Supporting Information

Site-selective chemoenzymatic glycoengineering of Fab and Fc glycans of a therapeutic antibody

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SI Materials, Methods, and Figures

Materials. Phosphate buffered saline (PBS) and isopropyl β-D-1-thiogalactopyranoside (IPTG) were supplied from Quality Biological, Inc (Gaithersburg, MD). Tris buffer, mini-PROTEAN cast gels, and Tris glycine SDS buffer were supplied from Bio-Rad (Hercules, CA). Sodium chloride, sodium citrate, imidazole, anthranilic acid (2-AA), dimethyl sulfoxide (DMSO), sodium cyanoborohydride, acetonitrile, trifluoracetic acid, and citric acid were purchased from Sigma-Aldrich (St. Louis, MO). PNGase F, NdeI and BamHI were purchased from New England Biolabs (Ipswich, MA). HiTrap MabSelect SuRe column (1 mL), DEAE-cellulose column (1 mL), HisTrap HP column (5 mL), Series S Sensor Chip CM5, and HBS-EP+ Buffer 10x (100 mM HEPES, 1.5M NaCl, 3 mM EDTA, and 0.5% v/v Surfactant P20 (Tween 20) were purchased from GE Healthcare Life Sciences (Amersham, UK). BL21 (DE3) competent cells, B-Per bacterial protein extraction reagent, Tris (2-carboxyethyl) phosphine hydrochloride (TCEP), Hypersep Hypercarb SPE Porous Graphitic Carbon (PGC) column and immobilized papain were purchased from Thermo Fisher Scientific (Waltham, MA). Oasis HLB cartridge was obtained from Waters Corporation (Milford, MA). Poroshell 300SB-C8 column was obtained from Agilent Technologies (Santa Clara, CA). Recombinant human FcγRIIIa (CD16a 176 Val) was purchased from Sino Biological (Beijing, China).

Expression and purification of the α-L-fucosidase (AlfC) from *Lactobacillus casei.* The AlfC gene (GenBank CAQ67984.1) was codon optimized for *E. coli* expression and synthesized by Genscript (Piscataway Township, NJ). The plasmid includes restriction sites NdeI and BamHI at the 5' and 3' ends, respectively, for subcloning into the expression plasmid pCPD-Lasso (1). AlfC-CPD-L was transformed into BL21 (DE3) competent cells plated on LB + Amp plates. Protein was expressed at 16 ºC overnight after induction with 0.2 mM IPTG. Cells were pelleted at 7855 x *g* and lysed with B-PER protein extraction solution. Protein was purified by immobilized metal affinity chromatography (IMAC) over a 5 mL nickel column. Column was washed with 10 CV of PBS, pH 7.4 with 20 mM imidazole then eluted at 150 mM imidazole in PBS. Fractions of AlfC were combined and buffer was exchanged into PBS. Protein fractions were concentrated 20 mg/mL and stored at -80 ºC. The enzyme activity was confirmed by assaying with p-nitrophenyl-α-L-fucopyranoside following the previously reported procedures (2).

Expression and purification of IdeS from *Streptococcus pyogenes.* The IdeS gene sequence corresponding to the amino acid residues 30-341 (GenBank JN035367.1) was codon optimized for *E. coli*

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expression and synthesized by Genscript (Piscataway Township, NJ). The plasmid includes restriction sites NdeI and BamHI at the 5' and 3' ends, respectively, used for subcloning into the expression plasmid pCPD-Lasso. IdeS-CPD was transformed into BL21 (DE3) competent cells plated on LB + Amp plates. Protein was expressed at 16 ºC overnight after induction with 0.2 mM IPTG. Cells were pelleted at 7855 x *g* and lysed with B-PER protein extraction solution. Protein was purified by immobilized metal affinity chromatography (IMAC) over a 5 mL nickel column. Column was washed with 10 CV of PBS, pH 7.4 with 20 mM imidazole then eluted at 150 mM imidazole in PBS. Fractions of IdeS were combined and buffer was exchanged into PBS. Protein fractions were concentrated 10 mg/mL and stored at -80 ºC. The enzyme activity was confirmed by assaying a rituximab IgG standard followed by LC-MS and SDS-PAGE.

LC-MS analysis of cetuximab glycoforms. LC-MS analyses was performed on an Ultimate 3000 HPLC system coupled to an Exactive Plus Orbitrap mass spectrometer (Thermo Fischer Scientific). For light chain and heavy chain analysis, 2 µg of cetuximab was treated with 50 mM TCEP at 37 ºC for 30 min to disconnect the heavy and light chains. Reduced IgG samples were analyzed at 60 ºC running a linear gradient of 25 - 35% ACN in a 0.1% formic acid solution for 8 min at 40 µL/min using an Agilent Poroshell 300SB-C8 column (5 µm, 1.0 x 75 mm).

For IdeS analysis, 3 µg of cetuximab was treated with recombinant IdeS at 37 ºC for 1 hou, following the previously described procedure (3, 4). After cleavage, 50 mM TCEP was added to perform reduction to give Fc/2, Fd and light chain subunits of cetuximab. IdeS-generated IgG subunits were analyzed at 60 ºC running a linear gradient of 15 - 45% ACN in a 0.1% formic acid solution for 30 min at 400 µL/min using a BEH300 C4 column (Waters).

