Stability of 5(6)-Carboxyfluorescein in Microwave-Assisted Synthesis of Fluorescein-Labelled O-Dimannosylated Peptides

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Abstract: Methodology for the efficient, automated and microwave-assisted Fmoc solid-phase synthesis of a 5(6)-carboxyfluorescein-labelled Lys(Dde)-Gly-Wang resin that can be further elongated through the lysine N^{ε} amino group is described. Incorporation of O-dimannosylated peptides onto this resin using Fmoc-[α-D-Man(OBz)$_4$-(1→6)-α-D-Man(OBz)$_3$α1-]Ser-OH and PEG-[α-D-Man(OBz)$_4$-(1→6)-α-D-Man(OBz)$_3$α1-]-OH building blocks is demonstrated. Conditions were optimised to enable the efficient automated synthesis of several carboxyfluorescein-labelled dimannosylated peptides. 5(6)-Carboxyfluorescein was shown to be stable to the microwave conditions used for glycopeptide synthesis. The methodology described provides a robust, flexible synthetic platform for the preparation of a variety of fluorescently labelled glycopeptides (especially O-dimannosylated peptides) for biological evaluation.

Key words: carbohydrates, glycopeptides, mannosylated amino acids, glycosylations, solid-phase synthesis

Post-translational glycosylation of proteins has a significant influence on a number of biologically important processes such as cell growth, cell adhesion, cell differentiation, and immune defence.[1] Abnormal glycosylation of proteins may lead to diseases including cancer and rheumatoid arthritis.[1] Tumour cells and infectious agents containing unique carbohydrates on their cell surface are recognised by the immune system therefore, synthetic peptides of similar structure can be used as vaccines to stimulate the immune system. Despite problems associated with proteolysis and delivery to the immune system,[2] peptide-based vaccines have enormous potential due to their ease of synthesis and purification. Carbohydrate bearing antigens are recognised by mannose receptors (MRs) which play an important role in binding antigens, migration of dendritic cells (DCs) and interaction of DCs with lymphocytes.[3]

The work reported herein focuses on developing synthetic technology to allow optimisation of a suitable pattern recognition ligand (PRL) to target human mannose receptors found on human antigen-presenting cells (APCs), for use in synthetic vaccines. The exact binding requirements for the mannose receptor are, as yet, unknown, however, polymannan derivatives,[4] mannosylated dendrimers,[5] and compounds that mimic the cluster effect[6] are most often used as antigenic moieties. Some of these constructs were prepared via mannosylation through the side chain N^{ε} amino group of a lysine residue.[7] There is also evidence that some receptors are able to recognise less complex sugar units[8] such as terminal single and dimannose units,[9] fucose, and *N*-acetylglucosamine moieties. Given that the optimal characteristics of sugar spacing for binding and uptake, and the mechanism of action of human mannose receptors, are still not fully described[8b,10] we wished to develop synthetic technology to allow optimisation of a suitable PRL to target human mannose receptors. Previously, we focused on the synthesis of monomannosylated peptides[11] incorporated into a polyalanine scaffold that also contained 5(6)-carboxyfluorescein as a fluorescent label to facilitate biological screening. Microwave technology was not used in this preliminary work. This initial synthetic strategy has now been extended to allow the automated microwave-enhanced solid-phase synthesis of 5(6)-carboxyfluorescein labelled O-dimannosylated constructs under uniform conditions and the results of this work are reported herein.

Synthesis of glycopeptides has been recently comprehensively reviewed by Davis et al.[12] 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. The second approach involves preparation of a glycosylated amino acid building block, which is then incorporated into stepwise on-resin peptide synthesis.[13] This latter approach has found broad utility for the preparation of O-glycosylated peptides. The limited success realized trying to effect the direct O-glycosylation of serine or threonine hydroxy groups on resin[14] established that stepwise solid-phase synthesis of glycopeptides is in fact the method of choice. Glycopeptide synthesis using a solid support is advantageous over solution-phase chemical methods due to shorter reaction times, ease of synthesis, the ability to drive acylation to completion using a large excess of building blocks, and the potential to automate the process due to its repetitive nature.[13c,15]

Recently, microwave irradiation has gained popularity for the solid-phase peptide synthesis of difficult sequences.[16] Previous reports on the use of microwave-enhanced solid-phase synthesis of glycopeptides,[17] albeit in the absence of the fluorescent label, established the convenience of us-

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ing microwave irradiation to effect peptide coupling using sterically demanding residues. Bradley et al.[18] have incorporated 5(6)-carboxyfluorescein into a simple peptide chain lacking glycosylated residues at the final stage of the synthesis using microwave irradiation whilst Katritzky et al.[19] have recently described the microwave-assisted solid-phase peptide synthesis (SPPS) of coumarin-based fluorescently labelled simple peptides using benzotriazole derivatives. To date no studies have provided a fast and reliable method for the preparation of 5(6)-carboxyfluorescein-labelled glycopeptides using microwave irradiation, thus establishing the ability of the fluorescent label to withstand microwave irradiation over prolonged periods of SPPS. Herein, we describe a convenient method for the microwave-assisted synthesis of O-dimannosylated peptides that also bear a 5(6)-carboxyfluorescein label, thus providing ready access to a focused library of labelled glycopeptides for biological assays.

A carbohydrate moiety can be attached to the peptide chain via an amino acid residue or a linker via an O- or N-glycosidic bond. The purpose of the linker is to keep the sugar and peptide backbone apart thus spacing the newly incorporated sugar molecules at a distance from the peptide chain thereby minimising unfavourable steric interactions. By employment of a lipophilic or hydrophilic spacer unit, molecules with the desired physical properties can be prepared.[20] A review on the use of chemical linkages to attach sugar moieties has been reported by Davis.[21] Polyethyleneoxy linkers are often used for the introduction of a carbohydrate unit[20a,22] with their increased hydrophilicity improving the desired water solubility of the final compounds. Moreover, they possess greater flexibility compared to natural oligosaccharides.[23] For the purposes of this study, serine and polyethyleneglycol were used as mannose carriers.

A carboxyfluorescein label can be introduced either at the initial or final stage of solid-phase glycopeptide synthesis (SPGS) however, previous experience has shown[11] that initial preparation of fluorescently labelled Wang resin with the fluorescent label attached to the N^{α} amino group of a lysine residue followed by glycopeptide chain elongation via the N^{ε} amino group of the same lysine residue is the strategy of choice affording better yields of the desired glycopeptides compared to peptide elongation via the N^{α} amino group.

In order to develop synthetic technology for the routine incorporation of glycosylated residues into fluorescently labelled peptides using solid-phase synthesis, several 5(6)-carboxyfluorescein-labelled O-dimannosylated peptides incorporated into a simple alanine-based peptide scaffold were prepared. The use of microwave-assisted SPGS was critical to the successful outcome of this work.

Preparation of O-Dimannosylated Building Blocks

Initial preparation of the appropriate dimannosylated building blocks, namely protected Fmoc-Ser[diMan(OBz)$_7$]-OH **12** and PEG-[diMan(OBz)$_7$]-OH **15** (Figure 1), that could be readily incorporated into SPPS was undertaken.

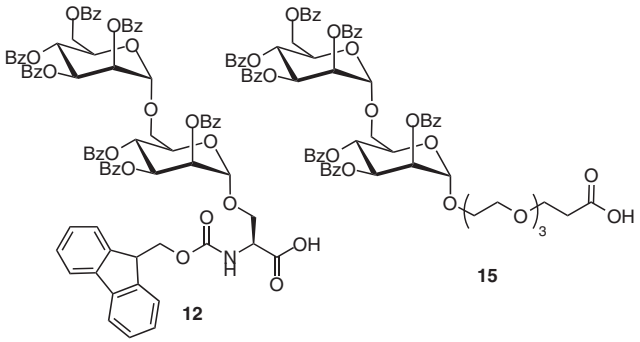


Figure 1 Dimannosylated building blocks **12** and **15**

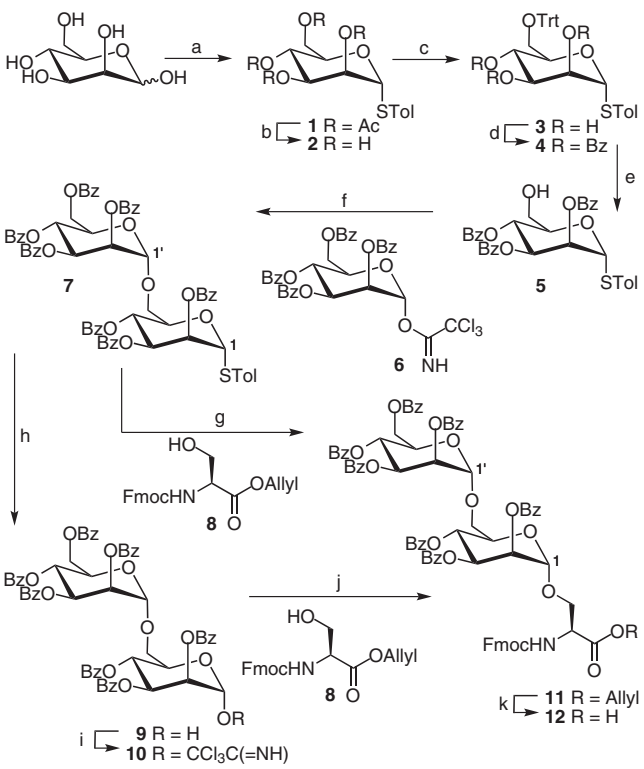
A Fmoc group was used to protect the N^{α} amino group and the sugar hydroxys could be protected with either benzyl, acetate, or benzoyl groups. The presence of protecting groups such as acetate or benzoyl on the hydroxy groups of the sugar moiety stabilises the glycosidic linkage during the final trifluoroacetic acid assisted glycopeptide cleavage from the resin.[24]

Different methods for the synthesis of the α(1→6) linked mannosides are reported in the literature.[25] Meldal et al.[26] have synthesized benzoyl-protected dimannosylated building blocks attached to N^{α}-Fmoc-protected serine, threonine, and hydroxyproline. The carboxy groups were protected as pentafluorophenyl esters. The preparation of the α(1→6) benzoyl-protected dimannosylated building block was achieved via Koenigs–Knörr glycosylation[27] using a benzoyl-protected dimannosylated bromide as the glycosyl donor. Glycosyl halides are unstable intermediates[28] requiring the use of in situ generated donors resulting in lower yields for the glycosylation step.[29]

Zhu and Kong[30] used 6-*O*-(2,3,4,6-tetra-*O*-acetyl-α-D-mannopyranosyl)-2,3,4-tri-*O*-benzoyl-α-D-mannopyranosyl trichloroacetimidate as the glycosyl donor for the preparation of a variety of α(1→6)-linked mannose polysaccharides. Trichloroacetimidates, are stable, easily prepared crystalline products which are advantageous over unstable glycosyl halides[28] used for Koenigs–Knörr glycosylation.[27] In our case the trichloroacetimidate method was adopted as this had already proven a reliable method for our previous preparation[11] of the simple Fmoc-[Man(OBz)$_4$α1-]Ser building block.

Preparation of Fmoc-Ser[diMan(OBz)$_7$]-OH 12

The synthetic route used for the synthesis of an α(1→6) linked dimannose unit attached to serine **12** is depicted in Scheme 1. 2,3,4,6-Tetra-*O*-benzoyl-α-D-mannopyranosyl trichloroacetimidate (**6**)[11] was used as a glycosyl donor together with versatile thioglycosides[31] which, depending on the requirements, served as either a mannosyl acceptor **5** or a mannosyl donor **7**.



