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Synthesis and CD structural studies of CD52 peptides and glycopeptides

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Dedicated to Professor Yongzheng Hui on the occasion of his 70th birthday

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ABSTRACT

The syntheses of five natural and N-terminal acetylated peptides and glycopeptides of the CD52 antigen are described. Solid phase peptide synthesis was employed in the construction of the target compounds from Fmoc-protected commercial amino acids and synthetic glycan–asparagine conjugates. Circular dichroism studies of the synthetic targets showed that they exist as random coils in solution, and no significant change in secondary structure was observed when the CD52 peptide was either acetylated at the N-terminus or glycosylated at the Asn^3 residue with a disaccharide or a fucose-containing branched trisaccharide.

1. Introduction

Asparagine glycosylation is a ubiquitous co-translational protein modification that occurs in eukaryotic and some prokaryotic systems.[1] The cellular functions of N-linked glycosylation in eukaryotes have been extensively studied and include molecular recognition, immune responses, modulation of protein function, protein folding, and protein stabilization.[2] The N-glycan, an oligosaccharide that is covalently linked to asparagine in the sequence Asn-Xaa-Ser/Thr (where X is any amino acid except proline), has the ability to affect protein structure and, consequently, activity.[3–5] This observation has provided the impetus to design studies that combine synthesis and spectroscopic analysis to probe the effects of N-glycosylation on protein conformation and function.[5–10] Such studies require access to pure and structurally defined samples, a task made challenging by the microheterogeneity of biosynthetic glycoproteins, which generally exist as mixtures of glycoforms. However, advances in carbohydrate and peptide synthesis have enabled chemists to obtain significant quantities of homogenous glycopeptides and small glycoproteins for comparative structural analyses.[11–13]

Our interest in the effect of N-glycosylation on protein structure lies in the human CD52 antigen, a glycosylphosphatidylinositol (GPI)-anchored glycopeptide that is expressed on lymphocyte and sperm cells.[14–16] Although both lymphocyte and sperm CD52 share an identical 12 amino acid peptide, structural variations in their GPI anchor and N-linked glycan lead to distinct biological functions, with the former playing a role in the human immune system and the latter being involved in the human reproductive process.[15,17] Lymphocyte CD52 antibodies have been used to treat immune-related diseases such as leukemia,[18] whereas sperm CD52-specific antibodies have been isolated from infertile women and suggest the potential for immunocontraceptive development.[19,20]

Structurally, the two forms of CD52 have the same remarkably short peptide and exhibit one N-glycosylation site at the Asn^3 residue, to which complex types of glycans are linked.[15,16] Interestingly, the makeup of the N-linked glycans is one of the main structural differences between the two forms. For example, bi- and tri-antennary N-glycans, peripheral fucosylation, and the $\alpha(2\rightarrow3)$-linkage of sialic acid residues are male-specific modifications of CD52.[17] The structural differences of the N-glycans between sperm and lymphocyte CD52 seem to have a critical influence on their bioactivity. Sperm CD52-specific antibodies bind to the N-glycan of the antigen and may inhibit sperm-egg binding.[19,20] Lymphocyte CD52 antibodies, however, recognize a sequence of the peptide chain,[18] suggesting a possible difference in peptide structure between the two forms of CD52 and therefore the potential for conformational change induced by N-glycosylation or GPI anchoring.

The present study aims to compare the structures of non-glycosylated CD52 peptides and their glycosylated forms to determine

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what effect a model N-glycan may have on the conformation of the CD52 peptide backbone. For this purpose, we prepared the natural and N-terminal acetylated CD52 peptides **1a** and **1b** (Fig. 1), as well as glycopeptides **2** and **3a**–**b** that contain a disaccharide β-D-Glc*p*NAc(1→4)-β-D-Glc*p*NAc and a fucose-containing branched trisaccharide β-D-Glc*p*NAc(1→4)[α-L-Fuc*p*(1→6)]-β-D-Glc*p*NAc, respectively, using 9-fluorenylmethoxycarbonyl (Fmoc)-based solid phase peptide synthesis (SPPS). Next, circular dichroism (CD) was used to examine the solution structures of the synthetic targets and determine whether Asn-N-glycosylation or N-terminal acetylation resulted in any significant secondary structure changes.

2. Results and discussion

2.1. Synthesis of peptides 1a and 1b

The straightforward preparation of peptides **1a** and **1b** was carried out using standard Fmoc-SPPS on an automatic peptide synthesizer. Commercially available Ser-loaded resin and Fmoc-protected amino acids were used to synthesize the target peptide on a 0.1 mmol scale (84% yield based on weight). A portion of the resin-bound peptide was N-acetylated, and another portion was left unaltered at the N-terminus. Concomitant release of the peptides from the solid support and deprotection of the amino acid side chains was accomplished using a 95% trifluoroacetic acid (TFA) aqueous solution containing 2.5% triethylsilane (Et_3SiH). Purification of the products by RP-HPLC gave the desired peptides **1a** and **1b**. All spectroscopic data gathered for **1a** were in agreement with previously reported values,[21] and **1b** was positively characterized using NMR and MS.

2.2. Synthesis of glycopeptides 2, 3a, and 3b

The most widely adopted method for glycopeptide synthesis is solid phase synthesis. However, due to the acid lability of *O*-glycosidic bonds, particularly the fucosidic linkage,[22] and the potential for strong base induced β-elimination or epimerization,[23] care must be taken in the selection of protecting groups and overall synthetic strategy. Thus, partially or fully benzyl ether-protected oligosaccharide-asparagine conjugates were used in our target syntheses to facilitate late-stage deprotection under mild conditions, and measures were taken to ensure that acid sensitive functionalities would remain intact throughout each synthesis.

The primary challenge of synthesizing **3a** and **3b** is the α-fucosidic linkage, which is particularly acid sensitive when it is fully benzylated.[22] Although acyl-protected fucosides are more stable to acidic conditions,[24] protection of the 2-O-position by an acyl group, which has the neighboring group participation effect, must be avoided in order to achieve α-fucosylation. Our group developed the 'solution-phase synthesis with solid-phase workup' strategy, which employs unprotected carbohydrate and peptide building blocks,[25,26] to address the problem in an earlier synthesis of **3b**.[27] Meanwhile, it was observed that an unprotected fucoside intermediate could withstand treatment with 20% TFA, which is strong enough to deprotect peptide side chains. Based on these results, our group developed a strategy for solid phase glycopeptide synthesis that is compatible with all glycosidic bonds.[28,29] The strategy uses the 2-chlorotrityl resin,[30] a hyper acid sensitive resin that can release fully protected peptides upon treatment with 10% acetic acid, as the solid phase support, and it was applied to our synthesis of the target compounds.

