Electronic Supplementary Information

Engineering a Bacterial Sialyltransferase for Di-Sialylation of a Therapeutic Antibody

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Materials. All reagents and chemicals were purchased from commercially suppliers and used without further purification. Commercial Herceptin was purchased from Roche (Shanghai, China). Peptide *N*-glycosidase F was purchased from New England BioLabs (Beverly, MA). SNA (*Sambucus nigra agglutinin*) lectin was from Vector (Burlingame, CA). *E. coli* chemically competent DH5α was from Genstar (Beijing, China). *E. coli* chemically competent BL21(DE3) cells, *TransTaq* HiFi PCR SuperMix II and Fast Site-Directed Mutagenesis Kit were from TransGen (Beijing, China). *Nde*I, *Xho*I restriction enzymes as well as T4 DNA ligase were from Takara (Dalian, China). The plasmid pET-22b (+) bearing synthetic codon-optimized Psp2,6ST gene was from Genscript (Nanjing, China). PCR Purification Kit was from Qiagen, Plasmid Miniprep Kit was from Tiangen (Beijing, China). Ni^{2+}-Sepharose 6 Fast Flow and Protein A Sepharose CL-4B were from GE Healthcare. Protease inhibitor cocktail (EDTA-free) was purchased from Abcam. Bicinchoninic acid (BCA) protein assay kit was from Pierce Biotechnology Inc. (Thermo Fisher). Acetonitrile (HPLC) was from Fisher Chemical (Thermo Fisher Scientific, MA). Ammonium formate (HPLC) was from Fluka. High resolution mass spectra (HRMS) were obtained using a nanoACQUITY UPLC/SYNAPT G2-Si Q-TOF MS instrument with ES ionization (Waters). 2-AB released glycan analysis was carried out using 2695 HPLC system with a 2475 Multi-fluorescence detector (Waters). UDP-Gal, Bovine β1,4 galactosyl transferase, MOPS, $MnCl_2$ and Sodium pyruvate were purchased from Sigma-Aldrich. Psp2,6ST(15-501)-His_6, Pmaldolase, NmCSS and CTP were generously provided by Dr. Hongzhi Cao group.

Native intact MS for glycoform analysis

An aliquot of Herceptin was buffer exchanged thrice into NH_4HCO_3 (50 mM, pH 8.0) using an Amicon 30 kDa cutoff spin concentrator (Millipore) for LC-MS analysis, the analytes were separated by a 60 min gradient elution at a flow rate 0.5 μL/min with a nano-ACQUITY UPLC system, which was directly interfaced with a SYNAPT-G2-Si mass spectrometer. The analytical column was a Protein BEH C4 silica capillary column (150 μm ID, 100 mm length; made in Ireland) packed with C-4 resin (300 Å, 1.7 μm) purchased from Waters company. Mobile phase A consisted of 0.1% formic acid aqueous solution, and mobile phase B consisted of 100% acetonitrile and 0.1% formic acid. Aliquots of 2 μL analytes were loaded into an autosampler for nanoelectrospray ionization and analyzed on a Q-TOF mass spectrometer instrument optimized for high-mass protein

analysis. The measurements were performed with capillary 3000V and data were collected over the m/z range of 500–4000. The raw spectrum was deconvoluted by MaxEnt 1 (Waters) to generate a spectrum (relative intensity versus mass).

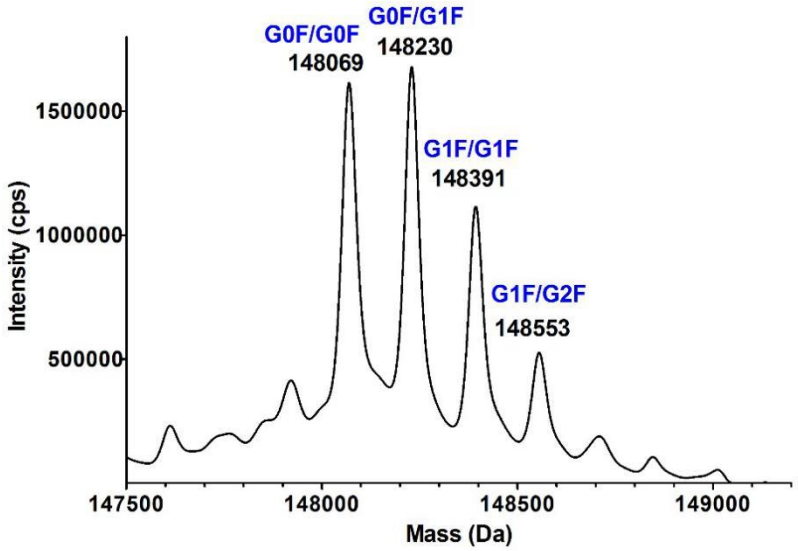


Fig. S1. Deconvoluted MS data for intact commercial Herceptin.

Conversion to the homogeneous G2F glycoform of the heterogeneous Herceptin

The commercial Herceptin (40 mg) solution was buffer exchanged to MOPS buffer (50 mM, pH 7.2) containing 20 mM $MnCl_2$ using an Amicon 30 kDa cutoff spin concentrator. The full galactosylation of *N*-glycans was catalyzed by the bovine β-1,4-galactosyl transferase (0.2 U/mL, Sigma) in the presence of UDP-Gal (15 mM, Sigma) and calf intestine alkaline phosphatase (1 U/mL, Promega) in final 4 mL solution incubated at 37 °C for 24 h, supplemented with an additional aliquot of UDP-galactose (5 mM) and galactosyltransferase (0.2 U) for another 24 h for the complete galactosylation.[1] An aliquot of the reaction solution was exchange repeated thrice to NH_4HCO_3 (50 mM, pH 8.0) for intact MS or released glycan analysis.

