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Solid-phase synthesis of a glycosylated hexapeptide of human sialophorin, using the trichloroacetimidate method ☆

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Abstract

A hexapeptide containing a β-D-Gal*p*-(1 → 3)-α-D-Gal*p*NAc-(1 → O)-L-threonine unit was synthesized using glycosylated pentafluorophenyl esters in an Fmoc-based strategy. In all of the glycosylation reactions, trichloroacetimidates were successfully employed. The disaccharide moiety was prepared from tetra-*O*-acetyl-α-D-galactopyranosyl trichloroacetimidate and *tert*-butyldimethylsilyl 2-azido-6-*O*-benzoyl-2-deoxy-β-E-galactopyranoside with boron trifluoride etherate as a catalyst. The glycosylated active esters were obtained in the reaction of α and β 2,3,4,6-tetra-*O*-acetyl-β-D-galactopyranosyl-(1 → 3)-4-*O*-acetyl-2-azido-6-*O*-benzoyl-2-deoxy-D-galactopyranosyl trichloroacetimidates with Fmoc-protected pentafluorophenyl esters of L-serine and L-threonine in the presence of trimethylsilyl trifluoromethanesulfonate as Lewis acid. The glycosylated pentafluorophenyl ester of L-threonine was transformed into glycopeptides via a solid-phase synthesis. Azide reduction and *N*-acetylation were performed on the solid phase with a thioacetic acid–pyridine mixture. The glycopeptide was then cleaved from the resin with strong acid, also removing the acid-labile protecting groups of the peptide chain. Finally, the acyl groups used for sugar protection were cleaved with sodium methoxide, affording the completely deprotected *N*-acetyl-L-leucyl-L-glutamyl-*O*-[β-D-galactopyranosyl-(1 → 3)-2-acetamido-2-deoxy-α-D-galactopyranosyl]-L-threonyl-L-seryl-L-threonyl-glycinamide (**1**) in high purity.

Keywords: O-Glycopeptides; Glycosyl trichloroacetimidates; Peptide synthesis, solid phase; N-Fmoc protection; Pentafluorophenyl esters

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1. Introduction

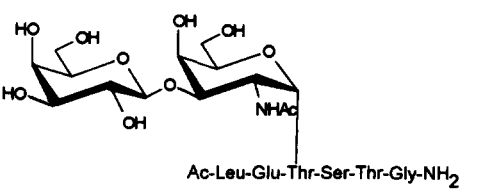
Sialophorin is a major outer-surface glycoprotein of human leucocytes and platelets. Biological studies suggest it to play a critical role in independent T-cell activation; possibly it also has a regulatory function in T-cell maturation and the development of the thymic reservoir [1]. Patients expressing a defective gene product of this molecule suffer from an immunodeficiency disease, the Wiskott–Aldrich syndrome. Sialophorin is highly glycosylated: the mature glycoprotein carries, on 84 of the 93 hydroxy amino acids of the outer-membrane region, O-linked glycosyl units. These mucin-type oligosaccharides are expected to mediate the biological functions of the molecule. Therefore partial glycopeptidic structures of sialophorin accessible by chemical synthesis can be useful in further investigations to determine the biological importance of specific sugar moieties.

As a target molecule, compound **1** was chosen (Scheme 1). It contains the core I unit, β-D-Gal*p*-(1 → 3)-D-Gal*p*NAc, linked α-glycosidically to the hydroxy group of an L-threonine residue. The glycosylated amino acid is incorporated in a peptide structure representing a partial sequence of human sialophorin. Efforts to synthesize the sugar moiety representing the epitopes of T- and T_N-antigen have been reported [2].

The crucial step in the preparation of O-linked glycopeptides is the synthesis of α-glycosylated active esters [3] which can be directly incorporated into a peptide chain either in a solid-phase approach or by classical synthesis in solution [3,4]. In this contribution the efficiency of the trichloroacetimidate method [5] in the preparation of glycosylated active esters and the subsequent synthesis of a mucin-type glycopeptide is demonstrated.

