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Chemoenzymatic Synthesis of Sialylated Glycopeptides Derived from Mucins and T-Cell Stimulating Peptides

Shaji K. George,[†] Tilo Schwientek,[‡] Björn Holm,[†] Celso A. Reis,[‡,§] Henrik Clausen,[‡] and Jan Kihlberg*[,†]

Contribution from the Department of Organic Chemistry, Umeå University, SE-901 87 Umeå, Sweden, School of Dentistry, University of Copenhagen, Nörre Allé 20, DK-2200 Copenhagen N, Denmark, and Institute of Molecular Pathology and Immunology, University of Porto, IPATIMUP, Rua Dr. Roberto Frias s/n, 4200 Porto, Portugal

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Abstract: The Tn, T, sialyl-Tn, and 2,3-sialyl-T antigens are tumor-associated carbohydrate antigens expressed on mucins in epithelial cancers, such as those affecting the breast, ovary, stomach, and colon. Glycopeptides carrying these antigens are of interest for development of cancer vaccines and a short, chemoenzymatic strategy for their synthesis is reported. Building blocks corresponding to the Tn (GalNAcα-Ser/Thr) and T [Galβ(1→3)GalNAcα-Ser/Thr] antigens, which are relatively easy to obtain by chemical synthesis, were prepared and then used in the synthesis of glycopeptides on the solid phase. Introduction of sialic acid to give the sialyl-Tn [Neu5Acα(2→6)GalNAcα-Ser/Thr] and 2,3-sialyl-T [Neu5Acα(2→3)Galβ(1→3)GalNAcα-Ser/Thr] antigens is difficult when performed chemically at the building block level. Sialylation was therefore carried out with recombinant sialyltransferases in solution after cleavage of the Tn and T glycopeptides from the solid phase. In the same manner, the core 2 trisaccharide [Galβ1→3(GlcNAcβ1→6)GalNAc] was incorporated in glycopeptides containing the T antigen by using a recombinant *N*-acetylglucosaminyltransferase. The outlined chemoenzymatic approach was applied to glycopeptides from the tandem repeat domain of the mucin MUC1, as well as to neoglycosylated derivatives of a T cell stimulating viral peptide.

Introduction

Most epithelial cells produce mucins, i.e. glycoproteins in which the polypeptide backbone consists of highly conserved tandem repeats with complex carbohydrates linked to multiple serine and threonine residues.[1−4] Biosynthesis of mucins occurs in the Golgi apparatus and is initiated by addition of *N*-acetylgalactosamine to serines and threonines by a family of *N*-acetylgalactosaminyltransferases, which show overlapping but somewhat different specificities with regard to peptide sequence.[5] When the mucin MUC1 is produced by the normal human mammary gland, galactosyl residues are added to the *N*-acetylgalactosamine moieties to form the type 1 core Galβ1→3GalNAcα-Ser/Thr (also called the T antigen). The type 1 core, in turn, acts as a substrate for core 2 β1,6GlcNAc transferases, so that the type 2 core trisaccharide [Galβ1→3(GlcNAcβ1→6)GalNAc] is formed.[3,4] Finally, oligo *N*-acetyl-

[†] Umeå University.
[‡] University of Copenhagen.
[§] University of Porto.

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lactosamine moieties are added, after which addition of sialic acid or fucose residues terminates growth of the mucin linked oligosaccharide structure.

In epithelial cancers, such as those affecting the breast, ovary, lung, and colon, low expression of core 2 β1,6GlcNAc transferases together with increased levels of sialyltransferases result in that mucins display simpler *O*-linked carbohydrates.[3,4] The Tn (GalNAcα-Ser/Thr), T [Galβ(1→3)GalNAcα-Ser/Thr], sialyl-Tn [Neu5Acα(2→6)GalNAcα-Ser/Thr] and 2,3-sialyl-T [Neu5Acα(2→3)Galβ(1→3)GalNAcα-Ser/Thr] antigens are important examples of such saccharides which constitute tumor-associated carbohydrate antigens.[3,6−10] The presence of these antigens on the surface of common human malignant tumors has led to intense studies directed toward development of synthetic carbohydrate-based anticancer vaccines.[11,12] Recently, studies in mice showed that short Tn-based glycopeptides coupled to the protein KLH induced a strong IgM response and a moderate IgG response, both of which were reactive to colon cancer cells.[12,13] Moreover, a sialyl-Tn KLH conjugate vaccine used in combination with cyclophosphamide was indicated to have a therapeutic effect when evaluated in clinical trials involving breast cancer patients.[14−16] These vaccines elicit an antibody response, but it would also be an advantage if T cells could be directed to tumor-associated carbohydrate antigens. Studies performed during recent years have indeed shown that T cells can recognize glycopeptides bound by MHC molecules on the surface of antigen-presenting cells.[17−20] Interestingly, in one case a set of T cells reactive only to the carbohydrate moiety of a neoglycopeptide antigen was obtained, suggesting that it may be possible to target T cells to carbohydrate antigens on tumor cells.[21]

Today, the most general synthetic route to *O*-linked glycopeptides employs N^{α}-Fmoc protected glycosylated amino acids as building blocks in stepwise solid-phase peptide synthesis.[22−25] Synthesis of building blocks corresponding to the structurally less complex Tn and T antigens has been described by several groups during recent years (reviewed in refs 11, 12, and 26). However, incorporation of sialic acid in glycoconjugates by chemical means is a substantially more demanding task due to poor control of the anomeric configuration of the sialic acid residue and the requirement for multistep synthetic schemes.[27,28] This may explain why only a few building blocks corresponding to the sialyl-Tn[29−33] and 2,3-sialyl-T[34,35] antigens have been prepared chemically and then employed in glycopeptide synthesis. Recently, enzymatic synthesis of 2,3-sialyl-T antigen building blocks has been described,[36,37] but the carboxyl group of the sialic acid residue and the different hydroxyl groups must be protected before these building blocks can be used for synthesis of glycopeptides.[38] Herein we describe the use of an alternative approach for preparation of glycopeptides in which building blocks corresponding to the simple Tn and T antigens are first assembled into glycopeptides on the solid phase. Sialyltransferases and core 2 β1,6GlcNAc transferases, which operate with complete regio- and stereocontrol, are then used to convert these readily available glycopeptides into more complex ones that contain the sialyl-Tn and 2,3-sialyl-T antigens, as well as the core 2 trisaccharide. This cassette-like strategy[13,32] is illustrated by synthesis of glycopeptides from the tandem repeat domain of the mucin MUC1, as well as neoglycosylated derivatives of a T cell stimulating viral peptide. To accomplish syntheses of the target glycopeptides novel routes to Tn and T antigen building blocks have also been developed.

Results and Discussion

Synthesis of building blocks corresponding to the Tn (GalNAcα-Thr) and T [Galβ(1→3)GalNAcα-Thr] antigens started from **2** which was obtained by silylation of azido galactose **1**.[39] *N*-Bromosuccinimide/tetrabutylammonium triflate promoted[40] glycosylation of Fmoc-threonine allyl ester (**3**) with thioglycoside **2** gave **4** (50%, Scheme 1). The Tn building block **6**, which carries acid labile protective groups on the carbohydrate moiety, was then obtained by reductive acetylation of the azido

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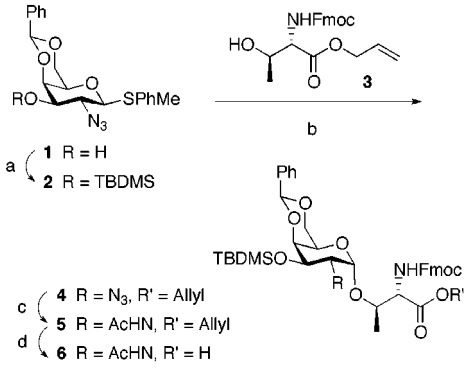
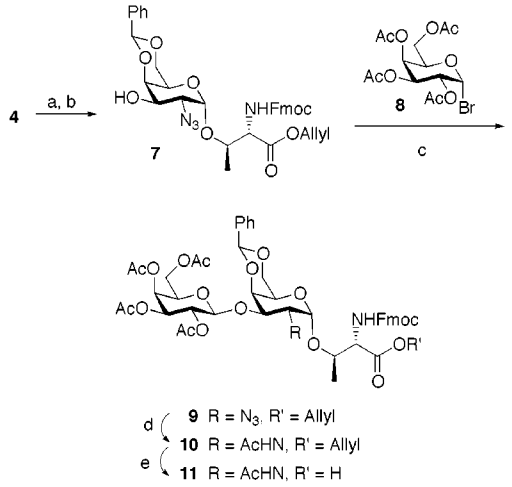
Scheme 1[a]



[a] Reagents (yields): (a) TBDMSOTf, pyridine, room temperature (93%); (b) NBS, QOTf, CH_2Cl_2, −28 °C (50%); (c) AcSH, pyridine, room temperature (80%); (d) $(PPh_3)_4Pd(0)$, *N*-methylaniline, THF, room temperature (85%).

