Supporting Information

Site-Specific Chemoenzymatic Conjugation of High-Affinity M6P Glycan Ligands to Antibodies for Targeted Protein Degradation

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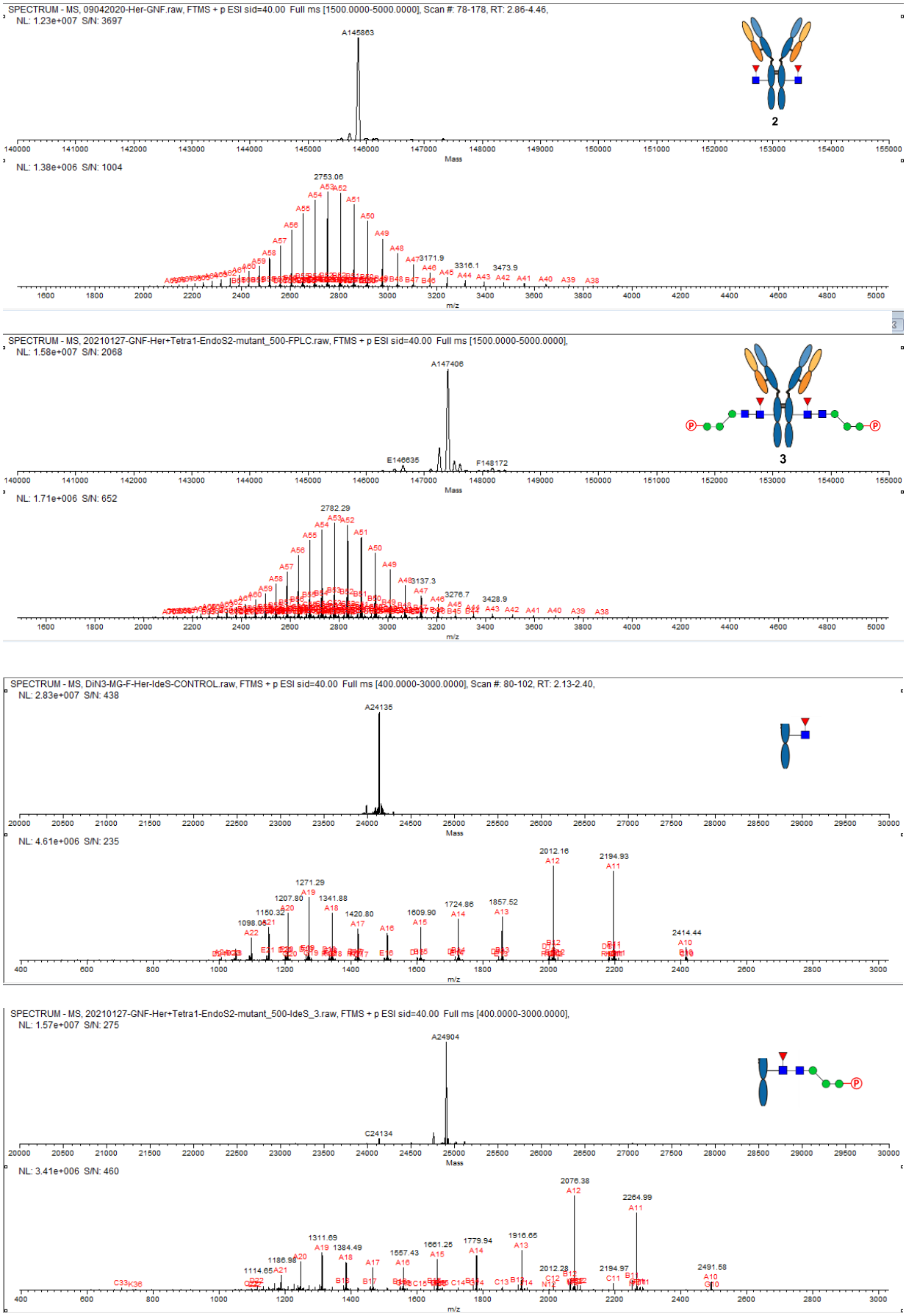


Fig. S1. LC-MS analysis of compound **3**.