2. Results and discussion

Previous publications have shown the efficiency of acyl protecting groups in solid-phase glycopeptide synthesis [6] as well as in classical synthesis in solution [7]. For the synthesis of the required disaccharidic active ester **12a** (Scheme 2), *tert*-butyldimethylsilyl 2-azido-2-deoxy-β-D-galactopyranoside (**3**) was obtained by a known procedure [8]. This azido sugar could be selectively benzoylated at the OH-6 position, using benzoyl cyanide at −35°C [9] to provide a 78% yield of **4** which was used



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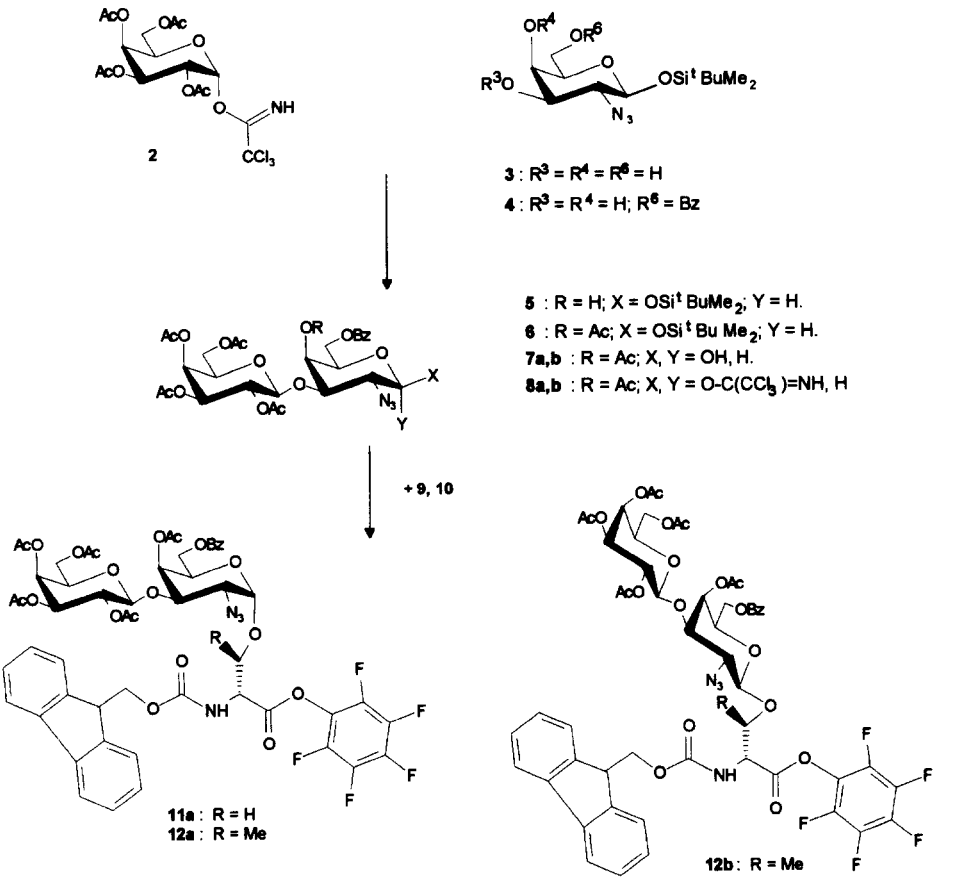
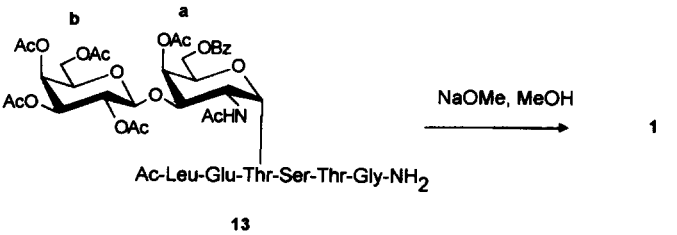
Scheme 1.



Scheme 2.

directly for the following step as glycosyl acceptor with OH-3 and OH-4 free. When treated with 2,3,4,6-tetra-*O*-acetyl-α-D-galactopyranosyl imidate [10] (**2**) as donor, the (1 → 3)-linked disaccharide **5** could be obtained as a single product with boron trifluoride etherate as Lewis acid. When the donor compound was used only in slight excess the formation of a 3,4-di-*O*-glycosylated byproduct could be avoided. The formation of the β-(1 → 3) linkage was favoured in this reaction both by the neighbouring-group effect and the nonpolar solvent mixture (3:1 hexane–dichloromethane) supporting an S_N2-type reaction. Three further steps were required to obtain the disaccharide donor **8a,b**. After acetylation of OH-4 (→ **6**), the $^tBuMe_2Si$ group was removed with tetrabutylammonium fluoride (TBAF) to afford compound **7**. The trichloroacetimidates **8a,b** were then generated with trichloroacetonitrile and potassium carbonate as base, yielding an excess of the β-product. The conversions from **5** to **8** were accomplished with a total yield of 73%. The N-Fmoc-protected pentafluorophenyl (Pfp) esters of L-serine (**9**) and L-threonine (**10**) were synthesized as previously reported [11]. The best results in the active ester glycosylation were achieved with **8b** as donor and



Scheme 3.

trimethylsilyl trifluoromethanesulfonate (TMSOTf) as catalyst. Products **11a** and **12a** were obtained as single anomers in 42 and 68% yield, respectively. Donor **8a** reacted with the L-threonine derivative **10** to give a 3:1 mixture of **12a,b**. A problem to be solved in these active ester glycosylations was the low stability of the active esters which are highly susceptible towards hydrolytic cleavage [12]. Products **11** and **12** decomposed over untreated silica gel.

