Supplementary Information for:

Harnessing Acylhydrazone-oxime Exchange Reaction to Achieve Diverse Synthesis of Glycosite-specific Antibody-Drug Conjugates

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1. General information

Abbreviations

r.t.: Room temperature

h: hour

DMF: Dimethylformamide

TFA: Trifluoroacetic acid

HATU: Hexafluorophosphate azabenzotriazole tetramethyl uranium

DIPEA: N,N-Diisopropylethylamine

Et_3N: Triethylamine

CDMBI: 2-chloro-1,3-dimethyl-1H-benzimidazol-3-ium chloride

Materials and methods

Endo-S2[1] and Endo-S D233Q[2] were expressed in E. coli following the reported procedure. NHS, Fmoc-Lys-OH were purchased from bidepharm (Shanghai, China). 2-Azidoacetic acid was purchased from Sigma-Aldrich (Shanghai, China). MMAE was purchased from Resuperpharmtech (Shanghai, China). NH_2-VC-PAB-MMAE was synthesized following previously reported procedure[3]. Trastuzumab was purchased from Roche (Shanghai, China). CHO-LacNAc, GN-trastuzumab was get following the reported procedure[4]. CHO-SCT was extracted, purified, and prepared from egg yolk following the reported procedure[4]. Protein-A was purchased from yeasen (Shanghai, China). Centrifugel filtration was purchased from Millipore Corporation. Other chemical reagents and solvents were purchased from Sinopharm Chemical Reagent Co. (Shanghai, China). Nuclear magnetic resonance (NMR) spectra were measured on a Varian- MERCURY Plus-400 or 500 instrument.

Cell culture

SK-BR-3, NCI-N87 and MDA-MB-231 cell lines were obtained from Cell Bank of Chinese Academy of Sciences (Shanghai, China). All cell cultures were grown in RPMI-1640 medium supplemented with Penicillin (50 units/mL), Streptomycin (50 μ/mL) and 10% FBS (fetal bovine serum) and were incubated in a water-saturated cell incubator (Thermo Scientific) at 37 ℃ under 5% CO_2.

High Performance Liquid Chromatography (HPLC).

Method A: Analytical RP-HPLC was performed on a Thermo ultimate 3000 instrument with a C18 column (Agilent, 4 μm, 4.6 x 150 mm) at 40 ℃. The column was eluted with a linear gradient of 2-60.7% acetonitrile containing 0.25% TFA in 20 min at a flow rate of 1 mL/min.

Method B: Semi-preparative HPLC was performed on a Beijing ChuangXinTongHeng LC3000 (preparative) instrument with a preparative C-18 column (Waters, 5 μm, 19 x 250mm). The column was eluted with a suitable gradient of aqueous acetonitrile containing 0.1% TFA or 0.1% $NH_3 \cdot H_2O$ at a flow rate of 8 mL/min.

Electron Spray Ionization Mass Spectrometry (ESI-MS).

Electrospray ionization mass spectrometry (ESI-MS) was performed on a Waters Xevo G2-XS Q-TOF. **Method A:** mobile phase A = water containing 0.1% formic acid; mobile phase B = acetonitrile containing 0.1% formic acid; gradient 0 - 1.3 min, 10 %-70% phase B; flow rate = 0.3 mL/min. Detecting absorbance is 214 nm. **Method B:** mobile phase A = water containing 0.1% formic acid; mobile phase B = water containing 0.1% formic acid; gradient 0-4 min, 5% - 90% phase B; flow rate = 0.3 mL/ min. Detecting absorbance is 280 nm. The small molecules were analyzed using a Waters C18 column (ACQUITY UPLC BEH C18, 1.7 μm, 2.1x50 mm) with method A. Mass spectra of small molecule were recorded in the mass range of 50-3000 under high resolution mass-spec mode (HRMS, standard 3200 m/z, 4 GHz). Key source parameters: Cone Gas of 50 L/h; Desolvation Gas of 800 L/h; source temperature of 120 ℃; Desolvation temperature of 200 ℃; Capillary voltage of 3000 V; Collision Cell RF Offset of 150 V; Collision Cell RF Gain of 10. The proteins samples were measured with a Waters C4 column (ACQUITY UPLC Protein BEH C4, 1.7 μm, 2.1 mm x 50 mm) with method B at 80 ℃. The mass spectra of proteins were performed under the extended mass range mode (High 20,000 m/z, 1 GHz) and the data were collected in the mass range of 500-3500. Key source parameters: Cone Gas of 50 L/h; Desolvation Gas of 800 L/h; source temperature of 120 ℃; Desolvation temperature of 600 ℃; Capillary voltage of 3000 V; Collision Cell RF Offset of 600 V; Collision Cell RF Gain of 0.

Calculation of DAR and yield for modified antibody and ADC

According to the signal response of the mass spectrum, different modification ratio was caculated, X% for starting antibody, Y% for one modification, Z% for two modifications(X+Y+Z=100). The DAR is equal to [(0 * X%) + (1* Y%) + (2 * Z%)]. The yield is equal to {[(0 * X%) + (1* Y%) + (2 * Z%)]/2}.

Purification for modified antibody and ADC

LC–MS monitoring indicated the complete transglycosylation. The antibody/ADC was subjected to protein A-agarose resin (2 mL) that was pre-equilibrated with a phosphate buffer (0.2 mol/L, pH 7.4). The column was washed with phosphate buffer (0.2 mol/L, pH 7.4, 6 mL) and phosphate buffer (0.2 mol/L, pH 5.0, 6 mL) respectively to remove the impurities. The bound IgG was released with glycine-HCl (100 mmol/L, pH 2.7, 1.6 mL) and the elution fractions were immediately neutralized with Tris-HCl buffer(0.2 mol/L, pH 8.0). The fractions containing the antibody component were combined and concentrated by centrifugal filtration (Millipore) to give the corresponding antibody/ADC.

Hydrophobic interaction chromatography (HIC) analysis

20 μg of each sample was dissolved in 20 μL of PBS. HIC analysis was performed with a Butyl-NPR column (MAbPac, 4.6x10 cm, 5 μm particle size). **Method:** mobile phase A = 50 mM sodium phosphate containing ammonium sulfate (1.5 M), 5% isopropanol, pH 7.0; mobile phase B = 50 mM sodium phosphate, 20% isopropanol, pH 7.0; gradient 0-25 min, 20-100% phase B; flow rate = 0.6 mL/min. Detecting absorbance is 280 nm.

Aggregation stability (SEC analysis)

Method: 200 μg of each sample was dissolved in 100 μL of PBS and heated at 37 ℃ for 28 days. Aliquots were taken at intervals and were analyzed by a SEC-300 column (BioCore, 7.8x300 mm, 5 μm) with a gradient of 100% mobile phase (150 mM Sodium phosphate, pH 6.8) in 15 min. 280 nm was set as the wavelength of detecting absorbance.

***In vitro* efficiency and cytotoxicity**

The MTT assay was performed to measure the in vitro efficiency of site-specific ADCs. HER2 positive tumor cell lines (SK-BR-3, NCI-N87) and HER2 negative tumor cell lines (MDA-MB-231) were cultured in a 10% FBS-containing RPMI 1640 medium and were planted into 96-well plates with 6000 cells per well. The plates were incubated overnight at 37 °C in a 5% CO_2 cell incubator. All ADCs were diluted 5-fold with RPMI 1640 medium from an initial concentration of 100 nmol/L, totally nine concentration gradients. The ADC samples were added to three wells with every single concentration

and the 96-well plates were cultured at 37 °C in a 5% CO_2 cell incubator for three days. Then MTT solution was added and incubated at 37 °C for 4 h, followed by adding 10% SDS solution to dissolve the formazan. Optical density (OD) value was measured at 570 nm using an BioTek Epoch. EC50 values and the cell viability curve were calculated by GraphPad software.

***In vivo* anti-tumor activity**

Five-weeks old female athymic BALB/c nu/nu mice were purchased from Shanghai Institute of Materia Medica and randomly divided into groups of six mice each. Animal handling and procedures were approved and performed according to the requirements of the Institutional Animal Care and Use Committee (IACUC) of Shanghai Institute of Materia Medica, CAS (license: SYXK (Shanghai) 2015-0027 and 2019-0032). All xenograft models were established by s.c. inoculation in the flanks of the mice. NCI-N87 cells were established by injecting 3×10^6 cells suspended in a Matrigel matrix. After tumor volume reached about 150 mm^3, tumor-bearing mice were randomized based on tumor volumes. The xenograft mice were injected i.p. with each ADC sample or PBS at a dose of 1 mg/kg, dosing on Days 1, 8, and 15. The tumor volume was calculated by formula: length × $width^2$ × 1/2. The in vivo activity was calculated by GraphPad software.

2. Supplementary figures

2.1 Glycoengineered antibodies based on acylhydrazone-functionalized LacNAc derivatives

A solution of wild-type trastuzumab (5 mg/mL) and acylhydrazone-functionalized LacNAc oxazolines (**G1**-**G6**, 0.5 mM) in 50 mM PB, pH 7.0 was incubated with Endo-S2 (0.4 mg/mL) at 30 °C for 2 h, the reaction mixture was monitored by LC-MS.

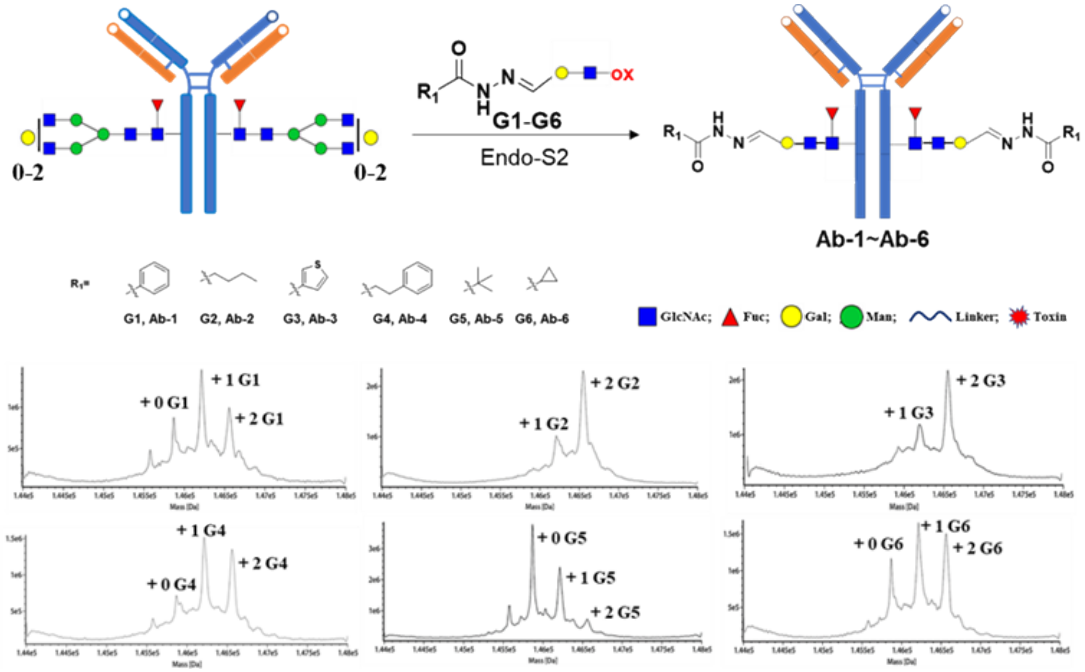


Figure S1. LC-MS profiles of glycoengineered antibodies **Ab-1**~**Ab-6** synthesized with **G1**-**G6** substrates.

2.2 Exploring Endo-S2 concentration conditions for G2 and G3 transglycosylation

A solution of wild-type trastuzumab (5 mg/mL) and acylhydrazone-functionalized LacNAc oxazolines (**G2**, **G3**, 0.5 mM) in 50 mM PB, pH 7.0 was incubated with Endo-S2 (0.64-400 μg/mL) at 30 °C for 2h, the reaction mixture was monitored by LC-MS.

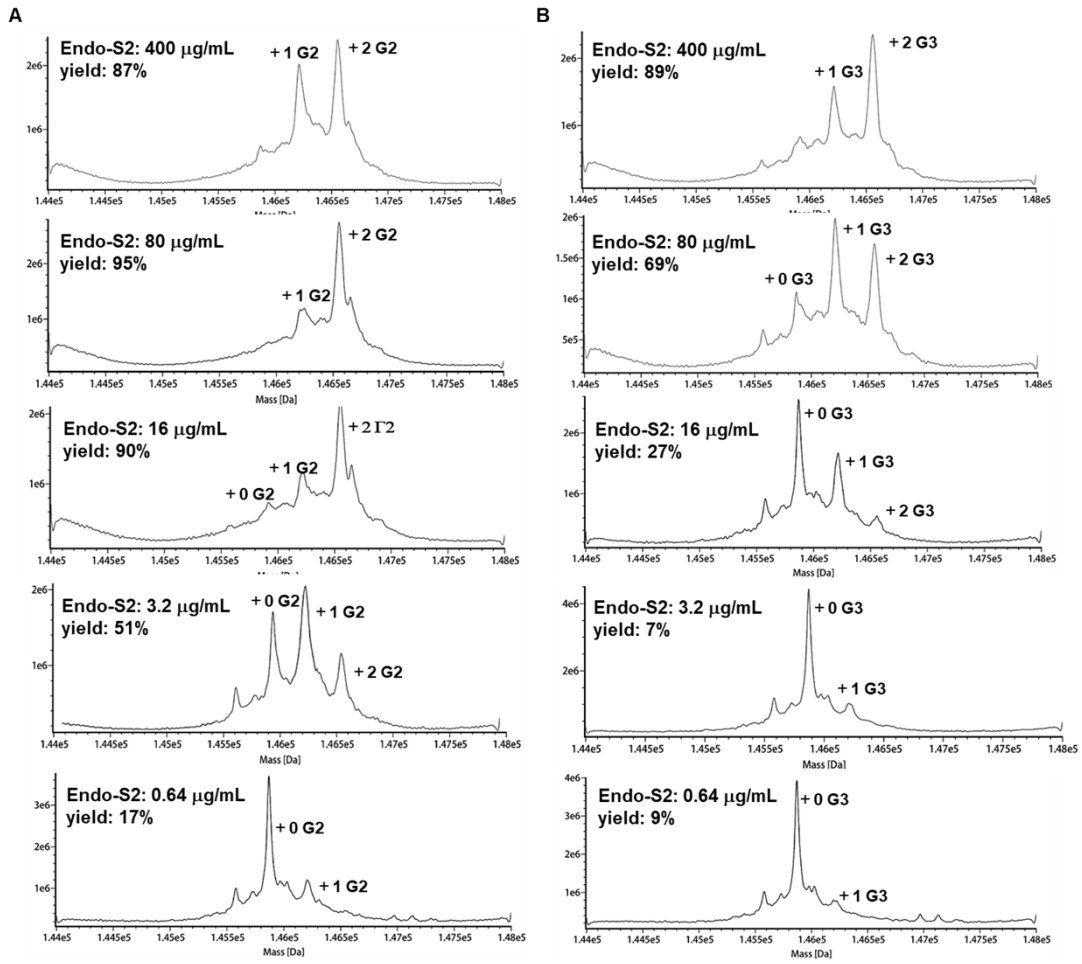


Figure S2. Endo-S2 concentration optimization of transglycosylation reaction using **G2** and **G3** as substrates. A), LC-MS profiles of **G2**-involved transglycosylation reaction under different Endo-S2 concentrations; B), LC-MS profiles of **G3**-involved transglycosylation reaction under different Endo-S2 concentrations. Note: DAR value was calculated by LC-MS data.