Scheme 2[a]

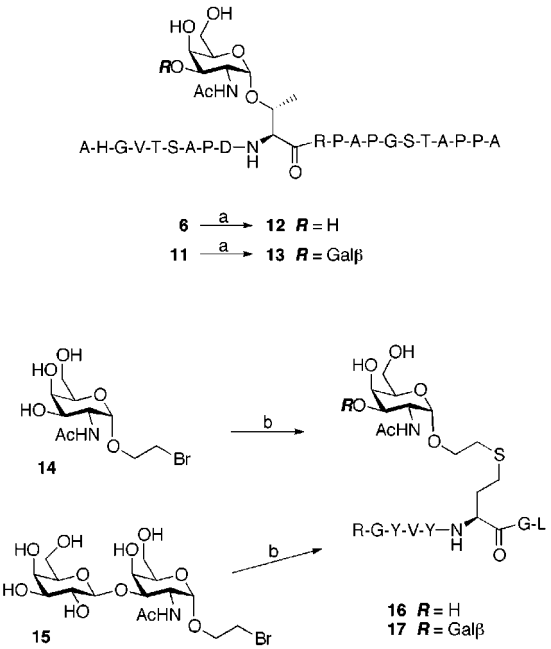


[a] Reagents (yields): (a) 80% aqueous TFA, 0 °C (97%); (b) α,α-dimethoxytoluene, *p*-TsOH (cat.), CH_3CN, room temperature (87%); (c) AgOTf, CH_2Cl_2-toluene (1:1), −30 °C (59%); (d) AcSH, pyridine, room temperature (86%); (e) $(PPh_3)_4Pd(0)$, *N*-methylaniline, THF, room temperature (88%).

group with thioacetic acid in pyridine,[41] followed by $(PPh_3)_4$-Pd(0) catalyzed[42] cleavage of the allyl ester. Glycoside **4** also served as a precursor for synthesis of the T antigen building block **11** (Scheme 2). Unfortunately, attempted cleavage of the TBDMS group in **4** with tetrabutylammonium fluoride in acetic acid,[13] or using zinc tetrafluoroborate,[43] failed. Instead, hydrolysis of both the TBDMS and the benzylidene group with aqueous TFA followed by reprotection with α,α-dimethoxytoluene gave **7**. Silver triflate mediated glycosylation of **7** with peracetylated galactosyl bromide **8** proceeded with excellent stereoselectivity to afford *β*-glycoside **9** as the only product (59%). Reductive acetylation and deprotection of the allyl ester was performed as for **4** to afford T building block **11**,[44,45] which is ready for use in solid-phase peptide synthesis.

After preparing the two building blocks we turned our attention to incorporating them, as well as the Tn and T antigen

Scheme 3[a]



[a] Reagents (yields): (a) Fmoc solid-phase peptide synthesis (23% for **12**, 19% for **13**); (b) R−G−Y−V−Y−**X**−G−L (**X** = homocysteine), Cs_2CO_3, DMF, room temperature (74% for **16**, 58% for **17**).

2-bromoethyl glycosides **14** and **15**,[46,47] into the two selected peptide backbones (Scheme 3). The first peptide represents the tandem repeat domain of the mucin MUC1 in which the glycosylated threonine is located in the center of the immunodominant region.[3] The second peptide is an analogue of a class I MHC restricted epitope from vesicular stomatitis virus nucleocapsid protein,[48] where the native Gln6 has been replaced by homocysteine. Incorporation of the Tn and T antigen building blocks **6** and **11** in the first peptide to give glycopeptides **12** and **13** was accomplished by Fmoc solid phase peptide synthesis on a TentaGel resin, using conditions reported previously by us.[31,49] Neoglycopeptides **16** and **17**, which carry the carbohydrate moieties of the Tn and T antigens, were prepared in solution by a convergent strategy involving akylation of the homocysteine residue of the viral peptide with 2-bromoethyl glycosides **14** and **15**.[46]

Having sufficient quantities of the Tn and T glycopeptides in hand, enzymatic extension of the carbohydrate moieties to provide the sialyl-Tn and 2,3-sialyl-T antigens, as well as the core 2 trisaccharide, was investigated (Schemes 4−6). First, recombinant mouse *N*-acetylgalactosamine α2−6 sialyltransferase[50] (ST6GalNAc-I) expressed in insect cells[5] was used to convert Tn glycopeptides **12** and **16** into the corresponding sialyl-Tn analogues (Scheme 4). On a semipreparative scale, incubation of **12** and **16** (each 0.5 mg) with recombinant ST6GalNAc-1 and CMP-Neu5Ac at pH 6.0 gave sialylated glycopeptides **18** and **19** with >90−95% conversion of **12** and

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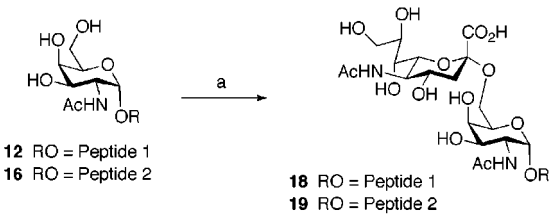
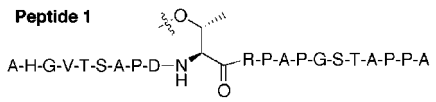
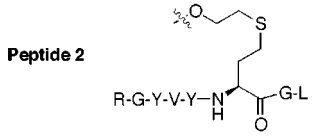
Scheme 4[a]

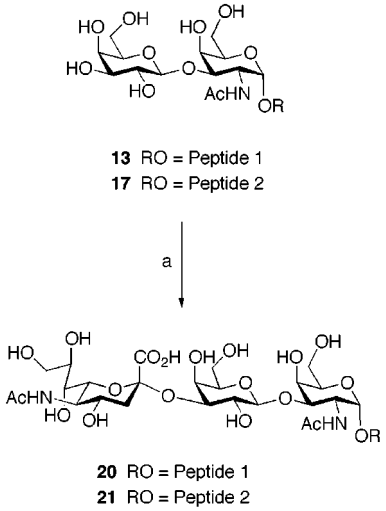






[a] Reagents (conversion and yield): (a) CMP-Neu5Ac, α2-6-sialyltransferase (ST6GalNAc-I), Bis-Tris buffer (20 mM, pH 6.0), 37 °C, <6 h (for **18**: >95% conversion of **12**, 74% isolated yield; for **19**: 90−95% conversion of **16**).

Scheme 5[a]

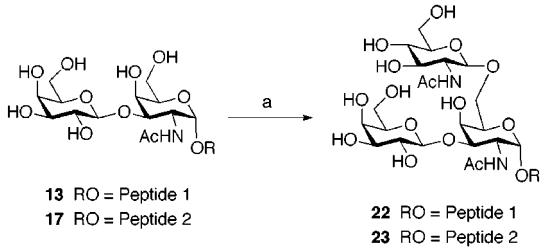


[a] Reagents (conversion and yield): (a) CMP-Neu5Ac, α2-3-sialyltransferase (ST3Gal-I), calf intestinal phosphatase, Tris-HCl buffer (25 mM, pH 6.5), 37 °C, <3 h (For **20**: >95% conversion of **13**, 94% isolated yield; for **21**: ∼95% conversion of **17**, 64% isolated yield).

16, as determined by nanoscale reversed-phase HPLC[51] in combination with MALDI-TOF mass spectrometry. When glycopeptide **12** was sialylated on a preparative scale (5.0 mg) **18** was obtained in 74% yield after purification by reversed-phase HPLC. To the best of our knowledge this is the first report describing the preparation of glycopeptides which contain the sialyl-Tn antigen based on enzymatic incorporation of the sialic acid moiety. Moreover, synthesis of **19** reveals that glycopeptides with nonnatural linkages between GalNAcα and the peptide moiety can be sialylated by ST6GalNAc-I.

Synthesis of glycopeptides **20** and **21** reveals that enzymatic α-(2→3)-sialylation of glycopeptides which contain the T antigen is also facile (Scheme 5). Incubation of either of glycopeptides **13** or **17** with human core 1-specific α2−3 sialyltransferase (ST3Gal-I,[37] obtained by expression in Sf9 cells), calf intestinal phosphatase, and CMP-Neu5Ac at pH 6.5 gave **20** and **21** with almost quantitative conversion (>95%) of the starting materials on a semipreparative scale (0.5 mg). When **13** and **17** were sialylated on a larger scale (2.0 and 3.5 mg, respectively) glycopeptides **20** and **21** could be isolated in 94 and 64% yields, respectively, after purification by reversed-phase HPLC. This reveals that ST3Gal-I, just as ST6GalNAc-I, accepts glycopeptide substrates in which the carbohydrate moiety does not need to be linked via serine or threonine to the peptide backbone.

Scheme 6[a]



[a] Reagents (conversion): (a) UDP-GlcNAc, core 2 *O*-glycan β1−6 *N*-acetylglucosaminyltransferase (C2GnT3), calf intestinal phosphatase, MES buffer (100 mM, pH 6.5) containing EDTA, D-galactono-1,5-lactone, and 2-acetamido-2-deoxy-D-glucono-1,5-lactone, 37 °C, ∼24 h for **22** and <3 h for **23** (>95% conversion of both **13** and **17**).