Scheme 1 *Reagents and conditions*: (a) 1. $Cu(OTf)_2$, Ac_2O, 0 °C to r.t. 2. $BF_3{\cdot}OEt_2$, TolSH, CH_2Cl_2, 0 °C to r.t., 24 h, 57% over 2 steps; (b) 1 M NaOMe, MeOH, r.t., 3 h, 96%; (c) TrtCl, py, DMAP, 0 °C to r.t., 24 h (crude); (d) BzCl, py, DMAP, 0 °C to r.t., 12 h, 98% (from crude); (e) TFA–CH_2Cl_2–*i*-Pr_3SiH (1:94:5), r.t., 1.5 h, 85%; (f) TMSOTf, CH_2Cl_2, –40 °C to r.t., 3 h, 88%; (g) NIS, AgOTf, CH_2Cl_2, 0 °C, 3 h, 58%; (h) NBS, EtOAc–H_2O (1:1), r.t., 24 h, 64%; (i) K_2CO_3, CH_2Cl_2, Cl_3CCN, r.t., 12 h, 71%; (j) TMSOTf, CH_2Cl_2, –40 °C to r.t., 3 h, 74%; (k) $Pd(PPh_3)_4$, $PhSiH_3$, CH_2Cl_2, Ar, 67%.

The synthesis started with the preparation of benzoyl-protected acceptor **5** containing a free 6-OH group that was successfully executed in six steps. Initial per-O-acetylation of D-mannose using copper triflate with a stoichiometric quantity of acetic anhydride, followed by anomeric substitution with *p*-thiocresol under boron trifluoride–diethyl ether complex activation gave thioglycoside **1**[32] in a one-pot synthesis.[33] Alternative strategies for this one-pot thioglycoside step are also available.[34] The next steps required deacetylation of **1**[32] using catalytic sodium methoxide in methanol (Zemplén conditions)[35] followed by selective formation of a primary trityl ether at C6, and benzoylation to afford benzoyl-protected thioglycoside **4**. Removal of the trityl protecting group was then achieved under acidic conditions.[36] Initial deprotection using *p*-toluenesulfonic acid in dichloromethane only afforded the desired product in low yield with substantial recovery of starting material. Longer reaction times and an increased quantity of *p*-toluenesulfonic acid did not improve the yield. Gratifyingly, trifluoroacetic acid with triisopropylsilane as a carbocation scavenger removed the trityl ether to afford the desired thioglycoside acceptor **5** in 85% yield.

The next step required glycosylation of thioacceptor **5** using mannosyl trichloroacetimidate **6**[11] as a glycosyl donor using the Schmidt protocol[37] to afford benzoyl-protected α(1→6)dimannosylated sugar donor **7**. Hence, glycosylation of **5** with benzoyl-protected mannosyl trichloroacetimidate **6**[11] with trimethylsilyl triflate activation in dichloromethane afforded the desired dimannosylated thioglycoside **7** in good yield (88%). The presence of a participating benzoyl group at C2 in **6**[11] led to formation of the desired O-dimannosylated α-anomer **7**. The ^1H NMR spectrum exhibited two doublets at $\delta_H = 5.15$ and $\delta_H = 5.76$ corresponding to H1′ and H1, respectively, and the anomeric configuration of the product was established by the magnitude of the $J_{1,2}$ and $J_{1',2}$ coupling constant (1.4 Hz and 1.6 Hz, respectively) and the $J_{C1,H1}$ and $J_{C1',H1'}$ heterocoupling constants which were 171.0 Hz and 173.8 Hz, respectively. Tvaroska and Taravel[38] reported that larger $^1J_{C1,H1}$ coupling constants (approximately 170 Hz) are exhibited by α-anomers of pyranoses and oligo- and polysaccharides while smaller $^1J_{C1,H1}$ coupling constants are characteristic of β-anomers (~160 Hz).

Subsequent glycosylation of the Fmoc-Ser-O-Allyl (**8**)[11] acceptor by thioglycoside donor **7** with activation by *N*-iodosuccinimide and silver triflate in dichloromethane afforded protected O-dimannosylated serine **11** in moderate yield (58%).

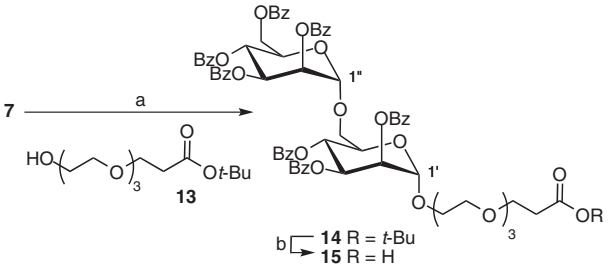
A better synthesis of **11** involved preparation of the benzoyl-protected dimannosyl trichloroacetimidate **10** as the glycosyl donor to which Fmoc-Ser-O-Allyl (**8**)[11] was coupled using Schmidt methodology.[37] Dimannosyl trichloroacetimidate **10** was synthesized by anomeric deprotection of thioglycoside **7** using *N*-bromosuccinimide in a mixture of ethyl acetate–water to afford dimannoside **9** in 64% yield. Subsequent conversion into trichloroacetimidate **10** in a similar fashion to that used for monomannosylated unit **6**[11] took place upon treatment with trichloroacetonitrile and potassium carbonate affording benzoyl-protected dimannosylated Schmidt donor **10** in good yield (71%). Glycosylation of Fmoc-Ser-O-Allyl (**8**)[11] using trimethylsilyl triflate (20 mol%) in dichloromethane at –40 °C for three hours afforded Fmoc-[α-D-Man(OBz)$_4$-(1→6)-α-D-Man(OBz)$_3$α1-]Ser-O-Allyl (**11**) in 74% yield. This yield provided a significant improvement compared to glycosylation using thioglycoside donor **7** (58%).

The identity of the product **11** was confirmed by the presence of an $[M + H]^+$ ion in the FAB spectrum (*m*/*z* calcd for $C_{82}H_{70}NO_{22}$: 1420.4390; found: 1420.4392). In addition, formation of the glycosylated product was confirmed by the presence of a signal due to the anomeric proton H1 at $\delta_H = 5.20$ which was shifted upfield ($\delta_H = 6.58$ for compound **10** before glycosylation) and a signal at $\delta_C = 98.5$ due to the anomeric C1 which was shifted downfield ($\delta_C = 94.8$ for compound **10** before glycosylation). Finally, removal of the allyl group using freshly prepared tetrakis(triphenylphosphine)palladium(0)[39] and phenylsilane as the allyl group acceptor[40] in dichloromethane gave the Fmoc-[α-D-Man(OBz)$_4$-(1→6)-α-D-

Man(OBz)$_3$α1-]Ser-OH (**12**) building block in 67% yield ready for incorporation into a peptide chain.

Preparation of PEG-[diMan(OBz)$_7$]-OH 15

In a similar fashion to the method used for the preparation of Fmoc-[α-D-Man(OBz)$_4$-(1→6)-α-D-Man(OBz)$_3$α1-]Ser-OH (**12**), the synthesis of PEG-[α-D-Man(OBz)$_4$-(1→6)-α-D-Man(OBz)$_3$α1-]-OH (**15**) was also undertaken (Scheme 2).



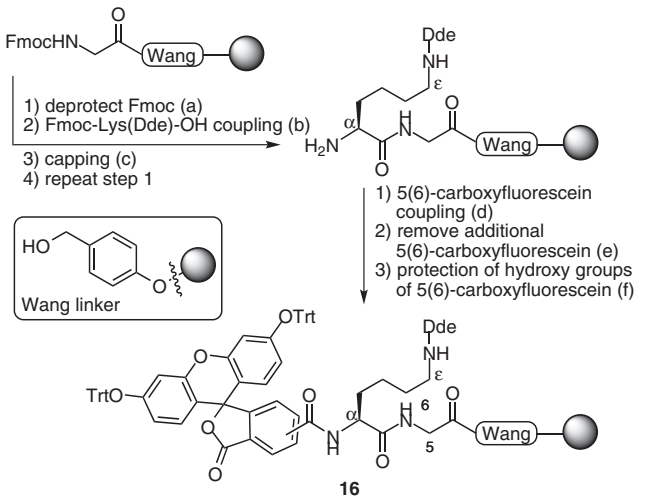
Scheme 2 *Reagents and conditions*: (a) NIS, AgOTf, CH_2Cl_2, 0 °C, 77%; (b) TFA, anisole (10:1), r.t., 1.5 h, 80%.

The synthesis of **15** started with the preparation of benzoyl-protected dimannosylated thiomannoside donor **7** (Scheme 1). Subsequent glycosylation of the *tert*-butyl-protected linker **13**[41] with thioglycoside donor **7** by activation with *N*-iodosuccinimide and silver triflate afforded glycoside **14** in good yield (77%). Thus incorporation of the PEG linker unit **13**[41] facilitated the glycosylation step compared to direct glycosylation of Fmoc-Ser-O-Allyl (**8**)[11] by dimannosylated thioglycoside donor **7**. The structure of the dimannosylated PEG product **14** was confirmed by the presence of the $[M + H]^+$ ion in the FAB spectrum (*m*/*z* calcd for $C_{74}H_{75}O_{23}$: 1331.4699; found: 1331.4719). ^1H and ^{13}C NMR spectroscopy also confirmed the desired structure of **14** by the characteristic resonances for the anomeric proton H1′ at $\delta_H = 5.19$ (shifted upfield compared to the anomeric proton H1 at $\delta_H = 5.76$ in donor **7**) and the anomeric carbon C1′ at $\delta_C = 97.7$ (shifted downfield compared to the anomeric carbon C1 at $\delta_C = 86.6$ in donor **7**). The magnitude of the $J_{1',2'}$ and $J_{1'',2''}$ coupling constants (1.3 Hz and 1.5 Hz, respectively) together with the $J_{C1',H1'}$ and $J_{C1'',H1''}$ heterocoupling constants (172.3 Hz and 170.4 Hz, respectively) confirmed the α-configuration of the product **14**.

Deprotection of the *tert*-butyl ester using trifluoroacetic acid and anisole afforded the desired dimannosylated PEG linker **15** building block (80%) ready for further elaboration into a peptide chain.

Microwave-Assisted Solid-Phase Synthesis of 5(6)-Carboxyfluorescein-Labelled Wang Resin

With protected glycosylated serine building blocks **12** and **15** in hand, Fmoc solid-phase glycopeptide synthesis could be performed. It was decided to attach the 5(6)-carboxyfluorescein label to the N^{α} amino group of a lysine with further incorporation of the mannosylated peptide chain through the side chain N^{ε} amino group.[11] The stability of the 5(6)-carboxyfluorescein to the microwave conditions was an important issue to be addressed in this study. Initial preparation of 5(6)-carboxyfluorescein-labelled Wang resin was undertaken. The microwave-assisted automated SPGS was carried out starting from commercially available pre-loaded Fmoc-Gly-Wang resin using a CEM Liberty microwave peptide synthesizer (Scheme 3).



Scheme 3 *Reagents and conditions*: (a) 20% piperidine–DMF, MW, 80 °C, 0.5 min plus 3 min; (b) HBTU, DIPEA, DMF, MW, 80 °C, 5 min; (c) 20% Ac_2O–DMF, MW, 70 °C, 2 min; (d) HBTU, DIPEA, DMF, MW, 80 °C, 30 min; (e) 6 cycles of 20% piperidine–DMF, MW, 80 °C, 0.5 min plus 3 min; (f) TrtCl, DIPEA, CH_2Cl_2, r.t., overnight.

The Fmoc protecting group was removed from Fmoc-Gly-Wang resin using 20% piperidine in *N*,*N*-dimethylformamide under microwave conditions followed by incorporation of Fmoc-Lys(Dde)-OH [Dde = 1-(4,4-dimethyl-2,6-dioxocyclohexylidene)ethyl] and subsequent Fmoc removal to allow for incorporation of the 5(6)-carboxyfluorescein via the N^{α} amino group of the lysine. This procedure was based on a conventional SPPS protocol employed by Brock et al.[42] for the preparation of fluorescently labelled Wang resin using diisopropylcarbodiimide (DIC) and 1-hydroxybenzotriazole (HOBt) as coupling reagent. Bradley et al.[18] reported an improved method for coupling 5(6)-carboxyfluorescein to the side chain of a lysine residue under microwave conditions, also using diisopropylcarbodiimide and 1-hydroxybenzotriazole as coupling reagent, however in this case the fluorescent label was introduced at the final stage of the synthesis. The reaction time was shortened to ten minutes compared to use of an overnight reaction that was required when conventional coupling of the label[42] was used. In our case the use of uniform reagents for all coupling steps was preferred hence, *N*-[(1*H*-benzotriazol-1-yl)(dimethylamino)methylene]-*N*-methylmethanaminium

hexafluorophosphate *N*-oxide (HBTU) was used for introduction of the 5(6)-carboxyfluorescein label. Since the Kaiser test[43] performed after ten minutes of microwave irradiation revealed incomplete coupling the reaction was continued with complete coupling requiring a longer time of 30 minutes with microwave heating at 80 ° C compared to the ten minute cycle at 60 °C reported by Bradley et al.[18] Inefficient 5(6)-carboxyfluorescein acylation was also found using a 10-minute cycle[18] under diisopropylcarbodiimide and 1-hydroxybenzotriazole activation (manual addition of reagents) and again 30 minutes was required for complete reaction.