Solid-phase peptide synthesis was carried out using a poly(ethylene glycol) resin [13] functionalized with the acid-labile linker 4-(4-aminomethyl-3,5-dimethoxyphenoxy)valeric acid [14]. For the removal of the Fmoc-groups, 50% morpholine in *N,N*-dimethylformamide was used [15]. Two equivalents of all activated amino acids were employed; in the case of pentafluorophenyl esters, two equivalents of 1-hydroxybenzotriazole were added to accelerate the coupling reaction [16]. The completion of all acylation steps was monitored by a UV/VIS spectrophotometer and a flow cell. In the case of the Pfp-esters Acid Violet 17 was added as counterion. For the reduction of azide sugars the resin was treated with 2:1 thioacetic acid–pyridine. The completion of the reaction could be observed in the IR spectrum of a resin sample when the azide vibration at 2117 cm^{-1} vanished. Cleavage from the resin was achieved with aq 95% trifluoroacetic acid. The resulting glycopeptide **13** was then fully deprotected with a catalytic amount of sodium methoxide in methanol to yield compound **1** (Scheme 3).

3. Experimental

General methods.—Optical rotations were determined with a Büchi polarimeter for solutions in $CHCl_3$ at 20°C, unless noted otherwise. Column chromatography was performed on Silica Gel 60 (0.04–0.063 mm, 230–400 mesh ASTM) and on Florisil (Fluka). MPLC was performed on Lichroprep Si 60 (Merck), and preparative HPLC on Lichrosorb RP 18. TLC was performed on Silica Gel 60 F_{254} (Merck) and analytical HPLC on Lichrospher 100 RP 18. Petroleum ether (PE) was used with a boiling range of 35–60°C. The ^1H NMR spectra were recorded on a Bruker AM 250 instrument. The values of δ are expressed in ppm relative to the solvent signal as internal standard ($CHCl_3$, 7.24 ppm; MeOD, 3.35 ppm; HOD, 4.63 ppm). Solid-phase peptide synthesis was carried out with a semi-automatic peptide synthesizer (Novabiochem). As synthesis resin, Fmoc-PAL-PEG-PS (Millipore) was used.

tert-*Butyldimethylsilyl 2-azido-6-O-benzoyl-2-deoxy-β-D-galactopyranoside* (**4**)

[9].—Compound **3** [8] (12 g, 38 mmol) was dissolved in MeCN (240 mL) and triethylamine (80 mL). The mixture was cooled to −35°C under N_2, and a solution of benzoyl cyanide (5.5 g, 42 mmol) in MeCN (30 mL) was added dropwise. After 2 h, solvents were removed in vacuo, the mixture was subjected to flash chromatography (3:1 toluene–acetone), and product **4** was obtained as a colourless oil (12.6 g, 78%); $[\alpha]_D$ +57° (*c* 1); R_f 0.5 (1:1 PE–EtOAc); ^{1}H NMR (250 MHz): δ 0.13, 0.14 [2 s, 6 H, $Si(CH_3)_2$], 0.91 [s, 9 H, $SiC(CH_3)_3$], 3.19 (br s, 2 H, 2 OH), 3.43–3.57 (m, 2 H, H-2,3), 3.79 (t, 1 H, $J_{5,6a} = J_{5,6b}$ = 5.7 Hz, H-5), 3.96 (d, 1 H, $J_{3,4}$ 2.2 Hz, H-4), 4.49–4.56 (m, 2 H, H-1,6a), 4.63 (dd, 1 H, $J_{6b,6a}$ 11.6 Hz, H-6b), 7.41–7.47 (m, 2 H, *m*-Ph), 7.54–7.61 (m, 1 H, *p*-Ph), 8.02–8.05 (m, 2 H, *o*-Ph). Anal. Calcd for $C_{19}H_{29}N_3O_6Si$ (423.6): C, 53.88; H, 6.90; N, 9.92. Found: C, 53.73; H, 6.81; N, 9.74.

