Supplementary information for

Enhanced Transglycosylation Activity of Endo-F3 Mutant by Ligand-directed Localization

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1. Supplementary figures:

**Figure S1.** Determination of conjugation site of FcBP. **A**, SDS-PAGE profile of D165A and FcBP-D165A conjugates. **B**, LC-MS analysis of free D165A and FcBP3-7c treated by 100 μM InsP₆ (inositol hexakisphosphate, a small molecule promoting CPD self-cleavage) at 4°C for 2 h.

**Figure S2.** LC-MS analysis of D165A and FcBP-D165A conjugates.
Figure S3. Affinity analysis of free D165A and FcBP3 7c to GN (F)-Rituximab. The primary concentration of enzymes was 2 μg/ml. And the x axis means the concentration of GN (F)-Rituximab.

Figure S4. Transglycosylation effect of different enzymes for GN(F)-Rituximab (9). Reaction condition: 5 mg/ml GN(F)-Rituximab (9), 0.1 mg/ml enzyme, 1 mM SCT-ox in 50 mM PB (pH 7.4) at 30 °C for 2 h; Lane 0: Marker; Lane 1: 9; Lane 2: 9 + D165A; Lane 3: 9 + 7a; Lane 4: 9 + 7c; Lane 5: 9 + 7b; Lane 6: 9 + 5a; Lane 7: 9 + 5b; Lane 8: 9 + 5c; Lane 9: 9 + 6a; Lane 10: 9 + 6b; Lane 11: 9 + 6c.
Figure S5. Screening of the enzyme concentration and pH. Reaction conditions were listed as follows: (A) 5 mg/ml GN(F)-Herceptin (14), 0.1 mg/ml enzymes, 1 mM Az-SCT-ox in 50 mM PB (pH 7.1) at 30 °C. (B) 5 mg/ml GN(F)-Herceptin (14), 0.1 mg/ml enzymes, 1 mM Az-SCT-ox in 50 mM PB (pH 7.4) at 30 °C. (C) 5 mg/ml GN(F)-Herceptin (14), 0.2 mg/ml enzymes, 1 mM Az-SCT-ox in 50 mM PB (pH 7.1) at 30 °C. (D) 5 mg/ml GN(F)-Herceptin (14), 0.2 mg/ml enzymes, 1 mM Az-SCT-ox in 50 mM PB (pH 7.4) at 30 °C. (E) 5 mg/ml GN(F)-Herceptin (14), 0.3 mg/ml enzymes, 1 mM Az-SCT-ox in 50 mM PB (pH 7.1) at 30 °C. (F) 5 mg/ml GN(F)-Herceptin (14), 0.3 mg/ml enzymes, 1 mM Az-SCT-ox in 50 mM PB (pH 7.4) at 30 °C.

Lane 0: Marker; Lane 1: 14; Lane 2: 14 + D165A, 0.5 h; Lane 3: 14 + 5a, 0.5 h; Lane 4: 14 + 5b, 0.5 h; Lane 5: 14 + D165A, 1 h; Lane 6: 14 + 5a, 1 h; Lane 7: 14 + 5b, 1 h; Lane 8: 14 + D165A, 1.5 h; Lane 9: 14 + 5a, 1.5 h; Lane 10: 14 + 5b, 1.5 h; Lane 11: 14 + D165A, 2 h; Lane 12: 14 + 5a, 2 h; Lane 13: 14 + 5b, 2 h.
Figure S6. Screening of the reaction temperature. (A) 5 mg/ml GN(F)-Herceptin (14), 0.2 mg/ml enzyme, 1 mM Az-SCT-ox in 50 mM PB (pH 7.4) at 25 °C. (B) 5 mg/ml GN(F)-Herceptin (14), 0.2 mg/ml enzyme, 1 mM Az-SCT-ox in 50 mM PB (pH 7.4) at 30 °C. (C) 5 mg/ml GN(F)-Herceptin (14), 0.2 mg/ml enzyme, 1 mM Az-SCT-ox in 50 mM PB (pH 7.4) at 37 °C.
Lane 0: Marker; Lane 1: 14; Lane 2: 14 +D165A, 0.5 h; Lane 3: 14 + 5a, 0.5 h; Lane 4: 14 + 5b, 0.5 h; Lane 5: 14 + D165A, 1 h; Lane 6: 14 + 5a, 1 h; Lane 7: 14 + 5b, 1 h; Lane 8: 14 + D165A, 2 h; Lane 9: 14 + 5a, 2 h; Lane 10: 14 + 5b, 2 h; Lane 11: 14 + D165A, 3 h; Lane 12: 14 + 5a, 3 h; Lane 13: 14 + 5b, 3 h.