2.3 Exploring the hydrolytic activity of Endo-S2

A solution of wild-type trastuzumab (5 mg/mL) and acylhydrazone-functionalized LacNAc oxazolines (**G2**, **G3**, 0.5 mM) in 50 mM PB, pH 7.0 was incubated with Endo-S2 (400 μg/mL) at 30 °C, the reaction mixture was monitored by LC-MS at 2 h/4 h/8 h.

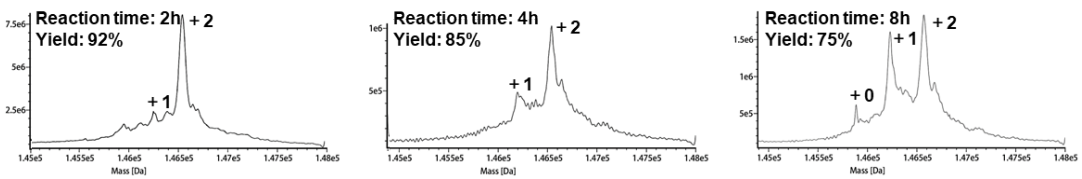


Figure S3. Transglycosylation efficiency at different time.

2.4 Exploring equivalent about G2 and G3 transglycosylation

A solution of wild-type trastuzumab (5 mg/mL) and acylhydrazone-functionalized LacNAc oxazolines (**G2**, **G3**, 0.16-0.5 mM) in 50 mM PB, pH 7.0 was incubated with Endo-S2 (**G2**: 80 μg/mL, **G3**: 400μg/mL) at 30 °C for 2 h, the reaction mixture was monitored by LC-MS.

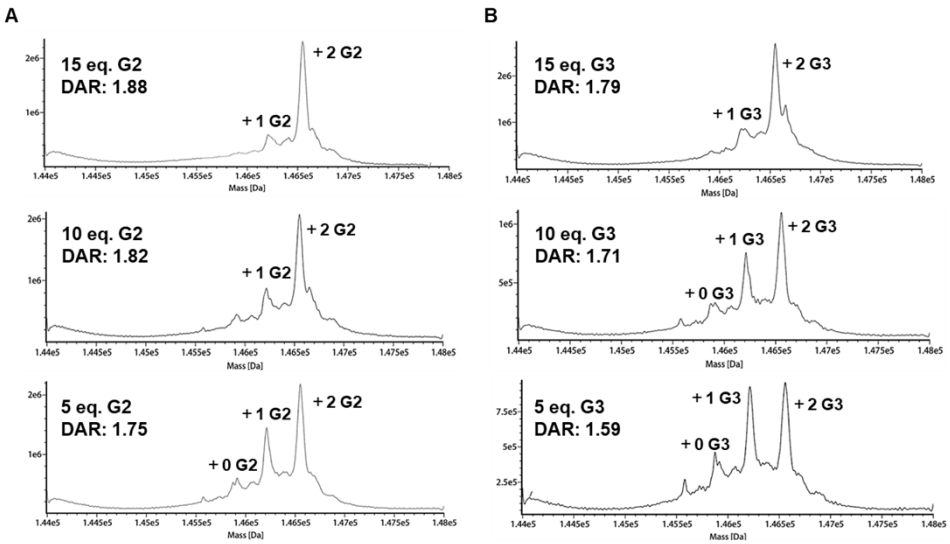


Figure S4. Optimization of LacNAc derivatives equivalents. A), LC-MS profiles of **G2**-involved transglycosylation reaction; B), LC-MS profiles of **G3**-involved transglycosylation reaction. Note: DAR value was calculated by LC-MS data.

2.5 Exchange reaction between Ab-2/Ab-3 with *O*-benzylhydroxylamine

A solution of glycoengineered antibody (**Ab-2** or **Ab-3**, 5 mg/mL) in 50 mM PB with pH range 3.0-6.0 was incubated with *O*-benzylhydroxylamine (0.67 mM) at 30°C for 8 h, the reaction mixture was monitored by LC-MS.

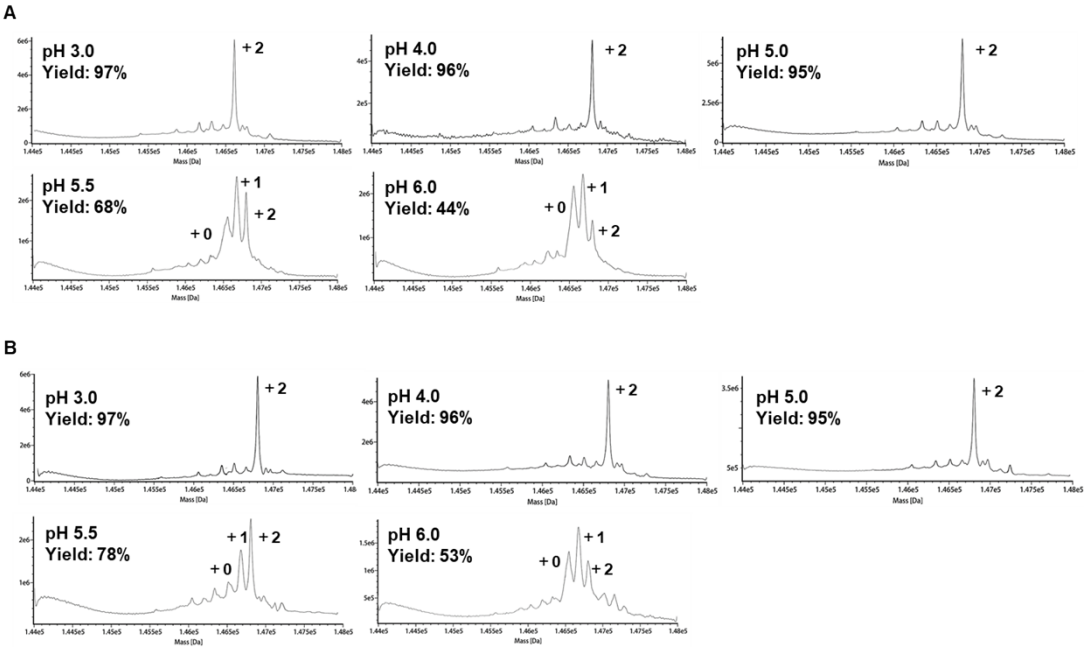


Figure S5. Exchange efficiency of **Ab-2** or **Ab-3** under different pH values. A), LC-MS profiles of exchange reaction based on **Ab-2** and *O*-benzylhydroxylamine under pH ranges 3.0-6.0; B), LC-MS profiles of exchange reaction based on **Ab-3** and *O*-benzylhydroxylamine under pH ranges 3.0-6.0.

2.6 Exchange reaction by exploring *O*-benzylhydroxylamine equivalent

A solution of glycoengineered antibody (**Ab-2** or **Ab-3**, 5 mg/mL) in 50 mM PB, pH 4.0 was incubated with *O*-benzylhydroxylamine (0.16-0.67 mM, 5-20 equivalents) at 30°C for 8 h, the reaction mixture was monitored by LC-MS.

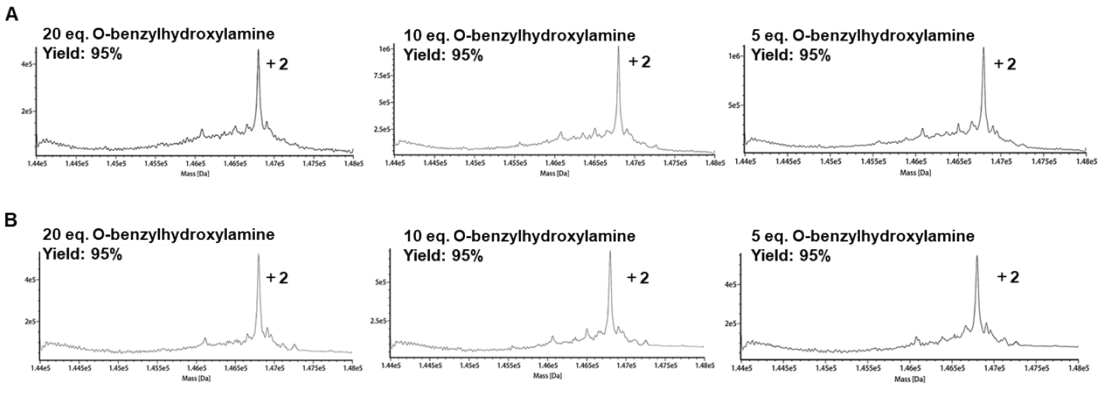


Figure S6. Exchange efficiency of **Ab-2** or **Ab-3** with different *O*-benzylhydroxylamine equivalents. A), LC-MS profiles of exchange reaction based on **Ab-2** with *O*-benzylhydroxylamine 5-20 equivalents; B), LC-MS profiles of exchange reaction based on **Ab-3** with *O*-benzylhydroxylamine 5-20 equivalents.

2.7 Exchange reaction in different buffer systems

A solution of glycoengineered antibody (**Ab-2** or **Ab-3**, 5 mg/mL) in 50 mM buffer (pH 5.5) was incubated with *O*-benzylhydroxylamine (0.33 mM) at 30°C for 24 h, the reaction mixture was monitored by LC-MS.

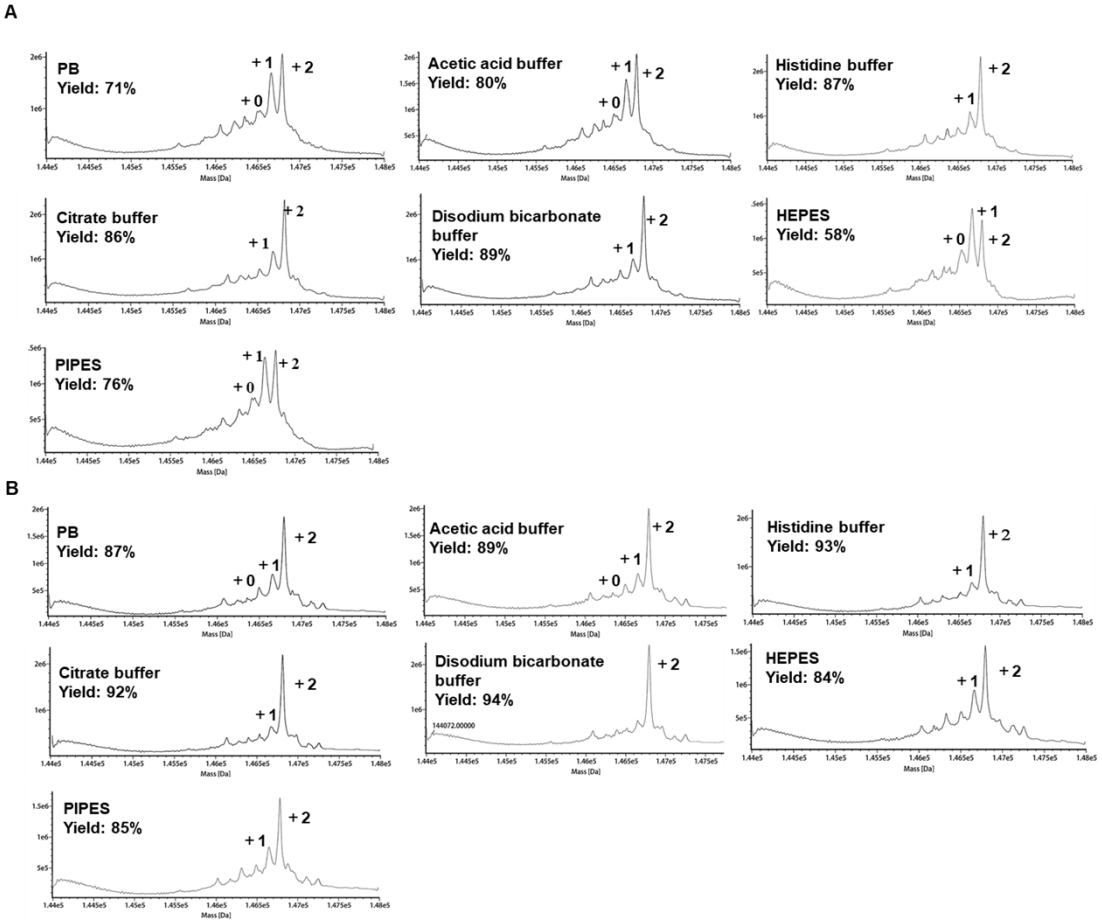


Figure S7. Exchange efficiency of **Ab-2** or **Ab-3** in different reaction buffers. A), LC-MS profiles of exchange reaction based on **Ab-2** and *O*-benzylhydroxylamine in different reaction buffers; B), LC-MS profiles of exchange reaction based on **Ab-3** and *O*-benzylhydroxylamine in different reaction buffers.

2.8 Applied on fluorescent antibody

Ab-2 (5 mg/mL) and **FITC-ONH$_2$** (0.68 mM) in 50 mM His-HCl, pH 6.0 (containing 136 mM **2c** and 20% DMF) were incubated at 30°C for 4h. LC–MS monitoring indicated the complete reaction of **Ab-2** to obtain **Ab-7**.

