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Site-selective sulfation of *N*-glycans by human GlcNAc-6-*O*-sulfotransferase 1 (CHST2) and chemoenzymatic synthesis of sulfated antibody glycoforms

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Abstract

Sulfation is a common modification of glycans and glycoproteins. Sulfated *N*-glycans have been identified in various glycoproteins and implicated for biological functions, but *in vitro* synthesis of structurally well-defined full length sulfated *N*-glycans remains to be described. We report here the first *in vitro* enzymatic sulfation of biantennary complex type *N*-glycans by recombinant human CHST2 (GlcNAc-6-*O*-sulfotransferase 1, GlcNAc6ST-1). We found that the sulfotransferase showed high antennary preference and could selectively sulfate the GlcNAc moiety located on the Man α 1,3Man arm of the biantennary *N*-glycan. The glycan chain was further elongated by bacterial β 1,4 galactosyltransferase from *Neisseria meningitidis* and human β 1,4 galactosyltransferase IV (B4GALT4), which led to the formation of different sulfated *N*-glycans. Using rituximab as a model IgG antibody, we further demonstrated that the sulfated *N*-glycans could be efficiently transferred to an intact antibody by using a chemoenzymatic Fc glycan remodeling method, providing homogeneous sulfated glycoforms of antibodies. Preliminary binding analysis indicated that sulfation did not affect the apparent affinity of the antibody for Fc γ IIIa receptor.

Keywords

Sulfated *N*-glycans; Sulfation; Sulfotransferase; Sulfated antibody; Chemoenzymatic synthesis; Glycoforms

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Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Appendix A. Supplementary material

Supplementary data to this article can be found online at <https://doi.org/10.1016/j.bioorg.2022.106070>.

1. Introduction

Sulfated *N*-glycans are a group of biologically important oligosaccharides, which are commonly found on glycoproteins formed by enzymatic modifications of *N*-glycans. Sulfation of *N*-glycans in glycoproteins occurs in the Golgi apparatus, where sulfotransferases catalyze the transfer of a sulfate group from 3'-phosphoadenosine-5'-phosphosulfate (PAPS) to glycoprotein substrates. Among the *N*-glycans, complex type structures are the most commonly sulfated, where the sulfate group is attached on the C-3 or C-6 position of galactose (Gal), the C-6 position of *N*-acetylglucosamine (GlcNAc), or the C-4 position of *N*-acetylgalactosamine (GalNAc) of the non-reducing terminal Gal β 1,4GlcNAc (LacNAc) and GalNAc β 1,4GlcNAc (LacdiNAc) disaccharide moieties [1].

A variety of biologically important glycoproteins have been shown to possess sulfated *N*-glycans. For example, 20 sulfated *N*-glycans of IgG antibodies as trace species were identified from human sera, which are implicated as biomarkers of *rheumatoid arthritis* [2]. Although the influence of sulfated *N*-glycans on IgG functions remains to be characterized, it is speculated that sulfated *N*-glycans might alter the ligand specificity and biological functions of IgG antibodies [2]. Large structural diversity of sulfated *N*-glycans have been found on influenza glycoproteins, e.g., the viral hemagglutinin (HA) and neuraminidase (NA) [3], which might significantly affect viral replication and receptor binding [4–6]. Moreover, sulfated *N*-glycans play a critical role in lymphocyte homing and recruitment mediated by L-selectins [7]. The half-life of pituitary hormone is regulated by sulfated *N*-glycans [8]. Besides, sulfated *N*-glycans participate in peripheral nervous system (PNS) myelination both in porcines and murine tissues [9]. Sulfated di-, tri- and tetra-antennary complex type *N*-glycans are identified in human Tamm-Horsfall glycoprotein [10], which are involved in the binding to neutrophils [11] and immunosuppressive properties [12]. Sulfated *N*-glycans have also been reported in porcine thyroglobulin, which are the major iodinated glycoprotein formed in the thyroid gland [13]. Additionally, sulfated *N*-glycans have been reported in erythropoietin expressed in baby hamster kidney (BHK) cells [14], hen egg albumin [15], lysosomal enzyme [16], urokinase [17], and recombinant L-selectins produced in HEK293F cells [18].

The diverse biological functions of sulfated glycans have stimulated tremendous interest in the synthesis of sulfated carbohydrates. For example, sulfated oligosaccharides containing HNK-1 epitope, sulfated core 2 *O*-GalNAc glycans and *O*-sulfated sialyl Lewis X with *O*-sulfation at different sites have been synthesized by a chemoenzymatic approach, in which respective sulfate group is added on the glucuronic acid, *N*-acetylglucosamine, galactose and *N*-acetylglucosamine moieties chemically followed by enzymatic sugar chain elongation [19–21]. Nevertheless, *in vitro* chemoenzymatic synthesis of structurally well-defined sulfated full-length *N*-glycans has not been described so far. For the biosynthesis of sulfated complex type *N*-glycans in mammalian systems, three types of sulfotransferases, including the galactose-3-*O*-sulfotransferases, the *N*-acetylglucosamine-6-*O*-sulfotransferases and the *N*-acetylgalactosamine-4-*O*-sulfotransferases, are responsible for the enzymatic sulfation. In human, four galactose-3-*O*-sulfotransferases have been identified and characterised, i.e., Gal3ST1, Gal3ST2, Gal3ST3 and Gal3ST4) [22–26], and two homologous enzymes of *N*-acetylgalactosamine-4-*O*-sulfotransferases have been cloned and expressed, which are able

to transfer sulfate group to a GalNAc β 1,4 GlcNAc (LacdiNAc) moiety [27]. In addition, five isoenzymes of GlcNAc-6-*O*-sulfotransferases have been produced and characterized, i.e., CHST2 (GlcNAc6ST-1), CHST4 (GlcNAc6ST-2), CHST5 (GlcNAc6ST-3), CHST7 (GlcNAc6ST-4), and CHST6 (GlcNAc6ST-5) (gene names followed by alternate names in parenthesis), which transfer a sulfate group to terminal GlcNAc leading to the formation of different cell surface epitopes including biologically important 6-sulfo sLe^x [28–33]. However, none of those five GlcNAc-6-*O*-sulfotransferases has been examined for *in vitro* sulfation of full-length intact *N*-glycans. We report here the first *in vitro* enzymatic sulfation of biantennary complex type *N*-glycans by recombinant human GlcNAc-6-*O*-sulfotransferase 1 (CHST2). We found that sulfotransferase CHST2 showed antennary preference for sulfation and could selectively add a sulfate group on the terminal GlcNAc of the Man- α 1,3-Man arm. Further elongation by two different β 1,4 galactosyltransferases gave additional sulfated *N*-glycans. Using rituximab as a model IgG antibody, we further demonstrated that the sulfated *N*-glycans could be successfully transferred to deglycosylated rituximab using an endoglycosidase mutant, Endo-S2 D184M, to provide homogeneous sulfated glycoforms of antibodies. We have previously shown that the Endo-S2 mutant (D184M) could use different glycan oxazolines as donor substrates for antibody Fc glycan remodeling [34–37]. The present study provided the first example showing that the Endo-S2 mutant is equally efficient to transfer sulfated *N*-glycans to antibodies to give structurally well-defined sulfated antibody glycoforms.

