Microwave-Assisted Synthesis of Fluorescein-Labelled GalNAcα1-*O*-Ser/Thr (Tn) Glycopeptides as Immunological Probes

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Abstract: Fluorescently labelled glycopeptides containing GalNAcα1-*O*-Ser/Thr residues provide valuable immunological probes for the development of cancer vaccines. The microwave-assisted automated Fmoc solid-phase synthesis of a series of 5(6)-carboxyfluorescein-labelled GalNAcα1-*O*-Ser/Thr peptides is described. Lys(Dde)-Gly-Wang polystyrene resin was elongated using Fmoc SPPS with incorporation of several GalNAcα1-*O*-Ser/Thr residues. Deprotection of the Lys(Dde) then allowed attachment of the 5(6)-carboxyfluorescein label. The synthetic methodology described is flexible and suitably robust enabling the incorporation of three contiguous GalNAcα1-*O*-Ser residues into the peptide chain.

Key words: carbohydrates, glycopeptides, GalNAcα1-*O*-Ser/Thr amino acids, glycosylations, solid-phase peptide synthesis

'Mucin-type' O-linked glycoproteins are one of the most prevalent O-linked glycoproteins found in higher eukaryotes and are characterized by the presence of *N*-acetyl-α-D-galactosamine (α-D-GalNAc) glycans attached to the hydroxy group of serine/threonine side chains.[1] The important role of these glycoproteins in biological processes[2] renders them attractive targets for therapeutic approaches.[3] A good example is the MUC1 glycosylated tumour-associated antigen.[4] MUC1 expressed by breast cancer cells bear shortened *O*-glycans such as GalNAcα1-*O*-Ser/Thr (Tn) and is an attractive tumour target for immunotherapy because MUC1 is highly expressed in several epithelial and non-epithelial cancers.[5] The development of efficient methods to assemble GalNAcα1-*O*-Ser/Thr glycopeptides is an essential step to facilitate the rational design of cancer vaccines.[6]

Dendritic cells (DC) are antigen-presenting cells (APCs) critical for the induction of adaptive immune responses.[7] After capturing and internalizing antigen in peripheral organs, they efficiently associate antigenic peptides resulting from proteolysis with MHC class I protein, and expose these MHC class I peptide complexes on their surface in a process called cross presentation. Cross presentation of tumour epitopes to cytotoxic T cell (CTL) precursors in peripheral lymphoid organs[8] then initiates cytotoxic responses to microbes and tumours.[9]

In order to perform their function, DC are equipped with specialized receptors, including adhesion receptors and several pattern recognition receptors, such as lectin-like receptors and Toll-like receptors.[10] C-Type lectins that recognize specific carbohydrate structures in a Ca^{2+}-dependent manner include DC-SIGN, mannose receptor, Langerin, DEC205, and the macrophage galactose-type C-type lectin (MGL). These lectin-like receptors can take up antigen, with the cytoplasmic tail of C-type lectins containing signalling or internalization motifs for antigen processing.[11] C-Type lectin receptors participate in the detection of carbohydrate structures specifically expressed during tumour progression.[12] Specifically, the tumour-associated Tn-MUC1 glycoform has been shown to be bound and internalized by the MGL C-type lectin and be delivered to MHC class I and II compartments in DC.[13] These studies provide further impetus for the synthesis of GalNAcα1-*O*-Ser/Thr glycopeptides to underpin the development of cancer vaccines.

There are two general approaches for the preparation of glycopeptides. The first strategy requires initial synthesis of the peptide chain followed by attachment of the carbohydrate unit.[14] The second approach involves preparation of a glycosylated amino acid building block, which is then incorporated into stepwise on-resin peptide synthesis.[14] The limited success realised trying to effect the direct O-glycosylation of serine or threonine hydroxy groups on resin[15] established that stepwise on resin synthesis of glycopeptides is the method of choice.

We herein report the convenient synthesis of a series of fluorescein-labelled glycopeptides containing GalNAcα1-*O*-Ser/Thr residues using microwave-enhanced solid-phase glycopeptide synthesis (SPGS). The boundaries of glycopeptide synthesis are thus extended to the incorporation of the immunologically relevant GalNAcα1-*O*-Ser/Thr residues that have hitherto not been introduced using microwave irradiation. Notably, in the present work three contiguous sterically demanding GalNAcα1-*O*-Ser residues were introduced in combination with a 5(6)-carboxyfluorescein label. Previous reports on the use of microwave-enhanced solid-phase synthesis to assemble glycopeptides in the absence,[16] or presence[17] of a fluorescent label, have not focused on GalNAcα1-*O*-Ser/Thr residues or addressed the issue of introducing contiguous glycosylated residues as required for MUC1 sequences.

