Supporting Information

A common glycan structure on immunoglobulin G for enhancement of effector functions

Chin-Wei Lin[1,2,3], Ming-Hung Tsai[4], Shiou-Ting Li[1], Tsung-I Tsai[1], Kuo-Ching Chu[4], Ying-Chih Liu[4], Meng-Yu Lai[1,4], Yung-Chieh Tseng[1,5], Chia-Yu Wu[1], Sachin S. Shivatare[4], Chia-Hung Wang[4], Ping Chao[4], Shi-Yun Wang[4], Hao-Wei Shih[4], Yi-Fang Zeng[4],Tsai-Hong You[4], Jung-Yu Liao[4], Yu-Chen Tu[4], Yih-Shyan Lin[4], Hong-Yang Chuang[4], Chia-Lin Chen[1,2,6], Charng-Sheng Tsai[4], Chiu-Chen Huang[4], Nan-Horng Lin[4], Che Alex Ma[1], Chung-Yi Wu[1,2]* and Chi-Huey Wong[1,2,3]*

1. Genomics Research Center, Academia Sinica, 128 Academia Road, Section 2, Nankang, Taipei 115, Taiwan.
2. Chemical Biology and Molecular Biophysics program, Taiwan International Graduate Program, Academia Sinica, Taiwan.
3. Department of Chemistry, National Taiwan University, Taipei 106, Taiwan.
4. CHO Pharma Inc., Park Street, Nangang District, Taipei-11503, Taiwan.
5. Graduate Institute of Life Sciences, National Defense Medical Center, Taipei 114, Taiwan
6. Institute of Biochemical Sciences, National Taiwan University, Taipei 106, Taiwan.

SI Materials and Methods

General Procedures in preperation of N-glycans

Method A: Glycosylation by Glycan Thioglycoside Donor

The MS-4Å molecular sieves were activated in a vacuum system and heated for 1 h. for glycosylation. After the activated molecular sieves were cooled to room temperature, they were added to a flask containing a **Donor** (1.5~2.0 eq. for one position glycosylation) and an **Acceptor** (1.0 eq.). Dichloromethane was added to the mixture, and then the mixture was stirred at room temperature for 3 h. *N*-iodosuccinimide (NIS, 1.7~2.2 eq.) and trimethylsilyl trifluoromethanesulfonate (TMSOTf, 0.1 eq.) were added to the mixture at -78 ^{o}C, and then stirred at -20 ^{o}C. The reaction was monitored by thin-layer chromatography (TLC) analysis on silica gel plates

(Merck DC Kieselgel 60F_{254}) and visualized by UV light (254 nm) and acidic ceric ammonium molybdate. After the acceptor was consumed completely, the reaction was quenched with sat. $NaHCO_{3(aq)}$ and 20% $Na_2S_2O_{3(aq)}$, and then the mixture was filtered through a pad of Celite. After the aqueous layer was extracted with two portions of dichloromethane, the combined organic layers were washed with brine, dried over $MgSO_4$, and concentrated. The crude product was purified by silica gel column chromatography (toluene/ethyl acetate as elution system) to give the desired product (the yield was shown on the Figure S1).

Method B: Glycosylation by Glycan Fluoride Donor

A mixture of silver triflate (5 eq.), bis (cyclopentadienyl) hafnium dichloride (3.5 eq.) and 4Å activated molecular sieves in dry toluene was stirred at room temperature for 1 h. The reaction mixture was then cooled to -50 °C, and a solution of **Acceptor** (1.0 eq.) and **Donor** (1.2~1.5 eq.) in toluene was added. The mixture was stirred at -10 ^{o}C for 2-8 h. After TLC indicated a complete consumption of acceptor, the reaction was quenched with Et_3N, diluted with EtOAc and filtered through Celite. The filtrate was washed with aqueous $NaHCO_3$, and a brine solution. The organic layer was dried over Na_2SO_4 and concentrated in *vacuo*. The crude product was purified by silica gel column chromatography (toluene/ethyl acetate as elution system) to give the desired product (the yield was shown on the Figure S1).

Mathod C: Deprotection of the *O*-Acetyl Group

NaOMe (0.25 eq.) was added to a solution of starting material (1.0 eq.) in THF/Methanol (2/3). The reaction was stirred at room temperature and monitored by TLC analysis. After the acetyl group was de-protected completely, the solution was neutralized by IR-120, filtered, and concentrated. The crude product was purified by silica gel column chromatography (hexanes/ethyl acetate as elution system) to obtain the desired product (the yield was shown on the Figure S1).

Method D: Deprotection of the *O*-Troc Group

Zn powder (20 eq.) and **acetic acid** (0.2 eq.) were added to a solution of **starting material** (1.0 eq.) in THF. The reaction was stirred at room temperature and monitored by thin-layer chromatography (TLC) analysis. After the Troc group was de-protected completely, the solution was filtered and concentrated. The crude product was purified by silica gel column chromatography (hexanes/ethyl acetate as elution system) to give the desired product (the yield was shown on the Figure S1).

Method E: Deprotection of the Benzylidene Group

***p*-Toluenesulfonic acid** (**pTSA**, 1.5 eq.) was added to a solution of **starting material** (1.0 eq.) in ACN/MeOH (2/1). The reaction was stirred at room temperature and monitored by thin-layer chromatography (TLC) analysis. After the benzylidene group was removed completely, the reaction was quenched by trimethylamine and then concentrated. The crude product was purified by silica gel column chromatography (hexanes/ethyl acetate as elution system) to give the desired product (the yield was shown on the Figure S1).

Method F: Global Deprotection

A mixture of **protected oligosaccharide** (50 mmol*)* and 10 mL of ethylene diamine : *n*BuOH (1/4) was stirred at 90 ^{o}C overnight. The volatiles were evaporated, and the crude product was reacted with 10 mL Ac_2O/pyridine (1/2) overnight. The solvent was removed under high vacuum, and the product was purified by flash column chromatography (acetone/toluene as elute system). The products were then de-acetylated using sodium methoxide in MeOH (10 mL) overnight. The reaction mixture was neutralized by IR-120, then, filtered and concentrated in vacuum. The residues were purified by flash column chromatography (acetone/toluene as elute system). The products were dissolved in 10 mL MeOH : H_2O : HCOOH (6/3/1), $Pd(OH)_2$ (50% by weight) was added, and the mixture was hydrogenated overnight. The reaction mixture was filtered through Celite and concentrated in *vacuo.* The residues were purified by G-15 gel column chromatography using water as eluent. The collected solution containing the product was lyophilized to obtain the product as white powder (the yield was shown on the Figure S1).

Method G: Enzymatic Glycosylation: Galactosylation and Sialylation

The enzymatic glycosylation was carried out using the corresponding glycosyltransferase and sugar nucleotide cofactor, and if necessary, regeneration of the cofactor was incorporated. For enzymatic sialylation, CMP-Neu5Ac and 2,3- or 2,6-sialyltransferase were used. CMP-Neu5Ac could also be generated in situ from Neu5Ac and regeneration of CMP-Neu5Ac could be used for the sialylation on preparative scales. Thus, the starting material (10 μmol), CTP (1 μmol), Neu5Ac (1.2 μmol), PEP (44 μmol), and ATP (1 μmol) were dissolved in 50 mM Tris-HCl buffer. After adjusting the pH of the solution to 7.5, α-(2,6)-sialyltransferase (150 units), CMP-Neu5Ac kinase (CMK) (80 units), pyruvate kinase (PK) (40 units), pyrophosphatase (PPA) (40 units), and CMP-sialic acid synthase (CSS) (40 units) were added to the solution. The reaction mixture was incubated at 37 °C for 24 h. and monitored by TLC. After heating the solution at 80 ^{o}C for 30 min., the denatured enzyme was removed by filtration and then the solution was concentrated in vacuo and the crude product was

purified by G-15 gel chromatography (eluent H_2O), followed by lyophilization to obtain the desired product as white solid.

Preparation of 2,6-NSCT glycan from isolated sialylglycopeptide (SGP). Isolation of the sialylglycopeptide (SGP) from hen's egg yolk was according to the published method. Briefly, the phenol extract of hen's egg yolk was centrifuged, filtrated, and purified by column chromatography, including Sephadex G-50, Sephadex G-25, DEAE-Toyoperarl 650M, CM-Sephadex C-25 and Sephadex G-25. A solution of sialylglycopeptide (SGP) (52 mg) in a sodium phosphate buffer (50 mM, pH 6.0, 5mM) was incubated with Endo M (53 μg) at 37 °C. After 7 h., the reaction mixture was subjected to gel filtration chromatography on a Sephadex G-25 column and eluted by water. The fractions containing the product were combined and lyophilized to give the product 2,6-NSCT as a white powder (30 mg, yield 82%).

Preparation of 2,3-NSCT glycan. The 2,3-NSCT was prepared by α-(2,3)-sialylation of SG1 (complex type oligosaccharide). The SG1 was obtained by treating a solution of 2,6-NSCT glycan (25 μmol dissolved in 50 mM Tris-HCl buffer, pH 7.4) with α-(2,6) sialidase (75 units) at 37 ^{o}C for 24 h. and monitored by TLC. After heating the solution at 80 ^{o}C for 30 min., the denatured enzyme was removed by filtration and the solution was concentrated in vacuo, and the crude SG1 oligosaccharide was purified by G-20 gel chromatography (elute water, 90%). The 2,3-NSCT was prepared by α-(2,3)-sialylation of SG1 via enzymatic method. SG1 (10 μmol), CTP (3 μmol), Neu5Ac (30 μmol), PEP (66 μmol), ATP (3 μmol) were dissolved in 50 mM Tris-HCl buffer. After adjusting the pH value of the solution to 7.5, α-(2,3)-sialyltransferase (150 units), CMK (80 units), PK (40 units), PPA (40 units), and CSS (40 units) were added to the solution. The reaction was incubated at 37 °C for 24 h. and monitored by TLC. After heating the solution at 80 ^{o}C for 30 min., the denatured enzyme was removed by filtration and the solution was concentrated in vacuo, and the desired 2,3-NSCT was purified by chromatography using LH-20 (eluted with water) and DEAE (eluted with $NaCl_{(aq)}$ from 5 mM to 15 mM) columns to give the product in 90% yield.

