Efficient syntheses of core 1, core 2, core 3 and core 4 building blocks for SPS of mucin *O*-glycopeptides based on the *N*-Dts-method

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The structures β-D-Gal-(1→3)-α-D-GalNAc-(1→*O*)-L-Thr, β-D-Gal-(1→3)-[β-D-GlcNAc-(1→6)]-α-D-GalNAc-(1→*O*)-L-Thr, β-D-GlcNAc-(1→3)-α-D-GalNAc-(1→*O*)-L-Thr and β-D-GlcNAc-(1→3)-[β-D-GlcNAc-(1→6)]-α-D-GalNAc-(1→*O*)-L-Thr represent the core 1, core 2, core 3 and core 4 structures, respectively, of mucin-type *O*-glycoproteins. Efficient syntheses of the corresponding building blocks 8, 12, 14 and 17 are described. Stereoselective glycosylation of different N^{α}-Fmoc-Thr(α-D-GalN$_3$)-OPfp derivatives with the 2-dithiasuccinimido glycosyl donor 10, followed by simultaneous *in situ* reduction of the *N*-dithiasuccinoyl (*N*-Dts) and azido functionalities with zinc dust in tetrahydrofuran/acetic acid in the presence of acetic anhydride afforded the protected building blocks 8, 12, 14 and 17. These building blocks can be used directly in solid-phase synthesis (SPS) of core 1, core 2, core 3 and core 4 mucin *O*-glycopeptides. Further modification of the carbohydrate moiety on the solid phase is not required.

Introduction

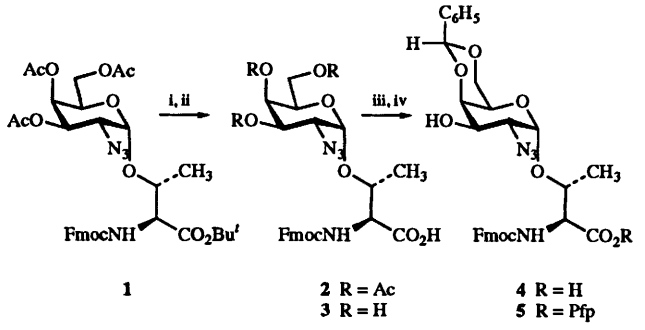
Mucin glycoproteins, the main class of glycoproteins containing *N*-acetylgalactosamine α-glycosidically linked to the hydroxy side chains of serine or threonine, are widely distributed in living organisms and are responsible for the gel-forming properties of mucus.[1,2] Mucins are large molecules (usually over 10^6 Da) containing from 50–80% in weight of carbohydrates. The carbohydrate moieties range in size from a single GalNAc residue to larger oligosaccharides with up to 20 monomers. Mucins are defined by their characteristic O-glycosylated domains. Typically, these domains contain a semi-repetitive protein backbone with a particularly high content of serine and threonine residues interspersed by proline residues.[3] A large variety of glycans are attached by stepwise assembly to the polypeptide backbones, leading to an impressive heterogeneity of mucins. A number of different core structures have been identified.[4] Only a few of the enzymes responsible for the biosynthesis of the different core structures have been identified and characterized.[5] Once the first *N*-acetylgalactosamine moiety has been attached to the polypeptide backbone by the *N*-acetylgalactosamine polypeptide transferase, the different mucin-type core structures are assembled by other specific glycosyltransferases. The heterogeneity and often low abundance of the glycopeptides lead to severe difficulties in the isolation and characterization of homogeneous glycans. Therefore the synthesis of well defined glycopeptide fragments remains a valuable approach to study the biosynthesis, structure and function of glycosylation of proteins and peptides.

The study of the various biosynthetic processes and functions of differential expression of β-1→6 GlcNAc transferases and the corresponding carbohydrate branching pattern in T-cell activation and malignancy requires well defined mucin glycopeptides. These glycoconjugates can be used as substrates and reference compounds for investigations of biosynthetic enzymic reactions and immune-system recognition. In order for a comprehensive biological study to be carried out, a number of synthetic glycopeptides with variation of the core structures, the peptide sequence and the glycosylation site on the peptide are required. Currently the most efficient strategy to build up a number of different glycopeptides is the multiple-column solid-phase synthesis (MCPS) method using glycosylated threonine and serine amino acids as building blocks.[6–9] The solid-phase synthesis of core 1, 2, 3 and 4 glycopeptides involves initially the preparation of the suitably protected building blocks. The fluoren-9-ylmethoxycarbonyl (Fmoc) group serves as a selectively cleavable amino-protecting group and the pentafluorophenyl (Pfp) ester as a carboxylate-protecting group during glycosylation and activating group during peptide synthesis. For the introduction of amino glycans the azido group has been employed for temporary amino-group protection and to obtain α-glycosylation, while acetyl or benzoyl groups are used as easily removable protecting groups for the carbohydrate hydroxy groups. Subsequent to solid-phase synthesis reduction of the azide group is performed on the resin with thioacetic acid.[10–12] This method is cumbersome and frequently results in formation of corresponding thioacetamido derivatives, leading to difficulties in the parallel reduction of several azido groups.[13] In order to circumvent these difficulties we reported the use of the dithiasuccinoyl (Dts) group for amino sugar protection in the synthesis of cytosol *O*-GlcNAc glycopeptides.[14–18] In the present work we describe the convenient use of the properties of the *N*-dithiasuccinoyl (*N*-Dts)-group and the azido-reduction approach[14] in the synthesis of the more complex building blocks **8**, **12**, **14** and **17** for use in the synthesis of core 1, core 2, core 3 and core 4 glycopeptides.

Results and discussion

Previously described N^{α}-Fmoc-Thr-(α-D-GalN$_3$)-OBu^t-galactosyl amino acid **1**[19] was transformed into the 4,6-*O*-benzylidene derivative N^{α}-Fmoc-Thr(α-D-GalN$_3$)-OPfp **5** by a four-step synthesis sequence: the *tert*-butyl ester was removed from ester

1 by formic acid hydrolysis to yield acid **2** in 94% yield, and then the acetates were removed by treatment of compound **2** with hydrazine hydrate. The introduction of the 4,6-benzylidene group was performed by reaction of the triol **3** with benzylidene dimethyl acetal in nitromethane under toluene-*p*-sulfonic acid *catalysis to yield* compound **4** in 73% yield. The final introduction of the pentafluorophenyl ester was achieved by treatment of acid **4** with pentafluorophenol in ethyl acetate under dicyclohexylcarbodiimide (DCC) activation (Scheme 1).