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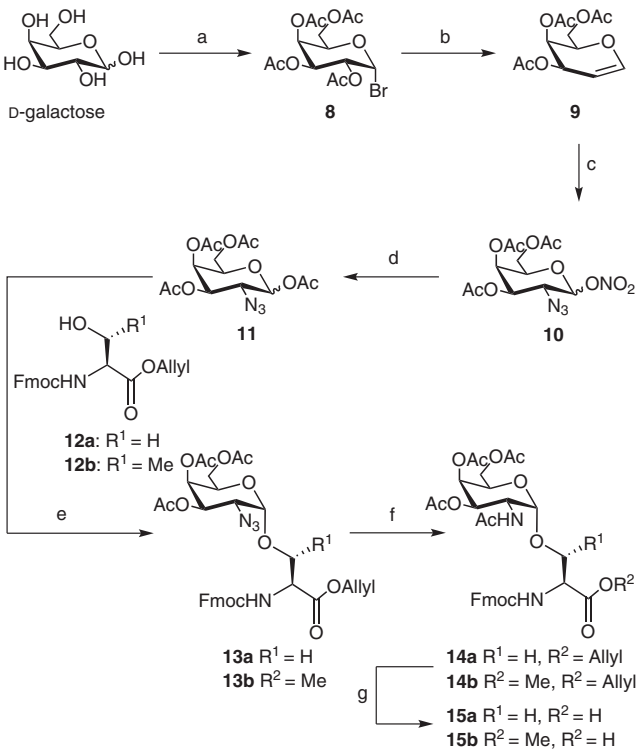
In order to develop synthetic methodology for the routine incorporation of GalNAcα1-*O*-Ser/Thr residues into fluorescently labelled peptides using solid-phase synthesis a series of 5(6)-carboxyfluorescein-labelled peptides containing GalNAcα1-*O*-Ser/Thr residues, specifically glycopeptides **1**–**7** (Figure 1), were prepared based on sequences emanating from the MUC1 glycoprotein. Notably, the successful synthesis of glycopeptide **3** tested the boundaries of the synthetic strategy requiring the introduction of three sterically hindered GalNAcα1-*O*-Ser residues in a contiguous fashion. A 5(6)-carboxyfluorescein label was introduced at the final stage of SPGS using WANG polystyrene resin with the fluorescent label attached to the N^{ε}-amino group of a lysine residue after glycopeptide chain elongation via the N^{α}-amino group of the same lysine residue. The use of microwave-assisted SPGS was critical to the successful outcome of this work.

The synthetic route used for the synthesis of Fmoc-[D-GalNAc(OAc)$_3$(α1-*O*)]Ser-OH (**15a**) and Fmoc-[D-GalNAc(OAc)$_3$(α1-*O*)]Thr-OH (**15b**) is depicted (Scheme 1) in which the key step required successful glycosylation of an acetyl-protected azidogalactosyl donor **11** with the protected amino acid derived glycosyl acceptors **12a** and **12b**. The glycosyl acceptors **12a** and **12b** were prepared from L-serine and L-threonine respectively. Initially the amino group was protected with Fmoc using Fmoc-succinimide,[18,19] followed by conversion of the carboxylic acid into an allyl ester using caesium carbonate and allyl bromide.[20]

After extensive experimentation it was established that azidogalactosyl donor **11** bearing an acetate as the leaving group at the anomeric position, proved to be the optimum glycosyl donor. Use of the azidogalactosyl bromide (Koenigs–Knorr conditions)[21] and the azidogalactosyl trichloroacetimidate (Schmidt protocol)[22] resulted in poor yields (5–11%) of the desired α-azidogalactosides **13a** and **13b**. The glycosylation of Fmoc-Ser-*O*-Allyl (**12a**) with tetra-*O*-acetyl-2-azido-2-deoxygalactose **11** has not been reported to date. In the present work glycosylation of **12a** with glycosyl donor **11** using boron trifluoride–diethyl ether complex with gentle heating for 60 hours afforded α-anomer **13a** exclusively in good yield (65%). Use of the same procedure to effect glycosylation of Fmoc-Thr-*O*-Allyl (**12b**) afforded the desired α-glycoside **13b** in 40% yield together with 10% of the β-anomer.

With α-azidoglycosides **13a** and **13b** in hand, reductive acetylation of the azide using pyridine and thioacetic acid[23] afforded the *N*-acetylgalactosides **14a** and **14b** in 87% and 74% yields, respectively.[23] Finally deprotection of the allyl esters using freshly prepared tetrakis(triphenylphosphine)palladium(0)[17,22] with *N*-methylmorpholine as the allyl scavenger afforded the desired Fmoc-[D-GalNAc(OAc)$_3$(α1-*O*)]Ser-OH (**15a**) and Fmoc-[D-GalNAc(OAc)$_3$(α1-*O*)]Thr-OH (**15b**) building blocks ready for subsequent SPGS.

The identity of the products **15a** and **15b** was confirmed by the presence of an $[M + H]^+$ ion in the FAB spectrum



Scheme 1 *Reagents and conditions:* (a) 1. Cu(OTf)$_2$, Ac$_2$O, 0 °C, 16 h; 2. 33% HBr–AcOH, 0 °C, 9 h, 67% (2 steps); (b) activated Zn, *N*-methylimidazole, EtOAc, reflux, 3 h, 72%; (c) NaN_3, CAN, anhyd MeCN, –20 °C, 6 h, 54%; (d) NaOAc, AcOH, 100 °C, 1 h, 64%; (e) $BF_3{\cdot}OEt_2$, CH_2Cl_2, 4 Å molecular sieves, 40 °C, 60 h, 65% (**13a**) and 40% (**13b**); (f) pyridine, AcSH, r.t., 16 h, 87% (**14a**) and 74% (**14b**); (g) Pd(PPh$_3$)$_4$, NMM, THF, 3 h, 92% (**15a** and **15b**).