Raw data was deconvoluted using MagTran (Amgen). The molecular mass of cetuximab was calculated using the amino acid sequence found in the literature and included below. For calculation of the heavy chain mass the following modifications were assumed: N-terminal pyroglutamic acid formation, incomplete C-terminal lysine cleavage (the major product having complete cleavage of lysine and the minor product with the lysine intact), two N-glycosylation sites (N-88 and N-300), complete reduction of 2 interchain disulfide bonds, and incomplete reduction of intrachain bonds. For calculation of the light chain the following modifications were assumed: complete reduction of interchain disulfide bond and incomplete reduction of intrachain disulfide bonds.

Sequence of cetuximab heavy chain

QVQLKQSGPGLVQPSQSLSITCTVSGFSLTNYGVHWVRQSPGKGLEWLGVIWSGGNTDYNTPFTSRLSIN
KDNSKSQVFFKMNSLQS**N**DTAIYYCARALTYYDYEFAYWGQGTLVTVSAASTKGPSVFPLAPSSKSTSGG
TAALGCLVKDYFPEPVTVSWNSGALTSGVHTFPAVLQSSGLYSLSSVVTVPSSSLGTQTYICNVNHKPSN
TKVDKRVEPKSCDKTHTCPPCPAPELLGGPSVFLFPPKPKDTLMISRTPEVTCVVVDVSHEDPEVKFNWY
VDGVEVHNAKTKPREEQY**N**STYRVVSVLTVLHQDWLNGKEYKCKVSNKALPAPIEKTISKAKGQPREPQV
YTLPPSREEMTKNQVSLTCLVKGFYPSDIAVEWESNGQPENNYKTTPPVLDSDGSFFLYSKLTVDKSRWQ
QGNVFSCSVMHEALHNHYTQKSLSLSPG<u>K</u>

The commercial cetuximab has two C-terminal lysine (underscored) variants: one carrying a C-terminal lysine residue and the other with C-terminal lysine truncation; the N-glycan sites Asn-88 and Asn-300 are bolded.

Sequence of cetuximab light chain

DILLTQSPVILSVSPGERVSFSCRASQSIGTNIHWYQQRTNGSPRLLIKYASESISGIPSRFSGSGSGTD
FTLSINSVESEDIADYYCQQNNNWPTTFGAGTKLELKRTVAAPSVFIFPPSDEQLKSGTASVVCLLNNFY
PREAKVQWKVDNALQSGNSQESVTEQDSKDSTYSLSSTLTLSKADYEKHKVYACEVTHQGLSSPVTKSFN
RGEC

Preparation of globally deglycosylated cetuximab (1). A solution of cetuximab (3 mg) in PBS (pH 7.4, 2mL) was treated with Endo-F3 WT (100 µg) and Endo-S2 WT (30 µg) at 37 °C for 6 h. LC-MS and SDS-PAGE

analysis were used to confirm glycan removal. Deglycosylated cetuximab was purified from the reaction mixture using a protein A column (1 mL) pre-equilibrated in PBS. Bound cetuximab was eluted using a citric acid solution (100 mM, pH 3.2) and quickly neutralized with 1.5 M tris buffer (pH 8.8). Eluted material underwent buffer exchange to a citrate solution (10 mM, pH 5.6, 150 mM NaCl) to give the deglycosylated cetuximab glycoform (**1**). LC-MS of the heavy chain of **1**: calculated for the heavy chain minus lysine, M = 49925 Da; found, 49919 Da (deconvoluted data) and calculated for the heavy chain with lysine, M=50053 Da; found, 50050 Da (deconvoluted data).

Preparation of fully glycosylated cetuximab glycoform (3). The deglycosylated cetuximab (**1**) (1 mg) was dissolved in a tris solution (100 mM, pH 7.4, 100 µL). Endo-F3 D165A was then added to the reaction along with SCTox (**2**) (1.5 mg). Reaction was incubated at 37 ºC for 1 h. Another 500 µg of SCTox (**2**) was added and left to incubate for another 45 min at 37 ºC to push the reaction towards completion. LC-MS and SDS-PAGE analyses were used to confirm glycan transfer. Remodeled cetuximab (**3**) was purified from the reaction mixture using a protein A column (1 mL) pre-equilibrated in PBS. Bound cetuximab was eluted using a citric acid solution (100 mM, pH 3.2) and quickly neutralized with 1.5 M tris pH 8.8 buffer. Eluted material underwent buffer exchange to a citrate solution (10 mM, pH 5.6, 150 mM NaCl) to give the remodeled cetuximab glycoform (**3**). LC-MS of the heavy chain of **3**: calculated for the heavy chain minus lysine, M = 53928 Da; found, 53922 Da (deconvoluted data) and calculated for the heavy chain with lysine, M=54056 Da; found: 54050 Da (deconvoluted data).

