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Synthesis of an *N*-linked glycopeptide from vitamin K-dependent Protein S

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Abstract

A novel asparagine building block having an *N*-linked chitobiose moiety protected with acid-labile TBDMS groups has been prepared. The building block was used in Fmoc solid-phase synthesis of a glycopeptide fragment corresponding to residues 447-460 of protein S which has a potential *N*-glycosylation site at Asn^{458}. The TBDMS groups of the chitobiose moiety were removed during cleavage of the glycopeptide from the solid phase, thus simplifying synthesis as compared to when using acetyl protection for the carbohydrate. Protein S is an anticoagulant which may be inactivated by complexation by C4b binding protein (C4BP). The protein S 447-460 glycopeptide was found to be a more efficient inhibitor of complex formation than the non-glycosylated parent peptide, indicating that protein S may carry an *N*-linked glycan at Asn^{458}.

Keywords: Glycopeptides; Protecting groups; Solid-phase synthesis; Protein S

1 Introduction

Vitamin K-dependent protein S is a cofactor in the regulatory system of blood coagulation which forms a strong complex with C4b binding protein (C4BP), one of the proteins of the complement system [1]. This interaction affects the concentration of free protein S, which can act as an anticoagulant. One region on protein S involved in binding to C4BP was recently found to reside in the Ser^{447}-Ser^{460} fragment [2], a sequence which contains a

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potential *N*-glycosylation site at Asn^{458}. It would therefore be interesting to probe if glycosylation at Asn^{458} plays a role for binding of the protein S 447-460 fragment to C4BP. An influence on binding to C4BP could be a result of a direct involvement of the *N*-glycan in binding to C4BP, or be due to a conformational effect in the proximity of the glycosylation site [3]. *N*-Linked carbohydrates of glycoproteins are large [4], but they all share the common $Man_3(GlcNAc)_2$ core linked by an amide bond to the side-chain of asparagine. Since it is known that it is the innermost residues of the carbohydrate moiety that exerts the greatest influence on peptide conformation [3], we chose chitobiose [GlcNAcβ(1-4)GlcNAc] as an approximation of the true carbohydrate structure found at Asn^{458} in C4BP.

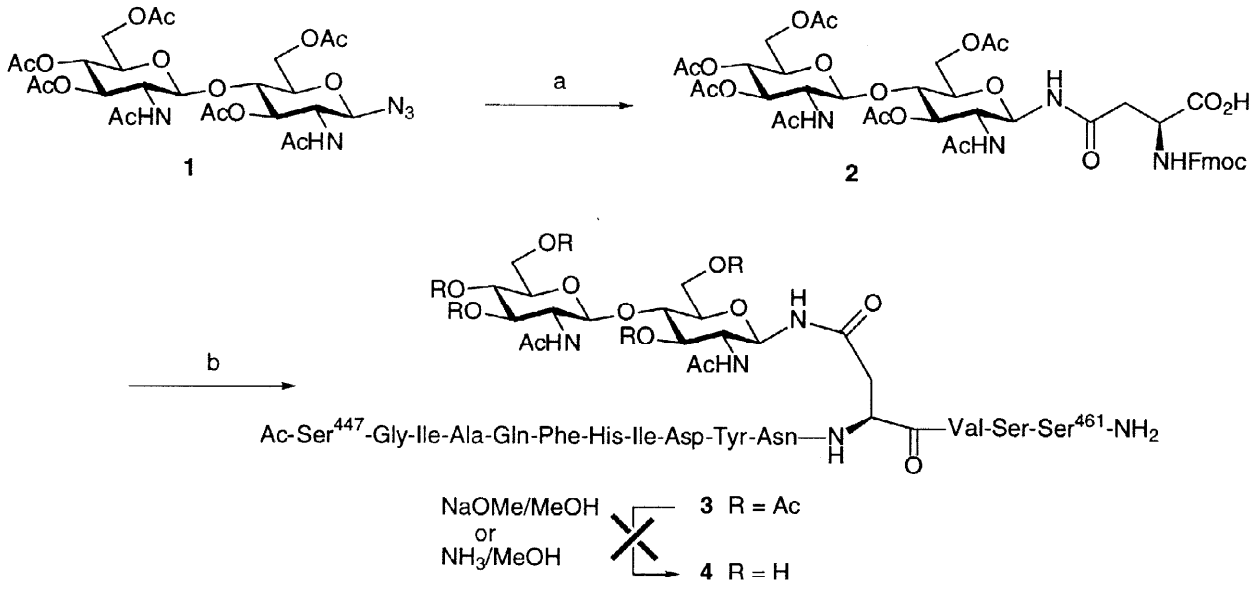
N-Linked glycopeptides may be prepared by either of two different strategies. Glycosyl amines can be coupled directly to the side-chain of an aspartic acid residue incorporated in a peptide [5-7], or they can be linked to a suitably protected derivative of aspartic acid to give a building block which is subsequently used to assemble the target glycopeptide [8,9]. It is, however, known that glycosylamines may undergo anomerization and dimerization during coupling to aspartic acid [10]. We therefore decided to prepare the glycosylated protein S fragment by the building block approach, hoping that removal of the undesired α-anomer would be more facile at the building block level.