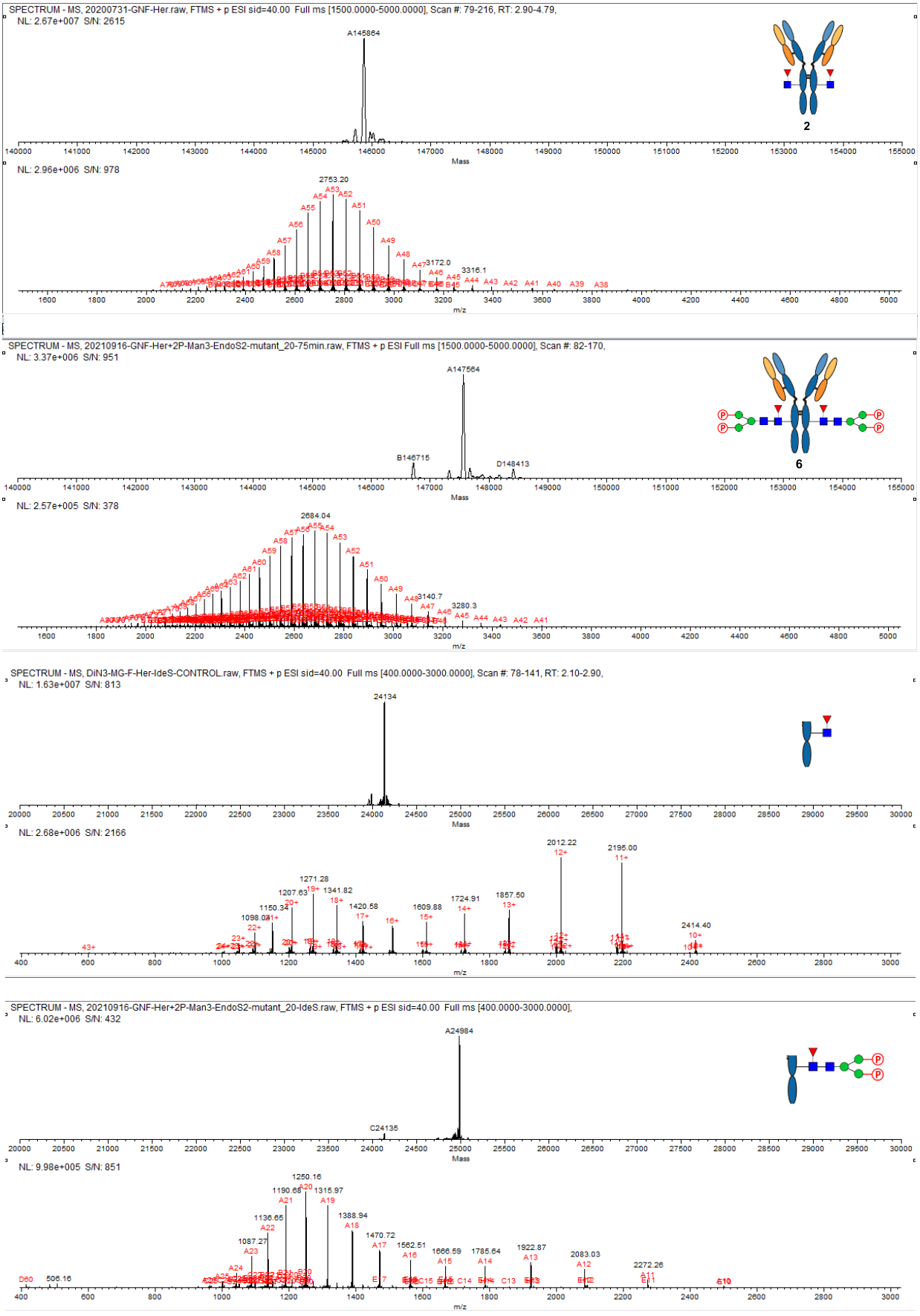


Fig. S2. LC-MS analysis of compound **6**.