Representative synthesis of glycan oxazolines. A solution of 2,6-NSCT ($Sia_2Gal_2GlcNAc_2Man_3GlcNAc$) (30 mg), 2-chloro-1,3-dimethylimidazolinium chloride (DMC) (62.7 mg) and Et_3N (89 μL) in water (593 μL) was stirred at 4 ^{o}C for 1 h. The reaction mixture was subjected to gel filtration chromatography on a Sephadex G-25 column eluted by 0.05% aqueous

Et_3N. The fractions containing the product (**2,6-NSCT oxazoline**) were combined and lyophilized to give the desired product as white powder (26 mg, yield 87.4%).

Expression of enzymes. The endo-glycosidases EndoF1 (1), EndoS (2), EndoS-mutants (D233Q and D233A) (3), and (D233N), and the α-L-fucosidase cloned from *Bacteroides fragilis* NCTC 9343 were transfected to *Escherichia coli* for enzyme expressions and purified with Ni-NTA agarose beads.

Preparation of mono-GlcNAc-Rituximab. Rituximab (2.5 mg; Roche) in a sodium phosphate buffer (50 mM, pH 7.0, 1.25 mL) was incubated with EndoS (125 μg) and BfFucH (2.5 mg) at 37 °C for 22 h. LC-MS and SDS-PAGE analyses indicated the complete cleavage of the N-glycans on the heavy chain. The reaction mixture was subjected to affinity chromatography on a column of protein A-agarose resin (1 mL; GE Healthcare) pre-equilibrated with a sodium phosphate buffer (20 mM, pH 7.0). Then, the column was washed with a sodium phosphate buffer (20 mM, pH 7.0, 10 mL). The bound IgG was released with glycine-HCl (50 mM, pH 3.0, 10 mL), and the elution fractions were immediately neutralized with Tris-Cl buffer (1.0 M, pH 8.3). The fractions containing the antibody were combined and concentrated by centrifugal filtration (Amicon Ultra centrifugal filter, Millipore, Billerica, MA) to give mono-GlcNAc Rituximab (1.93 mg). The product was trypsinized, and the glycopeptides, TKPREEQYNSTYR and EEQYNSTYR were analyzed using nanospray LC/MS to confirm the glycosylation pattern of mono-GlcNAc.

Transglycosylation of mono-GlcNAc Rituximab with glycan oxazolines. A glycan oxazoline was added to the mixture of an EndoS mutant and Mono-GlcNAc Rituximab in 50 mM Tris buffer (pH 7.4). The solution was incubated for 60 to 150 min. at either 30 or 37 ℃. Then, the reaction mixture was purified with protein A affinity column, followed by an anionic exchange column of Capto Q (GE Healthcare) to collect the desired product. The corresponding detailed reaction conditions and yields were list in Table S3. The degree of target glycosylation was evaluated by both the PAGE and the ratios of target glycosylated peptides to all glycosylated and non-glycosylated glycopeptides were listed in **Table S3 and Fig. S3**.

Preparation of mono-GlcNAc Herceptin. A mixture of EndoS (10 ug), EndoF1 (10 ug), BfFucH (2 mg) and Herceptin (2 mg; Roche) in 50mM sodium phosphate buffer (pH 7.0) was shaken at 37 ℃

for 24 hr. Then, the defucosylated product was subjected to a protein A affinity column and purified according to the method previously mentioned to give the product in 96.6%. Both trypsinized glycopeptides, TKPREEQYNSTYR and EEQYNSTYR, showed 100% mono-GlcNAc Herceptin in nanospray LC/MS **(Fig. S4)**.

Transglycosylation of mono-GlcNAc Herceptin with glycan oxazolines. A glycan oxazoline was added to the mixture of an EndoS mutant and mono-GlcNAc Herceptin in 50 mM Tris buffer (pH7.8). The solution was incubated for 0.5-1.5 h. at room temperature, 30 ℃ or 37 ℃. After the reaction was completed, the transglycosylated Herceptin was purified with protein A affinity column and the anion exchange column Capto Q (GE Healthcare) for further purification. The purified glycoengineered Herceptin was trypsinized and analyzed by nanospray LC/MS for relevant glycopeptides, TKPREEQYNSTYR and EEQYNSTYR. The corresponding transglycosylation conditions of each glycan and the ratio of target glycosylated peptide to all glycosylated/non-glycosylated peptides were listed in **Table S4**.

Expression and purification of FI6 antibody. The FI6 antibody expression plasmid was a kind gift from Dr. An-Suei Yang at Academia Sinica, Taiwan. The expression was carried out by transient transfection in HEK293-EBNA cells (ATCC number CRL-10852) with Freestyle 293 expression medium (Invitrogen) supplemented with 0.5% bovine calf serum. The antibody was purified by Protein A beads (Roche Diagnostics), and further purified by size exclusion Superdex 200 10/300 column (GE Healthcare Life Sciences) in PBS.

Preparation of mono-GlcNAc FI6. Mono-GlcNAc FI6 was prepared with similar method described in the preparation of mono-GlcNAc Herceptin. The mixture of EndoS, EndoF1, BfFucH and FI6 mAb in 50 mM sodium phosphate buffer (pH7) was shaken at 37 ℃ for one day, and then purified with protein A affinity column to give mono-GlcNAc FI6 in 85% yield; PAGE **(Fig. S1C)**. Both trypsinized glycopeptides, TKPREEQYNSTYR (m/z=1391.58) and EEQYNSTYR (m/z=1873.88), showed 100% mono-GlcNAc Herceptin in nanospray LC/MS.

Preparation of FI6m. The oxazoline-2,6-SCT (9.5mg) was added to the mixture of an EndoS mutant (2.5mg) and mono-GlcNAc FI6 (9.4mg) in 50 mM Tris buffer (pH7.8) and incubated for 40 min. at 37 ℃. After the reaction was completed, the FI6m obtained was purified with protein A

affinity column and Capto Q column as mentioned previously to give the product in 88% yield. The intensity ratio of TKPREEQYN(2,6-NSCT)STYR to all glycosylated/non-glycosylated TKPREEQYNSTYR is 96% and the corresponding value in minor peptide EEQYNSTYR is 93%. PAGE image also indicated the success of transglycosylation **(Fig. S3C)**.

Expression and purification of FcγRIIIa. The DNA encoding the extracellular domain of human FcγRIIIa (16-208) was fused to a hexa-histidine tag in pcDNA3.1 vector. FcγRIIIa was produced by transient transfection in HEK293-EBNA cells (ATCC number CRL-10852) with Freestyle 293 expression medium (Invitrogen) supplemented with 0.5% bovine calf serum. The FcγRIIIa protein was purified with Nickel-chelation column following standard protocol. The eluted proteins were further purified by size exclusion Superdex 200 10/300 column (GE Healthcare Life Sciences) with 0.02 M Tris buffer containing 0.15 M NaCl, pH 8.0 and concentrated to obtain a product solution of 1 mg/mL.

MS spectrometry analysis of glycoengineered mAb. For the analysis of trypsinized glycopeptides, high resolution and high mass accuracy nanoflow LC-MS/MS experiments were performed on a LTQFT Ultra (linear quadrupole ion trap Fourier transform ion cyclotron resonance) mass spectrometer (Thermo Electron, San Jose, CA) equipped with a nanoelectrospry ion source (New Objective, Inc.), an Agilent 1100 Series binary high-performance liquid chromatography pump (Agilent Technologies, Palo Alto, CA), and a Famos autosampler (LC Packings, San Francisco, CA). The digestion solution was injected (6µl) at the 10 µl/min flow rate to a self-packed precolumn (150 µm I.D. x 20 mm, 5 µm, 100 Å). The chromatographic separation was performed on a self-packed reversed phase C18 nano-column (75 µm I.D. x 300 mm, 5 µm, 100 Å) using 0.1% formic acid in water as mobile phase A and 0.1% formic acid in 80% acetonitrile as mobile phase B operated at 300 nl/min flow rate. Survey of full-scan MS conditions: mass range m/z 320-2000, resolution 100,000 at m/z 400. The ten most intense ions were sequentially isolated for MS2 by LTQ. Electrospray voltage was maintained at 1.8 kV and the capillary temperature was set at 200 ^{o}C. The results were shown in **Table S3** and **S4**.

SDS-PAGE detection of glycoengineered Herceptin antibodies. All the SDS –PAGE analyses were performed with NuPAGE® Novex® 4–12% Bis-Tris gel (Invitrogen) in MOPS buffer either with or without DTT addition in samples.

Binding to FcγIIIa, IIa, IIb ,I and C1q. FcγRIIIa or FcγI (0.5 μg/ml; R&D system) in a carbonate coating buffer (pH 10.0) was coated onto a 96-well plate (Corning) at 4℃ for overnight. Then, after a wash, 2% bovine serum albumin in PBST (assay buffer) was added to block the plate for 2 hours. Subsequently, after another wash, a serial dilution of Rituximabs ranging from 0.00192 nM to 150 nM in a 5-fold dilution of assay buffer was included to incubate for 1hr. Finally, after the incubation of anti-human IgG HRP (abcam) for 1 hour, the addition of 100 μl of substrate, 3, 3', 5, 5'-tetramethylbenzidine (TMB) for signal development and the stop by the adding 100 μl of 2.5N sulfuric acid, absorbance at 450 nm was measured using microplate reader Spectra Max M5 (Molecular Devices) and data were analyzed using the four-parameter nonlinear regression equation (4PL) to fit the curves of results. With similar coating methods, blocking procedures, developments of substrate, and the fitting equation, the binding assay towards FcγRIIa and FcγIIb (0.25 μg/ml and 0.125 μg/ml respectively; Sino Biological Inc.) were performed using the complexes of Rituximabs (0.0096 nM- 750 nM) and goat anti-κ antibody (AbD Sterotec) with a weight ratio of 1 to 2 under the incubation of 1 hour and subsequently anti-human IgG HRP was added for another incubation of 1 hour.