(**15a**: *m*/*z* calcd for $C_{32}H_{36}N_2O_{13}$: 657.2296; found: 657.2301; **15b**: *m*/*z* calcd for $C_{33}H_{38}N_2O_{13}$: 671.2447; found: 671.2451). In addition, formation of the glycosylated product was confirmed by the presence of doublets at δ = 4.98 and 5.30 in the ^{1}H NMR spectrum assigned to the anomeric protons H1 for compounds **15a** and **15b**, respectively. The magnitude of coupling constant $J_{1,2}$ of the anomeric protons ($J_{1,2}$ = 2.9 Hz for **15a** and $J_{1,2}$ = 1.9 Hz for **15b**) established the α-configuration at the anomeric carbon.

With protected glycosylated serine and threonine building blocks **15a** and **15b** in hand, Fmoc solid-phase glycopeptide synthesis could be performed (Scheme 2). It was decided to attach the 5(6)-carboxyfluorescein label to the N^{ε}-amino group of a lysine residue with further incorporation of the *N*-acetylgalactosylated peptide chain through the N^{α}-amino group of the same lysine. Thus attachment of the 5(6)-carboxyfluorescein-label was performed after microwave-assisted automated SPGS thereby obviating the need to carry the fluorescein tag through the peptide synthesis. The synthesis of glycopeptides **1**–**7** thus started from commercially available pre-loaded Fmoc-Gly-Wang PS resin with initial attachment of Fmoc-Lys(Dde) followed by a Thr residue. It should be noted that Boc-Ile was used for the N-terminus at the end of chain elongation. Fmoc-Ile was incompatible as removal of Fmoc would occur during Dde removal using hydrazine and the