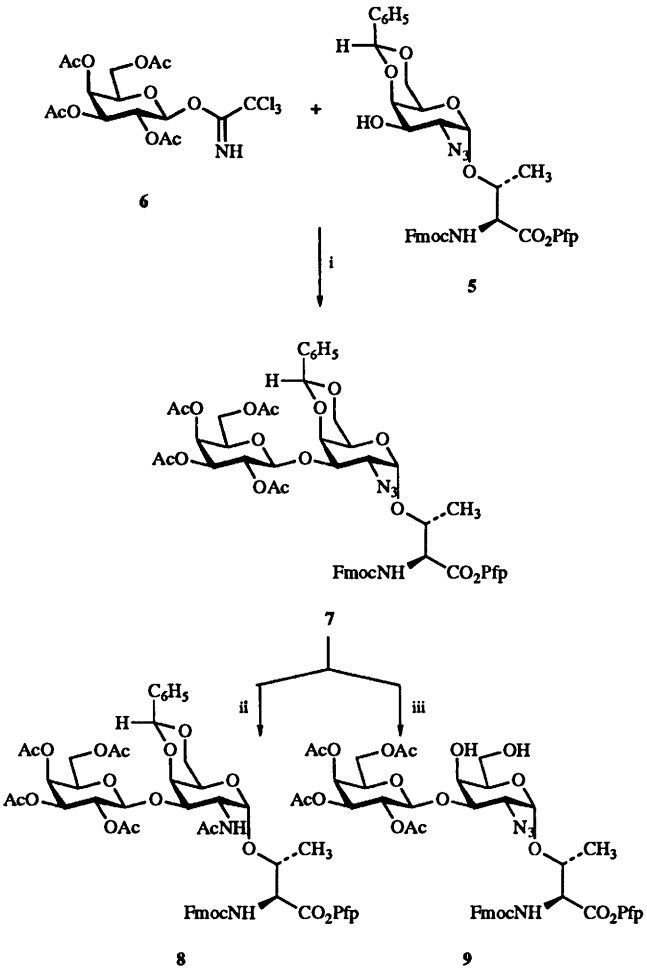


1 **2** R = Ac, **3** R = H, **4** R = H, **5** R = Pfp

Scheme 1 *Reactions and conditions:* i, HCO_2H; ii, $N_2H_4{\cdot}H_2O$, MeOH; iii, $PhCH(OMe)_2$, *p*-TsOH, $MeNO_2$; iv, PfpOH, DCC, EtOAc

Core **1** building block **8** and the precursor to core 2 building block **12** were obtained by stereoselective glycosylation of the 4,6-*O*-benzylidene-N^{α}-Fmoc-Thr(α-D-GalN_3)-OPfp derivative **5** with two equivalents of the peracetylated galactose imidate **6** at 0 °C to afford the N^{α}-Fmoc-Thr[β-D-Gal-(1→3)-α-D-GalN_3]-OPfp derivative **7** in 89% yield. The ^{1}H NMR spectrum of product **7** showed the anomeric 1′-H resonance at δ 4.73 with a coupling constant $J_{1',2'}$ = 8.1 Hz. Compound **7** represents a very versatile intermediate for the synthesis of the core 1 and core 2 building blocks **8** and **12**. Either it can be directly converted into the 2-acetamido compound **8**, which is a building block for the synthesis of core 1 glycopeptides, or it can be further modified to the core 2 building block **12**. Thus compound **7** was converted into building block **8** by reduction of the azido group with simultaneous N-acetylation using zinc (activated with 2% aq. $CuSO_4$) in tetrahydrofuran (THF), acetic anhydride and acetic acid (3:2:1) in 76% yield (Scheme 2). Transformation of the azido group into the N-acetamido functionality resulted in a shift of the dd-signal of 2-H from δ 3.85 into a ddd-signal at δ 4.52.

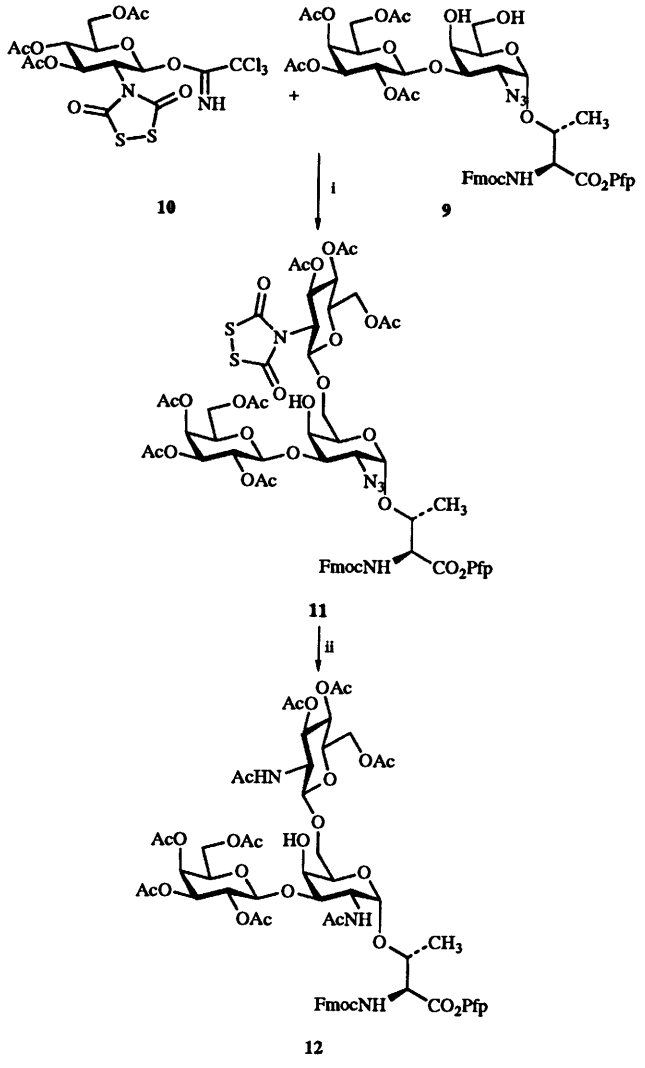
The conversion of compound **7** into the core 2 building block **12** began with the acid-catalysed hydrolysis of the benzylidene group in **7** into diol **9** with warm aq. acetic acid in 75% yield. The diol **9** was used without further purification in the subsequent glycosylation. Condensation of 1.1 equivalent of imidate **10**[17] with diol **9**, using trimethylsilyl triflate (TMSOTf) as a catalyst in dichloromethane at −40 °C afforded completely regioselectively the β-(1→6)-linked trisaccharide **11** ($J_{1'',2''}$ 8.5 Hz at δ 5.46) in 67% yield after purification by chromatography on pre-dried silica gel. The trisaccharide–threonine derivative **11** could be successfully converted into the core 2 building block **12** by simultaneously reducing the *N*-Dts and the azido group with *in situ* N-acetylation by dissolution of compound **11** in a mixture of THF acetic anhydride and acetic acid (3:2:1) and addition of activated zinc (Scheme 3). The reduction was complete within 4 h. Crude **12** was purified by chromatography on pre-dried silica gel to afford the core 2 building block **12** in 65% yield. The dd resonance of the 2-H protons was deshielded from δ 3.54 (**11**) to δ 4.42 (**12**) and now presented as a ddd-signal (azido into N-acetamido), and the dd-resonance for the 2″-H proton at δ 4.37 (**11**) shifted to a ddd-resonance at δ 3.93 (N-Dts into NHAc).