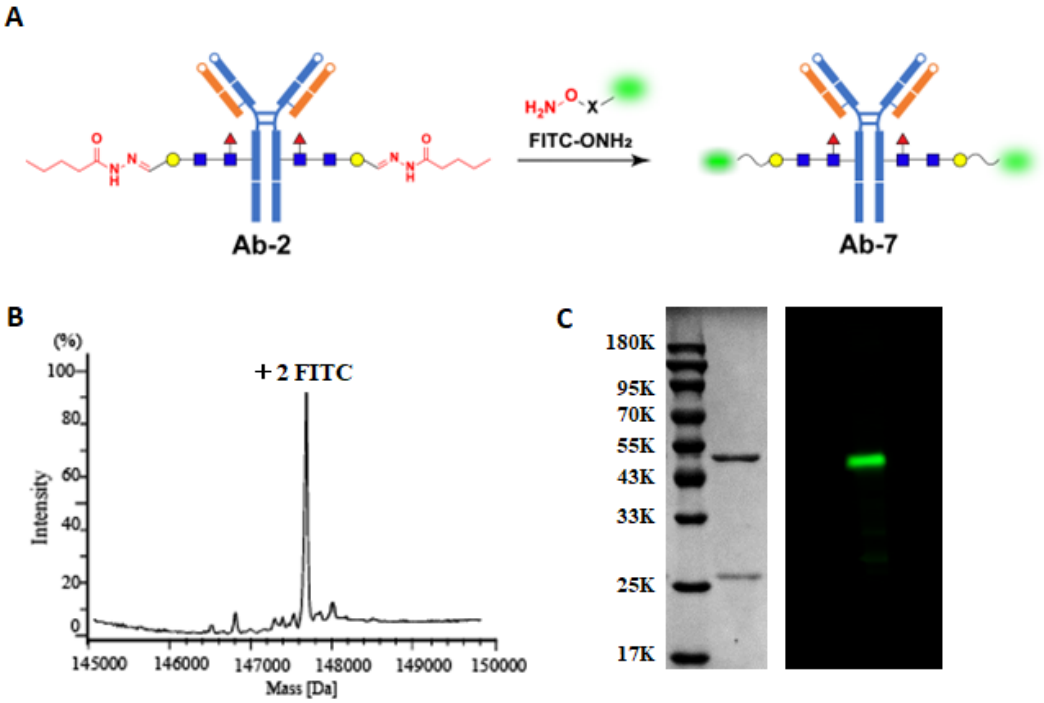


Figure S8. A) **Ab-7** prepared from **Ab-2** and **FITC-ONH$_2$** by exchange chemistry; B) LC-MS profiles of **Ab-7**; C) SDS-PAGE analysis of **Ab-7**.

2.9 Exchange reaction between Ab-2/Ab-3 and D1/D2

A solution of glycoengineered antibody (**Ab-2** or **Ab-3**, 5 mg/mL) in 50 mM His-HCl, pH 5.5 was incubated with **D1** or **D2** (0.67 mM) at 30°C for 24 h, the reaction mixture was monitored by LC-MS.

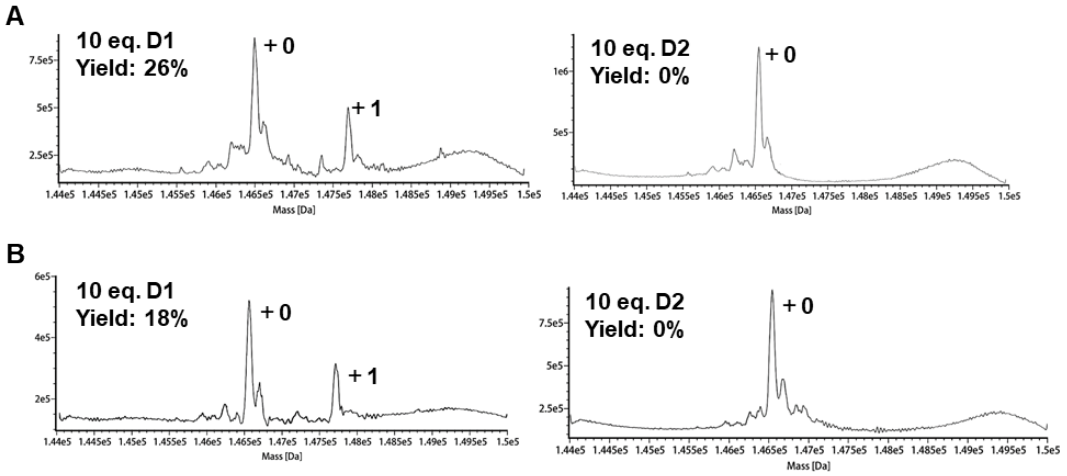


Figure S9. Exchange efficiency of **Ab-2** or **Ab-3** with different alkoxyamine-functionalized drug-linkers. A), LC-MS profiles of exchange reaction between **Ab-2** and drug-linkers **D1** or **D3**; B), LC-MS profiles of exchange reaction between **Ab-3** and drug-linkers **D1** or **D3**.

2.10 Exchange reaction under different aniline catalysts

A solution of glycoengineered antibody (**Ab-2**, 5 mg/mL) in 50 mM His-HCl, pH 6.0 was incubated with *O*-benzylhydroxylamine or **D1** (0.33 mM) and aniline catalyst **2a**-**2i** (136 mM) at 30°C for 8 h, the reaction mixture was monitored by LC-MS.

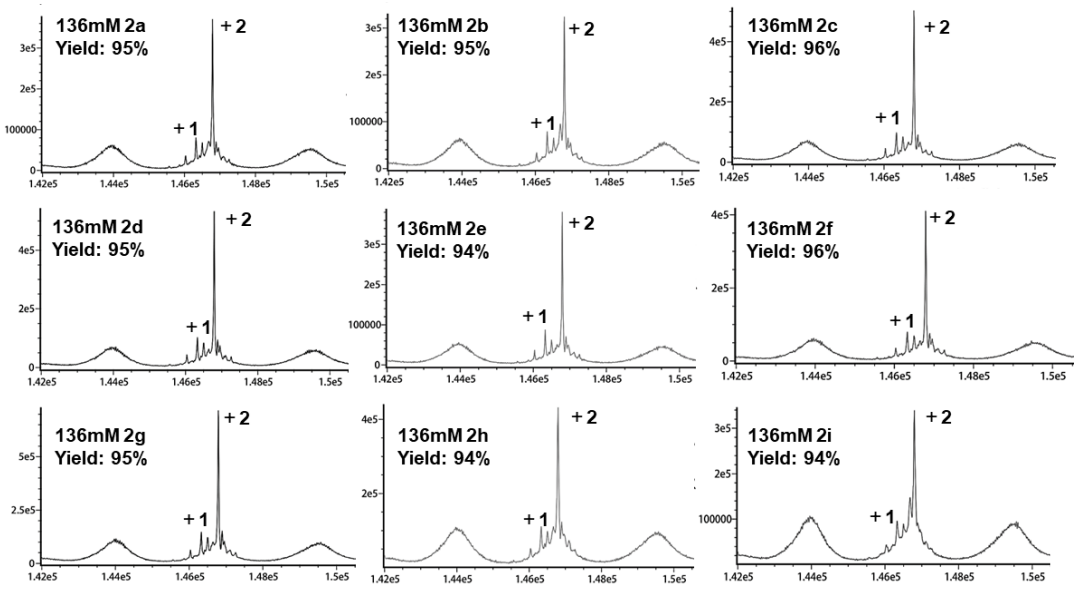


Figure S10. LC-MS profiles of exchange chemistry between **Ab-2** and *O*-benzylhydroxylamine under different catalysts.

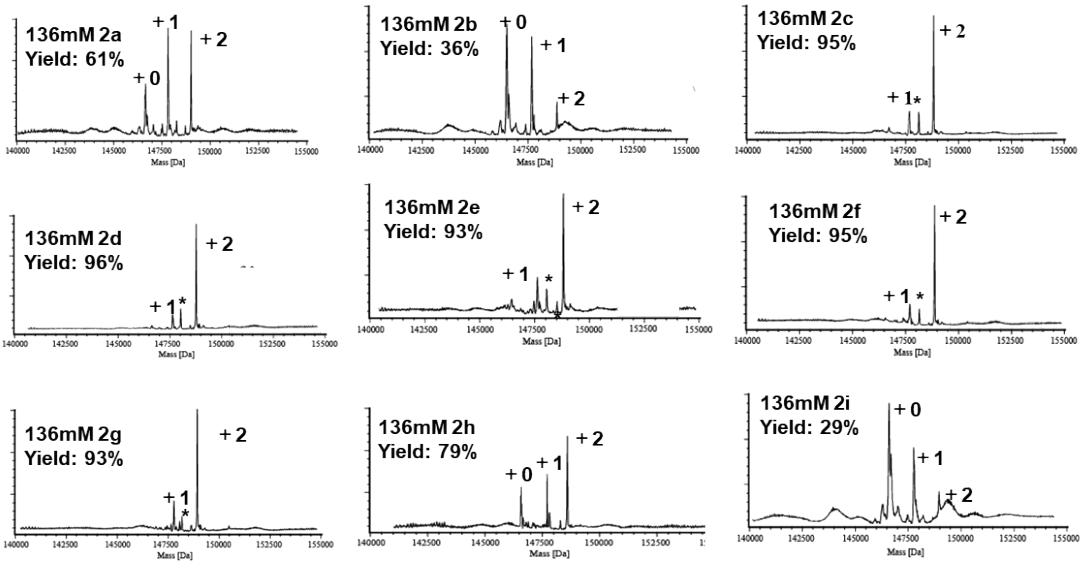


Figure S11. LC-MS profiles of exchange chemistry between **Ab-2** and **D1** under different catalysts.

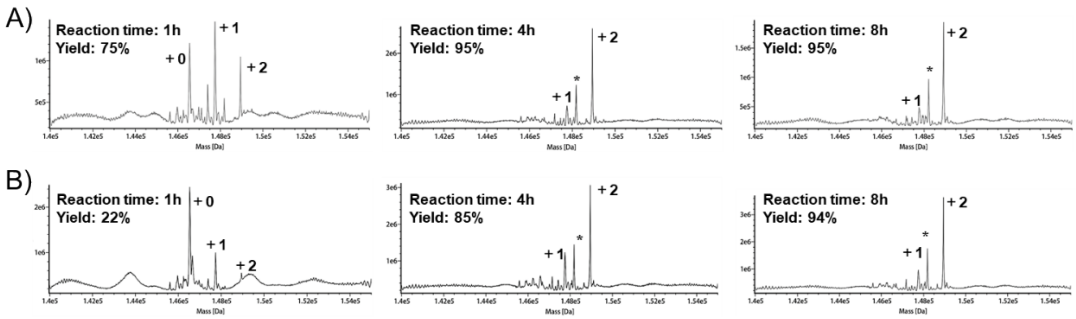


Figure S12. Catalyst equivalent optimization of aniline-catalyzed exchange reaction. A), the exchange activity between **Ab-2** and **D1** with **2c** (136 mM); B), the exchange activity between **Ab-2** and **D1** with **2c** (34 mM).

2.11 Size Exclusion Chromatography (SEC) for ADC1-ADC4

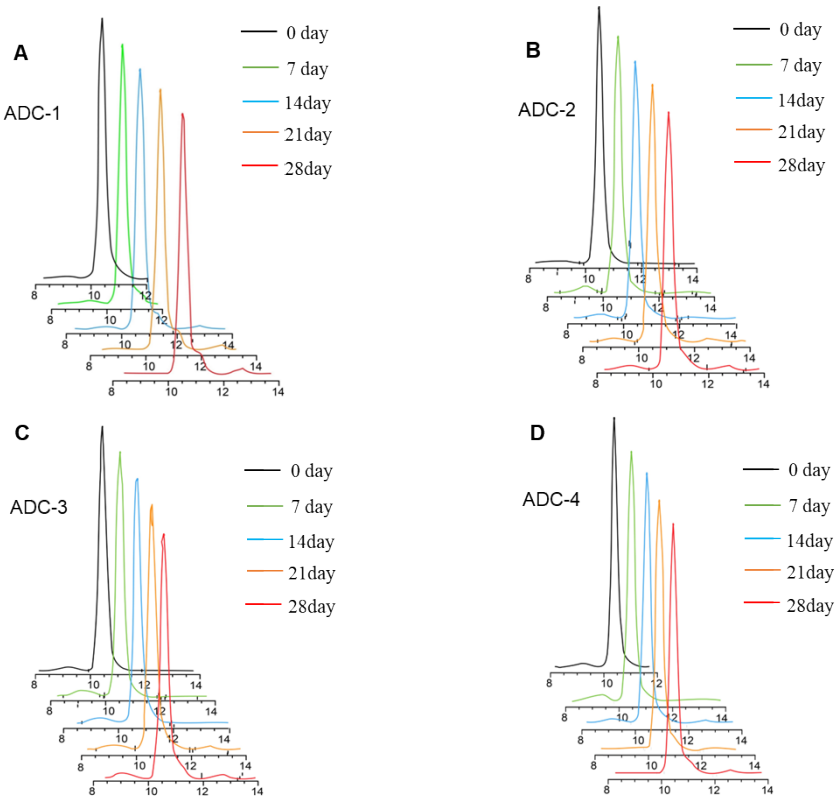
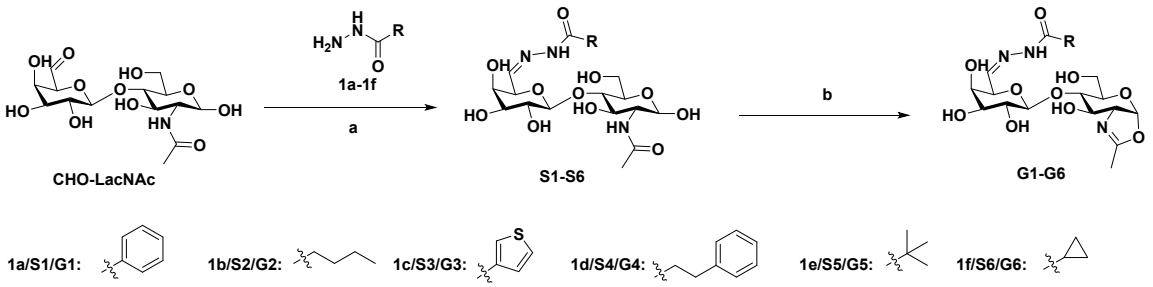


Figure S13. Aggregation stability of all ADCs with defined DARs.