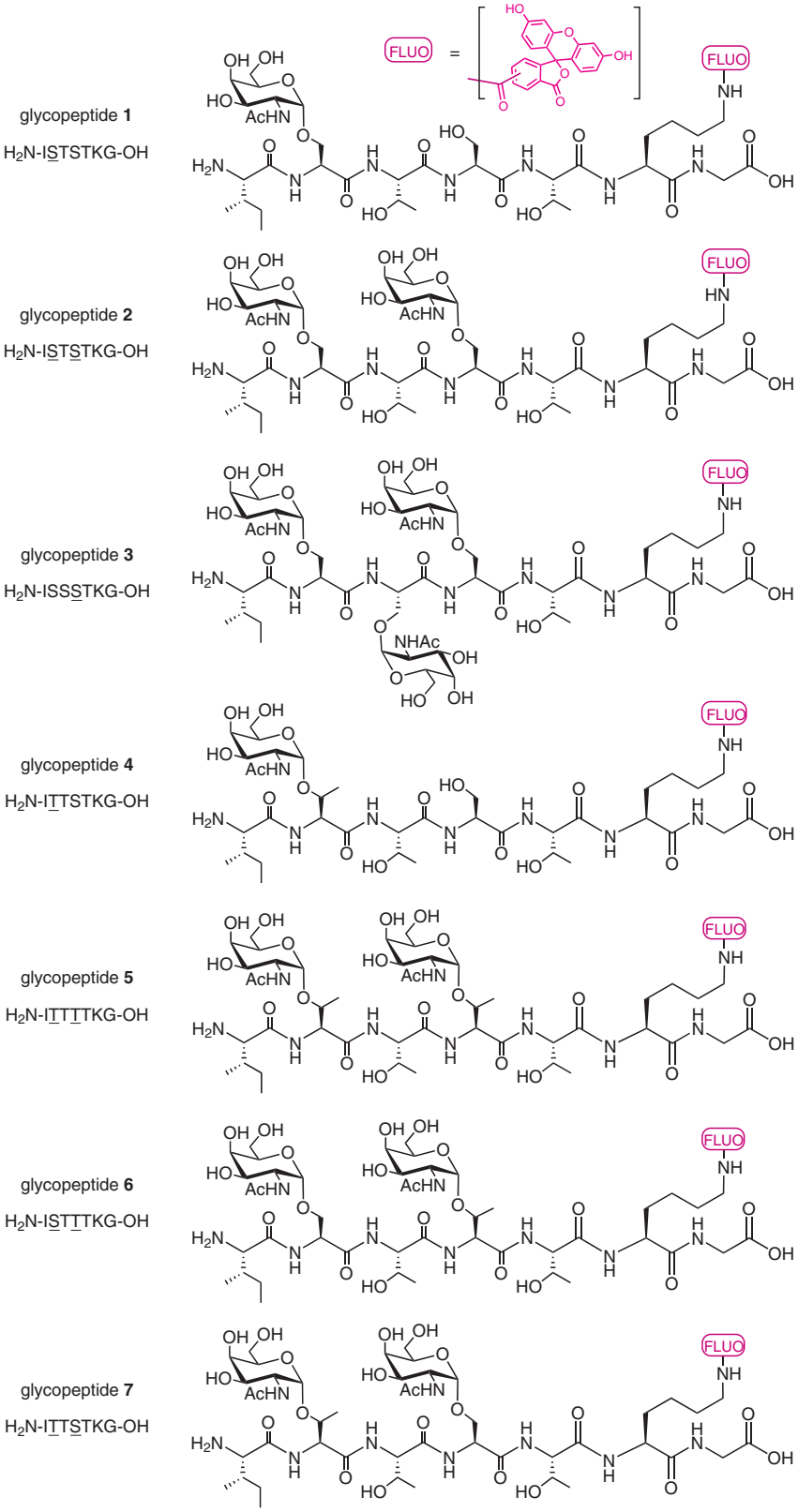
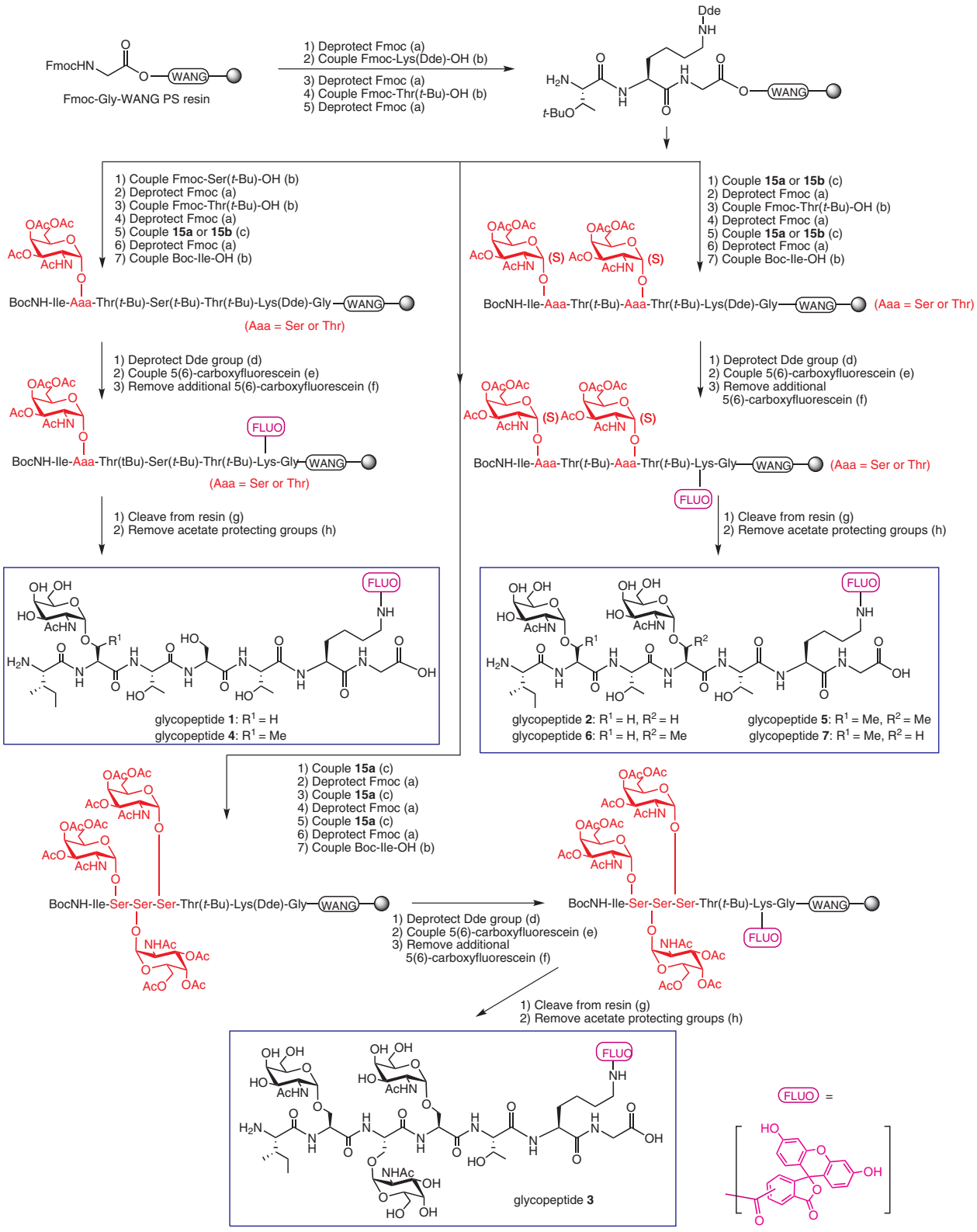


Figure 1 Structures of synthesized glycopeptides **1**–**7**

resulting free amino group would interfere with 5(6)-carboxyfluorescein coupling. The Boc protecting group was simultaneously removed during cleavage of glycopeptides from the resin.