2 Results and Discussion

Our initial approach was to use the glycosylated asparagine building block **2** [11] in solid-phase peptide synthesis (SPPS) of a protein S fragment according to the Fmoc protocol (scheme 1). Compound **2** was synthesized by a route similar to that used previously [12] for preparation of a chitobiose-asparagine conjugate which carried a tBoc group at the α-amino group. Thus, the peracetylated chitobiosyl azide **1** [12] was reduced, the resulting glycosyl amine was coupled with Fmoc-Asp(OH)-OtBu after which the *tert*-butyl ester was cleaved under acidic conditions. An anomeric mixture (β:α, >10:1) was obtained in the coupling but the desired β-anomer could be purified by flash column chromatography before removal of the *tert*-butyl ester. Building block **2** was then used in SPPS of glycopeptide **3** which corresponds to residues Ser^{447}-Ser^{461} of human protein S. Glycopeptide **3**, carrying an *O*-acetyl protected chitobiosyl moiety, was only sparingly soluble in water and methanol. Attempted deacetylation (NaOMe/MeOH or NH_3/MeOH) of **3** gave several, more polar products as determined by analytical reversed-phase HPLC. However, a single product could not be obtained and use of more basic conditions resulted in degradation of the glycopeptide.

To avoid the problems encountered during deacetylation of glycopeptide **3**, we decided to replace the base-labile *O*-acetyl protective groups of building block **2** with acid-labile TBDMS groups. The TBDMS group is removed under the acidic conditions employed for cleavage of the glycopeptide from the solid phase and deprotection of amino acid side chains. This renders a separate carbohydrate deprotection step, and the subsequent purification, unnecessary and saves both time and effort once the peptide has been synthesized. We have

previously employed acid-labile protective groups for carbohydrate moieties in the synthesis of *O*-linked glycopeptides containing the Tn-antigen [13] and glycopeptides from type II collagen which are glycosylated on hydroxylysine [14-16]. We then found that the glycosidic linkages were sufficiently stable to withstand the acidic conditions used for deprotection and cleavage from the solid phase, an observation which has also been made for unprotected chitobiose [17].



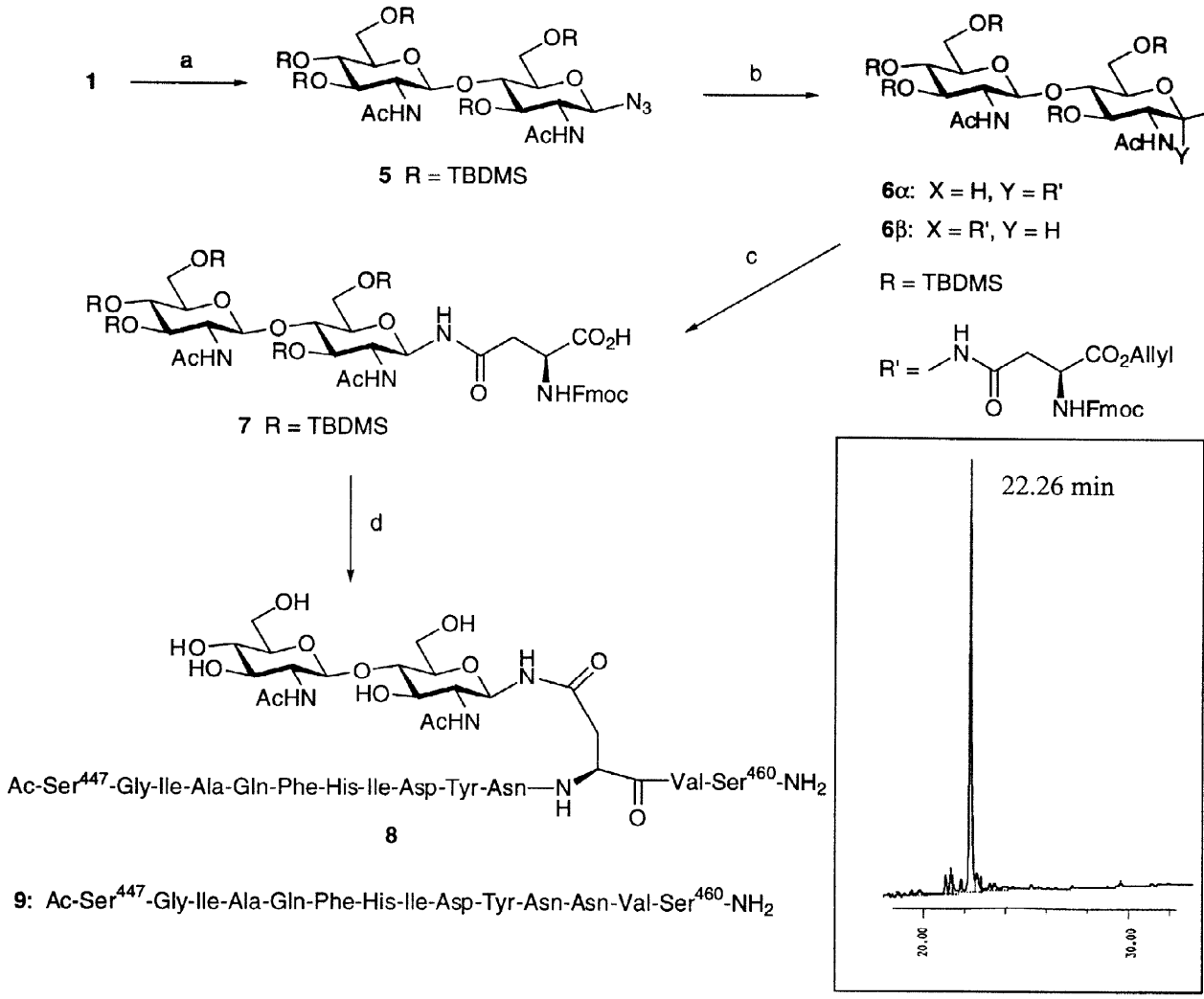
Scheme 1. Attempted synthesis of glycopeptide **4** from building block **2** [11] carrying acetyl protective groups on the carbohydrate moiety. (a) i) H_2, PtO_2 in EtOH, ii) EEDQ, Fmoc-Asp(OH)-OtBu in CH_2Cl_2, iii) TFA/H_2O 9:1; (b) solid-phase peptide synthesis.