2.2.1. Synthesis of glycopeptide 2

Perbenzylated disaccharide–Asn conjugate **10** was chosen as the glycosyl amino acid to be employed in Fmoc-SPPS en route to glycopeptide **2** (Scheme 1). Starting from D-glucosamine, reported methods were used to arrive at **4**,[31,32] which was deacetylated and then benzylated to afford thioglycoside **5**. Glycosylation of **6**[33] by **5** was promoted by *N*-iodosuccinimide (NIS) and triflic acid (TfOH) to give disaccharide **7** in a 75% yield. After phthalimide removal and subsequent N-acetylation to afford **8**, Lindlar's catalyst was used under a H_2 atmosphere to reduce the anomeric azide to

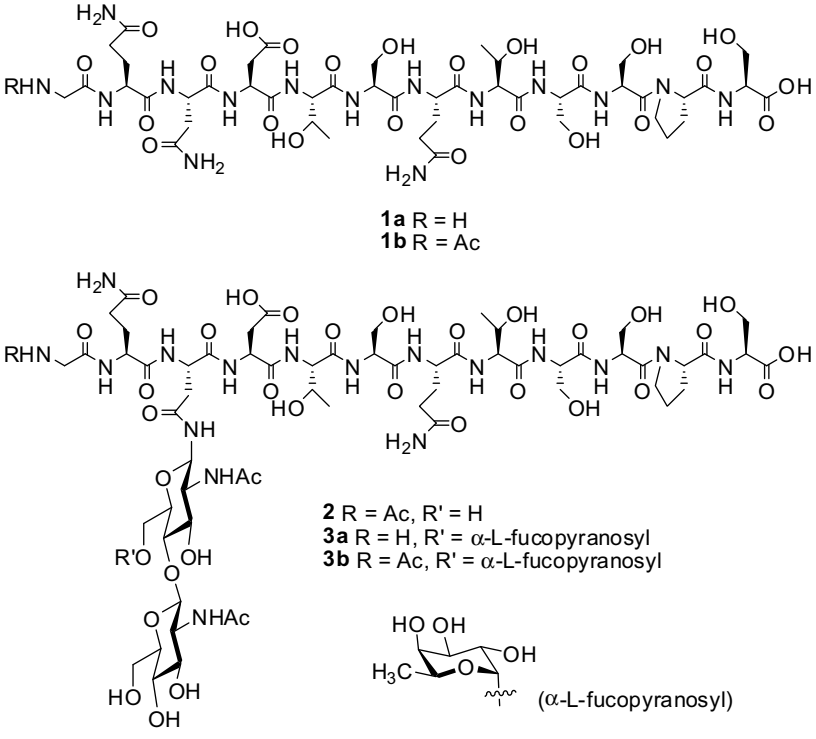


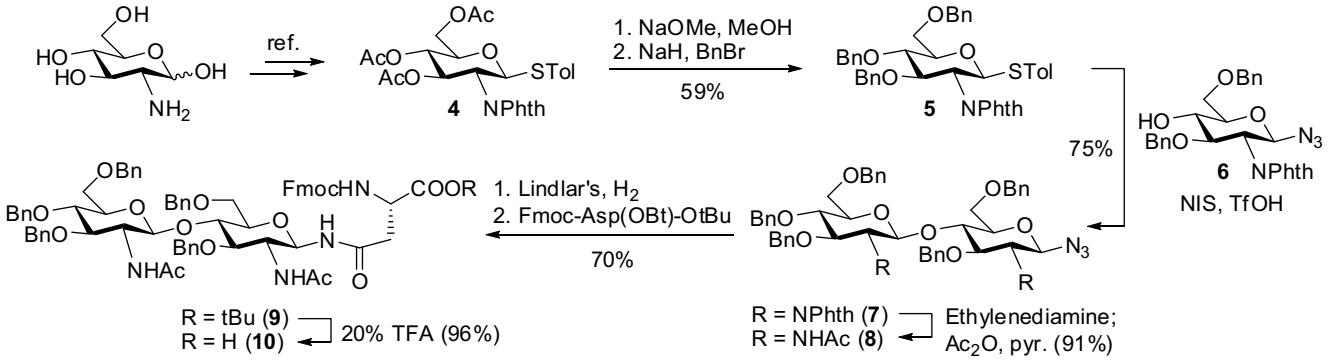
Figure 1. CD52 peptide and glycopeptide synthetic targets.

give a glycosyl amine, which immediately underwent amide bond formation using the active ester Fmoc-Asp(OB*t*)-O*t*Bu to furnish **9**. Finally, treatment of the product with 20% TFA to remove the C-terminal *t*-butyl group resulted in the desired disaccharide–Asn conjugate **10**.

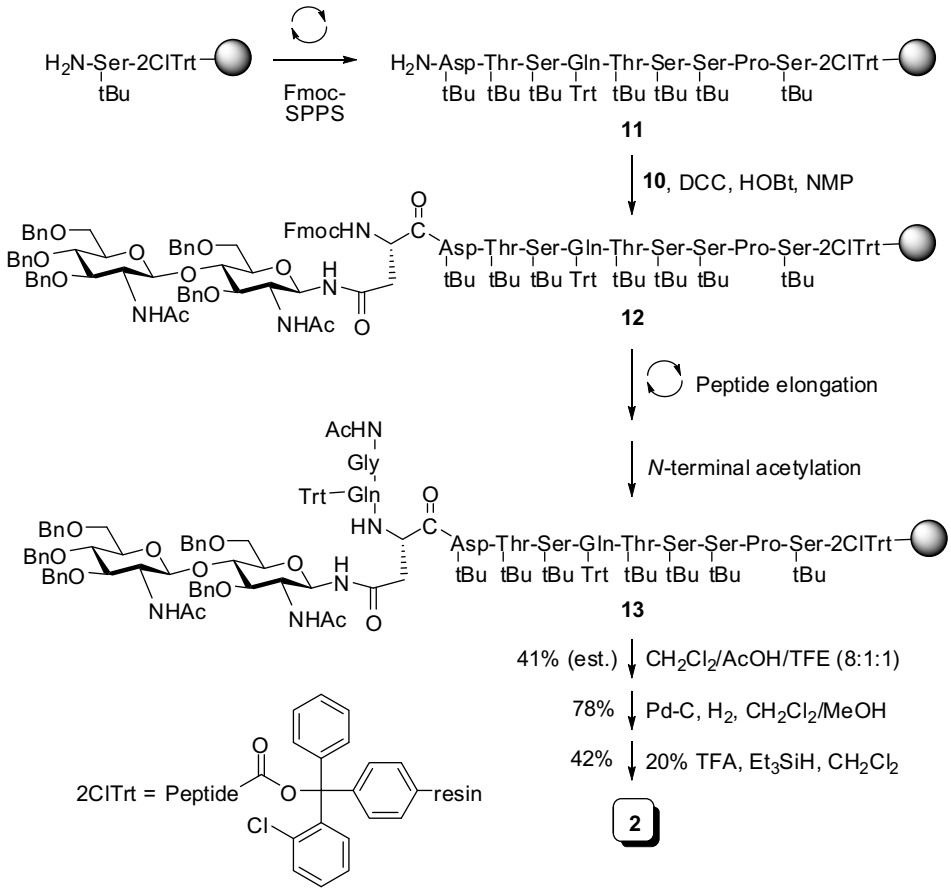
With glycosyl amino acid **10** in hand, solid phase construction of glycopeptide **2** was commenced (Scheme 2). The preparation of resin-linked nonapeptide **11** was carried out on an automated peptide synthesizer using standard Fmoc-SPPS chemistry starting from Ser-loaded 2-chlorotrityl resin. Coupling of the benzotriazole ester of **10** with **11** was completed manually. In this case, only 1.5 equiv of **10** was used in the synthesis. The mixture of **11** and the active ester of **10** was shaken under an argon atmosphere overnight to give **12**. The subsequent peptide chain elongation and N-terminal acetylation followed to afford resin-bound glycopeptide **13**. Release of the glycopeptide from the solid support with 10% acetic acid was followed by palladium-catalyzed N-glycan debenzylation. Finally, 20% TFA was used to deprotect the amino acid side chains to produce the target glycopeptide **2**, which was purified by RP-HPLC.