2. Results and discussion

2.1. Synthesis of sulfated *N*-glycans

To obtain biantennary complex type *N*-glycans, sialoglycopeptide (SGP) was isolated from egg yolk powder on a gram-scale according to the method reported previously [38–39]. SGP was treated with pronase to give the asparagine-linked (*N*-linked) bi-antennary *N*-glycan [40]. The free amino group in the asparagine moiety was protected by a fluorenylmethyloxycarbonyl (Fmoc) group to facilitate purification of products by preparative HPLC in each enzymatic transformation. The terminal sialic acid and galactose moieties were then removed by sialidase from *Micromonospora Viridifaciens* [41] and a β -1,4 galactosidase, respectively. The resulting product (G0-Asn-Fmoc, **1**) was utilized as substrate to evaluate enzymatic sulfation by recombinant human GlcNAc-6-*O*-sulfotransferase 1 (CHST2) expressed in HEK293F cells (Scheme 1). Incubation of **1** with CHST2 yielded a new product (**2**) that eluted later than the starting material (**1**) on reverse phase analytical HPLC, and MALDI-TOF MS analysis indicated that it was a monosulfated *N*-glycan. Afterwards, preparative scale synthesis was executed, which resulted in 80 % isolated yield of product **2**. To validate the sulfation position, we initially treated the product with commercial β -*N*-acetylglucosaminidase S and found that both sulfated and non-sulfated terminal GlcNAc moieties were removed, thus the β -*N*-acetylglucosaminidase S could not distinguish between the sulfated and free terminal GlcNAc moieties. It has been previously reported that the bacterial β -1,4-galactosyltransferase from *Neisseria meningitidis* was able to catalyze galactosylation specifically on free terminal GlcNAc rather than a sulfated GlcNAc [42]. Therefore, the free terminal GlcNAc could be temporarily blocked by galactosylation. Thus, treatment of **2** with the *Neisseria meningitidis*

β -1,4-galactosyltransferase gave a monogalactosylated *N*-glycan (**3**), as shown by MALDI-TOF MS analysis (Fig. S1). Then the intermediate was treated with the commercial β -*N*-acetylglucosaminidase S and the MALDI-TOF MS indicated that a sulfated GlcNAc moiety was removed to give a truncated *N*-glycan (Fig. S2). Finally, the resulting product was treated with a commercial α -1,3-mannosidase, which could specifically remove free terminal α 1,3-linked mannose. MALDI-TOF MS analysis showed that a mannose moiety was removed from the substrate upon treatment with the α -1,3-mannosidase to give a truncated *N*-glycan (**4**) (Fig. S3). The experimental data confirm that in product **2**, the Man-1,3-Man arm carries a sulfated GlcNAc moiety that could not be galactosylated by the *Neisseria meningitidis* β -1,4-galactosyltransferase, and would be removed selectively by the β -*N*-acetylglucosaminidase S, thus freeing the mannose moiety on the α -1,3Man arm (the lower arm in the *N*-glycan). These results clearly show a high-regioselectivity of the CHST2 at the low-arm antennal GlcNAc moiety. Nevertheless, we also found that use of excess amount of donor substrate PAPS and enzyme CHST2 could slowly drive the addition of a second sulfate group on the other terminal GlcNAc, as shown by the MALDI-TOF MS monitoring of the reaction, although a complete double sulfation would need further optimization of the reaction condition (Fig. S4).

It should be mentioned that treatment of the fully galactosylated *N*-glycan with sulfotransferase CHST2 did not give any sulfated *N*-glycan product (data not shown), suggesting that CHST2 only transfers sulfate group to a terminal instead of internal GlcNAc moiety in an *N*-glycan [43]. To synthesize additional sulfated *N*-glycan, the sulfated *N*-glycan (**2**) was elongated with human β 1,4 galactosyltransferase IV (B4GALT4) to give a fully galactosylated *N*-glycan (**5**). This result suggests that the human B4GALT4 is promiscuous and efficient to galactosylate both non-sulfated and the 6-sulfated GlcNAc moieties. Thus, the use of the bacterial and human galactosyltransferases gave the two differentially galactosylated and sulfated *N*-glycans (**3** and **5**), respectively, in excellent yields. (Scheme 1). All the sulfated *N*-glycans were purified by preparative HPLC and their identity was confirmed by HRMS and NMR analysis.

2.2. Synthesis of sulfated *N*-glycan oxazolines as enzyme substrates

In order to synthesize novel antibody glycoforms carrying sulfated *N*-glycans, we sought to apply our chemoenzymatic Fc glycan remodeling method [44] to investigate whether an endoglycosynthase mutant such as Endo-S2 D184M could recognize sulfated glycan oxazolines for transglycosylation. We have previously demonstrated that the Endo-S2 mutant (D184M) can take different glycan oxazolines as donor substrates for antibody Fc glycan remodeling [34–37]. For that purpose, we first synthesized the corresponding sulfated *N*-glycan oxazolines. Treatment of the sulfated Fmoc-*N*-glycans (**2**, **3**, and **5**) with wild-type Endo-S2 gave the free *N*-glycans, which were subsequently treated with DMC and TEA in water to afford the sulfated glycan oxazolines (**6**, **7**, and **8**), respectively, in excellent yields (Scheme 2). The sulfated glycan oxazolines were desalted by G10 size exclusion chromatography and lyophilized. All three sugar oxazolines were analysed by ^1H NMR. The anomeric protons at reducing termini of **6**, **7**, and **8** appeared as a doublet at *ca.* 6.0 ppm with a coupling constant ($J_{1,2}$) of *ca.* 7.0 Hz, which are a signature signal of the anomeric proton

of a glycan oxazoline (H-1 for **6**, 5.98 ppm, 7.28 Hz; H-1 for **7**, 5.96 ppm, 7.26 Hz; H-1 for **8**, 5.96 ppm, 7.26 Hz. Figs. S5–7).