tert-*Butyldimethylsilyl 2,3,4,6-tetra*-O-*acetyl-β*-D-*galactopyranosyl-(1 → 3)-2-azido-6*-O-*benzoyl-2-deoxy-β*-D-*galactopyranoside* (**5**).—At room temperature, compound **4** (11.5 g, 27 mmol) and trichloroacetimidate **2** [10] (16.5 g, 33 mmol) were suspended in CH_2Cl_2 (25 mL) and hexane (75 mL). 0.1 M Boron trifluoride etherate in CH_2Cl_2 (6 mL) was added dropwise under N_2 over a period of 30 min. After 2 h of stirring, TLC (3:1 toluene–acetone) showed quantitative conversion of the imidate. Further BF_3 solution (4 mL) was added to ensure the rearrangement of the orthoester product into compound **5**. After 1 h, the mixture was diluted with EtOAc (250 mL), washed with 1 M $NaHCO_3$ (50 mL), dried over $MgSO_4$, and concentrated under reduced pressure; **5** was purified by flash chromatography (8:1 toluene–acetone), and recrystallized from PE–EtOAc (13.3 g, 64%); $[\alpha]_D$ +31° (*c* 1); R_f 0.42; ^{1}H NMR (250 MHz): δ 0.11, 0.14 [2 s, 6 H, $Si(CH_3)_2$], 0.91 [s, 9 H, $SiC(CH_3)_3$], 2.01, 2.04, 2.11, 2.18 (4 s, 12 H, 4 Ac), 2.66 (s, 1 H, OH), 3.38 (dd, 1 H, $J_{2,3}$ 10.2, $J_{3,4}$ 3.3 Hz, H-3), 3.57 (dd, 1 H, $J_{1,2}$ 7.6 Hz, H-2), 3.78 (m, 1 H, H-5), 3.94 (m, 1 H, H-5′), 4.04 (m, 1 H, H-4), 4.06–4.16 (m, 2 H, 2 H-6), 4.48 (d, 1 H, H-1), 4.53–4.66 (m, 2 H, 2 H-6′), 4.69 (d, 1 H, $J_{1',2'}$ 7.9 Hz, H-1′), 5.04 (dd, 1 H, $J_{2',3'}$ 10.5, $J_{3',4'}$ 3.4 Hz, H-3′), 5.27 (dd, 1 H, H-2′), 5.40 (dd, 1 H, H-4′), 7.41–7.48 (m, 2 H, *m*-Ph), 7.55–7.58 (m, 1 H, *p*-Ph), 8.02–8.06 (m, 2 H, *o*-Ph). Anal. Calcd for $C_{33}H_{47}N_3O_{15}Si$ (753.8): C, 52.58; H, 6.28; N, 5.57. Found: C, 52.38; H, 6.14; N, 5.26.

tert-*Butyldimethylsilyl 2,3,4,6-tetra*-O-*acetyl-β*-D-*galactopyranosyl-(1 → 3)-4*-O-*acetyl-2-azido-6*-O-*benzoyl-2-deoxy-β*-D-*galactopyranoside* (**6**) [9].—Compound **5** (13 g, 17.2 mmol) was stirred in pyridine (40 mL) and Ac_2O (20 mL) at room temperature for 18 h. The mixture was concentrated in vacuo, and coevaporated twice with 20 mL of toluene. The residue was purified by flash chromatography (9:1 toluene–acetone) to yield **6** (12.1 g, 88%); $[\alpha]_D$ +22° (*c* 1); R_f 0.65 (3:1 toluene–acetone); ^{1}H NMR (250 MHz): δ 0.11, 0.13 [2 s, 6 H, $Si(CH_3)_2$], 0.90 [s, 9 H, $SiC(CH_3)_3$], 1.99, 2.04, 2.09, 2.16, 2.17 (5 s, 15 H, 5 Ac), 3.52–3.61 (m, 2 H, H-2,3), 3.88–3.92 (m, 2 H, H-5,5′), 4.09–4.16 and 4.32–4.41 (m, 4 H, 2 H-6, 2 H-6′), 4.51 (d, 1 H, $J_{1,2}$ 6.8 Hz, H-1), 4.70 (d, 1 H, $J_{1',2'}$ 7.7 Hz, H-1′), 5.01 (dd, 1 H, $J_{2',3'}$ 10.6, $J_{3',4'}$ 3.4 Hz, H-3′), 5.18 (dd, 1 H, H-2′), 5.37 (d, 1 H, H-4), 5.41 (d, 1 H, H-4′), 7.41–7.48 (m, 2 H, *m*-Ph), 7.55–7.58 (m, 1 H, *p*-Ph), 8.02–8.06 (m, 2 H, *o*-Ph). Anal. Calcd for $C_{35}H_{49}N_3O_{16}Si$ (795.9): C, 52.82; H, 6.21; N, 5.28. Found: C, 52.38; H, 6.21; N, 4.87.