Figure S7. Transglycosylation effect of different substrates for GN(F)-Rituximab (9). Reaction
conditions: 5 mg/ml GN(F)-Rituximab (9), 0.1 mg/ml enzyme, 1 mM oxazoline in 50 mM PB (pH 7.4) at 30 °C. (A) 15 min; (B) 30 min; (C) 1 h; (D) 2 h; Lane 0: Marker; Lane 1: 9; Lane 2: 9 + D165A + SCT-ox; Lane 3: 9 + 6c + SCT-ox; Lane 4: 9 + D165A + Man3-ox; Lane 5: 9 + 6c + Man3-ox; Lane 6: 9 + D165A + GN2M3-ox; Lane 7: 9 + 6c + GN2M3-ox; Lane 8: 9 + D165A + CT-ox; Lane 9: 9 + 6c + CT-ox.

Figure S8. Stability analysis of the glycoengineering antibody. 3.31 mg/ml G2F-Rituximab (10b) and FeBP2-PEG$_8$-SMCC-D165A (6c) in 1X PBS at 37 °C. (A) 12 h and 1 day. Lane 0: Marker; Lane 1: 9; Lane 2: 10b, 12 h; Lane 3: 10b + 0.01 mg/ml 6c, 12 h; Lane 4: 10b + 0.05 mg/ml 6c, 12 h; Lane 5: 10b + 0.1 mg/ml 6c, 12 h; Lane 6: 10b + 0.2 mg/ml 6c, 12 h; Lane 7: 10b, 1 day; Lane 8: 10b + 0.01 mg/ml 6c, 1 day; Lane 9: 10b + 0.05 mg/ml 6c, 1 day; Lane 10: 10b + 0.1 mg/ml 6c, 1 day; Lane 11: 10b + 0.2 mg/ml 6c, 1 day. (B) 2.5 days and 5 days. Lane 0: Marker; Lane 1: 9; Lane 2: 10b, 2.5 days; Lane 3: 10b + 0.01 mg/ml 6c, 2.5 days; Lane 4: 10b + 0.05 mg/ml 6c, 2.5 days; Lane 5: 10b + 0.1 mg/ml 6c, 2.5 days; Lane 6: 10b + 0.2 mg/ml 6c, 2.5 days; Lane 7: 10b, 5 days; Lane 8: 10b + 0.01 mg/ml 6c, 5 days; Lane 9: 10b + 0.05 mg/ml 6c, 5 days; Lane 10: 10b + 0.1 mg/ml 6c, 5 days; Lane 11: 10b + 0.2 mg/ml 6c, 5 days.
**Figure S9.** One-pot glycoengineering of IgGs by FcBP-D165A conjugates. 4 mg/ml WT-Rituximab (8) and 0.1 mg/ml or 0.2 mg/ml FcBP2-PEG8-SMCC-D165A (6c) or D165A were added in 1X PBS at 37 °C for 24 h. Then, a half of solution was supplied with 1 mM CT-ox, while the left solution supplied with 1 mM CT-ox and 0.1 mg/ml 6c or D165A for transglycosylation evaluation (30°C).

(A) Lane 0: Marker; Lane 1: 9; Lane 2: 0.1 mg/ml D165A + 8, 24 h; Lane 3: 0.1 mg/ml 6c + 8, 24 h; Lane 4: 0.2 mg/ml D165A + 8, 24 h; Lane 5: 0.2 mg/ml 6c + 8, 24 h; Lane 6: 0.1 mg/ml 6c + 8 + 1 mM CT-ox, 15 min; Lane 7: 0.1 mg/ml 6c + 8 + 1 mM CT-ox + 0.1 mg/ml 6c, 15 min; Lane 8: 0.1 mg/ml D165A + 8 + 1 mM CT-ox, 15 min; Lane 9: 0.1 mg/ml D165A + 8 + 1 mM CT-ox + 0.1 mg/ml D165A, 15 min; Lane 10: 0.2 mg/ml 6c + 8 + 1 mM CT-ox, 15 min; Lane 11: 0.2 mg/ml 6c + 8 + 1 mM CT-ox + 0.1 mg/ml 6c, 15 min; Lane 12: 0.2 mg/ml D165A + 8 + 1 mM CT-ox, 15 min; Lane 13: 0.2 mg/ml D165A + 8 + 1 mM CT-ox + 0.1 mg/ml D165A.