Finally, recombinant human core 2 *O*-glycan β1−6 *N*-acetylglucosaminyltransferase[52] (C2GnT3), expressed in insect cells, was used to convert the T antigen of glycopeptides **13** and **17** into the core 2 trisaccharide (Scheme 6). Initial studies with mucin derived glycopeptide **13** led to the formation of a 3:1 mixture of the expected product **22** and glycopeptide **12** which carries the Tn antigen. This was assumed to be due to a competing β-galactosidase activity in the C2GnT3 enzyme preparation leading to degradation of **13** in parallel with conversion of the T antigen into the core 2 trisaccharide. However, by inclusion of the galactosidase inhibitor, D-galactono-1,5-lactone, as well as the hexosaminidase inhibitor 2-acetamido-2-deoxy-D-glucono-1,5-lactone, degradation of **13** could be avoided. In the presence of these two inhibitors **13** and **17** could be transformed to **22** and **23**, respectively, by incubation with C2GnT3, UDP-GlcNAc, and calf intestinal phosphatase. On a semipreparative scale (0.6 mg) both **13** and **17** were completely converted to **22** and **23**, as determined by nanoscale reversed-phase HPLC in combination with MALDI-TOF mass spectrometry. Interestingly, conversion of neoglycopeptide **17** into **23** with C2GnT3 was found to be substantially faster than transformation of mucin-derived **13**. This is in contrast to use of the α2−6 and α2−3 sialyltransferases, where conversion of mucin glycopeptides **12** and **13** was faster than for neoglycopeptides **16** and **17**. The observation that all three transferases were able to glycosylate neoglycopeptides **16** and **17** suggests that these enzymes may find wide use for synthesis of (neo)glycopeptides with substantial structural variation in the peptide moiety.

Previously, chemoenzymatic approaches to glycopeptides have predominantly concerned synthesis of *N*-linked glycopeptides[53−56] and glycopeptides which carry the sialyl Lewis

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X tetrasaccharide, or fragments thereof.[57−61] As described herein chemoenzymatic synthesis is an attractive approach also for preparation of glycopeptides which contain the tumor associated sialyl-Tn and 2,3-sialyl-T antigens, as well as the core 2 trisaccharide. As revealed by the examples given above, both natural and nonnatural glycopeptides can be prepared since the glycosyl transferases show little, if any, specificity for the peptide moiety. The approach is based on the fact that cassette-like building blocks corresponding to the Tn and T antigens can be readily prepared via chemical synthesis. Such building blocks allow site-specific incorporation of the carbohydrate moieties at selected positions in a polypeptide, in contrast to enzymes belonging to the family of GalNAc-transferases[5] which constitute potential alternatives. Introduction of sialic acid, which is difficult when performed chemically at the building block level, is then carried out in solution after cleavage of the glycopeptide from the solid phase. Performing the enzymatic step in solution avoids problems associated with penetration of the enzyme into the cross-linked solid support,[62] as well as incompatibilities between enzyme and solid support or the linker used as attachment for the glycopeptide.[57] Studies directed toward chemoenzymatic synthesis of more complex mucin-derived glycopeptides, as well as attempts to use the glycopeptides described herein for development of cancer vaccines, are underway in our laboratories.

Experimental Section

General Methods and Materials. All reactions were carried out under an inert nitrogen atmosphere with dry solvents, under anhydrous conditions, unless otherwise stated. CH_2Cl_2 was dried by distillation from calcium hydride whereas THF and toluene were distilled from sodium benzophenone. Pyridine was dried over 4 Å molecular sieves. DMF was distilled and then dried over flame dried 3 Å molecular sieves. Organic solutions were dried over anhydrous Na_2SO_4 before being concentrated. TLC was performed on silica gel 60 F_{254} (Merck) with detection by UV light or charring with aqueous sulfuric acid (10%). Flash column chromatography was performed on silica gel (Matrex, 60 Å, 35−70 μm, Grace Amicon) with distilled solvents. Analytical reversed-phase HPLC was performed on a Kromasil C-8 column (250 × 4.6 mm, 5 μm, 100 Å), eluted with a linear gradient of MeCN in H_2O containing 0.1% TFA (flowrate of 1.5 mL/min, detection at 214 nm). Preparative reversed-phase HPLC was performed on a Kromasil C-8 column (250 × 20 mm^2, 5 μm, 100 Å) with the same solvent system (flowrate of 11 mL/min, detection at 214 nm).

The ^{1}H and ^{13}C NMR spectra for **2**−**11** were recorded at 400 and 100 MHz, respectively, for solutions in $CDCl_3$ [residual $CHCl_3$ (δ_H 7.26 ppm) or $CDCl_3$ (δ_C 77.0 ppm) as internal standard], CD_3OD [residual CD_2HOD (δ_H 3.35 ppm) or CD_3OD (δ_C 49.0 ppm) as internal standard], or in a 1:1 mixture of CD_3OD and $CDCl_3$ [residual CD_2HOD (δ_H 3.35 ppm) or CD_3OD (δ_C 49.0 ppm) as internal standard] or DMSO-d_6 [residual $(CH_3)_2SO$ (δ_H 2.50 ppm) or $(CH_3)_2SO$ (δ_C 39.5 ppm) as internal standard] at 298 K. Proton resonances were assigned from appropriate combinations of COSY, NOESY, and TOCSY experiments. Optical rotations were recorded on a Perkin-Elmer 343 polarimeter.

4-Methylphenyl 2-azido-2-deoxy-1-thio-β-D-galactopyranoside was prepared from 3,4,6-tri-*O*-acetyl-2-azido-2-deoxy-α-D-galactopyranosyl bromide[63,64] as described previously.[31] 2,3,4,6-Tetra-*O*-acetyl-β-D-galactopyranosyl bromide **8** was prepared from peracetylated galactose by treatment with HBr in $HOAc/Ac_2O$ at 0 °C. L-Arginylglycyl-L-tyrosyl-L-valyl-L-tyrosyl-L-homocysteinylglycyl-L-leucine[46] was prepared manually by Fmoc solid-phase synthesis in a mechanically agitated reactor with standard conditions.[65] 2-Bromoethyl 2-acetamido-2-deoxy-α-D-galactopyranoside (**14**) and 2-bromoethyl 2-acetamido-2-deoxy-3-*O*-β-D-galactopyranosyl-α-D-galactopyranoside (**15**) were prepared as described.[47] Recombinant mouse *N*-acetylgalactosamine α2−6 sialyltransferase[50] (ST6-GalNAc-I) was expressed in insect cells. The enzyme was purified by sequential ion-exchange chromatographies on Amberlite (IRA95, Sigma) and SP-sepharose (Sigma) essentially as described.[5] Purified samples were concentrated in a centrifugation cartridge (Sigma) before use. Human core 1-specific α2−3 sialyltransferase[66] (ST3Gal-I) was expressed and partially purified as described.[37] Final purification of ST3Gal-I was performed on MiniS (PC3.2/3) with use of the Smart system (Pharmacia). Recombinant human core 2 *O*-glycan β1−6 *N*-acetylglucosaminyltransferase (C2GnT3) was expressed in insect cells and purified by sequential ion-exchange chromatography as described.[52] Purified samples were concentrated in Biomax-10 Ultrafree cartridges (Millipore) before use.

4-Methylphenyl 2-Azido-4,6-*O*-benzylidene-2-deoxy-1-thio-β-D-galactopyranoside (1). A solution of 4-methylphenyl 2-azido-2-deoxy-1-thio-β-D-galactopyranoside[31] (2.98 g, 9.6 mmol) and α,α-dimethoxytoluene (2.00 g, 13.1 mmol) in acetonitrile (60 mL) was stirred at room temperature in the presence of a catalytic amount of *p*-toluenesulfonic acid (monohydrate, 50 mg) for 2 h. The solution was concentrated and flash column chromatography of the residue (toluene/EtOAc, 9:1→3:2) gave **1** (3.69 g, 97%) as a white solid. ^{1}H and ^{13}C NMR data were consistent with those reported previously.[39]

4-Methylphenyl 2-Azido-4,6-*O*-benzylidene-3-*O*-*tert*-butyldimethylsilyl-2-deoxy-1-thio-β-D-galactopyranoside (2). *tert*-Butyldimethylsilyl triflate (1.73 g, 1.5 mL, 6.55 mmol) was added dropwise to an ice cold and stirred solution of **1** (1.47 g, 3.68 mmol) in pyridine (15 mL), containing a catalytic amount of DMAP (15 mg). After 2 h, the solution was allowed to attain room temperature and was then stirred for a further 24 h. It was diluted with methanol (25 mL) and the solvents were evaporated under vacuum after which the residue was dissolved in CH_2Cl_2 (150 mL). The solution was washed with cold aqueous saturated $NaHCO_3$ (50 mL), followed by brine (50 mL), and then dried. Concentration followed by flash column chromatography of the residue (toluene/EtOAc, 100:1) gave **2** (1.75 g, 93%) as a white amorphous solid. $[\alpha]_D^{20}$ −40.8° (*c* 0.99, $CHCl_3$); ^{1}H NMR ($CDCl_3$) δ 7.60 (2H, d, ArH), 7.47−7.36 (5H, m, ArH), 7.01 (2H, d, ArH), 5.59 (1H, s, C*H*Ph), 4.43−4.35 (2H, m, H-1 and H-6), 4.05−3.96 (2H, m, H-3 and H-6), 3.66−3.58 (2H, m, H-4 and H-2), 3.47 (1H, m, H-5), 2.32 (3H, s, $ArCH_3$), 0.88 (9H, s, t-BuSi), 0.13 and 0.09 (6H, 2s, $Si(CH_3)_2$); ^{13}C NMR ($CDCl_3$) δ 138.4, 137.8, 134.3, 129.7, 128.9, 128.0, 126.6, 126.3, 100.7, 85.4, 75.6, 74.4, 69.8, 69.3, 61.7, 25.6, 21.3; HRMS (FAB): calcd for $C_{26}H_{35}N_3O_4SSi$ ($M + H^+$) 514.2196, found 514.2194.