2.2.2. Synthesis of glycopeptides 3a and 3b

Partially benzylated trisaccharide–Asn conjugate **22** was chosen as the glycosylated asparagine to be employed in Fmoc-SPPS en route to glycopeptides **3a** and **3b** (Scheme 3). Our previous studies suggested that the remaining free hydroxyl groups in **22** would remain inert during the peptide elongation process.[25] Glycosyl azide **20** was previously prepared by Shao et al.,[27] while a slightly shorter pathway leading to **20** was developed in the work presented herein. Starting from D-glucosamine, *p*-methoxybenzylidene acetal



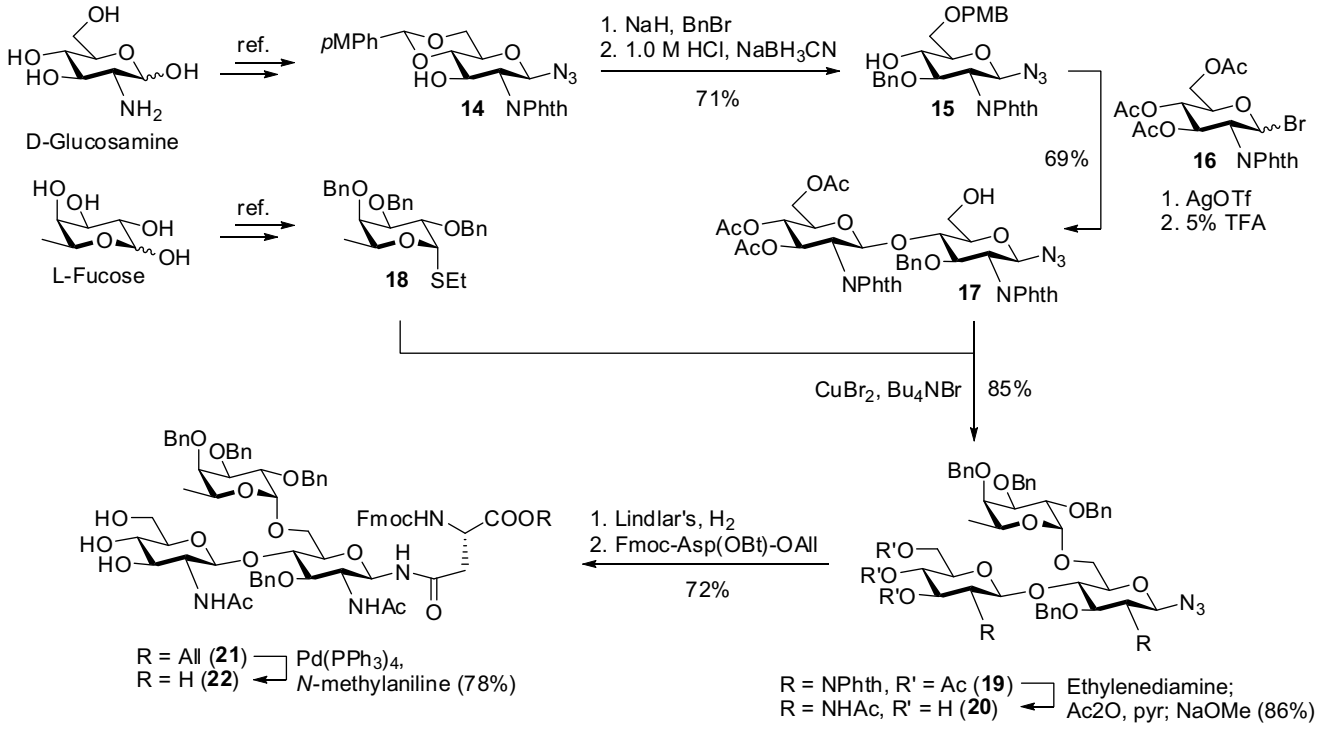
Scheme 1. Synthesis of disaccharide–Asn conjugate **10**.



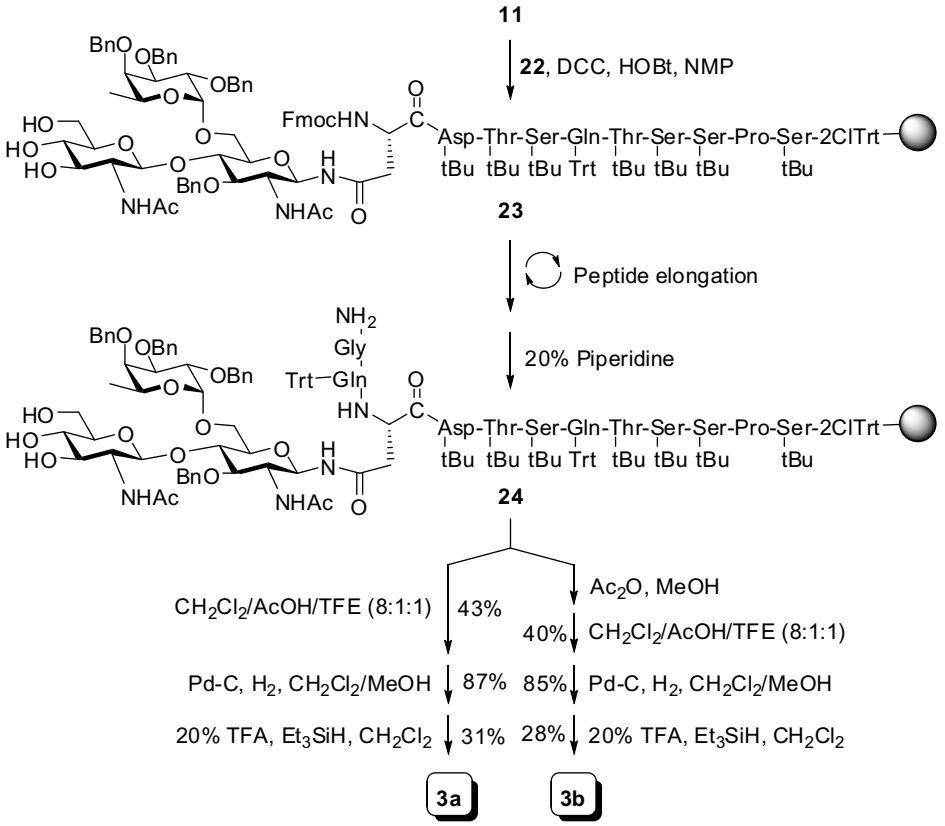
Scheme 2. Solid phase synthesis of glycopeptide **2**.

14[34] was synthesized in four steps. 3-O-Benzylation of **14** followed by regioselective acetal ring opening gave glycosyl acceptor **15** directly. The resultant *p*-methoxybenzyl (PMB) group on the 6-O-position can be easily and selectively removed under mild conditions.

Alcohol **15** underwent glycosylation with glycosyl bromide **16**[35] in the presence of silver triflate (AgOTf). The disaccharide product contained a small quantity of an inseparable impurity, so full characterization of the products was completed after acidic removal of



Scheme 3. Synthesis of trisaccharide–Asn conjugate **22**.



