Expeditious chemoenzymatic synthesis of CD52 glycopeptide antigens†

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CD52 is a glycosylphosphatidylinositol (GPI)-anchored glycopeptide antigen found on sperm cells and human lymphocytes. Recent structural studies indicate that sperm-associated CD52 antigen carries both a complex type N-glycan and an O-glycan on the polypeptide backbone. To facilitate functional and immunological studies of distinct CD52 glycoforms, we report in this paper the first chemoenzymatic synthesis of homogeneous CD52 glycoforms carrying both N- and O-glycans. The synthetic strategy consists of two key steps: monosaccharide primers GlcNAc and GalNAc were first installed at the pre-determined N- and O-glycosylation sites by a facile solid-phase peptide synthesis, and then the N- and O-glycans were extended by respective enzymatic glycosylations. It was found that the endoglycosidase-catalyzed transglycosylation allowed efficient attachment of an intact N-glycan in a single step at the N-glycosylation site, while the recombinant human T-synthase could independently extend the O-linked GalNAc to form the core 1 O-glycan. This chemoenzymatic approach is highly convergent and permits easy construction of various homogeneous CD52 glycoforms from a common polypeptide precursor. In addition, the introduction of a latent thiol group in the form of protected cysteamine at the C-terminus of the CD52 glycoforms will enable site-specific conjugation to a carrier protein to provide immunogens for generating CD52 glycoform-specific antibodies for functional studies.

Introduction

CD52 is a GPI-anchored glycopeptide antigen found on human lymphocytes and sperm cells. Structural studies have shown that CD52 antigen is a glycopeptide consisting of only 12 amino acid residues but carrying a large complex type N-glycan at the Asn-3 residue.[1–3] Detailed structural analysis suggests that the complex N-glycans from the sperm and lymphocyte CD52 antigens are highly heterogeneous and are cell type-specific in terms of branching and composition.[1,2,4–8] More recently, it has been demonstrated that the CD52 antigen from sperm cells also carries an O-glycan that was putatively assigned at the Thr-8 residue.[9] The different glycoforms revealed by these studies implicate distinct biological functions of the two types of CD52 antigens. Functional studies suggest that the CD52 antigen on human lymphocytes might be involved in signal transduction during lymphocyte activation.[10,11] By contrast, the CD52 antigen on sperm cell surface may be involved in sperm–egg interactions and associated with fertilization.[12,13] For example, the acquisition of the CD52 antigen by sperm cells from the male genital tract coincides with the acquisition of fertilizing capacity and, thus, CD52 is also called the "major maturation-associated sperm membrane antigen".[4,5,14] Several anti-CD52 antibodies were reported. The anti-CD52 antibody CAMPATH-1 reacts to both lymphocyte and sperm CD52 antigens, the epitope of which includes the last three amino acid residues and part of the GPI anchor.[15] In contrast, two other monoclonal antibodies, the S-19[5] and MAb H6-3C4,[16] which were generated in the reproductive system against the sperm CD52-specific N-glycans, are not cross-reactive to lymphocyte CD52 but are able to inhibit sperm–egg interactions.[17] Moreover, polyclonal antibodies against the gp20 antigen, a GPI-anchored protein of the human sperm surface that is homologous to leukocyte antigen CD52, were shown to be specific for the O-glycans of CD52 antigen.[18,19] These studies suggest that the sperm cell surface CD52 glycopeptide antigen may serve as a novel target for antibody-based immune contraception.

Such an immunological pursuit will rely on the preparation of structurally well-defined glycoforms of sperm CD52 antigens. However, isolation of distinct homogeneous glycoforms from natural sources in sufficient quantities for functional studies will be extremely difficult because of the structural heterogeneity of natural CD52 antigens. There are several commonly used methods for glycopeptide synthesis,[20–26] including the use of glycoamino acid as the building block in solid-phase peptide synthesis, the convergent coupling of selectively protected Asp-containing peptide with free glycosylamine, and chemoenzymatic synthesis using enzymes to extend the sugar chains. CD52 glycopeptides carrying an N-linked core tri- or penta-saccharide were previously synthesized by a glycoamino acid building block approach.[27–29] A CD52 glycoform carrying an N-linked undecasaccharide mimic was constructed using a convergent chemoselective ligation method.[30] We have previously reported a chemoenzymatic synthesis of CD52 antigen containing an intact bi-antennary complex type N-glycan.[31] But the use of wild type endo-N-acetylglucosaminidase from *Mucor hiemalis* (Endo-M) resulted in a low transglycosylation yield due to significant product hydrolysis. In this paper, we report a convergent chemoenzymatic synthesis of homogeneous CD52 glycopeptides that carry both N- and O-glycans (Fig. 1), the synthesis of which has not been achieved before. Our synthetic strategy consists of two key steps: 1) the installation of the monosaccharide primers

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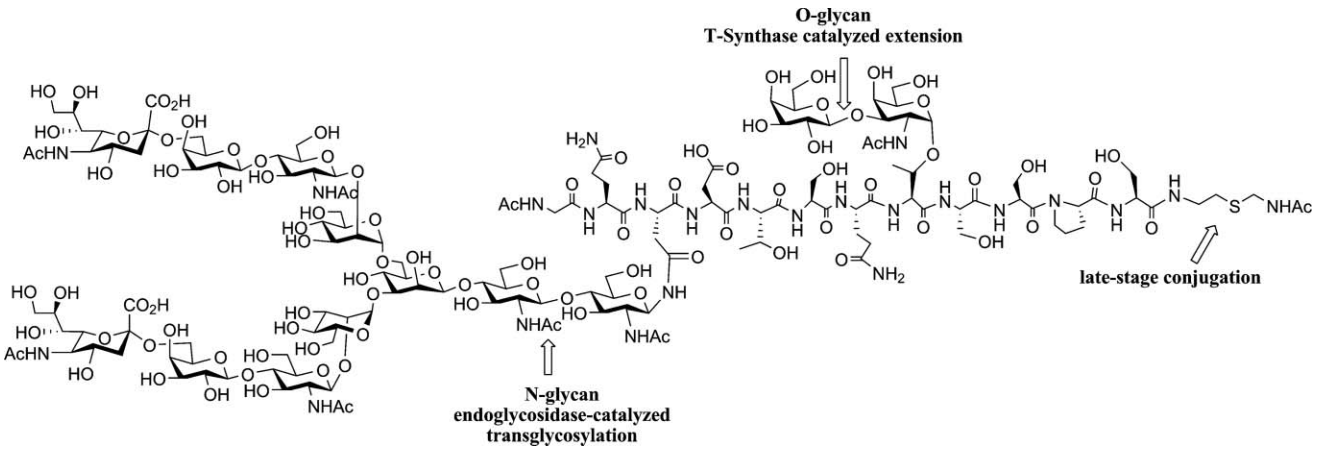


Fig. 1 The structure of a CD52 glycoform carrying natural N- and O-glycans.