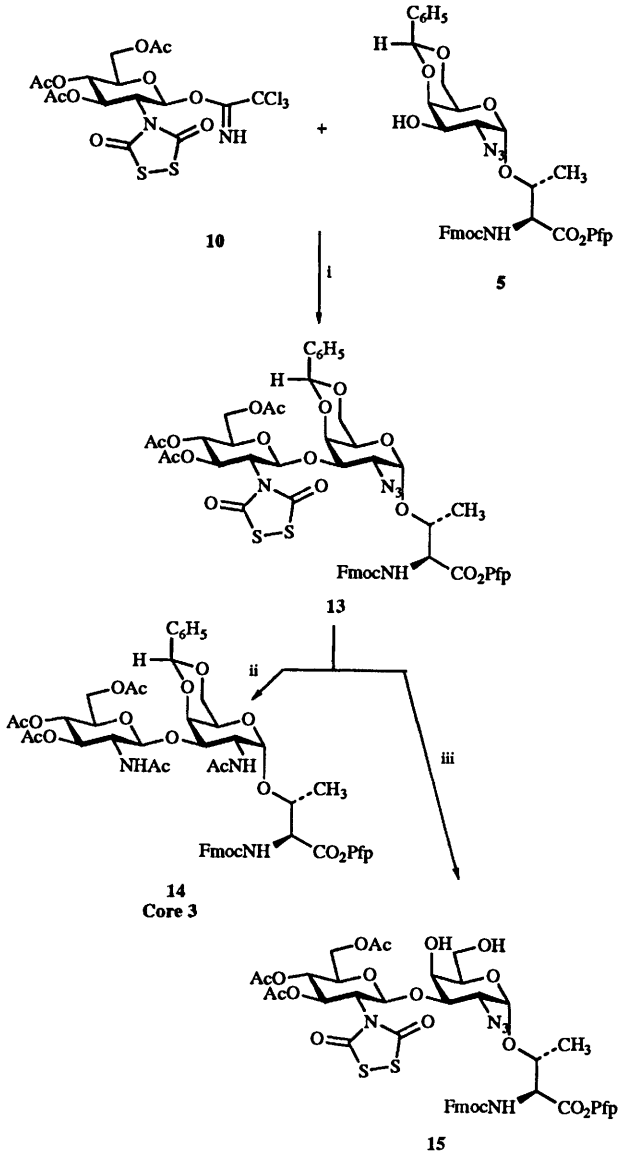
Scheme 2 *Reactions and conditions:* i, TMSOTf, molecular sieves, CH_2Cl_2 (0 °C); ii, Zn in THF–Ac_2O–HOAc (3:2:1); iii, 80% HOAc (70 °C)

Similarly, the core 3 building block **14** could be obtained by glycosylation of the 3-position of azide **5** with mole equivalents of imidate **10** at 0 °C in dichloromethane in the presence of TMSOTf to afford exclusively the β-(1→3)-linked disaccharide derivative **13** in 98% yield. The ^{1}H NMR spectrum of product **13** shows the characteristic anomeric signal for 1′-H at δ 5.61 with a coupling constant of $J_{1',2'}$ = 8.1 Hz indicating the desired β-linkage. The disaccharide **13** was then directly converted into the core 3 building block **14** by simultaneous reduction and N-acetylation of the azido and Dts groups with activated zinc in THF, acetic anhydride and acetic acid (3:2:1) at room temperature in an overall yield of 68% (Scheme 4). The ^{1}H NMR spectrum of compound **14** showed the two characteristic ddd-resonances of 2-H and 2′-H at δ 4.50 and 3.53, respectively. Noteworthy is the opposite direction of the shift changes for the dd- to the ddd-resonances of 2-H and 2′-H in the ^{1}H NMR spectrum of compound **14** due to formation of the acetamido group. The 2-H signal shifted from δ 3.84 (azido derivative) to δ 4.50 (acetamido derivative) whereas the 2′-H dd-resonance shifted from δ 4.45 (*N*-Dts derivative) to δ 3.53 (acetamido derivative).

Hydrolysis of the 4,6-*O*-benzylidene group of disaccharide **13** with an 80% aq. acetic acid solution at 70 °C afforded the 4,6 diol compound **15** in 98% yield. The primary alcoholic position of diol **15** could selectively be glycosylated with 1.1 equivalent amounts of imidate **10** in dichloromethane under TMSOTf catalysis at −30 °C to give the trisaccharide **16** in 65% overall yield after silica gel chromatography ($J_{1'',2''}$ 8.3 Hz at δ 5.33). Finally the core 4 trisaccharide precursor **16** containing two *N*-Dts groups, an azido group, an Fmoc group and an



Scheme 3 *Reactions and conditions:* i, TMSOTf, molecular sieves, CH_2Cl_2 (−40 °C); ii, Zn in THF–Ac_2O–HOAc (3:2:1)



Scheme 4 *Reactions and conditions:* i, TMSOTf, molecular sieves, CH_2Cl_2 (0 °C); ii, Zn in THF–Ac_2O–HOAc (3:2:1); iii, 80% HOAc (70 °C)

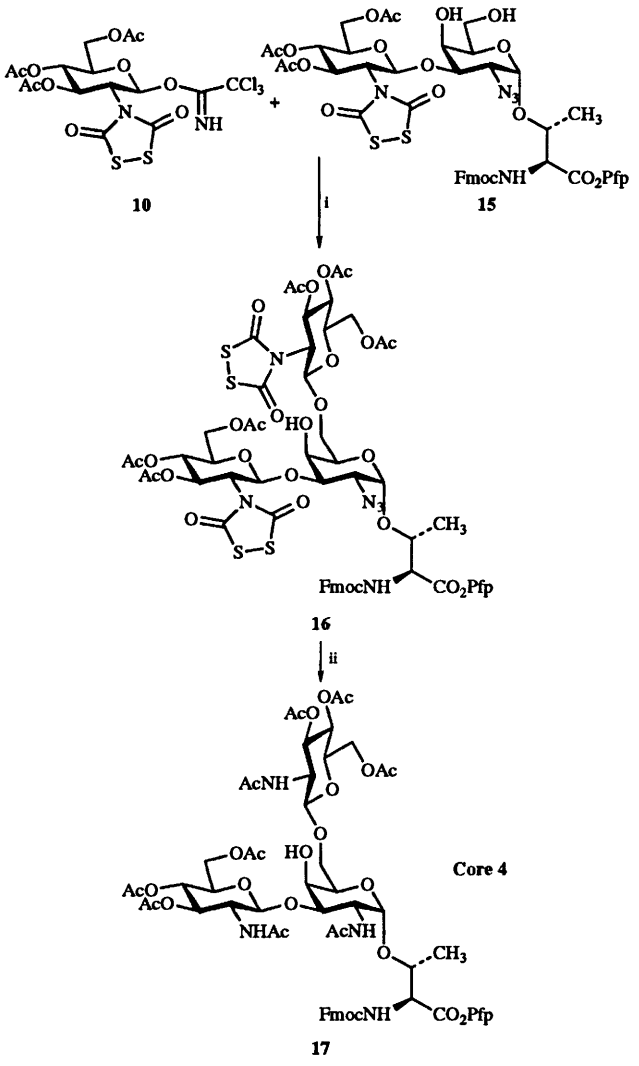
active Pfp ester was converted by simultaneous reduction of the two *N*-Dts groups and the azido group, by utilization of activated-zinc reduction in THF, acetic anhydride and acetic acid (3:2:1). The core 4 building block **17** was isolated in 61% overall yield after purification on pre-dried silica gel (Scheme 5). The characteristic ^1H shifts were observed for 2-H from δ 3.58 (dd) (**16**) to δ 4.27 (ddd) (**17**), for 2′-H from δ 4.46 (dd) to δ 3.44 (ddd) and for 2″-H from δ 4.35 (dd) to δ 3.92 (ddd). Characteristic shifts could also be observed in the ^{13}C NMR spectra. Owing to the conversion of the azido into the acetamido functionality, the resonance for C-2 shifted from about δ_C 59.03 (**16**) to δ_C 48.05 (**17**). Conversion of the *N*-Dts into the acetamido group resulted in a shift of C-2′ from δ_C 60.84 to δ_C 55.31 and of C-2″ from δ_C 61.28 to δ_C 53.89.