The discrepancy in the reaction time required to effect complete coupling of the 5(6)-carboxyfluorescein label might be attributed to the different solid supports used by Bradley et al.[18] and our laboratory. Tentagel[44] resin was used during the study by Bradley et al.[18] while polystyrene cross-linked with 1% DVB resin was used during the present work. Tentagel[44] resin is prepared by grafting PEG to low (1% to 2%) cross-linked polystyrene. The swelling properties of Tentagel[44] are much improved compared to polystyrene resin.[45]

Subsequent treatment of the peptidyl-resin with 20% piperidine in *N*,*N*-dimethylformamide (6 cycles) ensured cleavage of additional fluorophore esters.[42] Attempts to effect microwave-assisted introduction of *O*-trityl-protecting groups on the resin-bound label using trityl chloride and *N*,*N*-diisopropylethylamine were disappointing. Neither prolonged reaction time (CH_2Cl_2, 40 °C, 15 min) compared to the standard coupling cycle (80 °C, 5 min), nor the use of *N*,*N*-dimethylformamide instead of dichloromethane and higher temperature (80 °C, 15 min) effected the tritylation. Thus, trityl protection was achieved using standard manual conditions at room temperature upon treatment with trityl chloride and *N*,*N*-diisopropylethylamine in dichloromethane overnight to afford fluorescently labelled Wang resin **16** ready for further elongation.

Microwave-Assisted Solid-Phase Synthesis of Glycopeptides

Subsequent manual treatment of the peptidyl-resin **16** with 2% hydrazine hydrate effected removal of the Dde-protecting group in order to extend microwave-enhanced automated SPPS through the side chain N^{ε} amino group of the lysine residue (Scheme 4).

Because O-tritylation was undertaken manually it was also more convenient to perform the subsequent Dde deprotection step manually. From this point on the synthesis was once again carried out in the CEM Liberty peptide synthesizer. Thus, HBTU was used as a coupling reagent for incorporation of the Fmoc-Aaa (5 min cycle) and the Fmoc group was removed using 20% piperidine solution in *N*,*N*-dimethylformamide (0.5 min then 3 min cycles). 20% Acetic anhydride–*N*,*N*-dimethylformamide solution was used to cap the unreacted amino groups (2 min cycle) and 95% trifluoroacetic acid with triisopropylsilane and water as scavengers were used to cleave the peptides from resin (20 min cycle). *N*-[(dimethylamino)-1*H*-1,2,3-triazolo[4,5-*b*]pyridin-1-ylmethylene]-*N*-methylmethanaminium hexafluorophosphate *N*-oxide/1-hydroxy-7-azabenzotriazole (HATU/HOAt) was used as the coupling reagent for coupling the mannosylated building block and the coupling cycle was carried out for 20 minutes.

The free amino group present on the side chain of the N^{ε}-lysine residue allowed for coupling of a specific number of Fmoc-protected alanine residues (3 or 4) and finally either Fmoc-[α-D-Man(OBz)$_4$-(1→6)-α-D-Man(OBz)$_3$α1-]Ser-OH (**12**) (compound **18**) or PEG-[α-D-Man(OBz)$_4$-(1→6)-α-D-Man(OBz)$_3$α1]-OH (**15**) (compounds **20** and **22**) were introduced into the peptide chain. In order to minimise the use of the dimannosylated constructs **12** and **15**, only 1.5 equivalents of the building blocks were used per coupling step and the dimannosylated serine was pre-activated with HATU/HOAt outside the synthesizer. The solution was then added manually to the reaction vessel and subjected to microwave irradiation for 20 minutes (80 °C). The Kaiser test[43] performed after coupling of the glycosylated serine (for compound **18**) and glycosylated PEG linker (for compounds **20** and **22**) was negative hence, final Fmoc deprotection was undertaken. Subsequent cleavage from the resin afforded crude benzoyl-protected glycopeptides **17**, **19**, and **21**.

Glycopeptide **17** was purified by reverse-phase HPLC to afford the desired product in 8% yield. Pleasingly, reverse-phase HPLC analysis of the crude glycopeptides **19** and **21** obtained after cleavage from the resin revealed the presence of one major product (ca. 66% and ca. 73%, respectively, based on HPLC analysis of the crude products). Further analysis by MALDI-TOF spectroscopy identified the major peaks in the reverse-phase HPLC spectra as the desired glycopeptides **19** (*m*/*z* calcd: 2030.6750; found: 2030.6453) and **21** (*m*/*z* calcd: 2101.7121; found: 2101.9402;) by the presence of their corresponding [M^+] ions.

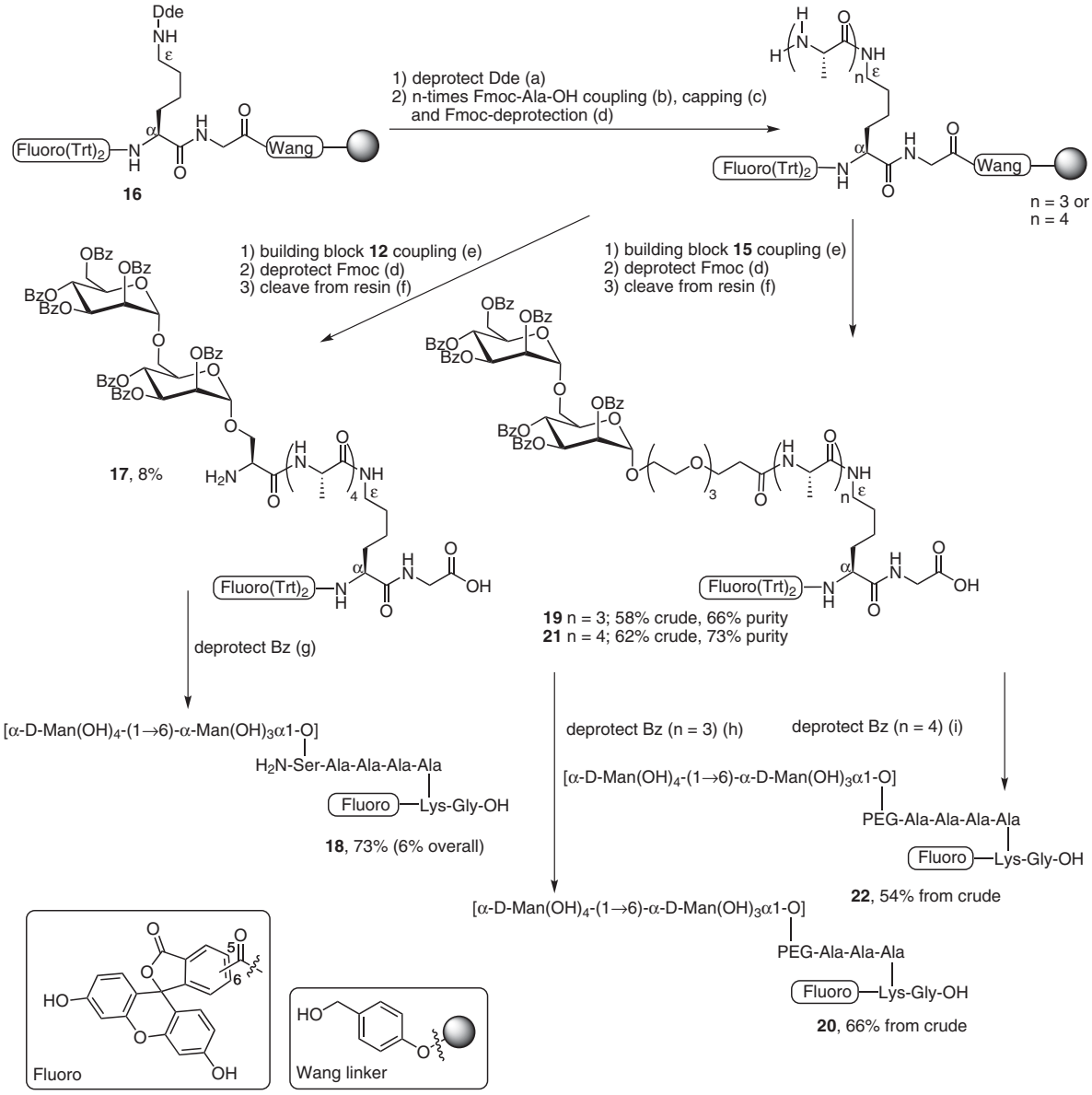
Finally, deprotection of the benzoate esters on the mannose residues of glycopeptide **17** using catalytic sodium methoxide in methanol and final purification by reverse-phase HPLC gave the desired glycopeptide 5(6)-carboxyfluorescein-{[D-Man(OH)$_4$-(α1→6)-D-Man(OH)$_3$(α1-O)]Ser-(Ala)$_4$-}Lys-Gly-OH (**18**) in 73% yield (6% overall). The identity of the product was confirmed by the presence of the $[M + Na]^+$ ion in the MALDI-TOF mass spectrum (*m*/*z* calcd: 1279.4501; found: 1278.9909).

As the crude glycopeptides **19** and **21** were of significant purity (ca. 66% and ca. 73%, respectively) removal of the benzoate protecting groups from the protected glycopeptides was performed without prior purification. Reverse-phase HPLC monitoring of the progress of O-debenzoylation of the crude **19** and **21** indicated almost quantitative conversion to the desired deprotected products 5(6)-carboxyfluorescein-{[D-Man(OH)$_4$-(α1→6)-D-Man(OH)$_3$(α1-O)]PEG-(Ala)$_3$-}Lys-Gly-OH (**20**) and 5(6)-carboxyfluo-

rescein-{[D-Man(OH)$_4$-(α1→6)-D-Man(OH)$_3$(α1-O)]PEG-(Ala)$_4$-}Lys-Gly-OH (**22**) after three and four hours, respectively.

Finally reverse-phase HPLC purification afforded pure glycopeptides **20** and **22** in good yield (66% and 54%, respectively, based on the benzoate removal step). The identity of the products was confirmed by the presence of the $[M]^+$ ion in the MALDI-TOF spectrum at *m*/*z* 1302.6882 (calcd: 1302.4915) for glycopeptide **20** and by the presence of the $[M + H]^+$ ion (*m*/*z* calcd: 1374.5359; found: 1374.1804), for glycopeptide **22**.

In conclusion, the microwave-assisted solid-phase synthesis of 5(6)-carboxyfluorescein-labelled Wang resin with the label attached to N^{α} amino group of lysine residue has been optimised and improved. Ready access to this resin allows a more convenient and efficient synthesis of fluorescently labelled glycopeptides and the fluorescently labelled resin itself. Use of HBTU as a coupling reagent instead of DIC/HOBt for incorporation of the 5(6)-carboxyfluorescein enables the use of uniform reagents in microwave-enhanced automated process. However, an additional study needs to be performed to find suitable conditions for protection of the hydroxy groups on the fluorescein label under microwave conditions. Related work by Bradley et al.[18] focused on coupling the carboxyfluorescein label at the final stage of the synthesis, using a microwave vessel using DIC and HOBt whereas in the present work HBTU was used to introduce the label onto the resin at an early stage. The latter method thus offers the possibility to readily access a range of glycopeptides using this pre-labelled resin in SPGS.