Release of *N*-glycans

An aliquot of IgG (50 μg) solution was buffer exchanged to 50 mM ammonium bicarbonate (pH 8.0) using an Amicon 30 kDa cutoff spin concentrator (Millipore), and then supplemented with 10 units of PNGase F (NEB) to constitute the 50-100 μL reaction system allowed to proceed overnight at 37 °C. The complete digestion was verified by reducing SDS-PAGE, giving the result that the heavy chains (HC) slightly shift down less than 2 kDa, while not the light chains (LC) (Fig. S2). Next, the *N*-deglycosylated proteins was precipitated by the addition of 3 volumes of ice-cold ethanol and stored at -20 °C for more than 2 h, and the supernatant containing released *N*-glycans separated from the precipitated proteins by centrifuge was transferred and dried by rotatory evaporation. The *N*-glycans were re-dissolved for the purification using a Sep-Pak C18 column (Waters).

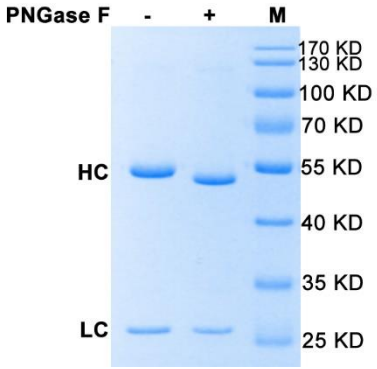
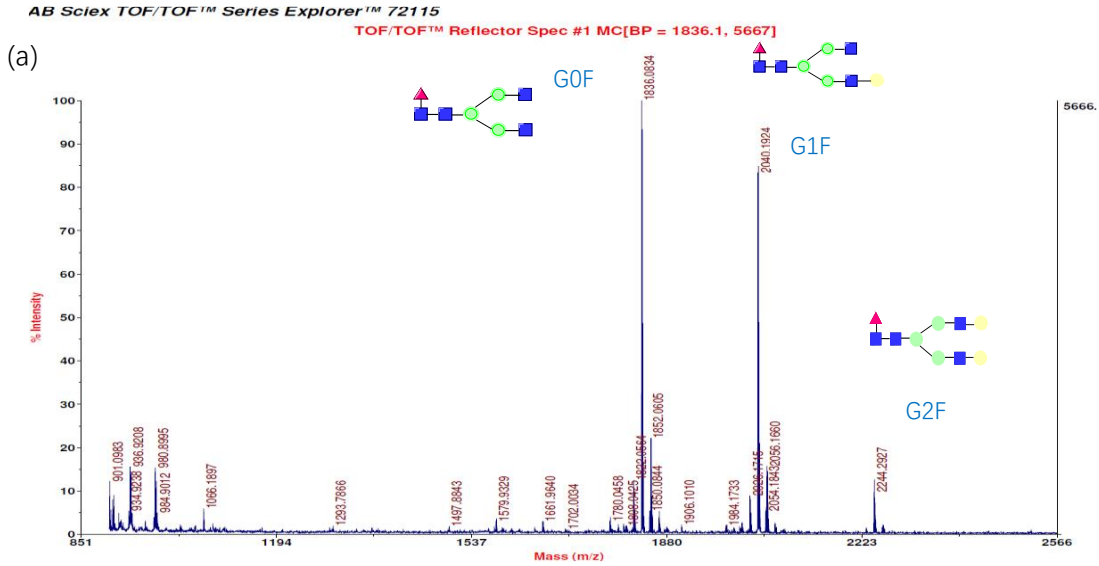


Fig. S2. The 12% SDS-PAGE analysis of the release of *N*-glycans from HC (heavy chain), LC (light chain) as an internal reference.

Glycan analysis by MALDI-TOF-MS (matrix-assisted laser-desorption ionization time-of-flight mass spectrometry)

The purified *N*-glycans were permethylated with NaOH and methyl iodide and purified using Sep-Pak C18 according to the report by Morelle and Michalski.[2] The purified permethylated glycans were then analyzed by MALDI-TOF-MS using DHBA (2,3-dihydroxybenzoic acid) as the matrix. The spectrum acquisitions were in the positive ion mode.



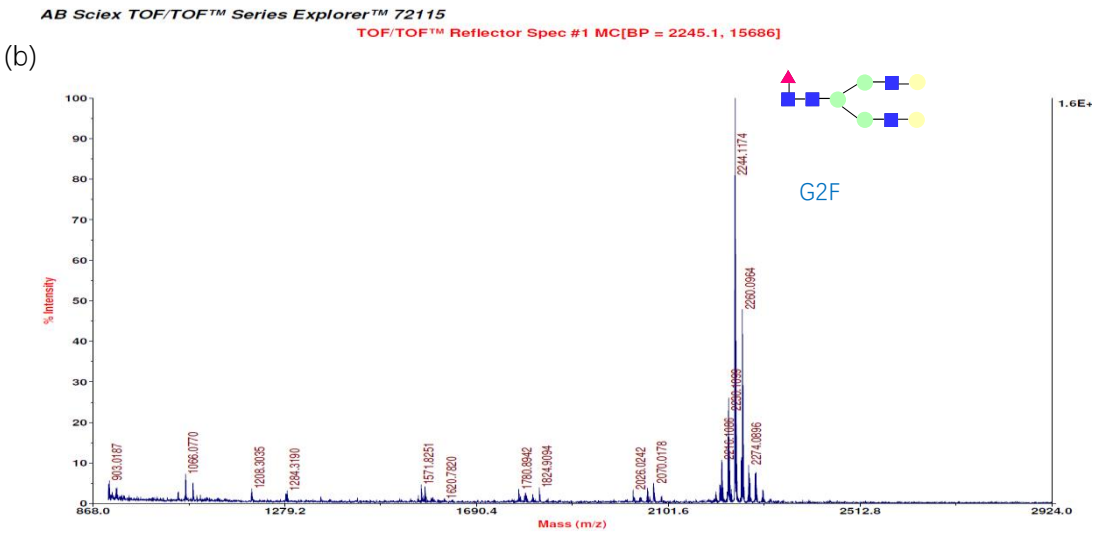


Fig. S3. The glycoforms of Herceptin galactosylation was determined by MALDI-TOF-MS. (a) commercial Herceptin before the galactosylation reaction. (b) Herceptin after being catalyzed by β-1,4-galactosyl transferase for 48 h.