Scheme 4. Solid phase synthesis of glycopeptides **3a** and **3b**.

the PMB group. The resulting alcohol **17** was fucosylated by **18**[36] stereoselectively according to the conditions ($CuBr_2$–*n*-Bu_4NBr) set forth by Lemieux et al.[37] The transformation from **19** to **20** was achieved in a 3-step sequence, including removal of the *N*-phthalyl and *O*-acetyl groups, acetylation, and then O-deacetylation. O-Deacetylation at this stage was preferential due to potential complications in late-stage basic deprotection. Next, Lindlar's catalyst was employed under a H_2 atmosphere to reduce the anomeric azide and produce a glycosyl amine, which underwent amide bond formation with the active ester Fmoc-Asp(OBt)-OAll to furnish **21**. In contrast to the previous trisaccharide–Asn conjugate,[27] in which the carboxylic group was protected by a *t*-butyl group, an allyl group was used to protect the carboxylic group in **21**.[38] The allyl ester could be deprotected without acid, thus allowing fucose to remain benzylated. Indeed, treatment of **21** with $Pd(PPh_3)_4$ and *N*-methylaniline resulted in the desired trisaccharide–Asn conjugate **22**, which was subsequently used in Fmoc-SPPS.[39]

Fmoc-SPPS construction of glycopeptides **3a** and **3b** (Scheme 4) began with the manual coupling of the benzotriazole active ester of glycosyl-Asn conjugate **22** to the 2-chlorotrityl-resin anchored nonapeptide **11**. The resulting glycodecapeptide was then elongated at the N-terminus by manual Fmoc-SPPS to give fully protected, resin-bound glycopeptide **24**. Both the natural and N-terminal acetylated trisaccharide glycopeptides, **3a** and **3b**, were accessible from **24**. Glycopeptide **3a** was obtained via cleavage of **24** from the solid support followed by palladium-catalyzed N-glycan debenzylation and TFA-mediated side chain deprotection as described above. Care was taken during the debenzylation of **3a** to use aldehyde-free solvents in order to prevent N-terminal methylation via reductive amination. After the benzyl protecting groups were removed, the fucosidic linkage became stable to 20% TFA treatment, which was used to achieve amino acid side chain deprotection. Glycopeptide **3b** was afforded by first carrying out solid phase N-terminal acetylation, then completing the same protocols of glycopeptide release and global deprotection described for **3a**. Purification by RP-HPLC gave the desired glycopeptides **3a** and **3b**. Characterization of **3a** was carried out using NMR and MS, while all spectroscopic data gathered for **3b** were in agreement with previously reported values.[27]

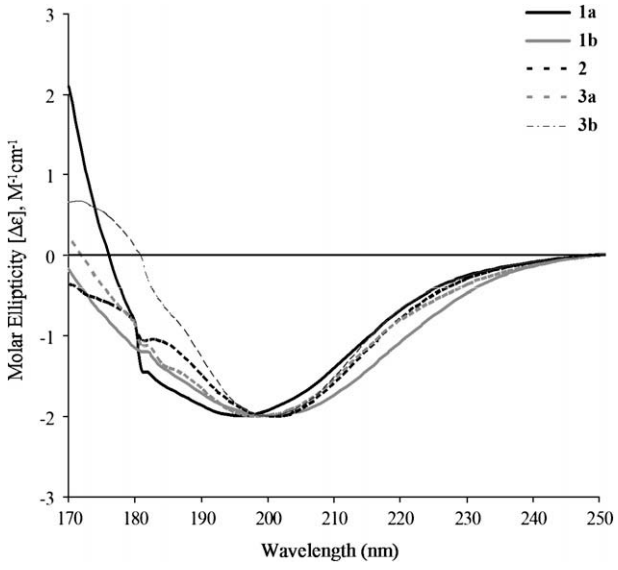


Figure 2. CD spectra of **1a**, **1b**, **2**, **3a**, and **3b** in H_2O at room temperature. The molar ellipticities of **1a**, **1b**, **2**, **3a**, and **3b** were normalized from concentrations of 6.6×10^{-2}, 3.2×10^{-2}, 8.0×10^{-2}, 6.2×10^{-2}, and 9.2×10^{-2} mM, respectively. Each curve represents the average of five scans.

2.3. CD structural studies

The aqueous solution conformations of the synthetic CD52 peptides and glycopeptides **1–3** were studied using CD spectroscopy, which has previously been used to probe the effects of glycosylation on peptide structure.[40–42] The CD spectra of **1a**, **1b**, **2**, **3a**, and **3b** are very similar with peak minima at 196, 201, 201, 198, and 200 nm, respectively (Fig. 2). These features are suggestive of random coil conformations for all derivatives as clearly described in the literature.[40,43] It was thus concluded that, in this case, neither N-glycosylation nor N-terminal acetylation led to any ordered secondary conformation of the CD52 peptide backbone.

2.4. Conclusion

The free peptide of the human CD52 antigen and four N-terminal acetylated and/or Asn-glycosylated derivatives were synthesized by SPPS. Benzylated glycosyl asparagine conjugates were employed as building blocks in the solid phase synthesis of glycopeptides. To avoid strong acid treatment that may affect the α-fucosidic bond, 2-chlorotrityl resin, which is particularly acid-labile, was used as the solid phase support so that the glycopeptides could be released under mildly acidic conditions compatible with all glycosidic linkages. CD structural studies of the synthetic peptides and glycopeptides led us to conclude that neither N-glycosylation nor N-terminal acetylation had a significant influence on the random coil conformation of the CD52 peptide, even though the fucose-containing branched trisaccharide is relatively sterically hindered. We believe that this is probably a result of the unique structure and properties of the CD52 peptide, as it is extremely short and hydrophilic. However, the conformation of the CD52 antigen when it is GPI-anchored onto the cell surface is still unknown.

3. Experimental

3.1. General methods

^1H NMR spectra were recorded at 400 or 500 MHz with chemical shifts reported in ppm (δ) downfield from tetramethylsilane (TMS) or relative to $CHCl_3$ (7.26 ppm) unless otherwise noted. Coupling constants (J) are reported in hertz (Hz). ^{13}C NMR spectra were recorded at 125 MHz. Thin layer chromatography (TLC) was performed on Silica Gel GF_{254} plates with detection by UV or charring with a 5% H_2SO_4 in EtOH solution. Molecular sieves 4 Å (4 Å MS) were dried in high vacuum at 170–180 °C for 6–10 h immediately before use. Anhydrous solvents were obtained from a solvent purification system, while commercial anhydrous reagents were used without further purification. Automated peptide synthesis was carried out on an Applied Biosystems 433A peptide synthesizer using fresh reagents and solvents purchased from Applied Biosystems. 2,5-Dihydroxybenzoic acid (DHB) was used as the MALDI TOFMS matrix, and samples were dissolved in either H_2O–CH_3CN (1:1) containing 1% TFA or CH_2Cl_2–MeOH (1:1) for unprotected and fully protected compounds, respectively.