In the binding assay of C1q, a serial dilution of Rituximabs ranging from 0.00192 nM to 150 nM was first coated onto 96-well plates, and with similar blocking method previously mentioned, the binding of C1q (2 μg/ml in assay buffer; Prospec) was performed for incubation of 1 hour and subsequently detected through the incubation of anti-C1q HRP (Biorad) for another 1 hour followed by addition of TMB substrate as mentioned previously. Data of C1q were also fitted with 4PL.

Complement-dependent cytotoxicity (CDC). CDC was performed using the CytoTox-Glo cytotoxicity assay (Promega). Ramos B lymphocytes (Bioresource Colletion and Research Center, Taiwan) were seeded to 96-well round microplates with a population of $5x10^4$ cells/well. Rabbit serums were divided into two aliquots; one aliquot was heat-inactivated at 56℃ for 30 min and the other aliquot was untreated. After the incubation of 100 μl of antibody dilutions and 100 μl of cell suspension in either heat-inactivated or nonheat-inactivated serum at 37℃ and 5% CO_2 for 4 hours, the 50 μl of supernatant from each well was transferred to a 96-well white flat-bottom plate for subsequent incubation with a 25 μl of the reconstituted substrate at room temperature for 5 to 15 minutes. Finally, the luminescence was read by a Spectra Max M5 to calculate the percentage of cell lysis with the following formula: The percentage of cell lysis = (luminescence of the experimental – luminescence of the spontaneous)/ (luminescence of the maximum – luminescence of the

spontaneous) x 100. The maximum was determined by incubating target cells with 30 μg/mL digitonin and the data was fitted with the four parameter nonlinear regression model in SoftMax Pro.

ELISA-based Antibody-dependent cellular cytotoxicity (ADCC) to B-lymphoma cells. The ADCC assay was also performed using the CytoTox-Glo cytotoxicity assay. Peripheral blood mononuclear cells (PBMCs) were separated from the blood of normal volunteers using Ficoll-Paque (GE healthcare). Three target cells were utilized in this ELISA based ADCC assay, including Raji (Bioresource Colletion and Research Center, Taiwan), Ramos and SKW6.4 (Fountain Biopharma Inc.).The target cells (10,000 cells/50 μl) were seeded into a 96-well round bottom plate and preincubated with 100 μl of antibody dilution for 10 minutes before adding the effector cells (2.5 x 10^5/ 50 μl) in a 25:1 E/T ratio. The cells were incubated for an additional 16 hours at 37℃, 5% CO_2. After incubation, the plate was spun down at 400g for 5 minute to pellet the cells. Then, a supernatant of 50 μl from each well was transferred to a 96-well white flat-bottom plate, and a reconstituted substrate of 25 μl was added into each assay well. The plate was incubated at room temperature for 5 to 15 minutes, and the luminescence was read by the Spectra Max M5. The percentage of cell lysis was calculated as described above for the CDC assay.

Surface Plasmon Resonance (SPR) Analysis. All the SPR experiments were performed in the single cycle kinetic methods with BIACORE T200 at 25℃ using HBS-EP (10mM HEPES pH7.4, 0.15M NaCl, 3mMEDTA, 0.005% surfactant P20) as running buffer. FcγRIIIa was transfected into HEK-293 cell line to express complex-type glycosylated recombinant protein as analyte. For the ligands of Herceptins, the $(Fab')_2$ fragment of goat anti-human $(Fab')_2$ (Jackson Immuno Research Inc.) was immobilized onto both the reference and active channels of CM5 sensor chip, and then Herceptins were captured on the active channel for interacting with the serial dilutions of FcγRIIIa analyte (1, 3, 9, 27, 81nM for G1,G2,2,6-NSCT and2,3-NSCT; 3, 9, 27, 81, 243nM for G3, G4, G5, G6, G7, G8 and G9; 8, 24, 72, 216, 648 nM for the commercial Herceptin;) at 30 μl/min for association of 240 seconds followed by dissociation time of 420 seconds. Herceptin Data were processed with double-referencing for background subtraction. For the ligands of Rituximabs, anti-human Fab antibodies in human Fab capture kit (GE Healthcare) were immobilized with the similar aforementioned method; interactions between the captured Rituximabs and the serial dilutions of FcγRIIIa analyte (12.5, 25, 50, 100 and 200 nM) were recorded as well. Both data of Rituximabs and Herceptins were fitted to 1:1 Langmuir binding model in BiaEvaluation software (GE Healthcare) to get kinetic/affinity constants.

Depletion of human B cells. Human whole blood from healthy volunteer donors was collected and aliquoted to 180 μl per well in a U-bottom 96-well plate. Then, after a 20 μl of antibody dilution (3000-0.001 ng/ml) was added, mixed well and incubated at 37^{o}C and 5 % CO_2 for 24 hr, a 50-μl aliquot of the blood was subsequently stained with anti-CD45-PECy7 (lymphocyte population), anti-CD3-FITC (T cells), and anti-CD19-PE (B cells) in the dark at 4^{o}C for 15 min. Finally, a FACS lysis solution (450 μl) was added to deplete erythrocytes and fix cells for analyzing in the flow cytometer. Results were evaluated by displaying 20000 cells in the CD45-positive gate. CD3-positive and CD19 positive populations were gated to represent T cells and B cells. The evaluation of relative B-cell depletion was obtained using the B/T cell ratio (numbers of B cells/ numbers of T cells). The percentage of B-cell depletion = [B/T ratio (antibody untreated)- B/T ratio (antibody treated)]/ [B/T ratio (antibody untreated)]x100.

The generation of Rituximab-resistant cell lines. The Rituximab-resistant cell lines were evolved from Ramos and Raji cells by serially exposed to an escalating dose of Rituximab (0.1-128 μg/ml) for 24 hrs under the presence of rabbit serum (dilution 1:200 up to 1:12.5) as a source of complement. Then, the medium was replaced with normal culture medium to propagate cells for 3-4 days. After repeating the aforementioned procedures for 17 passages, we got the Rituximab-resistant cell lines which showed the maximal resistance to the commercial Rituximab in ADCC assay.

Detect the CD20 expression of Rituximab-resistant cells by flow cytometry. For detecting the CD20 expression on cell surfaces, 1x10^{5} cells of parental Ramos, Raji and Rituximab-resistant cells (Ramos-R, Raji-R) were mixed with 1 μg/ml of Rituximab (Roche) or 2,6-NSCT-Rituximab in 100 μl of FACS buffer (PBS with 2 % FBS and 0.1 % NaN_3) and incubated on ice for 1 hr. After washing twice with 200 μl of FACS buffer, these cells were further stained with anti-human IgG-PE in 100 μl of FACS buffer on ice for 0.5 hr. The PE signals on each cell were analyzed by the flow cytometer.

PBMC mediated Antibody-dependent cellular cytotoxicity (ADCC) towards the Rituximab-resistant B-lymphocytes. The CD20^{+} B lymphoma cell lines, Raji, Ramos and their corresponding Rituximab-resistant cells were utilized as target cells in the experiments. The ratio of target cells to effector cells was set at 25:1. The target B cells in the PBS containing 0.1% BSA were first labeled with CFSE at 37 ^{o}C for 5 minutes and then washed in RPMI 1640 medium for subsequent incubation with both the serial dilution of antibodies and freshly prepared PBMC effector cells at 37□ for 16 hr.

Finally, resultant mixtures were stained in the dark for 5 minutes with the PI reagent and sent to the flow cytometry to analyze the proportion of cell deaths in ADCC.

The FcγRIIIa mediated ADCC reporter assay of Herceptin. ADCC reporter assay was performed with Promega reporter bioassay kit. Target cell, SK-BR-3 cancer cell line was obtained from ATCC and maintained in McCoy's 5A (modified) medium (Gibco) supplemented with 10% heat-inactivated fetal bovine serum and 1% Antibiotic-Antimycotic(Gibco). When ready for ADCC asssy, SK-BR-3 cells were harvested and plated in 384 well microplates for 2days. Then, serial dilutions of the glycoengineered Herceptins were added and followed by an incubation with the NFAT engineered Jerkat cells expressing FcγIIIa receptor with V158 variant for 6 hrs at 37℃.The ratio of effector cell to target cell is 6. The commercial Rituximab was chosen as the negative control while the commercial Herceptin was set as the positive control. The readout was done through adding a Bio-Glow luciferase substrate to plates and recorded with the victor3 luminescence plate reader. Fold of inductions was calculated with this equation: Fold of induction= (induced luminescence－background of buffer only)/ (induction of no antibody control－background of buffer only). Data was fitted with the four-parameter logistic equation to get the EC50.

Anti-influenza virus ADCC measurement. HEK293T cells (the target cells) were transfected with pIRES plasmid (Agilent) with a dual-expression construct of hemagglutinin (HA)-IRES-GFP sequence for 48 hours then they were trypsinized and sorted to obtain cells with high GFP expression levels by flow cytometry (FACS Aria IIU). The high HA expression level which correlates with high GFP expressing cells were assessed by anti-HA antibody immunostaining for flow cytometry (FACS Canto II). The cells with high HA expression were seeded in 96-well U-bottom plates, with 5,000 cells per well in 50 ul DMEM medium (Gibco). Peripheral blood mononuclear cells (PBMCs, the effector cells) were prepared by Ficoll-Paque separation of whole blood obtained from healthy volunteers and used as effector cells in the ADCC assay. Briefly, whole blood was diluted with an equal volume of HBSS, layered over Ficoll-Paque plus (GE Healthcare) and centrifuged at 400 g for 40 min. The PBMC cells were harvested, washed twice with HBSS and mixed with HA-expressing HEK293T cells using an effector-to-target cells ratio of 50/1. Mixture of PBMCs and HA-expressing HEK293T cells were added with various concentrations of antibodies and incubated at 37°C for 5 hour. Cytotoxicity was monitored by measuring the lactate dehydrogenase (LDH) released using cytoTox96 Non-Radioactive Cytotoxicity Assay kit (Promega) when killing of target cells takes place.