2,3,4,6-Tetra-O-*acetyl-β*-D-*galactopyranosyl-(1 → 3)-4*-O-*acetyl-2-azido-6*-O-*benzoyl-2-deoxy*-D-*galactopyranose* (**7**).—Compound **6** (8.0 g, 10 mmol) was dissolved

in THF (120 mL) and glacial AcOH (1 mL). The mixture was cooled to −20°C and 1.1 M TBAF solution in THF (20 mL) was added. The reaction was completed in 6 h. The mixture was then poured into ice–water (400 mL) and extracted three times with ether (200 mL). The organic phases were collected and dried over $MgSO_4$. Solvents were finally removed in vacuo. Crude product **7** (6.9 g, 10.0 mmol) was obtained quantitatively and employed in the following reaction without further purification.

*2,3,4,6-Tetra-*O*-acetyl-β-*D*-galactopyranosyl-(1 → 3)-4-O-acetyl-2-azido-6-*O*-benzoyl-2-deoxy-α,β-*D*-galactopyranosyl trichloroacetimidate* (**8a,b**).—A solution of **7** (6.9 g, 10.0 mmol) in CH_2Cl_2 (150 mL) was stirred vigorously with dry K_2CO_3 (6.5 g, 47 mmol). Trichloroacetonitrile (6 mL, 60 mmol) was added. After 2 h, more K_2CO_3 (3 g, 21 mmol) was added and after 4 h the reaction was completed. The mixture was filtered, concentrated under reduced pressure, dried with $MgSO_4$, and subjected to flash chromatography (8:1 toluene–acetone). Yield: 6.4 g (78%), α anomer **8a** (2.3 g, 28%), β anomer **8b** (4.1 g, 50%).

Compound **8a** had $[\alpha]_D$ +47.2° (*c* 1); R_f 0.63 (3:1 toluene–acetone); ^{1}H NMR (250 MHz): δ 1.99, 2.05, 2.08, 2.17, 2.18 (5 s, 15 H, 5 Ac), 3.93–4.47 (m, 8 H, H-2,3,5,5′, 2 H-6, 2 H-6′), 4.77 (d, 1 H, $J_{1',2'}$ 7.8 Hz, H-1′), 5.02 (dd, 1 H, $J_{2',3'}$ 10.6, $J_{3',4'}$ 3.4 Hz, H-3′), 5.24 (dd, 1 H, H-2′), 5.38 (d, 1 H, H-4′), 5.67 (d, 1 H, $J_{3,4}$ 2.9 Hz, H-4), 6.50 (d, 1 H, $J_{1,2}$ 3.6 Hz, H-1), 7.39–7.96 (m, 3 H, Ph), 7.96–7.99 (m, 2 H, *o*-Ph), 8.72 (s, 1 H, NH). Anal. Calcd for $C_{31}H_{35}Cl_3N_4O_{16}$ (825.99): C, 45.08; H, 4.27; N, 6.78. Found: C, 45.15; H, 4.17; N, 6.52.

Compound **8b** had $[\alpha]_D$ +18.9° (*c* 1); R_f 0.58 (3:1 toluene–acetone); ^{1}H NMR (250 MHz): δ 1.97, 2.01, 2.07, 2.15, 2.16 (5 s, 15 H, 5 Ac), 3.67 (dd, 1 H, $J_{2,3}$ 10.2, $J_{3,4}$ 3.5 Hz, H-3), 3.86–4.42 (m, 7 H, H-2,5,5′, 2 H-6, 2 H-6′), 4.72 (d, 1 H, $J_{1',2'}$ 7.8 Hz, H-1′), 4.99 (dd, 1 H, $J_{2',3'}$ 10.6, $J_{3',4'}$ 3.4 Hz, H-3′), 5.18 (dd, 1 H, H-2′), 5.35 (d, 1 H, H-4′), 5.52 (d, 1 H, $J_{3,4}$ 2.9 Hz, H-4), 5.63 (d, 1 H, $J_{1,2}$ 8.3 Hz, H-1), 7.39–7.96 (m, 3 H, Ph), 7.96–7.99 (m, 2 H, *o*-Ph), 8.71 (s, 1 H, NH). Anal. Calcd for $C_{31}H_{35}Cl_3N_4O_{16}$ (825.99): C, 45.08; H, 4.27; N, 6.78. Found: C, 45.13; H, 4.51; N, 6.16.