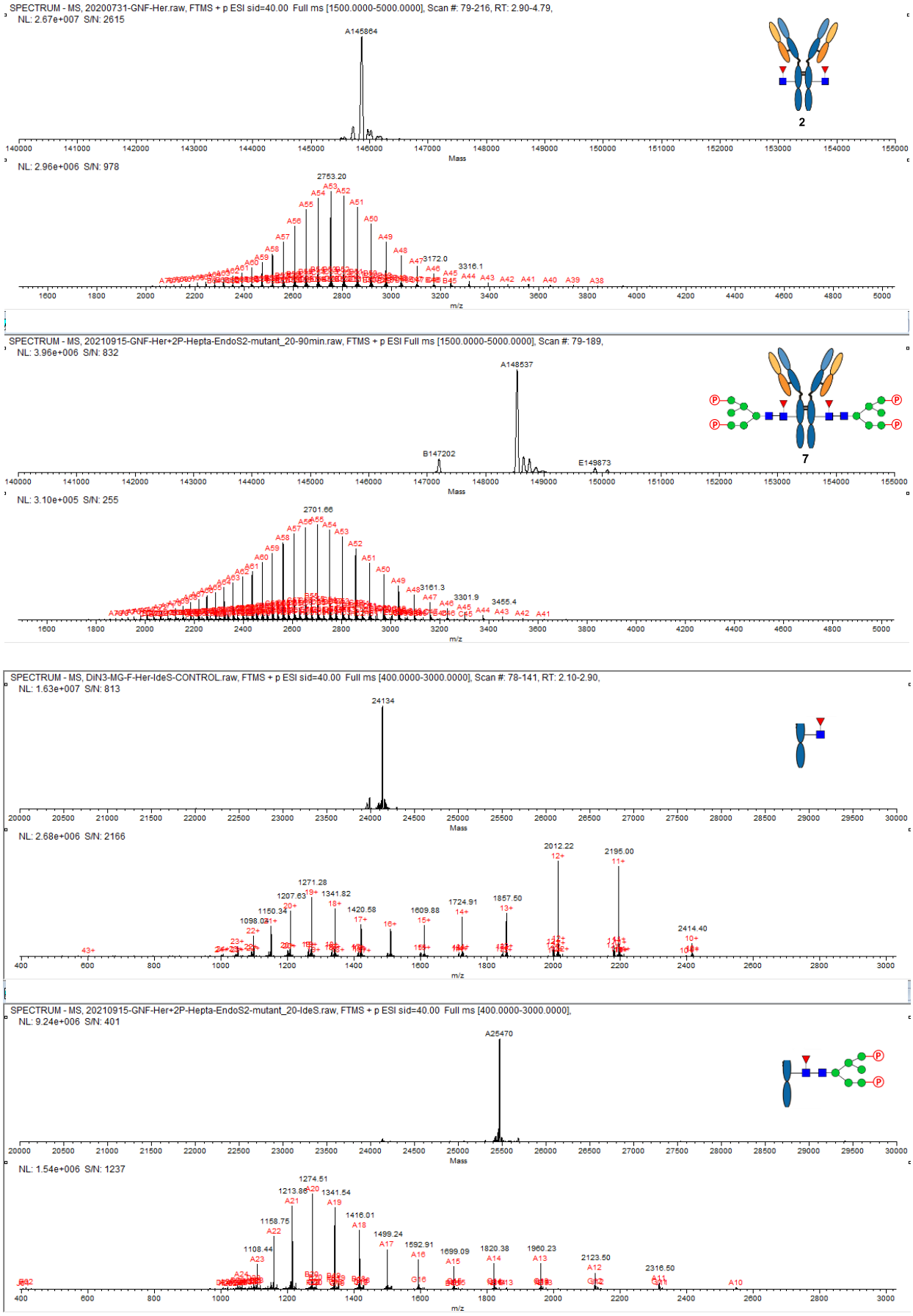


Fig. S3. LC-MS analysis of compound **7**.

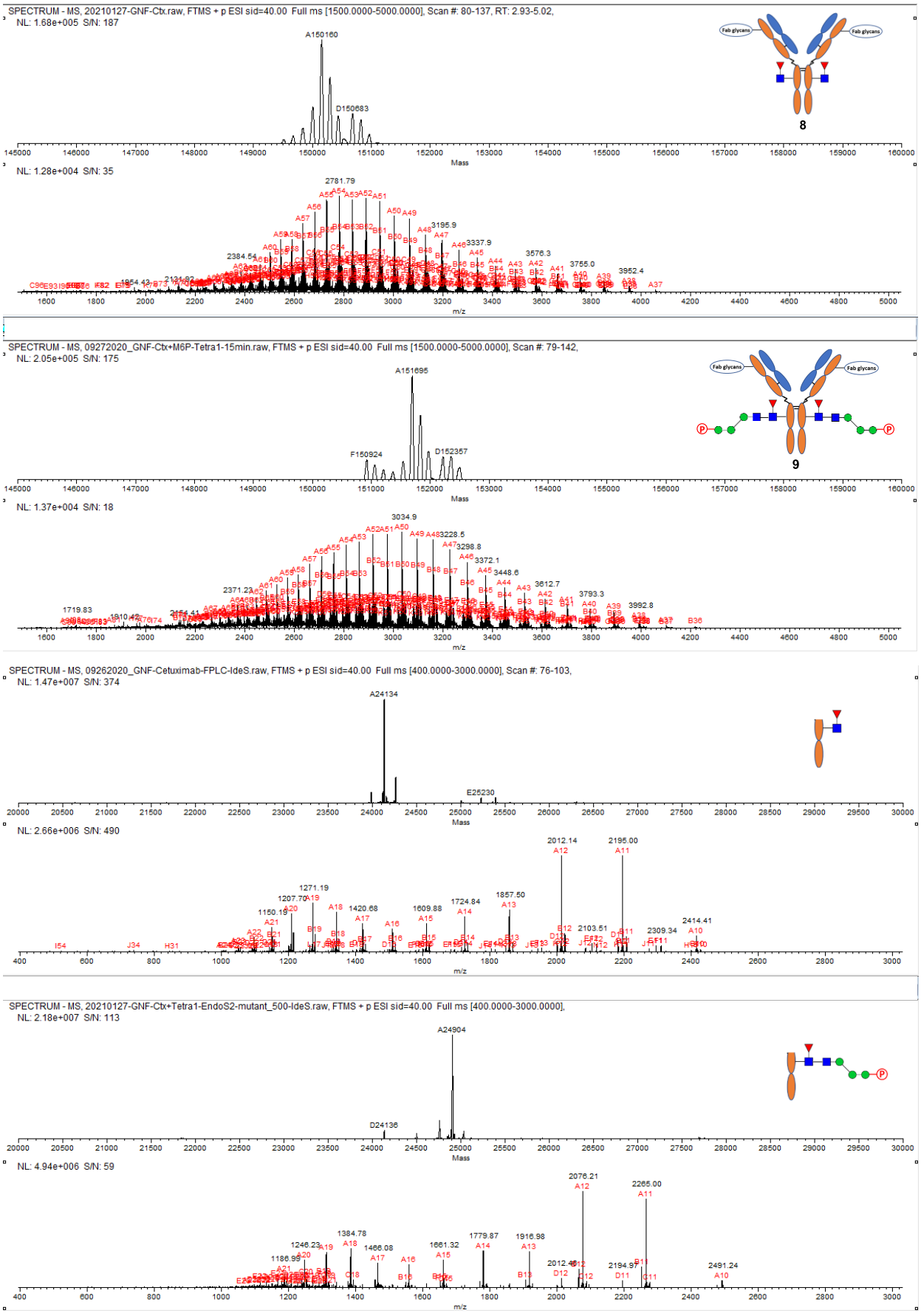


Fig. S4. LC-MS analysis of compound **9**.