Subsequent glycopeptide chain elongation then proceeded as outlined (Scheme 2) using CEM Liberty microwave peptide synthesizer. HBTU was used as a coupling reagent for incorporation of non-glycosylated Fmoc-Aaa (5 min cycle) and the Fmoc group was removed using 20%



Scheme 2 *Reagents and conditions:* (a) 20% piperidine–DMF, MW, 80 °C, 0.5 min plus 3 min; (b) HBTU, *i*-Pr_2EtN, DMF, MW, 80 °C, 5 min; (c) HATU, HOAt, collidine, DMF, MW, 80 °C, 20 min; (d) 2% hydrazine hydrate–DMF, r.t., 2 × 3 min; (e) DIC, HOBt, r.t., 24 h; (f) 20% piperidine–DMF, r.t., 5 × 20 min; (g) TFA, *i*-Pr_3SiH, H_2O, r.t., 3 h; (h) NaOMe–MeOH, r.t., pH 11.5–12.0, 3–4 h.

piperidine solution in *N*,*N*-dimethylformamide (0.5 min then 3 min cycles at 80 °C).

Notably, HATU/HOAt with 4-(dimethylamino)pyridine and collidine were used as the coupling reagent for incorporation of the glycosylated amino acids **15a** and **15b** and the coupling cycle was carried out for 20 minutes at 80 °C under microwave irradiation. This represents a substantial improvement over existing methods to introduce Fmoc-[D-GalNAc(OAc)$_3$(α1-*O*)]Ser-OH (**15a**) and Fmoc-[D-GalNAc(OAc)$_3$(α1-*O*)]Thr-OH (**15b**) into peptide sequences that required a coupling time of seven hours using HBTU/HOBt.[24] Notably, these conditions proved effective to introduce the three contiguous *N*-acetylgalactosyl residues present in glycopeptide **7**.

Subsequent manual treatment of the glycopeptidyl-resin with 2% hydrazine hydrate in *N*,*N*-dimethylformamide effected removal of the Dde-protecting group in order to introduce the 5(6)-carboxyfluorescein label. Manual coupling of the fluorescent label was subsequently carried out using DIC/HOBt in *N*,*N*-dimethylformamide[25] for 24 hours. Additional 5(6)-carboxyfluorescein label[26] was removed by five treatments of 20% piperidine in *N*,*N*-dimethylformamide (20 min each cycle). Cleavage of the crude glycopeptides from the resin was carried out using trifluoroacetic acid (95%) with triisopropylsilane (2.5%) and water (2.5%) as scavengers (3 h). Finally, acetate protecting groups of sugar hydroxys were removed using sodium methoxide in methanol (pH 11.5–12.0) for 3–4 hours.

Crude glycopeptides **1**–**7** were purified by reverse-phase HPLC to afford the desired products in 25–30% yield. Analysis by ESI-MS spectroscopy identified the major peaks in the reverse-phase HPLC spectra as the desired glycopeptides **1** (*m*/*z* calcd: 1254.8; found: 1254.9), **2** (*m*/*z* calcd: 1457.8; found: 1458.3), **3** (*m*/*z* [M + 2 H]$^{2+}$ calcd: 823.9; found: 824.0), **4** (*m*/*z* calcd: 1268.8; found: 1268.7), **5** (*m*/*z* calcd: 1485.8; found: 1486.3), **6** (*m*/*z* calcd: 1471.8; found: 1472.3), **7** (*m*/*z* calcd: 1471.8; found: 1472.1) attributed to their respective [M + H]$^+$ ions.

In summary, the synthetic protocol developed herein, provides a fast and effective tool to prepare a diverse library of fluorescein-labelled peptides bearing GalNAcα1-*O*-Ser/Thr residues. This methodology provides a reliable synthetic tool for the construction of potential ligands to evaluate in MGL lectin-binding assays. The development of effective ligands that bind to the MGL lectin is an important step for the rational design of cancer vaccines.