2.3. Chemoenzymatic synthesis of antibody glycoforms carrying sulfated N-glycans

To test the feasibility of the sulfated *N*-glycan oxazolines as substrates for enzymatic glycan remodeling of antibodies, we chose rituximab, a therapeutic monoclonal antibody, as a model for antibody Fc glycan remodeling. Thus, rituximab was first deglycosylated with Endo-S2 to give the Fuc α 1,6 GlcNAc-rituximab intermediate [34]. Then Endo-S2 mutant D184M from *Streptococcus pyogenes* was used for examining the transglycosylation with the sulfated *N*-glycan oxazolines, as the D184M mutant has shown excellent activity to transfer different types of *N*-glycans [34]. We found that all the three sulfated *N*-glycan oxazolines acted as excellent substrates of the D184M mutant to give the corresponding sulfated glycoforms of rituximab (**9**, **10**, and **11**), respectively, in high yields (Scheme 3). Interestingly, an attempt to perform direct sulfation of the terminal GlcNAc moiety in the G0F antibody glycoform (**12**) by CHST2 failed to give detectable sulfated antibody, as monitored by LC-MS analysis (Fig. S8), although the corresponding free *N*-glycan served as an excellent substrate of human CHST2. These results suggest that other human GlcNAc-6-*O*-sulfotransferases may be involved in the biosynthesis of such sulfated antibody glycoforms *in vivo*.

The antibody glycoforms were isolated by protein A affinity chromatography and their identity was confirmed by LC-ESI-MS analysis (Fig. 1). Calcd for intact antibody **9**, $M = 147234$ Da, found, 147236 Da; calcd for intact sulfated antibody **10**, $M = 147558$ Da, found, 147560 Da; calcd for intact sulfated antibody **11**, $M = 147882$ Da, found, 147884 Da; calcd for antibody **12**, $M = 147076$ Da, found, 147074 Da. All data were deconvoluted. To further confirm the structural homogeneity, the glycoengineered antibodies were treated with protease IdeS which can specifically digest immunoglobulin G to generate the monomeric Fc domain. The product Fc domain was analysed by LC-ESI-MS (Fig. S9), which indicate that the sulfated *N*-glycans have been transferred specifically to the Fc domain of antibody rituximab.

2.4. Binding of the sulfated glycoforms of antibody with Fc γ receptor Fc γ RIIIa-V158

Next, we investigated the impact of sulfate group on the binding between glycans on antibody rituximab and Fc receptors. Surface plasmon resonance (SPR) analysis was utilized to assess the binding affinity of Fc γ RIIIa-V158 to sulfated glycoforms and corresponding non-sulfated glycoforms. Rituximab with different glycoforms were site-specifically immobilized on the CM5 sensor chip which was pre-functionalised with protein A. Different concentrations of Fc γ RIIIa-V158 were injected as analyte following the procedures reported previously [35]. The SPR sensorgrams for the interaction between different glycoforms of rituximab and Fc γ RIIIa-V158 were shown in Fig. 2. The dissociation constant (K_D) between the binding of rituximab with various glycoforms to Fc γ RIIIa-V158 was estimated to be 198 nM (G0F-rituximab), 172 nM (SulfG0F-rituximab), 108 nM (G2F-rituximab) and 92 nM (SulfG2F-rituximab), respectively. Apparently, the complex-type glycoforms (G2F-rituximab, G0F-rituximab) showed the similar binding affinity compared to the corresponding sulfated glycoforms. Furthermore, the K_D value of the binding between

sulfated G1F-rituximab and Fc γ RIIIa-V158 was also obtained, which was 84 nM (Fig. S10). These results suggest that the introduction of a sulfate group at the lower arm GlcNAc moiety of the Fc glycans does not significantly affect the affinity of the antibody for the Fc γ receptor Fc γ RIIIa-V158. The present study represents the first *in vitro* enzymatic sulfation of full-length *N*-glycans by the human sulfotransferase CHST2. An expanded chemoenzymatic synthesis of diverse sulfated *N*-glycans using other sulfotransferases and the evaluation of the binding of the synthetic sulfated *N*-glycans with different lectins are currently underway in our laboratory and the results will be reported in due course.

3. Conclusion

The *in vitro* enzymatic sulfation of biantennary complex type *N*-glycans by recombinant human GlcNAc-6-*O*-sulfotransferase 1 (CHST2) and the first chemoenzymatic synthesis of sulfated antibody glycoforms are described. The high site-selectivity of sulfation at the lower arm of *N*-glycans by human sulfotransferase CHST2 is an unexpected discovery. The availability of structurally well-defined sulfated *N*-glycans and sulfated glycoforms of antibodies provides an exciting opportunity to investigate their biological functions. The next step is to expand this approach to evaluation of other sulfotransferases for the construction of a library of specific *N*-glycans and antibody glycoforms with diverse sulfation patterns, which will be highly valuable for structural and functional studies.

4. Experimental section

4.1. Materials

The Endo-S2 and Endo-S2 D184M glycosynthase mutant were prepared by our lab as described previously [34]. The plasmids bearing human CHST2 and B4GALT4 genes were generated as described previously [45]. PAPS was kindly provided by Prof. Jian Liu (the University of North Carolina, Chapel Hill, US). Bacterial β 1,4-galactosyltransferase from *Neisseria meningitidis* was expressed following the established procedure [46]. Polyethylenimine (PEI), Fmoc-Cl, formic acid and acetonitrile were purchased from Sigma-Aldrich. *E. coli* DH5 α , the α -1,3 mannosidase, the β -*N*-acetylglucosaminidase S, and the β 1,4-galactosidase were purchased from New England Biolab. The sialidase from *Micromonospora Viridifaciens* was expressed and purified following the previously described procedure [41]. 1 ml HisTrapTM FF column was purchased from GE Healthcare. NucleoBond[®]Xtra Midi EF kit was bought from MACHEREY-NAGEL.