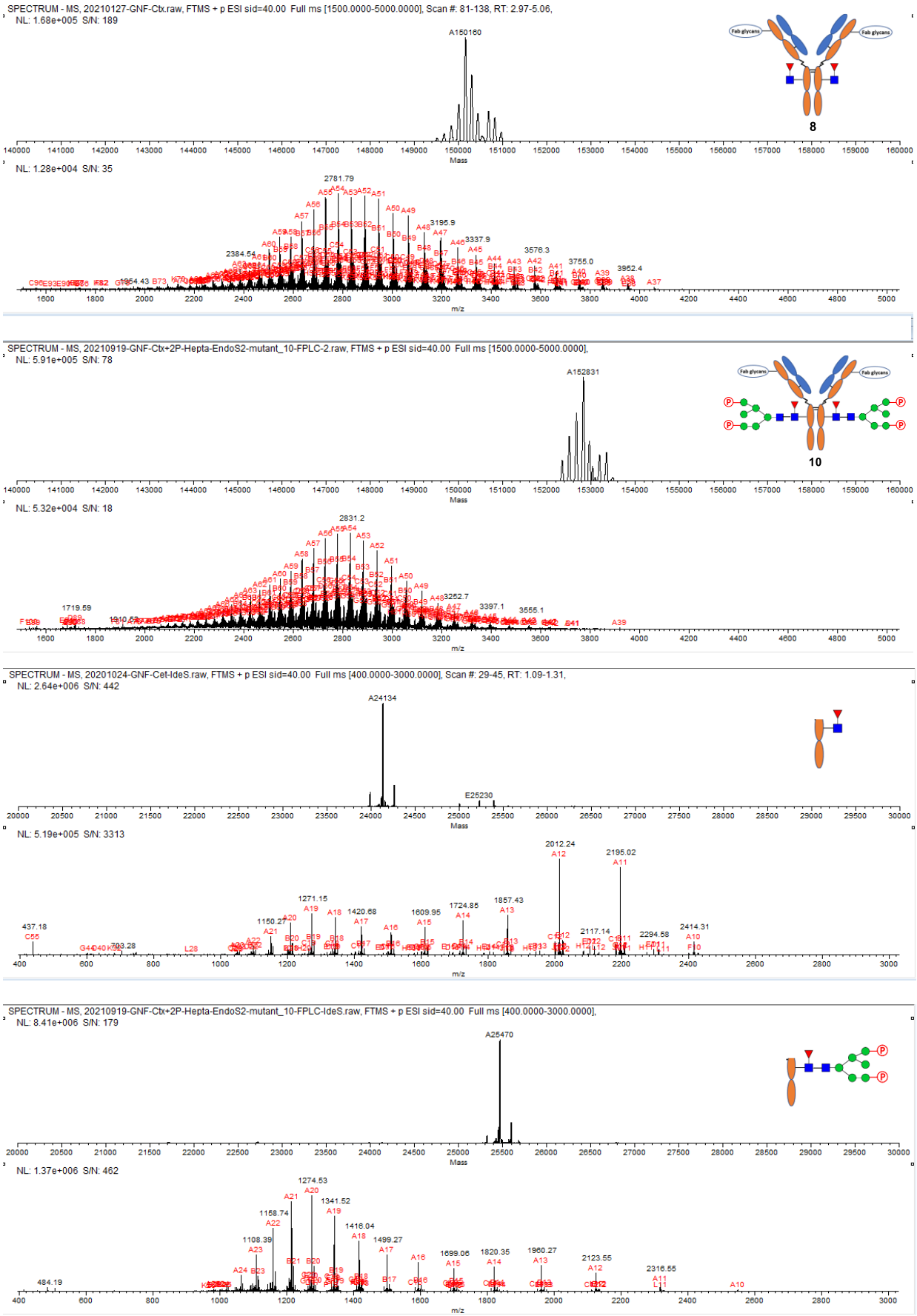
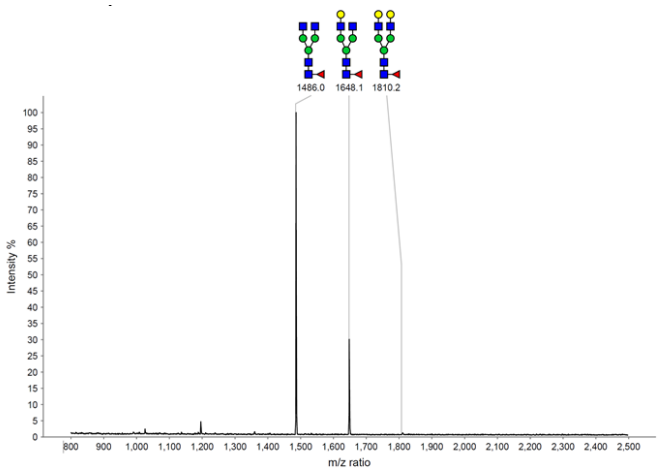
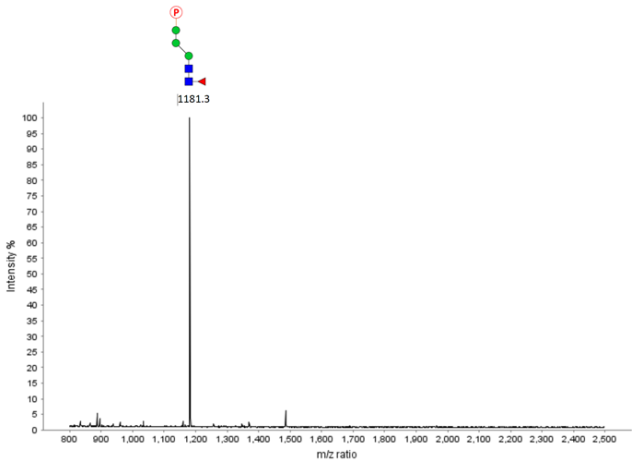


Fig. S5. LC-MS analysis of compound **10**.