***N*$^\alpha$-Fluoren-9-ylmethoxycarbonyl-L-threonine Allyl Ester (3).** Compound **3** was prepared by modification of a known procedure.[67] Thus, a solution of Fmoc-Thr-OH (2.0 g, 5.9 mmol) in aqueous ethanol (80%, 17 mL) was titrated with a solution of Cs_2CO_3 (1.0 g, 3.1 mmol, 25% in water) to pH 7 with bromothymol blue as indicator. The solvents were evaporated, the residue was co-concentrated with absolute ethanol (4 × 25 mL) and then dried under vacuum overnight. The cesium salt thus obtained was suspended in dry DMF (20 mL), cooled to 0 °C, and treated with allyl bromide (7.08 g, 58.6 mmol) by dropwise addition over 10 min. After 30 min the solution was allowed to attain room

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temperature and stirring was continued for a further 3 h. The solids were filtered off, the filtrate was concentrated, and the residue was suspended in water. The aqueous phase was extracted with CH_2Cl_2 (3 × 25 mL) and the combined organic layers were dried and concentrated. Flash column chromatography of the residue (toluene/EtOAc, 1:1) gave **3**[42,68] (2.0 g, 90%) as a white amorphous solid.

***N*α-Fluoren-9-ylmethoxycarbonyl-3-*O*-(2-azido-4,6-*O*-benzylidene-3-*O*-*tert*-butyldimethylsilyl-2-deoxy-α-D-galactopyranosyl)-L-threonine Allyl Ester (4).** A solution of *N*-bromosuccinimide (600 mg, 3.37 mmol) and tetrabutylammonium triflate[40] (328 mg, 0.84 mmol) in CH_2Cl_2 (30 mL) was added, dropwise, during 5 min to a stirred solution of **2** (1.72 g, 3.35 mmol) and **3** (2.68 g, 7.03 mmol) in CH_2Cl_2 (70 mL) at −28 °C. The solution was stirred at this temperature until TLC indicated completion of the reaction (toluene/EtOAc, 10:1, $R_F = 0.3$). The reaction was quenched by adding triethylamine (2.5 mL) and the solution was allowed to attain room temperature. Concentration followed by flash column chromatography of the residue (toluene/EtOAc, 10:1, containing 0.5% triethylamine) gave **4** (1.28 g, 50%) as a colorless amorphous solid. $[\alpha]_D^{20}$ +98.0° (*c* 1.1, $CHCl_3$); ^{1}H NMR ($CDCl_3$) δ 7.79 (2H, d, ArH), 7.67−7.59 (2H, m, ArH), 7.55−7.49 (2H, m, ArH), 7.45−7.31 (7H, m, ArH), 6.01−5.89 (1H, m, OCH_2CH═CH_2), 5.85 (1H, d, J = 9.3 Hz, NH-α), 5.39 (1H, dd, J = 17.2, 1.4 Hz, OCH_2CH═CH_2), 5.27 (1H, dd, J = 10.4, 1.2 Hz, OCH_2CH═CH_2), 5.04 (1H, d, J = 3.6 Hz, H-1), 4.69 (2H, d, J = 5.9 Hz, OCH_2CH═CH_2), 4.50 (1H, dd, J = 9.9, 6.6 Hz, $CHCH_2OCO$), 4.46 (1H, dd, J = 6.5, 2.4 Hz, H-β), 4.41 (1H, dd, J = 9.3, 2.4 Hz, H-α), 4.36−4.25 (3H, m, $CHCH_2OCO$, H-6 and H-4), 4.14−4.05 (3H, m, $CHCH_2OCO$, H-6 and H-3), 3.79−3.71 (2H, m, H-2 and H-5), 3.28 (3H, d, J = 6.4 Hz, γ-CH_3), 0.95 (9H, s, t-BuSi), 0.22 and 0.17 (6H, 2s, $Si(CH_3)_2$); ^{13}C NMR ($CDCl_3$) δ 176.5, 170.1, 143.8, 141.4, 137.7, 133.9, 131.5, 130.2, 129.0, 128.2, 127.8, 127.2, 127.1, 126.1, 125.3, 125.2, 120.1, 119.4, 100.7, 99.7, 76.5, 76.1, 69.4, 68.7, 67.4, 66.6, 63.6, 61.0, 58.9, 47.3, 28.7, 25.8, 19.0, 18.1, 4.2, 4.6; HRMS (FAB): calcd for $C_{41}H_{51}N_4O_9Si$ (M + H^+) 771.3425, found 771.3400.

***N*α-Fluoren-9-ylmethoxycarbonyl-3-*O*-(2-acetamido-4,6-*O*-benzylidene-3-*O*-*tert*-butyldimethylsilyl-2-deoxy-α-D-galactopyranosyl)-L-threonine Allyl Ester (5).** Freshly distilled (5 times) thioacetic acid (8 mL) was added dropwise to a stirred solution of **4** (285 mg, 370 μmol) in pyridine (8 mL) at 0 °C and the solution was allowed to attain room temperature. After 4 h, toluene (20 mL) was added and the solvents were evaporated. The residue was co-concentrated twice from toluene. Flash column chromatography of the residue (toluene/EtOAc, 6:2) gave **5** (233 mg, 80%) as a white amorphous solid. $[\alpha]_D^{20}$ +89.6° (*c* 0.66, $CHCl_3$); ^{1}H NMR (DMSO-d_6) δ 7.91 (1H, d, ArH), 7.73 (1H, d, ArH), 7.60 (1H, d, J = 9.7 Hz, NH-α), 7.47−7.23 (12H, m, ArH and $NHCOCH_3$), 5.94−5.81 (1H, m, OCH_2CH═CH_2), 5.83 (1H, s, C*H*Ph), 5.33 (1H, dd, J = 17.2, 1.6 Hz, OCH_2CH═CH_2), 5.25 (1H, dd, J = 10.4, 1.5 Hz, OCH_2CH═CH_2), 4.68 (1H, d, J = 3.8 Hz, H-1), 4.56−4.47 (4H, m, OCH_2CH═CH_2 and $CHCH_2OCO$), 4.37−4.24 (3H, m, $CHCH_2OCO$, H-α and H-β), 4.18 (1H, m, H-4), 4.12−4.99 (3H, m, H-2 and H-6), 3.88 (1H, dd, J = 10.9, 3.5 Hz, H-3), 3.68 (1H, bs, H-5), 1.81 (3H, s, $NHCOCH_3$), 1.10 (1H, d, J = 6.4 Hz, γ-CH_3), 0.80 (9H, s, t-BuSi), 0.03 and 0.02 (6H, 2s, $Si(CH_3)_2$); ^{13}C NMR (DMSO-d_6) δ 169.7, 168.7, 156.7, 143.7, 143.6, 140.8, 140.7, 138.4, 131.9, 128.8, 128.6, 128.2, 127.9, 126.6, 127.0, 125.9, 125.1, 120.2, 120.1, 118.6, 99.7, 99.6, 75.6, 74.4, 68.5, 67.9, 65.5, 65.2, 62.8, 58.5, 48.7, 46.8, 25.8, 22.9, 18.8, 17.8, −4.4, −4.6; HRMS (FAB): calcd for $C_{43}H_{54}N_2O_{10}SiNa$ (M + Na^+) 809.3445, found 809.3438.

***N*α-Fluoren-9-ylmethoxycarbonyl-3-*O*-(2-acetamido-4,6-*O*-benzylidene-3-*O*-*tert*-butyldimethylsilyl-2-deoxy-α-D-galactopyranosyl)-L-threonine (6).** A solution of allyl ester **5** (120 mg, 153 μmol), $(PPh_3)_4Pd(0)$ (16 mg, 14 μmol), and *N*-methylaniline (62 mg, 580 μmol) in THF (4 mL) was stirred at room temperature for 1 h in the absence of light. The solution was diluted with EtOAc (50 mL) and washed with saturated aqueous ammonium chloride (5 mL) and the aqueous phase was extracted with EtOAc (2 × 15 mL). The organic phases were combined, dried, and concentrated. Flash column chromatography of the residue ($CHCl_3$/MeOH, 40:1) gave **6** (97 mg, 85%) as an amorphous pale yellow solid. $[\alpha]_D^{20}$ +123° (*c* 0.72, $CHCl_3$); ^{1}H NMR ($CDCl_3$:CD_3OD) δ 7.82 (2H, d, ArH), 7.71 (3H, m, ArH), 7.55 (3H, m, ArH), 7.47−7.29 (8H, m, ArH), 5.61 (1H, s, C*H*Ph), 4.99 (1H, d, J = 3.0 Hz, H-1), 4.65−4.48 (2H, m, $CHCH_2OCO$), 4.48−4.36 (2H, m, H-2 and H-β), 4.35−4.12 (4H, m, $CHCH_2OCO$, H-6, H-6′, and H-4), 3.99 (1H, m, H-α), 3.79 (1H, bs, H-5), 1.99 (3H, s, $NHCOCH_3$), 1.25 (3H, d, J = 6.2 Hz, γ-CH_3), 0.89 (9H, s, t-BuSi), 0.11 (6H, s, $Si(CH_3)_2$); ^{13}C NMR ($CDCl_3$:CD_3OD) δ 173.4, 172.6, 158.8, 145.0, 144.8, 142.4, 139.1, 129.7, 128.8, 128.6, 128.0, 127.2, 125.9, 125.8, 120.8, 120.8, 101.9, 101.2, 79.1, 78.8, 78.5, 77.3, 76.6, 70.2, 69.6, 67.5, 64.4, 59.6, 56.7, 48.3, 26.1, 23.3, 19.4, 18.8, −3.9; HRMS (FAB): calcd for $C_{40}H_{50}N_2O_{10}SiNa$ (M + Na^+) 769.3132, found 769.3121. Anal. Calcd for $C_{40}H_{50}N_2O_{10}Si$: C 63.3; H 6.8; N 3.8. Found: C 63.6; H 6.8; N 3.8.