Deacetylation of chitobiosyl azide **1** with methanolic NaOMe followed by reprotection of the hydroxyl groups using *tert*-butyldimethylsilyl triflate in pyridine afforded the persilylated glycosyl azide **5** in 88% yield over two steps (Scheme 2). ^{1}H-NMR spectroscopy revealed that the $^3J_{1,2}$ coupling constants of the anomeric protons were significantly smaller than for compound **1**, an observation which can be explained by the bulky TBDMS groups that cause the carbohydrate rings to adopt conformations different from the normal $^4C_1$ chair conformation [16]. Hydrogenation of **5** over Pd/C in tetrahydrofuran and coupling of the resulting glycosyl amine with Fmoc-Asp(OH)-OAll using HBTU and diisopropylethylamine gave the β-linked building block **6β** (38% over two steps) together with the α-anomer **6α** (20%). Formation of the amide bond is known to be the most critical step in synthesis of glycosylated asparagines due to the instability of the glycosyl amine, which may lead to anomerisation and also formation of dimers [10]. In our hands, other

methods to form the amide bond, such as a Staudinger reaction between **5**, Fmoc-Asp(OH)-OAll and n-Bu_3P [18], a modified Staudinger reaction between **5**, Fmoc-Asp(Cl)-OPfp and PPh_3 [19], or reduction of **5** by $Et_3SiH/Pd(OH)_2$ in acetonitrile in the presence of Fmoc-Asp(OPfp)-OAll and 1-hydroxybenzotriazole all failed. Distortion of the pyranoside rings of **6α** and **6β** by the TBDMS groups made determination of the anomeric configuration from the $^3J_{1,2}$ coupling constants difficult. However, removal of the TBDMS groups using TFA/H_2O for 1.5 h and analysis of the crude products by ^{1}H-NMR spectroscopy revealed that **6β** was the ß-anomer ($J_{H1\text{-}H2}$=9.4 Hz) and **6α** the α-anomer ($J_{H1\text{-}H2}$=4.3 Hz). Deallylation of **6β** using $(PPh_3)_4Pd(0)$ and morpholine in tetrahydrofuran then afforded **7** (93%). The silylated compounds **5**, **6α/β** and **7** were all soluble in most organic solvents, an important advantage conferred by the TBDMS groups, as compared to the *O*-acetylated analogues which could only be dissolved in DMF and DMSO.

Glycosylated asparagine **7** was then used in SPPS of glycopeptide **8** which comprises residues Ser^{447}-Ser^{460} of protein S. Synthesis was performed in an automatic peptide synthesizer [20] on a polystyrene resin grafted with polyethylene glycol spacers (TentaGel^{TM} resin) and functionalized with the Rink amide linker [21,22]. In the peptide synthesizer N^{α}-Fmoc amino acids (4 equivalents, as compared to the capacity of the resin) having standard side-chain protective groups were activated as benzotriazolyl esters [23] with 1,3-diisopropyl carbodiimide in DMF. The glycosylated building block **7** (2 equivalents) was activated as an azabenzotriazolyl ester due to the higher coupling efficiency displayed by such esters as compared to benzotriazolyl esters in peptide synthesis [24]. Coupling of activated **7** was performed manually after removal of the peptide-resin from the synthesizer in order to allow use of a minimal volume of DMF as solvent. The resin was then reinserted in the automatic synthesizer and the remaining amino acids were attached. Couplings were monitored spectrophotometrically with bromophenol blue as an indicator [25] of unacylated amino groups. N^{α}-Fmoc deprotections were effected with piperidine in DMF and were monitored using the absorbance of the dibenzofulvene-piperidine adduct [26]. Cleavage of glycopeptide **8** from the solid phase by trifluoroacetic acid/water/thioanisole/ethanedithiol (35:2:2:1) for 3 h resulted in almost complete removal of all TBDMS groups without detectable degradation of the carbohydrate moiety, as determined by analytical reversed-phase HPLC. The observed hydrolytic stability is in agreement with previous observations [27,28,7,17]. Crude glycopeptide **8** was poorly soluble in water and acetic acid and the peptide was therefore dissolved in trifluoroacetic acid/water 3:1 to allow purification by reversed-phase HPLC. This also served to deprotect a small amount of monosilylated glycopeptide which remained after cleavage and **8** was obtained in 28% yield, based on the resin capacity, after purification. Due to the low solubility of **8** in water it was characterized by ^{1}H NMR spectroscopy in DMSO-d_6 containing 15 % H_2O (cf. Table 1 in Experimental). A 2D-NOESY experiment revealed strong NH_i-NH_{i+1} NOEs between Asp^{455} and Tyr^{456} and between Tyr^{456} and Asn^{457} indicating a non-random conformation near the chitobiosyl

moiety at Asn^{458}. The nature and origin of the conformational restriction of these residues was not established in greater detail.