Glycan analysis by 2-AB HILIC (hydrophilic interaction chromatography) HPLC

The purified dry glycan was fluorescently labeled with 2-AB (aminobenzamide), each glycan sample was dissolved in an aliquot (20 μL) of labeling solution prepared as the following conditions, 5 mg 2-AB was dissolved into 100 μL solution of acetic acid/DMSO (3:7, v/v), followed by addition of 6 mg $NaCNBH_3$ (sodium cyanoborohydride). Labeling reactions were performed at 65 °C in an isotherm incubator for 3 h, then labeled glycans were precipitated with acetonitrile (10 volumes) for purification by HILIC cartridge (Waters) to remove the unreacted dye and salts, the collected eluates were dried by vacuum evaporation and reconstituted in acetonitrile/water (1:1, v/v) before the loading. The labeled glycans were resolved by HILIC HPLC on a Shodex Asahipak NH2P column (5 μm, 4.6 mm ID, 150 mm length, Japan) using 2695 HPLC system (Waters) with a 2475 Multi-fluorescence detector ($\lambda_{ex}330/\lambda_{em}420$ nm), with the following LC conditions:[3] solvent A: 50 mM ammonium formate prepared by titrating ammonium formate solution with formic acid to pH 4.4; solvent B: acetonitrile. Elution: 70% B in 45 min, then with gradients: 70% to 55% B in 30 min and 55% to 0% B in 25 min; 0.8 mL/min flow rate. The relative yields were calculated using a single product peak area divided by the peak areas of the product and substrates.

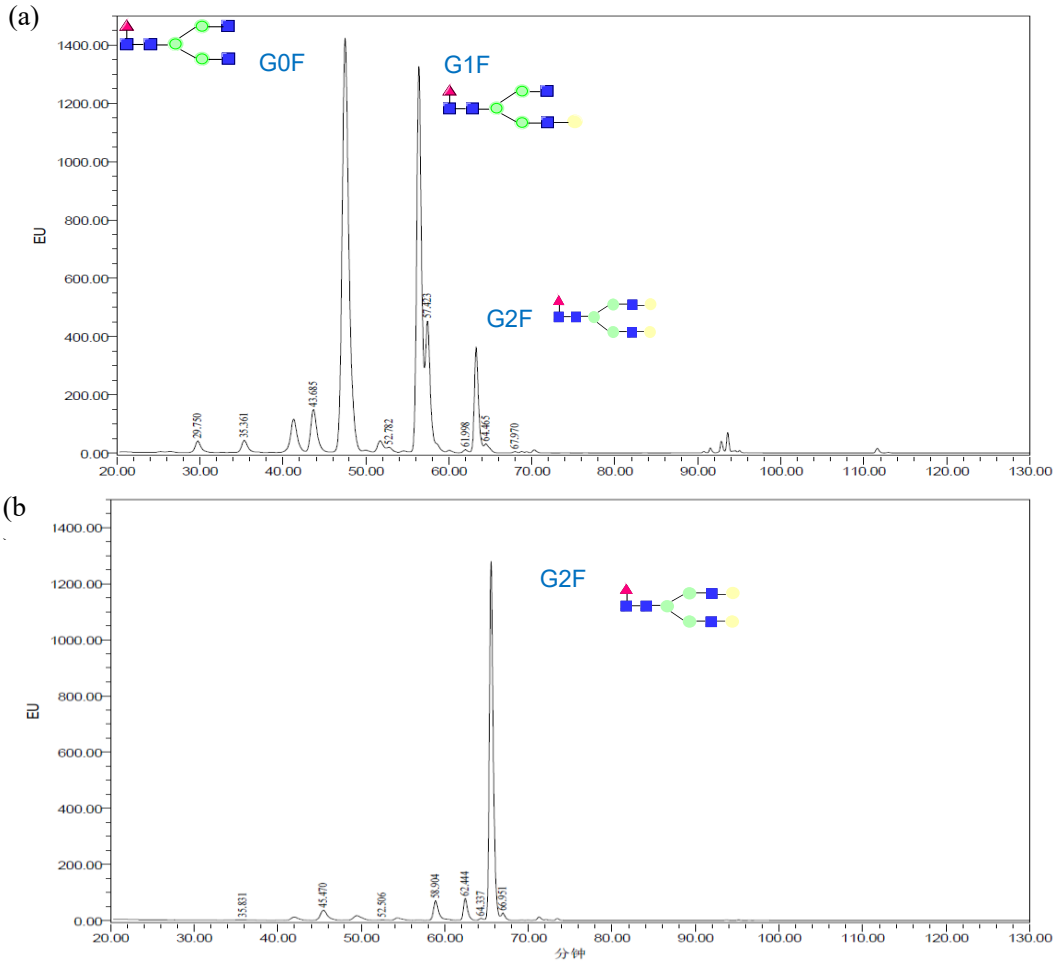


Fig. S4. 2-AB HILIC HPLC analysis of the glycoforms of Herceptin galactosylation. (a) Commercial Herceptin before the galactosylation reaction. (b) Herceptin after being catalyzed by β-1,4-galactosyltransferase for 48 h.

Psp2,6ST (15-501)-His$_6$ catalyzed one-pot three-enzyme sialylaion of Herceptin

The activity assay involving the three enzymes (Pmaldolase, NmCSS and Psp2,6ST(15-501)-His$_6$) was performed with ManNAc and lactose as substrates, the sialylated product was monitored using TLC (thin-layer chromatography) plate visualized by anisaldehyde sugar stain after dry.[3, 4] Afterward, the enzymes were employed to the one-pot three-enzyme sialylation of Herceptin. The reactions were performed as follows: ManNAc or its derivatives (final concentration 10 mM), sodium pyruvate (5 equiv.) and CTP (3 equiv.) were dissolved in Tris-HCl buffer (200 mM, pH 8.0, 50-100 μL) containing 20 mM $MgCl_2$, and the pH of the concentrated CTP solution was pre-adjusted to 7.5 with 2 M NaOH. Subsequently, PmAldolase (final concentration 20 μg/mL), NmCSS (final concentration 10 μg/mL), Psp2,6ST(15-501)-His$_6$ (final concentration 0.4 mg/mL) and Herceptin (final concentration 10 mg/mL) were added. The reaction was incubated at 37 °C, and the equivalent of donor mixture (excluding the sialyltransferase and Herceptin) was added at every 2 h intervals, followed by ultrafiltration every 6 h. By this means, the level of IgG sialylation can be raised gradually (Fig. S5). Finally, the highly di-sialylated Herceptin (≥80%) was acquired after 18 cycles of ultrafiltration (Fig. S6). The donor substrate promiscuity of Psp2,6ST(15-501)-His$_6$ was investigated with modified ManNAc derivatives (Fig. S7).