Scheme 4 *Reagents and conditions*: (a) 2% $NH_2NH_2{\cdot}H_2O$–DMF, r.t., 2 × 3 min; (b) HBTU, DIPEA, DMF, MW, 80 °C, 5 min; (c) 20% Ac_2O–DMF, MW, 70 °C, 2 min; (d) 20% piperidine–DMF, MW, 80 °C, 0.5 min plus 3 min; (e) HATU, HOAt, collidine, DMF, MW, 80 °C, 20 min; (f) TFA–*i*-Pr_3SiH–H_2O, MW, 40 °C, 20 min; (g) NaOMe, MeOH, r.t., pH 12.1, 3 h; (h) NaOMe, MeOH, r.t., pH 11.9, 3 h; (i) NaOMe, MeOH, r.t., pH 11.9, 4 h.

The successful synthesis of fluorescently labelled dimannosylated peptides **18**, **20**, and **22** with fluorescent label attached to the N^{α} amino group of the lysine residue established the stability of 5(6)-carboxyfluorescein label to microwave conditions.

The synthetic protocol developed herein, provides a fast and effective tool to prepare a diverse library of mannosylated peptides and is a reliable synthetic tool for the constructions of glycopeptide ligands for lectin-binding assays. A significant reduction in reaction time compared to manual or automated SPPS using conventional methods has been achieved using automated microwave-assisted SPPS. Employment of a better swelling resin, such as CLEAR resin[46] or ChemMatrix resin[47] rather than the polystyrene-based resin used in the present work may further improve this synthetic method.

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 resin and Fmoc-Lys(Dde) were purchased from IRIS Biotech GmbH. Fmoc-Ala-Wang resin, Fmoc-Ala, 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 in EtOH or ethanolic ninhydrin soln (0.3% ninhydrin–EtOH + 1% AcOH), followed by heating the plate for a few minutes. 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. Optical rotations were determined at the sodium 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) or Bruker Avance DRX400 spectrometer (^{1}H, 400 MHz, ^{13}C, 100 MHz) or on a Bruker Avance DRX600 spectrometer (^{1}H, 600 MHz, ^{13}C, 150 MHz); relative to TMS (δ_H 0.00) in $CDCl_3$/TMS or were referenced to the residual MeOH signal at δ_H = 3.34 in CD_3OD. The ^{13}C values were referenced to the residual $CHCl_3$ signal at δ_C = 77.0 in $CDCl_3$/TMS or residual MeOH signal at δ_C = 49.15 in CD_3OD solvent. Assignments were made with the aid of DEPT135, COSY, HSQC, and HMBC 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. ESI-MS were recorded on a Thermo Finnigan Surveyor MSQ Plus spectrometer. Accurate mass spectra were recorded using Matrix-assisted laser desorption/ionisation-time of flight mass spectrometry (MALDI-TOF MS) technique on a Voyager-DE spectrometer with the use of α-cyano-4-hydroxycinnamic acid (CHCA) as matrix. Analytical RP-HPLC was performed using Dionex P680 System using an analytical column (Phenomenex Jupiter C_4, 300 Å, 50 mm × 2.0 mm; 5 μm or Phenomenex Gemini C_{18}, 110 Å, 150 mm × 4.6 mm; 5 μm) at a flow rate of 0.5 mL or 1 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 Waters 600 System using a semipreparative column (Waters XTerra Prep. C_{18}, 300 mm × 19 mm; 10 μm or Phenomenex Jupiter C_4, 250 mm × 10 mm; 5 μm) at a flow rate of 10 mL or 7.5 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 O-Dimannosylated Building Blocks

4-Methylphenyl 1-Thio-α-D-mannopyranoside (2)

p-Methylphenyl 2,3,4,6-tetra-*O*-acetyl-1-thio-α-D-mannopyranoside[32] (**1**, 5.95 g, 13.1 mmol) was dissolved in MeOH (100 mL) and 1 M NaOMe in MeOH was added to adjust the pH to 10 (pH paper). The mixture was stirred for 3 h and a portion of dry ice was added to neutralise the mixture. After removal of the solvent in vacuo, the crude product was purified by flash chromatography (gradient elution, CH_2Cl_2–MeOH 9:1 to 7:3) to give **2** (3.62 g, 96%) as a white amorphous solid; R_f = 0.44 (CH_2Cl_2–MeOH, 8:2).

$[\alpha]_D^{20}$ +222.5 (*c* 0.35, MeOH) [Lit.[48] +279 (*c* 0.70, MeOH)].

IR (NaCl): 3428 cm^{-1} (s br, O–H).

^{1}H NMR (400 MHz, MeOD): δ = 2.28 (s, 3 H, C*H*$_3$-Tol), 3.66–3.82 (m, 4 H, H3, H4, H6), 4.01–4.05 (m, 1 H, H5), 4.06 (dd, *J* = 2.9, 1.5 Hz, 1 H, H2), 5.34 (d, *J* = 1.5 Hz, 1 H, H1), 7.08–7.39 (m, 4 H, Ph).

^{13}C NMR (100 MHz, MeOD): δ = 21.1 (*C*H$_3$-Tol), 62.6 (CH_2, C6), 68.7 (CH, C3), 73.2 (CH, C4), 74.2 (CH, C2), 76.2 (CH, C5), 90.8 (CH, C1), 130.7 (C_q, C1′), 130.8 (CH, C3′, C5′), 133.5 (CH, C2′, C6′), 138.9 (C_q, C4′).

NMR data was in agreement with that reported in the literature.[48]

4-Methylphenyl 6-*O*-Trityl-1-thio-α-D-mannopyranoside (3)

To an ice cold soln of **2** (0.51 g, 1.77 mmol) in pyridine (7 mL) was added TrtCl (2.96 g, 10.6 mmol) and a catalytic amount of DMAP. The mixture was stirred under N_2 for 24 h and allowed to warm to r.t. Ice-cold H_2O (5 mL) was added to quench the reaction and the crude product was extracted with EtOAc (3 × 5 mL), washed with 0.1 M aq HCl (2 × 5 mL) and sat. $NaHCO_3$ (2 × mL), dried (Na_2SO_4), and filtered. The solvent was removed in vacuo and the resulting yellow solid (1.30 g) was used in the subsequent benzoylation step without further purification; R_f = 0.21 (EtOAc–hexane, 1:1).

4-Methylphenyl 2,3,4-Tri-*O*-benzoyl-6-*O*-trityl-1-thio-α-D-mannopyranoside (4)

To an ice-cold soln of crude **3** (1.30 g, 2.46 mmol) and a catalytic amount of DMAP in pyridine–CH_2Cl_2 (1:4, 8 mL) was added BzCl (1.03 mL, 8.85 mmol) over 10 min. The mixture was stirred overnight, warmed to r.t., and diluted with EtOAc (10 mL) and H_2O (5 mL) was added. The organic layer was washed with 1 M aq HCl (2 × 5 mL), sat. $NaHCO_3$ (2 × 5 mL), H_2O (2 × 5 mL), brine (2 × 5 mL), dried (Na_2SO_4), and filtered. The solvent was removed in vacuo and the residue was purified by flash chromatography (EtOAc–hexane, 1:6) to give **4** (1.04 g, 98%) as a white foam; R_f = 0.23 (EtOAc–hexane, 1:2).

$[\alpha]_D^{20}$ –14.8 (*c* 1.40, $CHCl_3$).

IR (NaCl): 3060 (w), 3032 (w, ArH), 1730 (s, C=O), 1601 (m), 1584 (m, C–C, Ar), 1261 (s, C–O), 762 (s, C–H, Ar), 708 cm^{-1} (C–C, Ar).

^{1}H NMR (400 MHz, $CDCl_3$): δ = 2.03 (s, 3 H, C*H*$_3$-Tol), 3.34 (dd, *J* = 10.6, 4.6 Hz, 1 H, H6$_A$), 3.43 (dd, *J* = 10.6, 2.1 Hz, 1 H, H6$_B$), 4.76 (ddd, *J* = 10.1, 4.6, 2.1 Hz, 1 H, H5), 5.73–5.76 (m, 2 H, H1, H3), 5.95 (dd, *J* = 3.2, 1.6 Hz, 1 H, H2), 6.09 (t, *J* = 10.1 Hz, 1 H, H4), 7.07–8.14 (m, 34 H, Ph).

^{13}C NMR (100 MHz, $CDCl_3$): δ = 21.1 (*C*H$_3$-Tol), 62.2 (CH_2, C6), 67.1 (CH, C4), 70.9 (CH, C3), 71.4 (CH, C5), 72.2 (CH, C2), 86.2

(CH, C1), 86.7 (C_q, CPh_3), 126.8, 127.6, 127.9, 128.1, 128.3, 128.4, 128.6 (CH, Ph), 129.0, 129.2, 129.4 (C_q, Ph Bz), 129.7 (C_q, C1′), 129.9, 130.1, 132.4, 133.1, 133.4, 133.5 (CH, Ph), 138.0 (C_q, C4′), 143.7 (C_q, Ph Trt), 165.1, 165.5 (C_q, Bz).

MS (FAB): *m*/*z* (%) = 717 (3), 259 (1), 243 (100), 105 (56) [M + H]$^+$.

HRMS (FAB): *m*/*z* [M + H]$^+$ calcd for $C_{53}H_{45}O_{18}S$: 841.2835; found: 841.2833.

4-Methylphenyl 2,3,4-Tri-*O*-benzoyl-1-thio-α-D-mannopyranoside (5)

Compound **4** (1.94 g, 2.30 mmol) was dissolved in a mixture of anhyd CH_2Cl_2 (47 mL) and TFA (0.5 mL) under N_2 and then *i*-Pr_3SiH (2.50 mL, 12.2 mmol) was added. Upon completion of the reaction which was indicated by the disappearance of the yellow colour (1.5 h), H_2O (10 mL) was added and CH_2Cl_2 was removed in vacuo. The residue was diluted with EtOAc (30 mL), washed with sat. $NaHCO_3$ (3 × 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, 1:3) to give **5** (1.17 g, 85%) as a white foam; R_f = 0.19 (EtOAc–hexane, 1:3).

$[\alpha]_D^{20}$ –18.0 (*c* 0.97, $CHCl_3$).

IR (NaCl): 3523 (s br, O–H), 2923 (s), 2862 (s, C–H), 1728 (s, C=O), 1601 (m), 1584 (m, C–C, Ar), 1281 (s), 1262 (s, C–O), 1107 (s, O–C–C), 1093 (s, C–O–C), 709 cm^{-1} (s, C–C, Ar).

^{1}H NMR (300 MHz, $CDCl_3$): δ = 2.33 (s, 3 H, CH_3-Tol), 2.54 (br s, 1 H, OH), 3.82 (m, 2 H, H6), 4.60–4.63 (m, 1 H, H5), 5.71 (br s, 1 H, H1), 5.89–5.98 (m, 3 H, H3, H4, H2), 7.14–8.10 (m, 19 H, Ph).

^{13}C NMR (75 MHz, $CDCl_3$): δ = 21.1 (*C*H_3-Tol), 61.3 (CH_2, C6), 67.3 (CH, C4), 70.0 (CH, C2), 71.9 (CH, C3), 72.0 (CH, C5), 89.4 (CH, C1), 128.3, 128.5, 128.6 (CH, Ph), 128.8, 128.9, 129.1 (C_q, Ph Bz), 129.7 (C_q, C1′), 129.8, 129.9, 130.0, 132.7, 133.3, 133.6, 133.7 (CH, Ph), 138.5 (C_q, C4′), 165.3, 165.4, 166.4 (C_q, Bz).

MS (FAB): *m*/*z* (%) = 599 ([M + H]$^+$, 2), 475 (13), 355 (3), 233 (5), 105 (100).

HRMS (FAB): *m*/*z* [M + H]$^+$ calcd for $C_{34}H_{31}O_8S$: 599.1740; found: 599.1747.