Preparation of Fc selectively glycosylated cetuximab (4). The deglycosylated cetuximab (**1**) (1 mg) was dissolved in a tris solution (100 mM, pH 7.4, 100µL). Endo-S D233A was then added to the reaction along with SCTox (**2**) (1 mg). Reaction was incubated at 37 ºC for 1 hr. LC-MS and SDS-PAGE analyses were used to confirm glycan transfer. Remodeled cetuximab (**4**) was purified from the reaction mixture using a protein A column (1 mL) pre-equilibrated in PBS. Bound cetuximab was eluted using a citric acid solution (100 mM, pH 3.2) and quickly neutralized with 1.5 M tris pH 8.8 buffer. Eluted material underwent buffer exchange to a citrate solution (10 mM, pH 5.6, 150 mM NaCl) to give the remodeled cetuximab glycoform (**4**). LC-MS of the heavy chain of **4**: calculated for the heavy chain minus lysine, M = 51927 Da; found: 51921 Da (deconvoluted data) and calculated for the heavy chain with lysine, M=52055 Da; found: 52051 Da (deconvoluted data). Further analysis to confirm the site of SCT transfer was done by treating **4** with IdeS and then reduction with 50 mM TCEP. LC-MS of the Fc fragment of **4**: calculated for the Fc fragment minus lysine, M=26142 Da; found: 26137 Da (deconvoluted data) and calculated for the Fc fragment with lysine, M=26270 Da; found: 26269 Da (deconvoluted data). LC-MS of the Fd fragment of **4**: calculated for the Fd fragment, M=25804 Da; found: 25796 Da (deconvoluted data). LC-MS of the LC of **4**: calculated for the LC, M=23428 Da; found: 23422 Da (deconvoluted data).

Preparation of globally deglycosylated and Fc defucosylated cetuximab (5). A solution of the Endo-F3 deglycosylated cetuximab (**1**) (3 mg) was treated with the α-fucosidase (AlfC) (300 µg) in PBS (pH 7.4, 2 mL) at 37 ºC overnight. LC-MS and SDS-PAGE analyses were used to confirm glycan removal. Deglycosylated cetuximab was purified from the reaction mixture using a protein A column (1 mL) pre-equilibrated in PBS. Bound cetuximab was eluted using a citric acid solution (100 mM, pH 3.2) and quickly neutralized with 1.5 M tris pH 8.8 buffer. Eluted material underwent buffer exchange to a citrate solution (10 mM, pH 5.6, 150 mM NaCl) to give the selectively de-fucosylated glycoform (**5**). LC-MS of the heavy chain of **5**: calculated for the heavy chain minus lysine, M = 49779 Da; found: 49778 Da (deconvoluted data). Further analysis to confirm the site of defucosylation was done by treating **5** with IdeS and then reduction with 50 mM TCEP. LC-MS of the Fc fragment of **5**: calculated for the Fc fragment minus lysine, M=23995 Da; found: 23988 Da (deconvoluted data) and calculated for the Fc fragment with lysine, M=24123 Da; found: 24118 Da (deconvoluted data). LC-MS of the Fd fragment of **5**: calculated for the Fd fragment, M=25804 Da; found: 25797 Da (deconvoluted data). LC-MS of the LC of **5**: calculated for the LC, M=23428 Da; found: 23422 Da (deconvoluted data).

Preparation of Fab selectively glycosylated cetuximab (7). The deglycosylated cetuximab (**5**) (1 mg) was dissolved in a tris solution (100 mM, pH 7.4, 100 µL). Endo-F3 D165A (40 µg) was then added to the reaction

along with SCTox (**2**) (1 mg). Reaction was incubated at 37 ºC for 45 min. Product formation was monitored using LC-MS. Another 500 µg of SCTox (**2**) was added and left to incubate for another 40 min at 37 ºC to push the reaction towards completion. LC-MS and SDS-PAGE analyses were used to confirm glycan transfer. Remodeled cetuximab (**7**) was purified from the reaction mixture using a protein A column (1 mL) pre-equilibrated in PBS. Bound cetuximab was eluted using a citric acid solution (100 mM, pH 3.2) and quickly neutralized with 1.5 M tris pH 8.8 buffer. Eluted material underwent buffer exchange to a citrate solution (10 mM, pH 5.6, 150 mM NaCl) to give the remodeled cetuximab glycoform (**7**). LC-MS of the heavy chain of **7**: calculated for the heavy chain minus lysine, M = 51781 Da; found, 51774 Da (deconvoluted data) and calculated for the heavy chain with lysine, M=51909 Da; found: 51905 Da (deconvoluted data). Further analysis to confirm the site of SCT transfer was done by treating **7** with IdeS and then reduction with 50 mM TCEP. LC-MS of the Fc fragment of **7**: calculated for the Fc fragment minus lysine, M=23995 Da; found: 23988 Da (deconvoluted data) and calculated for the Fc fragment with lysine, M=24123 Da; found: 24118 Da (deconvoluted data). LC-MS of the Fd fragment of **7**: calculated for the Fd fragment, M=27805 Da; found: 27798 Da (deconvoluted data). LC-MS of the LC of **7**: calculated for the LC, M=23428 Da; found: 23422 Da (deconvoluted data).

