution of L-homoserine (4.7 g, 39.5 mmol) and $NaHCO_3$ (3.3 g) in water–acetone (124 cm^3; 1:1) and the mixture was stirred overnight at 25 °C. The reaction mixture was acidified to pH 2 and the solvents evaporated. The residue was dissolved in $CHCl_3$ and the solution was washed with HCl (0.1 M), dried over Na_2SO_4, and concentrated. The product was recrystallised from a mixture of $CHCl_3$–THF–hexane to yield **1** (6.3 g, 49%); δ_H [250 MHz; $(CD_3)_2CO$] 7.89–7.30 (8 H, m, Fmoc ArH), 6.81 (1 H, d, $J_{NH,CH\alpha}$ 7.81, NH), 4.51–4.42 (1 H, m, CH$^{\gamma}$-H^a), 4.38–4.34 (2 H, m, Fmoc CH_2), 4.29–4.24 (1 H, m, CH$^{\gamma}$-H^b), 3.77–3.72 (2 H, m, CH$^{\alpha}$ and Fmoc CH), 1.99–1.89 (2 H, m, CH$^{\beta}$); δ_C [75 MHz; $(CD_3)_2CO$] 173.0 (CO_2H), 156.1 (Fmoc CO), 144.0 (2), 143.9 (2), 140.9 (2), 127.4 (1), 126.8 (1), 125.7 (1), 125.1 (1) and 119.7 (2) (together 12 × Fmoc ArC), 66.4 (Fmoc CH_2), 57.9 (C$^{\gamma}$), 51.2 (C$^{\alpha}$), 46.9 (Fmoc CH), 34.2 (C$^{\beta}$) [Found: (ES-MS) m/z M$^+$, 341.9. $C_{19}H_{21}NO_5$ requires M, 343.3].

Table 1 ^{1}H NMR chemical-shifts assignment and coupling constants[a] (J/Hz, in parentheses) for compounds **3** and **4** measured in $CDCl_3$ at 300 K (aromatic signals are omitted)

	3	**4**
H-1′ ($J_{H1',H2'}$)	4.93 (8.3)	4.86 (8)
H-2′ ($J_{H2',H3'}$)	5.21 (9.5)	5.34 (10)
H-3′ ($J_{H3',H4'}$)	5.08 (2.5)	5.08 (3)
H-4′ ($J_{H4',H5'}$)	5.41 (<1)	5.45 (*m*)
H-5′ ($J_{H5',H6'a}$, $J_{H5',H6'b}$)	3.91 (8.7, 5.4)	3.99 (*m*, *m*)
H-6′^{a} ($J_{H6'a,H6'b}$)	4.13 (12.4)	4.17 (7, 11.5)
H-6′^{b}	4.27 (*m*, *m*)	4.26 (6.5, 11.5)
$COCH_3$	2.01, 2.01, 2.06, 2.19	2.21, 2.09, 2.09, 2.02
H-1 ($J_{H1,H2}$)	5.06 (<1)	5.13 (3)
H-2 ($J_{H2,H3}$)	3.97 (12.0)	4.07 (*m*)
H-3 ($J_{H3,H4}$)	4.12 (3.2)	4.18 (3)
H-4 ($J_{H4,H5}$)	4.41 (<1)	4.46 (*m*)
H-5 ($J_{H5,H6a}$, $J_{H5,H6b}$)	3.72 (*m*, *m*)	3.87 (*m*, *m*)
H-6^a ($J_{H6a,H5}$, $J_{H6a,H6b}$)	4.08 (*m*, *m*)	4.29 (<1, 12.5)
H-6^b ($J_{H6b,H5}$, $J_{H6b,H6a}$)	4.28 (*m*, *m*)	4.10 (<1, 12.5)
PhCH	5.57	5.61
OCH_2CO_2H		4.32, 4.37
hSer γ-H^a	3.59	
hSer γ-H^b	3.93	
β-H_2	2.32	
α-H	4.58	
NH	5.86 (5.4)	
CH_2^{a} Fmoc	4.42	
CH_2^{b} Fmoc	4.55	
CH Fmoc	4.29	

[a] Some coupling constants could not be determined at 250 MHz.