(GlcNAc and GalNAc) at the pre-determined N- and O-glycosylation sites by standard solid-phase peptide synthesis; and 2) the extension of the N- and O-glycans from the primers in context of free polypeptide by endoglycosidase-catalyzed transglycosylation and glycosyltransferase-catalyzed sugar transfer, respectively. This strategy permits independent synthetic manipulations of the N- and O-glycans, thus enabling a quick construction of various CD52 glycoforms from a common polypeptide precursor. In particular, recent advances in the endoglycosidase-catalyzed transglycosylation for N-glycopeptide/glycoprotein synthesis, including the use of the highly active sugar oxazolines as the donor substrates and the creation of novel endoglycosidase mutants (glycosynthases), has made it possible to introduce various N-glycans into a CD52 polypeptide in a convergent and highly efficient manner.[25,32–43]

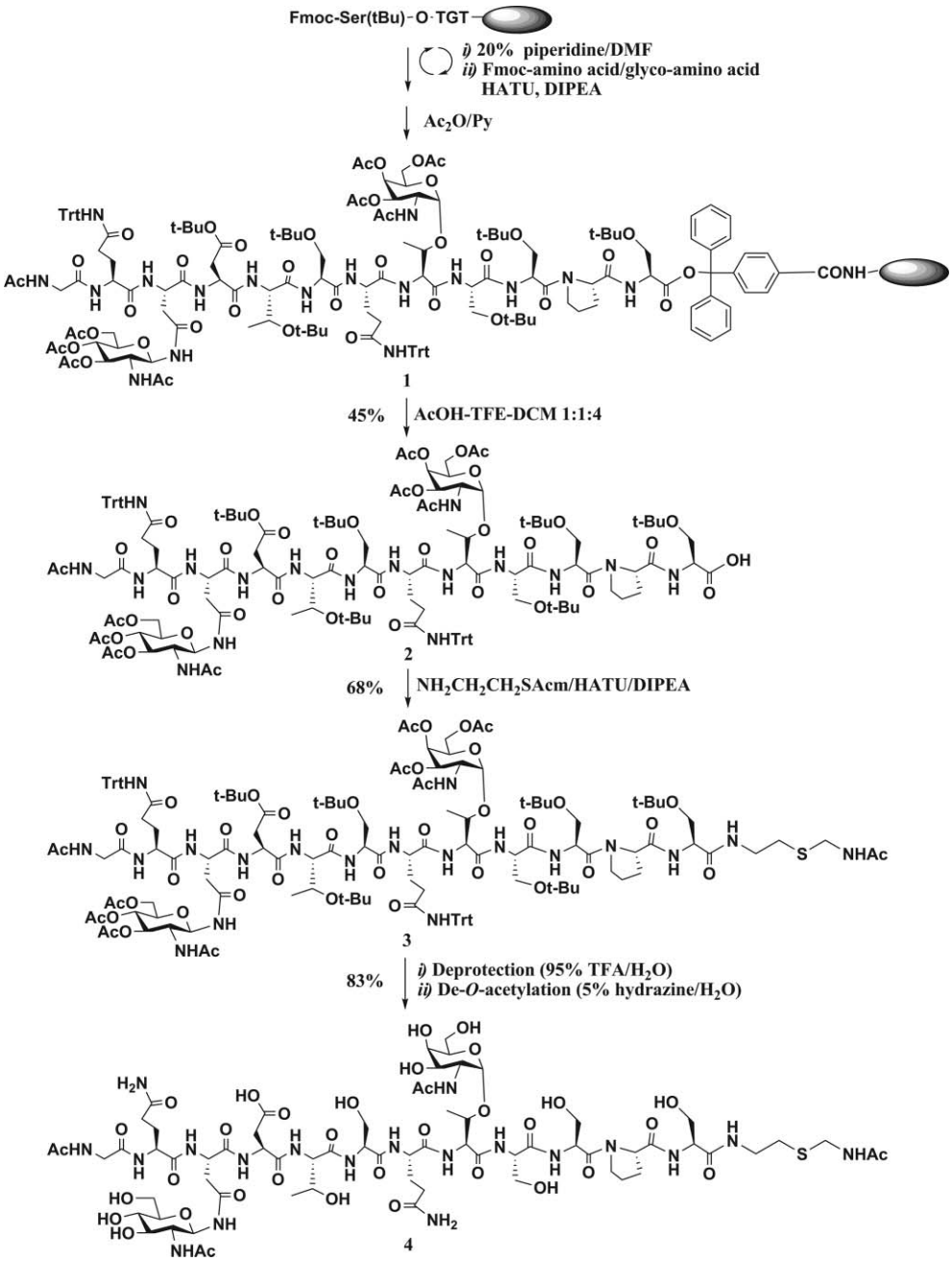
Results and discussion

Solid-phase synthesis of GlcNAc/GalNAc-containing CD52 peptide

Our synthesis started with the preparation of the CD52 polypeptide containing a GlcNAc moiety at the Asn-3 N-glycosylation site and a GalNAc moiety at the Thr-8 O-glycosylation site which will serve as primers for enzymatic sugar chain elongation. For late-stage site-specific conjugation to either a GPI anchor or a carrier protein for future functional and immunological studies, we chose to introduce an S-acetamidomethyl (Acm)-protected cysteamine at the C-terminus of the CD52 glycoforms (Fig. 1). By this approach, a free thiol group can be selectively generated after the glycopeptide assembly, ready for chemoselective conjugation. The solid-phase synthesis of monosaccharide-containing polypeptide was performed on the TGT resin with the C-terminal Ser residue attached through an acid-labile ester linkage. To introduce the GlcNAc and GalNAc moieties, the glyco-amino acid building blocks Fmoc-Asn(Ac_3GlcNAc)-OH and Fmoc-Thr(Ac_3GalNAc)-OH were used in the place of the Asn-3 and Thr-8 residues, respectively, during the solid-phase peptide synthesis (Scheme 1). After N-acetylation of the N-terminus, the polypeptide was selectively retrieved from the resin by mild acid treatment (AcOH/TFE/DCM; 1 : 1 : 4) to give the selectively protected polypeptide **2** in 45% overall yield after HPLC purification. Under these mildly acidic conditions, the protecting groups, including the O-*tert*-butyl, O-acetyl, and N-trityl groups, are stable. Coupling of the C-terminal free carboxyl group of **2** with an Acm-protected cysteamine using HATU gave compound **3** in 68% yield. Finally, global deprotection of the polypeptide backbone with 95% TFA, followed by de-O-acetylation of the carbohydrate moieties with 5% aqueous hydrazine, afforded the GlcNAc/GalNAc-containing CD52 polypeptide (**4**) in 83% yield (Scheme 1). The HPLC and MALDI-TOF MS analyses confirmed the purity and identity of the product (Fig. 2, panels a and b) (MALDI-TOF MS, calculated, M = 1785.72 Da; found (m/z), 1809.45 [M + Na]$^+$). ^{1}H NMR spectra of **4** showed that the H-1 of GlcNAc appeared at δ 4.98 as a doublet with a large coupling constant ($J_{1,2}$ 8.0 Hz) indicating a β-glycosidic linkage to the side chain of the Asn residue, while the H-1 of GalNAc appeared at δ 4.90 as a doublet with a small coupling constant ($J_{1,2}$ 2.0 Hz) suggesting an α-glycosidic linkage to the Thr residue.

Enzymatic extension of the N- and O-linked sugar chains to generate CD52 glycoforms

Upon the successful preparation of the GlcNAc/GalNAc-containing CD52 polypeptide precursor (**4**), we sought to extend the N- and O-glycans by respective enzymatic glycosylations. To introduce an N-glycan at the N-glycosylation site, we used the endoglycosidase-catalyzed transglycosylation to transfer an intact N-glycan specifically to the GlcNAc moiety in a single step by the desired β-1,4-glycosidic linkage. We have recently reported an Endo-M mutant, EndoM-Y217F, which showed much enhanced transglycosylation activity as well as significantly decreased product hydrolysis activity in comparison with the wild type Endo-M.[36] Therefore, we first used mutant Y217F as the catalyst and the sialoglycopeptide (SGP) (**5a**)[31,44] as the donor substrate in order to introduce a full-size, sialylated bi-antennary complex type N-glycan on the GlcNAc residue of precursor **4**. It was found that incubation of the GlcNAc/GalNAc-CD52 peptide (**4**), SGP (**5a**), and the mutant Y217F in a phosphate buffer (pH 7.0) led to a smooth transglycosylation of **4**, as monitored by HPLC analysis. After 1 h, the maximal formation of the transglycosylation product (**6**) was achieved and the product was



Scheme 1

isolated by HPLC in 55% yield. It should be noted that a similar transglycosylation by the wild type Endo-M using the natural sialoglycopeptide (**5a**) as the glycosyl donor gave only 8% yield of the corresponding product, because of product hydrolysis.[31]