4.2. Analytical methods

HPLC: Analytical RP-HPLC was carried out on a Thermo Scientific instrument with a YMC C18 column (5 μ m, 4.6 \times 250 mm). The column was eluted with a linear gradient of 10–50 % acetonitrile with 0.1 % formic acid (v/v) for 30 min at a flow rate of 1 ml/min under UV 266 nm. The retention time of sulfated *N*-glycans (**2**, **3**, **5**) were 23.5 min, 22.6 min, and 22.2 min, respectively. Reverse phase preparative HPLC was executed on a Waters 600 HPLC instrument with a SymmetryPrep C18 column (7 μ m, 19 \times 300 mm). The column was eluted with a linear gradient of 20–60 % acetonitrile with 0.1 % formic acid (v/v) for 30 min at a flow rate of 10 ml/min under UV 266 nm.

LC-ESI-MS: The core-fucosylated antibodies were analysed by liquid chromatography electrospray mass spectrometry (LC-ESI-MS). LC-ESI-MS was carried out on an Exactive Plus Orbitrap Mass Spectrometry (Thermo Scientific) with a C8 column (5 μm , 1.0×75 mm) for IdeS digested antibody and C4 column (3.5 μm , 2.1×50 mm) for intact antibody analysis.

HRMS: High resolution mass spectrometry was executed on an Exactive Plus Orbitrap Mass Spectrometry (Thermo Scientific).

NMR: ^1H , ^{13}C , ^1H - ^1H COSY and ^1H - ^{13}C HSQC NMR spectra were recorded on a 600 MHz spectrometer (Bruker, Tokyo, Japan) with D_2O as the solvent containing 1 % (v/v) DMSO- d_6 as an inner standard.

4.3. Enzyme expression and purification of human CHST2 and B4GALT4

The coding region of human CHST2 and B4GALT4 genes were inserted into pGen2 vector, which were utilized for HEK293F (ThermoFisher) cell transient expression as described previously [45,47]. For the purification, the culture supernatant was collected and used for Ni^{2+} -NTA column purification. The column was equilibrated with the binding buffer (Tris 50 mM, NaCl 500 mM, imidazole 10 mM, pH 7.5). The culture supernatant was loaded and washed with 20 times column volumes of washing buffer (Tris 50 mM, NaCl 500 mM, Imidazole 20 mM, pH 7.5), and eluted with a buffer containing Tris 50 mM, NaCl 500 mM, imidazole 300 mM, pH 7.5. The fractions containing proteins were collected and buffer exchanged using buffer containing HEPES 20 mM, NaCl 100 mM, pH 7.0, sodium azide 0.05 %, and glycerol 10 %. The protein concentrations were measured by Nanodrop. The two enzymes were analysed by SDS-PAGE (Fig. S11). The aliquots of the two enzymes were stored in -80 °C freezer until use.

4.4. The sulfation position determination

A reaction mixture composed of monosulfated G1-Asn-Fmoc (100 μg), Glycobuffer 1 (CaCl_2 5 mM, sodium acetate 50 mM), β -*N*-acetylglucosaminidase S was incubated at 37 °C. The product was analysed by MALDI-ToF. When the sulfated *N*-acetylglucosamine was hydrolysed completely, BSA (100 μg) and α 1-2,3 mannosidase (32 U) were added to the reaction mixture. The product was analysed by MALDI-ToF (Fig. S1-3).

4.5. Enzymatic synthesis of monosulfated G0-Asn-Fmoc

In total, 550 μl reaction mixture composed of G0-Asn-Fmoc (11 mg, 6.6 μmole), MES buffer (50 mM, pH 6), MgCl_2 (5 mM), PAPS (10 μmole), NaF (10 mM), ATP (1 mM) and human CHST2 (221 μg) was incubated at 37 °C. The reaction was monitored by HPLC. After the reaction was completed, the reaction was quenched by heating at 95 °C for 5 min. The product was purified by preparative HPLC. After lyophilisation, the product (9.1 mg, 80 %) was obtained as white powder. The compound was characterised by NMR and HRMS. ^1H NMR (D_2O + 1 % DMSO- d_6 , 600 MHz): δ = 7.73 (m, 2H, FmocH), 7.54 (m, 2H, FmocH), 7.34 (m, 2H, FmocH), 7.29 (m, 2H, FmocH), 5.04 (m, 1H), 4.91 (d, J = 9.6 Hz, 1H), 4.83 (m, 1H), 4.50-4.30 (m, 7H), 4.26 (d, J = 10.8 Hz, 1H), 4.15-3.40 (m, 55H, rest protons on the sugar rings), 1.97-1.80 (m, 12H, $-\text{COCH}_3$). ^{13}C NMR (D_2O + 1 % DMSO- d_6 , 151 MHz): δ

= 174.3, 174.25, 174.16, 157.9, 143.3, 140.5, 127.6, 127.0, 124.7, 119.7, 100.7, 99.8, 99.3, 99.2, 99.1, 96.6, 80.1, 79.2, 78.3, 77.8, 76.3, 76.0, 75.8, 75.4, 73.9, 73.3, 73.1, 73.0, 72.7, 72.4, 72.2, 71.5, 69.7, 69.5, 69.1, 69.0, 66.9, 66.8, 66.6, 66.1, 65.6, 65.3, 61.2, 61.1, 59.5, 54.9, 54.8, 54.5, 53.4, 50.0, 46.5, 36.8, 21.9, 21.8, 21.5. HRMS (ESI) m/z [M + H]⁺ calcd for C₆₉H₁₀₁N₆O₄₃S⁺: 1733.5616 found: 1733.5661.