Scheme 2. Synthetic route to glycopeptide **8** using TBDMS protective groups for the carbohydrate moiety. (a) NaOMe in MeOH then TBDMS-OTf and DMAP in pyridine, 88%; (b) H_2, Pd/C in THF then Fmoc-Asp(OH)-OtBu, HBTU, DIEA, 20% of **6α** and 38% of **6β**; (c) $(PPh_3)_4Pd(0)$ and morpholine in THF, 93%; (d) solid-phase peptide synthesis, 28%. The analytical HPLC chromatogram shows crude **8** immediately before purification.

The ability of **8** and the corresponding non-glycosylated peptide **9** (Scheme 2) to inhibit the binding of protein S to C4BP was determined as reported previously [2]. A four-fold lower concentration of glycopeptide **8**, as compared to peptide **9**, was sufficient to obtain 50% inhibition of complex formation between C4BP and protein S (Figure 1). Glycosylation

at position 458 thus enhances the inhibitory activity, an observation that may well be due to contacts between the glycan and C4BP. Alternatively, the residues interacting with C4BP may be partly shielded or unorganized in the non-glycosylated peptide, while glycosylation favours a conformation more ready to bind to C4BP. The results may tentatively be interpreted as supporting that Asn^{458} in Protein S carries an *N*-linked oligosaccharide moiety.

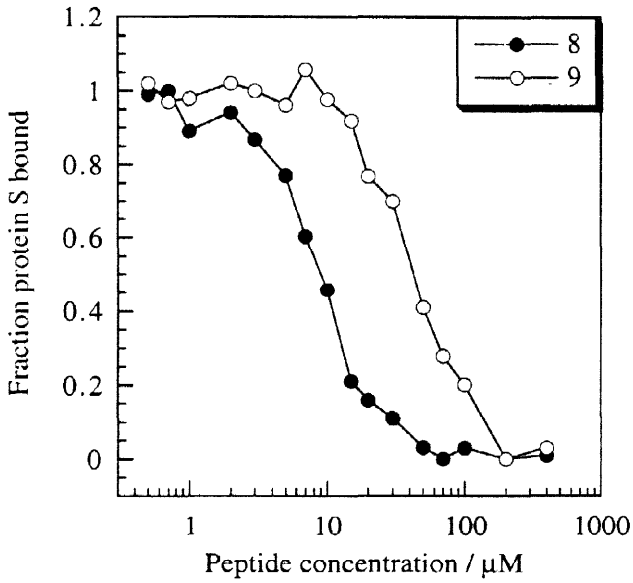


Figure 1. Inhibition of the binding of protein S to C4BP by glycopeptide **8** and the corresponding non-glycosylated peptide, **9**. C4BP was covalently immobilized on a BIAcore^{TM} sensorchip and the ability of **8** and **9** to inhibit binding of protein S was determined as described previously [2].

3 Experimental

General Methods and Materials. — TLC was performed on Silica Gel 60 F_{254} (Merck) with detection by UV light or phosphomolybdic acid/ceric sulphate in 6% aqueous sulfuric acid followed by heating. Flash column chromatography was performed on silica gel (Matrex, 60 Å, 35-70 μm, Grace Amicon) with distilled solvents. Dry THF was obtained by distilling from sodium and dry pyridine by distilling from CaH_2. DMF was distilled before use. The ^1H and ^{13}C NMR spectra were recorded at 400 MHz and 100 MHz, respectively, for solutions in $CDCl_3$ [residual $CHCl_3$ (δ_H 7.27 ppm) or $CDCl_3$ (δ_C 77.0 ppm) as internal standard]. First-order ^1H and ^{13}C chemical shifts, as well as ^1H coupling constants, were determined from one-dimensional spectra and resonances were assigned from COSY, TOCSY, HMQC, DEPT and NOESY experiments. Analytical reversed-phase HPLC was performed on a Kromasil C-8 column (250x4.6 mm, 5 μm, 100 Å, flowrate 1.5 mL/min, eluent: $MeCN/H_2O$ + 0.1% TFA with a linear gradient of 0 - 80% MeCN over 60 min, detection at 214 nm) and preparative reverse phase HPLC was performed on a Kromasil C-8

column (250x20 mm, 5 μm, 100 Å, flowrate 11 mL/min, eluent: MeCN/H_2O + 0.1% TFA with a linear gradient of 0 - 80% MeCN over 60 min, detection at 214 nm). 2-Acetamido-2-deoxy-4-*O*-(2-acetamido-2-deoxy-3,4,6-tri-*O*-acetyl-β-D-glucopyranosyl)-3,6-di-*O*-acetyl-β-D-glucopyranosyl azide (**1**) was synthesized as described previously [12]. N^4-[2-Acetamido-2-deoxy-4-*O*-(2-acetamido-2-deoxy-3,4,6-tri-*O*-acetyl-β-D-glucopyranosyl)-3,6-di-*O*-acetyl-β-D-glucopyranosyl]-N^2-(fluoren-9-ylmethoxycarbonyl)-L-aspartic acid (**2**) [11] was prepared by a route similar to that used for the corresponding N^2-tBoc protected derivative [12] with the modifications that Fmoc-Asp(OH)-OtBu was used instead of Boc-Asp(OH)-OAll and that cleavage of the OtBu ester was performed using TFA/H_2O (9:1).