In summary, 'one step' reduction of *N*-Dts and azide with *in situ* N-acetylation of azides **7**, **11**, **13** and **16** can be readily accomplished *via* reduction with activated zinc in THF acetic anhydride and acetic acid (3:2:1) to yield the building blocks **8**, **12**, **14** and **17**, which can be used directly in MCPS of core 1, core 2, core 3 and core 4 mucin *O*-glycopeptides. The simultaneous reduction and N-acetylation of the *N*-Dts and azide functionalities into acetamido groups circumvents the need for further reduction steps (thioacetic acid, dithiothreitol) during the solid-phase assembly of glycopeptides by MCPS. It can be concluded that the reactivity of the Pfp ester is sufficiently low for it to act as a protecting group which allows the intermediate formation of amino groups in the presence of acetic anhydride. Furthermore, the use of acetamido groups containing building blocks in SPS simplifies the final HPLC purification of the mucin *O*-glycopeptides by avoiding the potential formation of thioacetamido by-products during the thioacetic acid-promoted reduction of the azido group on the solid phase. Further studies of the application of the building blocks **8**, **12**, **14** and **17** in mucin *O*-glycopeptide synthesis are currently underway.

Experimental

General procedures

TLC was performed on Merck Silica Gel 60 F_{254} alumina sheets with detection by charring with sulfuric acid, and by UV light when applicable. Vacuum liquid chromatography (VLC) was performed on dried Merck Silica Gel 60 H, which was kept for several days at 125 °C before use. **Proper drying of silica gel is important because of the instability of Pfp esters on wet silica gel.** All solvents were purchased from Labscan Ltd. (Dublin, Ireland). Dichloromethane was distilled from P_2O_5 and was stored over 3 Å molecular sieves under argon in sealed vessels. Light petroleum was the 60–80 °C fraction. Concentrations were performed under reduced pressure at temperatures ≤40 °C. Round-bottomed flasks for glycosylation reactions were either flame dried or stored at 120 °C for 24 h prior to use.



Scheme 5 *Reactions and conditions:* i, TMSOTf, molecular sieves, CH_2Cl_2 (−30 °C); ii, Zn in THF–Ac_2O–HOAc (3:2:1)

Optical rotations were recorded on a Perkin-Elmer 241 polarimeter and $[\alpha]_D$-values are given in units of 10^{-1} deg cm^2 g^{-1}. ^{1}H NMR spectroscopy was performed on a Bruker DRX 250 or Bruker AMX 400 operating at 250 MHz or 400 MHz, respectively. ^{13}C NMR spectra were recorded at 62.9 MHz. Unless otherwise indicated all the NMR experiments were performed at 300 K in $CDCl_3$. Chemical shifts are given in ppm and referenced to internal $SiMe_4$ (0 ppm). Coupling constants are given in Hz (±0.3 Hz). For all compounds the assignment of the ^{1}H NMR spectra was based on 2D proton–proton shift-correlation spectra. The assignment of ^{13}C NMR spectra was based on carbon–proton shift-correlation spectra. The assigned ^{1}H NMR data are given in Tables 1, 3 and 5 and the corresponding ^{13}C NMR data in Tables 2, 4 and 6, respectively. MALDI-TOF MS was performed on a Finnigan MAT 2000 instrument with a matrix of α-cyano-4-hydroxy cinnamic acid. FAB mass spectra were recorded on a double-focused VG-Analytical 70-250 S mass spectrometer with a matrix of *m*-nitrobenzyl alcohol.

N^{α}-(fluoren-9-ylmethoxycarbonyl)-*O*-(3,4,6-tri-*O*-acetyl-2-azido-2-deoxy-α-D-galactopyranosyl)-L-threonine 2

Compound **1** (2 g, 2.81 mmol) was dissolved in formic acid (80 cm^3) and the solution was stirred for 12 h at room temperature. The mixture was then concentrated and several times co-concentrated with toluene. Product **2** was dried in oil-pump vacuum for 24 h to afford acid **2** (1.73 g, 94%), which was used without further purification in the next step; $[\alpha]_D^{22}$ +30.5 (*c* 0.50, $CHCl_3$) [Found: $(M + H)^+$ FAB-MS, 655.0. $C_{31}H_{34}N_4O_{12}$ requires M, 654.63]. ^{1}H and ^{13}C NMR data are presented in Tables 1 and 2.

Table 1 ^{1}H NMR chemical-shift assignments and coupling constants (Hz, in parentheses) for compounds **2**–**5** measured at 400.13 MHz on solutions in CD_3OD (compound **5** in $CDCl_3$) at 300 K

	Chemical shifts (δ)			
	2	**3**	**4**	**5**
1-H	5.15 (3.6)	5.03 (3.6)	5.13 (3.5)	5.18 (4.3)
2-H	3.80 (11.2)	3.49 (10.0)	3.62 (10.6)	3.69 (10.6)
3-H	5.32 (2.5)	3.97 (3.2)	4.13 (3.4)	4.15 (3.0)
4-H	5.44	3.92	4.29	4.10 (0.8)
5-H	4.25 (5.6/7.6)	3.92 (6.3/7.2)	4.40 (7.0/7.0)	4.27 (7.2/7.2)
6-H[a]	4.12 (10.7)	3.73 (11.5)	4.28 (10.5)	4.53 (10.3)
6-H[b]	4.08	3.69	4.26	4.41
Thr				
α-H	4.40 (3.0)	4.24 (2.8)	4.29 (2.9)	4.72 (2.4)
β-H	4.42 (6.6)	4.39 (6.6)	4.43 (5.9)	4.56 (6.4)
γ-H	1.32	1.33	1.29	1.41
NH				5.99 (9.0)
Fmoc CH	4.32	4.25	4.28	3.83
Fmoc CH_2	4.40	4.36	4.15	4.31
Aryl	7.82–7.28	7.82–7.28	7.81–7.25	7.80–7.28
OAc	2.13, 2.03, 2.00			
Bz CH			5.63	5.58
OH				2.46 (10.6)

Table 2 ^{13}C NMR chemical-shift assignments of compounds **2**–**5** measured at 100.57 MHz on solutions in CD_3OD (compounds **2** and **5** in $CDCl_3$) at 300 K

	Chemicals shifts (δ_C)			
	2	**3**	**4**	**5**
C-1	98.88	99.91	99.45	99.01
C-2	58.00	59.55	62.19	60.89
C-3	68.63	67.34	67.44	67.32
C-4	67.46	68.31	73.10	74.82
C-5	63.14	63.84	62.19	63.04
C-6	61.81	61.63	65.44	68.64
Thr				
C-α	58.01	61.05	59.19	58.17
C-β	76.50	75.96	74.83	75.19
C-γ	18.18	18.36	18.39	18.60
Fmoc CH	47.09	47.78	46.84	46.70
Fmoc CH_2	67.61	76.30	67.45	67.11
Fmoc arom. C + arom. C	120.01, 125.21, 127.19, 127.76	119.90, 125.27, 127.16, 127.79	118.13, 123.48, 125.42, 126.01, 127.13, 128.20	119.58, 124.65, 125.76, 127.35, 127.94, 129.02
OAc	20.63, 20.67 (2×)			

O-(2-Azido-2-deoxy-α-D-galactopyranosyl)-N^{α}-(fluoren-9-ylmethoxycarbonyl-L-threonine 3

Compound **2** (1.5 g, 2.29 mmol) was dissolved in methanol (36 cm^3) and hydrazine hydrate (2.23 cm^3, 44.5 mmol) was added. After the reaction mixture had been kept for 1.5 h at room temperature [TLC: $CHCl_3$–MeOH–water (10:10:1)] second