ADCC signaling of the effector cells was measured using the ADCC Reporter Bioassay, Core Kit according to the manufacturer's instructions (G7010, Promega). HEK293T cells as targeted cells were transfected with HA expression plasmid for 48 hour such that >80% of cells expressed surface HA. The HA-expressing HEK293T cells were trypsinized and seeded in white, flat-bottom 96-well assay plate, 5,000 cells per well in 25 ul RPMI medium supplied in ADCC Reporter Bioassay kit. The ADCC Bioassay Effector NK Cells were mixed with HA-expressing HEK293T cells using an effector-to-target cells ratio of 15/1. Mixture of NK cells and HA-expressing HEK293T cells were added with various concentrations of antibodies and incubated at 37°C for 6 hour. ADCC signaling nuclear factor of activated T-cell pathway is monitored by measuring the NFAT driven luciferase production using a bioluminescent substrate (Bio-Glo Luciferase Assay System, Promega) and detected by Luminescence Counter (TopCount NXT).

H1N1 virus challenge in mice. Female BALB/c mice 6–8 week old were given 2.5mg/kg monoclonal antibody FI6 or modified FI6 (FI6m) via intraperitoneal injections.Two hours later, mice was anesthetized and intranasally infected with 10 MLD_{50} (50% mouse lethal dose) influenza H1N1 virus (A/California/07/2009). The mice were euthanized when lost more than 25% of their initial body weight. All animal experiments were evaluated and approved by the Institutional Animal Care and Use Committee of Academia Sinica.

Figure legends

Figure S1. Experimental Procedures for the Synthesis of Asymmetric N-Glycans

Figure S2.Preparation of 2,3-NSCT glycan from isolated 2,6-NSCT.

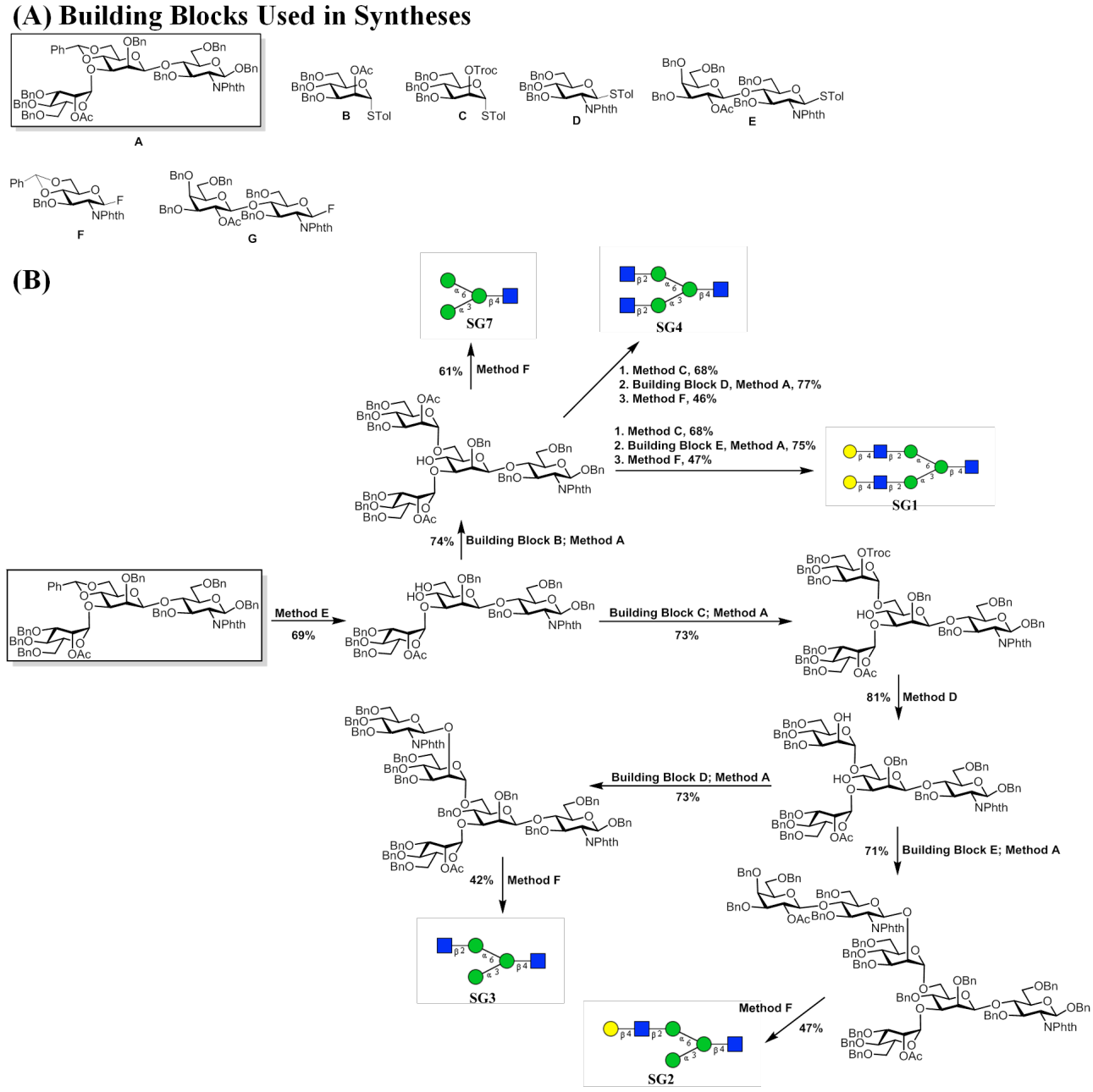
Figure S3. SDS-PAGE. (A) DTT-reduced Rituximab glycoforms; the upper bands represent heavy chain and the lower bands represent light chains.(B) Glycoengineered Herceptins; both the non-reduced and DTT-reduced PAGE are shown. (C) Glycoengineered FI6; both the non-reduced and DTT-reduced PAGE are shown.

Figure S4. Representative Xtract LTQFT Ultra MS spectra of trypsinized glycopeptides

Figure S5. Binding data of 2,3-NSCT- and 2,6-NSCT-Rituximab towards Fcγ receptors and C1q

Figure S6. CDC and ADCC assays of 2,3-NSCT and 2,6-NSCT-Rituximab glycoforms

Figure S1


(A) Building Blocks Used in Syntheses
A
B
C
D
E
F
G
(B)
SG7
SG4
61%
Method F
1. Method C, 68%
2. Building Block D, Method A, 77%
3. Method F, 46%
1. Method C, 68%
2. Building Block E, Method A, 75%
3. Method F, 47%
SG1
74%
Building Block B; Method A
Method E
69%
Building Block C; Method A
73%
81%
Method D
Building Block D; Method A
73%
71%
Building Block E; Method A
42%
Method F
SG3
Method F
47%
SG2

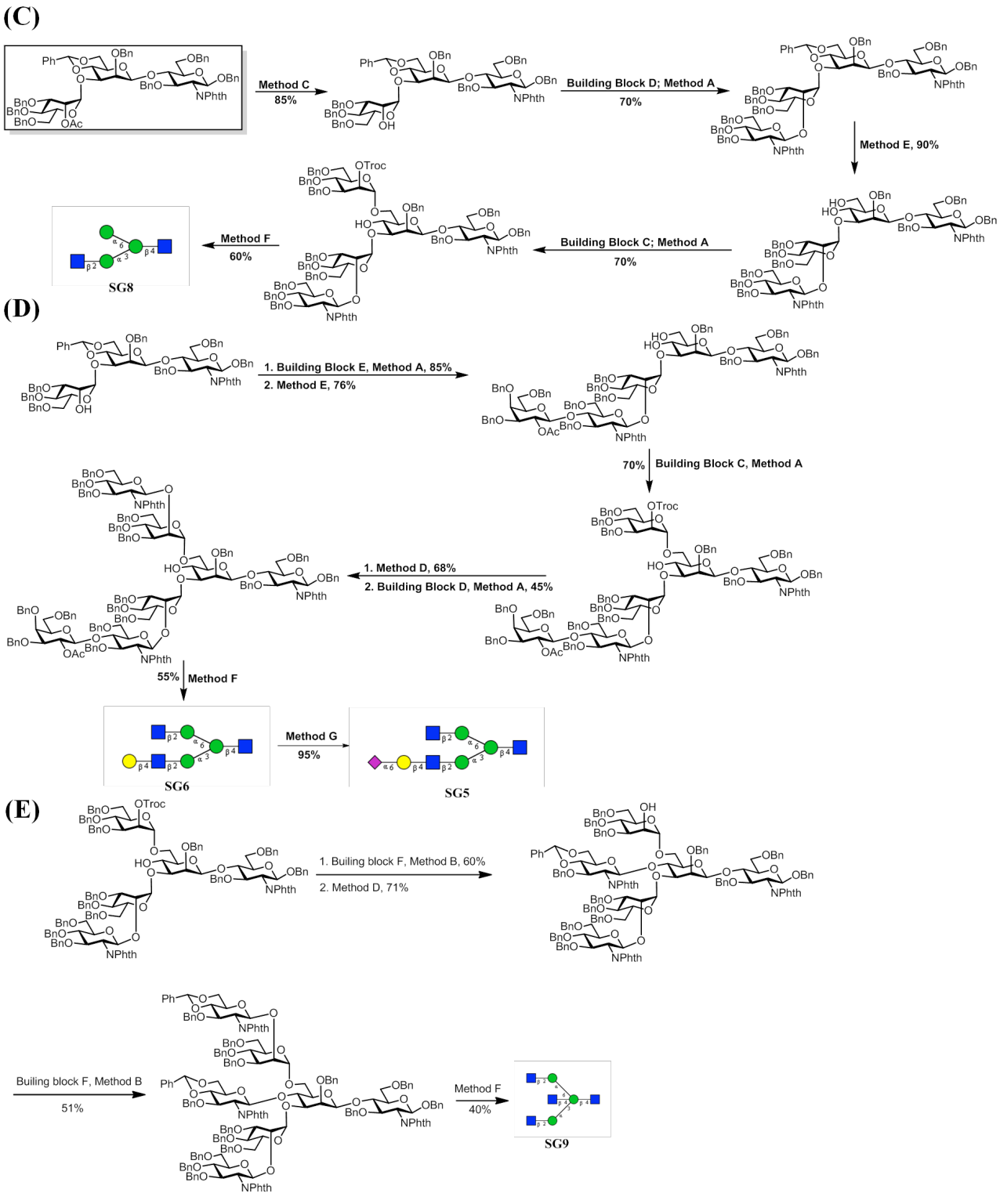

(C)
Method C
85%
Building Block D; Method A
70%
Method E, 90%
Building Block C; Method A
70%
Method F
60%
SG8
(D)
1. Building Block E, Method A, 85%
2. Method E, 76%
70% Building Block C, Method A
1. Method D, 68%
2. Building Block D, Method A, 45%
55% Method F
Method G
95%
SG6
SG5
(E)
1. Buiing block F, Method B, 60%
2. Method D, 71%
Builing block F, Method B
51%
Method F
40%
SG9