3.2. CD52 peptide 1b

Resin-bound, fully protected CD52 peptide **1a**[21] was subjected to acetylation conditions (pyridine–Ac_2O 2:1) for 4 h. After the resin was washed thoroughly and filtered, the desired N-terminal acetylated peptide **1b** was released by treatment with 95% aqueous TFA containing 2.5% Et_3SiH as the cation scavenger. After precipitation by cold ether and lyophilization, **1b** was purified by RP-HPLC (Supelco Discovery C18, 250 × 10 mm, eluent 5% CH_3CN in H_2O, 2 mL/min, t_R = 14.9 min). ^1H NMR (500 MHz, D_2O, DHO at δ 4.79 as

reference): δ 4.72 (t, J = 7.0 Hz, 1H), 4.54–4.43 (m, 4H), 4.41–4.39 (m, 3H), 4.35–4.31 (m, 2H), 4.25 (dd, J = 5.0, 6.5 Hz, 1H), 3.94–3.73 (m, 11H), 2.95 (dd, J = 6.5, 17.0 Hz, 1H), 2.87 (dd, J = 6.0, 15.5 Hz, 1H), 2.85 (dd, J = 7.0, 17.0 Hz, 1H), 2.77 (dd, J = 8.0, 15.5 Hz, 1H), 2.40–2.29 (m, 5H), 2.21–2.08 (m, 2H), 2.07 (s, 3H), 2.05–1.96 (m, 5H), 1.22 (d, J = 2.0 Hz, 3H), 1.20 (d, J = 1.5 Hz, 3H); ^{13}C NMR (D_2O, 125 MHz): δ 178.04, 177.99, 175.16, 174.97, 174.71, 174.54, 174.00, 173.61, 173.34, 173.18, 172.38, 172.27, 172.09, 172.00, 171.91, 171.53, 170.03, 67.19, 67.08, 61.74, 61.28, 61.03, 60.93, 60.74, 59.46, 59.18, 56.13, 56.05, 55.58, 53.85, 53.41, 53.37, 50.69, 48.26, 42.70, 36.17, 36.02, 31.23, 31.15, 29.52, 26.87, 26.72, 24.70, 21.90, 18.92, 18.87; HR ESIMS: $[M+Na]^+$ calcd for $C_{47}H_{75}N_{15}O_{25}Na$, 1272.4956; found: *m*/*z* 1272.4932.

3.3. *p*-Methylphenyl 3,4,6-tri-*O*-benzyl-2-deoxy-2-phthalimido-1-thio-β-D-glucopyranoside (5)

After **4** (5.00 g, 9.23 mmol) was treated with NaOMe in methanol (0.05 M, 40 mL) at rt for 2 h, the solution was neutralized to pH 6–7 by Amberlyst H^+ resin. After filtration of the resin, the filtrate was concentrated to afford a white powder that was directly used for the next step. To a solution of the triol intermediate (3.00 g, 7.22 mmol) in DMF (60 mL) were added tetrabutylammonium iodide (TBAI, 300 mg, 0.76 mmol) and benzyl bromide (5.71 mL, 45.9 mmol). The solution was cooled to 0 °C and then NaH (60% dispersion in mineral oil, 1.6 g, 40.0 mmol) was added slowly. The reaction mixture was stirred at 0 °C for 1 h and then stirred at rt overnight. The reaction mixture was quenched with aqueous NH_4Cl and extracted with CH_2Cl_2. The organic phase was washed with aqueous NH_4Cl and brine, then dried over Na_2SO_4 and filtered. The solvent was evaporated, and the crude product was purified by silica gel chromatography to give **5** as white foam (2.9 g, 59%). ^{1}H NMR (400 MHz, $CDCl_3$): δ 7.90–7.60 (m, 4 H), 7.42–7.26 (m, 10H), 7.04–6.82 (m, 5H), 5.51 (d, 1H), 4.76–4.90 (m, 2H), 4.74–4.56 (m, 3H), 4.50–4.36 (m, 2H), 4.26 (t, 1H), 3.90–3.66 (m, 4H), 2.29 (s, 3H); MALDI TOFMS: $[M+Na]^+$ calcd for $C_{42}H_{39}NO_6$-NaS, 708.2; found: *m*/*z* 708.4.

3.4. *O*-(-3,4,6-Tri-*O*-benzyl-2-deoxy-2-phthalimido-β-D-glucopyranosyl)-(1→4)-3,6-di-*O*-benzyl-2-deoxy-2-phthalimido-β-D-glucopyranosyl azide (7)

To a solution of **5** (1.0 g, 1.46 mmol) and **6** (514 mg, 1.00 mmol) in anhydrous CH_2Cl_2 (50 mL) was added 4 Å MS under an Ar atmosphere. The reaction mixture was cooled to −30 °C, and NIS (520 mg, 2.31 mmol) was added. TfOH (41 μL, 0.46 mmol) was then added dropwise over a 5-min period. The reaction mixture was stirred for 20 min, and was then filtered through Celite. The filtrate was washed with aqueous $NaHCO_3$, $Na_2S_2O_3$, and brine solution. The organic layer was dried over Na_2SO_4, filtered, and concentrated to give yellowish oil. Silica gel chromatography yielded **7** (800 mg, 75%) as clear syrup. NMR data were consistent with literature-reported values.[44] MALDI TOFMS: $[M-N_2+Na]^+$ calcd for $C_{63}H_{57}N_3O_{12}Na$, 1070.4; found: *m*/*z* 1070.2; $[M+Na]^+$ calcd for $C_{63}H_{57}N_5O_{12}Na$, 1098.4; found: *m*/*z* 1098.2.

3.5. *O*-(2-Acetamido-3,4,6-tri-*O*-benzyl-2-deoxy-β-D-glucopyranosyl)-(1→4)-2-acetamido-3,6-di-*O*-benzyl-2-deoxy-β-D-glucopyranosyl azide (8)

After a mixture of **7** (200 mg, 0.19 mmol), ethylenediamine (1 mL), and 1-butanol (5 mL) was stirred at 90 °C overnight, it was concentrated to dryness under vacuum. The resulting residue was dissolved in Ac_2O–pyridine (1:2, 3 mL) and stirred overnight at rt. After the reaction mixture was diluted with EtOAc (50 mL), the solution was washed with saturated $NaHCO_3$ and brine, dried over Na_2SO_4, filtered, and concentrated under vacuum. The residue was purified by silica gel chromatography to give **8** as white foam (156 mg, 91%). NMR data of **8** were consistent with literature-reported values.[44] MALDI TOFMS: $[M-N_2+Na]^+$ calcd for $C_{51}H_{57}N_3O_{10}Na$, 894.4; found: *m*/*z* 895.0; $[M+Na]^+$ calcd for $C_{51}H_{57}N_5O_{10}Na$, 922.4; found: *m*/*z* 922.8.

3.6. N^{α}-(9-Fluorenylmethoxycarbonyl)-N^{γ}-[2-acetamido-3,4,6-tri-*O*-benzyl-2-deoxy-β-D-glucopyranosyl)-(1→4)-2-acetamido-3,6-di-*O*-benzyl-2-deoxy-β-D-glucopyranosyl]-L-asparagine *tert*-butyl ester (9)