4-Methylphenyl 2,3,4-Tri-*O*-benzoyl-6-*O*-(2,3,4,6-tetra-*O*-benzoyl-α-D-mannopyranosyl)-1-thio-α-D-mannopyranoside (7)

2,3,4,6-Tetra-*O*-benzoyl-α-D-mannopyranosyl trichloroacetimidate[11] (**6**, 0.48 g, 0.64 mmol) and **5** (0.39 g, 0.66 mmol) were dried together under high vacuum for 8 h prior to the reaction. Activated powdered 4Å molecular sieves were added and the mixture was stirred in anhyd CH_2Cl_2 (12 mL) under N_2 for 30 min. The suspension was cooled to –40 °C and TMSOTf (0.02 mL, 0.13 mmol) was quickly added. The mixture was stirred for 3 h, Et_3N was added to neutralise the mixture (pH paper) and the temperature of the soln was slowly raised to r.t. The mixture was then filtered and the filtrate washed with sat. $NaHCO_3$ (2 × 5 mL), brine (2 × 5 mL), dried (Na_2SO_4), and filtered. The solvent was removed in vacuo and the crude product was purified by flash chromatography (EtOAc–hexane, 1:3) to give **7** (0.66 g, 88%) as a white foam; R_f = 0.50 (EtOAc–hexane, 1:1).

$[\alpha]_D^{20}$ –9.4 (*c* 0.81, $CHCl_3$).

IR (NaCl): 3064 (w), 3033 (w, C–H, Ar), 1729 (s, C=O), 1601 (m), 1584 (m, C–C, Ar), 1263 (s, C–O), 1107 (s, O–C–C), 1095 (s, C–O–C), 757 (s, C–H, Ar), 709 cm^{-1} (s, C–C, Ar).

^{1}H NMR (400 MHz, $CDCl_3$): δ = 2.23 (s, 3 H, CH_3-Tol), 3.80 (dd, *J* = 11.00, 1.8 Hz, 1 H, $H6_A$), 4.21 (dd, J_{AB} = 11.0 Hz, $J_{6B,5}$ = 4.8 Hz, 1 H, $H6_B$), 4.28 (1 H, dd, *J* = 12.2, 4.1 Hz, 1 H, $H6'_A$), 4.35 (ddd, *J* = 10.0, 4.1, 2.3 Hz, 1 H, H5′), 4.46 (dd, *J* = 12.2, 2.3 Hz, 1 H, $H6'_B$), 4.95–4.99 (m, 1 H, H5), 5.15 (d, *J* = 1.6 Hz, 1 H, H1′), 5.76 (d, *J* = 1.4 Hz, 1 H, H1), 5.85 (dd, *J* = 3.2, *J* = 1.6 Hz, 1 H, H2′), 5.93 (dd, *J* = 10.1, *J* = 3.2 Hz, 1 H, H3), 6.02 (dd, *J* = 10.0, 3.2 Hz, 1 H, H3′), 6.06 (dd, *J* = 3.2, 1.4 Hz, 1 H, H2), 6.13 (t, *J* = 10.0 Hz, 1 H, H4′), 6.23 (t, *J* = 10.1 Hz, 1 H, H4), 7.17–8.19 (m, 39 H, Ph).

^{13}C NMR (100 MHz, $CDCl_3$): δ = 21.0 (*C*H_3-Tol), 62.4 (CH_2, C6′), 66.5 (CH, C4), 66.8 (CH_2, C6), 66.9 (CH, C4′), 68.8 (CH, C5′), 70.1 (CH, C2′, C5), 70.5 (CH, C3, C3′), 71.9 (CH, C2), 86.6 (d, *J* = 171.0 Hz, CH, C1), 98.0 (d, *J* = 173.8 Hz, CH, C1′), 128.3, 128.5 (CH, Ph), 128.8 (C_q, C1″), 128.9, 129.0, 129.1, 129.2 (C_q, Ph), 129.6, 129.7, 129.8, 129.9, 130.1, 132.6, 132.9, 133.0, 133.2, 133.3, 133.4, 133.5 (CH, Ph), 138.4 (C_q, C4″), 165.1, 165.4, 165.5, 165.9 (C_q, Bz).

MS (FAB): *m*/*z* (%) = 1053 (2), 579 (9), 475 (1), 105 (100) [M + H]$^+$.

HRMS (FAB): *m*/*z* [M + H]$^+$ calcd for $C_{68}H_{57}O_{17}S$: 1177.3317; found: 1177.3332.

2,3,4-Tri-*O*-benzoyl-6-*O*-(2,3,4,6-tetra-*O*-benzoyl-α-D-mannopyranosyl)-α-D-mannopyranoside (9)

To a vigorously stirred soln of **7** (0.20 g, 0.17 mmol) in EtOAc–H_2O (1:1, 5 mL) was added NBS (0.08 g, 0.42 mmol) and the mixture stirred for 24 h. The mixture was diluted with EtOAc (5 mL) and neutralised with Et_3N (pH paper). The organic layer was separated, washed with brine (3 × 5 mL), and dried (Na_2SO_4) and the solvent removed in vacuo. The crude product was purified by flash chromatography (EtOAc–hexane, 2:3) to give **9** (0.12 g, 64%) as a white foam; R_f = 0.13 (EtOAc–hexane, 1:2).

$[\alpha]_D^{20}$ –84.1 (*c* 0.62, $CHCl_3$).

IR (NaCl): 3444 (s br, O–H), 3064 (w), 3034 (w, C–H, Ar), 1731 (s, C=O), 1601 (m), 1584 (m, C–C, Ar), 1265 (s, C–O), 1107 (s, O–C–C), 709 cm^{-1} (s, C–C, Ar).

^{1}H NMR (300 MHz, $CDCl_3$): δ = 3.86 (dd, *J* = 11.4, 1.8 Hz, 1 H, $H6_A$), 4.12–4.16 (m, 1 H, $H6_B$), 4.32 (dd, *J* = 12.2, 3.9 Hz, 1 H, $H6'_A$), 4.43–4.49 (m, 1 H, H5′), 4.61 (dd, *J* = 12.2, 2.5 Hz, 1 H, $H6'_B$), 4.66–4.72 (m, 1 H, H5), 5.18 (d, *J* = 1.7 Hz, 1 H, H1′), 5.59 (dd, *J* = 3.8, 1.5 Hz, 1 H, H1), 5.79–5.84 (m, 2 H, H2′, H2), 5.99–6.06 (m, 3 H, H3′, H3, H4), 6.13 (t, *J* = 10.0 Hz, 1 H, H4′), 7.23–8.17 (m, 35 H, Ph).

^{13}C NMR (75 MHz, $CDCl_3$): δ = 62.5 (CH_2, C6′), 66.7 (CH, C4′), 67.3 (CH, C4), 67.8 (CH_2, C6), 68.9 (CH, C5′), 69.5 (CH, C5), 69.9 (CH, C3), 70.2 (CH, C3′), 70.4 (CH, C2′), 71.1 (CH, C2), 92.5 (d, *J* = 175.0 Hz, CH, C1), 98.0 (d, *J* = 171.7 Hz, CH, C1′), 128.2, 128.3, 128.4, 128.5, 128.7 (CH, Ph), 129.0, 129.2, 129.4 (C_q, Ph), 129.7, 129.8, 130.0, 133.0, 133.1, 133.4 (CH, Ph), 165.3, 165.5, 165.6, 165.7, 166.2 (C_q, Bz).

MS (FAB): *m*/*z* (%) = 1053 ([M – H_2O], 1), 579 (9), 475 (1), 231 (5), 105 (100).

HRMS (FAB): *m*/*z* [M – OH] calcd for $C_{61}H_{49}O_{17}$: 1053.2970; found: 1053.2984.

2,3,4-Tri-*O*-benzoyl-6-*O*-(2,3,4,6-tetra-*O*-benzoyl-α-D-mannopyranosyl)-α-D-mannopyranosyl Trichloroacetimidate (10)

Compound **9** (2.53 g, 2.36 mmol) was dissolved in anhyd CH_2Cl_2 (50 mL) under N_2 and K_2CO_3 (0.47 g, 6.49 mmol) was added. The mixture was stirred for 10 min and trichloroacetonitrile (0.93 mL, 9.33 mmol) was added dropwise. The mixture was vigorously stirred overnight, filtered, concentrated in vacuo and the crude mixture purified by flash chromatography (EtOAc–hexane, 3:7) to give **10** (2.03 g, 71%) as a white foam; R_f = 0.36 (EtOAc–hexane, 1:2).

$[\alpha]_D^{20}$ –44.8 (*c* 0.99, $CHCl_3$).

IR (NaCl): 3441 (m), 3336 (m, =NH), 3064 (w), 3033 (w, C–H, Ar), 1730 (s, C=O), 1679 (v, C=N), 1601 (m), 1584 (m, C–C, Ar), 1264

(s, C–O), 1107 (s, O–C–C), 1092 (s, C–O–C), 756 (s, C–H, Ar), 708 cm^{-1} (C–C, Ar).

^1H NMR (300 MHz, $CDCl_3$): δ = 3.87 (dd, *J* = 11.0, 1.8 Hz, 1 H, $H6_A$), 4.15 (dd, *J* = 11.0, 5.3 Hz, 1 H, $H6_B$), 4.31 (dd, *J* = 12.1, 3.8 Hz, 1 H, $H6'_A$), 4.41–4.47 (m, 1 H, H5′), 4.51 (dd, *J* = 12.1, 2.3 Hz, 1 H, $H6'_B$), 4.58–4.63 (m, 1 H, H5), 5.14 (d, *J* = 1.7 Hz, 1 H, H1′), 5.76 (dd, *J* = 3.2, 1.7 Hz, 1 H, H2′), 5.96–6.04 (m, H3′, 3 H, H3, H2), 6.12 (t, *J* = 9.9 Hz, 1 H, H4′), 6.18 (t, *J* = 9.9 Hz, 1 H, H4), 6.58 (br s, 1 H, H1), 7.25–8.20 (m, 35 H, Ph), 9.01 (s, 1 H, NH).

^{13}C NMR (75 MHz, $CDCl_3$): δ = 62.5 (CH_2, C6′), 66.2 (CH, C4), 66.3 (CH_2, C6), 66.7 (CH, C4′), 68.9 (CH, C5′), 69.0 (CH, C3), 70.0 (CH, C2, C3′), 70.3 (CH, C2′), 72.2 (CH, C5), 90.7 [C_q, CCl_3C(=NH)], 94.8 (d, *J* = 180.7 Hz, CH, C1), 97.6 (d, *J* = 175.0 Hz, CH, C1′), 128.3, 128.4, 128.5, 128.7, 128.8, 128.9 (CH, Ph), 129.2, 129.3 (C_q, Ph), 129.7, 129.8, 129.9, 130.1, 132.9, 133.0, 133.3, 133.4, 133.6 (CH, Ph), 159.8 [C_q, CCl_3C(=NH)], 165.1, 165.2, 165.4, 165.5, 166.0 (C_q, Bz).

MS (ESI): *m*/*z* $[M + H + Na]^+$ calcd for $C_{63}H_{51}Cl_3NNaO_{18}$: 1237.21; found: 1237.43.

N-(9-Fluorenylmethoxycarbonyl)-*O*-[2,3,4-tri-*O*-benzoyl-6-*O*-(2,3,4,6-tetra-*O*-benzoyl-α-D-mannopyranosyl)-α-D-mannopyranosyl]-L-serine Allyl Ester (11)

Compound **10** (0.91 g, 0.75 mmol) and *N*-(9-fluorenylmethoxycarbonyl)-L-serine allyl ester[11] (**8**, 0.28 g, 0.77 mmol) were dried together under high vacuum for 8 h prior to the reaction. Activated powdered 4Å molecular sieves were added and the mixture was stirred in anhyd CH_2Cl_2 (7 mL) under N_2 for 30 min. The suspension was cooled to –40 °C and TMSOTf (0.03 mL, 0.15 mmol) was quickly added. The mixture was stirred for 3 h, Et_3N was added to neutralise the mixture (pH paper) and the temperature of the soln was slowly raised to r.t. The mixture was filtered, the soln was washed with sat. $NaHCO_3$ (2 × 3 mL), brine (2 × 3 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 1:1) to give **11** (0.79 g, 74%) as a white foam; R_f = 0.14 (EtOAc–hexane, 2:3).