Recently we have shown that a glycosynthase mutant of Endo-M, EndoM-N175A could take the highly activated sugar oxazolines of both asialoglycan and sialoglycan as glycosyl donors for transglycosylation without product hydrolysis.[39,41] Thus, we also examined the transglycosylation of **4** with the sialoglycan oxazoline **5b**[41] using EndoM-N175A as the catalyst. It was found that the combined use of the sugar oxazoline and the glycosynthase mutant resulted in a much improved synthesis of the glycopeptide **6**, which gave 85% yield (Scheme 2). In comparison with the EndoM-Y217F mutant, the glycosynthase mutant EndoM-N175A gave a much improved yield, mainly because the glycosynthase lacks product hydrolysis activity and allows the accumulation of the transglycosylation product. These results suggest that various N-glycans can be efficiently introduced into CD52 in a single transglycosylation step to form different CD52 glycoforms by using respective sugar oxazolines as the donor substrates. The HPLC and MS profiles of the purified glycopeptide **6** were shown in Fig. 2 (Panels c and d). The MALDI-TOF MS of **6** indicated the attachment of a single complex type N-glycan to the GlcNAc/GalNAc-CD52 peptide (MALDI-TOF MS of **6**, calculated, $M = 3787.41$ Da; found (m/z), 3810.28 [M + Na]$^+$). It was expected that the transferred N-glycan was attached

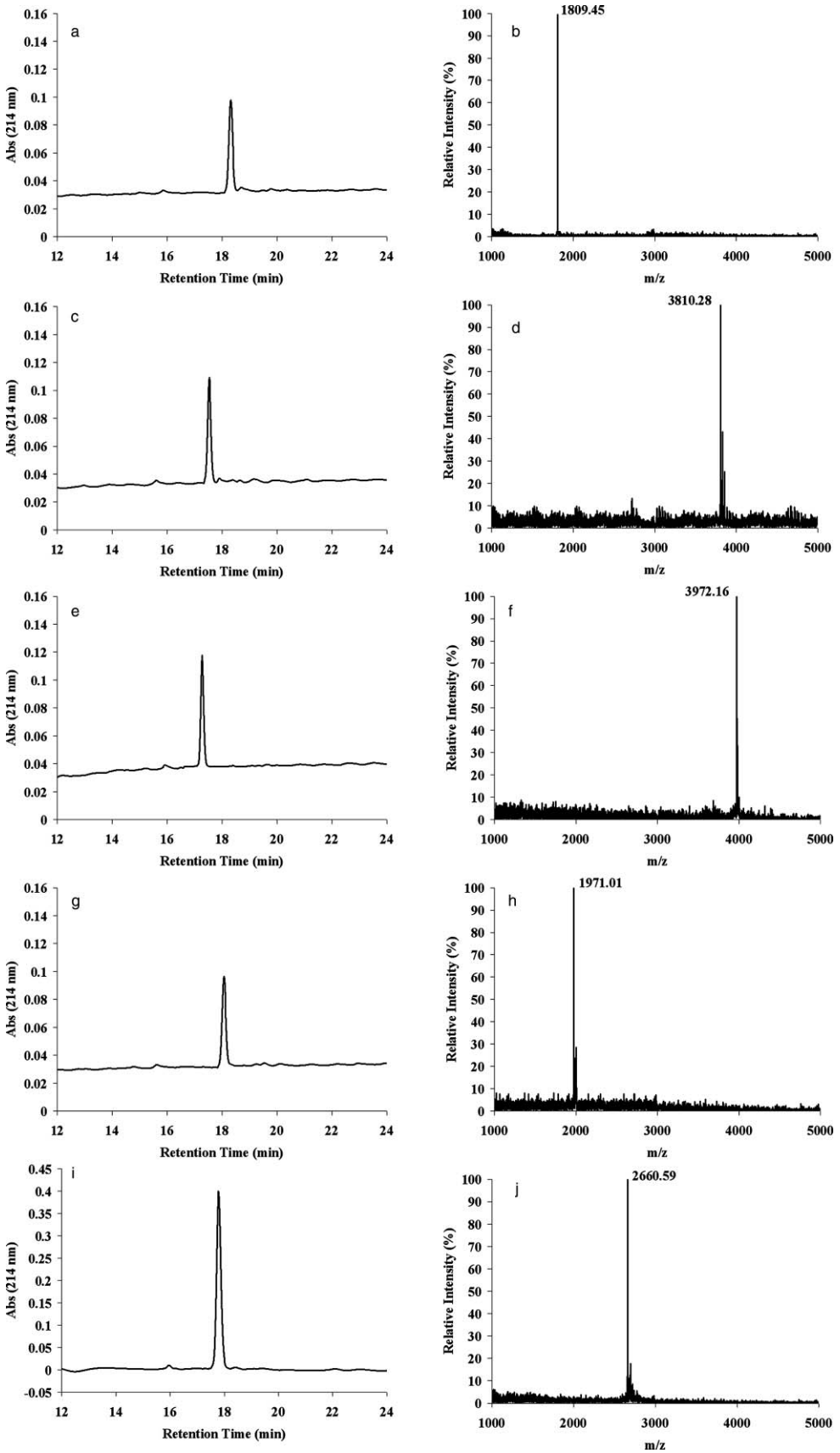
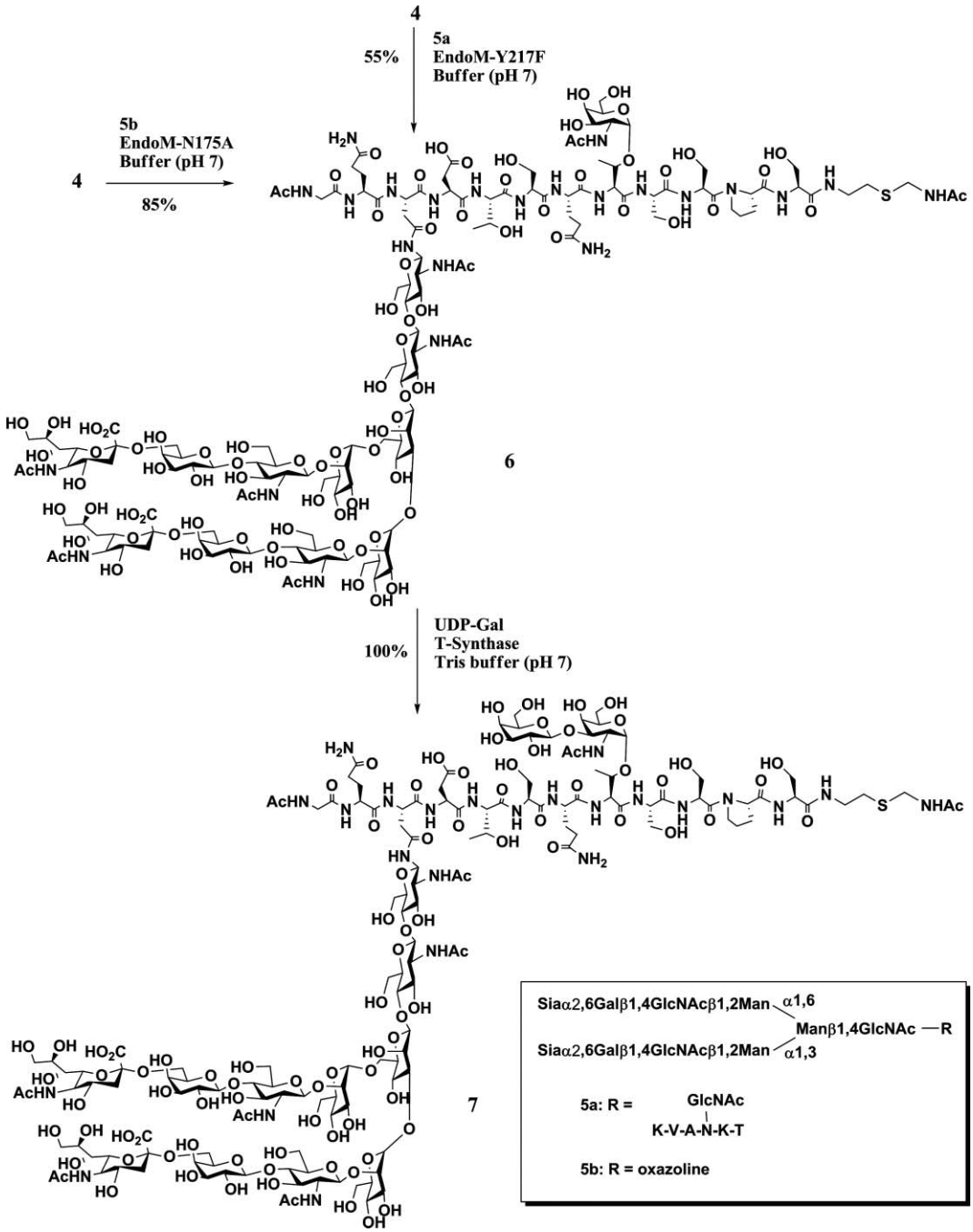
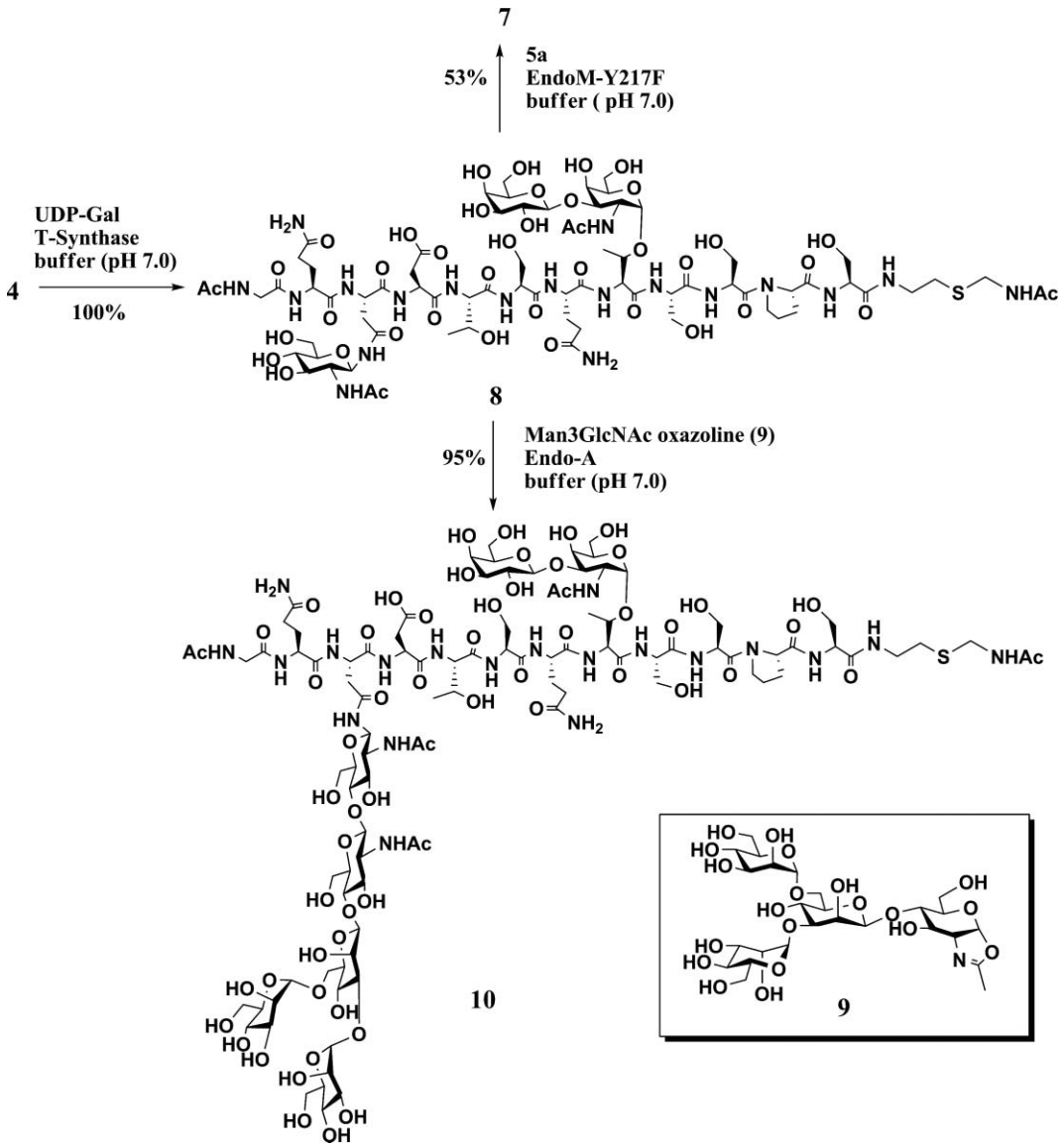