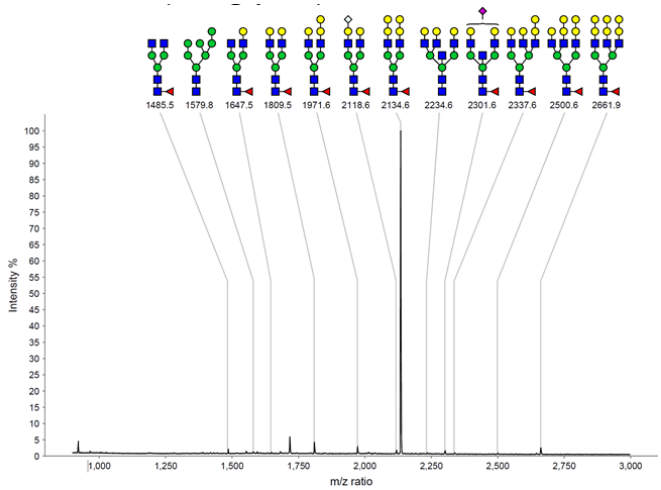
Trastuzumab (Fc glycans):



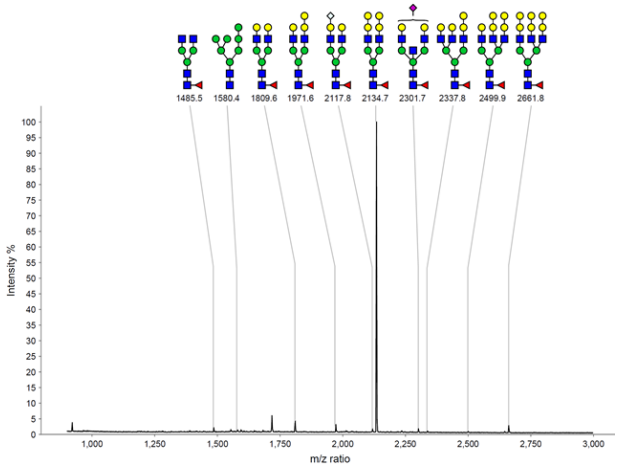
Compound **3** (Fc glycans):



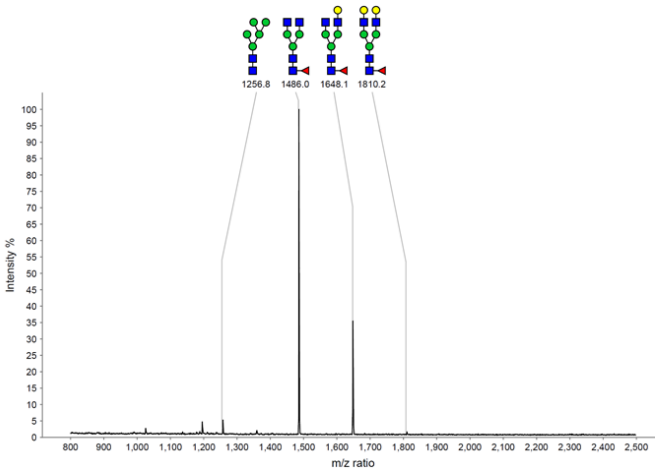
Cetuximab (Fab glycans):



Compound **9** (Fab glycans):



Cetuximab (Fc glycans):



Compound **9** (Fc glycans):

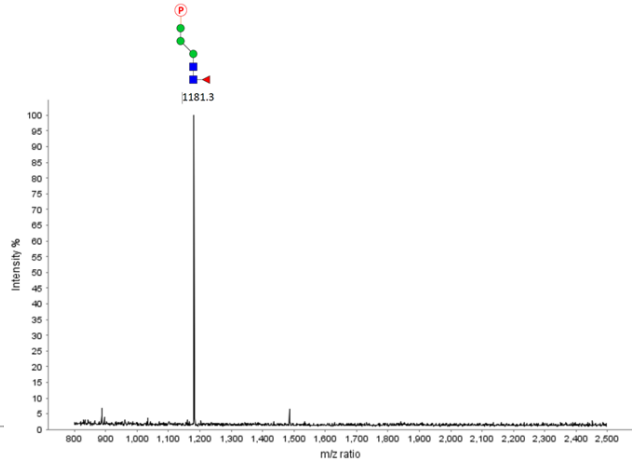


Fig. S6. MALDI-TOF-MS spectra (positive-ion mode) of free N-glycans from commercial antibodies and the glycoengineered antibodies (**3** and **9**) released by PNGase F treatment.