Figure S2

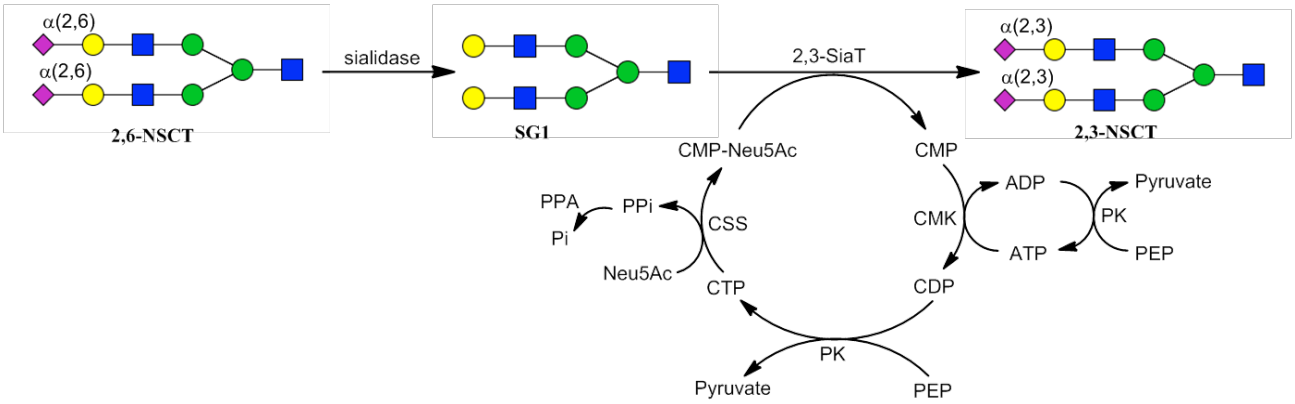

α(2,6)
α(2,6)
2,6-NSCT
sialidase
SG1
2,3-SiaT
α(2,3)
α(2,3)
2,3-NSCT
CMP-Neu5Ac
CMP
ADP
Pyruvate
PPA
PPi
CSS
CMK
PK
Pi
ATP
PEP
Neu5Ac
CTP
CDP
PK
Pyruvate
PEP

Figure S3

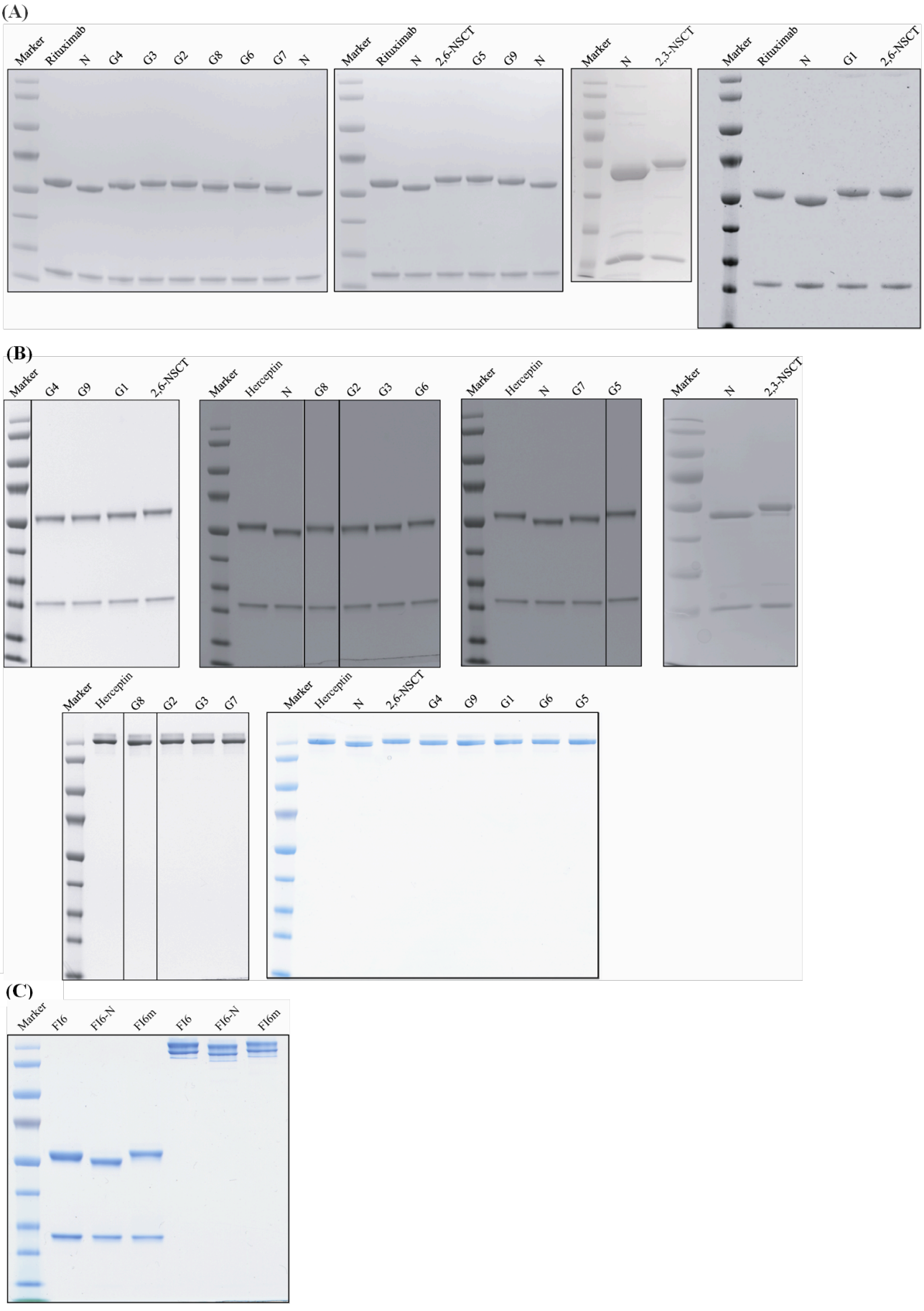

(A)
Marker
Rituximab
N
G4
G3
G2
G8
G6
G7
N
Marker
Rituximab
N
2,6-NSCT
G5
G9
N
Marker
N
2,3-NSCT
Marker
Rituximab
N
G1
2,6-NSCT
(B)
Marker
G4
G9
G1
2,6-NSCT
Marker
Herceptin
N
G8
G2
G3
G6
Marker
Herceptin
N
G7
G5
Marker
N
2,3-NSCT
Marker
Herceptin
G8
G2
G3
G7
Marker
Herceptin
N
2,6-NSCT
G4
G9
G1
G6
G5
(C)
Marker
Fl6
Fl6-N
Fl6m
Fl6
Fl6-N
Fl6m