Fig. 2 HPLC and MALDI-TOF MS profiles of synthetic glycoforms of the CD52 antigen. Panels a and b, compound **4**; Panels c and d, compound **6**; Panels e and f, compound **7**; Panels g and h, compound **8**; Panels i and j, compound **10**.



Scheme 2

to the Asn-linked GlcNAc rather than the O-linked GalNAc moiety, as it is known that enzyme Endo-M and its mutants (Y217F and N175A) could utilize GlcNAc as acceptor substrate for transglycosylation but do not recognize GalNAc moiety as the acceptor.[25] To further characterize the transglycosylation product, we treated glycopeptide **6** with PNGase F and analyzed the resulting products. It is well known that PNGase F releases N-glycan from N-glycopeptide or N-glycoprotein by hydrolyzing the amide linkage between the innermost GlcNAc of N-glycan and the Asn side chain, but does not hydrolyze O-glycans. It was found that treatment of **6** with PNGase F gave the intact complex type N-glycan (**6a**) and the resulting polypeptide (**6b**) in which the GlcNAc-attached Asn moiety was converted into an Asp residue (see Scheme S1, supporting information†). The ESI-MS of the released N-glycan (**6a**) gave a m/z species: 1112.36 for $[M + 2H]^{2+}$, which is in good agreement with the calculated molecular mass, $M = 2222.78$ Da, for the full-size bi-antennary complex N-glycan. The ESI-MS of the released Asp-glycopeptide (**6b**) showed two m/z species, 1584.69 for $[M + H]^{+}$ and 792.94 for $[M + 2H]^{2+}$, which are consistent with the calculated value of **6b** (M = 1583.63 Da). These results confirm that the transferred N-glycan was attached to the Asn-linked GlcNAc and the O-GalNAc remained intact.



Scheme 3

After extension of the N-glycan, we managed to extend the O-GalNAc to a core 1 O-glycan using a recombinant human UDP-Gal:glycoprotein-α-GalNAc β-1,3- galactosyltransferase (T-synthase).[45–48] We found that T-synthase was very efficient at transferring a galactose to the GalNAc moiety in glycopeptide **6** to form the core 1 structure, Galβ1,3GalNAc. Thus, incubation of glycopeptide **6** and UDP-Gal with T-synthase in a Tris buffer (pH 7.0) containing Mn^{2+} for 16 h gave a new glycopeptide **7** in a quantitative yield (Scheme 2). The MALDI-TOF MS of **7** gave a single *m/z* species at 3972.16 (Fig. 2, panel f), which matches well with the calculated mass, 3972.47 for $[M + Na]^+$, suggesting the addition of a galactose moiety to the precursor **6**. The CD52 glycopeptide **7** carries both a full-length complex type N-glycan and a core 1 O-glycan.