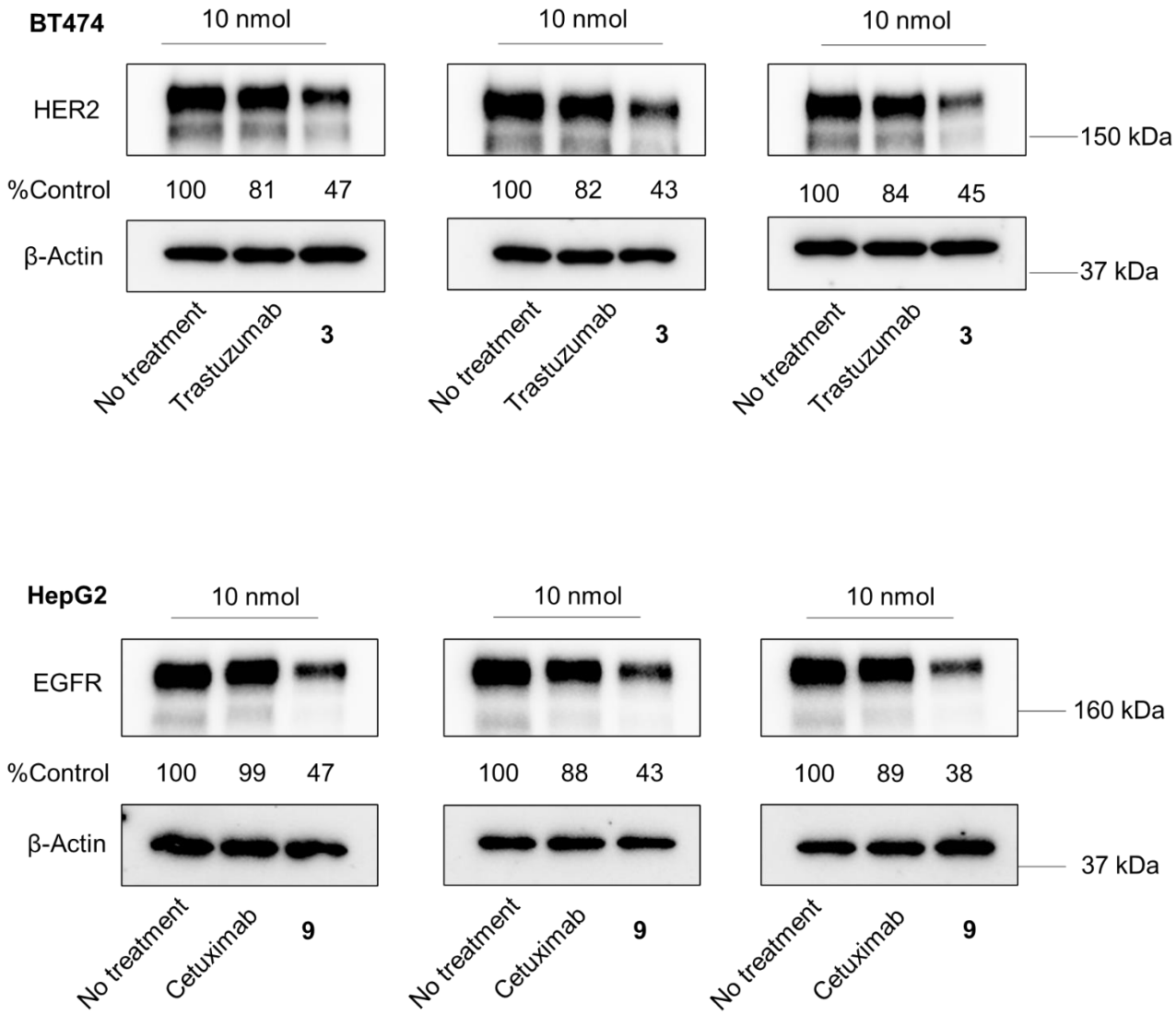


Fig. S7. Western blot analysis of HER2 and EGFR degradation after treatment with native or M6P-modified antibodies.

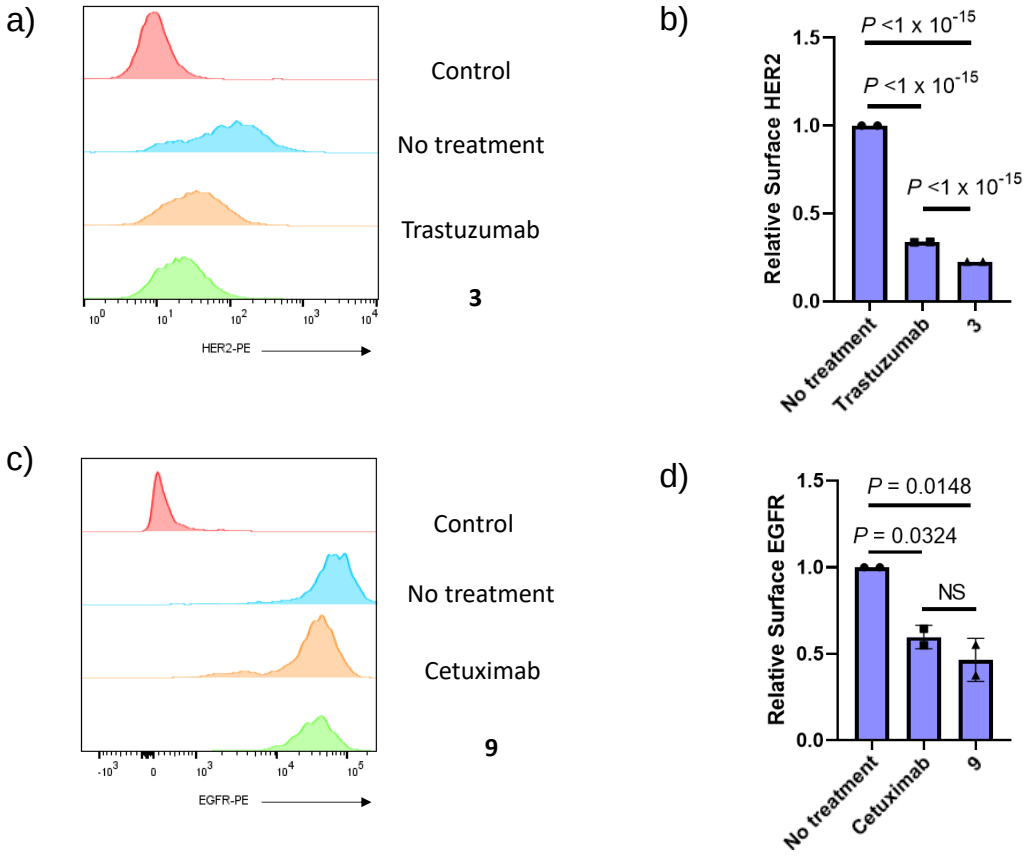


Figure S8. Degradation of cell surface HER2 or EGFR as determined by live-cell flow cytometry following 48 h of treatment with 10 nM native or M6P-modified antibodies. a) Representative FACS analysis of surface HER2 levels; b) Surface HER2 levels after treatment with trastuzumab or **3**; c) Representative FACS analysis of surface EGFR levels; d) Surface EGFR levels after treatment with cetuximab or **9**. All assays were performed in triplicate. Cells were stained with PE conjugated anti-human CD340 (erbB2/HER2) antibody (BioLegend, Cat.# 324405), PE conjugated anti-human EGFR (BioLegend, Cat.# 352903), or PE conjugated Isotype control antibody in PBS at 4°C for 30 min. *P* values were determined by one-way ANOVA multiple comparison tests. NS, not significant; *P* value of >0.05 was considered not significant.