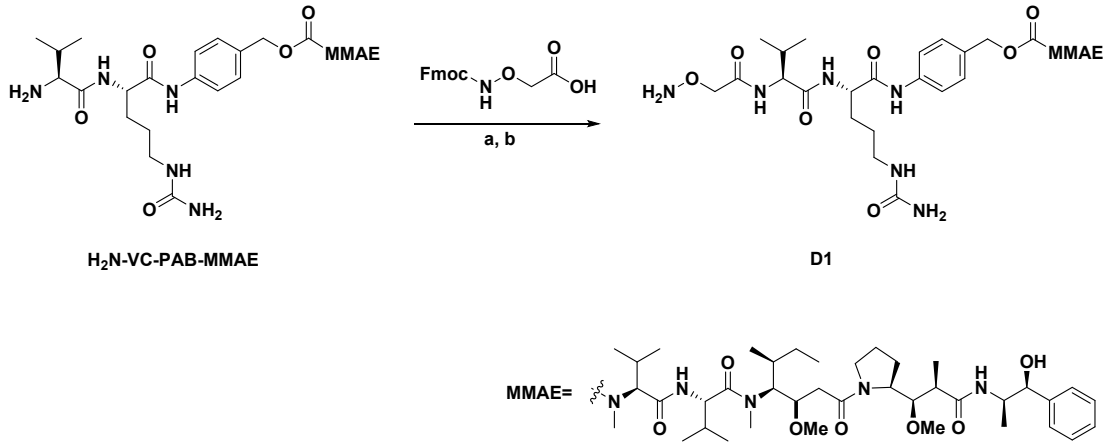
3. Supplementary schemes

Scheme S1. Synthesis of acylhydrazone-functionalized LacNAc derivatives



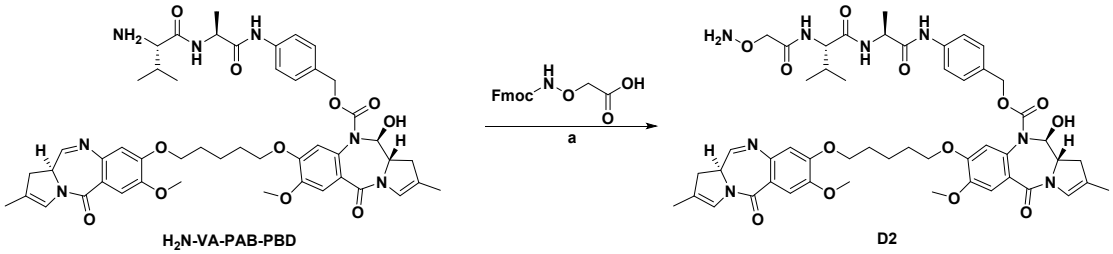
Reagents and conditions: a), 50 mM PB, pH 7.4, 30℃, 4h; b), CDMBI, K_3PO_4, 0 ℃, 2h.

Scheme S2. Synthesis of alkoxyamine-functionalized drug-linker **D1**



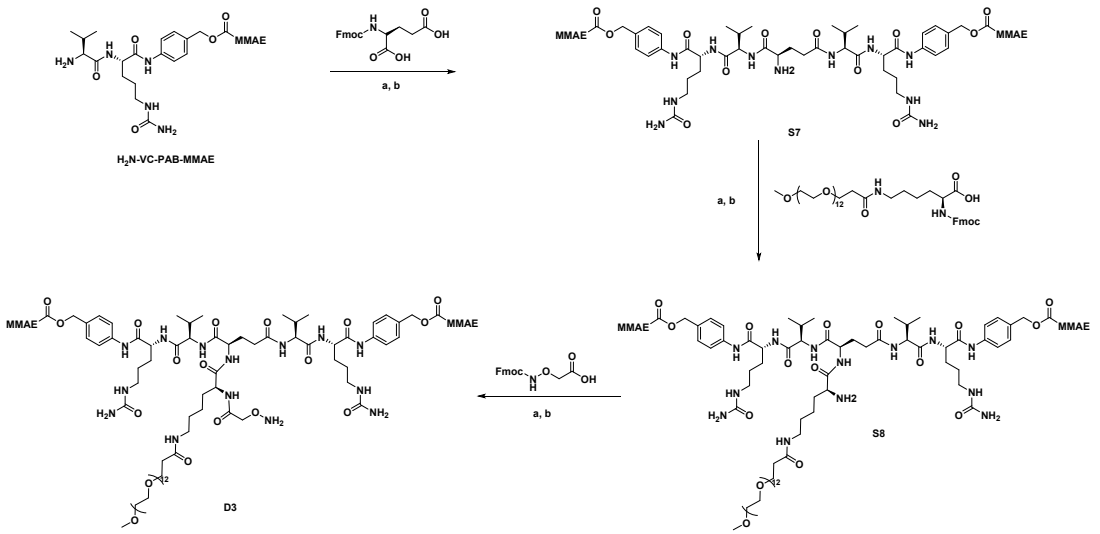
Reagents and conditions: a), HATU, DIPEA, DMF, r.t., 2h; b), 20% piperidine, r.t., 0.5h.

Scheme S3. Synthesis of alkoxyamine-functionalized drug-linker **D2**



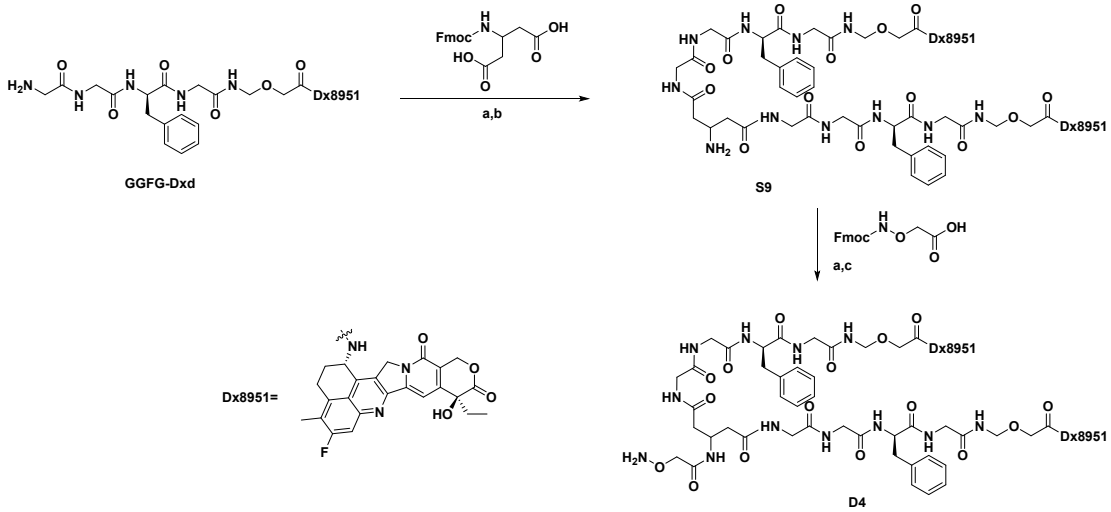
Reagents and conditions: a), NHS, DCC, DIPEA, DMF, 37 ℃, overnight.

Scheme S4. Synthesis of alkoxyamine-functionalized drug-linker **D3**



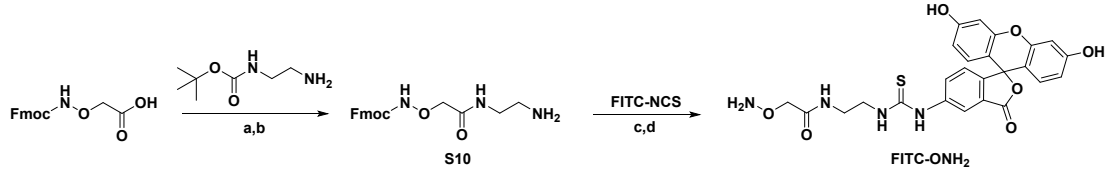
Reagents and conditions: a), HATU, DIPEA, DMF, r.t., 2h; b), 20% piperidine, r.t., 0.5h.

Scheme S5. Synthesis of alkoxyamine-functionalized drug-linker **D4**



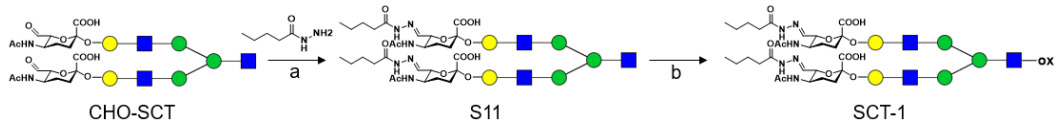
Reagents and conditions: a), HATU, DIPEA, DMF, r.t., 2h; b), 20% piperidine, r.t., 0.5h; b), 50% Et_3N, 2h.

Scheme S6. Synthesis of alkoxyamine-functionalized **FITC-ONH$_2$**



Reagents and conditions: a), HATU, DIPEA, DMF, r.t., 2h; b), TFA/H_2O/TIPES=95/2.5/2.5, ice bath, 0.5h; c), $NaHCO_3$, r.t., 3h; d), diethylamine, r.t., 3h.

Scheme S7. Synthesis of **SCT-1**

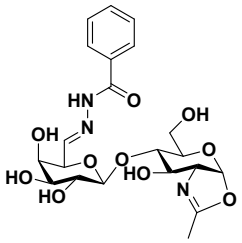


Reagents and conditions: a), 50 mM PB, pH 7.4, 30℃, 4h; b), CDMBI, K_3PO_4, 0 ℃, 2h.

5. Synthesis

Synthesis of glycan oxazolines G1-G6 and SCT-1

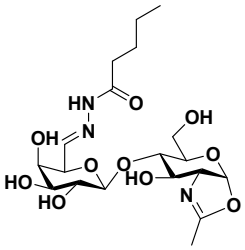
Synthesis of G1



*Synthesis of **S1**.* To a solution of CHO-LacNAc (20 mg, 52.5 μmol) in 50 mM PB, pH 7.4 (1 mL) was added benzoyl hydrazine (17.85 mg, 131.3 μmol). The mixture was stirred at 30 ℃ for 4 h. The reaction was purified by semi-preparative HPLC to get **S1** (21mg, 81%). ESI-MS calcd. for $C_{21}H_{29}N_3O_{11}$: $[M+H]^+$ *m/z* = 500.1880, found *m/z* = 500.1891.

*Synthesis of **G1**.* To a solution of **S1** (20 mg, 40.07 μmol) in 50 mM PB, pH 8.0 (2 mL) was added CDMBI (131 mg, 0.603 mmol) and K_3PO_4 (255 mg, 1.20 mmol), followed by adding ddH_2O to a total volume of 4 mL, the reaction mixture was stirred at 0 ℃ for 2 hours, the generated precipitation was removed by centrifuging. The reaction mixture was purified by semi-preparative HPLC to get **G1** (13mg, 70%). ^1H NMR (500 MHz, D_2O) δ 7.68 (d, J = 7.5 Hz, 2H), 7.54 (d, J = 4.7 Hz, 1H), 7.34 (dt, J = 14.5, 7.1 Hz, 3H), 5.95 (d, J = 7.3 Hz, 1H), 4.38 – 4.19 (m, 2H), 4.04 (s, 3H), 3.70 (d, J = 12.3 Hz, 1H), 3.65 – 3.49 (m, 3H), 3.44 (t, J = 8.9 Hz, 1H), 3.33 (t, J = 7.8 Hz, 1H), 2.05 – 1.82 (m, 6H).ESI-MS calcd. for $C_{21}H_{27}N_3O_{10}$: $[M+H]^+$ *m/z* = 482.1775, found *m/z* = 482.1739.

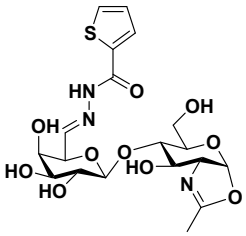
Synthesis of G2



*Synthesis of **S2**.* To a solution of CHO-LacNAc (20 mg, 52.5 μmol) in 50 mM PB, pH 7.4 (1 mL) was added pentyl hydrazone (17.85 mg, 131.3 μmol). The reaction mixture was stirred at 30 ℃ for 4 h. The mixture was purified by semi-preparative HPLC to get **S2** (22mg, 90%). ESI-MS calcd. for $C_{14}H_{33}N_3O_{11}$: $[M+H]^+$ *m/z* = 480.2193, found *m/z* = 480.2424.

*Synthesis of **G2***. To a solution of **S2** (20 mg, 41.8 μmol) in 50 mM PB, pH 8.0 (2 mL) was added CDMBI (136 mg, 0.627 mmol) and K_3PO_4 (265 mg, 1.25 mmol), followed by adding ddH_2O to a total volume of 4 mL, the reaction mixture was stirred at 0 ℃ for 2 hours, the generated precipitation was removed by centrifuging. The mixture was purified by semi-preparative HPLC to get **G2** (12mg, 63%). ^{1}H NMR (500 MHz, D_2O) δ 7.37 (dd, J = 12.1, 4.8 Hz, 1H), 5.95 (d, J = 7.2 Hz, 1H), 4.37 (d, J = 8.1 Hz, 1H), 4.17 (s, 1H), 3.98 (s, 1H), 3.67 (s, 1H), 2.00 (s, 2H), 1.99 – 1.90 (m, 6H), 1.88 (s, 1H), 1.77 (d, J = 1.2 Hz, 2H), 1.41 (s, 3H), 1.17 (s, 3H), 0.76 (t, J = 6.9 Hz, 4H).ESI-MS calcd. for $C_{19}H_{31}N_3O_{10}$: $[M+H]^+$ *m/z* = 462.2088, found *m/z* = 462.1957.

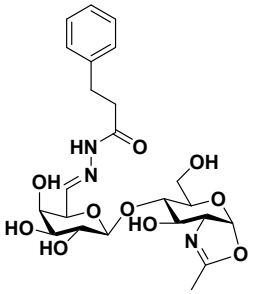
Synthesis of G3



*Synthesis of **S3**.* To a solution of CHO-LacNAc (20 mg, 52.5 μmol) in 50 mM PB, pH 7.4 (1 mL) was added thiophenyl hydrazone (18.65 mg, 131.3 μmol). The reaction mixture was stirred at 30 ℃ for 4 h. The mixture was purified by semi-preparative HPLC to get **S3** (24mg, 92%). ESI-MS calcd. for $C_{19}H_{27}N_3O_{11}S$: $[M+H]^+$ *m/z* = 506.1445, found *m/z* = 506.2019.