(B) 30 min; (C) 1 h; (D) 2 h. Lane 0: Marker; Lane 1: 9; Lane 2: 0.1 mg/ml 6c + 8 + 1 mM CT-ox; Lane 3: 0.1 mg/ml 6c + 8 + 1 mM CT-ox + 0.1 mg/ml 6c; Lane 4: 0.1 mg/ml D165A + 8 + 1 mM CT-ox; Lane 5: 0.1 mg/ml D165A + 8 + 1 mM CT-ox + 0.1 mg/ml D165A; Lane 6: 0.2 mg/ml 6c + 8 + 1 mM CT-ox; Lane 7: 0.2 mg/ml 6c + 8 + 1 mM CT-ox + 0.1 mg/ml 6c; Lane 8: 0.2 mg/ml D165A + 8 + 1 mM CT-ox; Lane 9: 0.2 mg/ml D165A + 8 + 1 mM CT-ox + 0.1 mg/ml D165A.
Figure S10. LC-MS analysis of the preparation of one-pot glycoengineering of IgGs by FcBP-D165A conjugates.

Figure S11. Synthesis of Az-S2G2F-Trastuzumab and LC-MS analysis of the Az-S2G2F-Trastuzumab and the deglycosylated trastuzumab. Owing to the quite low percentage of non-core-fucosylated IgG, the mass spectra of deglycosylated IgG only exhibited one major peak with 2 fucose and one small peak with 1 fucose, but without peak without fucose (see black box). We could clearly see that the molecular weight of deglycosylated IgG without core-fucose (145581) didn’t change after transglycosylation, and the molecular weight of deglycosylated IgG with 1 fucosylation shifted to 147774 (equals to 145581 + 146 (MW of fucose) + 2046 (MW of Az-SCT-ox)), and the
molecular weight of deglycosylated IgG with 2 fucosylation shifted to 149967 (equals to 145581 + 
2 * 146 (MW of fucose) + 2 * 2046 (MW of Az-SCT-ox)), indicating that the non-fucosylated heavy 
chain can’t be re-glycosylated by FcBP-D165A 6c.

Figure S12. Synthetic scheme of linker 1b and 1c.

General Procedure for Synthesis of linker 1b and 1c.
Acid-PEG-Amino (10 μmol, 1.0 eq), SMCC (12 μmol, 1.2 eq), Et3N (2.8 μl, 2.0 eq) and DMF (97.2 μl) were added to a tube successively at r.t. for 2 h and monitored by HPLC and LC-MS. The mixture was subjected to semi-preparation HPLC purification (Method A). The fractions containing the products were collected and lyophilized to obtain the product as a white powder. Then, the harvested Acid-PEG-MCC (10 μmol, 1.0 eq), NHS (20 μmol, 2.0 eq), EDCI (20 μmol, 2.0 eq), Et3N (2.8 μl, 2.0 eq) and DMF (97.2 μl) were added to a tube successively at r.t. for 6 h and monitored by HPLC and LC-MS. The mixture was subjected to semi-preparation HPLC for purification (Method A). The fractions containing the products were collected and lyophilized to obtain 1b and 1c as a white powder respectively.
2. Supplementary original figures:

**Figure S13.** Transglycosylation effect of different enzymes for GN(F)-Rituximab (9). Reaction condition: 5 mg/ml GN(F)-Rituximab (9), 0.15 mg/ml Endo S mutant D233Q/0.05 mg/ml Endo S2 D184M/0.15 mg/ml Endo F3 D165A, 1 mM SCT-ox in 50 mM PB (pH 7.4) at 30 °C. (A) 30 min; (B) 1 h; (C) 2 h; (D) 3 h; Lane 0: Marker; Lane 1: 8; Lane 2: 9; Lane 3: 9 + D233Q; Lane 4: 9 + D184M; Lane 5: 9 + D165A.