Alternatively, we also performed the transglycosylation in a reverse order, *i.e.*, installing the core 1 O-glycan first and then introducing the bulky N-glycan moiety (Scheme 3). As expected, glycosylation of the GlcNAc/GalNAc-polypeptide (**4**) by T-synthase using UDP-Gal as the donor substrate proceeded efficiently to give the CD52 glycopeptide **8**, in which the galactose was attached to the GalNAc residue to form the core 1 Galβ1,3GalNAc moiety (for HPLC and MS profiles, see Fig. 2, panels g and h). Then a complex type N-glycan was introduced at the GlcNAc moiety in compound **8** by EndoM-Y217F catalyzed transglycosylation using SGP as the donor substrate to provide the CD52 glycopeptide **7** in 53% yield. It was found that the presence or absence of the O-glycan had little effect on the Y217F-catalyzed transglycosylation of the GlcNAc moiety, as the transglycosylation of glycopeptide acceptors **4** and **8** to introduce the complex type N-glycan proceeded in about the same profile (as monitored by HPLC) to give the CD52 glycopeptide **7** in similar yields. In addition, when $Man_3GlcNAc$-oxazoline (**9**) was used as the donor substrate, Endo-A catalyzed transglycosylation of **8** gave another glycoform of the CD52 antigen (compound **10**) in 95% yield, in which a core N-pentasaccharide and a core 1 O-glycan (Galβ1,3GalNAc) were installed at the N- and O-glycosylation

sites in CD52, respectively (Scheme 3). The purity and identity of glycopeptide (**10**) were confirmed by its HPLC and MS analyses (Fig. 2, panels i and j).

Effects of neighboring N-linked oligosaccharide on the T-synthase catalyzed O-glycan extension

In the biosynthesis of glycoproteins, the N-linked oligosaccharide is attached to the asparagine (Asn) side chain as a co-translational event in the ER, which is subsequently processed in the ER and then in Golgi as a post-translational event.[49,50] On the other hand, O-glycosylation is a post-translational event occurring in the Golgi apparatus.[50] Therefore, N-glycosylation precedes O-glycosylation. In the case of CD52, we were interested in exploring whether the presence of the bulky N-glycan affects the extension of the O-glycan by the human T-synthase, a key enzyme essential for the core 1 O-glycan assembly.[45,46,48] To test this, we set up a competitive galactosylation experiment with the two acceptors, the GalNAc-containing CD52 with only an N-linked monosaccharide at the Asn-3 (**4**) and the GalNAc-containing CD52 with a pre-assembled, bulky N-glycan being attached at the Asn-3 residue (**6**), respectively. When a mixture of compounds **4**, **6**, and UDP-Gal (molar ratio, 1 : 1 : 1.2) in a buffer was incubated with the T-synthase at 37 °C, HPLC monitoring of the reaction clearly indicated that the glycopeptide **4** was a more favorable substrate than the glycopeptide **6**, as demonstrated by the much faster formation of the product **8** (from **4**) than that of product **7** (from **6**) (Fig. 3). After 4 h incubation, product **8** was formed in 41% yield while product **7** was formed in 25% yield. These results suggest that a bulky neighboring N-glycan next to the O-glycosylation site can affect the O-glycan elongation, probably due to steric hindrance for the glycosyltransferase-catalyzed reaction. A recent study on the peptide acceptor preferences for the human T-synthase also suggests that the neighboring amino acid motifs affect the transfer of galactose to the GalNAc moiety by this enzyme.[46]

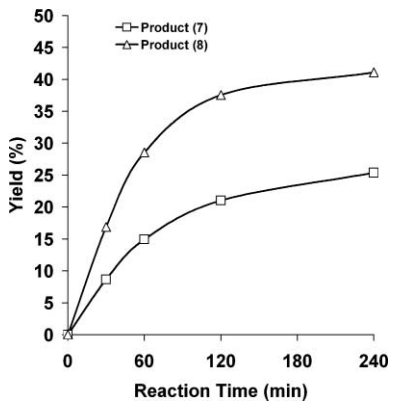


Fig. 3 Competitive galactosylation of the O-linked GalNAc CD52 glycopeptides **4** and **6** by T-synthase.

Conclusions

A convergent chemoenzymatic synthesis of CD52 glycoforms carrying both N- and O-glycans is described. The synthetic strategy features a facile solid-phase synthesis of a common CD52 polypeptide precursor that carries monosaccharide primers (GlcNAc and GalNAc) at the pre-determined N- and O-glycosylation sites, followed by enzymatic elongation of the N- and O-glycans by respective endoglycosidase- and glycosyltransferase-catalyzed glycosylations. The endoglycosidase-catalyzed transglycosylation enables the introduction of a large intact N-glycan at the N-glycosylation site in a single step, while the O-glycan can be extended independently and efficiently by the human T-synthase. This chemoenzymatic method provides a potentially general approach to constructing various homogeneous, cell-specific glycoforms of CD52 antigen. In addition, the introduction of a latent thiol group in the synthetic CD52 glycoforms permits a late-stage conjugation of the synthetic glycoforms to GPI anchor or a carrier protein for biological studies.

Experimental

Materials. The Fmoc-protected amino acids and Fmoc-Ser(tBu)-*O*-TGT resin were purchased from Novabiochem Corp (San Diego, CA). Fmoc-Asn(Ac_3GlcNAc)-OH and Fmoc-Thr(Ac_3GalNAc)-OH were prepared following the reported methods.[51,52] 2-(1-*H*-Azabenzotriazol-1-yl)-1,1,3,3-tetramethyluronium hexafluorophosphate (HATU) was purchased from GenScript Corp (Piscataway, NJ). Diisopropylethylamine (DIPEA) was purchased from Applied Biosystems (Carlsbad, CA). Piperidine (20% in DMF) was purchased from American Bioanalytical (Natick, MA). *N*,*N*-Dimethylformamide sequencing grade was purchased from Fisher Biotech (Pittsburgh, PA). Acetonitrile HPLC grade was purchased from Fisher Scientific (Pittsburgh, PA). 2-(Acetamidomethylthio)-ethylamine ($NH_2CH_2CH_2SAcm$) was prepared according to the reported method.[53] The bi-antennary complex-type sialoglycopeptide (SGP, **5a**) Lys-Val-Ala-Asn[(NeuAc-Gal-GlcNAc-Man)2Man-GlcNAc2)]-Lys-Thr was prepared from hen's egg yolks following the reported procedure.[31,44] Man_3GlcNAc oxazoline (**9**) was synthesized as reported.[32] Endo-A was overproduced in *E. coli* and purified following the previously reported procedure,[54] using the plasmid pGEX-2T/Endo-A that was kindly provided by Prof. Kaoru Takegawa. Endo-M mutant Y217F and mutant N175A were overproduced according to the previously reported method.[36] The recombinant human UDP-Gal:glycoprotein-α-GalNAc β-1,3- galactosyltransferase (T-synthase) was prepared as described previously.[45,46,48] All other reagents were purchased from Sigma/Aldrich and used as received.

Enzyme activity definition. The activity of Endo-A was defined as follows: 1 unit of Endo-A is the amount of enzyme required for the hydrolysis of 1 μmol $Man_9GlcNAc_2Asn$ (10 mM) in one minute at 30 °C in a phosphate buffer (50 mM, pH 6.5). The unit of Endo-M mutant Y217F was defined as follows: 1 unit of EndoM-Y217F is the amount of enzyme required to hydrolyze 1 μmol SGP (10 mM) in one minute at 30 °C in a phosphate buffer (50 mM, pH 6.5). The unit of Endo-M mutant N175A was defined as follows: 1 unit of EndoM-N175A is the amount of enzyme required to transfer 1 μmol Man_9GlcNAc oxazoline (10 mM) to GlcNAc-Asn(Fmoc) in one minute at 30 °C in a phosphate buffer (50 mM, pH 6.5). The unit of T-synthase was defined as the amount of enzyme required to transfer 1 μmol of UDP-Gal to 1-*O*-benzyl-GalNAc in one hour.[45,48]

High-performance liquid chromatography (HPLC). Analytical RP-HPLC was performed on a Waters 626 HPLC instrument with a Symmetry300™ C18 column (5.0 μm, 4.6 × 250 mm) at 40 °C. The column was eluted with a linear gradient of 0–10% MeCN containing 0.1% TFA within 20 min at a flow rate of 1 mL min^{-1} (*Method A*) or eluted with a linear gradient of 0–90% MeCN containing 0.1% TFA within 20 min, then at 90% MeCN containing 0.1% TFA for 10 min at a flow rate of 1 mL min^{-1} (*Method B*). Preparative HPLC was performed on a Waters 600 HPLC instrument with a preparative C18 column (Symmetry300, 19 × 250 mm). The column was eluted with a suitable gradient of aqueous acetonitrile containing 0.1% TFA at a flow rate of 12 mL min^{-1}.