*Synthesis of **G3**.* To a solution of **S3** (20 mg, 39.59 μmol) in 50 mM PB, pH 8.0 (2 mL) was added CDMBI (129 mg, 0.594 mmol) and K_3PO_4 (253 mg, 1.19 mmol), followed by adding ddH_2O to a total volume of 4 mL, the reaction mixture was stirred at 0 ℃ for 2 hours, the generated precipitation was removed by centrifuging. The mixture was purified by semi-preparative HPLC to get **G3** (13mg, 71%).^{1}H NMR (400 MHz, D_2O) δ 7.74 (s, 1H), 7.53 (d, J = 4.5 Hz, 1H), 7.32 (d, J = 3.3 Hz, 2H), 5.96 (d, J = 7.5 Hz, 1H), 4.41 (d, J = 8.0 Hz, 1H), 4.33 (s, 1H), 4.25 (s, 1H), 4.05 (s, 2H), 3.71 (d, J = 12.0 Hz, 1H), 3.58 (dd, J = 23.9, 10.5 Hz, 3H), 3.49 – 3.30 (m, 2H), 1.94 (s, 3H). ESI-MS calcd. for $C_{19}H_{25}N_3O_{10}S$: $[M+H]^+$ *m/z* = 488.1339, found *m/z* = 488.1459.

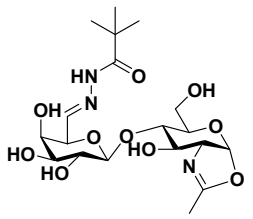
Synthesis of G4



*Synthesis of **S4***. To a solution of CHO-LacNAc (20 mg, 52.5 μmol) in 50 mM PB, pH 7.4 (1 mL) was added phenylpropionyl hydrazine (21.55 mg, 131.3 μmol). The reaction mixture was stirred at 30 ℃ for 4 h. The mixture was purified by semi-preparative HPLC to get **S4** (24mg, 89%). ESI-MS calcd. for $C_{23}H_{33}N_3O_{11}$: $[M+H]^+$ *m/z* = 528.2193, found *m/z* = 528.2027.

*Synthesis of **G4***. To a solution of **S4** (20 mg, 7.94 μmol) in 50 mM PB, pH 8.0 (2 mL) was added CDMBI (124 mg, 0.569 mmol) and K_3PO_4 (242 mg, 1.14 mmol), followed by adding ddH_2O to a total volume of 4 mL, the reaction mixture was stirred at 0 ℃ for 2 hours, the generated precipitation was removed by centrifuging. The mixture was purified by semi-preparative HPLC to get **G4** (14mg, 72%). ^{1}H NMR (400 MHz, D_2O) δ 7.18 (td, J = 17.2, 16.5, 7.0 Hz, 6H), 5.95 (d, J = 7.4 Hz, 1H), 4.39 – 4.26 (m, 2H), 4.15 (d, J = 4.6 Hz, 1H), 4.04 (s, 1H), 3.97 (s, 1H), 3.72 – 3.28 (m, 7H), 2.78 (t, J = 8.1 Hz, 2H), 2.31 (q, J = 8.9, 7.5 Hz, 2H), 1.93 (s, 3H).ESI-MS calcd. for $C_{23}H_{31}N_3O_{10}$: $[M+H]^+$ *m/z* = 510.2088, found *m/z* = 510.2107.

Synthesis of G5

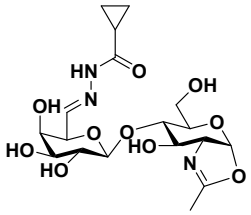


*Synthesis of **S5***. To a solution of CHO-LacNAc (20 mg, 52.5 μmol) in 50 mM PB, pH 7.4 (1 mL) was added 2,2-dimethylpropanoic acid hydrazide (15.24 mg, 131.3 μmol). The reaction mixture was stirred at 30 ℃ for 4 h. The mixture was purified by semi-preparative HPLC to get **S5** (22mg, 88%). ESI-MS calcd. for $C_{19}H_{33}N_3O_{11}$: $[M+H]^+$ *m/z* = 480.2193, found *m/z* = 480.1732.

*Synthesis of **G5***. To a solution of **S5** (20 mg, 41.74 μmol) in 50 mM PB, pH 8.0 (2 mL) was added CDMBI (135 mg, 0.626 mmol) and K_3PO_4 (265 mg, 1.25 mmol), followed by adding ddH_2O to a total

volume of 4 mL, the reaction mixture was stirred at 0 ℃ for 2 hours, the generated precipitation was removed by centrifuging. The mixture was purified by semi-preparative HPLC to get **G5** (13mg, 70%).^{1}H NMR (400 MHz, D_2O) δ 7.55 – 7.38 (m, 1H), 5.95 (d, J = 7.2 Hz, 1H), 4.37 (d, J = 7.9 Hz, 1H), 4.29 (s, 1H), 4.18 (d, J = 4.6 Hz, 1H), 4.10 – 3.93 (m, 2H), 3.74 – 3.25 (m, 6H), 1.93 (d, J = 2.1 Hz, 3H), 1.02 (d, J = 2.1 Hz, 9H). ESI-MS calcd. for $C_{19}H_{31}N_3O_{10}$: $[M+H]^+$ *m/z* = 462.2088, found *m/z* = 462.2106.

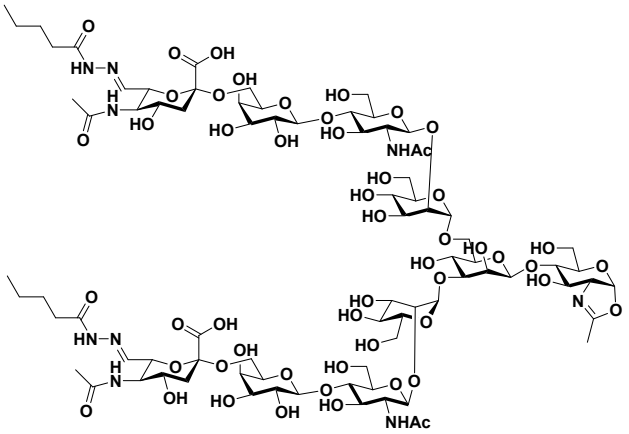
Synthesis of G6



*Synthesis of **S6***. To a solution of CHO-LacNAc (20 mg, 52.5 μmol) in 50 mM PB, pH 7.4 (1 mL) was added cyclopropanecarbohyd (13.14 mg, 131.3μmol). The reaction mixture was stirred at 30 ℃ for 4 h. The mixture was purified by semi-preparative HPLC to get **S6** (21mg, 87%). ESI-MS calcd. for $C_{18}H_{29}N_3O_{11}$: $[M+H]^+$ *m/z* = 464.1880, found *m/z* = 464.1795.

*Synthesis of **G6***. To a solution of **S6** (20 mg, 43.18 μmol) in 50 mM PB, pH 8.0 (2 mL) was added CDMBI (141 mg, 0.648 mmol) and K_3PO_4 (276 mg, 1.30 mmol), followed by adding ddH_2O to a total volume of 4 mL, the reaction mixture was stirred at 0 ℃ for 2 hours, the generated precipitation was removed by centrifuging. The mixture was purified by semi-preparative HPLC to get **G6** (14mg, 71%).^{1}H NMR (400 MHz, D_2O) δ 7.48 – 7.31 (m, 1H), 5.96 (d, J = 7.3 Hz, 1H), 4.42 – 4.27 (m, 2H), 4.17 (d, J = 4.9 Hz, 1H), 4.02 (d, J = 31.4 Hz, 2H), 3.73 – 3.28 (m, 6H), 2.10 – 1.68 (m, 3H), 1.40 (s, 1H), 0.72 – 0.56 (m, 4H). ESI-MS calcd. for $C_{18}H_{27}N_3O_{10}$: $[M+H]^+$ *m/z* = 446.1775, found *m/z* = 446.1291.

Synthesis of SCT-1

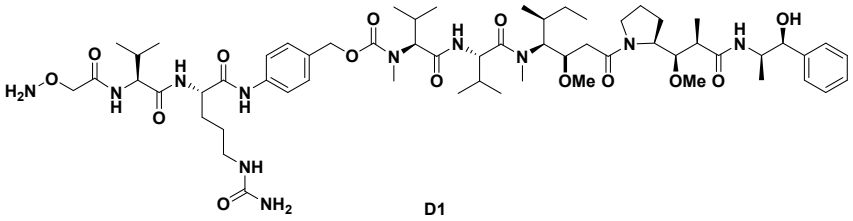


*Synthesis of **S11***. To a solution of CHO-SCT (10 mg, 5.3 μmol) in 50 mM PB, pH 7.4 (1 mL) was added pentyl hydrazone (3.7 mg, 31.8 μmol). The reaction mixture was stirred at 30 ℃ for 4 h. The mixture was purified by semi-preparative HPLC to get **S1** (8mg, 73%). ESI-MS calcd. for $C_{82}H_{133}N_9O_{53}$: $[M+H]^{2+}$ *m/z* =1046.9072, $[M+H]^{3+}$ *m/z*=698.2741, found *m/z*=1046.9045, 698.2789.

*Synthesis of **SCT-1***. To a solution of **S1** (5 mg, 2.4 μmol) in 50 mM PB, pH 8.0 (1 mL) was added CDMBI (7.74 mg, 36 μmol) and K_3PO_4 (9.8 mg, 72 μmol), added ddH_2O to a total volume of 2 mL, reacted at 0 ℃ for 2 hours, and observed a large amount of precipitation, centrifuged to remove the precipitation. The mixture was purified by G2 column to get **SCT-1** (3mg, 70%). ESI-MS calcd. for $C_{82}H_{131}N_9O_{52}$: $[M+H]^{2+}$ *m/z* =1037.9019, $[M+H]^{3+}$ *m/z*=692.2705, found *m/z*=1037.9076, 692.2759.

Synthesis of drug-linkers D1-D4, FITC-ONH$_2$

Synthesis of D1

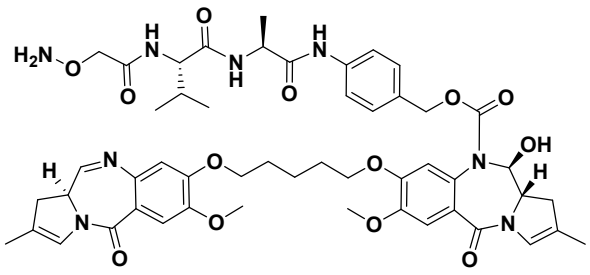


D1

*Synthesis of **D1***. To a solution of Fmoc-Aoa-OH (20 mg, 17.8 μmol) in DMF (200μL) was added HATU (13.5 mg, 35.6 μmol), H_2N-VC-PAB-MMAE (6.7 mg, 21.4 μmol) and N,N-diisopropylethylamine (93 μL, 53.4 μmol). The reaction mixture was stirred at room temperature for 2 h. Then 20% piperidine was added and the mixture was stirred at room temperature for another 0.5 h. The mixture was purified by semi-preparative HPLC to get **D1** as a white powder (16mg, 75%).^{1}H NMR (500 MHz, DMSO-d6) δ 10.06 (d, J = 6.7 Hz, 2H), 8.34 (d, J = 7.5 Hz, 2H), 8.26 (t, J = 8.5 Hz, 1H), 8.19 (d, J = 8.7 Hz, 2H), 8.02

(t, J = 6.9 Hz, 1H), 7.91 (d, J = 8.6 Hz, 1H), 7.66 (d, J = 8.5 Hz, 1H), 7.57 (t, J = 5.7 Hz, 4H), 7.36 – 7.22 (m, 12H), 7.16 (h, J = 6.6 Hz, 2H), 6.11 (s, 2H), 5.12 – 4.95 (m, 4H), 4.73 (t, J = 7.0 Hz, 1H), 4.66 – 4.60 (m, 1H), 4.47 (d, J = 7.1 Hz, 1H), 4.45 – 4.29 (m, 7H), 4.25 (dd, J = 14.8, 10.8 Hz, 2H), 3.98 (ddp, J = 20.7, 13.5, 6.7 Hz, 5H), 3.90 (s, 1H), 3.77 (dd, J = 9.3, 2.3 Hz, 1H), 3.60 – 3.51 (m, 2H), 3.47 (d, J = 7.8 Hz, 1H), 3.31 (d, J = 10.0 Hz, 1H), 3.25 – 3.15 (m, 14H), 3.11 (s, 3H), 3.04 (s, 1H), 3.04 (d, J = 12.1 Hz, 1H), 3.01 (d, J = 6.8 Hz, 1H), 2.97 (s, 4H), 2.97 – 2.91 (m, 1H), 2.90 – 2.75 (m, 6H), 2.40 (d, J = 16.1 Hz, 2H), 2.26 (ddd, J = 15.4, 9.5, 5.0 Hz, 2H), 2.12 (ddd, J = 17.3, 8.7, 4.7 Hz, 3H), 2.04 (d, J = 17.3 Hz, 1H), 2.03 – 1.96 (m, 2H), 1.94 (dd, J = 16.4, 7.7 Hz, 1H), 1.80 (d, J = 6.7 Hz, 1H), 1.78 (s, 2H), 1.71 (dq, J = 18.5, 7.5, 6.0 Hz, 2H), 1.60 (dq, J = 8.9, 4.2 Hz, 1H), 1.58 – 1.46 (m, 1H), 1.45 (dd, J = 10.4, 5.9 Hz, 2H), 1.41 – 1.34 (m, 1H), 1.31 (dd, J = 12.5, 6.6 Hz, 2H), 1.01 (ddd, J = 15.5, 13.0, 6.6 Hz, 11H), 0.81 (ddq, J = 41.5, 16.6, 8.0, 7.3 Hz, 46H), 0.62 (s, 3H). ESI-MS calcd. for $C_{60}H_{97}N_{11}O_{14}$: $[M+H]^+$ *m/z* = 1196.7295, $[M+2H]^{2+}$ 598.8686, found *m/z* = 1196.7263, 598.8622.