4.6. Enzymatic synthesis of monosulfated G1-Asn-Fmoc

In total, 260 μ l reaction mixture containing monosulfated G0-Asn-Fmoc (5.22 mg, 3 μ mol), Tris buffer (50 mM, pH 7.5), MnCl₂ (10 mM), UDP-Gal (3.9 μ mol), CIAP (5 U) and NmLgtB (109 μ g) was incubated at 37 °C. The reaction was monitored by HPLC. After the reaction was completed, the reaction was quenched by heating at 95 °C for 5 min. The product (5.1 mg, 90 %) was purified by preparative HPLC, which was subsequently lyophilised as white powder. The compound was characterised by NMR and HRMS. ¹H NMR (D₂O + 1 % DMSO-d₆, 600 MHz): δ = 7.78 (m, 2H, FmocH), 7.57 (m, 2H, FmocH), 7.37 (m, 2H, FmocH), 7.31 (m, 2H, FmocH), 5.03 (m, 1H), 4.90 (d, J = 9.6 Hz, 1H), 4.83 (m, 1H), 4.50–4.01 (m, 11H), 3.81 (d, J = 10.2 Hz, 1H), 3.68–3.41 (m, 45H, rest protons on the sugar rings), 1.98–1.95 (m, 12H, –COCH₃). ¹³C NMR (D₂O + 1 % DMSO-d₆, 151 MHz): δ = 174.2, 174.1, 143.3, 140.5, 127.6, 127.0, 124.6, 119.7, 102.5, 100.8, 99.9, 99.3, 99.1, 96.6, 80.0, 79.1, 78.3, 78.1, 77.8, 76.3, 75.9, 75.8, 74.9, 74.3, 73.9, 73.3, 73.1, 72.8, 72.4, 72.2, 72.1, 71.7, 71.5, 70.5, 69.7, 69.1, 68.1, 66.9, 66.8, 66.5, 66.0, 65.5, 65.3, 61.2, 60.6, 59.6, 54.8, 54.5, 54.4, 53.4, 49.9, 46.5, 36.7, 21.9. HRMS (ESI) m/z [M + H]⁺ calcd for C₇₅H₁₁₁N₆O₄₈S⁺: 1895.6144 found: 1895.6190.

4.7. Enzymatic synthesis of monosulfated G2-Asn-Fmoc

In total, 260 μ l reaction mixture composed of monosulfated G0-Asn-Fmoc (5.14 mg, 3 μ mol), Tris buffer (100 mM, pH 7.5), MnCl₂ (10 mM), UDP-Gal (6.9 μ mol), CIAP (5 U) and human B4GALT4 (257 μ g) was incubated at 37 °C. The reaction was monitored by HPLC. After the reaction was completed, the reaction was quenched by heating at 95 °C for 5 min. The product (5.6 mg, 90 %) was purified by preparative HPLC, which was obtained as white powder after lyophilisation. The compound was characterised by HRMS and NMR. ¹H NMR (D₂O + 1 % DMSO-d₆, 600 MHz): δ = 7.79 (m, 2H, FmocH), 7.57 (m, 2H, FmocH), 7.38 (m, 2H, FmocH), 7.31 (m, 2H, FmocH), 5.03 (m, 1H), 4.91 (m, 1H), 4.83 (m, 1H), 4.45–4.10 (m, 12H), (d, J = 1.8 Hz, 1H), 3.83–3.41 (m, 65H, rest protons on the sugar rings), 1.98–1.80 (m, 12H, –COCH₃). ¹³C NMR (D₂O + 1 % DMSO-d₆, 151 MHz): δ = 174.3, 174.2, 172.0, 143.2, 140.5, 127.6, 127.0, 124.6, 119.7, 102.5, 102.1, 100.8, 99.9, 99.1, 98.9, 96.6, 80.0, 79.0, 78.2, 78.1, 77.7, 77.0, 76.3, 75.9, 75.7, 74.8, 74.2, 73.9, 73.1, 72.4, 72.2, 71.6, 71.5, 71.4, 70.5, 69.7, 68.9, 68.1, 68.0, 66.9, 66.8, 66.0, 65.8, 65.4, 65.2, 61.2, 61.1, 60.5, 59.5, 54.5, 54.4, 54.3, 53.9, 49.9, 46.5, 21.9. HRMS (ESI) m/z [M + H]⁺ calcd for C₈₁H₁₂₁N₆O₅₃S⁺: 2057.6673 found: 2057.6723.

4.8. Synthesis of sugar oxazoline of monosulfated G0, G1 and G2 N-glycans

A solution containing monosulfated G0-Asn-Fmoc (11 mg) or monosulfated G1-Asn-Fmoc (5 mg) or monosulfated G2-Asn-Fmoc (5.9 mg) and Endo-S2 (300 μ g) in PBS buffer (pH7.4) was incubated at 37 °C. The reaction was monitored by LC-MS. When no substrate was found in LC-MS, the reaction was quenched by heating at 95 °C for 5 min.

Thereafter, the mixture was spun down at 12247 g for 5 min at room temperature. The supernatant was subsequently loaded on G15 column. The fractions containing *N*-glycans were pooled together and lyophilised. A solution composed of monosulfated glycans hydrolysed by Endo-S2, triethylamine (40 eq) and 2-chloro-1,3-dimethylimidazolium chloride (20 eq) was incubated on ice for 30 min. Thereafter, the solution was loaded on size exclusion chromatography G10 column. The fractions containing glycans were collected and lyophilised. The sugar oxazolines were characterised by ¹H NMR and HRMS. For monosulfated G0 oxazoline (**6**): ¹H NMR (400 MHz, D₂O) δ 5.98 (d, 1H, *J* = 7.28 Hz, H-1-GlcNAc-ox), 5.02 (s, 1H), 4.85–4.81 (m, 1H), 4.61 (s, 1H), 4.51–4.46 (m, 2H), 4.29–4.21 (m, 2H), 4.17–4.02 (m, 5H), 3.85–3.24 (m, 29H), 1.97–1.93 (m, 9H). HRMS (ESI) *m/z* [M + H]⁺ calcd for C₄₂H₇₀N₃O₃₃S⁺: 1176.3607, found: 1176.3529. For monosulfated G1 oxazoline (**7**): ¹H NMR (600 MHz, D₂O) δ 5.96 (d, 1H, *J* = 7.26 Hz, H-1-GlcNAc-ox), 5.00 (s, 1H), 4.83 (s, 1H), 4.62 (s, 1H), 4.50–4.44 (m, 2H), 4.34 (d, *J* = 7.86 Hz, 1H), 4.28–4.20 (m, 2H), 4.15–4.01 (m, 6H), 3.89–3.35 (m, 34H), 1.96–1.91 (m, 9H). HRMS (ESI) *m/z* [M + H]⁺ calcd for C₄₈H₈₀N₃O₃₈S⁺: 1338.4135, found: 1338.4068. For monosulfated G2 oxazoline (**8**): ¹H NMR (600 MHz, D₂O) δ 5.96 (d, 1H, *J* = 7.26 Hz, H-1-GlcNAc-ox), 5.00 (s, 1H), 4.84–4.79 (m, 1H), 4.62 (s, 1H), 4.52–4.45 (m, 2H), 4.41 (d, 1H, *J* = 7.8 Hz, 1H), 4.34 (d, 1H, *J* = 7.86 Hz, 1H), 4.31–4.19 (m, 3H), 4.15–3.97 (m, 6H), 3.89–3.26 (m, 39H), 1.96–1.90 (m, 9H). HRMS (ESI) *m/z* [M + H]⁺ calcd for C₅₄H₉₀N₃O₄₃S⁺: 1500.4663, found: 1500.4611.