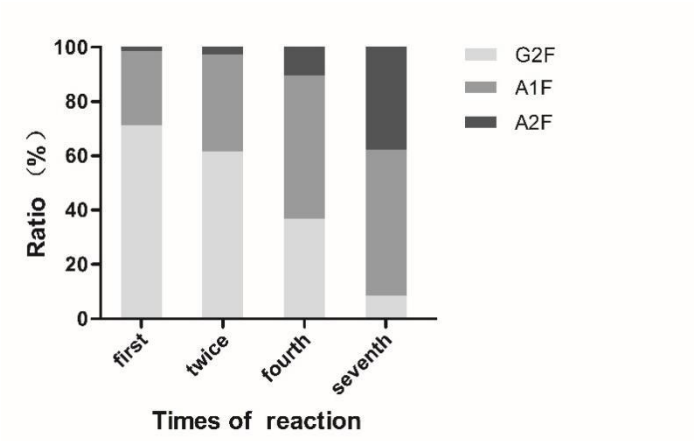


Fig. S5. Periodical ultrafiltration increased the level of Herceptin sialylation. Quantitative glycoforms distribution was determined by 2-AB HILIC HPLC. Additional reaction for 6 h was followed by the ultrafiltration at every 6 h interval and thrice addition of donor in every reaction at 37 °C.

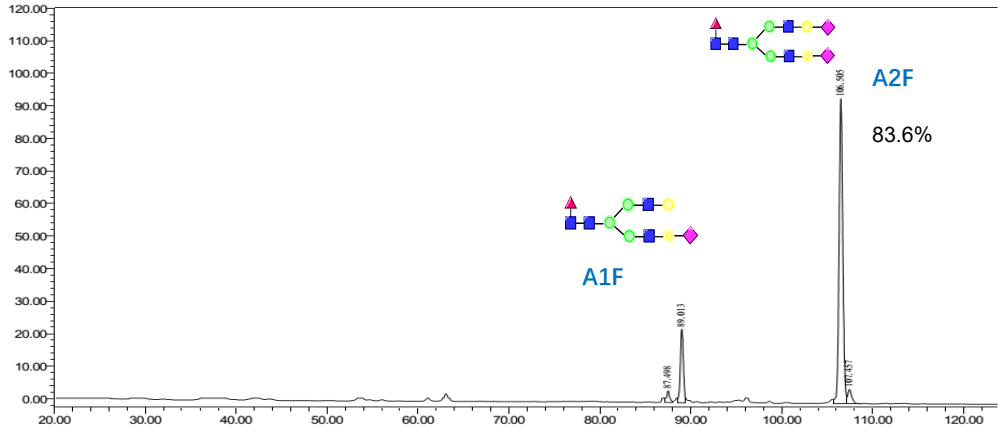


Fig. S6. The 2-AB HILIC HPLC quantitative analysis of Herceptin after 18 cycles of ultrafiltration catalyzed by Psp2,6ST(15-501)-His_6.

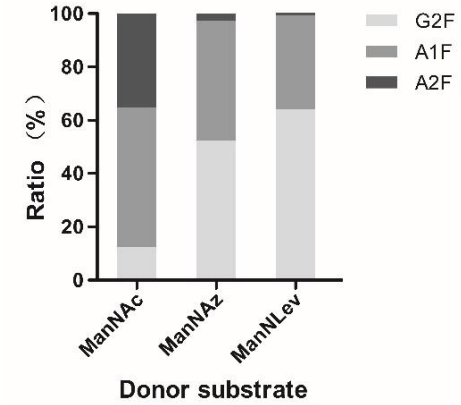


Fig. S7. The donor substrate promiscuity of Psp2,6ST (15-501)-His_6 examined with modified ManNAc derivatives. The 2-AB HILIC HPLC analyzed quantitatively the glycoforms in the OPTE reaction with individual ManNAc derivatives as substrate after 7 cycles of ultrafiltration at every 6

h reaction.

Optimizing the reaction conditions of Psp2,6ST (111-511)-His_6

In general, the 60 μL of donor mixture were prepared for each mutant, including 80 mM sodium phosphate (pH 7.0-7.5), 40 mM $MgCl_2$, 25 mM ManNAc (1 equiv.), 125 mM sodium pyruvate (5 equiv.), 75 mM CTP (3 equiv.), Pmaldolase (20 μg/mL) and NmCSS (10 μg/mL), an aliquot (20 μL) of the mixture was added to the 20 μL of Herceptin (20 mg/mL) solution containing an individual variant (0.2 mg/mL), and the equivalent addition of donor mixture was repeated twice every 2 h, the reactions were conducted under distinct conditions.

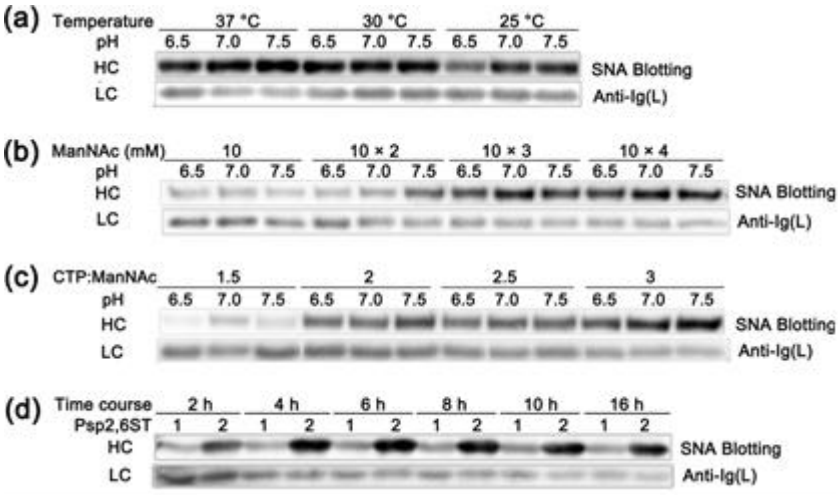


Fig. S8. Optimizing the reaction conditions of the truncated variant Psp2,6ST (111-511)-His_6 A366G (c-511) with Herceptin as substrate acceptor in OPTE system. The conditions: (a) temperature and pH value; (b) the times of equivalent addition of donor mixture at 37 ºC; (c) the ratio of CTP/ManNAc; (d) the time course of sialylation reaction. 1: Psp2,6ST (15-501)-His_6 A366G, 2: Psp2,6ST (111-511)-His_6 A366G.