*2-Acetamido-2-deoxy-4-*O*-(2-acetamido-2-deoxy-3,4,6-tri-*O*-tert-butyldimethylsilyl-β-D-glucopyranosyl)-3,6-di-*O*-tert-butyldimethylsilyl-β-D-glucopyranosyl azide (**5**).* — NaOMe in MeOH (0.02 M, 2 mL) was added to a suspension of **1** (461 mg, 720 μmol) in MeOH (40 mL) at room temperature. Additional portions of NaOMe/MeOH (0.02 M, 1 mL each) was added after 4 h and 17 h. After stirring for 3 days the solution was neutralized by addition of HOAc (pH paper). The solution was concentrated and the last traces of solvent were removed under vaccum. DMAP (9 mg, 70 μmol) and dry pyridine (15 mL) were added under N_2, the suspension was cooled to 0 °C and TBDMS-OTf (2.0 mL, 10.8 mmol) was slowly added. After 1 h the resulting solution was allowed to reach room temperature and after a further 20 h MeOH (≈2-3 mL) was added. The solution was then concentrated, toluene (≈15 mL) was added and the solution was concentrated again. Flash column chromatography of the residue (heptane/ethyl acetate; 5:1→3:1) afforded **5** (645 mg, 88%). Compound **5** had: R_f = 0.40 (heptane/ethyl acetate; 1:1); $[\alpha]_D^{20}$ -61° (*c* 1.0, $CHCl_3$); IR (KBr): ν 2310 cm^{-1} (N_3); ^1H NMR ($CDCl_3$) δ 6.73 (d, *J*=9.7 Hz, 1H, Ac<u>H</u>N), 5.79 (d, *J*=10.2 Hz, 1H, Ac<u>H</u>N'), 4.75 (d, *J*=5.1 Hz, 1H, H-1), 4.55 (d, *J*=6.8 Hz, 1H, H-1'), 4.13 (ddt, *J*=10.1, 6.8, ≈1 Hz, 1H, H-2'), 4.04 (m, 1H, H-2), 4.01 (dd, *J*=9.7, 8.0 Hz, 1H, H-6), 3.99 (m, 1H, H-4'), 3.96 (m, 1H, H-4), 3.91 (m, 1H, H-3), 3.85 (dd, *J*=9.7, 5.5 Hz, 1H, H-6), 3.81-3.75 (m, 3H, H-5, H-6',6'), 3.72 (m, 1H, H-5'), 3.68 (bd, *J*=4 Hz, 1H, H-3'), 2.04 and 1.92 (2 s, each 3H, NAc and NAc'), 0.95 and 0.92-0.89 (5 s, each 9H, t-Bu), 0.17, 0.15, 0.14, 0.14, 0.13, 0.12, 0.09, 0.08, 0.07 and 0.07 (10 s, each 3H, 10 Si-CH_3); ^{13}C NMR ($CDCl_3$) δ 170.2, 169.4, 99.5 (C1'), 88.7 (C1), 82.1, 77.4, 76.7, 72.4, 71.7, 70.3, 64.3, 63.2, 56.1, 53.0, 26.4, 26.2, 26.2, 24.1, 23.7, 18.8, 18.6, 18.6, 18.4, 18.3, -3.7, -3.9, -4.0, -4.0, -4.1, -4.1, -4.4, -4.5, -4.6, -4.6; HRMS (FAB): calcd for $C_{46}H_{97}N_5O_{10}Si_5Na$ $(M+Na)^+$ 1042.5978, found 1042.5992.

Allyl N^4*-[2-acetamido-2-deoxy-4-*O*-(2-acetamido-2-deoxy-3,4,6-tri-*O*-tert-butyldimethylsilyl-β-D-glucopyranosyl)-3,6-di-*O*-tert-butyldimethylsilyl-α- and β-D-glucopyranosyl]-*N*^{2}*-(fluoren-9-ylmethoxycarbonyl)-L-aspartate (**6α and 6β**).* — Compound **5** (1.16 g, 1.14 mmol) in dry THF (20 mL) was hydrogenated over Pd/C (10%, 400 mg) for 3.5 h at atmospheric pressure. The mixture was filtered (Celite) and the solution concentrated to a volume of about 10 mL. DIEA (490 μL, 2.85 mmol), Fmoc-Asp(OH)-