Nuclear magnetic resonance (NMR). The ^{1}H NMR spectra were measured with JEOL ECX 400 MHz or Inova 500 MHz NMR spectrometers. The ^{13}C NMR was measured at 100 MHz. All chemical shifts are assigned in ppm. The coupling constants (*J* values) are given in Hz.

Mass spectrometry (MS). The ESI-MS Spectra were measured on a Waters Micromass ZQ-4000 single quadruple mass spectrometer. MALDI-TOF MS measurement was performed on an Autoflex II MALDI-TOF mass spectrometer (Bruker Daltonics). The instrument was calibrated by using ProteoMass Peptide MALDI-MS calibration kit (MSCAL2, Sigma/Aldirich). The matrix used for glycans was 2,5-dihydroxybenzoic acid (DHB) and/or α-cyano-4-hydroxycinnamic acid (ACHA) (10 mg mL^{-1} in 50% acetonitrile containing 0.1% trifluoroacetic acid). The measuring conditions: 337 nm nitrogen laser with 100 μJ output; laser frequency 50.0 Hz; laser power 30–45%; linear mode; positive polarity; detection range 1000–10000; pulsed ion extraction: 70ns; high voltage: on; realtime smooth: high; shots: 500–2000.

Solid-phase synthesis of the protected GlcNAc/GalNAc-containing CD52 polypeptide (2). The CD52 peptide was synthesized on an automatic solid-phase peptide synthesizer (Pioneer system, Applied Biosystems) by the Fmoc-chemistry using Fmoc-protected amino acid derivatives. An O-link TGT resin (Novabiochem Corp) was used as the solid support, in which the first amino acid (Ser) was attached through the acid-labile ester linkage. To introduce a GlcNAc and a GalNAc residue at the respective glycosylation site, Fmoc-Asn(Ac_3GlcNAc)-OH and Fmoc-Thr(Ac_3GalNAc)-OH were used as building blocks to replace the residues at Asn-3 and Thr-8, respectively in the solid-phase peptide synthesis. HATU (0.5 M in DMF) and DIPEA (1.0 M in DMF) (1 : 1, v/v) were used as the coupling activator and piperidine (20% in DMF) was used as the deblocking reagent. Synthesis was carried out on a 0.2 mmol scale and 4-fold excess of Fmoc-protected building blocks were used for each coupling reaction cycle. The N-terminus amino group was protected with acetyl group by treatment with Ac_2O/Py. The resulted resin was cleaved by treatment with AcOH/TFE (trifluoroethanol)/DCM (dichloromethane) (1/1/4, v/v) at r.t. to give the crude, selectively protected peptide acid (**2**). HPLC purification of the crude peptide gave the selectively protected GlcNAc/GalNAc-containing CD52 polypeptide (**2**) in 45% overall yield. Analytical HPLC of **2** (*Method B*): t_R = 22.9 min; ^{1}H NMR (CD_3OD, 400 MHz): δ 7.34–7.26 (m, 30H, Ar–H), 5.44 (s, 1H, H-4 of GalNAc), 5.29 (m, 1H, H-3 of GalNAc), 5.25 (d, 1H, *J* = 8.4 Hz, H-1 of GlcNAc), 5.16 (t, 1H, *J* = 8.0 Hz, H-3 of GlcNAc), 5.07 (d, 1H, *J* = 1.2 Hz, H-1 of GalNAc), 5.02 (t, 1H, *J* = 8.0 Hz, H-4 of GlcNAc), 4.81–4.71 (m, 3H, Ser-αH, Asp-αH, Asn-αH), 4.66–4.52 (m, 6H, Ser-αH × 3, Pro-αH, Gln-αH × 2), 4.40 (dd, 1H, *J* = 2.0, 8.8 Hz, H-2 of GalNAc), 4.35 (m, 2H, Thr-βH, Thr-αH), 4.29 (dd, 1H, *J* = 2.4, 6.8 Hz, H-6a of GlcNAc), 4.25–4.18 (m, 3H, Thr-αH, Thr-βH, H-6a of GalNAc), 4.15–4.10 (m, 2H, H-6b of GlcNAc and GalNAc), 4.04 (t, 1H, *J* = 8.0 Hz, H-2 of GlcNAc), 3.92–3.61 (m, 12H, Ser-βH × 8, Gly-αH, H-5 of GlcNAc and GalNAc), 2.85–2.65 (m, 4H, Asp-βH, Asn-βH), 2.61–1.92 (m, 41H, Pro-H, Gln-H, Ac × 9), 1.49 (s, 9H, *t*-Bu), 1.34–1.25 (m, 48H, *t*-Bu × 5, Thr-CH_3), 1.20 (d, 3H, *J* = 7.6 Hz, Thr-CH_3); ^{13}C NMR (CD_3OD, 100 MHz): δ 172.9, 172.8, 172.7, 172.3, 172.1, 172.0, 171.8, 171.5, 171.4, 171.1, 171.0, 170.7, 170.6, 170.5, 170.4, 170.0, 169.9, 169.8, 144.6, 128.7, 128.4, 127.4, 126.5, 99.8, 81.3, 77.9, 77.6, 77.5, 74.9, 73.7, 73.6, 73.2, 73.1, 70.2, 68.4, 67.9, 67.2, 66.3, 62.2, 62.0, 61.8, 61.4, 60.2, 59.0, 57.0, 56.8, 56.7, 56.2, 54.1, 54.0, 53.8, 53.5, 53.2, 52.9, 52.7, 52.6, 52.5, 52.1, 51.2, 50.0, 49.9, 43.7, 43.2, 42.4, 36.4, 36.3, 32.8, 32.7, 32.2, 31.8, 29.4, 28.9, 27.4, 27.1, 26.6, 26.4, 24.2, 21.9, 21.6, 21.3, 19.4, 19.3, 18.2. ESI-MS: calculated for $C_{137}H_{189}N_{17}O_{41}$, *M* = 2728.32 Da; found (*m*/*z*), 910.72 $[M + 3H]^{3+}$, 1365.58 $[M + 2H]^{2+}$, 2729.78 $[M + H]^{+}$.

Coupling of the Acm-protected cysteamine to the free carboxyl group of the protected CD52 peptide 2. A solution of the protected CD52 glycopeptide **2** (20.0 mg, 7.32 μmol), $NH_2CH_2CH_2SAcm$ (10 mg, 67.6 μmol), HATU (0.5 M in DMF, 40.0 μL, 20.0 μmol), and DIPEA (1.0 M in DMF, 40.0 μL, 40.0 μmol) in MeCN–DMF (1 : 1, 6 mL) was shaken at r.t. for 1 h. The residue was subject to silica gel column chromatography eluted by $CHCl_3$–MeOH (5 : 1). The fractions containing the coupling product were pooled and concentrated. The crude product was purified by preparative HPLC to give the CD52 glycopeptide **3** (14.2 mg, yield 67.8%). ^{1}H NMR ($CDCl_3$, 400 MHz): δ 7.89–7.42 (m, 10H, NH), 7.36–7.13 (m, 30h, Ar–H), 5.34 (d, 1H, *J* = 1.2 Hz, H-4 of GalNAc), 5.18 (dd, 1H, *J* = 1.2, 8.8 Hz, H-3 of GalNAc), 5.10–5.01 (m, 2H, H-4 of GlcNAc, H-3 of GlcNAc), 4.98 (d, 1H, *J* = 8.8 Hz, H-1 of GlcNAc), 4.91 (d, 1H, *J* = 1.2 Hz, H-1 of GalNAc), 2.73–2.18 (m, 8H, Asp-βH, Asn-βH, $NHCH_2C\underline{H_2}SAcm$, Pro-H), 1.42 (s, 9H, *t*-Bu), 1.25–1.08 (m, 51H, *t*-Bu × 5, Thr-CH_3 × 2); ^{13}C NMR ($CDCl_3$, 100 MHz): δ 173.7, 173.6, 173.3, 172.9, 172.8, 172.2, 172.1, 172.0, 171.7, 171.5, 171.4, 171.3, 171.0, 170.9, 170.8, 170.6, 170.5, 170.5, 169.9, 169.5, 144.2, 128.8, 128.7, 128.2, 128.0, 127.4, 127.1, 98.9, 82.7, 78.4, 78.0, 77.3, 75.2, 74.8, 74.6, 74.2, 74.0, 73.7, 73.4, 71.2, 70.8, 68.7, 67.9, 67.6, 67.1, 66.2, 62.6, 62.1, 62.0, 61.7, 61.6, 61.0, 57.0, 55.6, 54.7, 54.3, 53.8, 53.7, 53.3, 53.1, 52.3, 51.0, 50.7, 48.3, 48,2, 43.4, 41.8, 39.5, 36.4, 35.9, 34.0, 33.5, 31.9, 31.0, 29.8, 28.9, 28.2, 28.1, 27.5, 27.4, 27.3, 27.2, 27.1, 25.0, 22.6, 22.5, 22.2, 22.1, 20.7, 20.6, 20.5, 18.2. analytical HPLC (*Method B*): t_R = 23.7 min; ESI-MS: calculated for $C_{142}H_{199}N_{19}O_{41}S$, *M* = 2858.38 Da; found (*m*/*z*), 954.17 $[M + 3H]^{3+}$, 1430.48 $[M + 2H]^{2+}$, 2859.84 $[M + H]^{+}$.