N-*(9-Fluorenylmethoxycarbonyl)-*O*-[2,3,4,6-tetra-*O*-acetyl-β-*D*-galactopyranosyl-(1 → 3)-4-*O*-acetyl-2-azido-6-*O*-benzoyl-2-deoxy-α-*D*-galactopyranosyl]-*L*-serine pentafluorophenyl ester* (**11**).—Compound **8b** (0.5 g, 0.6 mmol) and **9** [11] (0.4 g, 0.78 mmol) were suspended in CH_2Cl_2 (3 mL) and cooled to 0°C. Trimethylsilyl trifluoromethanesulfonate (TMSOTf) (6 μL) was dissolved in CH_2Cl_2 (0.5 mL) and added slowly to the mixture. After 2 h, the mixture was neutralized with pyridine (5 μL). The solution was concentrated at 40°C under reduced pressure and chromatographed over Florisil (12:1 toluene–acetone). Product **11** (694 mg, 42%) was finally isolated by MPLC (9:1 toluene–acetone) as a white lyophilisate; $[\alpha]_D$ +35.2° (c 1); R_f 0.58 (3:1 toluene–acetone); ^{1}H NMR (250 MHz): δ 1.96–2.15 (5 s, 15 H, 5 Ac), 3.71 (dd, 1 H, $J_{3,4}$ 2.7, $J_{2,3}$ 10.7 Hz, H-3), 3.84 (m, 1 H, H-5), 4.04–4.39 (m, 11 H, Fmoc-CH, Fmoc-CH_2, Ser-β-CH_2, 2 H-6, 2 H-6′, H-5′, H-2), 4.65 (d, 1 H, $J_{1',2'}$ 7.7 Hz, H-1′), 4.90 (m, 1 H, Ser-α-CH), 4.93 (dd, 1 H, $J_{2',3'}$ 10.5, $J_{3',4'}$ 3.1 Hz, H-3′), 4.99 (d, 1 H, $J_{1,2}$ 3.2 Hz, H-1), 5.17 (dd, 1 H, H-2′), 5.34 (d, 1 H, H-4), 5.56 (d, 1 H, H-4′), 5.96 (d, 1 H, *J* 8.7 Hz, NH), 7.25–8.04 (m, 13 H, 8 Fmoc-H, 5 Bz-H). Anal. Calcd for $C_{53}H_{49}F_5N_4O_{20}$ (1156.98): C, 55.02; H, 4.27; N, 4.84. Found: C, 55.69; H, 4.48; N, 4.62.

N-*(9-Fluorenylmethoxycarbonyl)-*O*-[2,3,4,6-tetra-*O*-acetyl-β-*D*-galactopyranosyl-(1*

→ *3)-4-O-acetyl-2-azido-6-O-benzoyl-2-deoxy-α,β-D-galactopyranosyl]-L-threonine pentafluorophenyl ester* (**12a,b**).—Compound **8a** (0.65 g, 0.78 mmol) and **10** [11] (0.5 g, 0.98 mol) were dissolved at 0°C in CH_2Cl_2 (3 mL). TMSOTf (7 μL, 0.039 mmol, 0.05 equiv) was dissolved in CH_2Cl_2 (1 mL) and added slowly. After 2 h, the mixture was neutralized by the addition of pyridine (3 μL, 0.039 mmol). Solvents were removed under reduced pressure. After chromatography over Florisil (12:1 toluene–acetone), both anomeric products were obtained by MPLC (9:1 toluene–acetone). Yield: 69%, 483 mg of **12a** (52%), 161 mg of **12b** (17%).

Alternative procedure. Compound **8b** (0.5 g, 0.6 mmol) and **10** (0.4 g, 0.8 mmol) were suspended in CH_2Cl_2 (3 mL) at room temperature. A freshly prepared 0.1 M solution of TMSOTf in CH_2Cl_2 (0.3 mL) was added slowly. After 2 h, the mixture was treated as described above to yield pure **12a** (442 mg, 68%).

Compound **12a** had $[\alpha]_D$ +19.4° (*c* 1); R_f 0.61 (3:1 toluene–acetone); ^{1}H NMR (250 MHz): δ 1.35 (d, 3 H, *J* 6.3 Hz, Thr-Me), 2.02–2.16 (5 s, 15 H, 5 Ac), 3.73 (dd, $J_{2,3}$ 10.8, $J_{3,4}$ 3.8 Hz, H-3), 3.90–3.94 (m, 1 H, H-5′), 4.0–4.2 (m, 3 H, Fmoc-CH, 2 H-6′), 4.2–4.5 (m, 5 H, Fmoc-CH_2, H-5, 2 H-6), 4.48–4.52 (m, 1 H, Thr-β-CH), 4.53 (dd, 1 H, $J_{1,2}$ 3.9 Hz, H-2), 4.71 (dd, 1 H, $J_{CH\alpha,NH}$ 9.2, $J_{\alpha,\beta}$ 3.1 Hz, Thr-α-CH), 4.74 (d, 1 H, $J_{1',2'}$ 7.8 Hz, H-1′), 5.03 (dd, 1 H, $J_{2',3'}$ 10.5, $J_{3',4'}$ 3.1 Hz, H-3′), 5.14 (d, 1 H, H-1), 5.19 (dd, 1 H, H-2′), 5.36 (d, 1 H, H-4′), 5.59 (d, 1 H, H-4), 5.82 (d, 1 H, $J_{NH,CH\alpha}$ 9.2 Hz, Thr-NH), 7.28–8.05 (m, 13 H, 8 Fmoc-H, 5 Bz-H). Anal. Calcd for $C_{54}H_{51}F_5N_4O_{20} \cdot H_2O$ (1189.01): C, 54.54; H, 4.49; N, 4.71. Found: C, 54.70; H, 4.46; N, 4.79.