O-[2-Azido-4,6-*O*-benzylidene-2-deoxy-3-*O*-(2,3,4,6-tetra-*O*-acetyl-β-D-galactopyranosyl)-α-D-galactopyranosyl]-*N*$^{\alpha}$-(fluoren-9-ylmethoxycarbonyl)-L-homoserine 3

Imidate **2** (1.13 g, 1.47 mmol) and **1** (753 mg, 2.21 mmol, 1.5 equiv.) were dissolved in a 3:1 mixture of dry CH_2Cl_2–THF (8 cm^3), and the solution was cooled to −50 °C under argon. A 0.05 M solution of TMSOTf in dry CH_2Cl_2 (52 mm^3) was added. After 1 h the mixture was warmed to room temperature, solid $NaHCO_3$ was added to neutrality (pH 7, pH paper) and the suspension filtered. The organic solution was concentrated, and purification of the residue by flash chromatography [chloroform–methanol 96:4 + 0.1% acetic acid] afforded compound **3** as a 20:1 α/β mixture (1.29 g, 92%); $[\alpha]_D$ +73.3 (c 1.5, $CHCl_3$); ^{1}H and ^{13}C NMR data are presented in Tables 1 and 2 [Found: (MALDI-MS) m/z (M + Na)$^+$, 970.46. $C_{46}H_{50}N_4O_{18}$ requires M, 946.92].

2-*O*-[2-Azido-4,6-*O*-benzylidene-2-deoxy-3-*O*-(2,3,4,6-tetra-*O*-acetyl-β-D-galactopyranosyl)-α-D-galactopyranosyl]glycolic acid 4

Glycolic acid (247 mg, 3.25 mmol) was dissolved in a 2:1 mixture of dry CH_2Cl_2–THF (3 cm^3), and the solution was cooled to −50 °C under argon. A 0.05 M solution of TMSOTf was injected (652 mm^3) and after 10 min a solution of imidate **2** (1.25 g, 1.63 mmol) in the same mixture (6 cm^3) was added dropwise. After 3 h the reaction mixture was allowed to reach room temperature and neutralised with solid $NaHCO_3$; the suspension was filtered and the solvent evaporated. Purification by flash chromatography [chloroform–methanol 96:4 + 0.1% acetic acid] afforded the title compound **4** (0.852 g,

Table 2 ^{13}C NMR chemical-shift assignment for compounds **3** and **4** measured in $CDCl_3$ at 300 K (aromatic signals are omitted)

	3	**4**
C-1′	102.9	102.8
C-2′	70.4	69.3
C-3′	71.6	71.6
C-4′	67.9	67.6
C-5′	71.8	71.6
C-6′	62.4	61.9
C-1	99.7	100.0
C-2	59.8	59.5
C-3	66.6	76.4
C-4	66.5	76.2
C-5	64.2	64.4
C-6	69.9	69.5
Ph*C*H	101.3	101.3
O*C*H_2CO_2H		65.6
hSer α-C	52.4	
hSer β-C	32.1	
hSer γ-C	65.5	
CH_2 Fmoc	67.9	
*C*H Fmoc	47.9	

77%); $[\alpha]_D$ +74.9 (*c* 0.7, $CHCl_3$); ^1H and ^{13}C NMR data are presented in Tables 1 and 2 [Found: (MALDI-MS) *m*/*z* $(M + Na)^+$, 705.56. $C_{29}H_{35}N_3O_{16}$ requires *M*, 681.62]. A second fraction contained compound **5** (250 mg, 12%) [Found: (MALDI-MS) *m*/*z* $(M + Na)^+$, 1308.8. $C_{56}H_{66}N_6O_{29}$ requires *M*, 1287.15].

Solid-phase synthesis of glycopeptides 6 and 7

The synthesis was carried out manually using a disposable plastic syringe fitted with a Teflon filter (pore size 70 μm) and connected to a vacuum waste bottle *via* a 2-way Teflon valve. Synthesis of peptides was carried out on $PEGA_{800}$ resin (308 mg, 0.055 mmol and 327 mg, 0.059 mmol of resin for peptides **6** and **7**, respectively) derivatised with an HMPA linker. The first amino acid was coupled as the Fmoc-protected amino acid (3 equiv.) activated with 1-(mesitylsulfonyl)-3-nitro-1,2,4-triazole (MSNT) (2.9 equiv.) in the presence of 1-methylimidazole (MeIm) (4.5 equiv.) in DMF (2 × 45 min).[23] Subsequent amino acids were synthesised using fully protected N^{α}-Fmoc amino acid OPfp esters (3 equiv.) in DMF with the addition of Dhbt-OH (1 equiv.) as an acylation catalyst and an indicator of the end-point of the coupling. The Fmoc group was removed by treatment with 20% piperidine (2 × 18 min). Resin was washed with DMF (6 × 2 min) between each coupling and deprotection step. The glycosyl amino acid building block **3** (2.0 equiv.) was coupled (2 × 3 h) after preactivation for 5 min with TBTU (1.1 equiv.) and NEM (1.8 equiv.) in DMF. The N-terminal amino acid was coupled as the N^{α}-Boc-protected amino acid (3 equiv.) under TBTU–NEM activation. The completeness of all reactions was monitored using the Kaiser test.[24]