Site-directed mutagenesis

Mutagenesis based on the protein crystal structure of Δ16Psp2,6ST (Fig. S9). Sialyltransferase activity-based screening of truncated mutants with Herceptin as substrate receptor in OPTE system at 37 ºC, the activities of mutants detected by SNA lectin blotting (Fig. S10).

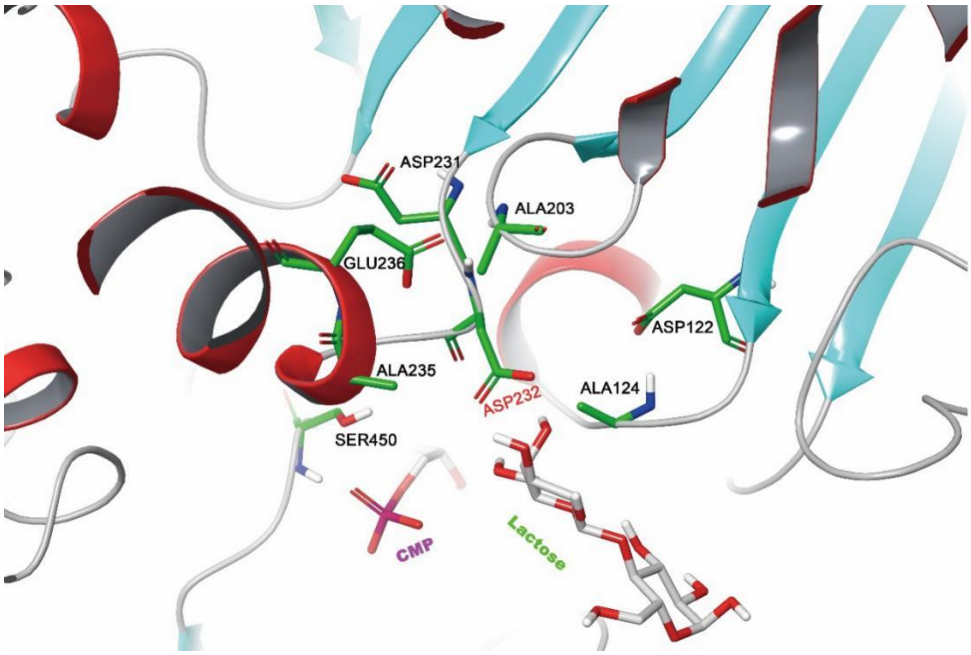


Fig. S9. Protein crystal structure guided the mutagenesis of Psp2,6ST (PDB id 2Z4T). The mutation sites are shown in green thick lines, surrounding the catalytic base D232. CMP and lactose were shown as sticks.

Table S1. Primers Used for Site-Directed Mutagenesis

mutant	Primer Sequence		Template
A235G	Forward 1	5’ CTCACCGCTGCCGTCATCGTACAGGTCAATGTTG-3’	Wild type
	Reverse 1	5’ GATGACGGCAGCGGTGAGTACGTTAACCTGTAT-3’	
A235H	Forward 2	5’ CTCATGGCTGCCGTCATCGTACAGGTCAATGT-3’	Wild type
	Reverse 2	5’ GATGACGGCAGCCATGAGTACGTTAACCTG-3’	
A235N	Forward 3	5’ CTCATTGCTGCCGTCATCGTACAGGTCAATGT-3’	Wild type
	Reverse 3	5’ GATGACGGCAGCAATGAGTACGTTAACCTG-3’	
A235D	Forward 4	5’ CTCATCGCTGCCGTCATCGTACAGGTCAATGT-3’	Wild type
	Reverse 4	5’ GATGACGGCAGCGATGAGTACGTTAACCTG-3’	
A235R	Forward 5	5’ CTCACGGCTGCCGTCATCGTACAGGTCAATGT-3’	Wild type
	Reverse 5	5’ GATGACGGCAGCCGTGAGTACGTTAACCTGTAT-3’	
A235S	Forward 6	5’ CTCACTGCTGCCGTCATCGTACAGGTCAATGT-3’	Wild type
	Reverse 6	5’ GATGACGGCAGCAGCGAGTACGTTAACCTGTAT-3’	
A235K	Forward 7	5’ CTCTTTGCTGCCGTCATCGTACAGGTCAATGT-3’	Wild type
	Reverse 7	5’ GATGACGGCAGCAAAGAGTACGTTAACCTGTAT-3’	