Synthesis of the free GlcNAc/GalNAc-containing CD52 peptide (4). The protected CD52 glycopeptide **3** (12.0 mg, 4.20 μmol) was dissolved in 95% aqueous TFA (10 mL) and the solution was shaken at r.t. for 2 h. The residue was concentrated and lyophilized. The resulting white powder was treated with 5% aqueous hydrazine (10 mL) at r.t. for 1 h, then the reaction mixture was neutralized by 1.0 M AcOH and was subject to preparative HPLC to afford the GlcNAc/GalNAc-containing CD52 peptide (**4**) (6.2 mg, yield

82.6%) as a white powder. ^{1}H NMR (D_2O, 400 MHz): δ 4.98 (d, 1H, J = 8.0 Hz, H-1 of GlcNAc), 4.90 (d, 1H, J = 2.0 Hz, H-1 of GalNAc), 4.75–4.66 (m, 3H, Asp-αH, Ser-αH, Asn-αH), 4.54 (m, 1H, Thr-αH), 4.48–4.40 (m, 4H, Ser-αH, Gln-αH, Ser-αH, Gln-αH), 4.30–4.21 (m, 7H, Ser-αH, Thr-βH, Thr-αH, Pro-αH, Thr-βH, SC$\underline{H_2}$NHAc), 4.01 (dd, 1H, J = 2.0, 8.0 Hz, H-2 of GalNAc), 3.97 (dd, 1H, J = 4.8, 4.8 Hz, H-6a of GalNAc), 3.91 (m, 1H, H-4 of GalNAc), 3.87 (m, 2H, Gly-αH), 3.83–3.79 (m, 10H, H-3 of GalNAc, Ser-βH × 8, H-6a of GlcNAc), 3.75 (t, 1H, J = 8.0 Hz, H-2 of GlcNAc), 3.70–3.66 (m, 3H, H-5 of GalNAc, H-6b of GalNAc, H-6b of GlcNAc), 3.53 (t, 1H, J = 8.0 Hz, H-3 of GlcNAc), 3.42–3.36 (m, 4H, H-5 of GlcNAc, H-4 of GlcNAc, NHC$\underline{H_2}$CH$_2$SAcm), 2.90 (dd, 1H, J = 4.8, 12.8 Hz, Asp-βH), 2.84–2.79 (m, 2H, Asp-βH, Asn-βH), 2.75 (m, 1H, Asn-βH), 2.71 (t, 2H, J = 9.2 Hz, NHCH$_2$C$\underline{H_2}$SAcm), 2.36–2.26 (m, 5H, Pro-H, Gln-H), 2.10 (m, 1H, Gln-H), 2.07–1.94 (m, 20H, Pro-H, Gln-H, Ac × 4), 1.22 (d, 3H, J = 5.2 Hz, Thr-CH$_3$), 1.16 (d, 3H, J = 5.2 Hz, Thr-CH$_3$); ^{13}C NMR (D_2O, 100 MHz): δ 177.9, 177.8, 174.9, 174.7, 174.2, 174.1, 173.9, 173.4, 173.2, 172.7, 172.4, 172.1, 171.9, 171.7, 171.6, 171.3, 171.1, 170.5, 99.0, 78.3, 78.0, 77.6, 76.6, 74.3, 71.3, 69.4, 68.5, 68.1, 66.8, 61.4, 61.3, 60.9, 60.6, 60.5, 59.4, 57.3, 55.9, 55.8, 55.2, 54.2, 53.7, 52.9, 50.2, 50.1, 49.7, 48,2, 42.5, 40.4, 38.7, 36.2, 35.2, 31.0, 29.6, 29.3, 26.8, 26.6, 22.3, 22.1, 22.0, 21.7, 18.7, 18.3. analytical HPLC (*Method A*): t_R = 18.4 min; MALDI-TOF MS: calculated for $C_{68}H_{111}N_{19}O_{35}S$, M = 1785.72 Da; found (m/z), 1809.45 [M + Na]$^+$.

Chemoenzymatic synthesis of CD52 glycopeptide (6) using SGP (5a) as the donor substrate and mutant Y217F as the enzyme for transglycosylation. A solution of **4** (2.0 mg, 1.1 μmol) and SGP (**5a**, 7.2 mg, 2.5 μmol) in a phosphate buffer (50 mM, pH 7.0, 120 μL) was incubated at 30 °C with Endo-M Y217F (20 mU) for 60 min. The reaction mixture was quenched by 10% TFA (2 μL) and the residue was subject to RP-HPLC purification to give **6** (2.3 mg, yield 55.2%). ^{1}H NMR (D_2O, 500 MHz): δ 5.01 (s, 1H, H-1 of Man^4), 4.92 (d, 1H, J = 8.0 Hz, H-1 of GlcNAc^1), 4.85 (d, 1H, J = 1.5 Hz, H-1 of GalNAc), 4.81 (s, 1H, H-1 of Man$^{4'}$), 4.76–4.58 (m, 4H, H-1 of Man^3, Asp-αH, Ser-αH, Asn-αH), 4.48 (m, 4H, H-1 of GlcNAc^2, H-1 of GlcNAc^5, H-1 of GlcNAc$^{5'}$, Thr-αH), 4.44–4.30 (m, 6H, Ser-αH, Gln-αH, Ser-αH, Gln-αH, H-1 of Gal^6, H-1 of Gal$^{6'}$), 4.27–4.16 (m, 7H, Ser-αH, Thr-βH, Thr-αH, Pro-αH, Thr-βH, SC$\underline{H_2}$NHAc), 4.14 (s, 1H), 4.09 (m, 1H), 2.79 (m, 6H,Asp-βH, Asn-βH, NHCH$_2$C$\underline{H_2}$SAcm), 2.55 (m, 2H, H3$_{ax}$ of Sialic acid), 2.31–2.22 (m, 5H, Pro-H, Gln-H), 2.08 (m, 1H, Gln-H), 2.00–1.84 (m, 35H, Pro-H, Gln-H, Ac × 9), 1.61 (t, 2H, J = 10.0 Hz, H3$_{eq}$ of Sialic acid), 1.17 (d, 3H, J = 5.0 Hz, Thr-CH$_3$), 1.10 (d, 3H, J = 5.0 Hz, Thr-CH$_3$). analytical HPLC (*Method A*): t_R = 17.6 min; MALDI-TOF MS: calculated for $C_{144}H_{234}N_{24}O_{91}S$, M = 3787.41 Da; found (m/z), 3810.28 [M + Na]$^+$.