***N*α-Fluoren-9-ylmethoxycarbonyl-3-*O*-(2-azido-4,6-*O*-benzylidene-2-deoxy-α-D-galactopyranosyl)-L-threonine Allyl Ester (7).** An ice cold solution of TFA in water (25 mL, 80%) was added to an ice cold and stirred solution of **4** (1.24 g, 1.61 mmol) in CH_2Cl_2 (5 mL) during 5 min. After 8 h at 0 °C, cold CH_2Cl_2 (50 mL) was added and the solution was neutralized with cold saturated aqueous $NaHCO_3$. The phases were separated and the aqueous phase was successively extracted with CH_2Cl_2 (3 × 50 mL) and EtOAc (3 × 50 mL). The combined organic phases were washed with brine (50 mL), dried, and concentrated. Flash column chromatography of the residue ($CHCl_3$/MeOH, 9:1) gave *N*α-fluoren-9-ylmethoxycarbonyl-3-*O*-(2-azido-2-deoxy-α-D-galactopyranosyl)-L-threonine allyl ester (881 mg, 97%) as a gum. A solution of *N*α-fluoren-9-ylmethoxycarbonyl-3-*O*-(2-azido-2-deoxy-α-D-galactopyranosyl)-L-threonine allyl ester (760 mg, 1.33 mmol), α,α-dimethoxytoluene (407 mg, 2.67 mmol), and a catalytic amount of *p*-toluenesulfonic acid (monohydrate, 30 mg) in CH_3CN (18 mL) was stirred at room temperature for 24 h. Concentration of the mixture followed by flash column chromatography of the residue (toluene/EtOAc, 5:1) gave **7** (762 mg, 84% from **4**) as a white amorphous solid. $[\alpha]_D^{20}$ +110° (*c* 0.90, $CHCl_3$); ^{1}H NMR ($CDCl_3$) δ 7.77 (2H, d, ArH), 7.64−7.60 (2H, m, ArH), 7.51−7.46 (2H, m, ArH), 7.45−7.30 (7H, m, ArH), 5.95 (1H, m, OCH_2CH═CH_2), 5.74 (1H, d, J = 9.4 Hz, NHFmoc), 5.59 (1H, s, C*H*Ph), 5.34 (1H, dd, J = 17.1, 1.1 Hz, OCH_2CH═CH_2), 5.28 (1H, dd, J = 10.4, 1.1 Hz, OCH_2CH═CH_2), 5.07 (1H, d, J = 3.4 Hz, H-1), 4.71 (2H, d, J = 5.9 Hz, OCH_2CH═CH_2), 4.59−4.41 (3H, m, $CHCH_2OCO$, H-α and H-β), 4.37 (1H, m, $FmocCH_2$), 4.33−4.20 (3H, m, H-6, $CHCH_2OCO$ and H-4), 3.80 (1H, bs, H-5), 3.62 (1H, dd, J = 10.6 and 3.5 Hz, H-2), 2.47 (1H, d, J = 11.0 Hz, 3-OH), 1.33 (3H, d, J = 6.4 Hz, γ-CH_3); ^{13}C NMR ($CDCl_3$) δ 169.9, 156.8, 143.9, 143.7, 141.3, 137.8, 131.3, 129.4, 129.0, 129.0, 128.6, 128.6, 128.4, 128.2, 127.7, 127.7, 127.1, 127.1, 126.2, 120.0, 119.9, 119.3, 101.3, 99.6, 76.4, 75.3, 69.1, 66.5, 63.3, 61.2, 58.8, 47.2, 18.8; HRMS (FAB): calcd for $C_{35}H_{36}N_4O_9Na$ (M + Na^+) 679.2380, found 679.2368.

***N*α-Fluoren-9-ylmethoxycarbonyl-3-*O*-[2-azido-4,6-*O*-benzylidene-2-deoxy-3-*O*-(2,3,4,6-tetra-*O*-acetyl-β-D-galactopyranosyl)-α-D-galactopyranosyl]-L-threonine Allyl Ester (9).** A solution of silver triflate (165 mg, 642 μmol) in toluene (3 mL) was added dropwise to a suspension of **7** (300 mg, 457 μmol), 2,3,4,6-tetra-*O*-acetyl-α-D-galactopyranosyl bromide **8** (245 mg, 596 mmol), and crushed molecular sieves (flame dried, 4 Å, 400 mg) in CH_2Cl_2 (6 mL) at −30 °C. After 1 h, the reaction was quenched by addition of pyridine (1 mL) while keeping the temperature below −30 °C. After 5 min, the mixture was allowed to attain room temperature and the solids were removed by filtration (Hyflow, Supercel) and washed with CH_2Cl_2 (5 × 10 mL). The combined filtrates were washed with a mixture of aqueous $Na_2S_2O_3$ (0.5 M) and saturated aqueous $NaHCO_3$ (1:1, 2 × 12 mL), dried, and concentrated. Flash column chromatography of the residue (toluene/EtOAc, 3:1) gave **9** (265 mg, 59%) as a white amorphous solid. $[\alpha]_D^{20}$ +51.7° (*c* 1.00, $CHCl_3$); ^{1}H NMR ($CDCl_3$) δ 7.78 (2H, d, ArH), 7.66−7.60 (2H, m, ArH), 7.56−7.50 (2H, m, ArH), 7.45−7.30 (7H, m, ArH), 5.96 (1H, m, OCH_2CH═CH_2), 5.75 (1H, d, J = 9.5 Hz, NHFmoc), 5.57 (1H, s, C*H*Ph), 5.43 (1H, m, H-4′), 5.41−5.26 (3H, m, OCH_2CH═CH_2 and H-2′), 5.10−5.05 (1H, m, H-3′), 5.04 (1H, d, J = 3.1 Hz, H-1), 4.82 (1H, d, J = 8.0 Hz, H-1′), 4.70 (2H, m, OCH_2CH═CH_2), 4.54 (1H, dd, J = 10.3, 6.8 Hz, $CHCH_2OCO$), 4.50 (1H, m, H−β), 4.46 (1H, m, H-α), 4.41 (1H, m, H-4), 4.36 (1H, m,

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CHCH_2OCO), 4.32−4.18 (3H, m, H-6, CHCH$_2$OCO and H-6′), 4.18−4.10 (1H, m, H-6′), 4.10−4.00 (2H, m, H-3 and H-6), 3.99−3.93 (1H, m, H-5′), 3.84 (1H, dd, J = 10.9, 3.6 Hz, H-2), 3.73 (1H, m, H-5), 2.18, 2.07, 2.05, and 2.00 (12H, 4s, 4 Ac), 1.35 (3H, d, J = 6.2 Hz, γ-CH$_3$); ^{13}C NMR (CDCl$_3$) δ 170.3, 170.3, 170.1, 169.9, 169.4, 156.7, 143.8, 143.6, 141.3, 137.5, 131.2, 128.2, 127.8, 127.1, 127.0, 126.1, 125.1, 125.1, 120.0, 119.5, 102.4, 100.7, 99.7, 76.2, 75.8, 75.6, 71.0, 70.9, 69.1, 68.6, 67.3, 66.9, 66.6, 63.5, 61.3, 59.1, 58.7, 53.8, 47.1, 29.2, 20.7, 20.6, 18.9; HRMS (FAB): calcd for $C_{49}H_{54}N_4O_{18}Na$ (M + Na$^+$) 1009.3331, found 1009.3337.