Preparation of Fc selectively glycosylated cetuximab (8). The deglycosylated cetuximab (**5**) (1 mg) was dissolved in a tris solution (100 mM, pH 7.4, 100 µL). Endo-S D233A (40 µg) was then added to the reaction along with CTox (**6**) (1 mg). Reaction was incubated at 37 ºC for 1 hr. LC-MS and SDS-PAGE analyses were used to confirm glycan transfer. LC-MS of the heavy chain of **8**: calculated for the heavy chain minus lysine, M = 51198 Da; found, 51190 Da (deconvoluted data) and calculated for the heavy chain with lysine, M=51326 Da; found: 51319 Da (deconvoluted data). Further analysis to confirm the site of CT transfer was done by treating **8** with IdeS and then reduction with 50 mM TCEP. LC-MS of the Fc fragment of **8**: calculated for the Fc fragment minus lysine, M=25414 Da; found: 25409 Da (deconvoluted data) and calculated for the Fc fragment with lysine, M=25542 Da; found: 25537 Da (deconvoluted data). LC-MS of the Fd fragment of **8**: calculated for the Fd fragment, M=25804 Da; found: 25797 Da (deconvoluted data). LC-MS of the LC of **7**: calculated for the LC, M=23428 Da; found: 23423 Da (deconvoluted data).

Preparation of selectively glycoengineered cetuximab (9) from glycoform 7. Fab glycoengineered cetuximab (**7**) (1 mg) was dissolved in a tris solution (100 mM, pH 7.4, 100 µL). Endo-S D233Q (40 µg) was then added to the solution along with CT glycan oxazoline (**6**) (1 mg). Reaction was incubated at 37 ºC for 1 hr. The mixture was purified using a protein A (1 mL) column pre-equilibrated PBS pH 7.4. Bound cetuximab was eluted using a citric acid solution (100 mM, pH 3.2) and quickly neutralized with 1 M tris buffer, pH 8.8. Eluted material buffer was exchanged to a citrate solution (10 mM, pH 5.6, 150 mM NaCl), giving the glycoengineered glycoform (**9**) (1 mg). LC-MS of the heavy chain of **9**: calculated for the heavy chain minus lysine, M = 53200 Da; found, 53195 Da (deconvoluted data); calculated for the heavy chain with lysine, M=53328 Da; found: 53323 Da (deconvoluted data). Further analysis to confirm the location of each glycan was done by treating **9** with IdeS and then with 50 mM TCEP. LC-MS of the Fc fragment of **9**: calculated for the Fc fragment minus lysine, M=25414 Da; found: 25410 Da (deconvoluted data); calculated for the Fc fragment with lysine, M=25542 Da; found: 25538 Da (deconvoluted data). LC-MS of the Fd fragment of **9**: calculated for the Fd fragment, M=27805 Da; found: 27797 Da (deconvoluted data). LC-MS of the LC of **9**: calculated for the LC, M=23428 Da; found: 23422 Da (deconvoluted data).

Preparation of selectively glycoengineered cetuximab (9) from glycoform 8. Fc glycoengineered cetuximab (**8**) (1 mg) was dissolved in a tris solution (100 mM, pH 7.4, 100 µL). Endo-F3 D165A (40 µg) was then added to the solution along with SCT glycan oxazoline (**2**) (1 mg). Reaction was incubated at 37 ºC for 45 min. Another 500 µg of **2** was added and the left to incubated for another 40 min at 37 ºC to push the reaction towards completion. The mixture was purified using a protein A (1 mL) column pre-equilibrated PBS pH 7.4. Bound cetuximab was eluted using a citric acid solution (100 mM, pH 3.2) and quickly neutralized with 1 M tris buffer, pH 8.8. Eluted material buffer was exchanged to a citrate solution (10 mM, pH 5.6, 150 mM NaCl), giving the

glycoengineered glycoform (**9**) (1 mg). the patterns of Fc and Fab glycosylations were verified by LC-MS analysis as demonstrated above, which confirmed the identity and homogeneity of the final product.

Separation of the Fab and Fc domains of cetuximab glycoforms for glycan analysis. Cetuximab (100 µg) was incubated with immobilized papain resin (1 µg) in a phosphate solution (20 mM, pH 7.0, 10 mM EDTA, 20 mM cysteine•HCl, 100 µL) at 37 ºC rotating overnight. Resin was pelleted at 10,600 x *g* removing the supernatant and washed 3 times with 500 µL PBS centrifuging each time. Supernatants were combined and loaded of a protein A column (1 mL). Unbound material containing the Fab fragment underwent buffer exchange into PBS. Fc and undigested cetuximab were eluted using the citric acid solution (pH 3.2) and quickly neutralized with 1.5 tris (pH 8.8) then switched into a 10 mM Tris-HCl solution. Sample was loaded over a 1 mL DEAE-cellulose then washed with 10 CV of 10 mM Tris-HCl solution to remove any undigested cetuximab. Fc portion was eluted using 100 mM NaCl. The isolated Fab and Fc domains were subjected to LC-MS analysis