OAll (1.05 g, 2.3 mmol) and HBTU (0.86 g, 2.3 mmol) were added and the solution was stirred overnight. The solution was then diluted with CH_2Cl_2, washed with $NaHCO_3$ (aq., satd.) and H_2O, dried (Na_2SO_4), filtered and concentrated. Flash column chromatography of the residue (CH_2Cl_2/EtOH; 30:1) afforded **6α** (308 mg, 20%) and **6β** (593 mg, 38%). Compound **6α** had; R_f = 0.29 (heptane/ethyl acetate; 1:1; 1% HOAc); $[\alpha]_D^{20}$ -23° (*c* 1.0, $CHCl_3$); ^1H NMR ($CDCl_3$) δ 7.77-7.32 (m, 8H, Fmoc-arom), 7.21 (d, *J*=10.0 Hz, 1H, AcHN), 6.63 (d, *J*=9.0 Hz, 1H, H-Nδ), 6.09 (d, *J*=8.8 Hz, 1H, H-Nα), 5.91 (m, 1H, OCH_2-CH=CH_2), 5.80 (d, *J*=10.1 Hz, 1H, AcHN'), 5.65 (bd, *J*=9.2 Hz, 1H, H-1), 5.33 (d, *J*=15.9 Hz, 1H, OCH_2-CH=$CH_{cis}H_{trans}$), 5.22 (dd, *J*=10.5, 1.3 Hz, 1H, OCH_2-CH=$CH_{cis}H_{trans}$), 4.63-4.70 (m, 3H, OCH_2-CH=CH_2, H-α), 4.51 (d, *J*=6.7 Hz, 1H, H-1'), 4.42 (dd, *J*=10.5, 7.4 Hz, 1H, $OCOCH_2CH$), 4.34 (dd, *J*=10.5, 7.2 Hz, 1H, $OCOCH_2CH$), 4.27 (t, *J*=7.2 Hz, 1H, $OCOCH_2CH$), 4.19 (t, *J*=9.0 Hz, 1H), 4.09 (m, 2H), 4.01 (bd, *J*=9 Hz, 1H, H-2), 3.99 (d, *J*=3.9 Hz, 1H), 3.68-3.92 (m, 7H), 3.01 (dd, *J*=16.4, 4.5 Hz, 1H, H-β), 2.97 (dd, *J*=16.4, 4.3 Hz, 1H, H-β), 2.17, 1.97 (2s, each 3H, NAc, NAc'), 0.88, 0.91, 0.91, 0.91, 0.94 (5s, each 9H, tBu), 0.16, 0.14, 0.13, 0.13, 0.12, 0.09, 0.07, 0.07, 0.06 and 0.06 (10s, each 3H, Si-CH_3); ^{13}C NMR ($CDCl_3$) δ 172.2, 170.7, 169.7, 169.4, 156.1, 143.9, 141.2, 131.7, 127.6, 127.0, 125.3, 119.8, 118.3, 99.0, 81.9, 77.2, 75.9, 71.0, 70.3, 69.6, 69.5, 67.3, 66.2, 63.7, 60.4, 55.6, 50.4, 47.0, 37.6, 25.9, 25.9, 25.8, 25.8, 25.7, 23.4, 23.1, 18.3, 18.1, 18.0, 17.9, 17.7, -4.6, -4.9, -5.0, -5.0 -5.2; HRMS (FAB): calcd for $C_{68}H_{118}N_4O_{15}Si_5Na$ 1393.7336 $(M+Na)^+$, found 1393.7307. Compound **6β** had; R_f = 0.37 (heptane/ethyl acetate, 1:1; 1% HOAc); $[\alpha]_D^{20}$ -14° (*c* 1.0, $CHCl_3$); ^1H NMR ($CDCl_3$) δ 7.77-7.27 (m, 8H, Fmoc-arom), 6.85 (d, *J*=8.2 Hz, 1H, H-Nδ), 6.58 (d, *J*=9.3 Hz, 1H, AcHN), 6.14 (d, *J*=9.1 Hz, 1H, H-Nα), 5.91 (m, 1H, OCH_2-CH=CH_2), 5.75 (d, *J*=10.2 Hz, 1H, AcHN'), 5.33 (dd, *J*=17.2, 1 Hz, 1H, OCH_2-CH=$CH_{cis}H_{trans}$), 5.24 (dd, *J*=10.5, 1 Hz, 1H, OCH_2-CH=$CH_{cis}H_{trans}$), 4.97 (t, *J*=7.6 Hz, 1H, H-1), 4.65 (m, 3H, OCH_2-CH=CH_2, H-α), 5.56 (d, *J*=7 Hz, 1H, H-1'), 4.44 (dd, *J*=9.6, 6.6 Hz, 1H, $OCOCH_2CH$), 4.21-4.31 (m, 2H, $OCOCH_2CH$, $OCOCH_2CH$), 4.14 (m, 1H, H-2'), 3.98-4.03 and 3.65-3.83 (m, 11H), 3.02 (dd, *J*=16.7, 4.4 Hz, 1H, H-β), 2.97 (dd, *J*=16.5, 4.0 Hz, 1H, H-β), 2.04 and 1.97 (2s, each 3H, NAc, NAc'), 0.87-0.95 (5s, each 9H, tBu), 0.17, 0.16, 0.14, 0.14, 0.13, 0.10, 0.07, 0.07, 0.04 and 0.02 (10s, each 3H, Si-CH_3); ^{13}C NMR ($CDCl_3$) δ 171.4, 171.0, 170.9, 169.8, 156.7, 144.4, 144.3, 141.7, 132.1, 128.1, 127.5, 125.7, 125.7, 120.3, 119.0, 99.3, 82.3, 78.1, 77.7, 76.9, 73.5, 72.2, 70.5, 67.7, 66.6, 64.2, 63.0, 55.9, 53.9, 50.9, 47.5, 26.4, 26.2, 23.8, 23.4, 18.8, 18.6, 18.6, 18.6, 18.3, 18.3, -3.8, -3.9, -4.2, -4.3, -4.4, -4.6, -4.8, -4.9; HRMS (FAB): calcd for $C_{68}H_{118}N_4O_{15}Si_5Na$ $(M+Na)^+$ 1393.7336, found 1393.7308.