N^{α}-Fluoren-9-ylmethoxycarbonyl-3-*O*-[2-acetamido-4,6-*O*-benzylidene-2-deoxy-3-*O*-(2,3,4,6-tetra-*O*-acetyl-β-D-galactopyranosyl)-α-D-galactopyranosyl]-L-threonine Allyl Ester (10). Freshly distilled (5 times) thioacetic acid (5 mL) was added dropwise to an ice cold solution of **9** (210 mg, 213 μmol) in pyridine (5 mL) and the solution was allowed to attain room temperature. After 8 h, the solvents were evaporated and the residue was co-concentrated with toluene (3 × 20 mL). Flash column chromatography of the residue (toluene/EtOAc, 3:2) gave **10** (184 mg, 86%) as a white amorphous solid. $[\alpha]_D^{20}$ +73.1° (*c* 0.70, CHCl$_3$); ^{1}H NMR (CDCl$_3$) δ 7.78 (2H, d, ArH), 7.65−7.58 (2H, m, ArH), 7.54 (2H, dd, ArH), 7.45−7.30 (7H, m, ArH), 5.88 (1H, m, OCH$_2$CH=CH$_2$), 5.72 (1H, d, J = 9.2 Hz, NHCOCH$_3$), 5.64 (1H, d, J = 9.3 Hz, NHFmoc), 5.56 (1H, m, H-4′), 5.39−5.24 (2H, m, OCH$_2$CH=CH_2), 5.20 (1H, dd, J = 10.4, 7.9 Hz, H-2′), 5.00 (1H, m, H-3′), 4.98 (1H, d, J = 2.9 Hz, H-1), 4.77 (1H, d, J = 7.7 Hz, H-1′), 4.73−4.46 (3H, m, H-2 and H-α), 4.36−4.18 (2H, m, H-β and H-4), 4.01−3.86 (2H, m, H-5′ and H-3), 2.16 (3H, s, NHCOCH$_3$), 2.15, 2.04, 1.98, 1.95, and 1.90 (15H, 5s, 5 Ac), 1.29 (3H, d, J = 6.4 Hz, γ-CH$_3$); ^{13}C NMR (CDCl$_3$) δ 170.6, 170.5, 170.3, 170.2, 169.8, 169.5, 156.5, 143.6, 141.3, 137.5, 130.8, 128.9, 128.2, 127.9, 127.1, 126.2, 124.9, 120.1, 120.1, 120.0, 101.0, 100.7, 100.4, 76.4, 75.5, 73.8, 71.0, 70.8, 69.1, 68.8, 67.0, 66.4, 63.6, 61.4, 58.6, 48.0, 47.2, 20.7, 20.6, 18.7; HRMS (FAB): calcd for $C_{51}H_{58}N_2O_{19}Na$ (M + Na$^+$) 1025.3532, found 1025.3551.

N^{α}-Fluoren-9-ylmethoxycarbonyl-3-*O*-[2-acetamido-4,6-*O*-benzylidene-2-deoxy-3-*O*-(2,3,4,6-tetra-*O*-acetyl-β−D-galactopyranosyl)-α−D-galactopyranosyl]-L-threonine (11). A solution of allyl ester **10** (160 mg, 160 μmol), (PPh$_3$)$_4$Pd(0) (19 mg, 16 μmol), and *N*-methylaniline (62 mg, 580 μmol) in THF (4 mL) was stirred at room temperature for 1 h in the absence of light. The solution was diluted with EtOAc (50 mL) and washed with saturated aqueous ammonium chloride (5 mL). The aqueous phase was extracted with EtOAc (2 × 15 mL) and the combined organic phases were dried and then concentrated. Flash column chromatography of the residue (CHCl$_3$/MeOH/AcOH, 92:7:1) gave **11** (135 mg, 88%) as an amorphous pale yellow solid. $[\alpha]_D^{20}$ +98.4° (*c* 0.63, CHCl$_3$); ^{1}H NMR (CDCl$_3$) δ 7.82 (1H, d, ArH), 7.69 (1H, d, ArH), 7.56−7.51 (2H, m, ArH), 7.47−7.30 (8H, m, ArH), 5.61 (1H, s, CHPh), 5.39 (1H, m, H-4′), 5.16 (1H, dd, J = 10.5, 8.0 Hz, H-2′), 5.04−4.86 (2H, m, H-3′ and H-1), 4.73 (1H, d, J = 7.9 Hz, H-1′), 4.61 (2H, m, H-2 and H-α), 4.46−4.42 (1H, m, H-β), 3.89 (1H, dd, J = 11.3, 3.13 Hz, H-3), 3.78−3.71 (1H, m, H-5), 2.18, 2.06, 2.04, 1.99, and 1.98 (15H, 5s, 5CH$_3$CO), 1.25 (1H, d, J = 6.4 Hz, γ-CH$_3$); ^{13}C NMR (CDCl$_3$) δ 173.6, 171.8, 171.3, 170.3, 170.1, 169.4, 157.1, 143.4, 143.2, 140.8, 137.2, 128.3, 127.6, 127.4, 127.2, 126.6, 125.7, 124.3, 119.4, 101.3, 100.2, 99.5, 70.6, 69.9, 68.6, 68.3, 66.6, 66.1, 62.9, 60.7, 57.9, 46.8, 21.9, 19.9, 19.8, 19.7, 19.6, 18.2; HRMS (FAB): calcd for $C_{48}H_{54}N_2O_{19}Na$ (M + Na$^+$) 985.3219, found 985.3248. Anal. Calcd for $C_{48}H_{54}N_2O_{19}$: C 59.8; H 5.7; N 2.9. Found C 59.3; H 5.7; N 2.8.

General Procedure for Solid-Phase Synthesis of Glycopeptides 12 and 13. A TentaGel S Trt-Ala-Fmoc resin (Rapp Polymere, Germany) was used for the synthesis of glycopeptides **12** and **13**. N^{α}-Fmoc-amino acids (Bachem, Switzerland) with the following side chain protecting groups were used in the synthesis: 2,2,5,7,8-pentamethylchroman-6-sulfonyl (Pmc) for arginine; triphenylmethyl (Trt) for histidine, and *tert*-butyl (*t*Bu) for aspartic acid, serine, and threonine. DMF was distilled before being used.

Glycopeptides **12** and **13** were synthesized with 78 and 90 mmol of resin, respectively**.** Couplings were performed manually in a mechanically agitated reactor. Fmoc amino acids (4 equiv) were activated as benzotriazolyl esters by using 1,3-diisopropylcarbodyimide (DIC, 3.9 equiv) and 1-hydroxybenzotriazole (HOBt, 6 equiv) in dry DMF. Acylations were monitored by using bromophenol blue as indicator[69] (the color of the resin changes from blue to yellow). N^{α}-Fmoc deprotections were performed by a flow of piperidine in DMF (20%) for 3 min and then by shaking for 7 min. Before and after treatment with piperidine the resin was washed 5 times with DMF. Glycosylated amino acid building blocks **6** (70 mg, 94 mmol) and **11** (110 mg, 114 mmol) were activated in dry DMF (1 mL) at room temperature during 35 min by addition of 1,3-diisopropylcarbodiimide (1.1 equiv) and 1-hydroxy-7-azabenzotriazole (HOAt, 3 equiv), respectively. The activated esters were then coupled to the peptide resins during 24 h. After coupling of building blocks **6** and **11**, unreacted peptide N^{α} amino groups were capped by addition of a 1:1 mixture of acetic anhydride and DMF.

After completion of the synthesis, the resin was washed with CH$_2$Cl$_2$ (5 times) and dried under vacuum. Cleavage from the resin and removal of acid labile protective groups was performed with TFA/H$_2$O/thioanisole/ethanedithiol (87.5:5:5:2.5, 20 mL/200 mg of resin) for 3−3.5 h, followed by filtration. Acetic acid (15 mL) was added to the filtrate which was then concentrated. The residue was co-concentrated several times with acetic acid until it formed a thin film. It was then washed with diethyl ether (3 times), dissolved in a mixture of water and acetic acid (6:1), and freeze-dried. Purification by preparative reversed-phase HPLC gave glycopeptides **12** and **13**.

L-Alanyl-L-histidinylglycyl-L-valyl-L-threonyl-L-seryl-L-alanyl-L-prolyl-L-aspartyl-3-*O*-(2-acetamido-2-deoxy-α-D-galactopyranosyl)-L-threonyl-L-arginyl-L-prolyl-L-alanyl-L-prolylglycyl-L-seryl-L-threonyl-L-alanyl-L-prolyl-L-prolyl-L-alanine (12). Synthesis, cleavage of the resin-bound glycopeptide (78 mmol) with simultaneous deprotection, and then purification by reversed-phase HPLC (gradient 0→100% CH$_3$CN in H$_2$O, both containing 0.1% TFA, during 80 min), according to the general procedure, gave **12** (35 mg, 78% peptide content, 23% overall yield). MS (FAB): calcd for $C_{91}H_{145}N_{27}O_{34}$ (M + H)$^+$ 2161, found 2162. Amino acid analysis: Ala 5.00 (5), Arg 1.00 (1), Asp 1.02 (1), Gly 2.08 (2), His 1.01 (1), Pro 5.00 (5), Ser 1.99 (2), Thr 2.91 (3), Val 1.00 (1).

L-Alanyl-L-histidinylglycyl-L-valyl-L-threonyl-L-seryl-L-alanyl-L-prolyl-L-aspartyl-3-*O*-(2-acetamido-2-deoxy-3-*O*-β-D-galactopyranosyl-α-D-galactopyranosyl)-L-threonyl-L-arginyl-L-prolyl-L-alanyl-L-prolylglycyl-L-seryl-L-threonyl-L-alanyl-L-prolyl-L-prolyl-L-alanine (13). Synthesis, cleavage of the resin-bound glycopeptide (90 μmol), with simultaneous deprotection, and then purification by reversed-phase HPLC (gradient 0→100% CH$_3$CN in H$_2$O, both containing 0.1% TFA, during 60 min), according to the general procedure, gave the target glycopeptide which carried *O*-acetyl groups on the Galβ-moiety [56 mg, MS (FAB): calcd (M + H)$^+$ 2490.14 , found 2492.14]. Methanolic sodium methoxide (233 μL, 0.2 M) was added to a solution of this *O*-acetylated glycopeptide (23 mg) in methanol (23 mL) and the solution was stirred under nitrogen for 1 h. It was neutralized with a solution of acetic acid in methanol (2:5, dry pH paper) and concentrated under vacuum. The residue was purified by reversed-phase HPLC (gradient 0→100% CH$_3$CN in H$_2$O, with 0.1% TFA, during 60 min) to give **13** (16 mg, 75% peptide content, 19% overall yield). MS (ES): calcd for $C_{97}H_{155}N_{27}O_{39}$ 2322.1 (M)$^+$, found 2322.2. Amino acid analysis: Ala 5.13 (5), Arg 1.01 (1), Asp 1.02 (1), Gly 2.03 (2), His 1.02 (1), Pro 4.78 (5), Ser 2.02 (2), Thr 2.98 (3), Val 1.02 (1).