MALDI-TOF MS analysis of PNGase F released N-glycans. Fc and Fab samples were denatured and reduced following the PNGAse F protocol (NEB) separately and the N-glycans were released using PNGase F at 37 ºC overnight. After release, samples were dried down and resuspended in a 0.1% TFA solution (500 µL). Samples were loaded over a 1 mL SPE PGC (50 mg) prewashed with 10 mL methanol, 10 mL 90% ACN + 0.1% TFA, and 0.1% TFA. Column was washed with 0.1%TFA for 10 mL then glycans were eluted in 20% ACN + 0.1%TFA (3 mL total). Eluted fractions were lyophilized. Released oligosaccharides were treated with 2-AA and sodium cyanoborohydride (3:2) in an acidified DMSO solution for 3 h at 65 ºC. Excess material was removed using an OASIS HLB column (1 mL) under normal phase conditions. Column was pre-equilibrated in 95% ACN. Glycan labeling mixture was diluted 100 fold then loaded over the column and washed with 10 mL of 95% ACN. 2-AA labeled glycan were eluted at 20% ACN. After speedVac drying, the 2-AA labelled glycans were dissolved in water. The samples were mixed 1:1 with 2,5-dihydroxybenzoic acid (DHB) matrix (20 mg/ mL) in water/ ACN (70:30). Samples were spotted on MALDI target plate and let dry. Glycans were analyzed using a Bruker Autoflex II in negative reflectron mode. Laser power was adjusted to 15%.

Surface Plasmon Resonance (SPR) binding assay. Experiments were performed using a Biacore T200 system (GE Healthcare) with analysis temperature set to 25 °C and sample compartment temperature set to 15 °C. FcγRIIIa binding measurements were conducted using capture method via a protein A immobilized CM5 chip. Protein A was immobilized to a level of 6000 RU on a CM5 biosensor chip (GE Healthcare) using amine coupling chemistry at pH 4.5. Both cetuximab and remodeled cetuximab were injected at 10 µL/min over the protein A surface to a capture level of 50 RU. The recombinant FcγRIIIa was buffer exchanged into the running buffer (HBS-EP+ pH 7.4) and flowed over the cetuximab coated protein A CM5 chip at 40 µL/min starting with 1 µM to 1.953 nM. Following the association and dissociation phases of the experiment, the surface of chip was regenerated by a 30 s injection of glycine HCl buffer (10 mM pH 3.0) prior to the next cycle. The assay was repeated and the average Kd value and standard deviation for each were calculated. The resulting sensograms were double reference subtracted and fit to a 1:1 interaction model using the Biacore T200 Evaluation Software.

EGFR binding assay and ADCC assay. The anti-EGFR mAbs (glycoengineered cetuximab **9** and the commercial cetuximab) were used to trigger ADCC in vitro against the A431 cell line, which is an EGFR+ epidermoid carcinoma. The A431 cells were stained with various concentrations of the glycoengineered cetuximab, the commercial cetuximab, and the isotype negative control and washed 3 times before staining with a fluorescently-labeled anti-human IgG secondary antibody (Southern Biotech, Birmingham, AL). The cells were analyzed by flow cytometry analysis (BD LSR-II) and the mean fluorescence intensity (MFI) values were recorded. For ADCC assay, the $EGFR^+$ A431 epidermoid carcinoma cells were labeled with various concentrations of the glycoengineered cetuximab, the commercial cetuximab, and the isotype negative control for 30 min, were washed three times, and 300,000 cells were cultured 1:1 with PBMCs purified from one of four different leukocyte donors (New York Blood Center, New York, NY) for 3.5 h in 96-well U-bottom plates. Surface CD107a expression by $CD56^+CD3^-$ NK cells was assessed by flow cytometry analysis. IRB approval is granted

under Rockefeller IRB registration number IRB00000385. Dose-response curves were generated by plotting the mean % of CD107a+ cells against the antibody concentration. The EC_{50} values were calculated by determining the response halfway between the baseline and maximum response. For comparison, relative activity was calculated by inversely normalizing the EC_{50} value of the glycoengineered cetuximab with that of the control cetuximab using the following formula: Relative Activity = EC_{50} Cetuximab Control/ EC_{50} Glycoengineered Cetuximab.