Table 3 ^{1}H NMR chemical-shift assignments and coupling constants (Hz, in parentheses) for compounds **7**–**12** measured at 250.13 MHz on solutions in $CDCl_3$ at 300 K

	Chemical shifts (δ)				
	7	**8**	**9**	**11**	**12**
1-H	5.14 (3.2)	5.10 (3.3)	5.08 (3.5)	4.95 (3.2)	4.85 (3.2)
2-H	3.85 (10.4)	4.52 (10.2)	3.62 (10.7)	3.54 (10.5)	4.42 (10.1)
3-H	3.97	3.94	3.91	3.89	3.62
4-H	4.31	4.24	4.16	4.12 (0.5/2.1)	4.07
5-H	3.63	3.62	3.81	3.87	3.83
6-H^a	4.19	4.18	3.83	4.13	3.98
6-H^b	3.98	3.98	3.71	3.98	3.72
NH		5.54 (8.8)			5.59 (9.5)
1′-H	4.73 (8.1)	4.66 (7.9)	4.69 (8.1)	4.68 (7.9)	4.46 (8.0)
2′-H	5.23 (9.1)	5.12 (10.3)	5.23 (9.1)	5.24 (9.3)	5.09
3′-H	4.97 (3.1)	4.89 (3.3)	4.95 (3.0)	4.95 (3.2)	4.86
4′-H	5.33 (0.5/3.1)	5.31 (0.5)	5.35 (0.5/3.0)	5.33 (0.5/3.1)	5.29
5′-H	3.88	3.82	3.87	3.89	3.83
6′-H^a	4.21	4.18	4.23	4.24	4.07
6′-H^b	4.07	4.09	4.06	4.04	4.01
N′H					
1″-H				5.46 (8.5)	4.51 (8.2)
2″-H				4.37 (8.8)	3.93
3″-H				5.65 (9.3)	5.08
4″-H				5.09 (9.8)	4.85
5″-H				3.70	3.63
6″-H^a				4.27	4.21
6″-H^b				4.07	4.09
N″H					5.67 (8.9)
Thr					
α-H	4.67 (2.1/9.1)	4.65 (2.0/9.0)	4.68 (2.1/9.2)	4.67 (2.1/9.2)	4.56 (2.0/9.3)
β-H	4.51 (6.3)	4.38 (6.2)	4.52 (6.3)	4.42 (6.1)	4.29 (6.2)
γ-H	1.34	1.31	1.33	1.33	1.33
NH	5.88 (9.1)	5.81 (9.0)	5.78 (9.2)	5.71 (9.2)	6.05 (9.3)
Fmoc CH	4.19	4.20	4.18	4.19	4.19
Fmoc CH_2	4.50, 4.32	4.52, 4.48	4.51, 4.33	4.51, 4.35	4.49, 4.42
Aryl	7.72–7.23	7.72–7.23	7.72–7.23	7.72–7.23	7.72–7.23
OAc	2.08, 1.95 (2 ×), 1.91	2.07, 1.94 (2 ×), 1.90	2.11, 1.99, 1.98, 1.93	2.10, 2.05, 2.04, 1.99, 1.98, 1.96, 1.93	2.08, 2.02 (2 ×), 1.95 (3 ×), 1.91
NHAc		1.71			1.83, 1.70
Bz CH	5.49	5.48			
Bz arom.	7.55–7.28	7.55–7.28			
OH			2.87, 2.71	2.57	2.71

portion of hydrazine hydrate (1.5 cm^3, 29.7 mmol) was injected and the mixture was kept for another 6 h. After neutralization with acetic acid, the solution was filtered through Celite and concentrated. Purification by chromatography on silica gel [$CHCl_3$–MeOH (5:1)] afforded *compound* **3** (783 mg, 65%), $[\alpha]_D^{22}$ +70.6 (*c* 0.50, MeOH) [Found: $(M + H)^+$ FAB-MS, 529.0. $C_{25}H_{28}N_4O_9$ requires M, 528.52]. ^{1}H and ^{13}C NMR data are presented in Tables 1 and 2.

O-(2-Azido-4,6-*O*-benzylidene-2-deoxy-α-D-galactopyranosyl)-N^{α}-(fluoren-9-ylmethoxycarbonyl)-L-threonine 4

To a solution of compound **3** (783 mg, 1.48 mmol) and benzaldehyde dimethyl acetal (7.1 cm^3, 47.3 mmol) in nitromethane (100 cm^3) was added toluene-*p*-sulfonic acid (90 mg). After being stirred at room temperature for 3 h the mixture was neutralized by addition of triethylamine, concentrated and purified. Chromatography on silica gel [$CHCl_3$–MeOH (20:1)] gave *compound* **4** (669 mg, 73%) $[\alpha]_D^{22}$ +88.8 (*c* 0.50, $CHCl_3$) [Found: $(M + H)^+$ FAB-MS, 618.8. $C_{32}H_{32}N_4O_9$ requires M, 616.63]. ^{1}H and ^{13}C NMR data are presented in Tables 1 and 2.

O-(2-Azido-4,6-*O*-benzylidene-2-deoxy)-α-D-galactopyranosyl)-N^{α}-(fluoren-9-ylmethoxycarbonyl)-L-threoninepentafluorophenyl ester 5

To a solution of acid **4** (540 mg, 0.877 mmol) in ethyl acetate (3 cm^3) was added pentafluorophenol (181 mg, 0.988 mmol). The mixture was cooled to 0 °C, DCC (202 mg, 0.988 mmol) was

Table 4 ^{13}C NMR shift assignments of compounds **7**–**12** measured at 62.9 MHz on solutions in $CDCl_3$ at 300 K

	Chemical shifts (δ_C)				
	7	**8**	**9**	**11**	**12**
C-1	99.84	100.48	99.34	99.90	99.68
C-2	58.48	48.85	59.35	59.39	48.21
C-3	75.93	74.01	76.56	76.30	77.78
C-4	76.30	75.83	68.42	69.87	69.10
C-5	64.11	64.19	70.24	69.82	71.06
C-6	69.41	69.50	62.98	63.41	70.28
C-1′	102.81	101.34	102.34	102.43	102.15
C-2′	69.09	69.26	69.49	68.94	69.15
C-3′	71.46	71.22	71.07	70.53	71.00
C-4′	67.36	67.40	67.37	67.27	67.21
C-5′	71.75	71.45	71.94	72.13	71.06
C-6′	61.74	61.81	61.92	62.31	61.76
C-1″				98.26	101.88
C-2″				61.32	54.63
C-3″				69.67	72.85
C-4″				68.82	68.70
C-5″				72.53	72.38
C-6″				62.17	62.41
Thr					
C-α	58.98	58.94	58.88	58.96	58.65
C-β	75.96	76.32	75.97	75.97	75.39
C-γ	19.43	18.90	19.29	19.62	18.91
Fmoc CH	47.56	47.61	47.57	47.60	47.53
Fmoc CH_2	67.88	67.40	67.85	67.97	67.82
Fmoc arom. C	120.47, 125.31, 127.52, 128.24	120.49, 125.42, 127.54, 128.29	120.46, 125.41, 127.51, 128.56	120.52, 125.52, 127.58, 128.30	120.35, 125.26, 127.62, 128.20
OAc	21.09 (2 ×), 20.95 (2 ×)	21.07 (2 ×), 20.94 (2 ×)	21.03 (2 ×), 20.93 (2 ×)	21.18 (2 ×), 21.08 (2 ×), 21.05 (2 ×), 20.85	21.03, 20.95 (2 ×), 20.79(3 ×) 20.76
NHAc		23.34			23.48, 23.17
Benzylidene C	101.11	101.18			
Arom. C	125.43, 129.39, 129.46	125.23, 128.59, 129.34			

added, and the solution was stirred overnight at room temperature. After addition of a second portion of DCC (67 mg, 0.33 mmol) the mixture was stirred for another 6 h [TLC: toluene–MeOH (8:1)]. The solution was filtered through Celite, rinsed several times with ethyl acetate, concentrated and purified. Chromatography on pre-dried silica gel [toluene–acetone (50:1→20:1)] and crystallization from toluene–light petroleum (10:1) afforded *compound* **5** (509 mg, 75%), $[\alpha]_D^{22}$ +30.6 (*c* 0.50, $CHCl_3$) [Found: $(M + H)^+$ FAB–MS, 783.5. $C_{38}H_{31}F_5N_4O_9$ requires M, 782.68]. ^1H and ^{13}C NMR data are presented in Tables 1 and 2.