$[\alpha]_D^{20}$ –41.0 (*c* 1.31, $CHCl_3$).

IR (NaCl): 3427 (m), 3366 (m, N–H), 2952 (s), 289 1 (s, C–H), 1728 (s, C=O), 1601 (m), 1584 (m, C–C, Ar), 1519 (w, N–H), 1263 (s, C–O), 1108 (s, O–C–C), 1095 (s, C–O–C), 759 (s), 742 (s, C–H, Ar), 709 cm^{-1} (s, C–C, Ar).

^1H NMR (400 MHz, $CDCl_3$): δ = 3.81–3.83 (m, 1 H, $H6_A$), 4.14–4.18 (m, 2 H, $H6_B$, Serβ-H_AH_B), 4.24–4.36 (m, 7 H, CH Fmoc, $H6'_A$, CH_2 Fmoc, Serβ-H_AH_B, H5, H5′), 4.52 (dd, *J* = 12.2, 2.2 Hz, 1 H, $H6'_B$), 4.74–4.87 (m, 3 H, $OCH_2CH{=}CH_2$, Serα-H), 5.17 (br s, 1 H, H1′), 5.20 (br s, 1 H, H1), 5.29–5.44 (m, 2 H, $OCH_2CH{=}CH_2$), 5.75 (m, 1 H, H2′), 5.84 (m, 1 H, H2), 5.90 (dd, *J* = 10.1, 3.0 Hz, 1 H, H3′), 6.00–6.10 (m, 3 H, H4′, H3, $OCH_2CH{=}CH_2$), 6.13 (t, *J* = 9.9 Hz, 1 H, H4), 6.30 (d, *J* = 8.7 Hz, 1 H, NH), 7.19–8.18 (m, 43 H, Ph).

^{13}C NMR (100 MHz, $CDCl_3$): δ = 47.0 (CH, Fmoc), 54.1 (CH, Serα), 62.5 (CH_2, C6′), 66.4 (CH_2, C6), 66.6 (CH, CH_2, C4, $OCH_2CH{=}CH_2$), 66.8 (CH, C4′), 67.5 (CH_2, Fmoc), 68.8 (CH_2, Serβ), 69.0 (CH, C5′), 69.9 (CH, C3′, C5), 70.0 (CH, C3), 70.2 (CH, C2′), 70.3 (CH, C2), 97.6 (d, *J* = 171.3 Hz, CH, C1′), 98.5 (d, *J* = 173.4 Hz, CH, C1), 119.6 (CH_2, $OCH_2CH{=}CH_2$), 119.8, 124.9, 125.2, 127.3, 127.6, 128.2, 128.3, 128.4, 128.5, 128.7 (CH, Ph), 129.0, 129.1, 129.2 (C_q, Ph), 129.6, 129.7, 129.8, 130.0 (CH, Ph), 131.4 (CH, $OCH_2CH{=}CH_2$), 132.9, 133.1, 133.2, 133.4, 133.5 (CH, Ph), 141.2, 143.8 (C_q, Fmoc), 156.0 (C_q, CONH), 165.2, 165.4, 165.5, 166.0 (C_q, Bz), 169.9 (C_q, COOAllyl).

MS (FAB): *m*/*z* (%) = 1053 (2), 579 (8), 231 (6), 178 (20), 105 (100) $[M + H]^+$.

HRMS (FAB): *m*/*z* $[M + H]^+$ calcd for $C_{82}H_{70}NO_{22}$: 1420.4390; found: 1420.4392.

N-(9-Fluorenylmethoxycarbonyl)-*O*-[2,3,4-tri-*O*-benzoyl-6-*O*-(2,3,4,6-tetra-*O*-benzoyl-α-D-mannopyranosyl)-α-D-mannopyranosyl]-L-serine (12)

Compound **11** (0.73 g, 0.52 mmol) was dissolved in CH_2Cl_2 (10 mL) and the mixture was degassed under an argon atmosphere. $Pd(PPh_3)_4$ (0.02 g, 20.6 μmol) was added and argon was bubbled through the yellow soln for 10 min. $PhSiH_3$ (0.51 mL, 4.12 mmol) was then added and the mixture was stirred under argon for 1.5 h during which time the colour of the mixture turned black. Upon completion of the reaction (TLC: EtOAc–hexane, 2:1 + 10% AcOH), the solvent was removed in vacuo and the crude product was purified twice by flash chromatography (CH_2Cl_2–MeOH, 8:2). The resultant brown solid was lyophilised (*t*-BuOH) to give **12** (0.48 g, 67%) as a white amorphous powder; R_f = 0.34 (EtOAc–hexane, 1:1 + 10% AcOH).

$[\alpha]_D^{20}$ –24.2 (*c* 0.89, $CHCl_3$).

IR (NaCl): 3300–2500 (s br, O–H), 3423 (m), 3350 (m, N–H), 2951 (s), 2873 (s, C–H), 1727 (s, C=O), 1601 (m), 1585 (m, C–C, Ar), 1451 (m, C–O–H), 1263 (s, C–O), 1108 (s, O–C–C), 1095 (s, C–O–C), 759 (s, C–H, Ar), 709 cm^{-1} (s, C–C, Ar).

^1H NMR (400 MHz, $CDCl_3$): δ = 3.82 (m, 1 H, $H6_A$), 4.10–4.21 (m, 2 H, $H6_B$, Serβ-H_AH_B), 4.25–4.31 (m, 3 H, CH Fmoc, $H6'_A$, Serβ-H_AH_B), 4.37–4.50 (m, 5 H, H5′, CH_2 Fmoc, $H6'_B$, H5), 4.79 (m, 1 H, Serα-H), 5.17 (d, *J* = 1.6 Hz, 1 H, H1′), 5.19 (br s, 1 H, H1), 5.76–5.78 (m, 1 H, H2), 5.84 (dd, *J* = 2.9, 1.6 Hz, 1 H, H2′), 5.95 (dd, *J* = 10.0, 3.0 Hz, 1 H, H3), 6.05 (dd, *J* = 10.2, 2.9 Hz, 1 H, H3′), 6.09–6.10 (m, 2 H, H4, H4′), 6.41 (d, *J* = 8.2 Hz, 1 H, NH), 7.20–8.14 (m, 43 H, Ph).

^{13}C NMR (100 MHz, $CDCl_3$): δ = 47.1 (CH, Fmoc), 54.2 (CH, Serα), 62.4 (CH_2, C6′), 66.5 (CH_2, C6), 66.7 (CH, C4′), 67.4 (CH_2, Fmoc), 68.9 (CH_2, Serβ), 69.0 (CH, C5′), 69.9 (CH, C5), 70.1 (CH, H3, H3′), 70.2 (CH, C2, C2′), 97.8 (d, *J* = 174.0 Hz, CH, C1′), 98.5 (d, *J* = 171.0 Hz, CH, C1), 119.8, 125.2, 127.1, 127.6, 128.4, 128.5, 128.6, 128.7, 128.8 (CH, Ph), 129.0, 129.1, 129.2 (C_q, Ph), 129.6, 129.7, 129.8, 130.0, 132.0, 132.1, 132.2, 133.0, 133.1, 133.2, 133.4, 133.5 (CH, Ph), 141.2, 143.8 (C_q, Fmoc), 156.1 (C_q, CONH), 165.2, 165.4, 165.5, 165.6, 166.0 (C_q, Bz), 172.3 (C_q, COOH).

MS (FAB): *m*/*z* (%) = 1054 (1), 579 (5), 231 (4), 178 (15), 165 (4), 105 (100).

HRMS (FAB): *m*/*z* $[M + H]^+$ calcd for $C_{79}H_{66}NO_{22}$: 1380.4077; found: 1380.4065.

tert-Butyl 12-{[2,3,4-Tri-*O*-benzoyl-6-*O*-(2,3,4,6-tetra-*O*-benzoyl-α-D-mannopyranosyl)-α-D-mannopyranosyl)]oxy}-4,7,10-trioxadodecanoate (14)

Compound **7** (2.00 g, 1.70 mmol) and *tert*-butyl 12-hydroxy-4,7,10-trioxadodecanoate[41] (**13**, 0.36 g, 1.31 mmol) were dried together under high vacuum for 8 h prior to the reaction. Activated powdered 4Å molecular sieves were added and the mixture was stirred in anhyd CH_2Cl_2 (7 mL) under N_2 for 30 min. The suspension was cooled to 0 °C, NIS (0.76 g, 3.40 mmol) and AgOTf (0.10 g, 0.39 mmol) were added under a flow of N_2. This resulted in a slight pink mixture that developed continuously during the progress of the reaction to finally change to yellow indicating that the all starting material had been consumed.[49] The mixture was neutralised with Et_3N (pH paper), diluted with CH_2Cl_2 (5 mL), filtered, washed with 10% aq $Na_2S_2O_3$ (3 × 5 mL), sat. $NaHCO_3$ (2 × 5 mL), brine (2 × 5 mL), dried (Na_2SO_4), filtered and the solvent was removed in vacuo. The crude product (amber oil) was purified by flash chromatography (EtOAc–hexane, 2:3) to give **14** (1.74 g, 77%) as a white foam; R_f = 0.26 (EtOAc–hexane, 1:1).

$[\alpha]_D^{20}$ –45.4 (*c* 0.97, $CHCl_3$).

IR (NaCl): 2927 (s), 2869 (s, C–H), 1728 (s, C=O), 1601 (m), 1584 (m, C–C, Ar), 1263 (s, C–O), 1108 (s, O–C–C), 710 cm^{-1} (s, C–C, Ar).

^1H NMR (400 MHz, $CDCl_3$): δ = 1.43 [s, 9 H, $(CH_3)_3C$], 2.48–2.51 (m, 2 H, H2), 3.60–3.81 (m, 10 H, H5, H6, H3, H8, H9), 3.83–3.90 (m, 3 H, H11, $H12_A$), 4.05–4.16 (m, 3 H, $H12_B$, H6′), 4.30 (dd, *J* = 12.2, 4.0 Hz, 1 H, H6″$_A$), 4.41 (ddd, *J* = 10.0, 4.0, 2.4 Hz, 1 H, H5″), 4.46–4.51 (m, 2 H, H5′, H6″$_B$), 5.18 (d, *J* = 1.5 Hz, 1 H, H1″), 5.19 (d, *J* = 1.3 Hz, 1 H, H1′), 5.80–5.82 (m, 2 H, H2′, H2″), 5.97 (dd, *J* 10.2, 3.4 Hz, 1 H, H3′), 6.02 (dd, *J* = 10.0, 3.2 Hz, 1 H, H3″), 6.11 (t, *J* = 10.2 Hz, 1 H, H4′), 6.12 (t, *J* = 10.0 Hz, 1 H, H4″), 7.25–8.19 (m, 35 H, Ph).

^{13}C NMR (100 MHz, $CDCl_3$): δ = 28.0 [CH_3, $(CH_3)_3C$], 36.2 (CH_2, C2), 62.5 (CH_2, C6″), 66.6 (CH_2, CH, C3, C4″), 66.8 (CH_2, C6′), 66.9 (CH, C4′), 67.5 (CH_2, C12), 68.8 (CH, C5″), 69.3 (CH, C5′), 70.0 (CH, C2″), 70.1 (CH_2, C11), 70.2 (CH, C3′, C3″), 70.3 (CH_2, C5), 70.4 (CH, C2′), 70.5 (CH_2, C6), 70.6 (CH_2, C8), 70.7 (CH_2, C9), 80.4 [C_q, $(CH_3)_3C$], 97.7 (d, *J* = 172.3 Hz, CH, C1′), 97.9 (d, *J* = 170.4 Hz, CH, C1″), 128.2, 128.4, 128.5 (CH, Ph), 128.9, 129.0, 129.1, 129.2, 129.3 (C_q, Ph), 129.6, 129.7, 129.8, 130.0, 133.0, 133.3, 133.4 (CH, Ph), 165.1, 165.4, 165.5, 165.6, 165.9 (C_q, Bz), 170.9 (C_q, C1).