References

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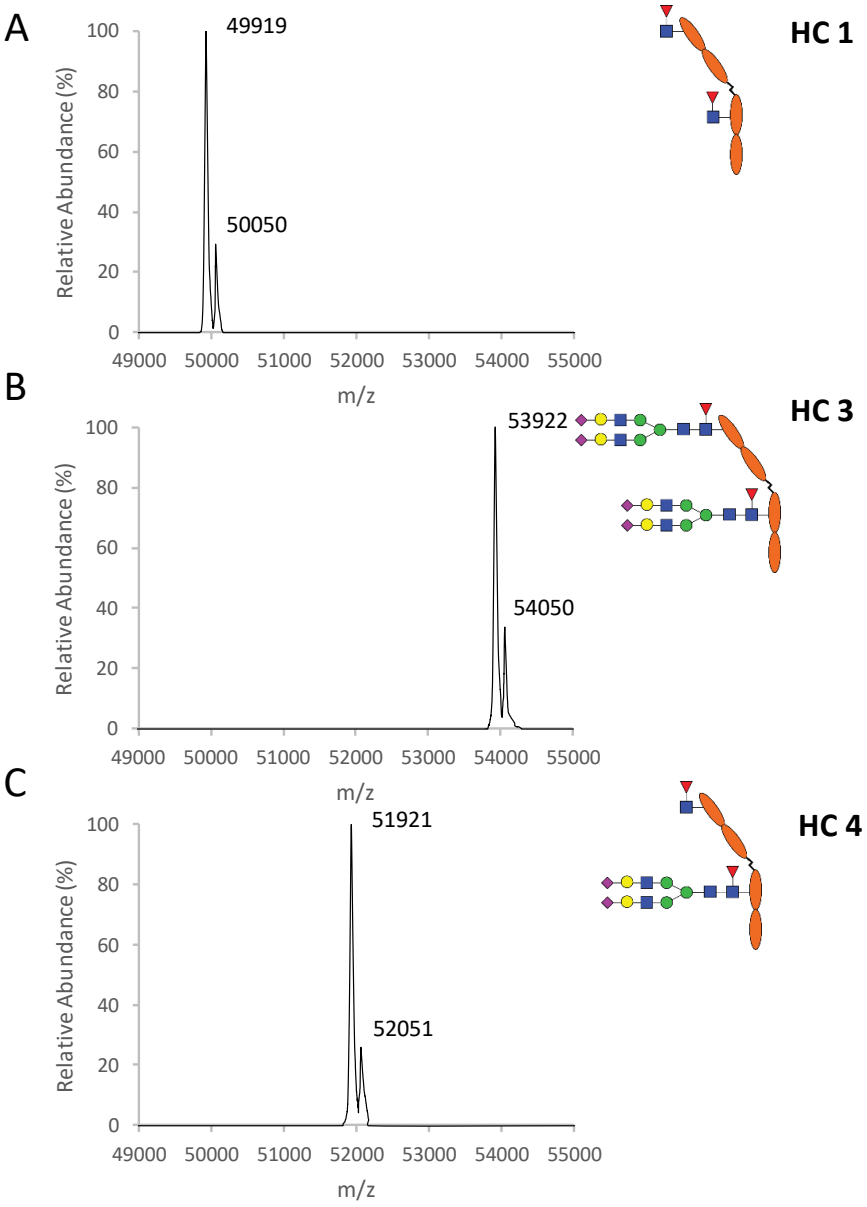

Figure S1. LC-ESI-MS analysis of the glycan remodeled heavy chain of cetuximab. The antibody was reduced and the heavy chain was subjected to ESI-MS analysis. A) the deconvoluted spectrum of heavy chain of **1**; B) heavy chain of **3**; and C) heavy chain of **4.**

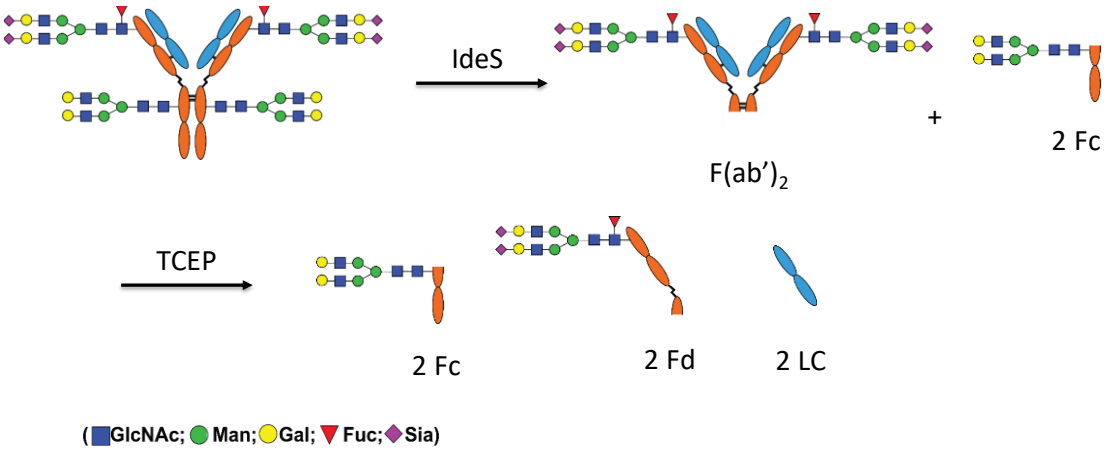

Figure S2. Diagram of IdeS treatment of cetuximab followed by reduction with TCEP to give the Fc, Fd and LC (light chain) fragments