4.9. Enzymatic synthesis of rituximab with sulfated N-Glycans

Synthesis of 9.—The reaction mixture containing Fuca1,6 GlcNAc-rituximab (1.28 mg), sugar oxazoline of **6** (750 μg) and glycosynthase mutant Endo-S2 D184M (40 μg) in 1 × PBS buffer (pH7.4) was incubated at 30 °C. The reaction was monitored by Thermo LC-ESI-MS. After the reaction was completed, the product of **9** (1.19 mg, 92 %) was purified by protein A chromatography. LC-ESI-MS analysis: calcd for intact antibody of **9**, *M* = 147234 Da, found, 147236 Da; after IdeS digestion of **9**, LC-ESI-MS calcd for the monomeric Fc part 25283 Da, found, 25279 Da (deconvolution data).

Synthesis of 10.—The reaction mixture containing Fuca1,6 GlcNAc-rituximab (1.3 mg), sugar oxazoline of **7** (750 μg) and glycosynthase mutant Endo-S2 D184M (40 μg) in 1 × PBS buffer (pH7.4) was incubated at 30 °C. The reaction was monitored by Thermo LC-ESI-MS. After the reaction was completed, the product of **10** (1.18 mg, 89 %) was purified by protein A chromatography. LC-ESI-MS analysis: calcd for intact antibody of **10**, *M* = 147558 Da, found, 147560 Da; after IdeS digestion of **10**, LC-ESI-MS calcd for the monomeric Fc part 25445 Da, found, 25441 Da (deconvolution data).

Synthesis of 11.—The reaction mixture containing Fuca1,6 GlcNAc-rituximab (1.3 mg), sugar oxazoline of **8** (750 μg) and glycosynthase mutant Endo-S2 D184M (40 μg) in 1 × PBS buffer (pH7.4) was incubated at 30 °C. The reaction was monitored by Thermo LC-ESI-MS. After the reaction was completed, the product of **11** (1.19 mg, 90 %) was purified by protein A chromatography. LC-ESI-MS analysis: calcd for intact antibody of **11**, *M* = 147882 Da, found, 147884 Da; after IdeS digestion of **11**, LC-ESI-MS calcd for the monomeric Fc part 25607 Da, found, 25603 Da (deconvolution data).

Synthesis of 12.—The reaction mixture containing Fuca 1,6 GlcNAc-rituximab (900 µg), the G0 *N*-glycan oxazoline [48] (750 µg), and glycosynthase mutant Endo-S2 D184M [34] (40 µg) in 1 × PBS buffer (pH7.4) were incubated at 30 °C. The reaction was monitored by Thermo LC-ESI-MS. After the reaction was completed, the product of **12** (822 µg, 90 %) was purified by protein A chromatography. LC-ESI-MS analysis: calcd for intact antibody of **12**, *M* = 147076 Da, found, 147074 Da; after IdeS digestion of **12**, LC-ESI-MS calcd for the monomeric Fc domain, *M* = 25204 Da, found, 25199 Da (deconvolution data).

4.10. Enzymatic activity of CHST2 with rituximab G0F glycoform (**12**).

The reaction mixture containing the rituximab G0F glycoform (**12**) (391 µg), MgCl₂ (5 mM), PAPS (1 mM), NaF (10 mM), ATP (1 mM) and CHST2 (5.2 µg) in MES buffer (100 mM, pH 6) was incubated at 37 °C. The reaction was monitored by LC-ESI-MS. No enzymatic sulfation of the sulfation was observed after incubation for 16 h at 37 °C (Fig. S8).

4.11. Surface plasmon resonance assays

Biacore T200 (GE Healthcare, USA) surface plasmon resonance (SPR) was utilised to determine the binding affinity between various glycoforms of rituximab and FcγRIIIa-V158. To capture a variety of rituximab glycoforms, a standard primary amine coupling chemistry at pH4.5 was utilised to immobilise protein A on a CM5 biosensor chip (GE Healthcare, USA). A flow cell prepared with BSA was used as reference. Each rituximab glycoform in HBS-P buffer (10 mM HEPES pH 7.4, 150 mM NaCl, 0.05 % surfactant P20) was injected individually at 10 µl/min. The antibodies were captured at 200 RU for FcγRIIIa-V158. 2 × serial dilutions of FcγRIIIa receptor in HBS-P buffer was injected at 30 µl/min. After each cycle, the surface was regenerated by injecting glycine HCl buffer (10 mM, pH 2.0). To obtain the equilibrium constant *K_D*, the experimental data was fitted into a 1:1 Langmuir binding model utilising the BIAcore T200 evaluation software (GE Healthcare).

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Data availability

All the data and experimental procedures are described in the main text and the supporting information.