MS (FAB): *m*/*z* (%) = 1053 (4), 579 (8), 105 (100).

HRMS (FAB): *m*/*z* [M + H]$^+$ calcd for $C_{74}H_{75}O_{23}$: 1331.4699; found: 1331.4719.

12-{[2,3,4-Tri-*O*-benzoyl-6-*O*-(2,3,4,6-tetra-*O*-benzoyl-α-D-mannopyranosyl)-α-D-mannopyranosyl]oxy}-4,7,10-trioxadodecanoic Acid (15)

Compound **14** (0.87 g, 0.65 mmol) was dissolved in TFA (15 mL) then anisole (1.5 mL) was added and the mixture was stirred for 1.5 h. The solvent was removed in vacuo and the crude product was purified by flash chromatography (EtOAc–hexane 2:1) to give **15** (0.66 g, 80%) as a white foam; R_f = 0.57 (EtOAc–hexane, 1:1 + 10% AcOH).

$[\alpha]_D^{20}$ –49.7 (*c* 0.65, $CHCl_3$).

IR (NaCl): 3300–2500 (s br, O–H), 2925 (s), 2881(s, C–H), 1728 (s, C=O), 1601 (m), 1584 (m, C–C, Ar), 1451 (m, C–O–H), 1263 (s, C–O), 1108 (s, O–C–C), 756 (s, C–H, Ar), 710 cm^{-1} (s, C–C, Ar).

^1H NMR (400 MHz, $CDCl_3$): δ = 2.59–2.62 (m, 2 H, H2), 3.62–3.82 (m, 10 H, H5, H6, H3, H8, H9), 3.84–3.91 (m, 3 H, H11, $H12_A$), 4.04–4.16 (m, 3 H, $H12_B$, H6′), 4.29 (dd, *J* = 12.2, 4.0 Hz, 1 H, H6″$_A$), 4.41 (ddd, *J* = 10.0, 4.0, 2.6 Hz, 1 H, H5″), 4.46–4.52 (m, 2 H, H6″$_B$, H5′), 5.18 (d, *J* = 1.5 Hz, 1 H, H1″), 5.21 (d, *J* = 1.5 Hz, 1 H, H1′), 5.80–5.82 (m, 2 H, H2′, H2″), 5.97 (dd, *J* = 10.1, 3.4 Hz, 1 H, H3′), 6.02 (dd, *J* = 10.0, 3.2 Hz, 1 H, H3″), 6.08–6.14 (m, 2 H, H4′, H4″), 7.25–8.20 (m, 35 H, Ph).

^{13}C NMR (100 MHz, $CDCl_3$): δ = 34.7 (CH_2, C2), 62.5 (CH_2, C6″), 66.3 (CH_2, C3), 66.6 (CH_2, CH, C6′, C4″), 67.0 (CH, C4′), 67.6 (CH_2, C12), 68.9 (CH, C5″), 69.4 (CH, C5′), 70.1 (CH, C2″), 70.2 (CH_2, CH, C11, C3″, C3′), 70.3 (CH, C2′), 70.4 (CH_2, C5), 70.5 (CH_2, C6), 70.6 (CH_2, C8), 70.8 (CH_2, C9), 97.7 (CH, C1′), 97.9 (CH, C1″), 128.3, 128.4, 128.5, 128.8 (CH, Ph), 129.0, 129.1, 129.3 (C_q, Ph), 129.7, 129.8, 129.9, 130.0, 130.1, 130.4, 130.5 (CH, Ph), 165.2, 165.5, 165.6, 165.7, 166.0 (C_q, Bz), 171.2 (C_q, C1).

MS (FAB): *m*/*z* (%) = 1297 (M + Na^+, 1), 1053 (4), 579 (8), 105 (100).

HRMS (FAB): *m*/*z* [M + H]$^+$ calcd for $C_{70}H_{67}O_{23}$: 1275.4073; found: 1275.4075.

Solid-Phase Synthesis of Glycopeptides 17–22; General Procedures

Glycopeptides were assembled using SPGS on pre-loaded Fmoc-Gly-Wang resin with the use of a CEM Liberty microwave peptide synthesizer using the following conditions: *Fmoc removal*: 20% piperidine–DMF, 0.5 min plus 3 min cycle at 80 °C; *Fmoc-Aaa coupling*: Fmoc-Aaa (5.0 equiv), HBTU (4.5 equiv), DIPEA (10 equiv), DMF, 5 min cycle at 80 °C; *5(6)-carboxyfluorescein coupling*: 5(6)-carboxyfluorescein (5.0 equiv), HBTU (4.5 equiv), DIPEA (10 equiv), DMF, 30 min cycle at 80 °C; *O-dimannosylated building block coupling*: O-dimannosylated building block (1.5 equiv), HATU (1.45 equiv), HOAt (1.5 equiv), collidine (4.5 equiv), DMF, 20 min cycle at 80 °C; *capping*: 20% Ac_2O–DMF, 2 min cycle at 70 °C; *cleavage*: TFA–*i*-Pr_3SiH–H_2O (95:2.5:2.5), 20 min cycle at 40 °C; *removal of additional ester bound 5(6)-carboxyfluorescein*: 20% piperidine–DMF, six cycles of 0.5 min plus 3 min at 80 °C.

Glycosylated building blocks were pre-activated before placement in the synthesizer. 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 was shaken until dissolved. The soln was transferred to the reaction vessel, followed by the addition of collidine (4.5 equiv). The mixture was microwaved for 20 min at 80 °C, and a sample of the resin (1.0 mg) was taken for the Kaiser test.[43]

Removal of the Dde protecting group from the N^{ε} amino group of the lysine residue was performed manually using 2% $NH_2NH_2{\cdot}H_2O$–DMF (2 × 3 min).

5(6)-Carboxyfluorescein(Trt)$_2$-Lys(Dde)-Gly-Wang Resin (16)

Peptidyl-resin **16** was synthesised on a CEM Liberty microwave peptide synthesizer using Fmoc-Gly-Wang resin (625 mg, 0.80 mmol g^{-1}) and the conditions outlined in the general procedure. After removal of the additional ester bound 5(6)-carboxyfluorescein, the 5(6)-carboxyfluorescein-Lys(Dde)-Gly-Wang resin was transferred to the fritted glass reaction vessel and dried under a flow of N_2. TrtCl (12 equiv) was then added, followed by the addition of CH_2Cl_2 and DIPEA (12 equiv). The mixture was shaken overnight, filtered, washed with CH_2Cl_2 (5 ×), dried under a flow of N_2 and a fresh portion of the tritylation reagents was added and the procedure repeated to yield yellow-coloured 5(6)-carboxyfluorescein(Trt)$_2$-Lys(Dde)-Gly-Wang resin (**16**), which was used for further synthesis. A sample of the trityl-protected fluorescein-labelled resin (25.0 mg) was cleaved from the resin using TFA–*i*-Pr_3SiH–H_2O (95:2.5:2.5) for 2 h, precipitated from cold Et_2O, isolated by centrifuge and lyophilised from MeCN–H_2O + 0.1% TFA to yield 5(6)-carboxyfluorescein-Lys(Dde)-Gly-OH; HPLC (Phenomenex Jupiter C_4 analytical column, 50 mm × 2.0 mm, 0.5 mL min^{-1}, linear gradient of 1% B to 50% B over 15 min): t_R = 12.78 min.

MS (ESI): *m*/*z* [M + H]$^+$ calcd for $C_{39}H_{40}N_3O_{11}$: 726.26; found: 726.55.

5(6)-Carboxyfluorescein-{[D-Man(OBz)$_4$-(α1→6)-D-Man(OBz)$_3$(α1-O)]Ser-(Ala)$_4$-}Lys-Gly-OH (17)

Fmoc-Gly-Wang resin (120 mg, 0.75 mmol g^{-1}) was used for the synthesis of glycopeptide **17** using a CEM Liberty microwave peptide synthesizer and the conditions outlined in the general methods. The crude benzoyl-protected product was purified by RP-HPLC (semi-preparative Waters XTerra Prep. C_{18} column, flow rate: 10 mL min^{-1}; linear gradient 10% B to 100% B over 35 min) and lyophilised to give **17** (15.9 mg, 8%) as a yellow amorphous solid; HPLC (Phenomenex Gemini C_{18} analytical column, 150 mm × 4.6 mm, 1 mL min^{-1}, linear gradient of 10% B to 100% B over 35 min): t_R = 29.47 min.

HRMS (MALDI-TOF): *m*/*z* [M + Na]$^+$ calcd for $C_{105}H_{100}N_8NaO_{32}$: 2007.6336; found: 2005.7562.

NMR data was not recorded.

5(6)-Carboxyfluorescein-{[D-Man(OH)$_4$-(α1→6)-D-Man(OH)$_3$(α1-O)]Ser-(Ala)$_4$-}Lys-Gly-OH (18)

Compound **17** (9.5 mg, 4.8 μmol) was dissolved in MeOH (5 mL) and 1 M NaOMe soln in MeOH was added to adjust the pH to 12.1 (pH meter). When the all starting material had disappeared as judged by analytical RP-HPLC (3 h), the soln was neutralised with a portion of dry ice and the solvent was removed in vacuo. The crude product was purified by RP-HPLC (semi-preparative Phenomenex Jupiter C_4 column, flow rate: 7.5 mL min^{-1}, linear gradient: 1% B to 50% B over 35 min) and lyophilised to give the **18** (4.4 mg, 73%) as a yellow amorphous solid; HPLC (Phenomenex Jupiter C_4 analytical column, 50 mm × 2.0 mm, 0.5 mL min^{-1}, 3 min of 1% B followed by linear gradient of 1% B to 50% B over 15 min): t_R = 15.31 min.

^{1}H NMR (600 MHz, MeOD): δ = 1.31–1.42 (m, 12 H, 4 × Alaβ-H$_3$), 1.44–1.69 (m, 4 H, Lysγ-H$_2$, Lysδ-H$_2$), 1.78–2.04 (m, 2 H, Lysβ-H$_2$), 3.18–3.28 (m, 2 H, Lysε-H$_2$), 3.65–4.08 (m, 15 H, H2, H2′, H3, H3′, H4, H4′, H5, H5′, H6, H6′, Glyα-H$_2$, Serβ-H_AH$_B$), 4.11–4.18 (m, 3 H, Serβ-H$_A$$H_B$, Alaα-H, Serα-H), 4.27–4.37 (m, 2 H, 2 × Alaα-H), 4.45–5.00 (m, 1 H, Alaα-H), 4.57–4.60 (m, 0.4 H, Lysα-H 6-CF), 4.69–4.72 (m, 0.6 H, Lysα-H 5-CF), 4.82 (br s, 1 H, H1′), 4.83 (br s, 1 H, H1), 6.66–6.83 (m, 6 H, CH$_{Fluoro}$), 7.37–7.39 (m, 0.6 H, CH$_{Fluoro}$ 5-CF), 7.79 (br s, 0.4 H, CH$_{Fluoro}$ 6-CF), 8.17–8.19 (m, 0.4 H, CH$_{Fluoro}$ 6-CF), 8.24–8.26 (m, 0.4 H, CH$_{Fluoro}$ 6-CF), 8.30–8.32 (m, 0.6 H, CH$_{Fluoro}$ 5-CF), 8.61 (s, 0.6 H, CH$_{Fluoro}$ 5-CF).

^{13}C NMR (150 MHz, MeOD): δ = 17.8, 17.9, 18.2, 18.3 (CH$_3$, 4 × Alaβ), 24.1, 24.2 (CH$_2$, Lysγ), 29.7, 29.9 (CH$_2$, Lysδ), 32.5, 32.7 (CH$_2$, Lysβ), 39.9, 40.0 (CH$_2$, Lysε), 41.8, 41.9 (CH$_2$, Glyα), 50.5, 50.7, 50.8, 50.9 (CH, 4 × Alaα), 54.3 (CH, Serα), 55.6 (CH, Lysα), 63.0 (CH$_2$, C6′), 67.2 (CH$_2$, Serβ), 67.4 (CH$_2$, C6), 68.5, 68.7, 71.4, 72.1, 72.5, 72.6, 73.7, 74.6 (CH, C2, C2′, C3, C3′, C4, C4′, C5, C5′),101.3 (CH, C1′), 102.6 (CH, H1), 103.6 (CH, Fluoro Ph), 114.7 (C$_q$, Fluoro), 118.1, 126.4, 129.1, 130.7, 131.0, 135.6 (CH, Fluoro Ph), 137.6, 155.1, 160.7, 161.0 (C$_q$, Fluoro), 167.4, 168.5, 168.6, 170.1, 170.3, 172.9, 173.0, 174.4, 174.5, 174.7, 174.8, 174.9, 175.1, 175.2 (C$_q$, CONH, CO$_2$H).