All reagents were purchased as reagent grade and used without further purification. Solvents were dried and purified prior to use. Solvents for RP-HPLC were purchased as HPLC grade and used without further purification. Fmoc-Gly-Wang PS resin (0.63 mmol/g) and Fmoc-Lys(Dde) were purchased from IRIS Biotech GmbH. Fmoc-Thr, Fmoc-Ser, Boc-Ile, HOBt, and HBTU were purchased from Advanced ChemTech. HOAt was purchased from Acros Organics. HATU and 5(6)-carboxyfluorescein (isomeric mixture) were purchased from Fluka. Analytical TLC was performed using 0.2 mm plates of Kieselgel F_{254} (Merck) and compounds were visualised by UV fluorescence or by staining with 4% H_2SO_4–EtOH or ethanolic ninhydrin soln (0.3% ninhydrin–EtOH + 1% AcOH), followed by heating the plate for a few min. Flash chromatography was carried out on Kieselgel F_{254} S 0.063–0.1 mm (Riedel de Hahn) silica gel with the solvents indicated. IR spectra were obtained using a Perkin Elmer Spectrum One Fourier Transform infrared spectrophotometer; v = varying. Optical rotations were determined at the Na D line (589 nm), at 20 °C with a Perkin-Elmer 341 polarimeter and are given in units of 10^{-1} deg cm^2 g^{-1}. NMR spectra were recorded as indicated on either a Bruker Avance DRX300 (^{1}H, 300 MHz, ^{13}C, 75 MHz) spectrometer, relative to TMS signal recorded at δ = 0.00 (^{1}H NMR, $CDCl_3$/TMS). and residual $CHCl_3$ at δ = 77.0 (^{13}C NMR, $CDCl_3$/TMS). ^{13}C values are reported as chemical shift (δ_C), degree of hybridisation, and assignment. Assignments were made with the aid of DEPT135, COSY, and HSQC experiments. The ratio of 5-carboxyfluorescein and 6-carboxyfluorescein regioisomers was assumed to 60:40 for the assignment of ^{1}H NMR data. LR-MS were recorded on a VG-70SE mass spectrometer operating at a nominal accelerating voltage of 70 eV (FAB). HRMS were recorded using a VG-70SE spectrometer at a nominal resolution of 5000 to 10,000 as appropriate. Major and significant fragments are quoted in the form x (y%), where x is the mass to charge ratio and y is the percentage abundance relative to the base peak. ESI-MS were recorded on a Thermo Finnigan Surveyor MSQ Plus spectrometer. Accurate mass spectra were recorded using Analytical RP-HPLC was performed using Dionex P680 System using an analytical column (Phenomenex Gemini C_{18}, 110 Å, 150 mm × 4.6 mm; 5 μm) at a flow rate of 1.0 mL min^{-1}. A linear gradient of 0.1% TFA–H_2O (buffer A) and 0.1% TFA–MeCN (buffer B) was used with detection at 210 nm or 254 nm. Gradient systems were adjusted according to the elution profiles. Semi-preparative RP-HPLC was performed using a Gilson System using a semipreparative column (Phenomenex Gemini C_{18}, 250 mm × 10 mm, 5 μm) at a flow rate of 5 mL min^{-1}. A linear gradient of 0.1% TFA–H_2O (buffer A) and 0.1% TFA–MeCN (buffer B) was used with detection at 254 nm. Gradient systems were adjusted according to the elution profiles and peak profiles obtained from the analytical RP-HPLC chromatograms.

Synthesis of Building Blocks Fmoc-[D-GalNAc(OAc)$_3$(α1-*O*)]Ser-OH (15a) and Fmoc-[D-GalNAc(OAc)$_3$(α1-*O*)]Thr-OH (15b)

2,3,4,6-Tetra-*O*-acetyl-α-D-galactopyranosyl Bromide (8)[27,28]

D-Galactose (10.0 g, 56.2 mmol), Ac_2O (27.0 mL, 286.2 mmol), and $Cu(OTf)_2$ (0.61 g, 1.68 mmol) were stirred at 0 °C for 16 h. MeOH (20 mL) was added to quench the reaction, then the mixture was concentrated in vacuo to afford an oil. The crude oil was extracted with EtOAc (2 × 200 mL) and the extracts were washed consecutively with sat. $NaHCO_3$ (100 mL), H_2O (50 mL), and brine (50 mL), dried (Na_2SO_4), filtered, and concentrated in vacuo to afford a yellowish viscous oil [R_f = 0.63 (EtOAc–hexane, 1:2)]. Without purification, the crude 2,3,4,6-tetra-*O*-acetyl-α,β-D-galactopyranosyl acetate was dissolved in 33% HBr–AcOH (70 mL) and stirred at 0 °C for 9 h. The mixture was diluted with CH_2Cl_2 (400 mL), washed consecutively with ice cold H_2O (200 mL), sat. $NaHCO_3$ (2 × 100 mL), H_2O (100 mL), and brine (50 mL), dried (Na_2SO_4), filtered, and concentrated in vacuo to afford **8** (15.5 g, 67% over 2 steps) as a viscous orange oil; R_f = 0.59 (EtOAc–hexane, 1:2).

^{1}H NMR data was in agreement with that reported in the literature.[28]

3,4,6-Tri-*O*-acetyl-D-galactal (9)[28]

Galactopyranosyl bromide **8** (8.30 g, 21.2 mmol) was dissolved in EtOAc (200 mL), then activated Zn (8.0 g, 122.3 mmol) and *N*-methylimidazole (1.59 mL, 20.0 mmol) were added. The mixture was refluxed for 3 h. After cooling to r.t. the mixture was filtered through Celite and the filtrate washed with 10% HCl (50 mL), 1 M HCl (50 mL), sat. $NaHCO_3$ (50 mL) and brine (50 mL), dried

(Na_2SO_4), filtered, and concentrated in vacuo. The crude product was purified by flash chromatography (EtOAc–hexane, 1:2) to afford **9** (3.96 g, 72%) as a light green oil; R_f = 0.37 (EtOAc–hexane, 1:2).