To a solution of Fmoc-Asp-O*t*Bu in CH_2Cl_2 (10 mL) and NMP (1 mL) were subsequently added HOBt and DCC. The mixture was stirred at rt for 1 h to give the active ester. Meanwhile, a mixture of **8** and Lindlar's catalyst in CH_2Cl_2–MeOH was stirred at rt for 2.5 h under an H_2 atmosphere. After filtration through Celite and concentration in vacuum, the resulting residue was dissolved in CH_2Cl_2 (2 mL), and then the freshly prepared active ester solution was added (DCU was filtered off before addition). The mixture was stirred at rt overnight, concentrated, and purified directly by silica gel chromatography to give **9** as white foam (70%). ^{1}H NMR (500 MHz, CD_3OD–$CDCl_3$ 1:5): δ 7.71 (d, 2H, J = 8.0 Hz), 7.56 (d, 2H, J = 7.5 Hz), 7.40–7.06 (m, 32H), 4.79–4.67 (m, 4H), 4.61–4.53 (m, 3H), 4.51 (d, 1H, J = 10.5 Hz), 4.42 (t, 1H, J = 5 Hz), 4.40–4.31 (m, 5H), 4.23 (t, 1H, J = 7.5 Hz), 4.16 (t, 1H, J = 7.0 Hz), 4.04 (t, 1H, J = 7.0 Hz), 3.96 (t, 1H, J = 8.5 Hz), 3.84 (t, 1H, J = 10.0 Hz), 3.64–3.49 (m, 7H), 3.49–3.41 (m, 2H), 3.28–3.23 (m, 1H), 2.77 (ddd, 2H, J = 16 Hz, 3.5 Hz), 1.78 (s, 3H), 1.73 (s, 3H), 1.37 (s, 9H); MALDI TOFMS: $[M+Na]^+$ calcd for $C_{74}H_{82}N_4O_{15}Na$, 1289.6; found: *m*/*z* 1289.9.

3.7. N^{α}-(9-Fluorenylmethoxycarbonyl)-N^{γ}-[2-acetamido-3,4,6-tri-*O*-benzyl-2-deoxy-β-D-glucopyranosyl)-(1→4)-2-acetamido-3,6-di-*O*-benzyl-2-deoxy-β-D-glucopyranosyl]-L-asparagine (10)

To a solution of **9** (55 mg, 43 μmol) in CH_2Cl_2 (4 mL) were added triethylsilane (0.25 mL) and TFA (1 mL). After the mixture was stirred for 2.5 h at rt, Et_3N was added to quench the reaction, which was then concentrated and subjected to silica gel chromatography to give **10** as white foam (50 mg, 96%). ^{1}H NMR (400 MHz, CD_3OD–$CDCl_3$ 1:5): δ 7.76 (d, J = 7.2 Hz, 2H), 7.63 (d, J = 7.2 Hz, 2H), 7.37–7.16 (m, 32H), 5.71 (d, J = 2.4 Hz, 1H), 4.78–4.52 (m, 9H), 4.49–4.44 (4H), 4.39–4.31 (m, 2H), 4.26–4.16 (m, 3H), 3.93–3.73 (m, 6H), 3.66–3.53 (m, 5H), 3.37 (s, br, 1H), 2.86 (dd, J = 5.6, 16.4 Hz, 1H), 2.76 (dd, J = 7.2, 15.2 Hz, 1H), 2.04 (s, 3H), 1.86 (s, 3H); MALDI TOFMS: $[M+Na]^+$ calcd for $C_{70}H_{74}N_4O_{15}Na$, 1233.5; found: *m*/*z* 1233.4.

3.8. Resin-bound glycopeptide 11

Ser-loaded 2-chlorotrityl resin (417 mg, 0.25 mmol) was subjected to eight cycles of Fmoc-SPPS on an automated peptide synthesizer to prepare resin-bound nonapeptide **11** (661 mg, 93% by weight) using HBTU-DIEA as the condensation reagent and 20% piperidine as the deprotection reagent. The product was then used in the SPPS construction of glycopeptides **2**, **3a**, and **3b**.

3.9. CD52 glycopeptide 2

A solution of glycosyl amino acid **10** (37 mg, 30 μmol), HOBt (8 mg, 60 μmol), and DCC (12 mg, 60 μmol) in NMP (1 mL) was stirred at rt for 1 h and then transferred to a SPPS vessel containing resin-bound nonapeptide **11** (56 mg, 20 μmol). The mixture was shaken at rt overnight under an Ar atmosphere, after which the resin was filtered off and washed thoroughly with NMP to give

solid supported glycopeptide **12**. After Fmoc removal with 20% piperidine in NMP, the resin was subjected to two manual Fmoc-SPPS cycles using the benzotriazole active esters of Fmoc-Gln(Trt)-OH and Fmoc-Gly-OH to afford the desired peptide sequence. After final Fmoc deprotection and N-terminal acetylation of half of the sample with Ac_2O to give **13**, the resin was treated with CH_2Cl_2–AcOH–TFE (8:1:1) at rt for 2 h to release the glycopeptide. The resin was filtered off and the cleavage mixture was diluted with hexanes and concentrated under vacuum to give a white solid (13 mg, 41% based on **11**). To the resulting fully protected glycopeptide (13 mg, 4 μmol) were added solvents CH_2Cl_2–MeOH (1:1, 1 mL) and 10% Pd–C (13 mg) under a H_2 atmosphere. After MALDI MS showed complete reaction, the catalyst was filtered off and the solvent was evaporated to get the debenzylated product (8 mg, 78% based on previous step), which was treated with 20% TFA in CH_2Cl_2 (1 mL) at rt for 2.5 h. After co-evaporation with toluene and thorough washing of the residue with diethyl ether, the product **2** was lyophilized and then purified by RP-HPLC (Supelco Discovery C18, 250 × 10 mm, eluent 2.0% *i*-PrOH in H_2O, 2 mL/min, t_R = 16.1 min, 2.2 mg, 42% based on the previously given yield). ^{1}H NMR (500 MHz, D_2O, DHO at δ 4.79 as reference): δ 5.05 (d, *J* = 10.0 Hz, 1H), 4.69 (t, *J* = 7.0 Hz, 1H), 4.61 (d, *J* = 8.0 Hz, 1H), 4.55 (t, *J* = 5.0 Hz, 1H), 4.51 (t, *J* = 5.0 Hz, 1H), 4.47–4.40 (m, 4H), 4.37–4.32 (m, 2H), 4.28–4.26 (m, 2H), 4.01–3.81 (m, 15H), 3.78–3.74 (m, 4H), 3.69–3.64 (m, 2H), 3.60–3.55 (m, 2H), 3.52–3.49 (m, 2H), 2.89 (dd, *J* = 6.0, 16.0 Hz, 1H), 2.77 (dd, *J* = 7.5, 15.0 Hz, 2H), 2.69 (dd, *J* = 6.5, 16.0 Hz, 1H), 2.42–2.30 (m, 5H), 2.22–2.11 (m, 2H), 2.08 (s, 6H), 2.06–2.04 (m, 2H), 2.02 (s, 3H), 2.01–1.99 (m, 2H), 1.24 (s, 3H), 1.23 (s, 3H); ^{13}C DEPT NMR CH carbons (D_2O, 125 MHz): 101.53, 78.91, 78.38, 76.35, 76.08, 73.62, 73.12, 69.87, 67.20, 66.85, 61.08, 59.44, 59.19, 57.35, 56.45, 55.76, 55.56, 53.83, 53.46, 53.29, 51.80, 50.22; HR ESIMS: $[M+2H]^{2+}$ calcd for $C_{63}H_{103}N_{17}O_{35}$, 828.8401; found: *m*/*z* 828.8363.