**Figure S14.** Optimization of the substrate concentration. (A) 5 mg/ml GN(F)-Herceptin (14), 0.2 mg/ml enzyme, 1 mM Az-SCT-ox in 50 mM PB (pH 7.4) at 30 °C. (B) 5 mg/ml GN(F)-Herceptin (14), 0.2 mg/ml enzyme, 1.5 mM Az-SCT-ox in 50 mM PB (pH 7.4) at 30 °C. (C) 5 mg/ml GN(F)-Herceptin (14), 0.2 mg/ml enzyme, 2 mM Az-SCT-ox in 50 mM PB (pH 7.4) at 30 °C. Lane 0: Marker; Lane 1: 14; Lane 2: 14 + D165A, 0.5 h; Lane 3: 14 + 5a, 0.5 h; Lane 4: 14 + 5b, 0.5 h; Lane 5: 14 + D165A, 1 h; Lane 6: 14 + 5a, 1 h; Lane 7: 14 + 5b, 1 h; Lane 8: 14 + D165A, 1.5 h; Lane 9: 14 + 5a, 1.5 h; Lane 10: 14 + 5b, 1.5 h; Lane 11: 14 + D165A, 2 h; Lane 12: 14 + 5a, 2 h; Lane 13: 14 + 5b, 2 h.
**Figure S15.** Transfer efficiency of conjugates with distinct linker length. 5 mg/ml GN(F)-Herceptin (14), 0.2 mg/ml enzyme, 1 mM Az-SCT-ox in 50 mM PB (pH 7.4) at 30 °C. Lane 0: Marker; Lane 1: 14; Lane 2: 14 + D165A, 0.5 h; Lane 3: 14 + 5b, 0.5 h; Lane 4: 14 + 5c, 0.5 h; Lane 5: 14 + D165A, 1 h; Lane 6: 14 + 5b, 1 h; Lane 7: 14 + 5c, 1 h; Lane 8: 14 + D165A, 2 h; Lane 9: 14 + 5b, 2 h; Lane 10: 14 + 5c, 2 h; Lane 11: 14 + D165A, 3 h; Lane 12: 14 + 5b, 3 h; Lane 13: 14 + 5c, 3 h.

**Figure S16.** Transfer efficiency of conjugates with different mutation and distinct linker length. 5 mg/ml GN(F)-Herceptin (14), 0.3 mg/ml enzyme, 2 mM Az-SCT-ox in 50 mM PB (pH 7.4) at 30 °C. (A) Lane 0: Marker; Lane 1: 14; Lane 2: 14 + D165A, 0.5 h; Lane 3: 14 + 5b, 0.5 h; Lane 4: 14 + 5c, 0.5 h; Lane 5: 14 + 6b, 0.5 h; Lane 6: 14 + 6c, 0.5 h; Lane 7: 14 + D165A, 1 h; Lane 8: 14 + 5b, 1 h; Lane 9: 14 + 5c, 1 h; Lane 10: 14 + 6b, 1 h; Lane 11: 14 + 6c, 1 h; (B) Lane 0: Marker; Lane 1: 14; Lane 2: 14 + D165A, 2 h; Lane 3: 14 + 5b, 2 h; Lane 4: 14 + 5c, 2 h; Lane 5: 14 + 6b, 2 h; Lane 6: 14 + 6c, 2 h; Lane 7: 14 + D165A, 3 h; Lane 8: 14 + 5b, 3 h; Lane 9: 14 + 5c, 3 h; Lane 10: 14 + 6b, 3 h; Lane 11: 14 + 6c, 3 h.
Figure S17. Cleavage efficiency of free D165A and conjugates. (A) 5 mg/ml WT-Herceptin (15) and 0.2 mg/ml enzymes in 50 mM PB (pH 6.5) at 37 °C. Lane 0: Marker; Lane 1: 15; Lane 2: 15 + D165A, 0.5 h; Lane 3: 15 + 5a, 0.5 h; Lane 4: 15 + 5b, 0.5 h; Lane 5: 15 + D165A, 1 h; Lane 6: 15 + 5a, 1 h; Lane 7: 15 + 5b, 1 h; Lane 8: 15 + D165A, 2 h; Lane 9: 15 + 5a, 2 h; Lane 10: 15 + 5b, 2 h; (B) 5 mg/ml WT-Herceptin (15) and 0.2 mg/ml enzymes in 50 mM PB (pH 6.5) at 37 °C. Lane 0: Marker; Lane 1: 15; Lane 2: 15 + D165A, 3 h; Lane 3: 15 + 5a, 3 h; Lane 4: 15 + 5b, 3 h; Lane 5: 15 + D165A, 4 h; Lane 6: 15 + 5a, 4 h; Lane 7: 15 + 5b, 4 h; Lane 8: 15 + D165A, 6 h; Lane 9: 15 + 5a, 6 h; Lane 10: 15 + 5b, 6 h.