A235M	Forward 8	5’ CTCCATGCTGCCGTCATCGTACAGGTCAATGTTG-3’	Wild type
	Reverse 8	5’ GATGACGGCAGCATGGAGTACGTTAACCTGTAT-3’	
A235C	Forward 9	5’ CTCGCAGCTGCCGTCATCGTACAGGTCAATGTTG-3’	Wild type
	Reverse 9	5’ GATGACGGCAGCTGCGAGTACGTTAACCTGTAT-3’	
A235Q	Forward 10	5’ CTCCTGGCTGCCGTCATCGTACAGGTCAATGT-3’	A235N
	Reverse 10	5’ GATGACGGCAGCCAGGAGTACGTTAACCTG-3’	
A235T	Forward 11	5’ CTCAGTGCTGCCGTCATCGTACAGGTCAATGTTG-3’	Wild type
	Reverse 11	5’ GATGACGGCAGCACTGAGTACGTTAACCTGTAT-3’	
A203S	Forward 12	5’ ACTGGTGTTGCTGCTCAGCTTCACGTTCAG-3’	Wild type
	Reverse 12	5’ AGCAGCAACACCAGTCACAGCTTCGATAAC-3’	
A203V	Forward 13	5’ AACGGTGTTGCTGCTCAGCTTCACGTTCAG-3’	Wild type
	Reverse 13	5’ AGCAGCAACACCGTTCACAGCTTCGATAAC-3’	
A203N	Forward 14	5’ TAAGGTGTTGCTGCTCAGCTTCACGTTCAG-3’	Wild type
	Reverse 14	5’ AGCAGCAACACCAATCACAGCTTCGATAAC-3’	
A203G	Forward 15	5’ ACCGGTGTTGCTGCTCAGCTTCACGTTCAG-3’	Wild type
	Reverse 15	5’ AGCAGCAACACCGGTCACAGCTTCGATAAC-3’	
A203C	Forward 16	5’ ACAGGTGTTGCTGCTCAGCTTCACGTTCAG-3’	Wild type
	Reverse 16	5’ AGCAGCAACACCTGTCACAGCTTCGATAAC-3’	
A203T	Forward 17	5’ TGTGGTGTTGCTGCTCAGCTTCACGTTCAG-3’	Wild type
	Reverse 17	5’ AGCAGCAACACCACACACAGCTTCGATAAC-3’	
A203D	Forward 18	5’ ATCGGTGTTGCTGCTCAGCTTCACGTTCAG-3’	Wild type
	Reverse 18	5’ AGCAGCAACACCGATCACAGCTTCGATAAC-3’	
S450T	Forward 19	5’ AAAATACAGTGTGCTCGCGATACCCGCCAC-3’	Wild type
	Reverse 19	5’ GCGAGCACACTGTATTTTACCATTCCGGCG-3’	
A235N/A203S	Forward 12/ Reverse 12		A235N
	Forward 3/ Reverse 3		A203S
A235N/A203C	Forward 16/ Reverse 16		A235N
	Forward 3/ Reverse 3		A203C

A235N/A203D	Forward 18/ Reverse 18	A235N
	Forward 3/ Reverse 3	A203D
A235N/A203N	Forward 14/ Reverse 14	A235N
	Forward 3/ Reverse 3	A203N
A203S/S450T	Forward 12/ Reverse 12	A203S
	Forward 19/ Reverse 19	S450T

The mutation sites were underlined

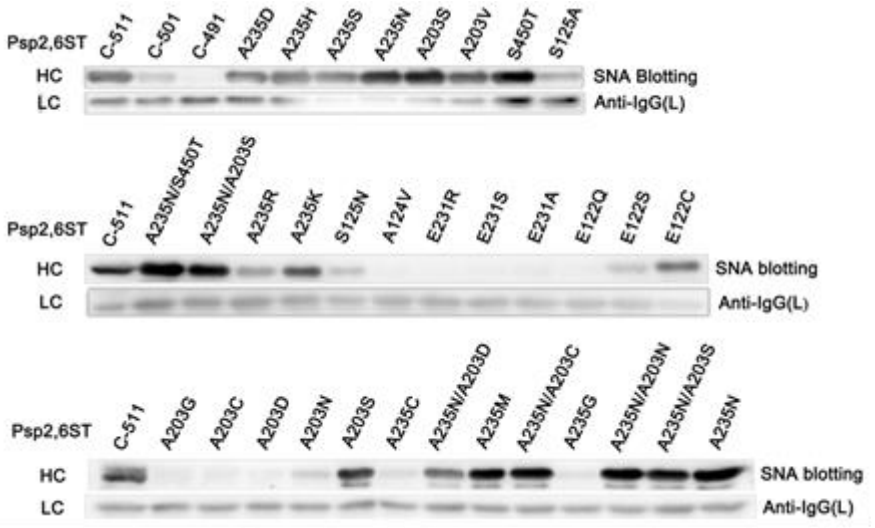


Fig. S10. Preliminary screening based activities of truncated mutants with Herceptin as acceptor in OPTE system at 37 ºC. The sialylation level of Herceptin was detected by SNA lectin blotting with Psp2,6ST (111-511)-His_6 A366G (C-511) as the wild type control.

Comparative study on the donor hydrolysis activity of the Psp2,6ST mutants

To evaluate the effects of truncations and mutations on the donor hydrolysis activity, the hydrolysis activity assay of each variant or mutant was performed in a total volume of 50 μL containing the CMP-Neu5Ac (2 mM) donor and the individual Psp2,6ST mutant (about 1 ng/μL) in sodium phosphate buffer (50 mM, pH 6.5) at 37 °C for 20 min in the absence of acceptor substrate. Ten-fold volume of ice-water was added immediately on ice to stop the reaction. An aliquot (100 μL) of the supernatant via centrifugation (13,000 g for 15 min) was taken out to load onto an Agilent 1260 Infinity HPLC system with a reverse-phase column (Zorba SB-C18 StableBond (4.6mm × 250 mm, Aglient)) preceded by a Zorbo SB-C18 analytical guard column (4.6 mm × 12.50 mm, Aglient), the distinct compositions were separated at a flow rate of 1 mL/min with the following gradient: 100% mobile phase A (50 mM KH_2PO_4/K_2HPO_4 and 4 mM *tetra*-butylammonium hydrogen sulfate at pH 7.0) for 10 min; 0% to 30% mobile phase B (methanol) for 10 min; 30% phase B for 10 min. Eluted CMP and CMP-Neu5Ac were monitored by absorbance at 270 nm with commercial CMP and CMP-Neu5Ac as standards. The yields were quantified based on the integrated areas of CMP product and CMP-Neu5Ac substrate, as follows (Fig. S11).