Compound **12b** had $[\alpha]_D$ +12.8° (*c* 1), R_f 0.58 (3:1 toluene–acetone); ^{1}H NMR (250 MHz): δ 1.38 (d, 3 H, *J* 6.3 Hz, Thr-Me), 1.98–2.16 (5 s, 15 H, 5 Ac), 3.52 (dd, 1 H, $J_{2,3}$ 7.8, $J_{3,4}$ 3.0 Hz, H-3), 3.66 (dd, 1 H, $J_{1,2}$ 7.5 Hz, H-2), 3.8–3.9 (m, 2 H, H-5,5′), 4.0–4.15 (m, 2 H, 2 H-6), 4.18–4.28 (m, 3 H, 2 H-6′, Fmoc-CH), 4.41 (d, 2 H, *J* 6.9 Hz, Fmoc-CH_2), 4.42 (d, 1 H, H-1), 4.62–4.68 (m, 1 H, Thr-β-CH), 4.70 (d, 1 H, $J_{1',2'}$ 7.7 Hz, H-1′), 4.78 (dd, 1 H, $J_{CH\alpha,NH}$ 9.0, $J_{\alpha,\beta}$ 3.0 Hz, Thr-α-CH), 5.05 (dd, 1 H, $J_{2',3'}$ 10.3, $J_{3',4'}$ 2.6 Hz, H-3′), 5.16 (dd, 1 H, $J_{1',2'}$ 7.7 Hz, H-2′), 5.36 (d, 1 H, H-4′), 5.42 (d, 1 H, $J_{3,4}$ 3.0 Hz, H-4), 5.76 (d, $J_{NH,CH\alpha}$ 9.0 Hz, 1 H, Thr-NH), 7.28–8.00 (m, 13 H, 8 Fmoc-H, 5 Bz-H). Anal. Calcd for $C_{54}H_{51}F_5N_4O_{20} \cdot H_2O$ (1189.01): C, 54.54; H, 4.49; N, 4.71. Found: C, 54.34; H, 4.51; N, 4.70.

N-*Acetyl-L-leucyl-L-glutamyl-O-[2,3,4,6-tetra-O-acetyl-β-D-galactopyranosyl-(1 → 3)-2-acetamido-4-O-acetyl-6-O-benzoyl-2-deoxy-α-D-galactopyranosyl]-L-threonyl-L-seryl-L-threonyl-glycinamide* (**13**).—Poly(ethylene glycol)–poly(styrene) resin (360 mg), which was functionalized with 0.14 mmol/g of the acid-labile Fmoc-protected peptide amide linker (PAL), was transferred in the continuous flow reactor. All deprotection steps were carried out with 1:1 morpholine–DMF. The glycopeptide chain was built up in sequence by the addition of *N*-(9-fluorenylmethoxycarbonyl)glycine pentafluorophenyl ester (Fmoc-Gly-OPfp) (46 mg), Fmoc-(O-*tert*-butyl)-Thr-Dhbt (56 mg), Fmoc-(O-*tert*-butyl)-Ser-OPfp (55 mg), compound **12a** (120 mg), Fmoc-(O-*tert*-butyl)-Glu-OPfp (60 mg), and Fmoc-Leu-OPfp (52 mg) to the solid-phase acylations. To the pentafluorophenyl esters was added hydroxybenzotriazole (13 mg). The acylation reactions were monitored with the dye Violet Acid 17 at 600 nm in the case of the pentafluorophenyl esters and at 360 nm for the Dhbt-esters. Reactions were completed