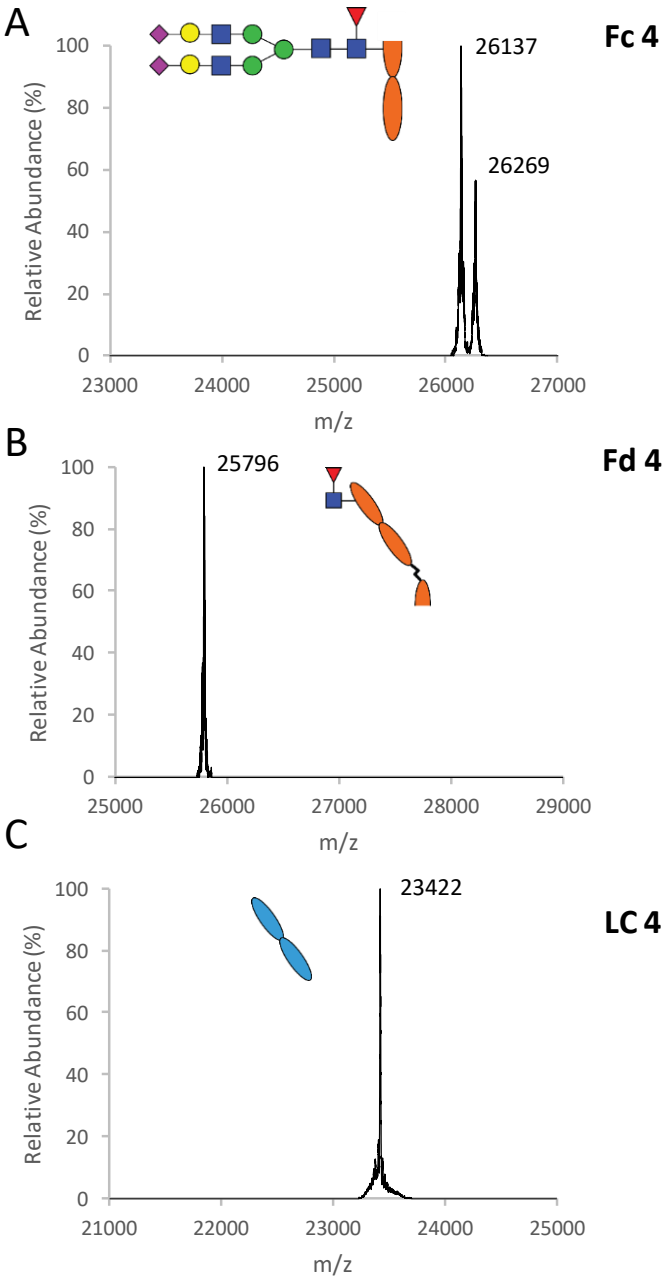

Figure S3. LC-ESI-MS analysis of IdeS produced fragments of EndoS2 remodeled cetuximab. The antibody was treated with IdeS and then reduced with TCEP. A) the deconvoluted spectrum of Fc of **4**; B) Fd of **4**; C) LC of **4.**

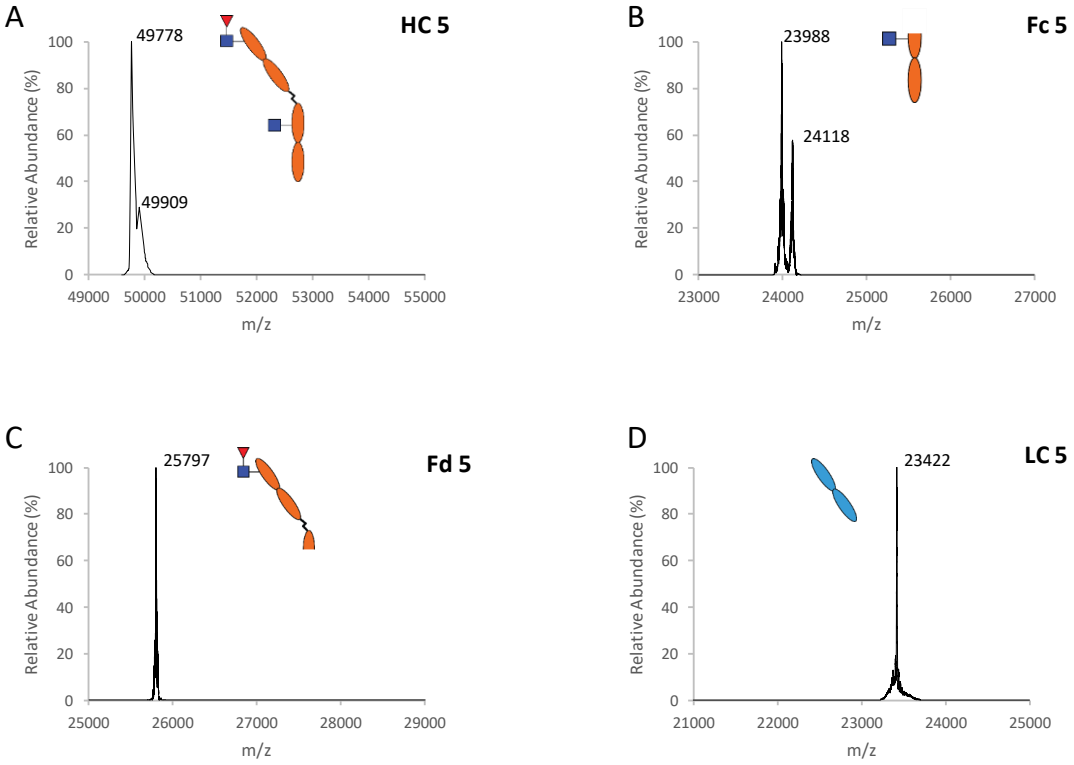

Figure S4. LC-ESI-MS analysis of IdeS produced fragments of cetuximab glycoform **5** (generated by treatment with EndoF3/EndoS2 WT and Alfc (α-fucosidase). A) the deconvoluted spectrum of HC of **5**; B) Fc of **5**; C) Fd of **5**; and D) LC of **5.**