Figure S18. Cleavage efficiency of free D165A and conjugates. (A) 5 mg/ml WT-Herceptin (15) and 0.3 mg/ml enzymes in 50 mM PB (pH 6.5) at 37 °C. Lane 0: Marker; Lane 1: 15; Lane 2: 15 + D165A, 0.5 h; Lane 3: 15 + 5a, 0.5 h; Lane 4: 15 + 5b, 0.5 h; Lane 5: 15 + D165A, 1 h; Lane 6: 15 + 5a, 1 h; Lane 7: 15 + 5b, 1 h; Lane 8: 15 + D165A, 2 h; Lane 9: 15 + 5a, 2 h; Lane 10: 15 + 5b, 2 h; (B) 5 mg/ml WT-Herceptin (15) and 0.3 mg/ml enzymes in 50 mM PB (pH 6.5) at 37 °C. Lane 0: Marker; Lane 1: 15; Lane 2: 15 + D165A, 4 h; Lane 3: 15 + 5a, 4 h; Lane 4: 15 + 5b, 4 h; Lane 5: 15 + D165A, 6 h; Lane 6: 15 + 5a, 6 h; Lane 7: 15 + 5b, 6 h; Lane 8: 15 + D165A, 12 h; Lane 9: 15 + 5a, 12 h; Lane 10: 15 + 5b, 12 h.
**Figure S19.** Cleavage efficiency of free D165A and conjugates. (A) 5 mg/ml WT-Herceptin (15) and 0.5 mg/ml enzymes in 50 mM PB (pH 6.5) at 37 ℃. Lane 0: Marker; Lane 1: 15; Lane 2: 15 + D165A, 0.5 h; Lane 3: 15 + 5a, 0.5 h; Lane 4: 15 + 5b, 0.5 h; Lane 5: 15 + D165A, 1 h; Lane 6: 15 + 5a, 1 h; Lane 7: 15 + 5b, 1 h; Lane 8: 15 + D165A, 2 h; Lane 9: 15 + 5a, 2 h; Lane 10: 15 + 5b, 2 h; (B) 5 mg/ml WT-Herceptin (15) and 0.5 mg/ml enzymes in 50 mM PB (pH 6.5) at 37 ℃. Lane 0: Marker; Lane 1: 15; Lane 2: 15 + D165A, 4 h; Lane 3: 15 + 5a, 4 h; Lane 4: 15 + 5b, 4 h; Lane 5: 15 + D165A, 6 h; Lane 6: 15 + 5a, 6 h; Lane 7: 15 + 5b, 6 h; Lane 8: 15 + D165A, 12 h; Lane 9: 15 + 5a, 12 h; Lane 10: 15 + 5b, 12 h.