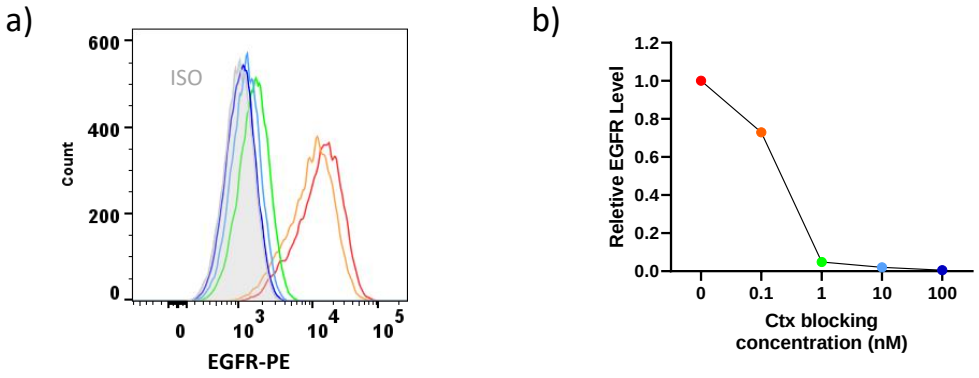
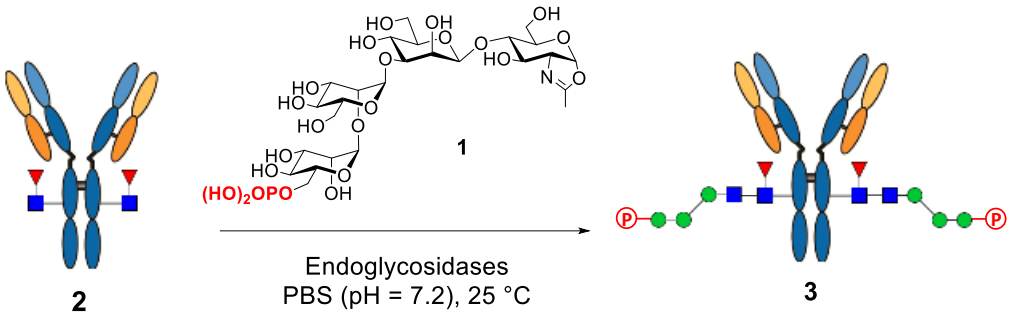


Figure S9. Inhibition of the clone AY13 EGFR detection of GFR by cetuximab. a) a representative FACS analysis of surface EGFR levels; b) Inhibition of surface EGFR detection by cetuximab. In a 96-well setting, freshly trypsinzed and FACS buffer washed HepG2 cells were pre-incubated with a 10-fold serial dilution of cetuximab starting from 100 nM on ice for 25 min. Cells were then washed and incubated with anti-EGFR PE (clone AY13) for 25 mins and washed. Cells were then incubated for 10 min with aqua live/dead stain and washed and re-suspended in FACS buffer for flow acquisition.

Materials and Methods.

All chemicals, reagents, and solvents were purchased from Sigma−Aldrich and Themo Fisher Scientific and unless specially noted applied in the reaction without further purification. MALDI-TOF MS analysis was performed on a Bruker Autoflex Speed Mass Spectrometer in positive reflectron mode with DHB (ACN/H_2O = 1:1) as the matrix. LC-ESI-MS analysis was performed on an Ultimate 3000 HPLC system coupled to an Exactive Plus Orbitrap mass spectrometer (Thermo Fischer Scientific) with C4 (whole antibody, gradient, 5−95% aq MeCN containing 0.1% FA for 6 min, 0.4 mL/min) or C8 (IdeS digestion, gradient, 25−35% aq MeCN containing 0.1% FA for 6 min, 0.4 mL/min) column. Deconvolution data was transformed by MagTran software.

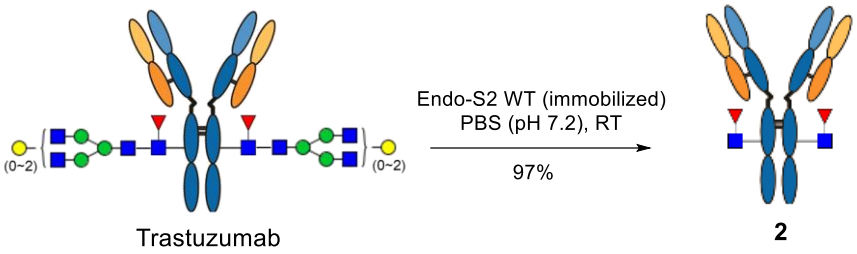
1. Screening of the transglycosylation conditions.



A solution of the deglycosylate trastuzumab (**2**) (100 µg) and the M6P tetrasaccharide oxazoline (**1**) (21.1 µg, 20 eq per Fc glycosylation site) was incubated with different endoglycosidases (0.1 µg ~ 10 µg) at 25 °C in 10 µL of 150 mM PBS buffer (pH 7.2). The reaction was monitored by LC-ESI-MS analysis of aliquots. The ratio of the starting material and the product species were determined by the peak intensities.