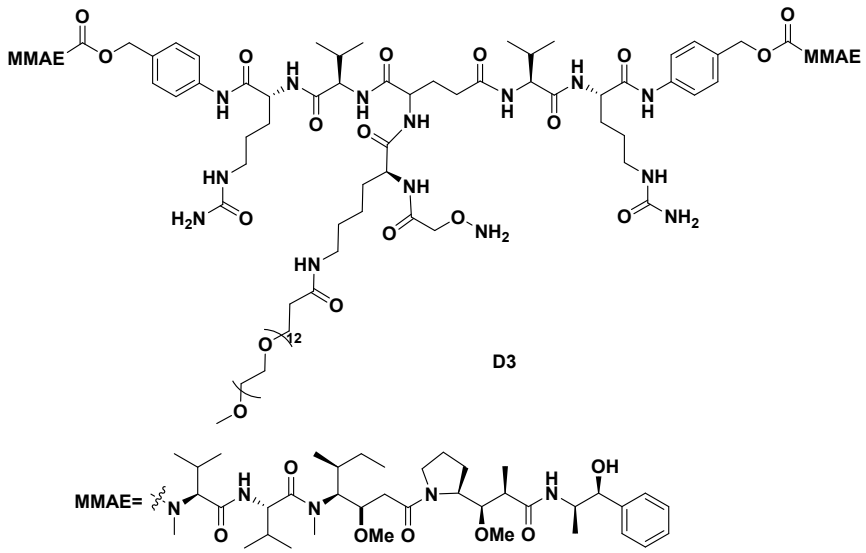
Synthesis of D2



D2

*Synthesis of **D2**.* To a solution of H_2N-VA-PAB-PBD (10 mg, 10.85 μmol) in DMF (68 μL) was added Fmoc-Aoa-OH (6.8 mg, 21.7 μmol), NHS (3.75 mg, 32.5 μmol), DCC (6.71 mg, 32.5 μmol) and N,N-diisopropylethylamine (11.3 μL, 65.1 μmol). The reaction mixture was stirred at 37 ℃ for overnight. Then we found the product by HPLC monitoring without extra deFmoc step. The mixture was purified by semi-preparative HPLC to get **D2** as a white powder (6mg, 60%).^{1}H NMR (500 MHz, DMSO-d6) δ 7.63 (s, 2H), 7.28 (s, 0H), 7.13 (s, 2H), 7.04 (d, J = 8.3 Hz, 2H), 4.06 (q, J = 11.4, 9.5 Hz, 6H), 3.84 (s, 7H), 3.80 (s, 1H), 3.57 (s, 1H), 3.53 – 3.44 (m, 2H), 3.46 (s, 1H), 2.82 (dd, J = 13.8, 4.6 Hz, 2H), 2.67 (dd, J = 13.7, 9.3 Hz, 2H), 2.55 (s, 6H), 1.37 (s, 12H), 1.31 (s, 4H), 0.85 (s, 1H), 0.77 (s, 1H). ESI-MS calcd. for $C_{51}H_{62}N_8O_{13}$: $[M+H]^+$ *m/z* = 995.4515, $[M+2H]^{2+}$ 498.2297, found *m/z* = 995.4408, 498.2190.

Synthesis of D3



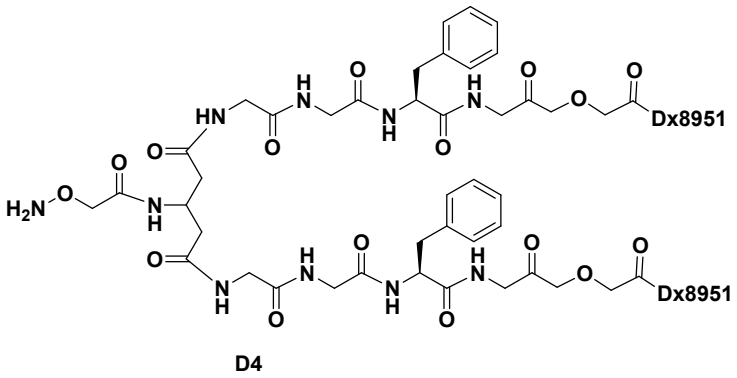
*Synthesis of **S7**.* To a solution of Fmoc-Glu-OH (30 mg, 81.2 μmol) in DMF (300 μL) was added HATU (92.8 mg, 0.244 mmol), H_2N-VC-PAB-MMAE (200 mg, 0.179 mmol) and N,N-diisopropylethylamine (141 μL, 0.812 mmol). The reaction mixture was stirred at room temperature for 2h. Then 20% piperidine was added and the mixture was stirred at room temperature for 0.5h. The mixture was purified by semi-preparative HPLC to get **S7** (153mg, 80%). ESI-MS calcd. for $C_{121}H_{192}N_{21}O_{26}$: $[M+2H]^{2+}$ *m/z* = 1179.2291, $[M+3H]^{3+}$ 786.4886, found *m/z* = 1179.2371, 786.4456.

*Synthesis of **S8**.* To a solution of Fmoc-Lys$(PEG)_{12}$-OH (191 mg, 81.2 μmol) in DMF (1mL) was added HATU (92.8 mg, 0.244 mmol), **S7** (97.7 mg, 97.44 μmol) and N,N-diisopropylethylamine (42.5 μL, 0.244 mmol). The reaction mixture was stirred at room temperature for 2h. Then 20% piperidine was added and the mixture was stirred at room temperature for 0.5h. The mixture was purified by semi-preparative HPLC to get **S8** (206mg, 82%). ESI-MS calcd. for $C_{155}H_{259}N_{23}O_{41}$: $[M+2H]^{2+}$ *m/z* = 1550.4522, $[M+3H]^{3+}$ 1033.9708, found *m/z* = 1550.3929, 1033.9491.

*Synthesis of **D3**.* To a solution of Fmoc-Aoa-OH (6.7 mg, 21.4 μmol) in DMF (200μL) was added HATU (13.5 mg, 35.6 μmol), **S8** (55.1 mg, 17.8 μmol) and N,N-diisopropylethylamine (93 μL, 53.4 μmol). The reaction mixture was stirred at room temperature for 2 h. Then 20% piperidine was added and the mixture was stirred at room temperature for 0.5 h. The mixture was purified by semi-preparative HPLC to get **D3** as a white powder (43mg, 76%).^{1}H NMR (500 MHz, DMSO-d6) δ 10.04 (s, 2H), 8.26 (dt, J = 15.6, 7.5 Hz, 4H), 8.04 (s, 2H), 7.97 (d, J = 8.0 Hz, 1H), 7.90 (t, J = 8.1 Hz, 1H), 7.83 (t, J = 5.6 Hz, 1H), 7.66 (d, J = 8.5 Hz, 1H), 7.57 (d, J = 8.3 Hz, 4H), 7.37 – 7.22 (m, 10H), 7.16

(dt, J = 11.9, 6.8 Hz, 2H), 6.06 (s, 2H), 5.11 (d, J = 13.5 Hz, 1H), 5.04 (s, 3H), 4.96 (d, J = 12.8 Hz, 1H), 4.73 (s, 1H), 4.63 (s, 1H), 4.49 (q, J = 7.4, 6.2 Hz, 2H), 4.46 – 4.37 (m, 3H), 4.40 (s, 4H), 4.34 (q, J = 7.9, 5.6 Hz, 1H), 4.30 – 4.20 (m, 4H), 4.15 (d, J = 7.7 Hz, 1H), 4.06 – 3.92 (m, 3H), 3.78 (dd, J = 9.5, 2.2 Hz, 1H), 3.60 (s, 1H), 3.60 – 3.52 (m, 3H), 3.50 (d, J = 5.0 Hz, 23H), 3.42 (dd, J = 5.9, 3.6 Hz, 2H), 3.31 (d, J = 10.0 Hz, 1H), 3.26 – 3.16 (m, 15H), 3.12 (s, 3H), 3.08 – 2.98 (m, 2H), 2.96 (d, J = 11.0 Hz, 4H), 2.86 (dd, J = 18.5, 5.7 Hz, 5H), 2.41 (d, J = 15.9 Hz, 2H), 2.30 (t, J = 6.5 Hz, 2H), 2.27 (s, 1H), 2.18 (d, J = 8.8 Hz, 1H), 2.16 – 2.05 (m, 2H), 1.98 (dp, J = 16.0, 8.7, 8.1 Hz, 3H), 1.85 – 1.75 (m, 3H), 1.71 (q, J = 7.9, 7.0 Hz, 2H), 1.57 – 1.47 (m, 1H), 1.49 – 1.42 (m, 1H), 1.36 (dt, J = 12.7, 7.0 Hz, 2H), 1.29 (s, 1H), 1.07 – 1.00 (m, 4H), 1.01 (dd, J = 7.4, 4.6 Hz, 5H), 0.98 (s, 1H), 0.93 (s, 1H), 0.87 (dd, J = 15.1, 6.3 Hz, 4H), 0.79 (dt, J = 23.6, 8.1 Hz, 41H), 0.13 (s, 4H). ESI-MS calcd. for $C_{157}H_{262}N_{24}O_{43}$: $[M+2H]^{2+}$ *m/z* = 1586.9609, $[M+3H]^{3+}$ 1058.3101, found *m/z* = 1586.9719, 1058.3296.

Synthesis of D4



D4

*Synthesis of **S9**.* To a solution of Fmoc-Glu-OH (4.2 mg, 11.4 μmol) in DMF (42μL) was added HATU (13 mg, 34.2 μmol), GGFG-Dxd (21 mg, 25 μmol) and N,N-diisopropylethylamine (11.88 μL, 68.2 μmol). The reaction mixture was stirred at room temperature for 2h. Then 20% piperidine was added and the mixture was stirred at room temperature for 0.5h. The mixture was purified by semi-preparative HPLC to get **S9** (21mg, 53%). ESI-MS calcd. for $C_{89}H_{96}F_2N_{17}O_{22}$: $[M+H]^+$ *m/z* = 1792.6884, $[M+2H]^{2+}$ 896.8481, found *m/z* = 1792.3795, 896.4799.

*Synthesis of **D4**.* To a solution of Fmoc-Aoa-OH (2.94 mg, 9.39 μmol) in DMF (30μL) was added HATU (10.71 mg, 28.2 μmol), **S9** (14 mg, 7.82 μmol) and N,N-diisopropylethylamine (4.08 μL, 28.2 μmol). The reaction mixture was stirred at room temperature for 2 h. Then 50% triethylamine was added and the mixture was stirred at room temperature for 2 h. The mixture was purified by semi-

preparative HPLC to get **D4** (10mg, 69%). ESI-MS calcd. for $C_{91}H_{98}F_2N_{18}O_{24}$: $[M+H]^+$ *m/z* = 1865.7048, $[M+2H]^{2+}$ 933.3563, found *m/z* = 1865.8045, 933.4976.

Synthesis of FITC-ONH$_2$

*Synthesis of **S10**.* To a solution of Fmoc-Aoa-OH (50 mg, 159.6 μmol) in DMF (500μL) was added HATU (121.4 mg, 319 μmol), N-Boc-Ethylenediamine (38.4 mg, 239.4 μmol) and N,N-diisopropylethylamine (61.9 mg, 478.8 μmol). The reaction mixture was stirred at room temperature for 2h. The mixture was purified by semi-preparative HPLC. Then 500 uL TFA/H_2O/TIPES=95/2.5/2.5 mixed solution was added and stirred in ice bath 0.5h to get **S10** (39mg, 69%). ESI-MS calcd. for $C_{19}H_{21}N_3O_4$: $[M+H]^+$ *m/z* = 356.1610, found *m/z* = 356.1759.

*Synthesis of **FITC-ONH$_2$**.* To a solution of **S10** (30 mg, 84.5 μmol) in DMF (300μL) was added FITC (66 mg, 169 μmol), $NaHCO_3$ (50 mg, 591.5 μmol). The reaction mixture was stirred at room temperature for 3h. Then diethylamine (123 mg, 1.7 mmol) was added and stirred at room temperature for 3h. The mixture was purified by semi-preparative HPLC to get **FITC-ONH$_2$** (22mg, 51%). ESI-MS calcd. for $C_{25}H_{22}N_4O_7S$: $[M+H]^+$ *m/z* = 523.1287, found *m/z* = 523.1398.

General procedure for ADCs with various drug-linkers and DAR values

A solution of glycoengineered antibody (**Ab-2**, 5 mg/mL) and **D1-D4** or **FITC-ONH$_2$** (0.34-0.68 mM) in 50 mM His-HCl, pH 6.0 (containing 136 mM **2c** or **2d** and 20% DMF) were incubated at 30°C for 4-12 h. LC–MS monitoring indicated the complete reaction of glycoengineered antibody to obtain **ADC-1**~**ADC-5** or **Ab-7**.

Synthesis of Ab-7

A solution of glycol engineered antibodies (**Ab-2**, 5 mg/mL) and **FITC-ONH$_2$** (0.68 mM) in 50 mM His-HCl, pH 6.0 (containing 136 mM **2c** and 20% DMF) were incubated at 30°C for 4h. LC–MS monitoring indicated the complete reaction of **Ab-2** to obtain **Ab-7**.

Synthesis of ADC-1

A solution of glycoengineered antibody (**Ab-2**, 5 mg/mL) and **D1** (0.34 mM) in 50 mM His-HCl, pH 6.0 (containing 136 mM **2c** and 20% DMF) were incubated at 30°C for 4 h. LC–MS monitoring indicated the complete reaction of glycoengineered antibody to obtain **ADC-1**.

Synthesis of ADC-2

A solution of glycoengineered antibody (**Ab-2**, 5 mg/mL) and **D2** (0.68 mM) in 50 mM His-HCl, pH 6.0 (containing 136 mM **2c** and 20% DMF) were incubated at 30°C for 24 h. LC–MS monitoring indicated the complete reaction of glycoengineered antibody to obtain **ADC-2**.

Synthesis of ADC-3

A solution of glycoengineered antibody (**Ab-2**, 5 mg/mL) and **D3** (0.68 mM) in 50 mM His-HCl, pH 6.0 (containing 136 mM **2d** and 20% DMF) were incubated at 30°C for 24 h. LC–MS monitoring indicated the complete reaction of glycoengineered antibody to obtain **ADC-3**.