Figure S4

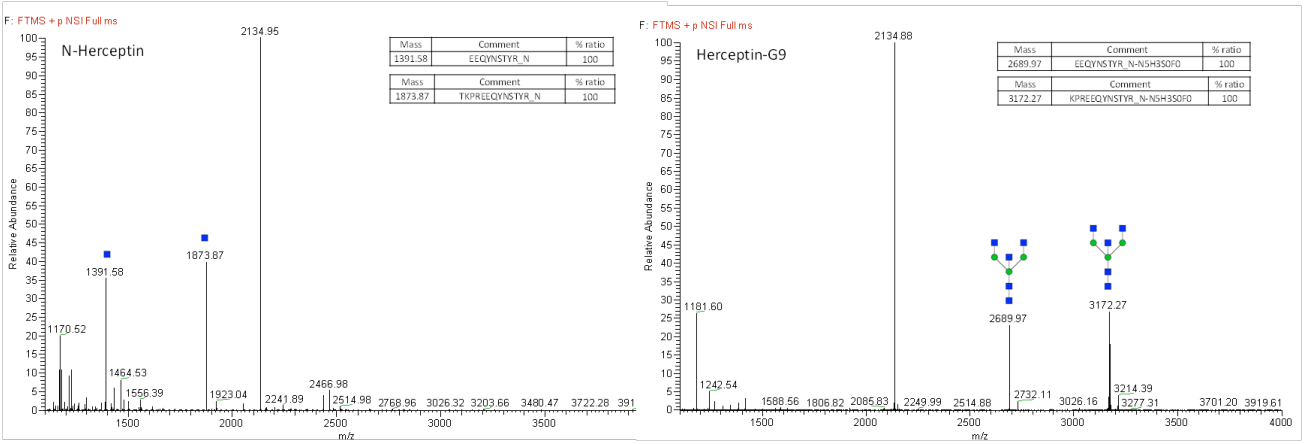
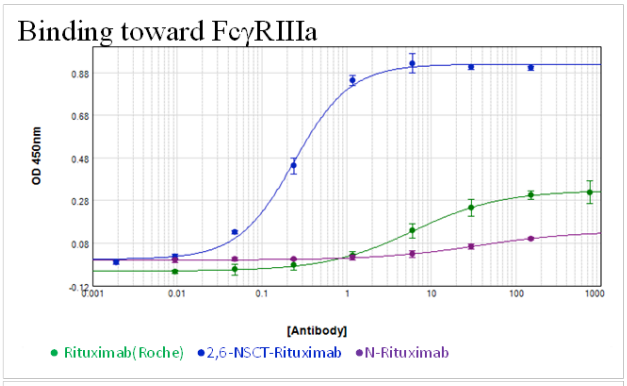
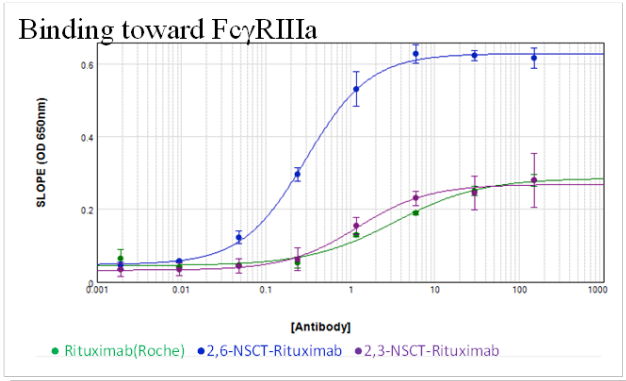
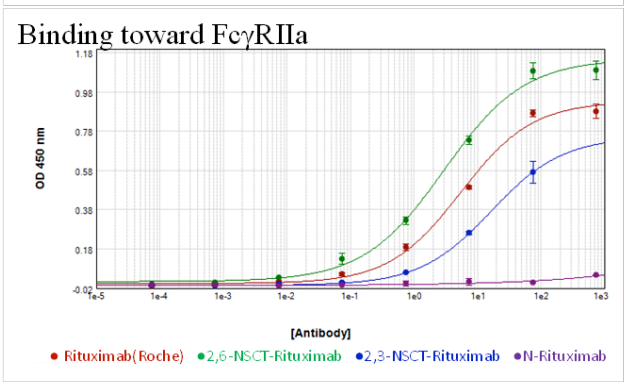
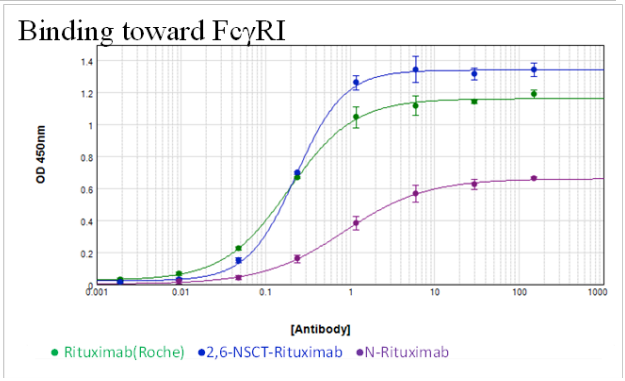


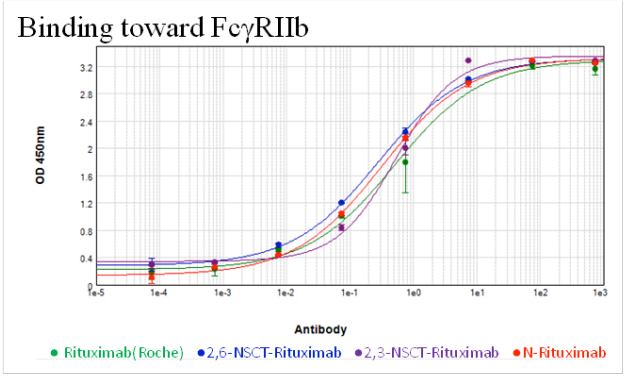
Figure S5











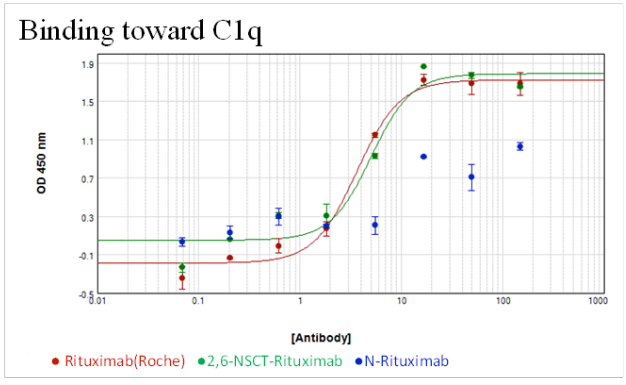
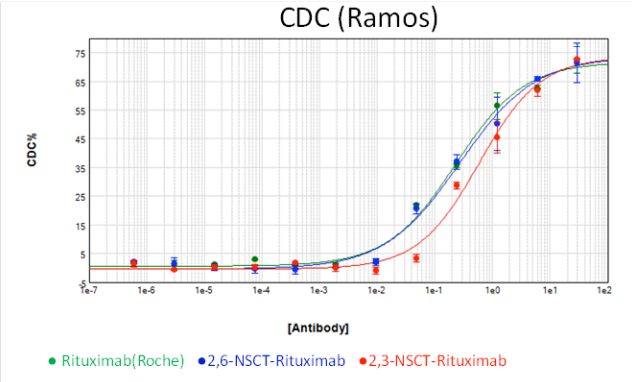
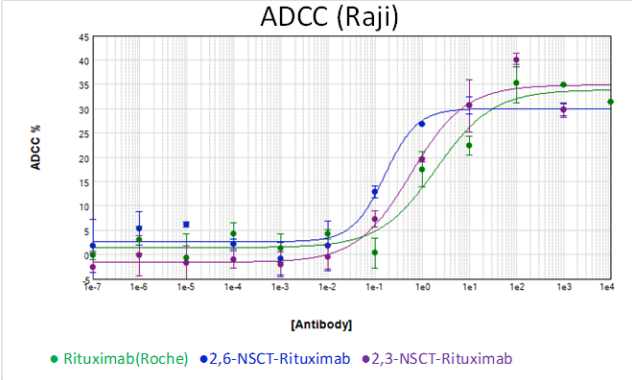
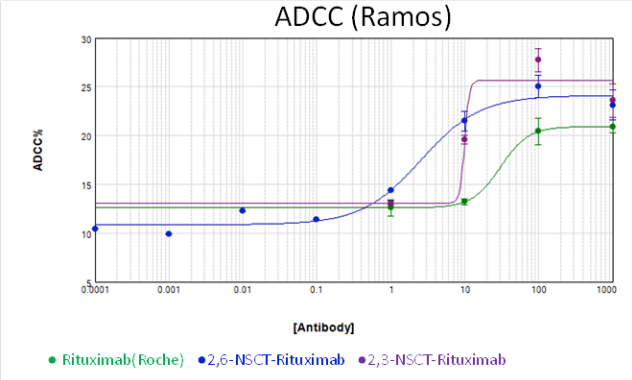


Figure S6


CDC (Ramos)
CDC%
[Antibody]
Rituximab(Roche)
2,6-NSCT-Rituximab
2,3-NSCT-Rituximab


ADCC (Raji)
ADCC %
[Antibody]
Rituximab(Roche)
2,6-NSCT-Rituximab
2,3-NSCT-Rituximab


ADCC (Ramos)
ADCC%
[Antibody]
Rituximab(Roche)
2,6-NSCT-Rituximab
2,3-NSCT-Rituximab

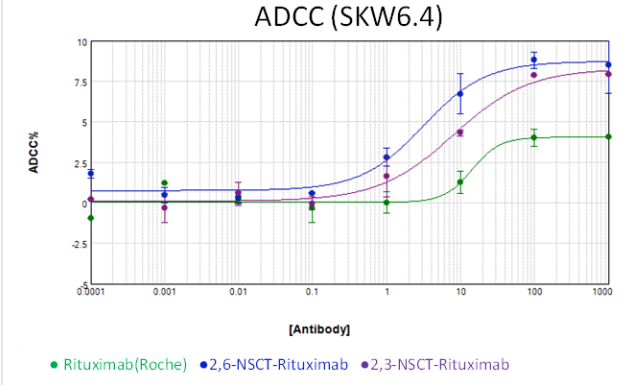
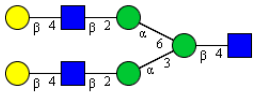
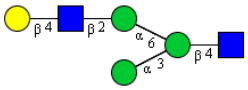
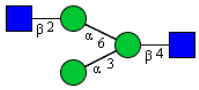
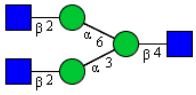
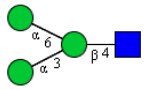
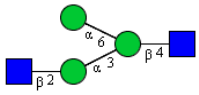
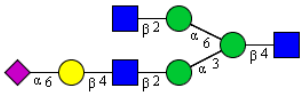
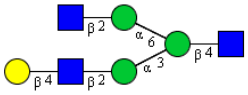
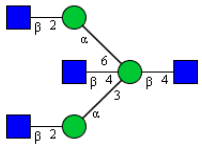
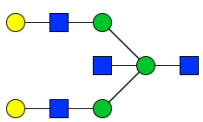

ADCC (SKW6.4)
ADCC%
[Antibody]
Rituximab(Roche)
2,6-NSCT-Rituximab
2,3-NSCT-Rituximab