Tn neoglycopeptide 16. l-Arginylglycyl-L-tyrosyl-L-valyl-L-tyrosyl-L-homocysteinylglycyl-L-leucine (15 mg, 13 μmol) was added to a mixture of 2-bromoethyl glycoside **14** (15 mg, 46 μmol) and cesium carbonate (50 mg, 154 μmol) in dry DMF (1.3 mL) under a nitrogen atmosphere. The solution was stirred at room temperature until analytical reversed-phase HPLC (gradient 0→100% CH$_3$CN in H$_2$O, both containing 0.1% TFA, during 60 min, R_T = 17.2 min) indicated that the peptide was consumed. The reaction was quenched by addition of 0.1% aqueous TFA (15 mL) and the mixture was freeze-dried. Purification of the residue by preparative reversed-phase HPLC (gradient 0→100% CH$_3$CN in H$_2$O, both containing 0.1% TFA, during

(69) Flegel, M.; Sheppard, R. C. *J. Chem. Soc., Chem. Commun.* **1990**, 536−538.

Table 1. ^{1}H NMR Data (δ, ppm) for Glycopeptide **18** in Water Containing 10% D_2O[a,b]

residue	NH	Hα	Hβ	Hγ	others
Ala[1]		4.01	1.42		
His[2]		4.63	3.21, 3.17		8.42 (H2), 7.24 (H4)
Gly[3]	8.62	3.91[c]			
Val[4]	8.25	4.19	2.06	0.89[c]	
Thr[5]	8.48	4.37	4.17	1.15	
Ser[6]	8.45	4.40	3.79[c]		
Ala[7]	8.45	4.55	1.31		
Asp[9]	8.59	4.69	2.72, 2.56		
Thr[10d]	8.74	4.46	4.27	1.24	
Arg[11]	8.47	4.46	1.79, 1.67	1.66[c]	3.17 (δ), 7.34 (NH)
Gly[15]	8.62	3.91[c]			
Ser[16]	8.24	4.46	3.87, 3.83		
Thr[17]	8.34	4.32	4.17	1.15	
Ala[18]	8.64	4.54	1.29		

[a] Recorded at 600 MHz, 278 K, and pH 5.6 with H_2O as internal standard (δ_H 4.98 ppm). [b] Resonances that could not be unambiguously assigned are not reported. This includes all resonances of Pro[8], Pro[12]-Pro[14], and Pro[19]-Ala[21]. [c] Degeneracy has been assumed. [d] Chemical shifts (δ, ppm) for the Neu5Acα(2→6)GalNAc moiety—GalNAc: 4.79 (H-1), 3.99 (H-2), 3.89 (H-4), 3.82 (H-3). Neu5Ac: 4.07 (H-8), 3.89 and 3.49 (H-9,9′), 3.79 (H-5), 3.64 (H-6), 3.59 (H-4), 2.64 (H-3_{eq}), 1.61 (H-3_{ax}).

60 min) gave **16** (14 mg, 77% peptide content, 74% yield). HRMS (FAB): calcd for $C_{53}H_{83}N_{12}O_{17}S$ ($M + H^+$) 1191.5720, found 1191.5728. Amino acid analysis: Arg 0.98 (1), Gly 1.99 (2), Leu 1.01 (1), Tyr 1.98 (2), Val 1.04 (1).

T neoglycopeptide 17. L-Arginylglycyl-L-tyrosyl-L-valyl-L-tyrosyl-L-homocysteinylglycyl-L-leucine (12 mg, 13 μmol) was added to a mixture of 2-bromoethyl glycoside **15** (10 mg, 20 μmol) and cesium carbonate (35 mg, 107 μmol) in dry DMF (1.0 mL) under a nitrogen atmosphere. The mixture was stirred at room temperature until analytical reversed-phase HPLC (gradient 0→100% CH_3CN in H_2O, both containing 0.1% TFA, during 60 min, R_T = 16.9 min) indicated that the peptide was consumed. The reaction was quenched by addition of 0.1% aqueous TFA (15 mL) and the mixture was freeze-dried. Purification of the residue by preparative reversed-phase HPLC (gradient 0→100% CH_3CN in H_2O, both containing 0.1% TFA, during 60 min) gave **17** (10 mg, 81% peptide content, 58% yield). HRMS (FAB): calcd for $C_{59}H_{93}N_{12}O_{22}S$ ($M + H^+$) 1353.6248, found 1353.6215. Amino acid analysis: Arg 1.00 (1), Gly 1.99 (2), Leu 1.03 (1), Tyr 2.00 (2), Val 0.99 (1).

L-Alanyl-L-histidinylglycyl-L-valyl-L-threonyl-L-seryl-L-alanyl-L-prolyl-L-aspartyl-3-*O*-[2-acetamido-2-deoxy-6-*O*-(5-acetamido-3,5-dideoxy-D-glycero-α-D-galacto-2-nonulopyranosylonic acid)-α-D-galactopyranosyl]-L-threonyl-L-arginyl-L-prolyl-L-alanyl-L-prolylglycyl-L-seryl-L-threonyl-L-alanyl-L-prolyl-L-prolyl-L-alanine (18). Glycopeptide **12** (0.50 mg, 0.18 μmol based on 78% peptide content) was added to purified ST6GalNAc-I (~2 mU) in 20 mM Bis-Tris buffer (pH 6.0, 1.0 mL) containing CMP-Neu5Ac (2mM), EDTA (20 mM), and dithiothreitol (1 mM). The solution was then incubated at 37 °C for 6 h, after which analysis[51] by nanoscale reversed-phase HPLC in combination with MALDI-TOF mass spectrometry indicated >95% conversion of **12**. This procedure was repeated so that 5.0 mg (1.8 μmol) of **12** was sialylated. Purification by HPLC on a Zorbax 300SB-C3 column (9.4 × 250 mm) with a gradient of 0→90% CH_3CN in water, both containing 0.1% TFA, gave **18** (4.5 mg, 73% peptide content, 74% yield) after freeze-drying. ^{1}H NMR data, see Table 1; MS (MALDI TOF) calcd for $C_{102}H_{162}N_{28}O_{42}$ $(M)^+$ 2451 , found 2451. Amino acid analysis: Ala 4.99 (5), Arg 1.01 (1), Asp 1.02 (1), Gly 1.98 (2), His 0.99 (1), Pro 5.08 (5), Ser 1.98 (2), Thr 2.93 (3), Val 1.01 (1).

Sialyl-Tn Neoglycopeptide 19. Neoglycopeptide **16** (0.50 mg, 0.32 μmol based on 77% peptide content) was added to purified ST6GalNAc-I (~2mU) in 20 mM Bis-Tris buffer (pH 6.0, 1.0 mL) containing CMP-Neu5Ac (2mM), EDTA (20 mM), and dithiothreitol (1 mM). The solution was then incubated at 37 °C for 6 h, after which analysis[51] by nanoscale reversed-phase HPLC in combination with MALDI-TOF mass spectrometry indicated 90−95% conversion of **16**. Purification by HPLC on a Zorbax 300SB-C3 column (9.4 × 250 mm) with a gradient of 0→90% CH_3CN in water, both containing 0.1% TFA, gave **19** (~250 mg) after freeze-drying; MS (MALDI-TOF) calcd for $C_{64}H_{100}N_{13}O_{25}S$ (M^+) 1483 , found 1483.

Table 2. ^{1}H NMR Data (δ, ppm) for Glycopeptide **20** in Water Containing 10% D_2O[a,b]

residue	NH	Hα	Hβ	Hγ	others
Ala[1]		4.08	1.49		
His[2]	8.95	4.69	3.29, 3.23		8.56 (H4), 7.30 (H2)
Gly[3]	8.65	3.98[c]			
Val[4]	8.26	4.23	2.10	0.96, 0.95	
Thr[5]	8.47	4.42	4.22	1.20	
Ser[6]	8.44	4.44	3.84[c]		
Ala[7]	8.47	4.55	1.36		
Pro[8]		4.40	2.28, 2.00	1.87[c]	3.81 and 3.66 (d)
Asp[9]	8.58	4.74	2.81, 2.64		
Thr[10d]	8.85	4.54	4.44	1.23	
Arg[11]	8.42	4.48	1.85, 1.76	1.73[c]	7.52, 6.90, 6.62 (NH); 3.23 (δ)[c]
Pro[12 b]		4.36	2.29[b]		3.76 and 3.61 (δ)
Ala[13]	8.63	4.62	1.38		
Pro[14 b]		4.43	2.29[b]		3.80 and 3.66 (δ)
Gly[15]	8.65	3.98[c]			
Ser[16]	8.27	4.52	3.93, 3.88		
Thr[17]	8.33	4.37	4.22	1.21	
Ala[18]	8.42	4.61	1.35		
Pro[19]		4.72	2.38, 1.92	2.05[c]	3.81 and 3.65 (δ)
Pro[20 b]		4.38	2.30[b]		3.83 and 3.65 (δ)
Ala[21]	8.11	4.10	1.34		

[a] Recorded at 600 MHz, 279 K, and pH 5.4 with H_2O (δ_H = 4.95) as internal standard. [b] All proline resonances could not be unambiguously assigned due to overlap. [c] Degeneracy has been assumed. [d] Chemical shifts (δ, ppm) for the Neu5Acα(2→3)Galβ(1′3)GalNAc moiety—GalNAc: 8.11 (NH), 4.84 (H-1), 4.21 (H-2), 4.02 (H-3), 2.02 (Ac). Gal: 4.51 (H-1), 4.06 (H-3), 3.50 (H-2). Neu5Ac: 8.14 (NH), 3.85 (H-5), 3.67 (H-4), 3.61 (H-6), 2.75 (H-3_{eq}), 1.78 (H-3_{ax}).