**Figure S20.** Cleavage efficiency of WT Endo F3. 5mg/ml WT-Herceptin (15) and 0.5mg/ml WT-F3 were incubated in 50 mM PB (pH 6.5) at 37 ℃. Lane 0: Marker; Lane 1: 15; Lane 2: 0.5 h; Lane 3: 1 h; Lane 4: 2 h; Lane 5: 4 h; Lane 6: 6 h; Lane 7: 12 h.
Figure S21. Transglycosylation effect of different enzymes for GN(F)-Rituximab. Reaction conditions were listed as follows: (A) 5 mg/ml GN(F)-Rituximab (9), 0.1 mg/ml enzyme, 0.5 mM SCT-ox in 50 mM PB (pH 7.4) at 30 ℃. (B) 5 mg/ml GN(F)-Rituximab (9), 0.1 mg/ml enzyme, 1 mM SCT-ox in 50 mM PB (pH 7.4) at 30 ℃. (C) 5 mg/ml GN(F)-Rituximab (9), 0.1 mg/ml enzyme, 2 mM SCT-ox in 50 mM PB (pH 7.4) at 30 ℃. Lane 0: Marker; Lane 1: 9; Lane 2: 9 + D165A, 15 min; Lane 3: 9 + 6b, 15 min; Lane 4: 9 + 6c, 15 min; Lane 5: 9 + D165A, 30 min; Lane 6: 9 + 6b, 30 min; Lane 7: 9 + 6c, 30 min; Lane 8: 9 + D165A, 1 h; Lane 9: 9 + 6b, 1 h; Lane 10: 9 + 6c, 1 h; Lane 11: 9 + D165A, 2 h; Lane 12: 9 + 6b, 2 h; Lane 13: 9 + 6c, 2 h.
Figure S22. Transglycosylation effect of different enzymes for GN(F)-Rituximab (9). Reaction conditions were listed as follows: (A) 5 mg/ml GN(F)-Rituximab (9), 0.2 mg/ml enzyme, 0.5 mM SCT-ox in 50 mM PB (pH 7.4) at 30 °C. (B) 5 mg/ml GN(F)-Rituximab (9), 0.2 mg/ml enzyme, 1 mM SCT-ox in 50 mM PB (pH 7.4) at 30 °C. (C) 5 mg/ml GN(F)-Rituximab (9), 0.2 mg/ml enzyme, 2 mM SCT-ox in 50 mM PB (pH 7.4) at 30 °C. Lane 0: Marker; Lane 1: 9; Lane 2: 9 + D165A, 15 min; Lane 3: 9 + 6b, 15 min; Lane 4: 9 + 6c, 15 min; Lane 5: 9 + D165A, 30 min; Lane 6: 9 + 6b, 30 min; Lane 7: 9 + 6c, 30 min; Lane 8: 9 + D165A, 1 h; Lane 9: 9 + 6b, 1 h; Lane 10: 9 + 6c, 1 h; Lane 11: 9 + D165A, 2 h; Lane 12: 9 + 6b, 2 h; Lane 13: 9 + 6c, 2 h.
**Figure S23.** Transglycosylation effect of different enzymes for GN(F)-Rituximab (9). Reaction conditions were listed as follows: (A) 5 mg/ml GN(F)-Rituximab (9), 0.1 mg/ml enzyme, 0.5 mM SCT-ox in 50 mM PB (pH 7.4) at 30 °C. (B) 5 mg/ml GN(F)-Rituximab (9), 0.1 mg/ml enzyme, 1 mM SCT-ox in 50 mM PB (pH 7.4) at 30 °C. (C) 5 mg/ml GN(F)-Rituximab (9), 0.1 mg/ml enzyme, 2 mM SCT-ox in 50 mM PB (pH 7.4) at 30 °C. (D) 5 mg/ml GN(F)-Rituximab (9), 0.2 mg/ml enzyme, 0.5 mM SCT-ox in 50 mM PB (pH 7.4) at 30 °C. (E) 5 mg/ml GN(F)-Rituximab (9), 0.2 mg/ml enzyme, 1 mM SCT-ox in 50 mM PB (pH 7.4) at 30 °C. (F) 5 mg/ml GN(F)-Rituximab (9), 0.2 mg/ml enzyme, 2 mM SCT-ox in 50 mM PB (pH 7.4) at 30 °C. Lane 0: Marker; Lane 1: 9; Lane 2: 9 + D165A, 15 min; Lane 3: 9 + 6b, 15 min; Lane 4: 9 + 6c, 15 min; Lane 5: 9 + D165A, 30 min; Lane 6: 9 + 6b, 30 min; Lane 7: 9 + 6c, 30 min; Lane 8: 9 + D165A, 1 h; Lane 9: 9 + 6b, 1 h; Lane 10: 9 + 6c, 1 h; Lane 11: 9 + D165A, 2 h; Lane 12: 9 + 6b, 2 h; Lane 13: 9 + 6c, 2 h.
Figure S24. Transglycosylation effect of different enzymes for GN(F)-Rituximab (9). Reaction conditions were listed as follows: (A) 5 mg/ml GN(F)-Rituximab (9), 0.1 mg/ml enzyme, 0.5 mM SCT-ox in 50 mM PB (pH 7.4) at 30 °C. (B) 5 mg/ml GN(F)-Rituximab (9), 0.1 mg/ml enzyme, 1 mM SCT-ox in 50 mM PB (pH 7.4) at 30 °C. (C) 5 mg/ml GN(F)-Rituximab (9), 0.1 mg/ml enzyme, 2 mM SCT-ox in 50 mM PB (pH 7.4) at 30 °C. (D) 5 mg/ml GN(F)-Rituximab (9), 0.2 mg/ml enzyme, 0.5 mM SCT-ox in 50 mM PB (pH 7.4) at 30 °C. (E) 5 mg/ml GN(F)-Rituximab (9), 0.2 mg/ml enzyme, 1 mM SCT-ox in 50 mM PB (pH 7.4) at 30 °C. (F) 5 mg/ml GN(F)-Rituximab (9), 0.2 mg/ml enzyme, 2 mM SCT-ox in 50 mM PB (pH 7.4) at 30 °C. Lane 0: Marker; Lane 1: 9; Lane 2: 9 + D165A, 15 min; Lane 3: 9 + 6b, 15 min; Lane 4: 9 + 6c, 15 min; Lane 5: 9 + D165A, 30 min; Lane 6: 9 + 6b, 30 min; Lane 7: 9 + 6c, 30 min; Lane 8: 9 + D165A, 1 h; Lane 9: 9 + 6b, 1 h; Lane 10: 9 + 6c, 1 h; Lane 11: 9 + D165A, 2 h; Lane 12: 9 + 6b, 2 h; Lane 13: 9 + 6c, 2 h.
Figure S25. Transglycosylation effect of different enzymes for GN(F)-Rituximab (9). Reaction conditions: 5 mg/ml GN(F)-Rituximab (9), 0.1 mg/ml enzyme, 1 mM SCT-ox in 50 mM PB (pH 7.4) at 30 °C. (A) 15 min; (B) 30 min; (C) 1 h; (D) 2 h; Lane 0: Marker; Lane 1: 9; Lane 2: 9 + D165A; Lane 3: 9 + 7a; Lane 4: 9 + 7c; Lane 5: 9 + 7b; Lane 6: 9 + 5a; Lane 7: 9 + 5b; Lane 8: 9 + 5c; Lane 9: 9 + 6a; Lane 10: 9 + 6b; Lane 11: 9 + 6c.
**Figure S26.** Transglycosylation effect of different enzymes for GN(F)-Rituximab (9). Reaction conditions: 5 mg/ml GN(F)-Rituximab (9), 0.1 mg/ml enzyme, 1 mM SCT-ox in 50 mM PB (pH 7.4) at 30 °C. (A) 15 min; (B) 30 min; (C) 1 h; (D) 2 h; Lane 0: Marker; Lane 1: 9; Lane 2: 9 + D165A; Lane 3: 9 + 7a; Lane 4: 9 + 7c; Lane 5: 9 + 7b; Lane 6: 9 + 5a; Lane 7: 9 + 5b; Lane 8: 9 + 5c; Lane 9: 9 + 6a; Lane 10: 9 + 6b; Lane 11: 9 + 6c.
Figure S27. Transglycosylation effect of different enzymes for GN(F)-Rituximab (9). Reaction conditions: 5 mg/ml GN(F)-Rituximab (9), 0.1 mg/ml enzyme, 1 mM SCT-ox in 50 mM PB (pH 7.4) at 30 °C. (A) 15 min; (B) 30 min; (C) 1 h; (D) 2 h; Lane 0: Marker; Lane 1: 9; Lane 2: 9 + D165A; Lane 3: 9 + 7a; Lane 4: 9 + 7c; Lane 5: 9 + 5a; Lane 6: 9 + 5b; Lane 7: 9 + 5c; Lane 9: 9 + 6a; Lane 10: 9 + 6b; Lane 11: 9 + 6c.