O-{2-Azido-4,6-O-benzylidene-2-deoxy-O-[2,3,4,6-tetra-O-acetyl-β-D-galactopyranosyl-(1→3)]-α-D-galactopyranosyl}-N^{α}-(fluoren-9-ylmethoxycarbonyl)-L-threonine pentafluorophenyl ester 7

Imidate **6** (630 mg, 1.28 mmol) and compound **5** (500 mg, 640 μmol) were dissolved in dry dichloromethane (20 cm^3), molecular sieves (3 Å) were added, and the solution was then stirred for 30 min at 0 °C under argon. A solution of TMSOTf in dichloromethane [500 mm^3; TMSOTf–dichloromethane-(1:50)] was injected. After 1 h the mixture was warmed to room temperature, dry dichloromethane (50 cm^3) was added and the mixture was filtered through Celite and concentrated. VLC [light petroleum–ethyl acetate (2:1→3:2)] yielded the title compound **7** (634 mg, 89%), $[\alpha]_D^{25}$ +39.9 (*c* 0.95, $CHCl_3$) [Found: $(M + Na)^+$ MALDI-MS, 1136.05. $C_{52}H_{49}F_5N_4O_{18}$ requires M, 1112.99]. ^1H and ^{13}C NMR data are presented in Tables 3 and 4.

O-{2-Acetamido-4,6-O-benzylidene-2-deoxy-O-[2,3,4,6-tetra-O-acetyl-β-D-galactopyranosyl-(1→3)]-α-D-galactopyranosyl}-N^{α}-(fluoren-9-ylmethoxycarbonyl)-L-threonine pentafluorophenyl ester 8

Compound **7** (200 mg, 180 μmol) was dissolved in THF–acetic anhydride–acetic acid (10 cm^3; 3:2:1) and zinc (150 mg, activated with 2% aq. $CuSO_4$ was added. The mixture was then stirred at room temperature for 2 h. After completion of the reaction, the mixture was diluted with THF (freshly distilled), filtered through Celite, concentrated and purified by VLC [ethyl acetate–light petroleum (2:1 ⟶ 3:1)] on pre-dried silica gel 60 to give title compound **8** (154 mg, 76%), $[\alpha]_D^{25}$ +40.3 (*c* 1.15, $CHCl_3$) [Found: $(M + Na)^+$ MALDI-MS, 1152.23. $C_{54}H_{53}F_5N_2O_{19}$ requires M, 1129.02]. ^1H and ^{13}C NMR data are presented in Tables 3 and 4.

O-{2-Azido-2-deoxy-O-[2,3,4,6-tetra-O-acetyl-β-D-galactopyranosyl-(1→3)]-α-D-galactopyranosyl}-N^{α}-(fluoren-9-ylmethoxycarbonyl)-L-threoninepentafluorophenyl ester 9

Compound **7** (400 mg, 360 μmol) was dissolved in 80% aq. acetic acid solution. The mixture was stirred at 70 °C for 7 h and then the solution was concentrated to dryness. Toluene was added, and the mixture was concentrated three times to yield *compound* **9**, which was directly used, without further purification, as an aglycone in the following glycosylation; $[\alpha]_D^{25}$ +23.4 (*c* 1.2, $CHCl_3$) [Found: $(M + H)^+$ MALDI-MS, 1026.05. $C_{45}H_{45}F_5N_4O_{18}$ requires M, 1024.87]. ^1H and ^{13}C NMR data are presented in Tables 3 and 4.

O-{2-Azido-2-deoxy-O-[2,3,4,6-tetra-O-acetyl-β-D-galactopyranosyl)-(1→3)]-O-[3,4,6-tri-O-acetyl-2-deoxy-2-dithiasuccinimido-β-D-glucopyranosyl-(1→6)]-α-D-galactopyranosyl}-N^{α}-(fluoren-9-ylmethoxycarbonyl)-L-threoninepentafluorophenyl ester 11

Diol **9** (200 mg, 195 μmol), imidate **10** (121 mg, 214 μmol) and molecular sieves (3 Å) were placed in a pre-dried flask. After evacuation with an oil-pump the flask was filled with argon and dry dichloromethane was injected (10 cm^3). The mixture was cooled to −40 °C, TMSOTf [100 mm^3; TMSOTf–dichloromethane (1:50)] was added, and the mixture was stirred for 30 min at −40 °C and then allowed to warm to room temperature, before being filtered, concentrated and purified by VLC on pre-

Table 5 ^{1}H NMR chemical-shift assignments and coupling constants (Hz, in parentheses) for compounds **13–17** measured at 250.13 MHz in $CDCl_3$ at 300 K

	Chemical shifts (δ)				
	13	**14**	**15**	**16**	**17**[a]
1-H	5.11 (3.4)	5.06 (3.4)	5.02 (3.6)	5.01 (3.5)	4.80 (3.4)
2-H	3.84 (10.6)	4.50 (9.6)	3.61 (10.5)	3.58 (10.6)	4.27 (10.6)
3-H	3.95	3.88 (0.5)	3.89	3.90	3.71
4-H	4.35	4.27	4.15	4.01	3.94
5-H	3.65	3.61	3.89	4.20	3.79
6-H^a	4.21	4.15 (11.9)	3.78	4.06	3.95
6-H^b	4.02	3.99	3.73	3.74	3.92
N-H		5.90 (8.8)			
1′-H	5.61 (8.1)	4.97 (8.4)	5.57 (8.2)	5.60 (8.3)	4.93 (8.2)
2′-H	4.45 (9.0)	3.53 (9.9)	4.42 (10.5)	4.46 (10.3)	3.44 (10.3)
3′-H	5.66 (9.6)	5.26 (9.6)	5.67 (9.1)	5.70 (9.2)	5.30 (9.4)
4′-H	5.12 (9.0)	4.99 (9.4)	5.06 (9.8)	5.11 (9.9)	4.88 (9.6)
5′-H	3.71 (2.3/3.8)	3.66 (1.9/3.9)	3.77 (5.1/6.8)	3.75	3.65
6′H^a	4.33 (10.8)	4.35 (12.3)	4.23 (12.3)	4.19	4.38
6′-H^b	4.07	4.03	4.08	4.10	4.02
NH		5.82 (8.0)			
1″-H				5.33 (8.3)	4.50
2″-H				4.35 (10.5)	3.92
3″-H				5.69 (9.2)	5.06
4″-H				5.11	4.98
5″-H				3.83	3.92
6″-H^a				4.27	4.28
6″-H^b				4.19	4.17
Thr					
α-H	4.66 (1.9/9.1)	4.66 (1.3/8.6)	4.68 (1.7/9.2)	4.71 (0.8/9.2)	4.59
β-H	4.50 (6.2)	4.39 (6.2)	4.51 (6.1)	4.46 (6.2)	4.30 (6.2)
γ-H	1.34	1.33	1.34	1.34	1.31
NH	5.94 (9.03)	6.20 (9.16)	5.82 (9.09)	5.78 (9.12)	
Fmoc CH	4.25	4.19	4.20	4.25	4.19
Fmoc CH_2	4.51 (10.5), 4.28	4.51, 4.48	4.47, 4.27	4.51, 4.33	4.45, 4.38
Aryl	7.75–7.23	7.75–7.23	7.72–7.24	7.72–7.24	7.78–7.19
OAc	1.98, 1.96, 1.92	2.03, 1.95 (2×)	2.04, 1.99, 1.94	2.07, 2.02, 2.00, 1.97, 1.96	2.02, 1.99, 1.95 (3×), 1.94
NHAc		1.80, 1.72			1.84, 1.82, 1.73
Bz CH	5.49	5.45			
Bz arom.	7.55–7.28	7.55–7.28			
OH			2.68 (4- and 6-H)	2.54 (4-OH)	