Solid-phase synthesis of glycopeptides 8 and 9

Synthesis of peptides was carried out on $PEGA_{800}$ resin (loading 0.23 mmol g^{-1}) (100 mg, 0.023 mmol and 140 mg, 0.032 mmol of resin for peptides **8** and **9**, respectively). The resin was derivatised with an HMBA linker as described for the HMPA linker. The first amino acid and subsequent amino acids were coupled as described above. The completed peptides were treated with 50% TFA in CH_2Cl_2 for 30 min to remove the Boc side-chain-protecting groups of Lys and Orn. The resin was then washed with CH_2Cl_2 (6×) and DMF (3×). The glycosylated building block **4** (2 equiv.) was coupled for 1 h to the side-chain amino function after preactivation for 5 min with TBTU–NEM. The N-terminal Fmoc protecting group was cleaved by treatment with 20% piperidine, the resin washed as previously described, then treated with Boc anhydride (10 equiv.) and NEM (2 equiv.) for 30 min. The resin was washed once more with DMF (6×).

Reduction of azide functionality on solid support

The resin-bound glycopeptides were treated for 30 min with 0.1 M DTT in dry DMF, 0.2 M DIPEA in dry DMF (volume required to cover the resin) and 1 equiv. of DBU for glycopeptides **6** and **7**, and DTT (3 equiv.) and DBU (1 equiv.) (premixed for 10 min prior to addition to the resin) for glycopeptides **8** and **9**. The free amino function was then acetylated by treatment with 20% acetic anhydride in DMF for 30 min. The resin was washed several times with DMF (6×) then CH_2Cl_2 (10×) and dried for at least 1 h. The Boc and benzylidene protecting groups of glycopeptides **8** and **9** were removed by treatment with 50% TFA in CH_2Cl_2 for 30 min before cleavage from the resin.

Cleavage of peptides

Glycopeptides **6** and **7** attached to the HMPA linker were cleaved by incubation with 95% TFA in water for 2 h. The resin was washed with AcOH (2×), glycopeptide solutions were concentrated *in vacuo*, and excess of TFA co-evaporated with toluene. The crude glycopeptides were treated with 0.05 M NaOMe (11 cm^3) at pH 10 for 30 min. The reaction mixture was neutralised by adding glacial acetic acid to pH 7, and concentrated. Glycopeptides **8** and **9** attached *via* the HMBA linker were cleaved by treatment with 0.1 M NaOH for 2 h, effecting simultaneous removal of acetyl protecting groups. The solutions were neutralised with 0.1 M HCl and lyophilised. Glycopeptides were purified by preparative-scale HPLC, the purity checked by analytical HPLC, and mass measured by MALDI-TOF-MS: glycopeptide **6**: 57.24 mg, 82.5% [Found: (ES-MS) *m*/*z* M^+, 1255.9. $C_{57}H_{94}N_{10}O_{21}$ requires *M*, 1255.44]; Analytical HPLC (eluent linear gradient 0–100% Buffer B; analysis time 50 min), t_R = 29.41 min; glycopeptide **7**: 19.0 mg, 30% [Found: (ES-MS) *m*/*z* M^+, 1082.8. $C_{44}H_{75}N_9O_{22}$ requires *M*, 1082.14]; Analytical HPLC (eluent linear gradient 0–100% Buffer B; analysis time 50 min), t_R = 26.11 min; glycopeptide **8**: 18.8 mg, 58.7% [Found: (MALDI-TOF-MS) *m*/*z* $(M + Na)^+$, 1362.2. $C_{61}H_{101}N_{11}O_{22}$ requires *M*, 1339.54]; Analytical HPLC (eluent linear gradient 0–100% Buffer B; analysis time 50 min), t_R = 29.84 min; glycopeptide **9**: 23 mg, 55.4% [Found: (MALDI-TOF-MS) *m*/*z* $(M + Na)^+$, 1348.5. $C_{60}H_{99}N_{11}O_{22}$ requires *M*, 1325.52]; Analytical HPLC (eluent linear gradient 0–100% Buffer B; analysis time 50 min), t_R = 29.55 min.

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