3.10. 3-*O*-Benzyl-2-deoxy-6-*O*-(*p*-methoxybenzyl)-2-phthalimido-β-D-glucopyranosyl azide (15)

To a solution of **14** (2.20 g, 4.86 mmol) in DMF (15 mL) at 0 °C was added NaH (60% dispersion in mineral oil, 1.24 g, 7.27 mmol). After stirring for 30 min, benzyl bromide (0.86 mL, 7.27 mmol) was added to the reaction mixture, which was stirred at rt overnight. After quenching excess NaH with MeOH, the reaction mixture was concentrated under vacuum to remove most of the DMF, then diluted with EtOAc and washed with saturated $NaHCO_3$, brine, dried over Na_2SO_4, and concentrated under vacuum. Purification by silica gel chromatography gave the benzylated intermediate (2.11 g, 3.9 mmol, 80%), which was then dissolved in THF and cooled to 0 °C. To this solution were added MS 4 Å (1.0 g), $NaBH_3CN$ (2.4 g, 39 mmol), and 1.0 M HCl in dry Et_2O (to pH 1–2). After 1 h, MS were filtered off, and the filtrate was diluted with EtOAc. The organic layer was washed with cold $NaHCO_3$ and brine, then dried over Na_2SO_4, filtered, and concentrated under vacuum. Silica gel chromatography gave **15** as colorless syrup (1.9 g, 89%). ^{1}H NMR (500 MHz, $CDCl_3$): δ 7.83–7.71 (m, 4H), 7.29 (d, *J* = 8.5 Hz, 2H), 6.89–7.04 (m, 7H), 5.36 (d, *J* = 9.5 Hz, 1H), 4.75 (d, *J* = 12.0 Hz, 1 H), 4.59 (d, *J* = 12.0 Hz, 1H), 4.53 (d, *J* = 11.5 Hz, 2H), 4.26 (dd, *J* = 8.5, 10.5 Hz, 1H), 4.08 (dd, *J* = 9.5, 10.5 Hz, 1H), 3.81–3.85 (m, 2H), 3.82 (s, 3H) 3.70–3.79 (m, 2H). HR ESIMS: $[M+Na]^+$ calcd for $C_{29}H_{28}N_4O_7Na$, 567.1856; found: *m*/*z* 567.1832.

3.11. *O*-(2-Acetamido-2-deoxy-β-D-glucopyranosyl)-(1→4)-[*O*-2,3,4-tri-*O*-benzyl-α-fucopyranosyl)-(1→6)]-2-acetamido-3-*O*-benzyl-2-deoxy-β-D-glucopyranosyl azide (17)

To a solution of 1,2,3,4-tetra-*O*-acetyl-2-deoxy-2-phthalimido-β-D-glucopyranose (75 mg, 0.15 mmol) in CH_2Cl_2 (2 mL) was added 33% HBr in HOAc (100 μL) at 0 °C. After being stirred at rt for 5 h, the reaction mixture was washed with saturated aqueous $NaHCO_3$ and brine, dried over Na_2SO_4, and concentrated under vacuum to give glycosyl bromide **16** as syrup that was directly used in the glycosylation reaction. After a mixture of **16**, **15** (40 mg, 0.07 mmol), and MS 4 Å (50 mg) in dry CH_2Cl_2 (2 mL) was stirred at rt for 1 h, it was cooled to −50 °C. To this mixture was added AgOTf (38 mg, 0.15 mmol). The reaction mixture was then warmed to rt and stirred for 3 h, at which point the MS were filtered off, and the resulting filtrate was washed with $NaHCO_3$ and brine, then dried over Na_2SO_4 and concentrated under vacuum. The residue was not fully separable by silica gel chromatography, as another compound co-eluted with the desired disaccharide. Thus, to a solution of the intermediate in CH_2Cl_2 (4 mL) were added TFA (0.2 mL) and Et_3SiH (0.5 mL). After the reaction mixture was stirred for 2.5 h, it was quenched with Et_3N, concentrated, and purified by silica gel chromatography to give pure **17** (43 mg, 69% yield over two steps). Spectroscopic data of **17** were identical to literature-reported values.[27]

3.12. N^{α}-(9-Fluorenylmethoxycarbonyl)-N^{γ}-[2-acetamido-3,4,6-tri-*O*-benzyl-2-deoxy-β-D-glucopyranosyl)-(1→4)-[*O*-2,3,4-tri-*O*-benzyl-α-fucopyranosyl)-(1→6)]-2-acetamido-3-*O*-benzyl-2-deoxy-β-D-glucopyranosyl]-L-asparagine allyl ester (21)

Glycosyl azide **20** (127 mg, 0.133 mmol) was dissolved in a mixture of CH_2Cl_2 (1 mL) and MeOH (3 mL). Lindlar's catalyst (127 mg) was then added and the reaction mixture was stirred under a H_2 atmosphere at rt for 3 h. After filtration of the catalyst by silica gel, the reaction mixture was concentrated under vacuum to give the glycosyl amine intermediate. Meanwhile, to a solution of Fmoc-Asp(OH)-OAll (79 mg, 0.20 mmol) in CH_2Cl_2 (3 mL) and NMP (0.1 mL) were added DCC (49 mg, 0.24 mmol) and HOBt (32 mg, 0.24 mmol). After stirring for 1 h, DCU was filtered off and the active ester solution was directly transferred to a solution of the glycosyl amine in CH_2Cl_2 (1 mL). The reaction mixture was stirred at rt under an Ar atmosphere overnight, then concentrated and purified by silica gel chromatography to give the glycosyl-Asn conjugate **21** (122 mg, 72%). ^{1}H NMR (400 MHz, $CDCl_3$–CD_3OD 3:1): δ 7.56 (d, *J* = 8.0 Hz, 2H), 7.40 (d, *J* = 7.2 Hz, 2H), 7.21–7.05 (m, 24H), 5.72–5.65 (m, 1H), 5.11 (d, *J* = 16.8 Hz, 1H), 5.02 (d, *J* = 10.4 Hz, 1H), 4.77 (d, *J* = 11.6 Hz, 1H), 4.72 (d, *J* = 11.2 Hz, 1H), 4.68–4.55 (m, 4H), 4.44–4.40 (m, 6H), 4.23 (dd, *J* = 7.6, 10.8 Hz, 1H), 4.04–3.99 (m, 2H), 3.87–3.84 (m, 2H), 3.74 (dd, *J* = 3.2, 9.6 Hz, 1H), 3.71–3.66 (m, 4H), 3.62–3.56 (m, 5H), 3.35–3.27 (m, 4H), 3.13–3.07 (m, 4H), 2.62 (dd, *J* = 6.0, 16.4 Hz, 1H) 2.49 (dd, *J* = 4.0, 16.4 Hz, 1H), 1.77 (s, 3H), 1.67 (s, 3H), 0.95 (d, *J* = 6.4 Hz, 3H); HR ESIMS: $[M+Na]^+$ calcd for $C_{72}H_{82}N_4O_{19}Na$, 1329.5471; found: *m*/*z* 1329.5414.