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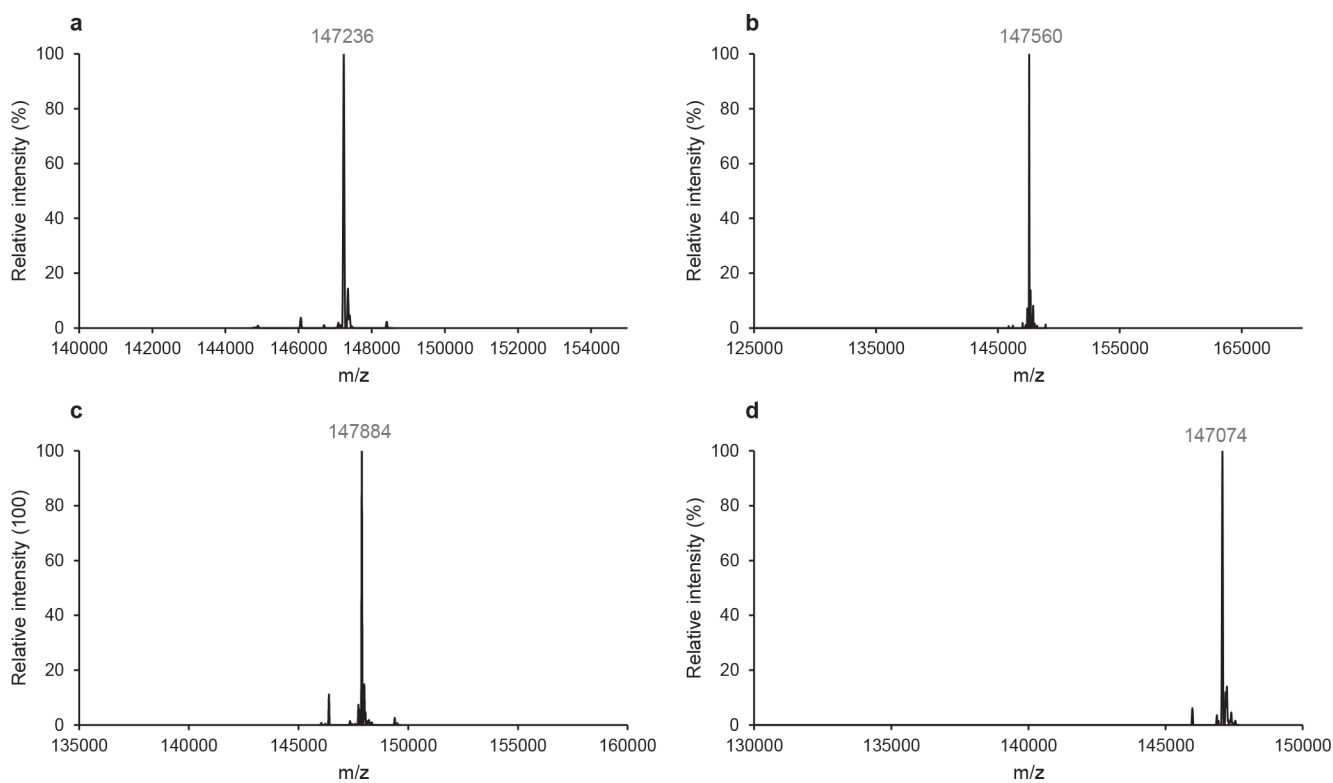


Fig. 1. LC-ESI-MS analysis of glycoengineered rituximab. (a) Intact antibody **9**. (b) Intact antibody **10**. (c) Intact antibody **11**. (d) Intact antibody **12**. The spectra are the deconvoluted spectra.

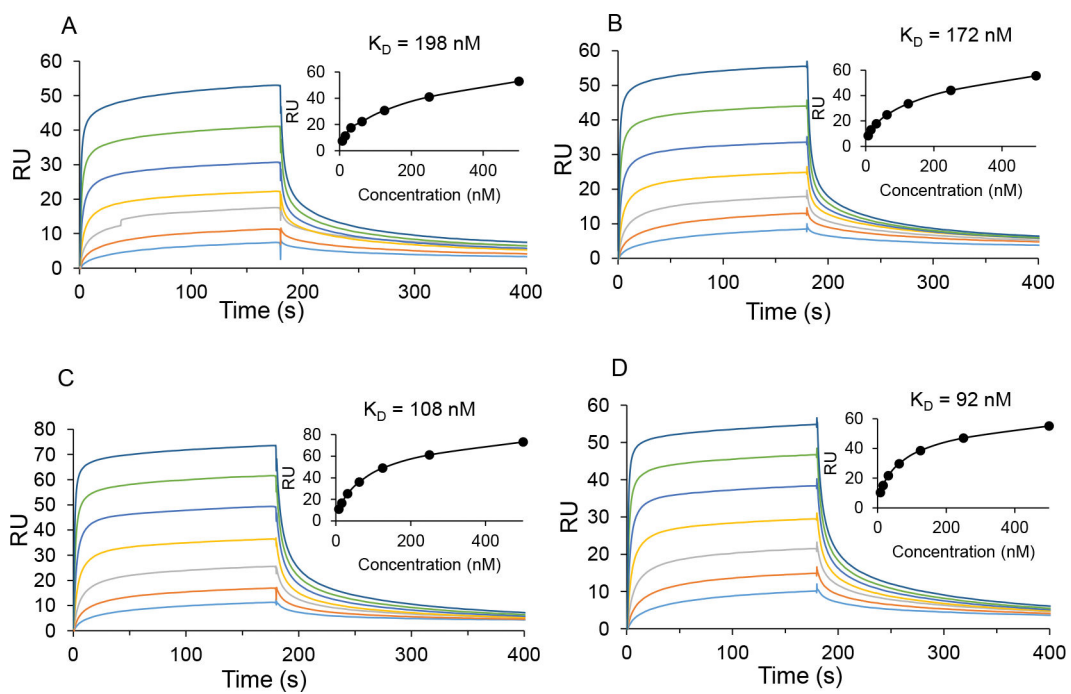
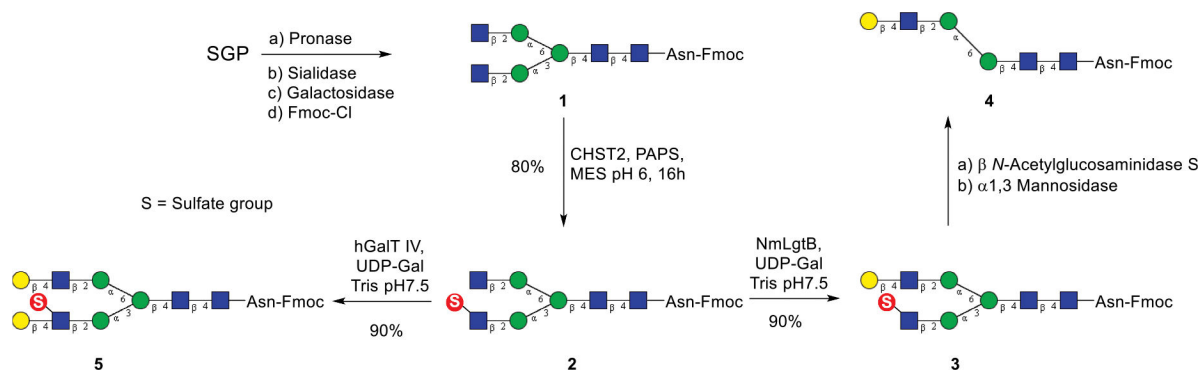
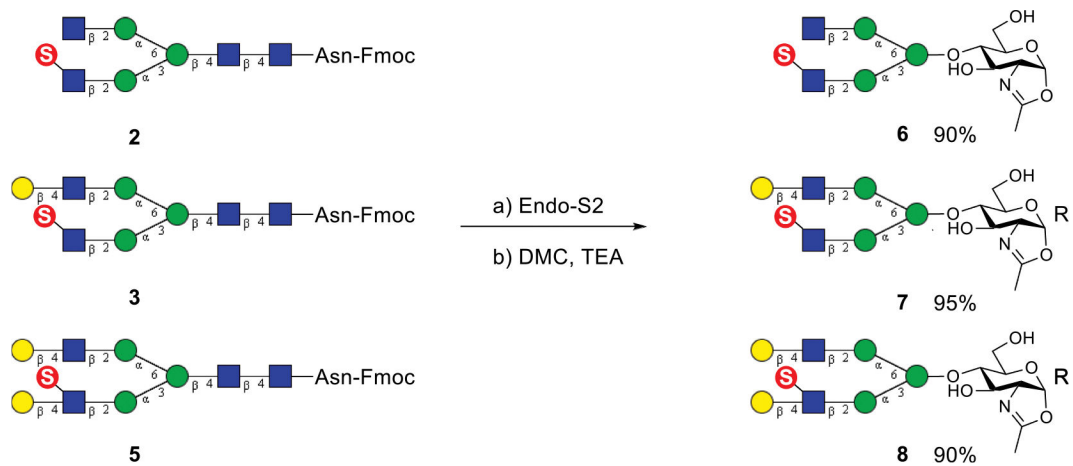