L-Alanyl-L-histidinylglycyl-L-valyl-L-threonyl-L-seryl-L-alanyl-L-prolyl-L-aspartyl-3-*O*-[(5-acetamido-3,5-dideoxy-D-glycero-α-D-galacto-2-nonulopyranosylonic acid)-(2→3)-*O*-β-D-galactopyranosyl-(1→3)-*O*-(2-acetamido-2-deoxy-α-D-galactopyranosyl)]-L-threonyl-L-arginyl-L-prolyl-L-alanyl-L-prolylglycyl-L-seryl-L-threonyl-L-alanyl-L-prolyl-L-prolyl-L-alanine (20). Purified ST3Gal-I (20 μL, ≈2 mU) was added to **13** (0.50 mg, 0.16 μmol based on 75% peptide content) in 25 mM Tris-HCl buffer (pH 6.5, 1.0 mL) containing CMP-Neu5Ac (2 mM), Triton X-100 (0.1%), and calf intestinal phosphatase (2 mU). The solution was then incubated at 37 °C for 3 h, after which analysis[51] by nanoscale reversed-phase HPLC in combination with MALDI-TOF mass spectrometry indicated >95% conversion of **13**. This procedure was repeated so that 2.0 mg (0.64 μmol) of **13** was sialylated. Purification by HPLC on a Zorbax 300SB-C3 column (9.4 × 250 mm) using a gradient of 0→90% CH_3CN in water, both containing 0.1% TFA, gave **20** (2.0 mg, 79% peptide content, 94% yield) after freeze-drying. ^{1}H NMR data, see Table 2; MS (ES) calcd for $C_{108}H_{173}N_{28}O_{47}$ ($M + H^+$) 2614.2, found 2614.5. Amino acid analysis: Ala 4.90 (5), Arg 1.00 (1), Asp 1.02 (1), Gly 2.02 (2), His 1.03 (1), Pro 5.13 (5), Ser 1.97 (2), Thr 2.90 (3), Val 1.04 (1).

2,3-Sialyl-T Neoglycopeptide 21. Purified ST3Gal-I (20 mL, ≈2 mU) was added to neoglycopeptide **17** (0.50 mg, 0.30 μmol based on 81% peptide content) in 25 mM Tris-HCl buffer (pH 6.5, 1.0 mL) containing CMP-Neu5Ac (2 mM), Triton X-100 (0.1%), and calf intestinal phosphatase (10 mU). The solution was then incubated at 37 °C for 3 h, after which analysis[51] by nanoscale reversed-phase HPLC in combination with MALDI-TOF mass spectrometry indicated ~95% conversion of **17**. This procedure was repeated so that 3.5 mg (2.1 mmol) of **17** was sialylated. Purification by HPLC on a Zorbax 300SB-C3 column (9.4 × 250 mm) with a gradient of 0→90% CH_3CN in water, both containing 0.1% TFA, gave **21** (2.9 mg, 77% peptide content, 64% yield) after freeze-drying. ^{1}H NMR data, see Table 3;

Table 3. ^{1}H NMR Data (δ, ppm) for Glycopeptide **21** in Water Containing 10% D_2O[a]

residue	NH	Hα	Hβ	Hγ	others
Arg		3.99	1.88[b]	1.62[b]	3.16[b] (Hδ,δ'); 7.19 (CH_2NH)
Gly	8.78	4.02, 3.90			
Tyr	8.43	4.48	2.94, 2.85		7.03 and 6.74 (ArH)
Val	7.97	3.94	1.83	0.82, 0.78	
Tyr	8.49	4.40	3.03, 2.87		7.16 and 6.78 (ArH)
Hcy[c]	8.37	4.42	2.04, 1.81	2.52, 2.36	2.72[b] (SCH_2CH_2O); 3.80and 3.60 (SCH_2CH_2O)
Gly	7.01	3.80[b]			
Leu	7.90	4.19	1.57[b]	1.57	0.88 and 0.84 (γ,γ'Me)

[a] Recorded at 500 MHz, 278 K, and pH 5.4 with H_2O (δ_H 4.98 ppm) as internal standard. [b] Degeneracy has been assumed. [c] Chemical shifts (δ, ppm) for the Neu5Acα(2→3)Galβ(1→3)GalNAc moiety—Neu5Ac: 8.15 (NH), 3.82 (H-5), 3.64 (H-4), 3.57 (H-6), 2.71 (H-3_{eq}), 2.00 (Ac), 1.76 (H-3_{ax}). Gal: 4.47 (H-1), 4.03 (H-3), 3.50 (H-2). GalNAc: 8.21 (NH), 4.87 (H-1), 4.27 (H-2), 4.19 (H-4), 3.98 (H-3), 1.98 (Ac).

MS (ES) calcd for $C_{70}H_{110}N_{13}O_{30}S$ (M^+) 1643.7, found 1643.6. Amino acid analysis: Arg 1.00 (1), Gly 2.01 (2), Leu 1.01 (1), Tyr 2.00 (2), Val 0.99 (1).

L-Alanyl-L-histidinylglycyl-L-valyl-L-threonyl-L-seryl-L-alanyl-L-prolyl-L-aspartyl-3-*O*-[2-acetamido-6-*O*-(2-acetamido-2-deoxy-*β*-D-glucopyranosyl)-3 *O*-(*β*-D-galactopyranosyl)-2-deoxy-α-D-galactopyranosyl]-L-threonyl-L-arginyl-L-prolyl-L-alanyl-L-prolylglycyl-L-seryl-L-threonyl-L-alanyl-L-prolyl-L-prolyl-L-alanine (22). Purified C2GnT3 (30 μL, ≈ 1.7 mU) was added to **13** (100 μg, 32 nmol based on 75% peptide content) in 100 mM MES buffer (pH 6.5, 0.50 mL) containing UDP-GlcNAc (2 mM), EDTA (2 mM), D-galactono-1,5-lactone (5 mM), and 2-acetamido-2-deoxy-D-glucono-1,5-lactone (2 mM). Calf intestinal phosphatase (100 mU) was added after 1 h incubation. The solution was then incubated at 37 °C for 6 h, after which additional C2GnT3 (10 μL, ≈ 0.6 mU) and UDP-GlcNAc (2 μL of a 0.4 mM solution in water) were added. After incubation for 18 h analysis[51] by nanoscale reversed-phase HPLC in combination with MALDI-TOF mass spectrometry indicated >95% conversion of **13**. This procedure was repeated so that 0.60 mg (0.19 mmol) of **13** was converted. Purification by HPLC on a Zorbax 300SB-C3 column (9.4 × 250 mm) with a gradient of 0→90% CH_3CN in water, both containing 0.1% TFA, gave **22** (~600 μg) after freeze-drying. MS (ES) calcd for $C_{105}H_{169}N_{28}O_{44}$ ($M + H^+$) 2526.2, found 2526.5.

Core 2 Neoglycopeptide 23. Purified C2GnT3 (30 μL, ≈ 1.7 mU) was added to neoglycopeptide **17** (100 μg, 60 nmol based on 81% peptide content) in 100 mM MES buffer (pH 6.5, 0.50 mL) containing UDP-GlcNAc (2 mM), EDTA (2 mM), D-galactono-1,5-lactone (5 mM), and 2-acetamido-2-deoxy-D-glucono-1,5-lactone (2 mM). Calf intestinal phosphatase (100 mU) was added after 1 h incubation. The solution was then incubated at 37 °C for 3 h, after which analysis[51] by nanoscale reversed-phase HPLC in combination with MALDI-TOF mass spectrometry indicated >95% conversion of **17**. This procedure was repeated so that 0.6 mg (3.6 μmol) of **17** was converted. Purification by HPLC on a Zorbax 300SB-C3 column (9.4 × 250 mm) using a gradient of 0→90% CH_3CN in water, both containing 0.1% TFA, gave **23** (~400 μg) after freeze-drying. MS (ES) calcd for $C_{67}H_{106}N_{13}O_{27}S$ ($M + H^+$) 1556.7, found 1556.8.

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Supporting Information Available: ^{1}H and ^{13}C NMR spectra for compounds **2**, **4**, **5**, **7**, **9**, and **10** and tabulated ^{1}H NMR data for glycopeptides **13**, **16**, and **17** (PDF). This material is available free of charge via the Internet at http://pubs.acs.org.

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