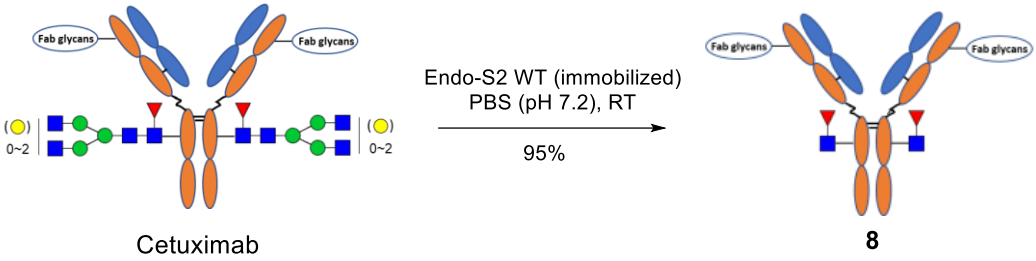
Enzymes	Sources
Endo-S	*Streptococcus pyogenes*
Endo-S D233Q	*Streptococcus pyogenes*
Endo-S2	*Streptococcus pyogenes M49*
Endo-S2 D184M	*Streptococcus pyogenes M49*
Endo-F3	*Elizabethkingia miricola*
Endo-F3 DA65A	*Elizabethkingia miricola*
Endo-A	*Arthrobacter protophormiae*
Endo-CC	*Coprinopsis cinerea*
Endo-D	*Streptococcus pneumoniae*

2. Preparation of the deglycosylated trastuzumab (2).



Trastuzumab was deglycosylated as previously described.[1] Briefly, the commercial trastuzumab (10 mg) was applied to a spin column (Pierce #69705) containing the immobilized wild-type Endo-S2 (Endo-S2 WT) (200 µg) in a total volume of 1.0 ml (PBS, pH = 7.2). The mixture was incubated for 1h at room temperature when LC-ESI-MS indicated the completion of deglycosylation. Then the reaction mixture was passed through the spin filter and separated from the agarose resins by centrifugation to afford the deglycosylated trastuzumab (**2**) (9.5 mg, 97%). ESI-MS: calculated for whole antibody, M = 145865 Da; found (m/z), 145863 (deconvolution data); calculated for the Fc monomer after IdeS digestion, M = 24134 Da; found (m/z), 24135 (deconvolution data).

3. Preparation of Fc deglycosylated trastuzumab (8).



The commercial Cetuximab (10 mg) was applied to a spin column (Pierce #69705) containing the immobilized Endo-S2 WT (400 µg) in a total volume of 5.0 ml (PBS, pH = 7.2). The mixture was incubated for 2 h at room temperature when LC-ESI-MS of the Fc domain after IdeS treatment indicated the completion of deglycosylation. The reaction mixture was passed through the spin filter and separated from the agarose resins by centrifugation to afford the Fc-deglycosylated cetuximab (**8**) (9.3 mg, 95%). ESI-MS: calculated for the Fc monomer after IdeS treatment, M = 24134 Da; found (m/z), 24134 (deconvolution data).

4. Release and enrichment of the N-glycans from the Fab and Fc domains.

The release of N-glycans from antibodies was performed following the NEB PNGase F Protocol [2] with modifications. For the analysis of trastuzumab and M6P-modified trastuzumab (**3**), the antibody (200 µg) and PNGase F (10 µg) were mixed in 20 µL of the PBS buffer (150 mM, pH = 7.2). the mixture was incubated at 37 °C for 5 h. LC-ESI-MS analysis indicated the completion of glycan release. The released N-glycans were purified on a porous graphitic carbon cartridge (The Hypersep Hypercarb PGC from the Themo Scientific). The fractions containing the N-glycans were pooled, lyophilized, and analyzed by MALDI-TOF MS.

For the analysis of Cetuximab and M6P-modified cetuximab (**9**), the antibodies were first treated with protease IdeS to disconnect the Fab and Fc domains. Briefly, the antibody (200 µg) was mixed with IdeS (10 µg) in 20 µL of PBS buffer (150 mM, pH = 7.2). The mixture was incubated at 37 °C for 1 h. Then the reaction mixture was subjected to Protein L (Cytiva, 1 mL). The Fc domain (released as the monomer) was collected in the flow through, and the Fab domain (as an intact dimer) was eluted with citric buffer (30 mM, pH = 2.6). Salts were removed by ultrafiltration and the proteins were extracted and lyophilized to give the separated Fc and Fab domains. The Fc N-glycans were released and analyzed following the procedures as described for the analysis of the trastuzumab Fc glycans. For Fab glycan release, the "Denaturing Reaction Conditions" of the NEB protocol [2] were used. Briefly, The Fab domain protein (100 µg) was dissolved in the Glycoprotein Denaturing Buffer (1X, 30 µL), then the mixture was heated at 100 °C for 10 min before adding 6 µL GlycoBuffer 2 (10X), 6 µL 10% NP-40 and 18 µL H_2O. PNGase F (10 µg) was added, and the reaction was incubated at 37 °C overnight. The released Fab N-glycans were purified and analyzed by MALDI-TOF MS as described above.

References:

1. T. Li, C. Li, D. N. Quan, W. E. Bentley, L.-X. Wang. *Carbohydr Res*, **2018**, *458-459*, 77-84.

2. https://www.neb.com/protocols/2014/07/31/pngase-f-protocol