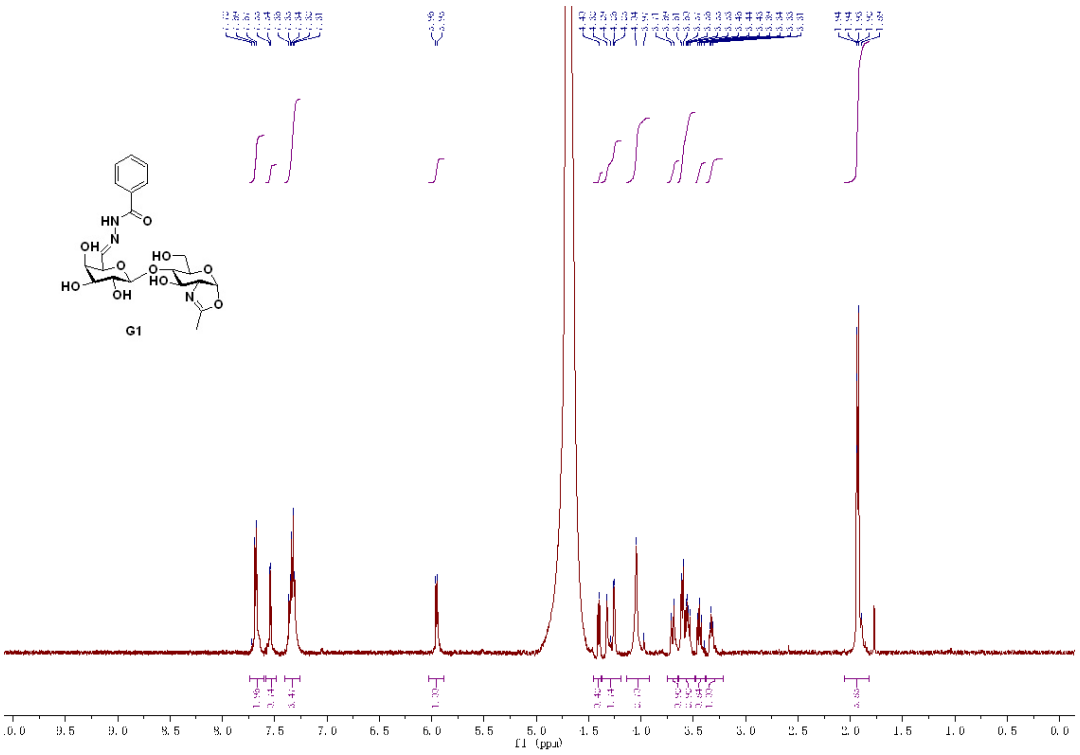
Synthesis of ADC-4

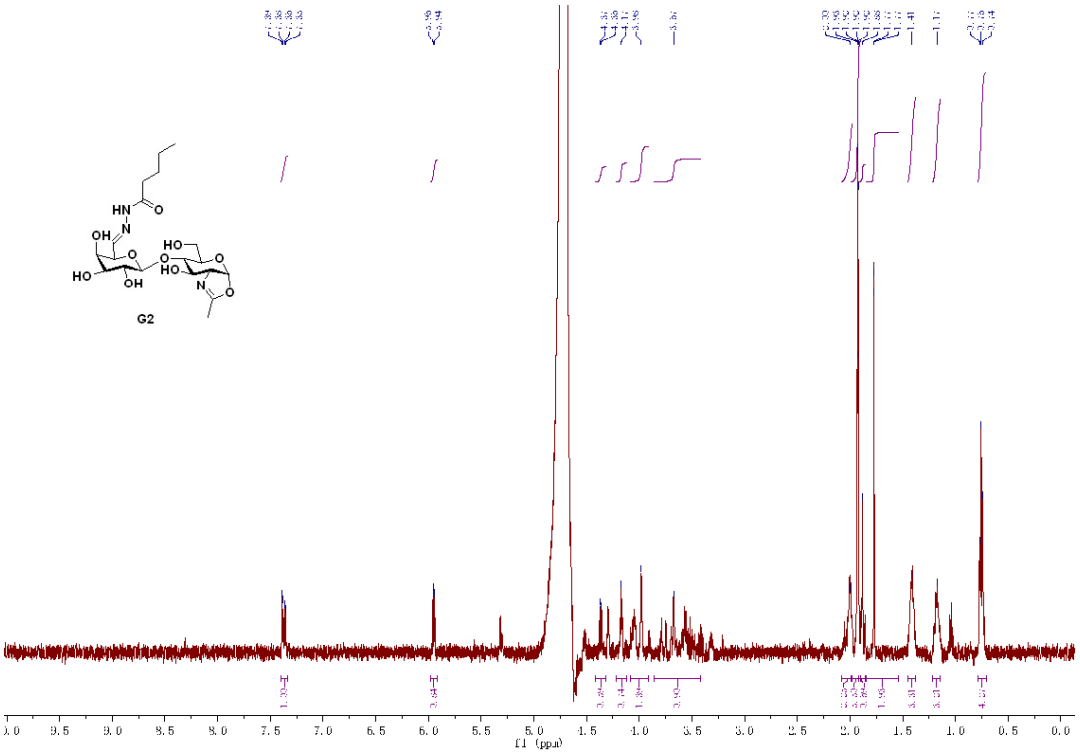
A solution of glycoengineered antibody (**Ab-2**, 5 mg/mL) and **D4** (0.68 mM) in 50 mM His-HCl, pH 6.0 (containing 136 mM **2d** and 20% DMF) were incubated at 30°C for 4 h. LC–MS monitoring indicated the complete reaction of glycoengineered antibody to obtain **ADC-4**.

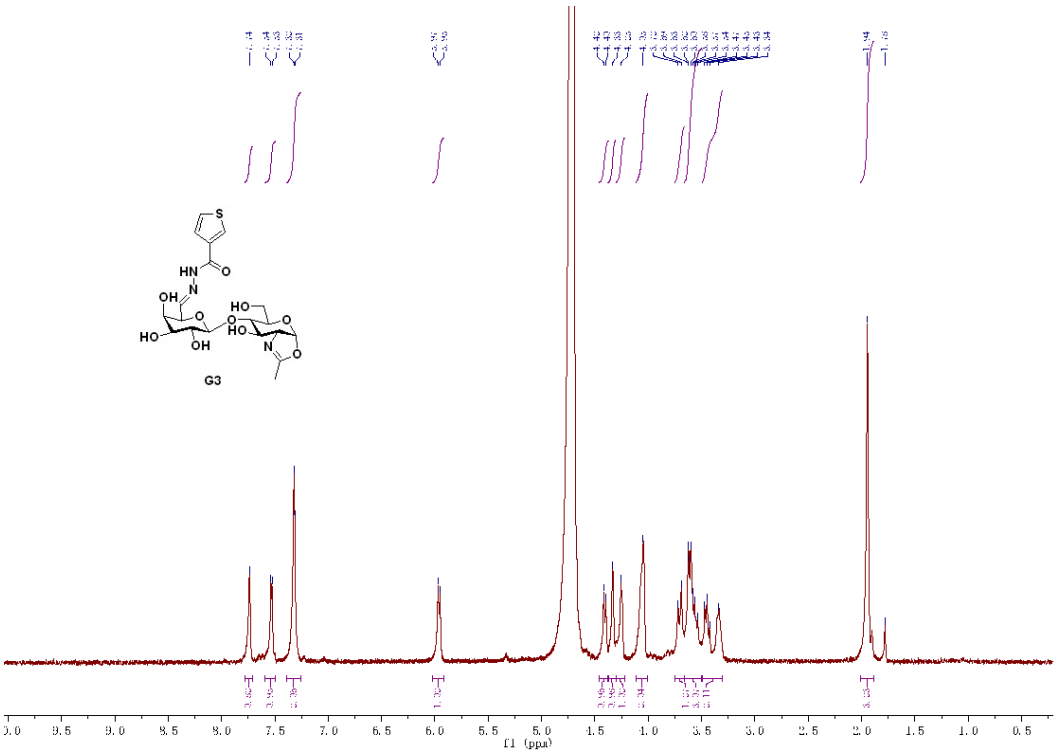
Synthesis of ADC-5

A solution of GN-trastuzumab (5 mg/mL) and **SCT-1** (0.5 mM) in 50 mM PB, pH 7.4 was added Endo-S D233Q (0.15 mg/mL), the reaction mixture was stirred at 30 ℃ for 2 h. LC–MS monitoring indicated the complete reaction, The corresponding antibody **Ab-8** was purified by protein A and ultrafiltration. Then, a solution of **Ab-8** (5 mg/mL) in 50 mM His-HCl, pH 6.0 (containing 136 mM **2c** and 20% DMF) was incubated with **D1** (0.68 mM) at 30°C for 6 h. LC–MS monitoring indicated the complete reaction, the mixture was subject to purification by protein A and ultrafiltration to get **ADC-5**.