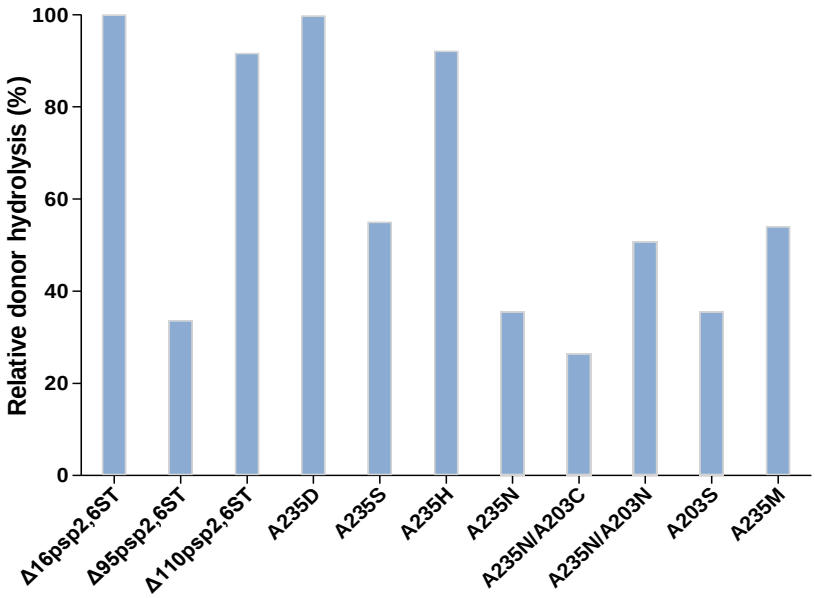


Fig. S11. Comparative study of donor (CMP-Neu5Ac) hydrolysis of various Psp2,6ST variants at 37 °C, detected by HPLC.

The temperature dependence of sialylation reactivity of Psp2,6ST truncated mutants

The reaction system was same as mentioned above. An aliquot of the reaction solution under distinct temperature was taken and cooled immediately at – 20 °C at various time point for Western blot analysis.

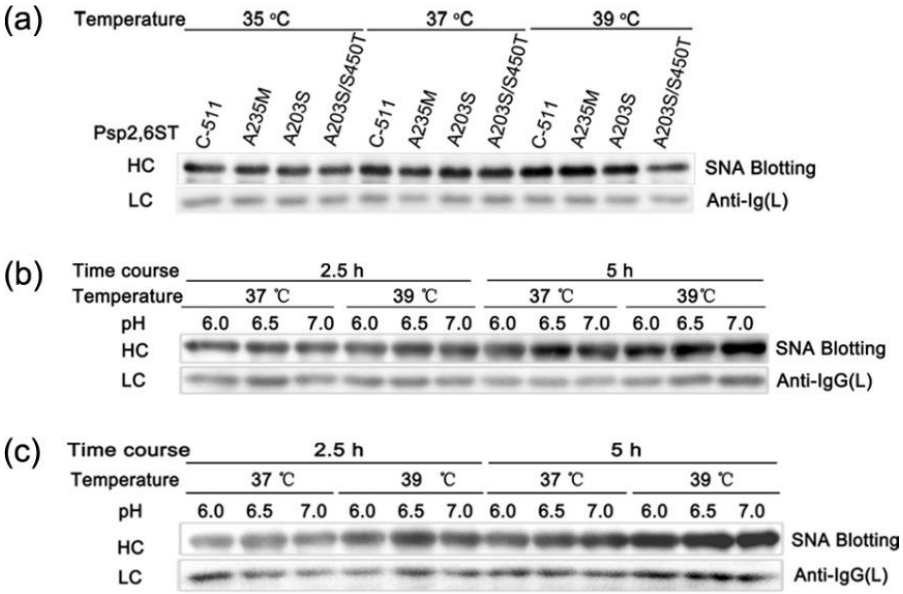


Fig. S12. Temperature impacts on the catalytic activities of Psp2,6ST truncated mutants in OPTE system in sodium phosphate buffer. (a) The activities of truncated mutants under the varying temperature in sodium phosphate (pH = 7.5) for 4 h; (b) the catalysis of A203S when varying pH, time and temperature; (c) the catalysis of A235M when varying pH, time and temperature.

Optimizing the sialylation reaction of Herceptin catalyzed by Psp2,6ST (111-511) A366G/A235M

The reactions catalyzed by the truncated mutant A366G/A235M were optimized, including higher temperature (Fig. S13 A), pH value (Fig. S13 B), the modes of donor addition (Fig. S13 C) and the time course: 4 h, 6 h, 8 h (Fig. S13 D).

In general, the donor reaction system was first prepared in a total volume of 60 μL containing 80 mM sodium phosphate (pH 7.0-7.5), 30 mM $MgCl_2$, 20 mM ManNAc (1 equiv.), 100 mM sodium pyruvate (5 equiv.), 60 mM CTP (3 equiv., pH 7.0), Pmaldolase (10-20 μg/mL) and NmCSS (5-10 μg/mL), an aliquot (20 μL) of the donor mixture was added every 2 h in thrice to the 20 μL of Herceptin (20-40 mg/mL) solution in the presence of the truncated mutant A366G/A235M (0.1 mg/mL) at 39 °C.

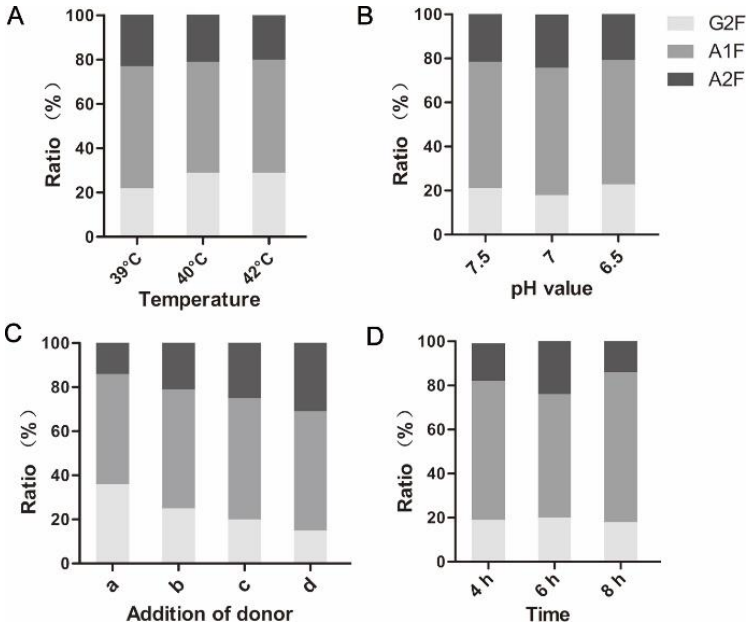


Fig. S13. Impacts of reaction conditions on the sialylation level of Herceptin catalyzed by Psp2,6ST (111-511) A366G/A235M at 39 °C, which were determined by 2-AB HILIC HPLC. (A) The degree of Herceptin sialylation at higher temperature than 39 °C for 4 h. (B) The degree of sialylation at different pH value for 4 h. (C) The degree of sialylation affected by the modes of donor addition: a) an aliquot (30 μL) of the donor mixture was added to 20-25 μL of Herceptin（20 mg/mL）in once addition; b) two aliquots of the donor mixture in once addition; c) two aliquots of the donor mixture in twice addition; d) two aliquots of the donor mixture in thrice addition. (D) The degree of sialylation at the different time course.