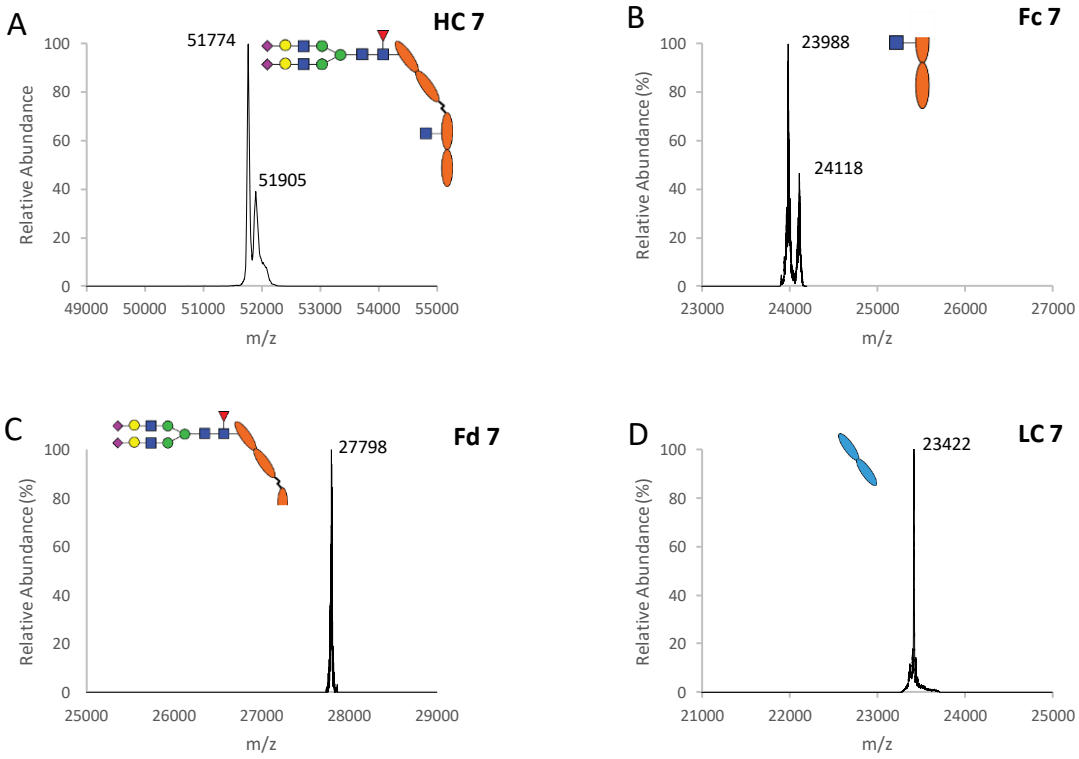

Figure S5. LC-ESI-MS analysis of IdeS produced fragments of 7 generated by the Endo-F3 D165A mediated transfer with SCT glycan oxazoline. A) the deconvoluted spectrum of HC of **7**; B) Fc of **7**; C) Fd of **7**; and D) LC of **7.**

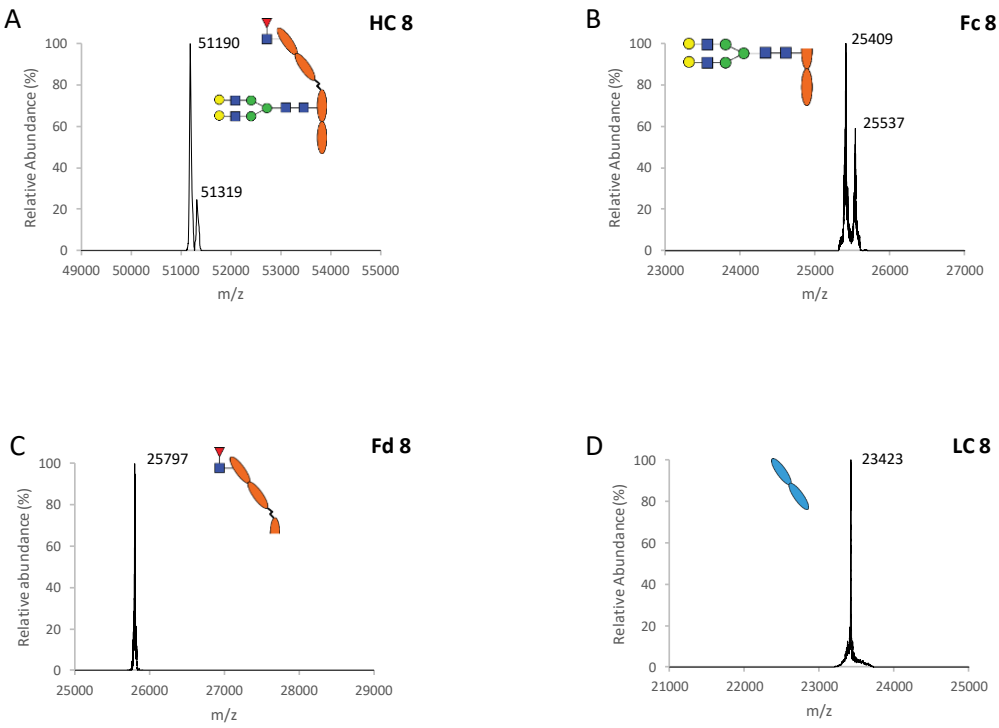

Figure S6. LC-ESI-MS analysis of IdeS produced fragments of **8** generated by Endo-S D233Q mediated transfer with CT glycan oxazoine. A) the deconvoluted spectrum of HC of **8**; B) Fc of **8**; C) Fd of **8**; and D) LC of **8.**