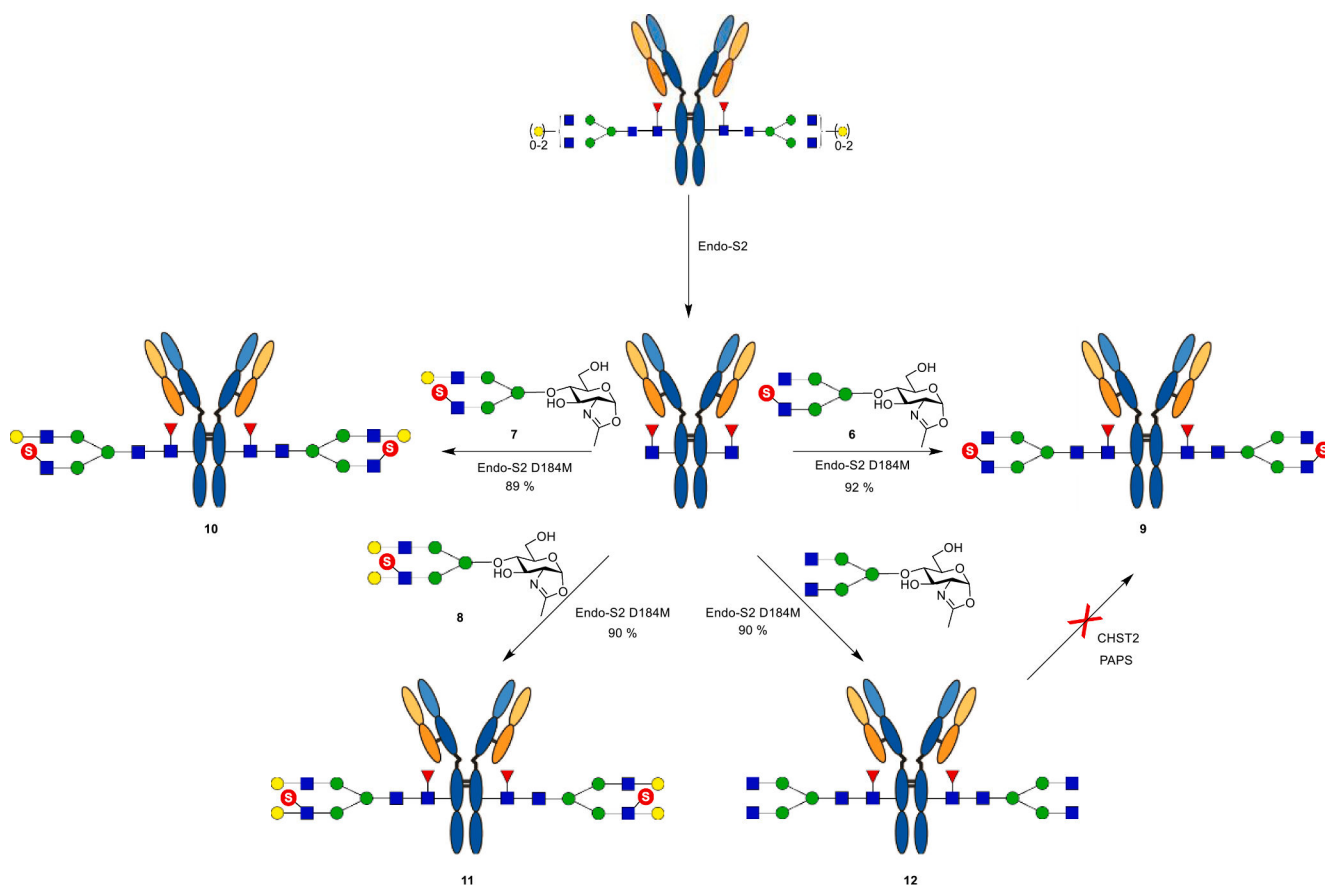
Fig. 2. SPR sensorgrams of the binding of Fc γ RIIIa-V158 with different glycoforms of rituximab. A) G0F-rituximab (**12**); B) Sulfated G0F-rituximab (**9**); C) G2F-rituximab; D) Sulfated G2F-rituximab (**11**).



Scheme 1.
Chemoenzymatic synthesis of sulfated *N*-glycans.



Scheme 2.
Synthesis of sulfated *N*-glycan oxazolines.



Scheme 3.
Chemoenzymatic synthesis of sulfated glycoforms of rituximab.