The sialidase activity assay of the Psp2,6ST(111-511)-His_6 A366G/A235M

The sialidase activity of this enzyme was tested in a volume of 20 μL containing 50 mM sodium phosphate (pH = 7.0), 20 mg/mL sialylated Herceptin (A1F-1,3 and A2F) and the enzyme or without enzyme at 39 °C for 4 h. Subsequently the glycoforms were analyzed by 2-AB HILIC-HPLC respectively. The results showed that the sialidase activity of the enzyme is very low for both sialylated substrates (A1F-1,3 or A2F) when excluding the generation of CMP.

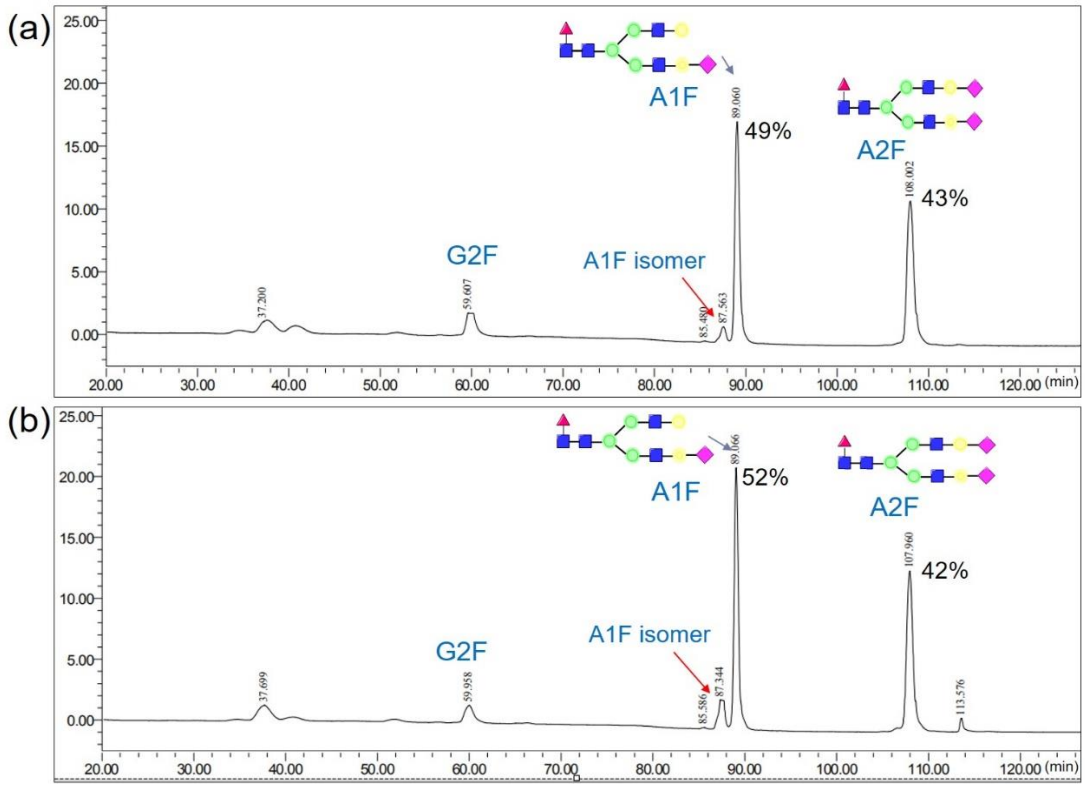


Fig. S14. The sialidase activity of Psp2,6ST(111-511)-His_6 A366G/A235M tested with mono-sialylated and di-sialylated Herceptin as the substrates. (a) The glycoforms in the absence of the enzyme (control). (b) The glycoforms in the presence of the enzyme.

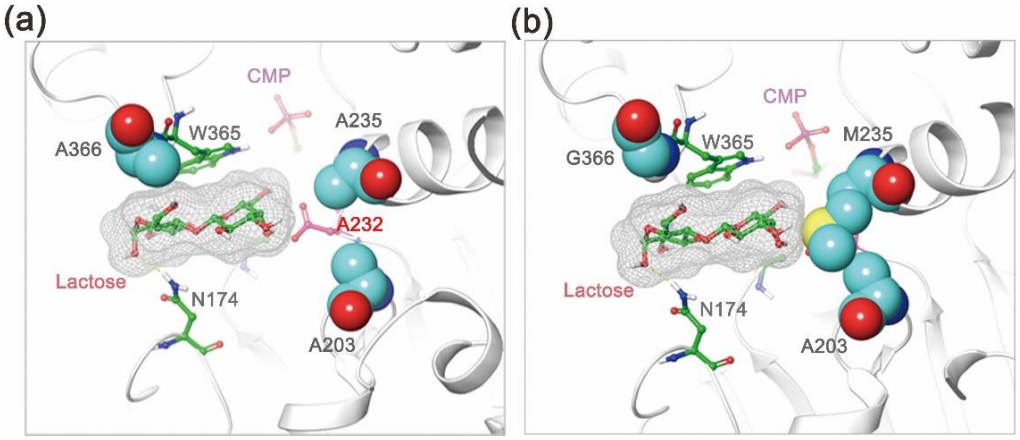


Fig. S15. Conformation change induced by Psp2,6ST mutant A366G/A235M based on protein crystal structure. (a) Active site region of the wild type Psp2,6ST complex with CMP and lactose (PDB ID: 2Z4T); (b) the substrate binding region of the double mutant A366G/A235M. Substrates and residues in proximity to the receptor binding pocket are drawn using green thick lines. The introduced A235M/ A366G mutations with A203 are shown in light blue surface mode.

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