^{1}H NMR data was in agreement with that reported in the literature.[28]

3,4,6-Tri-*O*-acetyl-2-azido-2-deoxy-α,β-D-galactopyranosyl Nitrate (10)[29]

Galactal **9** (12.0 g, 44.1 mmol) was dissolved in anhyd MeCN (200 mL) and stirred at –20 °C with 4Å molecular sieves (5.0 g) for 30 min. CAN (72.5 g, 132.2 mmol) and NaN_3 (4.3 g, 66.1 mmol) were added and the mixture was stirred at –20 °C for 6 h. The mixture was diluted with Et_2O (150 mL), H_2O (150 mL) was added and the mixture was filtered through celite, then further extracted with Et_2O (150 mL). The Et_2O extract was dried (Na_2SO_4), filtered, and concentrated in vacuo. The crude product was purified by flash chromatography (EtOAc–hexane, 1:2) to afford **10** (9.03 g, 54%) as a brownish gum as a mixture of α and β anomers; ratio α/β 4:1; R_f = 0.38 (EtOAc–hexane, 1:2).

^{1}H NMR data was in agreement with that reported in the literature.[29]

3,4,6-Tri-*O*-acetyl-2-azido-2-deoxy-α,β-D-galactopyranosyl Acetate (11)[29]

A mixture of galactopyranosyl nitrate **10** (3.22 g, 8.56 mmol) and NaOAc (1.40 g, 17.1 mmol) in AcOH (25 mL) was refluxed at 100 °C for 1 h. The mixture was diluted with CH_2Cl_2 (100 mL), washed consecutively with H_2O (50 mL), sat. $NaHCO_3$ (50 mL), and brine (50 mL), dried (Na_2SO_4), filtered, and concentrated in vacuo. The crude product was purified by flash chromatography (EtOAc–hexane, 1:2) to afford **11** (2.04 g, 64%) as a white solid as a mixture of α and β anomers; ratio α/β 8:1; R_f = 0.35 (EtOAc–hexane, 1:2).

^{1}H NMR data was in agreement with that reported in the literature.[28]

N^{α}-(Fluoren-9-ylmethoxycarbonyl)-3-*O*-(3,4,6-tri-*O*-acetyl-2-azido-2-deoxy-α-D-galactopyranosyl)-l-serine Allyl Ester (13a);[22,23] Typical Procedure

Galactopyranosyl acetate **11** (0.78 g, 2.09 mmol) and Fmoc-L-Ser-All (**12a**, 0.51 g, 1.39 mmol) were dried together in the presence of P_2O_5 under high vacuum for 16 h prior to the reaction. Activated powdered 4Å molecular sieves were added and the mixture was stirred in anhyd CH_2Cl_2 (20 mL) under argon at 0 °C for 30 min. $BF_3{\cdot}OEt_2$ (0.78 mL, 6.37 mmol) was added slowly and the mixture was stirred at 40 °C for 60 h [2 extra portions of $BF_3{\cdot}OEt_2$ (0.78 mL, 6.37 mmol) were added at 20 h and 40 h]. The soln was cooled to r.t., neutralised with sat. $NaHCO_3$ and filtered through Celite. The product was extracted with CH_2Cl_2 (50 mL) and the extract was washed with sat. $NaHCO_3$ (2 × 10 mL) and brine (10 mL), dried (Na_2SO_4), and filtered. The solvent was removed in vacuo and the crude product was purified by flash chromatography (EtOAc–hexane, 1:2) to give **13a** (0.62 g, 65%) as a yellow gum; R_f = 0.28 (EtOAc–hexane, 1:2).

^{1}H NMR data was in agreement with that reported in the literature.[23]

N^{α}-(Fluoren-9-ylmethoxycarbonyl)-3-*O*-(3,4,6-tri-*O*-acetyl-2-azido-2-deoxy-α-D-galactopyranosyl)-l-threonine Allyl Ester (13b)[22,23]

Following the typical procedure using galactopyranosyl acetate **11** (1.86 g, 5.00 mmol) and Fmoc-L-Thr-All (**12b**, 2.0 g, 5.24 mmol) in anhyd CH_2Cl_2 (15 mL) with $BF_3{\cdot}OEt_2$ (2.04 mL, 16.6 mmol) [2 extra portions of $BF_3{\cdot}OEt_2$ (2.04 mL, 16.6 mmol) were added at 20 h and 40 h]. Washing used sat. $NaHCO_3$ (2 × 15 mL) and brine (10 mL). Flash chromatography (EtOAc–hexane, 1:2) gave **13b** (1.39 g, 40%) as an off-white gum; R_f = 0.54 (EtOAc–hexane, 1:1).

^{1}H NMR data was in agreement with that reported in the literature.[23]

N^{α}-(Fluoren-9-ylmethoxycarbonyl)-3-*O*-(3,4,6-tri-*O*-acetyl-2-acetamido-2-deoxy-α-D-galactopyranosyl)-l-serine Allyl Ester (14a);[23] Typical Procedure

Fmoc-Ser(3,4,6-Ac_3-2-N_3Gla)-All **13a** (0.21 g, 0.31 mmol) was dissolved in distilled pyridine (2 mL) and cooled to 0 °C. AcSH (1.5 mL) was then added and the mixture was stirred at 0 °C for 16 h. The mixture was allowed to warm to r.t. and left stirring for 16 h, then was diluted with CH_2Cl_2 (20 mL), washed with H_2O (20 mL), 1 M HCl (20 mL), and sat. $NaHCO_3$ (10 mL), dried (Na_2SO_4), filtered, and concentrated in vacuo. The crude product was purified by flash chromatography (EtOAc–hexane, 4:1) to afford **14a** (0.18 g, 87%); R_f = 0.49 (EtOAc–hexane, 4:1).