[a] In $CDCl_3$–CD_3OD 5:1.

dried silica gel [ethyl acetate–light petroleum (3:2→1:1)] to yield the trisaccharide **11** (187 mg, 67%), $[\alpha]_D^{25}$ +8.7 (*c* 0.71, $CHCl_3$) [Found: $(M + Na)^+$ MALDI-MS, 1453.26. $C_{59}H_{60}F_5N_5O_{27}S_2$ requires M, 1430.28]. ^{1}H and ^{13}C NMR data are presented in Tables 3 and 4.

***O*-{2-Acetamido-*O*-[2-acetamido-3,4,6-tri-*O*-acetyl-2-deoxy-β-D-glucopyranosyl-(1→6)]-2-deoxy-*O*-[2,3,4,6-tetra-*O*-acetyl-β-D-galactopyranosyl-(1→3)]-α-D-galactopyranosyl}*N*$^\alpha$-(fluoren-9-ylmethoxycarbonyl)-L-threonine pentafluorophenyl ester 12**

Trisaccharide **11** (180 mg, 125 μmol) was dissolved in THF–acetic anhydride–acetic acid [10 cm^3 (3.2:1)], zinc (75 mg, activated with 2% aq. $CuSO_4$ was added and the mixture was stirred for 4 h at room temperature before being diluted with freshly distilled THF (50 cm^3), filtered through Celite, and concentrated. VLC [ethyl acetate–light petroleum (3:1)] yielded the *title trisaccharide* **12** (112 mg, 65%), $[\alpha]_D^{25}$ +8.1 (*c* 1.0, $CHCl_3$) [Found: $(M + H)^+$ MALDI-MS, 1371.27. $C_{61}H_{68}F_5N_3O_{27}$ requires M, 1370.22). ^{1}H and ^{13}C NMR data are presented in Tables 3 and 4.

***O*-{2-Azido-4,6-*O*-benzylidene-2-deoxy-*O*-[3,4,6-tri-*O*-acetyl-2-deoxy-2-dithiasuccinimido-β-D-glucopyranosyl-(1→3)]-α-D-galactopyranosyl}-*N*$^\alpha$-(fluoren-9-ylmethoxycarbonyl)-L-threonine pentafluorophenyl ester 13**

Imidate **10** (184 mg, 325 μmol), compound **5** (125 mg, 160 μmol)

Table 6 ^{13}C NMR shift assignments of compounds **13–17** measured at 62.9 MHz on solutions in $CDCl_3$ at 300 K

	Chemical shifts (δ_C)				
	13	**14**	**15**	**16**	**17**
C-1	99.11	100.18	99.20	99.48	99.85
C-2	59.19	48.23	59.13	59.03	48.05
C-3	76.54	72.52	79.14	78.92	77.11
C-4	75.22	75.22	68.93	68.99	69.46
C-5	63.65	63.78	70.18	69.53	70.87
C-6	69.04	69.20	62.81	70.59	69.88
C-1′	98.22	99.58	98.05	97.96	101.07
C-2′	60.75	55.38	60.87	60.84	55.31
C-3′	69.55	72.08	69.62	69.83	71.82
C-4′	68.59	68.62	68.93	68.82	68.81
C-5′	72.06	72.84	72.30	72.79	72.11
C-6′	61.42	61.85	61.99	61.93	62.48
C-1″				98.21	101.83
C-2″				61.28	53.89
C-3″				69.61	73.33
C-4″				68.22	68.80
C-5″				72.42	73.33
C-6″				62.04	62.31
Thr					
C-α	58.55	58.71	58.81	58.81	58.90
C-β	75.38	75.49	75.72	75.72	75.42
C-γ	19.07	19.07	19.30	19.58	18.93
Fmoc CH	47.20	47.34	47.55	47.53	47.77
Fmoc CH_2	67.64	67.22	68.01	68.22	67.68
Fmoc arom C	120.11, 125.21, 127.86, 128.23	120.18, 125.08, 127.47, 128.30	120.45, 125.59, 127.56, 128.21	120.46, 125.60, 127.57, 128.23	
Others					
OAc	20.79, 20.64, 20.40	20.76 (3 ×)	21.12, 20.97, 20.75	21.13, 20.97, 20.75	21.13, 21.01 (2 ×), 20.91 (2 ×), 20.73
NHAc		23.47, 23.12			23.12, 23.07, 22.98
Benzylidene C	100.68	101.10			
Arom. C	125.13, 129.02	124.47, 129.13			

and molecular sieves (4 Å) were placed in a pre-dried flask. After dissolution in dichloromethane (3 cm^3), the solution was cooled to 0 °C, stirred for 10 min, and TMSOTf [100 mm^3; TMSOTf–dichloromethane (1 : 50)] was injected. The solution was stirred for 30 min, warmed to room temperature, diluted with dichloromethane (20 cm^3), filtered through Celite, concentrated and purified. VLC [ethyl acetate–light petroleum (2 : 3)] gave *disaccharide* **13** (187 mg, 98%), $[\alpha]_D^{25}$ +31.2 (*c* 1.0, $CHCl_3$) [Found: $(M + H)^+$ MALDI-MS, 1189.25. $C_{52}H_{46}F_5N_5O_{10}S_2$ requires M, 1188.08]. ^1H and ^{13}C NMR data are presented in Tables 5 and 6.

O-{2-Acetamido-O-[2-acetamido-3,4,6-tri-O-acetyl-2-deoxy-β-D-glucopyranosyl-(1→3)]-4,6-O-benzylidene-2-deoxy-α-D-galactopyranosyl}-N^{α}-(fluoren-9-ylmethoxycarbonyl)-L-threonine pentafluorophenyl ester 14

To a stirred solution of compound **13** (50 mg, 42 μmol) in THF–acetic anhydride–acetic acid [5 cm^3 (3 : 2 : 1)] was added zinc (75 mg, activated with 2% aq. $CuSO_4$. After being stirred for 4 h, the mixture was diluted with freshly distilled THF (20 cm^3), filtered through Celite, rinsed several times with THF, and concentrated. Purification by chromatography on pre-dried silica gel [ethyl acetate–light petroleum (3 : 1)] afforded the title disaccharide **14** (32 mg, 68%), $[\alpha]_D^{25}$ +75.4 (*c* 0.8, $CHCl_3$) [Found: $(M + H^+)$ MALDI-MS, 1129.57. $C_{54}H_{54}F_5N_3O_{18}$ requires M, 1228.04]. ^1H and ^{13}C NMR data are presented in Tables 5 and 6.