after 1 h; the glycosylated active ester **12a** required a prolonged reaction time of 24 h. After deprotection of the last amino acid, the resin was thoroughly washed with DMF and CH_2Cl_2, and dried in vacuo. It was then treated with 2:1 thioacetic acid–pyridine (1.5 mL) for 18 h. After another wash-and-dry procedure the resin was treated with 19:1 CF_3CO_2H–water (2 mL) for 2 h. The resin was washed with the cleavage mixture (10 mL), CF_3CO_2H–water was removed in vacuo. The product **13** was isolated using preparative reversed-phase HPLC (4:6 MeCN–water, 0.1% CF_3CO_2H); t_R 12 min; 38 mg (58%); ^1H NMR (250 MHz, MeOD): δ 0.94, 0.99 (2 d, 6 H, $J_{\gamma,\delta}$ 6.3 Hz, 2 Leu-Me), 1.27 (d, 3 H, $J_{\beta,\gamma}$ 6.1 Hz, Thr-Me), 1.28 (d, 3 H, $J_{\beta,\gamma}$ 6.0 Hz, Thr-Me), 1.58–1.64 (m, 2 H, Leu-β-CH_2), 1.65–1.80 (m, 1 H, Leu-γ-CH), 1.98, 2.05, 2.08, 2.10, 2.18, 2.19 (m, 23 H, 7 Ac, Glu-β-CH_2), 2.45–2.62 (m, 2 H, Glu-γ-CH_2), 3.65–4.0 (m, 8 H, Ser-β-CH_2, H-5,5′, 2 H-6, 2 H-6′), 4.22 (dd, 1 H, $J_{2,3}$ 11.4 Hz, H-3), 4.23 (m, 1 H, 1 Thr-β-CH), 4.32 (d, Thr-α-CH), 4.36 (m, Thr′-β-CH), 4.47 (dd, 1 H, H-2), 4.58 (d, 1 H, Thr′-α-CH), 4.62 (m, 2 H, ser-α-CH, Glu-α-CH), 4.87 (d, 1 H, $J_{1',2'}$ 8.0 Hz, H-1′), 5.04 (d, 1 H, J 11.7 Hz, Thr-α-CH), 5.05 (dd, 1 H, $J_{2',3'}$ 10.7 Hz, H-2′), 5.09 (d, 1 H, H-1), 5.12 (dd, 1 H, H-3′), 5.42 (d, 1 H, H-4′), 5.58 (d, 1 H, H-4), 7.5–8.2 (m, 5 H, Bz-H). FABMS: Calcd for $C_{57}H_{82}N_8O_{28}$: 1326.52. Found: m/z 1328 (MH^+).

N-*Acetyl*-L-*leucyl*-L-*glutamyl*-O-[β-D-*galactopyranosyl*-(1 → 3)-2-*acetamido*-2-*deoxy*-α-D-*galactopyranosyl*]-L-*threonyl*-L-*seryl*-L-*threonyl*-*glycinamide* (**1**).—Compound **13** (10 mg) was dissolved in MeOH (15 mL). A 0.5 M solution of NaOMe in MeOH was added dropwise until a wet pH-paper showed a pH of 10.5. The reaction was monitored with analytical reversed-phase HPLC and was complete after 6 h. The mixture was neutralized with AcOH and, after removal of the solvents in vacuo, product **1** was isolated by preparative RP–HPLC (2:8 MeCN–water, 0.1% CF_3CO_2H; t_R 16 min) with 82% yield (6 mg); ^1H NMR (250 MHz, D_2O): δ 0.70, 0.74 (2 d, 6 H, $J_{\gamma,\delta}$ 6.0 Hz, 2 Leu-Me), 1.04 (d, 3 H, $J_{\beta,\gamma}$ 6.6 Hz, Thr-Me), 1.07 (d, 3 H, $J_{\beta,\gamma}$ 8.2 Hz, Thr-Me), 1.37–1.49 (m, 3 H, Leu-β-CH_2, Leu-γ-CH), 1.84 (s, 6 H, 2 NAc), 1.81–2.04 (2 m, 2 H, Glu-β-CH_2), 2.31–2.38 (m, 2 H, Glu-γ-CH_2), 2.98, 3.04 (2 d, 2 H, $J_{\alpha,\alpha'}$ 7.2 Hz, Gly-CH_2), 3.32 (dd, 1 H, H-2′), 3.43 (dd, 1 H, $J_{2',3'}$ 10.0 Hz, $J_{3',4'}$ 3.3 Hz, H-3′), 3.53–3.62 (m, 2 H, Ser-β-CH_2), 3.65–3.75 (m, 3 H, H-5′, 2 H-6′), 3.75 (d, 1 H, $J_{3',4'}$ 3.5 Hz, H-4′), 4.02–4.18 (m, 6 H, H-2,3,4,5, 2 H-6), 4.16 (m, 1 H, Thr-β-CH), 4.28 (d, 1 H, $J_{1',2'}$ 8.8 Hz, H-1′), 4.36–4.45 (m, 3 H, Thr′-β-CH, 2 Thr-α-CH, Glu-α-CH), 4.76 (d, 1 H, $J_{1,2}$ 3.8 Hz, H-1). FABMS: Calcd for $C_{40}H_{68}N_8O_{22}$: 1012.44. Found: m/z 1014 (MH^+).

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