N^4-*[2-Acetamido-2-deoxy-4-*O*-(2-acetamido-2-deoxy-3,4,6-tri-*O*-*tert*-butyldimethylsilyl-β-*D*-glucopyranosyl)-3,6-di-*O*-*tert*-butyldimethylsilyl-β-*D*-glucopyranosyl]-N^2-(fluoren-9-ylmethoxycarbonyl)-L-aspartic acid (7).* — Morpholine (68 μL, 0.780 mmol) and $(PPh_3)_4Pd(0)$ (43.5 mg, 0.039 mmol.) were added to a solution of **6β** (538 mg, 0.392 mmol) in dry THF (7 mL) under inert atmosphere and in the absence of light. After 40 min the solution was diluted with EtOAc (150 mL) and washed with NH_4Cl (aq., satd.), H_2O and

brine, dried (Na_2SO_4), filtered and concentrated. Flash column chromatography of the residue (toluene/EtOH, 20:1) afforded **7** (490 mg, 93%). Compound **7** had; $[\alpha]_D^{20}$ -2° (*c* 1.0, $CHCl_3$); ^1H NMR ($CDCl_3$) δ 7.75-7.30 (m, 8H, Fmoc-arom), 7.20 (d, *J*=8 Hz, 1H, H-Nδ), 6.21 (d, *J*=8.2 Hz, 1H, H-Nα), 5.85 (d, *J*=10.4 Hz, 1H, AcHN'), 4.92 (t, *J*=8.0 Hz, 1H, H-1), 4.60 (m, 1H, H-α), 4.58 (d, *J*=7.0 Hz, 1H, H-1'), 4.37 (dd, *J*=10.6, 7.3 Hz, 1H, OCOCH_2CH), 4.39 (dd, *J*=10.4, 7.6 Hz, 1H, OCOCH_2CH), 4.19 (t, *J*=7.4 Hz, 1H, $OCOCH_2CH$), 4.14 (m, 1H, H-2'), 4.05 (m, 2H), 3.99 (d, *J*=4.5 Hz, 1H), 3.66-3.88 (m, 7H), 2.93 (dd, *J*=15.8, 4.6 Hz, 1H, H-β), 2.70 (d, *J*=15.9, 5.3 Hz, 1H, H-β), 2.05 and 2.02 (2s, each 3H, NAc, NAc'), 0.94, 0.91, 0.90, 0.89, 0.89 (5s, each 9H, t-Bu), 0.15, 0.14, 0.15, 0.13, 0.13, 0.12, 0.09, 0.07, 0.07, 0.04 and 0.03 (10s, each 3H, Si-CH_3); ^{13}C NMR ($CDCl_3$) δ 171.5, 171.1, 171.0, 169.9, 156.7, 144.4, 144.2, 141.7, 141.7, 132.1, 128.1, 128.1, 127.5, 120.4, 119.0, 99.2, 81.9, 78.1, 77.7, 76.9, 73.5, 72.2, 70.4, 67.7, 66.6, 64.2, 63.0, 55.9, 53.9, 50.9, 47.5, 38.0, 26.4, 26.4, 26.3, 23.8, 23.4, 18.8, 18.7, 18.6, 18.4, 18.3, -3.7, -3.9, -4.1, -4.1, -4.3, -4.4, -4.6, -4.8, -4.9, -4.9.

Table 1.

^1H-NMR Data (δ, ppm) for glycopeptide **8**.[a]

Residue	NH	H-α	H-β	H-γ	H-δ	Others
Ser^{447}	8.06	4.19	3.60[b]			1.87 (NAc)
Gly^{448}	8.29	3.73[b]				
Ile^{449}	7.72	4.06	1.70	0.77, 1.03	1.35	0.77 (β-CH_3)
Ala^{450}	8.11	4.12	1.17			
Gln^{451}	7.82	4.09	1.64, 1.78	2.04[b]		6.80 and 7.32 ($CONH_2$)
Phe^{452}	7.92	4.41	2.77, 2.95			7.16-7.20 (arom.)
His^{453}	8.29	4.60	2.95, 3.08			7.24 (H-4), 8.79 (H-2)
Ile^{454}	7.85	4.09	1.64	0.75, 1.00	1.33	0.70 (β-CH_3)
Asp^{455}	8.30	4.53	2.51, 2.65			
Tyr^{456}	7.83	4.32	2.67, 2.88			6.59 and 6.95 (arom.)
Asn^{457}	8.12	4.48	2.47, 2.55			6.91 and 7.45 ($CONH_2$)
Asn^{458}	8.14	4.54	2.43, 2.65			[c], 8.60 (CONH)
Val^{459}	7.74	4.05	2.03	0.80, 0.82		
Ser^{460}	7.86	4.19	3.60[b]			7.06, 7.13 ($CONH_2$)

[a]Obtained at 600 MHz and 300 K in DMSO-D_6 containing 15% H_2O. Chemical shifts are referenced to residual DMSO-d_5 at 2.50 ppm.