Table S1. ^{1}H NMR of prepared glycans

Code	Structure	^{1}H NMR (400 MHz, D_2O)
SG1		δ 5.19 (d, J = 2.8 Hz, 1H), 5.10 (s, 1H), 4.91 (s, 1H), 4.75 (d, J = 1.0 Hz, 1H), 4.56 (d, J = 8.0 Hz, 2H), 4.44 (d, J = 8.0 Hz, 2H), 4.24~4.23 (m, 1H), 4.17 (d, J = 2.0 Hz, 1H), 4.09 (dd, J = 1.2, 3.2 Hz, 1H), 3.97~3.44 (m, 45H), 2.04~2.03 (m, 9H)
SG2		δ 5.21 (d, 2.8 Hz, 1H), 5.10 (s, 1H), 4.93 (s, 1H), 4.77 (s, 1H), 4.58 (d, J = 8.0 Hz, 1H), 4.47 (d, J = 8.0 Hz, 1H), 4.26 (t, J = 2.4 Hz, 1H), 4.11 (dd, J = 1.2, 3.2 Hz, 1H), 4.07 (dd, J = 1.6, 3.2 Hz, 1H), 3.99~3.4 (m, 33H), 2.06~2.05 (m, 6H).
SG3		δ 5.17 (d, J = 2.8 Hz, 1H), 5.10 (d, J = 1.6 Hz, 1H), 4.84 (d, J = 8.4 Hz, 1H), 4.78 (s, 1H), 4.68 (s, 1H), 4.27 (t, J = 2.8 Hz, 1H), 4.24 (t, J = 2.8 Hz, 1H), 4.16~4.12 (m, 1H), 4.06 (tt, J = 1.6, 3.2 Hz, 1H), 3.93~3.37 (m, 25H), 3.31~3.27 (m, 1H), 2.05 (d, J = 3.6 Hz, 3H), 2.02 (d, J = 2.0 Hz, 3H)
SG4		δ 5.18 (d, J = 3.0 Hz, 1H), 5,09 (s, 1H), 4.89 (s, 1H), 4.75 (s, 1H), 4.52 (d, J = 8.4 Hz, 1H), 4.24 (s, 1H), 4.16 (s, 1H), 4.08 (s, 1H).
SG7		δ 5.18 (d, J = 3.2 Hz, 1H), 5.07 (s, 1H), 4.88 (d, J = 1.6 Hz, 1H), 4.76 (d, J = 2.8 Hz, 1H), 4.23 (t, J = 2.8 Hz, 1H), 4.03 (dd, J = 1.6, 3.6 Hz, 1H), 3.95~3.60 (m, 22H), 2.02 (d, J = 1.6 Hz, 3H).
SG8		δ 5.18 (d, J = 2.8 Hz, 1H), 5.09 (s, 1H), 4.89 (d, J = 1.6 Hz, 1H), 4.77 (t, J = 2.8 Hz, 1H), 4.53 (d, J = 8.4 Hz, 1H), 4.24 (t, J = 2.4 Hz, 1H), 4.16 (dd, J = 0.8, 2.8 Hz, 1H), 3.96~3.42 (m, 28H), 2.034~2.030 (m, 6H)
SG5		δ 5.19 (d, J = 3.2 Hz, 1H), 5.12 (s, 1H), 4.90 (s, 1H), 4.77 (s, 1H), 4.59 (s, J = 8.0 Hz, 1H), 4.54 (d, J = 8.4 Hz, 1H), 4.43 (d, J = 8.0 Hz, 1H), 4.25~4.24 (m, 1H), 4.19~4.18 (m, 1H), 4.11~4.09 (m, 1H), 4.00~3.42 (m, 46H), 2.65 (dd, J = 4.8, 12.8 Hz, 1H), 2.06~2.03 (m, 9H), 2.01 (s, 3H), 1.71 (t, J = 12.4 Hz, 1H).
SG6		δ 5.18 (d, 3.2 Hz, 1H), 5.09 (s, 1H), 4.89 (s, 1H), 4.75 (d, J = 3.2 Hz, 1H), 4.56~4.52 (m, 2H), 4.43 (d, J = 8.0 Hz, 1H), 4.24~4.22 (m, 1H), 4.17~4.16 (m, 1H), 4.09~4.07 (m, 1H), 3.97~3.38 (m, 39H), 2.03~2.02 (m, 9H).
SG9		δ 5.17 (s, 1H), 5.03 (s, 1H), 4.97 (s, 1H), 4.67 (s, 1H), 4.53 (s, 1H), 4.51 (s, 1H), 4.43 (d, J = 7.6 Hz, 1H), 4.22 (s, 1H), 4.15 (s, 1H), 4.12 (s, 1H), 4.05 (t, J = 9.6 Hz, 1H), 3.96~3.16 (m, 39H), 2.02 (s, 12H).
		δ ^{1}H NMR (400 MHz, D_2O) δ 5.07 (d, *J* = 2.8 Hz, 1H), 4.89-4.93 (m, 2H), 4.58 (dd, *J* = 10.0, 7.7 Hz, 1H), 4.45 (d, *J* = 8.1 Hz, 2H), 4.33-4.35 (m, 3H), 4.13-4.14 (m, 1H), 4.04-4.06 (m, 1H), 4.01-4.03 (m, 1H), 3.96 (td, *J* = 9.5, 2.9 Hz, 1H), 3.76-3.87 (m, 13H), 3.67-3.74 (m, 4H), 3.58-3.67 (m, 15H), 3.53-3.57 (m, 5H), 3.49-3.50 (m, 3H), 3.45-3.46 (m, 1H), 3.37-3.45 (m, 5H), 3.34 (t, *J* = 9.7 Hz, 2H), 3.28 (t, *J* = 8.4 Hz, 1H), 3.13 (t, *J* = 9.2 Hz, 1H), 1.93 (s, 6H), 1.92 (s, 3H), 1.91 (s, 3H).

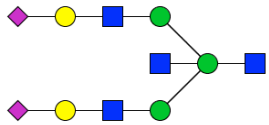
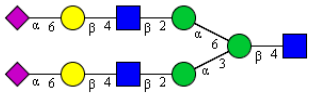
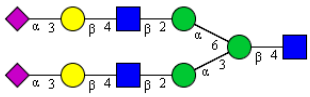
		δ 5.17 (d, J = 2.4 Hz, 1H), 4.96 (s, 1H), 4.92 (s, 1H), 4.67~4.66 (m, 2H), 4.61~4.59 (m, 2H), 4.51~4.47 (m, 2H), 4.37~4.32 (m, 3H), 4.15~3.28 (m, 64H), 3.15 (t, J = 9.2 Hz, 1H), 2.58~2.53 (m, 2H), 1.98~1.91 (m, 18H), 1.61 (t, J = 12.4 Hz, 1H), 1.60 (t, J = 12.0 Hz, 1H).
2,6-NSCT		δ 5.19 (d, *J* = 3.1 Hz, 1H), 5.11 (s, 1H), 4.93 (s, 1H), 4.76 (s, 1H), 4.58 (d, *J* = 7.9 Hz, 2H), 4.42 (d, *J* = 7.8 Hz, 2H), 4.24-4.23 (m, 1H), 4.18 (d, *J* = 2.6 Hz, 1H), 4.10 (d, *J* = 2.0 Hz, 1H), 3.41-4.02 (m, 59H), 2.64 (dd, *J* = 12.4, 4.3 Hz, 2H), 2.04~2.01 (s, 15H), 1.70 (t, *J* = 12.0 Hz, 2H).
2,3-NSCT		δ 5.33 (d, *J* = 2.8 z, 1H), 5.27(s, 1 H), 5.08 (s, 1H), 4.92 (d, 1H), 4.67 (d, *J* = 7.8 Hz, 2H),4.38 (m, 1H), 4.32 (d, *J* = 3.2 Hz, 1H), 4.23 (m, 3H), 4.15-3.55 (m, 59H), 2.86 (dd, *J* = 12.4, 4.4 Hz, 2H), 2.04~2.01 (m, 15H), 1.92 (t, *J* = 12.0 Hz, 2H).