Figure S28. Transglycosylation effect of unconjugated FcBPs and enzyme for GN(F)-Rituximab (9). Reaction conditions were listed as follows: 5 mg/ml GN(F)-Rituximab (9), 0.1 mg/ml enzyme, 1.8 μM FcBPs and 1 mM SCT-ox in 50 mM PB (pH 7.4) at 30 °C. Lane 0: Marker; Lane 1: 2; Lane 2: FcBP3 + D165A, 15 min; Lane 3: FcBP1 + D165A, 15 min; Lane 4: FcBP2 + D165A, 15 min; Lane 5: FcBP3 + D165A, 30 min; Lane 6: FcBP1 + D165A, 30 min; Lane 7: FcBP2 + D165A, 30 min; Lane 8: FcBP3 + D165A, 1 h; Lane 9: FcBP1 + D165A, 1 h; Lane 10: FcBP2 + D165A, 1 h; Lane 11: FcBP3 + D165A, 2 h; Lane 12: FcBP1 + D165A, 2 h; Lane 13: FcBP2 + D165A, 2 h.
**Figure S29.** The glycan hydrolysis on Az-S2G2F-Rituximab (10e) by PNGase F or Endo S. Reaction conditions were listed as follows: 2 mg/ml Az-SCT-Rituximab, 0.1 mg/ml PNGase F or 0.01 mg/ml Endo S in 1X PBS buffer at 37 °C. Lane 0: Marker; Lane 1: 9; Lane 2: 10e; Lane 3: 10e + PNGase F, 15 min; Lane 4: 10e + Endo S, 15 min; Lane 5: 10e + PNGase F, 30 min; Lane 6: 10e + Endo S, 30 min; Lane 7: 10e + PNGase F, 1 h; Lane 8: 10e + Endo S, 1 h; Lane 9: 10e + PNGase F, 2 h; Lane 10: 10e + Endo S, 2 h.