[b]Degeneracy has been assumed.

[c]Chemical shifts (δ, ppm) for the chitobiosyl moiety: 8.03 (NH), 7.90 (N'H), 4.80 (H-1), 4.35 (H-1'), 3.73 (H-6'), 3.57 (H-6), 3.57 (H-2), 3.51 (H-3), 3.49 (H-2'), 3.43 (H-6), 3.40 (H-6'), 3.28 (H-3'), 3.28 (H-4), 3.21 (H-5'), 3.20 (H-5), 3.08 (H-4'), 1.84 (N'Ac), 1.80 (NAc).

N^{α}-Acetyl-L-seryl-glycyl-L-isoleucyl-L-alanyl-L-glutaminyl-L-phenylalanyl-L-histidinyl-L-isoleucyl-L-aspart-1-yl-L-tyrosyl-L-asparaginyl-N^{4}-[2-acetamido-2-deoxy-4-O-(2-acetamido-2-deoxy-β-D-glucopyranosyl)-β-D-glucopyranosyl]-L-asparaginyl-L-valyl-L-serine amide (8). — Glycopeptide **8** was prepared using a custom made fully automatic continuous flow peptide synthesizer constructed essentially as described [20]. A Tenta-Gel™ (Rapp Polymere, Germany) resin [capacity: 0.26 mmol/g, 231 mg (60 μmol)] functionalized with the Rink amide linker [21,22] and N^{α}-Fmoc amino acids (Bachem, Switzerland) carrying triphenylmethyl (Asn, Gln, His) or *tert*-butyl (Ser, Tyr, Asp) side-chain protective groups were used for the synthesis. The following automatic procedure was used for every coupling except for glycosylated **7** which was coupled manually. The Fmoc-amino acid (240 μmol, 4.0 eq.) was activated as a 1-benzotriazolyl ester [23] using 1,3-diisopropylcarbodiimide (DIC, 234 μmol, 3.9 eq.) and 1-hydroxybenzotriazole (HOBt) (340 μmol, 6.0 eq.) in DMF (1.3 mL) for 45 min. Bromophenolblue in DMF (0.30 mL of a 0.15 mM solution) was then added and the solution was circulated through the column containing the resin. The acylation was monitored [25] using the absorption of bromophenol blue at 600 nm and when the reaction was complete the resin was washed with DMF. N^{α}-Fmoc deprotection of the peptide resin was performed by a flow of 20% piperidine in DMF (2 mL/min for 12.5 min) using the absorbance of the dibenzofulvene-piperidine adduct at 350 nm for monitoring [26]. The resin was then washed with DMF, after which the procedure was repeated for the remaining amino acids. After the coupling of Val^{459}, the resin was transfered to a mechanically agitated reactor. Removal of the Fmoc group was then performed using a slow flow of 20% piperidine in DMF for 4 min, followed by washing with DMF. Glycosylated asparagine **7** (120 μmol, 2 eq.) was activated separately using DIC (117 μmol, 1.95 eq.) and 1-hydroxy-7-azabenzotriazole [24] (HOAt, 180 μmol, 3.0 eq.) in a minimal amount of DMF for 1 h. This solution was added to the resin and bromophenol blue (75 μL of a 2 mM solution in DMF) was added for monitoring. After reaction overnight the resin was washed with DMF and any unreacted amino groups were capped by treatment with acetic anhydride (1 mL) for 1 h. The resin was then washed with DMF and transferred to the peptide synthesizer where the remaining amino acids were coupled. After completion of the peptide synthesis the *N*-terminal amino group was acylated with acetic anhydride for 1 h. The resin was washed with CH_2Cl_2 and dried under vacuum giving 371 mg of glycopeptide resin. A solution of TFA/H_2O/thioanisole/ethanedithiol (35:2:2:1, 8 mL) was added to a portion (110 mg) of the resin. After 3 h without stirring the resin was filtered off and washed with HOAc (8 mL) and the combined filtrates were co-concentrated with HOAc until near dryness. Trituration with diethyl ether (2 x 2 mL) gave a pale yellow solid (30.4 mg) which according to analytical HPLC contained mainly **8** but also a small amount of monosilylated glycopeptide. A mixture of HOAc and H_2O was added and the resulting cloudy solution was freeze-dried. The crude product was divided into two portions, each of which was dissolved in TFA/H_2O (3:1, 5 mL) for 1 h to remove the remaining silyl

group(s). The solution was then directly applied on a reversed-phase HPLC column and purification as described in "General Methods and Materials" gave **8** (11.8 mg, 85% peptide content, 28% yield based on resin capacity). Glycopeptide **8** had; HRMS (FAB) calcd for $C_{87}H_{131}N_{22}O_{33}$ ($M+H^+$) 2011.9250, found 2011.9256; amino acid analysis: Ala 0.99 (1), Asp 2.97 (3), Gly 0.99 (1), Glu 1.02 (1), His 1.03 (1), Ile 1.97 (2), Phe 0.98 (1), Ser 2.04 (2), Tyr 1.00 (1), Val 1.04 (1).

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