HRMS (MALDI-TOF): *m*/*z* [M + Na]$^+$ calcd for $C_{56}H_{72}N_8NaO_{25}$: 1279.4501; found: 1278.9909.

5(6)-Carboxyfluorescein-{[D-Man(OBz)$_4$-(α1→6)-D-Man(OBz)$_3$(α1-O)]PEG-(Ala)$_3$-}Lys-Gly-OH (19)

Fmoc-Gly-Wang resin (125 mg, 0.80 mmol g^{-1}) was used for the synthesis of glycopeptide **19** using a CEM Liberty microwave peptide synthesizer and the conditions outlined in the general methods. The crude benzoyl-protected glycopeptide **19** (118 mg, 58% yield, 66% purity (RP-HPLC)) was used in the subsequent debenzoylation step without further purification; HPLC (Phenomenex Jupiter C_4 analytical column, 50 mm × 2.0 mm, 0.5 mL min^{-1}, 3 min of 5% B followed by linear gradient of 5% B to 100% B over 15 min): t_R = 18.75 min.

HRMS (MALDI-TOF): *m*/*z* [M]$^+$ calcd for $C_{108}H_{106}N_6O_{34}$: 2030.6750; found: 2030.6453.

5(6)-Carboxyfluorescein-{[D-Man(OH)$_4$-(α1→6)-D-Man(OH)$_3$(α1-O)]PEG-(Ala)$_3$-}Lys-Gly-OH (20)

Crude **19** (19.5 mg, 9.61 μmol) was dissolved in MeOH (7 mL) and 1 M NaOMe soln in MeOH was added to adjust the pH to 11.9 (pH meter). When the all starting material had disappeared as judged by analytical RP-HPLC (3 h), the soln was neutralised with a portion of dry ice and the solvent was removed in vacuo. The crude product was purified by RP-HPLC (semi-preparative Waters XTerra Prep. C_{18} column; flow rate: 10 mL min^{-1}; linear gradient: 1% B to 50% B over 35 min) and lyophilised to give **20** (8.2 mg, 66%) as a yellow amorphous solid; HPLC (Phenomenex Jupiter C_4 analytical column, 50 mm × 2.0 mm, 0.5 mL min^{-1}, 3 min of 1% B followed by linear gradient of 1% B to 50% B over 15 min): t_R = 15.55 min.

^{1}H NMR (600 MHz, MeOD): δ = 1.34–1.42 (m, 9 H, 3 × Alaβ-H$_3$), 1.52–2.02 (m, 6 H, Lysγ-H$_2$, Lysδ-H$_2$, Lysβ-H$_2$), 2.46–2.60 (m, 2 H, H2), 3.16–3.31 (m, 2 H, Lysε-H$_2$), 3.65–4.07 (m, 28 H, H3, H5, H6, H8, H9, H11, H12, H2′, H2″, H3′, H3″, H4′, H4″, H5′, H5″, H6′, H6″, Glyα-H$_2$), 4.13–4.32 (m, 3 H, 3 × Alaα-H), 4.56–4.59 (m, 0.4 H, Lysα-H 6-CF), 4.68–4.70 (m, 0.6 H, Lysα-H 5-CF), 4.80 (br s, 1 H, H1″), 4.86 (br s, 1 H, H1′), 6.60–6.76 (m, 6 H, CH$_{Fluoro}$), 7.34–7.36 (m, 0.6 H, CH$_{Fluoro}$ 5-CF), 7.80 (br s, 0.4 H, CH$_{Fluoro}$ 6-CF), 8.13–8.14 (m, 0.4 H, CH$_{Fluoro}$ 6-CF), 8.23 (d, *J* = 8.0 Hz, 0.4 H, CH$_{Fluoro}$ 6-CF), 8.29–8.30 (m, 0.6 H, CH$_{Fluoro}$ 5-CF), 8.57 (s, 0.6 H, CH$_{Fluoro}$ 5-CF).

^{13}C NMR (150 MHz, MeOD): δ = 17.5, 17.6, 17.9, 18.0 (CH$_3$, 3 × Alaβ), 24.1, 24.2 (CH$_2$, Lysγ), 29.7, 29.9 (CH$_2$, Lysδ), 32.5, 32.6 (CH$_2$, Lysβ), 37.2 (CH$_2$, C2), 39.9 (CH$_2$, Lysε), 41.8, 41.9 (CH$_2$, Glyα), 50.9, 51.2, 51.3, 51.7, 51.8 (CH, 3 × Alaα), 55.7 (CH, Lysα), 62.9 (CH$_2$, C6″), 67.5 (CH$_2$, C12), 67.8 (CH$_2$, CH$_2$ PEG), 68.3 (CH$_2$, C6′), 68.7 (CH, C4′, C4″), 71.2, 71.3, 71.5, 71.6 (CH$_2$, 5 × CH$_2$ PEG), 72.1, 72.2 (CH, C2′, C2″), 72.7, 72.8 (CH, C3′, C3″), 73.2, 74.4 (CH, C5′, C5″), 101.4 (CH, C1′), 101.9 (CH, C1″), 103.7 (CH, Fluoro Ph), 111.2 (C$_q$, Fluoro), 114.0, 125.7, 125.9 (CH, Fluoro Ph), 128.7 (C$_q$, Fluoro), 130.4, 130.6, 130.8, 135.9 (CH, Fluoro Ph), 137.5, 140.5, 141.8, 154.4, 162.0 (C$_q$, Fluoro), 168.5, 168.6, 170.5, 172.8, 172.9, 174.7, 174.8, 174.9, 175.0 (C$_q$, CONH, COOH).

HRMS (MALDI-TOF): *m*/*z* [M]$^+$ calcd for $C_{59}H_{78}N_6O_{27}$: 1302.4915; found: 1302.6882.

5(6)-Carboxyfluorescein-{[D-Man(OBz)$_4$-(α1→6)-D-Man(OBz)$_3$(α1-O)]PEG-(Ala)$_4$-}Lys-Gly-OH (21)

Fmoc-Gly-Wang resin (125 mg, 0.80 mmol g^{-1}) was used for the synthesis of glycopeptide **21** using a CEM Liberty microwave peptide synthesizer and the conditions outlined in the general methods. The crude benzoyl-protected glycopeptide **21** [131 mg, 62% yield, 73% purity (RP-HPLC)] was used in the subsequent debenzoylation step without further purification; HPLC (Phenomenex Jupiter C_4 analytical column, 50 mm × 2.0 mm, 0.5 mL min^{-1}, 3 min of 5% B followed by linear gradient of 5% B to 100% B over 15 min): t_R = 18.75 min.

HRMS (MALDI-TOF): *m*/*z* [M]$^+$calcd for $C_{111}H_{111}N_7O_{35}$: 2101.7121; found: 2101.9402.

5(6)-Carboxyfluorescein-{[D-Man(OH)$_4$-(α1→6)-D-Man(OH)$_3$(α1-O)]PEG-(Ala)$_4$-}Lys-Gly-OH (22)

Crude **21** (32.8 mg, 15.60 μmol) was dissolved in MeOH (15 mL) and 1 M NaOMe soln in MeOH was added to adjust the pH to 11.9 (pH meter). When the all starting material had disappeared as judged by analytical RP-HPLC (4 h), the soln was neutralised with a portion of dry ice and the solvent was removed in vacuo. The crude product was purified by RP-HPLC (semi-preparative Waters XTerra Prep. C_{18} column; flow rate: 10 mL min^{-1}; linear gradient: 1% B to 50% B over 35 min) and lyophilised to give **22** (11.6 mg, 54%) as a yellow amorphous solid; HPLC (Phenomenex Jupiter C_4 analytical column, 50 mm × 2.0 mm, 0.5 mL min^{-1}, 3 min of 1% B followed by linear gradient of 1% B to 50% B over 15 min): t_R = 15.68 min.

^{1}H NMR (600 MHz, MeOD): δ = 1.34–1.44 (m, 12 H, 4 × Alaβ-H$_3$), 1.53–2.02 (m, 6 H, Lysγ-H$_2$, Lysδ-H$_2$, Lysβ-H$_2$), 2.48–2.53 (m, 1 H, H2$_A$), 2.60–2.64 (m, 1 H, H2$_B$), 3.15–3.26 (m, 2 H, Lysε-H$_2$), 3.63–3.87 (m, 25 H, H3, H5, H6, H8, H9, H11, H12$_A$, H2′, H2″, H3′, H3″, H4′, H4″, H5′, H5″, H6′, H6″), 3.89–4.07 (m, 3 H, H12$_B$, Glyα-H$_2$), 412–4.30 (m, 4 H, 4 × Alaα-H), 4.56–4.58 (m, 0.4 H, Lysα-H 6-CF), 4.67–4.70 (m, 0.6 H, Lysα-H 5-CF), 4.80 (br s, 1 H, H1″), 4.86 (br s, 1 H, H1′), 6.61–6.72 (m, 4 H, CH$_{Fluoro}$), 6.77–6.78 (m, 2 H,

CH_{Fluoro}), 7.36 (d, J = 8.0 Hz, 0.6 H, CH_{Fluoro} 5-CF), 7.80 (s, 0.4 H, CH_{Fluoro} 6-CF), 8.14 (m, 0.4 H, CH_{Fluoro} 6-CF), 8.23 (d, J = 8.0 Hz, 0.4 H, CH_{Fluoro} 6-CF), 8.30 (m, 0.6 H, CH_{Fluoro} 5-CF), 8.58 (s, 0.6 H, CH_{Fluoro} 5-CF).

^{13}C NMR (150 MHz, MeOD): δ = 17.5, 17.6, 17.7, 18.0 (CH_3, 4 × Alaβ), 24.2, 24.3 (CH_2, Lysγ), 29.8, 29.9 (CH_2, Lysδ), 32.6, 32.7 (CH_2, Lysβ), 37.2 (CH_2, C2), 40.0, 40.1 (CH_2, Lysε), 41.9, 42.0 (CH_2, Glyα), 51.1, 51.4, 51.5, 51.8, 51.9, 52.0, 52.2 (CH, 4 × Alaα), 55.8, 55.9 (CH, Lysα), 63.0 (CH_2, C6″), 67.6 (CH_2, C12), 67.9 (CH_2, CH_2 PEG), 68.3, 68.4 (CH_2, C6′), 68.7, 68.8 (CH, C4′, C4″), 71.5, 71.6, 71.7 (CH_2, 5 × CH_2 PEG), 72.2, 72.3 (CH, C2′, C2″), 72.8, 72.9 (CH, C3′, C3″), 73.2, 74.5 (CH, C5′, C5″), 101.5 (CH, C1′), 102.0 (CH, C1″), 103.8 (CH Fluoro Ph), 111.5 (C_q, Fluoro), 114.4, 125.2, 126.1, 126.8 (CH, Fluoro Ph), 128.9 (C_q, Fluoro), 130.6, 130.8, 131.0, 135.9 (CH, Fluoro Ph), 137.7, 141.9, 154.6, 162.0, 162.1, 162.3 (C_q, Fluoro), 168.6, 168.7, 170.4, 170.5, 172.9, 174.9, 175.0, 175.1, 175.2, 176.0, 176.1, 176.3, 176.4 (C_q, CONH, COOH).

HRMS (MALDI-TOF): m/z $[M + H]^+$ calcd for $C_{62}H_{85}N_7O_{28}$: 1374.5359; found: 1374.1804.

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