**Figure S30.** The hydrolysis activity of Endo F3 and FcBP2-PEG<sub>8</sub>-MCC-F3 on WT-Rituximab (8). Reaction conditions were listed as follows: 5 mg/ml WT-Rituximab, 0.05 mg/ml enzymes in 50 mM PB (pH 6.5) at 37 °C. Lane 0: Marker; Lane 1: 8; Lane 2: 8 + Endo F3, 15 min; Lane 3: 8 + FcBP2-PEG<sub>8</sub>-MCC-F3, 15 min; Lane 4: 8 + Endo F3, 30 min; Lane 5: 8 + FcBP2-PEG<sub>8</sub>-MCC-F3, 30 min; Lane 6: 8 + Endo F3, 1 h; Lane 7: 8 + FcBP2-PEG<sub>8</sub>-MCC-F3, 1 h; Lane 8: 8 + Endo F3, 2 h; Lane 9: 8 + FcBP2-PEG<sub>8</sub>-MCC-F3, 2 h.
Figure 31. (A) 4 mg/ml WT-Rituximab (8) and 0.1 mg/ml or 0.2 mg/ml FcBP2 6c were added in 1X PBS at 37 °C at first. Lane 0: Marker; Lane 1: 9; Lane 2: 0.1 mg/ml 6c + 9, 12 h; Lane 3: 0.2 mg/ml 6c + 9, 12 h; Lane 4: 0.1 mg/ml 6c + 9, 18 h; Lane 5: 0.2 mg/ml 6c + 9, 18 h; Lane 6: 0.1 mg/ml 6c + 9, 24 h; Lane 7: 0.2 mg/ml 6c + 9, 24 h. After 36 h, a half of solution was supplied with 1 mM CT-ox, while the left solution supplied with 1 mM CT-ox and 0.1 mg/ml 6c for transglycosylation evaluation (30 °C). (B) 15 min and 30 min; (C) 1 h and 3 h. Lane 0: Marker; Lane 1: 9; Lane 2: 0.1 mg/ml 6c + CT-ox; Lane 3: 0.1 mg/ml 6c + CT-ox + 0.1 mg/ml 6c; Lane 4: 0.2 mg/ml 6c + CT-ox; Lane 5: 0.2 mg/ml 6c + CT-ox + 0.1 mg/ml 6c; Lane 6: 0.1 mg/ml 6c + CT-ox; Lane 7: 0.1 mg/ml 6c + CT-ox + 0.1 mg/ml 6c; Lane 8: 0.2 mg/ml 6c + CT-ox; Lane 9: 0.2 mg/ml 6c + CT-ox + 0.1 mg/ml 6c.
Figure S32. LC-MS profiles of native antibodies, GN (F)-Rituximab and GN (F)-Herceptin.