3.13. N^{α}-(9-Fluorenylmethoxycarbonyl)-N^{γ}-[2-acetamido-3,4,6-tri-*O*-benzyl-2-deoxy-β-D-glucopyranosyl)-(1→4)-[*O*-2,3,4-tri-*O*-benzyl-α-fucopyranosyl)-(1→6)]-2-acetamido-3-*O*-benzyl-2-deoxy-β-D-glucopyranosyl]-L-asparagine (22)

To a solution of **21** (77 mg, 0.058 mmol) in CH_2Cl_2–MeOH (1:1, 2 mL) were added *N*-methylaniline (100 μL) and $Pd(PPh_3)_4$ (12 mg, 0.01 mmol). The reaction mixture was stirred overnight at rt, then concentrated under vacuum and purified by silica gel chromatography to give **22** (57 mg, 78%). ^{1}H NMR (500 MHz, CD_3OD, δ 4.78 as reference): δ 7.69 (d, *J* = 7.2 Hz, 2H), 7.55–7.52 (m, 2H), 7.29–7.15 (m, 24H), 4.98 (d, *J* = 11.6 Hz, 1H), 4.90 (d, *J* = 10.0 Hz, 1H), 4.84 (d, *J* = 2.4 Hz, 1H), 4.71–4.66 (m, 4H), 4.61 (d, *J* = 8.8 Hz, 1H), 4.54 (d, *J* = 10.4 Hz, 1H), 4.47 (d, *J* = 11.2 Hz, 1H), 4.24–4.23 (m, 3H), 4.11 (t, *J* = 6.4 Hz, 1H), 4.01 (d, *J* = 5.6 Hz, 1H), 3.90–3.81 (m,

4H), 3.78–3.68 (m, 5H), 3.53 (t, J = 9.6 Hz, 1H), 3.47–3.42 (m, 2H), 3.38 (t, J = 9.8 Hz, 1H), 3.31–3.28 (m, 3H), 3.19–1.17 (m, 1H), 2.65 (s, br, 2H), 1.91 (s, 3H), 1.84 (s, 3H), 1.05 (d, J = 5.6 Hz, 3H); HR ESIMS: $[M+Na]^+$ calcd for $C_{69}H_{78}N_4O_{19}Na$, 1289.5158; found: m/z 1289.5116.

3.14. CD52 glycopeptide 3a

A solution of glycosyl amino acid **10** (51 mg, 40 μmol), HOBt (12 mg, 90 μmol), and DCC (18 mg, 90 μmol) in NMP (1 mL) was stirred at rt for 1 h and then transferred to a SPPS vessel containing resin-bound nonapeptide **11** (56 mg, 20 μmol). The mixture was shaken at rt overnight under an Ar atmosphere, after which the resin was filtered off and washed thoroughly with NMP to give resin supported glycopeptide **12**. After Fmoc removal with 20% piperidine in NMP, the resin was subjected to two manual Fmoc-SPPS cycles using the benzotriazole active esters of Fmoc-Gln(Trt)-OH and Fmoc-Gly-OH to give the desired peptide sequence. After final Fmoc deprotection to give **24**, half of the resin was treated with CH_2Cl_2–AcOH–TFE (8:1:1) at rt for 2 h to release the glycopeptide. The resin was filtered off and the cleavage mixture was diluted with hexanes and concentrated under vacuum to give a white solid (13 mg, 43% based on **11**). To the resulting fully protected glycopeptide (13 mg, 4.3 μmol) were added CH_2Cl_2–MeOH (1:1, 1 mL) and 10% Pd–C (13 mg) under a H_2 atmosphere (MeOH used in the debenzylation was refluxed with $NaBH_4$ for several hours and then distilled to reduce any formaldehyde present and thus prevent N-methylation via reductive amination). After MALDI MS showed complete reaction, the catalyst was filtered off and the solvent was evaporated to get the debenzylated product (10 mg, 87% based on previous step), which was treated with 20% TFA in CH_2Cl_2 (1 mL) at rt for 2.5 h. After co-evaporation with toluene and thorough washing of the residue with diethyl ether, the product **3a** was lyophilized and then purified by RP-HPLC (Supelco Discovery C18, 250 × 10 mm, eluent 1.5% i-PrOH in H_2O, 2 mL/min, t_R = 17.9 min, 2.1 mg, 31% based on the previously given yield). ^{1}H NMR (500 MHz, D_2O, DHO at δ 4.79 as reference): δ 5.05 (d, J = 10.0 Hz, 1H), 4.90 (d, J = 4.0 Hz, 1H), 4.67 (d, J = 8.5 Hz, 1H), 4.54 (t, J = 5.5 Hz, 1H), 4.53–4.51 (m, 1H), 4.49–4.44 (m, 2H), 4.43–4.40 (m, 2H), 4.37–4.32 (m, 2H), 4.27 (t, J = 6.0 Hz, 1H), 4.14 (dd, J = 6.5, 13.0 Hz, 1H), 3.95–3.86 (m, 11H), 3.85–3.74 (m, 8H), 3.70–3.68 (m, 2H), 3.57 (t, J = 8.5 Hz, 1H), 3.53–3.46 (m, 2H), 2.90 (dd, J = 5.0, 16.5 Hz, 1H), 2.86–2.77 (m, 3H), 2.42–2.37 (m, 4H), 2.35–2.32 (m, 1H), 2.21–2.17 (m, 1H), 2.12–2.10 (m, 1H), 2.09 (s, 3H), 2.08–2.04 (m, 2H), 2.03 (s, 3H), 2.02–2.00 (m, 2H), 1.24–1.20 (m, 9H); ^{13}C NMR (D_2O, 125 MHz): δ 177.99, 177.78, 174.93, 174.74, 173.84, 173.58, 173.16, 172.59, 172.23, 172.13, 171.99, 171.89, 171.52, 170.05, 167.70, 101.29, 99.48, 78.66, 78.43, 76.10, 75.34, 73.67, 72.99, 72.02, 69.90, 69.70, 68.35, 67.19, 66.98, 66.69, 61.98, 61.28, 61.07, 61.00, 60.75, 59.41, 59.16, 56.07, 55.78, 55.58, 53.90, 53.84, 53.78, 53.33, 51.26, 50.24, 48.25, 40.54, 36.32, 31.22, 31.10, 29.51, 27.01, 26.87, 24.68, 22.41, 22.31, 18.94, 18.87, 15.61; MALDI TOFMS: $[M+Na]^+$ calcd for $C_{67}H_{109}N_{17}O_{38}Na$, 1782.7; found: m/z 1782.6.

3.15. CD52 glycopeptide 3b

Compound **3b** was prepared according to the procedure described for the synthesis of **3a**. The half of resin-bound glycopeptide **24** (10 μmol) remaining from the synthesis of **3a** was treated with Ac_2O–MeOH (1:2, 1 mL) at rt for 4 h. The resin was then treated with CH_2Cl_2–AcOH–TFE (8:1:1) at rt for 2 h to release the glycopeptide. The resin was filtered off and the cleavage mixture was diluted with hexanes and concentrated under vacuum to give a white solid (12 mg, 40% based on **11**). The remaining deprotection steps were carried out according to the protocol described for **3a** to arrive at **3b**, which was purified by RP-HPLC (Supelco Discovery C18, 250 × 10 mm, eluent 1.5% i-PrOH in H_2O, 2 mL/min, t_R = 17.9 min, 1.9 mg, 28% based on the previously given yield). Spectroscopic data of **3b** were consistent with literature-reported values.[27]

3.16. Circular dichroism spectroscopy

CD spectra of all derivatives were recorded on a Chirascan circular dichroism spectrometer equipped with a water bath to control the temperature at 25 °C. The solutions of **1a**, **1b**, **2**, **3a**, and **3b** were prepared from dry samples in ddH_2O to give concentrations of 6.6×10^{-2}, 3.2×10^{-2}, 1.6×10^{-2}, 4.2×10^{-2}, and 1.8×10^{-2} mM, respectively. The molar ellipticity was normalized using the equation $\Delta\varepsilon = \theta/(32.98 \times C)$, in which θ is the CD absorbance of each peptide analog.

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Supplementary data

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