Chemoenzymatic synthesis of CD52 glycopeptide (6) using sialoglycan oxazoline (5b) as the glycosyl donor and the glycosynthase mutant N175A as the enzyme for transglycosylation. A solution of **4** (0.5 mg, 0.28 μmol) and sialoglycan oxazoline (**5b**, 2.8 mg, 1.4 μmol) in a phosphate buffer (50 mM, pH 7.0, 50 μL) was incubated at 30 °C with Endo-M N175A (50 mU) for 4h. The reaction mixture was quenched by 10% TFA (2 μL) and the residue was subject to RP-HPLC purification to give **6** (0.9 mg, yield 85%).

Chemoenzymatic synthesis of CD52 glycopeptide (7). A solution of **6** (1.0 mg, 0.26 μmol) and UDP-Gal (0.5 mg, 0.82 μmol) in a Tris-Cl buffer (100 mM, pH 7.0, 30 μL) containing Mn^{2+} (20 mM) was incubated at 37 °C with T-synthase (60 mU) for 16 h. The residue was subject to RP-HPLC purification to give **7** (1.1 mg, quantitative yield). ^{1}H NMR (D_2O, 500 MHz): δ 5.12 (s, 1H, H-1 of Man^4), 5.03 (d, 1H, J = 8.0 Hz, H-1 of GlcNAc^1), 4.96 (d, 1H, J = 1.5 Hz, H-1 of GalNAc), 4.92 (s, 1H, H-1 of Man$^{4'}$), 4.84–4.68 (m, 4H, H-1 of Man^3, Asp-αH, Ser-αH, Asn-αH), 4.58–4.40 (m, 11H, H-1 of GlcNAc^2, H-1 of GlcNAc^5, H-1 of GlcNAc$^{5'}$, Thr-αH, Ser-αH, Gln-αH, Ser-αH, Gln-αH, H-1 of Gal^6, H-1 of Gal$^{6'}$, H-1 of *O*-Gal), 4.38–4.21 (m, 7H, Ser-αH, Thr-βH, Thr-αH, Pro-αH, Thr-βH, SC$\underline{H_2}$NHAc), 2.96 (dd, 1H, J = 4.0, 12.5 Hz, Asp-βH), 2.84 (m, 2H, Asp-βH, Asn-βH), 2.75 (m, 3H, Asn-βH, NHCH$_2$C$\underline{H_2}$SAcm), 2.65 (m, 2H, H3$_{ax}$ of Sialic acid), 2.43–2.32 (m, 5H, Pro-H, Gln-H), 2.16–1.94 (m, 36H, Pro-H, Gln-H, Ac × 9), 1.72 (t, 2H, J = 10.5 Hz, H3$_{eq}$ of Sialic acid), 1.28 (d, 3H, J = 5.0 Hz, Thr-CH$_3$), 1.20 (d, 3H, J = 5.0 Hz, Thr-CH$_3$). analytical HPLC (*Method A*): t_R = 17.3 min; MALDI-TOF MS: calculated for $C_{150}H_{244}N_{24}O_{96}S$, M = 3949.47 Da; found (m/z), 3972.16 [M + Na]$^+$.

Chemoenzymatic synthesis of CD52 glycopeptide (8). A solution of **4** (1.0 mg, 0.56 μmol) and UDP-Gal (1.0 mg, 1.6 μmol) in a Tris-Cl buffer (100 mM, pH 7.0, 30 μL) was incubated at 37 °C with T-synthase (100 mU) for 16 h. The residue was subject to RP-HPLC purification to give **8** (1.1 mg, quantitative yield). ^{1}H NMR (D_2O, 500 MHz): δ 5.03 (d, 1H, J = 8.0 Hz, H-1 of GlcNAc), 4.98 (d, 1H, J = 1.5 Hz, H-1 of GalNAc), 4.62 (m, 1H, Thr-αH), 4.58–4.50 (m, 2H, Ser-αH, Gln-αH), 4.48–4.42 (m, 3H, Ser-αH, Gln-αH, H-1 of Gal), 4.38–4.25 (m, 7H, Ser-αH, Thr-βH, Thr-αH, Pro-αH, Thr-βH, SC$\underline{H_2}$NHAc), 2.95–2.75 (m, 6H, Asp-βH, Asn-βH, NHCH$_2$C$\underline{H_2}$SAcm), 2.42–2.30 (m, 5H, Pro-H, Gln-H), 2.18 (m, 1H, Gln-H), 2.12–1.94 (m, 20H, Pro-H, Gln-H, Ac × 4), 1.32 (d, 3H, J = 5.0 Hz, Thr-CH$_3$), 1.21 (d, 3H, J = 5.0 Hz, Thr-CH$_3$). analytical HPLC (*Method A*): t_R = 18.1 min; MALDI-TOF MS: calculated for $C_{74}H_{121}N_{19}O_{40}S$, M = 1947.77 Da; found (m/z), 1971.01 [M + Na]$^+$.

Y217F-catalyzed transglycosylation of 8 using SGP as the glycosyl donor. A solution of **8** (1.0 mg, 0.51 μmol) and SGP (**5a**, 3.5 mg, 1.2 μmol) in a phosphate buffer (50 mM, pH 7.0, 50 μL) was incubated at 30 °C with EndoM-Y217F (10 mU) for 60 min. The reaction mixture was quenched by 10% TFA (2 μL) and the mixture was subject to RP-HPLC purification to give CD52 glycopeptide **7** (1.1 mg, yield 53.4%).

Chemoenzymatic synthesis of CD52 glycopeptide (10). A solution of **8** (1.0 mg, 0.51 μmol) and Man$_3$GlcNAc oxazoline (**9**, 1.0 mg, 1.5 μmol) in a phosphate buffer (50 mM, pH 7.0, 30 μL) was incubated at 30 °C with Endo-A (20 mU) for 3 h. Then the reaction mixture was subject directly to RP-HPLC purification to give the CD52 glycopeptide (**10**) (1.3 mg, 95.4%). ^{1}H NMR (D_2O, 500 MHz): δ 5.08 (s, 1H, H-1 of Man^4), 5.03 (d, 1H, J = 8.0 Hz, H-1 of GlcNAc^1), 4.98 (d, 1H, J = 1.5 Hz, H-1 of GalNAc), 4.92 (s, 1H, H-1 of Man$^{4'}$), 4.62 (m, 2H, Thr-αH, H-1 of GlcNAc^2), 4.58–4.53 (m, 2H, Ser-αH, Gln-αH), 4.48–4.42 (m, 3H, Ser-αH, Gln-αH, H-1 of Gal), 4.38–4.25 (m, 7H, Ser-αH, Thr-βH, Thr-αH, Pro-αH, Thr-βH, SC$\underline{H_2}$NHAc), 2.95–2.77 (m, 6H, Asp-βH, Asn-βH, NHCH$_2$C$\underline{H_2}$SAcm), 2.43–2.32 (m, 5H, Pro-H, Gln-H),

2.17 (m, 1H, Gln-H), 2.12–1.93 (m, 20H, Pro-H, Gln-H, Ac × 5), 1.31 (d, 3H, J = 5.0 Hz, Thr-CH_3), 1.22 (d, 3H, J = 5.0 Hz, Thr-CH_3). analytical HPLC (*Method A*): t_R = 17.8 min; MALDI-TOF MS: calculated for $C_{100}H_{164}N_{20}O_{60}S$, M = 2637.01 Da; found (m/z), 2660.59 $[M + Na]^+$.

Competitive galactosylation of CD52 glycopeptides 4 and 6 by T-synthase. A solution of **4** (0.2 mg, 0.11 μmol), **6** (0.4 mg, 0.11 μmol), and UDP-Gal (0.08 mg, 0.13 μmol) in a Tris-Cl buffer (100 mM, pH 7.0, 40 μL) containing Mn^{2+} (20 mM) was incubated at 37 °C with T-synthase (25 mU). The reaction was monitored by HPLC by taking aliquots at 0.5 h, 1 h, 2 h and 4 h. The yields of corresponding products **8** and **7** were calculated based on the individual product peak area divided by total CD52 peak area in HPLC.

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