O-{2-Azido-2-deoxy-O-[3,4,6-tri-O-acetyl-2-deoxy-2-dithiasuccinimido-β-D-glucopyranosyl-(1→3)]-α-D-galactopyranosyl}-N^{α}-(fluoren-9-ylmethoxycarbonyl)-L-threonine pentafluorophenyl ester 15

Compound **13** (100 mg, 84 μmol) was dissolved in aq. 80% acetic acid (5 cm^3) and the solution was warmed to 70 °C. After 7 h the solution was cooled to room temperature, concentrated, and co-concentrated several times with toluene. The product **15** was dried in oil-pump vacuum for 20 h to afford pure *title compound* **15** (90 mg, 98%), which was used without further purification for the next glycosylation step; $[\alpha]_D^{25}$ +34.1 (*c* 0.92, $CHCl_3$) [Found: $(M + H)^+$ MALDI-MS, 1101.23. $C_{45}H_{42}F_5N_5O_{18}S_2$ requires M, 1099.97]. ^1H and ^{13}C NMR data are presented in Tables 5 and 6.

O-{2-Azido-2-deoxy-O-[3,4,6-tri-O-acetyl-2-deoxy-2-dithiasuccinimido-β-D-glucopyranosyl-(1→3)]-O-[3,4,6-tri-O-acetyl-2-deoxy-2-dithiasuccinimido-β-D-glucopyranosyl-(1→6)]-α-D-galactopyranosyl}-N^{α}-(fluoren-9-ylmethoxycarbonyl)-L-threonine pentafluorophenyl ester 16

Aglycone **15** (90 mg, 81 μmol), imidate **10** (51 mg, 90 μmol), and molecular sieves (3 Å) were placed in a pre-dried flask and dried on oil-pump vacuum. After 4 h the flask was filled with argon and dry dichloromethane (3 cm^3) was injected. The mixture was cooled to −30 °C and TMSOTf [50 mm^3; TMSOTf–dichloromethane (1 : 50)] was added. After 30 min the solution was warmed to room temperature, diluted with dichloromethane, filtered, concentrated, and purified by VLC on pre-dried silica gel [ethyl acetate–light petroleum (1 : 2 ⟶ 1 : 1)]. This procedure afforded pure trisaccharide **16** (80 mg, 65%), $[\alpha]_D^{25}$ +10.3 (*c* 1.16, $CHCl_3$) [Found: $(M + Na)^+$ MALDI-MS, 1528.67. $C_{59}H_{57}F_5N_6O_{27}S_4$ requires M, 1505.38]. ^1H and ^{13}C NMR data are presented in Tables 5 and 6.

O-{2-Acetamido[2-acetamido-3,4,6-tri-O-acetyl-2-deoxy-β-D-glucopyranosyl-(1→3)]-O-[2-acetamido-3,4,6-tri-O-acetyl-2-deoxy-β-D-glucopyranosyl-(1→6)]-2-deoxy-α-D-galactopyranos yl}N^{α}-(fluoren-9-ylmethoxycarbonyl)-L-threonine pentafluorophenyl ester 17

Trisaccharide **16** (80 mg, 53 μmol) was dissolved in THF–acetic anhydride–acetic acid [5 cm^3 (3 : 2 : 1)] and activated zinc (75 mg) was added. After being stirred for 6 h, the reaction mixture was diluted with acetic acid, filtered through Celite, rinsed

several times with acetic acid, and evaporated. VLC [chloroform–methanol (20:1 ⟶ 10:1)] afforded the title building block **17** (44 mg, 61%), $[\alpha]_D^{25}$ +48.3 [*c* 1.0, $CHCl_3$–MeOH (20:1)] [Found: $(M + H)^+$ MALDI-MS, 1370.45. $C_{61}H_{69}F_5N_4O_{26}$ requires M, 1369.24]. ^1H and ^{13}C NMR data are presented in Tables 5 and 6.

Acknowledgements

This project was supported by the EU-Science program (grant MM-SCI*-CT92-0765).

References

1 *Glycoconjugates*, ed. H. J. Allen and E. C. Kisailus, Marcel Dekker, New York, 1992, p. 263.
2 D. K. Podolsky, *J. Biol. Chem.*, 1985, **260**, 8262.
3 K. L. Carraway and N. Fregien, *Trends Glycosci. Glycotechnol.*, 1995, **7**(33), 31.
4 K. L. Carraway and S. R. Hull, *Glycobiology*, 1991, **1**, 131.
5 I. Brockhausen, in *Glycoprotein: Biosynthesis of* O-*Glycans of* N-*Acetylgalactosamine-α-Ser/Thr Linkage Type*, ed. J. Montreuil, J. F. G. Vliegenthart and H. Schachter, Elsevier, Amsterdam, 1995, p. 201.
6 M. Meldal and K. J. Jensen, *J. Chem. Soc., Perkin Trans. 1*, 1990, 483.
7 S. Peters, T. Bielfeldt, M. Meldal, K. Bock and H. Paulsen, *J. Chem. Soc., Perkin Trans. 1*, 1992, 1163.
8 M. Meldal, *Curr. Opin. Struct. Biol.*, 1994, **4**, 710.
9 M. Meldal, in *Neoglyconjugate: Preparation and Applications*, eds. Y. C. Lee and R. T. Lee, Academic Press, San Diego, 1994, p. 145; H. Paulsen, S. Peters and T. Bielfeldt, in *Glycoproteins: Chemical Synthesis of Glycopeptides*, eds. J. Montreuil, J. F. G. Vliegenthart and H. Schachter, Elsevier, Amsterdam, 1995, p. 87.
10 T. Bielfeldt, S. Peters, M. Meldal, K. Bock and H. Paulsen, *Angew. Chem., Int. Ed. Engl.*, 1992, **31**, 857.
11 T. Bielfeldt, S. Peters, M. Meldal, K. Bock and H. Paulsen, *Liebigs Ann. Chem.*, 1994, **369**, 381.
12 P. Braun, H. Waldmann and H. Kunz, *Synlett.*, 1992, 39.
13 S. Rio-Anneheim, H. Paulsen, M. Meldal and K. Bock, *J. Chem. Soc., Perkin Trans. 1*, 1995, 1071.
14 K. Frische, T. Jensen, L. Galli-Stampino, S. Mouritsen, O. Werdelin and M. Meldal, in Proceedings of the Symposium on Peptide Immunology, ed. C. H. Schneider, Wiley, 1995, in the press.
15 K. Frische, M. Meldal, O. Werdelin, S. Mouritsen, T. Jensen, L. Galli-Stampino and K. Bock, *J. Pept. Sci.*, 1996, in the press.
16 E. Meinjohanns, M. Meldal, H. Paulsen and K. Bock, Abstracts of the XVIIth International Carbohydrate Symposium, Ottawa, 1994, p. 260.
17 E. Meinjohanns, M. Meldal, H. Paulsen and K. Bock, *J. Chem. Soc., Perkin Trans. 1*, 1995, 405.
18 E. Meinjohanns, A. Vargas-Berenguel, M. Meldal, H. Paulsen and K. Bock, *J. Chem. Soc., Perkin Trans. 1*, 1995, 2165.
19 H. Paulsen and K. Adermann, *Liebigs Ann. Chem.*, 1989, 751.

Paper 5/07235B
Received 2*nd November* 1995
Accepted 8*th January* 1996