Table S2. ^{1}H NMR of oxazoline glycans

Code	^{1}H NMR (400 MHz, D_2O)
2,6-NSCT-ox	δ 6.04 (d, *J* = 7.2 Hz, 1H), 5.08 (s, 1H), 4.91 (s, 1H), 4.70 (s, 1H), 4.51-4.61 (m, 2H), 4.39 (d, *J* = 7.8 Hz, 2H), 4.35 (dd, *J* = 2.8, 1.8 Hz, 1H), 4.09-4.20 (m, 4H), 3.41-3.99 (m, 56H), 3.37 (m, 1H), 2.61 (dd, *J* = 12.7, 3.5 Hz, 2H), 2.02 (s, 3H), 2.01 (s, 6H), 1.98 (s, 6H), 1.67 (t, *J* = 12.1 Hz, 2H)
2,3-NSCT-ox	δ 6.10 (d, *J* = 7.2 Hz, 1H), 5.12 (s, 1H), 4.95 (s, 1H), 4.53-4.65 (m, 4H), 4.4 (m, 1H), 4.32-3.4 (m, 62 H), 2.76 (dd, *J* = 12.8, 4.8 Hz, 2H), 2.01-2.13 (m, 15H), 1.82 (t, *J* = 12.1 Hz, 2H)
SG1-ox	δ 6.08 (d, J = 7.2 Hz, 1H), 5.02 (s, 1H), 4.94 (s, 1H), 4.74 (s, 1H), 4.61~4.56 (m, 2H), 4.47 (d, J = 1.2 Hz, 1H), 4.45 (d, J = 1.6 Hz, 1H), 4.38 (dd, J = 1.6, 3.2 Hz, 1H), 4.20~4.15 (m, 4H), 4.01~3.39 (m, 43H), 2.06~2.04 (m, 9H)
SG2-ox	δ 6.07 (d, J = 7.2 Hz, 1H), 5.07 (d, J = 1.2 Hz, 1H), 4.93 (d, J = 1.2 Hz, 1H), 4.73 (s, 1H), 4.58 (d, J = 7.6 Hz, 1H), 4.45 (d, J = 7.6 Hz, 1H), 4.37 (dd, J = 2.0, 3.2 Hz, 1H), 4.20~3.47 (m, 34H), 3.41 (ddd, J = 2.4, 6.0, 8.8 Hz, 1H), 2.05 (d, J = 1.6 Hz, 3H), 2.04 (s, 3H)
SG4-ox	δ 6.06 (d, J = 7.2 Hz, 1H), 5.09 (s, 1H), 4.93 (d, J = 1.2 Hz, 1H), 4.73 (s, 1H), 4.56 (d, J = 8.4 Hz, 1H), 4.54 (d, J = 8.4 Hz, 1H), 4.37 (dd, J = 2.0, 2.8 Hz, 1H), 4.19~4.13 (m, 3H), 4.00~3.39 (m, 32 H), 2.05~2.04 (m, 9H)
SG5-ox	δ 6.06 (d, J = 7.2 Hz, 1H), 5.09 (s, 1H), 4.91 (s, 1H), 4.72 (s, 1H), 4.56 (t, J = 8.4 Hz, 2H), 4.41 (d, J = 8.0 Hz, 1H), 4.35 (dd, J = 2.0, 3.2 Hz, 1H), 4.16~4.12 (m, 4H), 3.99~3.38 (m, 44H), 2.63 (dd, J = 4.8, 12.4 Hz, 1H), 2.031~2.029 (m, 9H), 1.99 (s, 3H), 1.69 (t, J = 12.0 Hz, 1H)
SG6-ox	δ 6.06 (d, J = 7.2 Hz, 1H), 5.08 (s, 1H), 4.91 (d, J = 1.2 Hz, 1H), 4.71 (s, 1H), 4.56 (s, 1H), 4.54 (s, 1H), 4.44 (d, J = 8.0 Hz, 1H), 4.36 (dd, J = 2.0, 3.2 Hz, 1H), 4.16~4.12 (m, 4H), 3.97~3.39 (m, 37H), 2.04~2.02 (m, 9H)
SG7-ox	δ 6.06 (d, J = 7.2 Hz, 1H), 5.06 (d, J = 1.2 Hz, 1H), 4.92 (d, J = 1.6 Hz, 1H), 4.72 (s, 1H), 4.27 (dd, J = 1.6, 3.2 Hz, 1H), 4.17~4.16 (m, 1H), 4.12 (d, J = 3.2 Hz, 1H), 4.04 (dd, J = 2.0, 3.6 Hz, 1H), 3.99 (dd, J = 2.0, 3.6 Hz, 1H), 3.97~3.54 (m, 18H), 3.38 (ddd, J = 2.4, 6.0, 8.8 Hz, 1H), 2.04 (d, J = 1.6 Hz, 3H)
SG8-ox	δ 6.06 (d, J = 7.2 Hz, 1H), 5.08 (d, J = 0.4 Hz, 1H), 4.92 (d, J = 1.6 Hz, 1H), 4.71 (s, 1H), 4.52 (d, J = 8.4 Hz, 1H), 4.36 (dd, J = 1.6, 2.8 Hz, 1H), 4.18~4.15 (m, 2H), 4.12 (d, J = 2.8 Hz, 1H), 3.99 (dd, J = 1.6, 3.2 Hz, 1H), 3.96~3.37 (m, 25H), 2.03 (d, J = 1.6 Hz, 3H), 2.02 (s, 3H)
SG9-ox	δ 6.06 (d, J = 7.2 Hz, 1H), 4.99 (d, J = 1.2 Hz, 1H), 4.96 (d, J = 1.2 Hz, 1H), 4.67 (s, 1H), 4.54 (dd, J = 8.4 Hz, 1H), 4.51 (d, J = 8.4 Hz, 1H), 4.44 (d, J = 8.4 Hz, 1H), 4.29 (t, J = 2.8 Hz, 1H), 4.21 (dd, J = 1.6, 2.8 Hz, 1H), 4.16~4.15 (m, 2H), 4.07 (d, J = 3.2 Hz, 1H), 4.01~3.37 (m, 37 H), 2.04~2.03 (m, 12H)

Table S3. Transglycosylation conditions and yields with mono-GlcNAc Rituximab as acceptor.

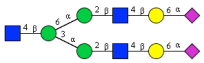
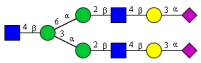
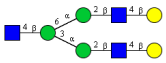
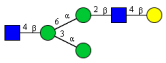
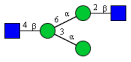
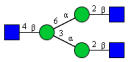
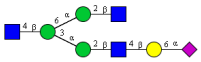
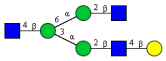
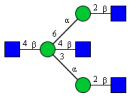
Structure of oxazoline sugars	EndoS mutant	Reaction time	Reaction temperature	Amount of Target Glycan form in LC/MS analysis	Recovery Yield after two chromatography purification
	D233Q	90mins	37 ℃	87.3%	54.6%
	D233Q	90mins	37 ℃	82.78%	82%
	D233Q	90mins	37 ℃	88.7%	58.2%
	D233Q	90mins	30 ℃	84.6%	56.5%
	D233Q	120mins	30 ℃	81.3%	51.1%
	D233Q	75mins	30 ℃	97.7%	49.9%
	D233Q	60mins	37 ℃	86.1%	42.7%
	D233Q	90mins	30 ℃	88.2%	44.2%
	D233Q	150mins	37 ℃	72.7%	48.9%
	D233Q	90min	37 ℃	69.3%	50.2%
	D233Q	120min	30 ℃	94.5%	53.8%

Table S4. Transglycosylation conditions and yields with N-Herceptin as acceptor.

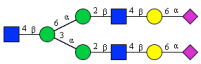
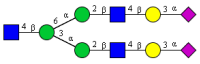
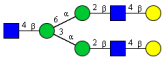
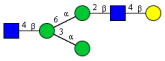
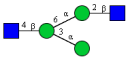
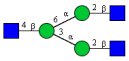
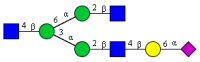
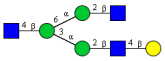
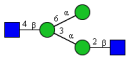
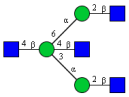
Structure of oxazoline sugars	EndoS mutant	Reaction time	Reaction temperature	Amount of Target Glycan form in LC/MS analysis TKPREEQYNSTYR	Amount of Target Glycan form in LC/MS analysis EEQYNSTYR	Recovery Yield after two chromatography purification
	D233Q	0.5hr	30℃	100%	80.7%	56.2%
	D233Q	90mins	37℃	85.8%	45.9%	85%
	D233A	40min	r.t.	97.3%	94.1%	35.4%
	D233A	0.5hr	r.t.	100%	94.9%	37.2%
	D233A	0.5hr	r.t.	100%	93.1%	65.8%
	D233A	40min	r.t.	100%	96.2%	80.6%
	D233Q	0.5hr	r.t.	100%	83.3%	77.1%
	D233Q	0.5hr	r.t.	100%	94.5%	52.0%
	D233Q	1hr	r.t.	98.7%	96.1%	77.4%
	D233Q	1hr	r.t.	100%	96.4%	59.1%
	D233Q	0.5hr	r.t.	100%	100%	52.1%

Table S5. Binding affinity of glycoengineered Rituximab IgG1 to FcγRIIIa measured by surface plasma resonance analysis. Analyzed antibodies were captured by the Human Fab capture kit and detected with the single cycle kinetic method.

Sample	ka (1/Ms)	kd (1/s)	KD (M)	Rmax (RU)	Fold
Rituximab	2.07E+05	0.03344	1.62E-07	49.29	1-fold
2,6-NSCT	6.86E+05	0.005681	8.28E-09	90.48	19.6-fold
G1	6.55E+05	0.006116	9.33E-09	93.4	17.4-fold
G7	2.22E+05	0.01391	6.27E-08	56.28	2.6-fold
G4	3.56E+05	0.01338	3.75E-08	67.01	4.3-fold
G9	2.67E+05	0.006993	2.62E-08	76.02	6.2-fold
G3	2.39E+05	0.01996	8.36E-08	51.03	1.9-fold
G8	4.44E+05	0.05322	1.20E-07	38.43	1.4-fold
G2	3.25E+05	0.004263	1.31E-08	72.12	12.4-fold
G6	3.67E+05	0.01	2.72E-08	70.8	6.0-fold
G5	3.33E+05	0.006284	1.89E-08	67.52	8.6-fold

*The fold number was calculated with the K_D value of the commercial Rituximab divided by the K_D value of the glycoengineered Rituximab

Table S6. Binding affinity of glycoengineered Herceptin IgG1 to FcγRIIIa using surface plasma resonance analysis. Analyzed antibodies were captured by the $F(ab')_2$ fragment of goat anti-human $F(ab')_2$ and detected by the single cycle kinetic method with double referencing. Data shown are represents of 2 replicates.

Sample	ka (1/Ms)	kd (1/s)	KD (M)	Rmax (RU)	Fold
Herceptin	1.45E+05	0.0131	9.09E-08	30.01	1-fold
2,6-NSCT	2.14E+05	0.00209	9.76E-09	44.98	9.3-fold
G1	2.04E+05	0.00192	9.37E-09	55.68	9.7-fold
G7	1.68E+05	0.0071	4.22E-08	41.54	2.2-fold
G4	1.59E+05	0.00447	2.81E-08	53.98	3.2-fold
G9	1.74E+05	0.00406	2.33E-08	39.88	3.9-fold
G3	1.61E+05	0.00498	3.08E-08	48.19	3.0-fold
G8	2.03E+05	0.0156	7.68E-08	18.15	1.2-fold
G2	2.15E+05	0.00207	9.61E-09	70.48	9.5-fold
G6	1.23E+05	0.00465	2.72E-08	52.82	2.4-fold
G5	1.67E+05	0.00318	1.89E-08	59.89	4.8-fold
2,3-NSCT	1.83E+05	0.00473	2.58E-08	26.95	3.5-fold

*The fold number was calculated with the K_D value of the commercial Herceptin divided by the K_D value of the glycoengineered Herceptin

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2. Collin M & Olsen A (2001) EndoS, a novel secreted protein from Streptococcus pyogenes with endoglycosidase activity on human IgG. *EMBO J* 20(12):3046-3055.
3. Huang W, Giddens J, Fan S-Q, Toonstra C, & Wang L-X (2012) Chemoenzymatic Glycoengineering of Intact IgG Antibodies for Gain of Functions. *J Am Chem Soc* 134(29):12308-12318.