^{1}H NMR data was in agreement with that reported in the literature.[23]

N^{α}-(Fluoren-9-ylmethoxycarbonyl)-3-*O*-(3,4,6-tri-*O*-acetyl-2-acetamido-2-deoxy-α-D-galactopyranosyl)-l-threonine Allyl Ester (14b)[23]

Following the typical procedure using Fmoc-Thr(3,4,6-Ac_3-2-N_3Gla)-All **13b** (1.39 g, 2.00 mmol), distilled pyridine (6 mL), and AcSH (7 mL) with washing with H_2O (30 mL), 1 M HCl (50 mL), and sat. $NaHCO_3$ (20 mL). Flash chromatography (EtOAc–hexane, 4:1) gave **14b** (1.05 g, 74%); R_f = 0.42 (EtOAc–hexane, 4:1).

^{1}H NMR data was in agreement with that reported in the literature.[23]

N^{α}-(Fluoren-9-ylmethoxycarbonyl)-3-*O*-(3,4,6-tri-*O*-acetyl-2-acetamido-2-deoxy-α-D-galactopyranosyl)-l-serine (15a);[23] Typical Procedure

Fmoc-Ser(3,4,6-Ac_3-2-AcHNGal)-All **14a** (0.92 g, 1.35 mmol) was dissolved in anhyd THF (10 mL) and degassed with argon for 30 min. $Pd(PPh_3)_4$ (0.15 g, 0.13 mmol) and NMM (1.43 mL, 13.2 mmol) were added and the mixture was stirred at r.t. for 3 h. The solvent was removed in vacuo and the product purified by flash column chromatography (CH_2Cl_2–MeOH, 95:5) to afford **15a** (0.87 g, 92%) as an off-white solid.

HRMS (FAB): *m*/*z* [M + H]$^+$ calcd for $C_{32}H_{36}N_2O_{13}$: 657.2296; found: 657.2301.

^{1}H NMR data was also in agreement with that reported in the literature.[23]

N^{α}-(Fluoren-9-ylmethoxycarbonyl)-3-*O*-(3,4,6-tri-*O*-acetyl-2-acetamido-2-deoxy-α-D-galactopyranosyl)-l-threonine (15b)[22,23]

Following the typical procedure using Fmoc-Thr(3,4,6-Ac_3-2-AcHNGal)-All **14b** (1.0 g, 1.41 mmol), anhyd THF (10 mL) and $Pd(PPh_3)_4$ (0.16 g, 0.14 mmol) and PhNHMe (1.52 mL, 14.1 mmol). Flash column chromatography (CH_2Cl_2–MeOH, 95:5) gave **15b** (0.85 g, 92%) as an off-white solid.

HRMS (FAB): *m*/*z* [M + H]$^+$ calcd for $C_{33}H_{38}N_2O_{13}$: 671.2447; found: 671.2451.

^{1}H NMR data was also in agreement with that reported in the literature.[23]

Solid-Phase Synthesis of Glycopeptides 1–7

Glycopeptides were assembled using SPGS on pre-loaded Fmoc-Gly-Wang PS resin (0.63 mmol/g) with the use of CEM Liberty microwave peptide synthesizer using the following conditions: (a) Fmoc removal: 20% piperidine–DMF, 80 °C, 0.5 min then 3 min; (b) Fmoc-Aaa coupling (5.0 equiv): HBTU (4.5 equiv), *i*-Pr_2EtN (10 equiv) in DMF, 80 °C for ; (e) 5(6)-carboxyfluorescein coupling (2.5 equiv): DIC (2.5 equiv), HOBt (2.5 equiv) in DMF, r.t. for 24 h; (f) removal of additional ester bound 5(6)-carboxyfluorescein: 20% piperidine–DMF, r.t., 5 × 20 min; (c) Fmoc-GalNAc-O-Ser/Thr building block coupling (1.5 equiv): HATU (1.45 equiv), HOAt (1.5 equiv), collidine (4.5 equiv), in DMF, 80 °C, 20 min; and (g) cleavage: TFA–*i*-Pr_3SiH–H_2O 95:2.5:2.5, r.t., 3 h.

Glycosylated building blocks were pre-activated before placement in the synthesizer. The glycosylated building block (1.5 equiv) was dissolved in DMF, HOAt (1.5 equiv), and HATU (1.45 equiv) were then added and the mixture shaken until dissolved. The soln was transferred to the reaction vessel, followed by the addition of collidine (4.5 equiv). The mixture was subjected to microwave irradiation at 80 °C for 20 min, and a sample of the resin (1.0 mg) was taken for the Kaiser test.

Removal of the Dde protecting group (d) from the N^{ε}-amino group of the lysine residue was performed manually using 2% hydrazine hydrate soln in DMF (2 × 3 min).

Supporting Information for this article is available online at http://www.thieme-connect.com/ejournals/toc/synthesis.

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