6. Profiles



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OH
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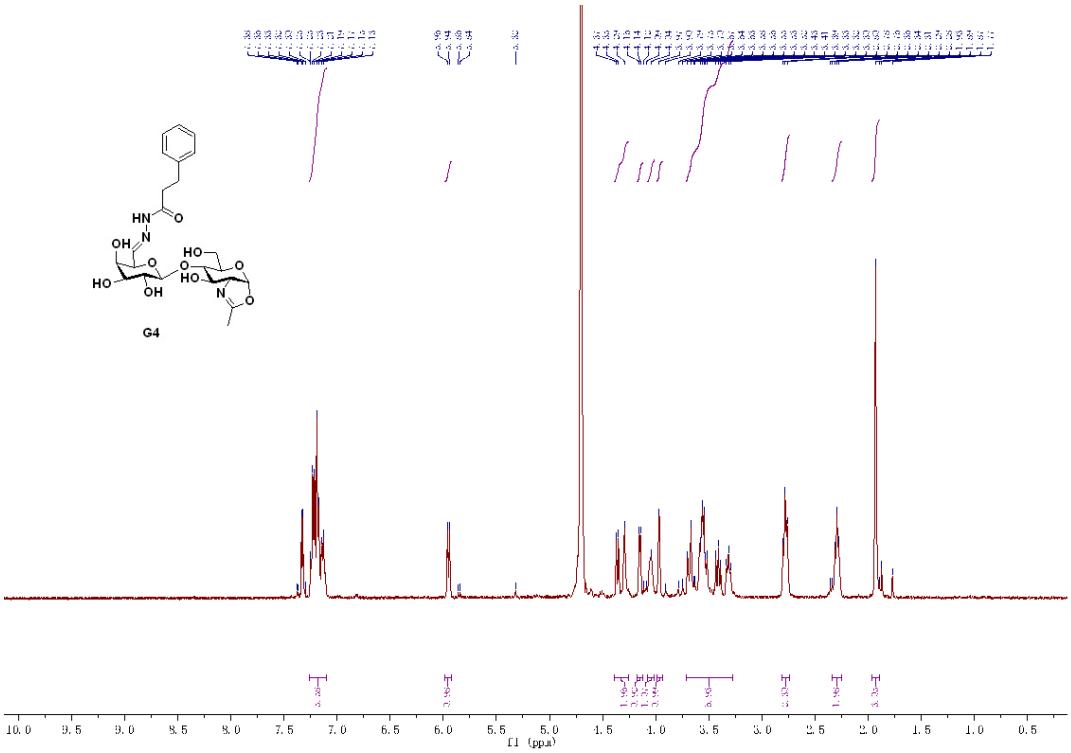
G3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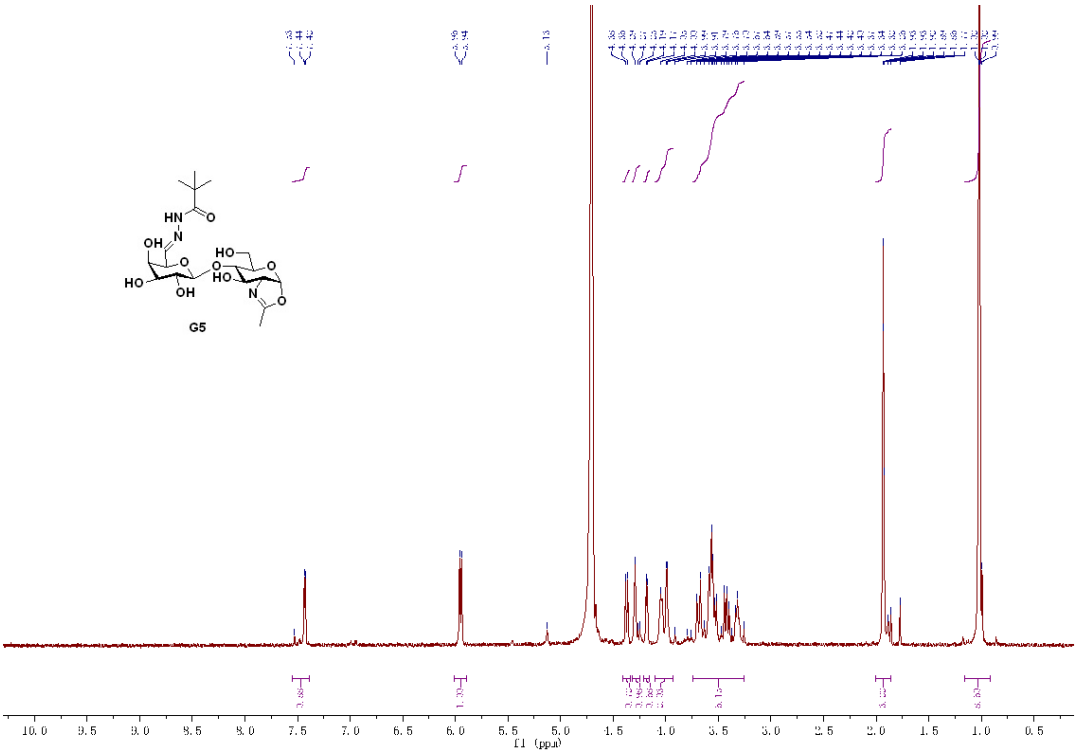
G4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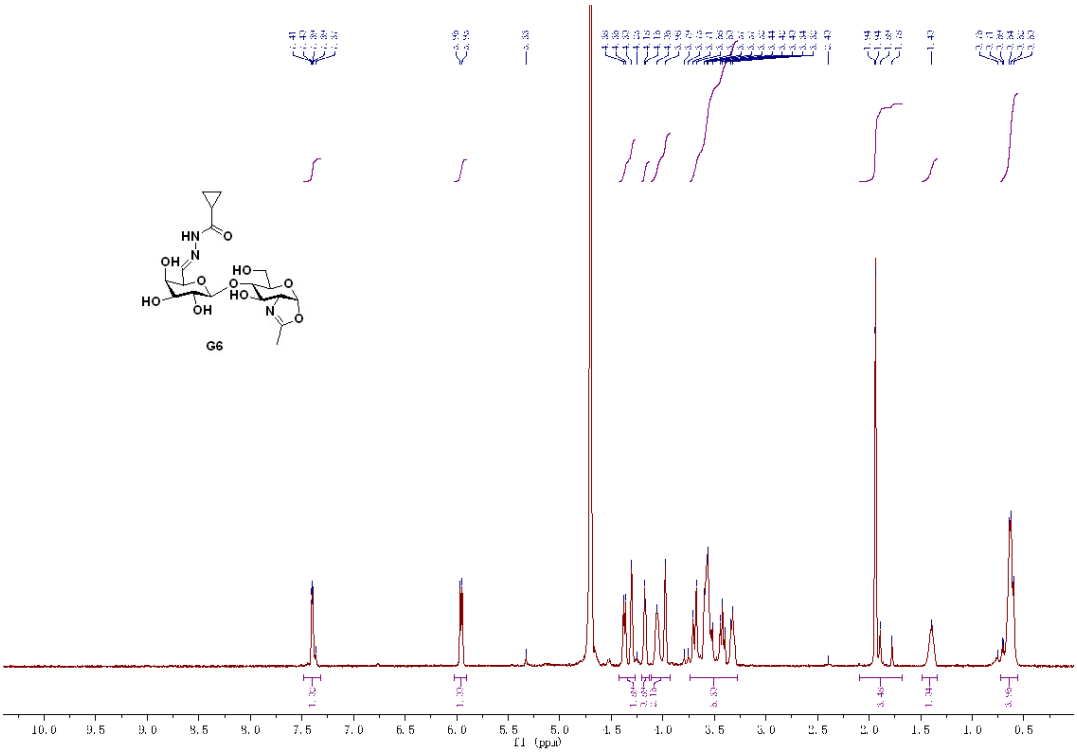
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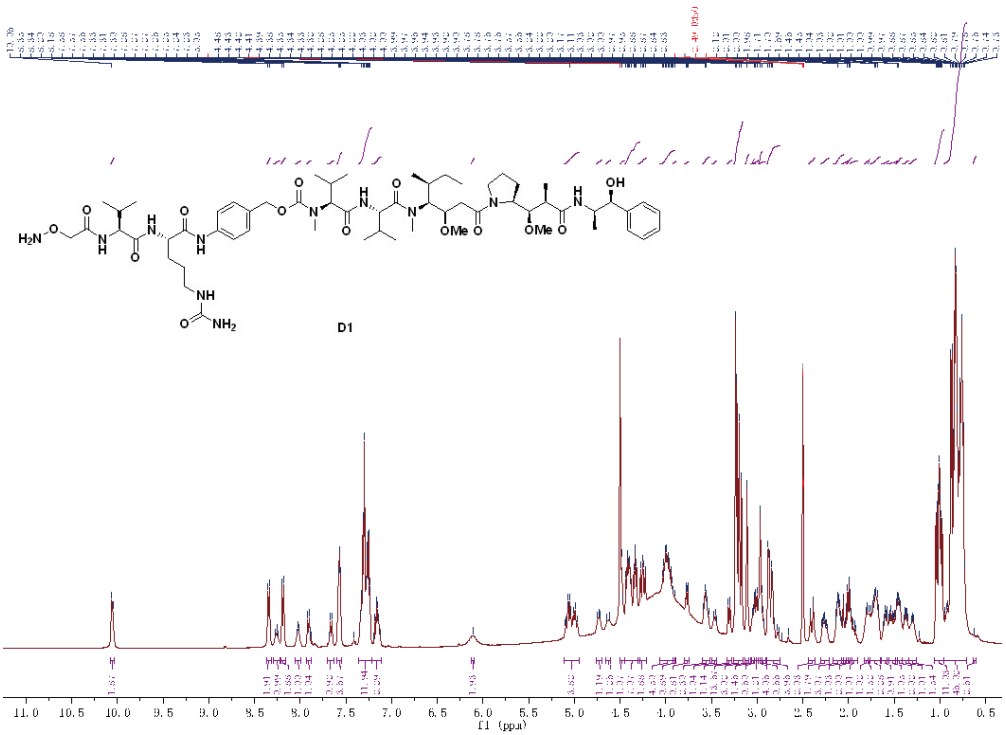
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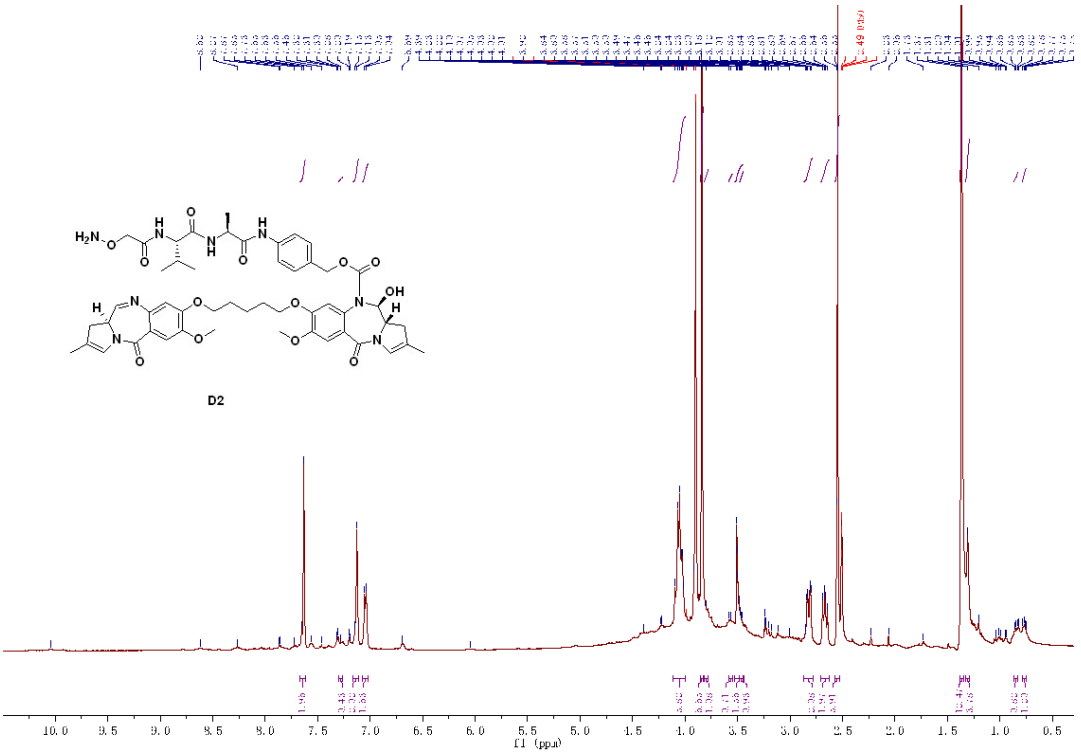
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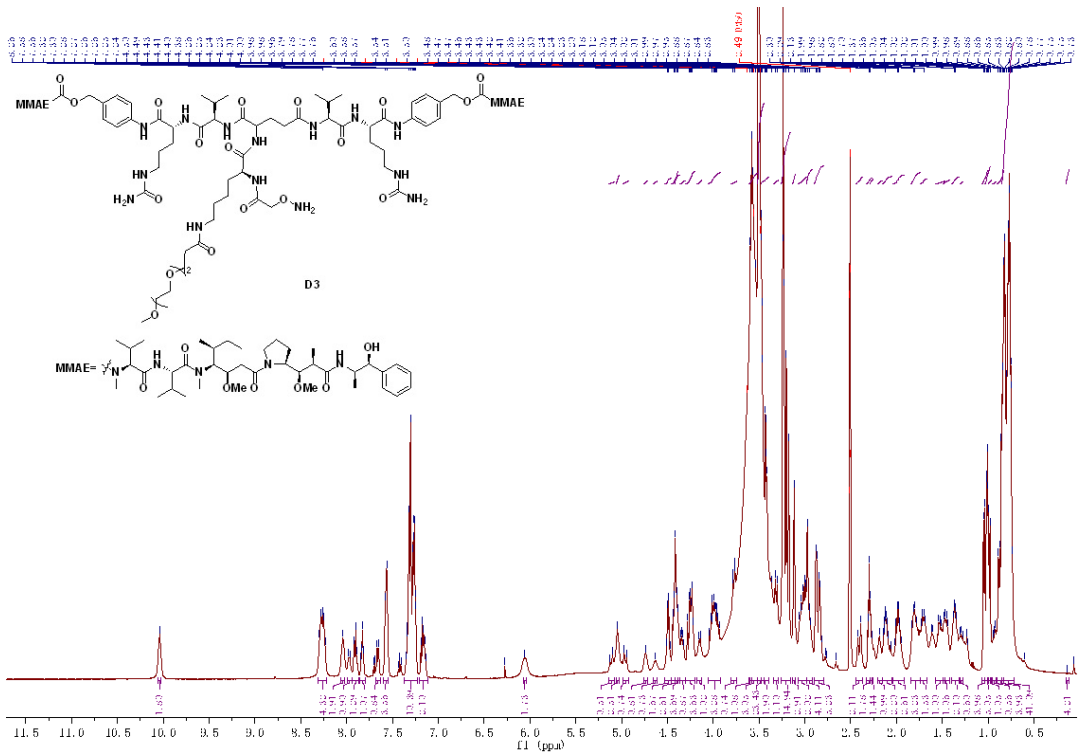
f1 (ppm)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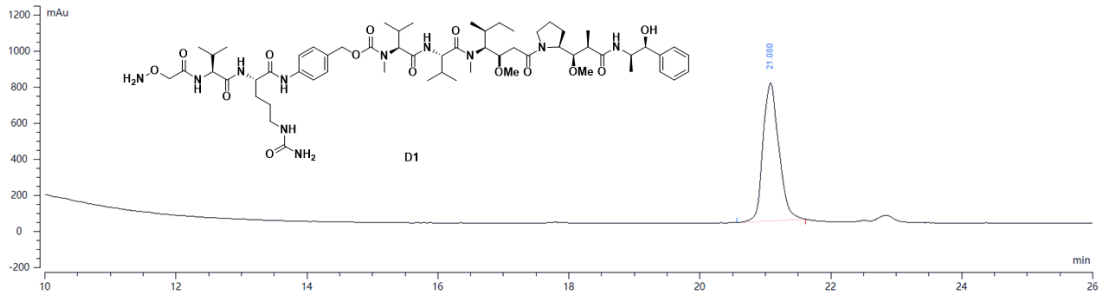
MMAE
D3
MMAE=
OMe
OMe
OH
f1 (ppm)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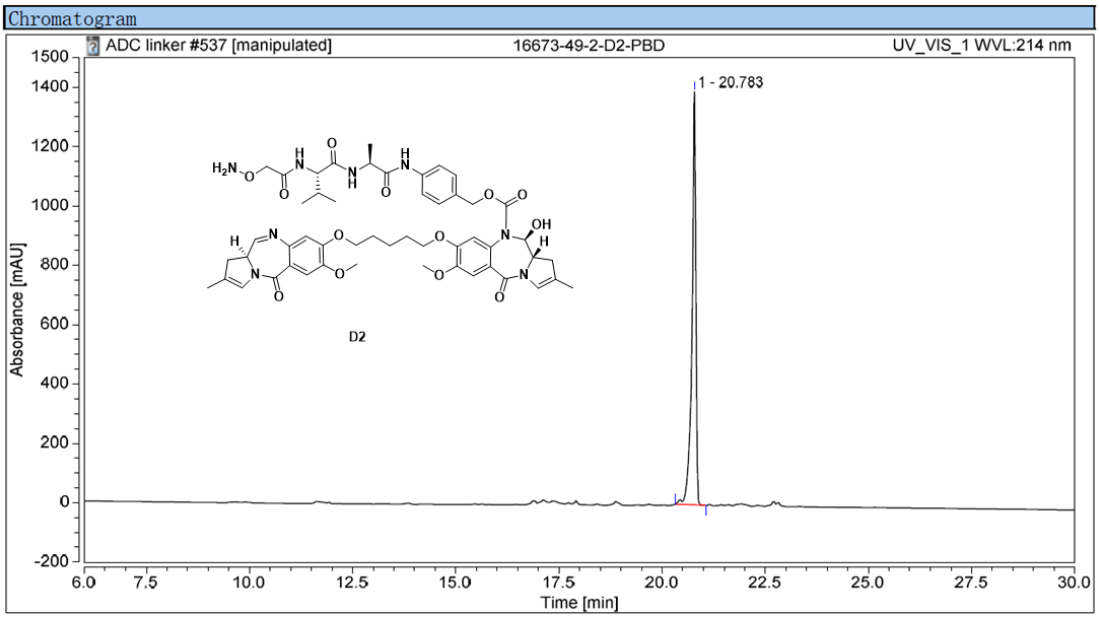
D1
21.000
min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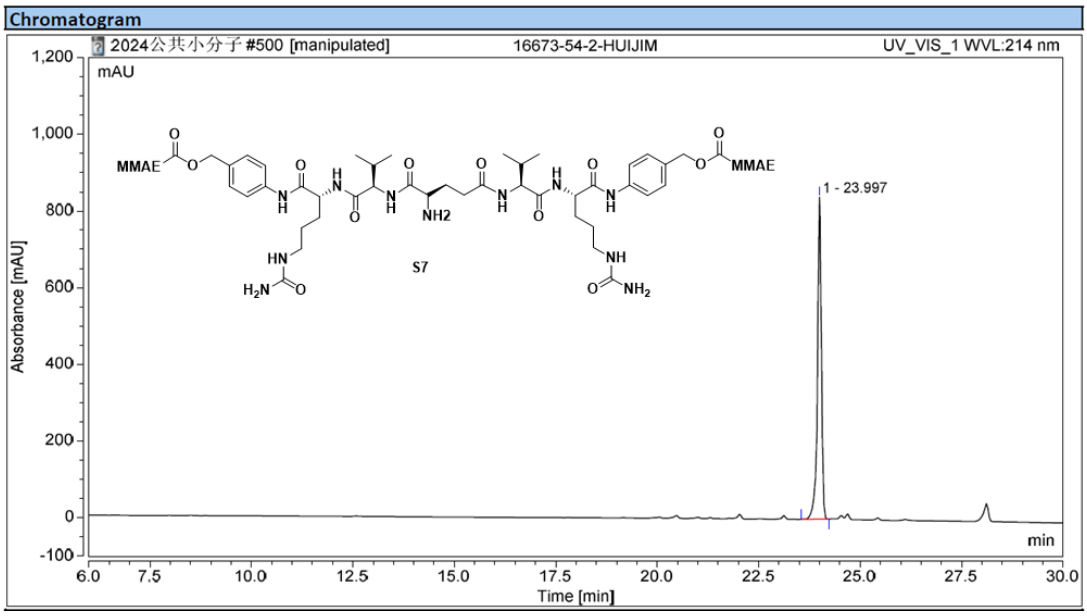
Absorbance [mAU]
Time [min]


Chromatogram
2024公共小分子 #500 [manipulated]
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mAU
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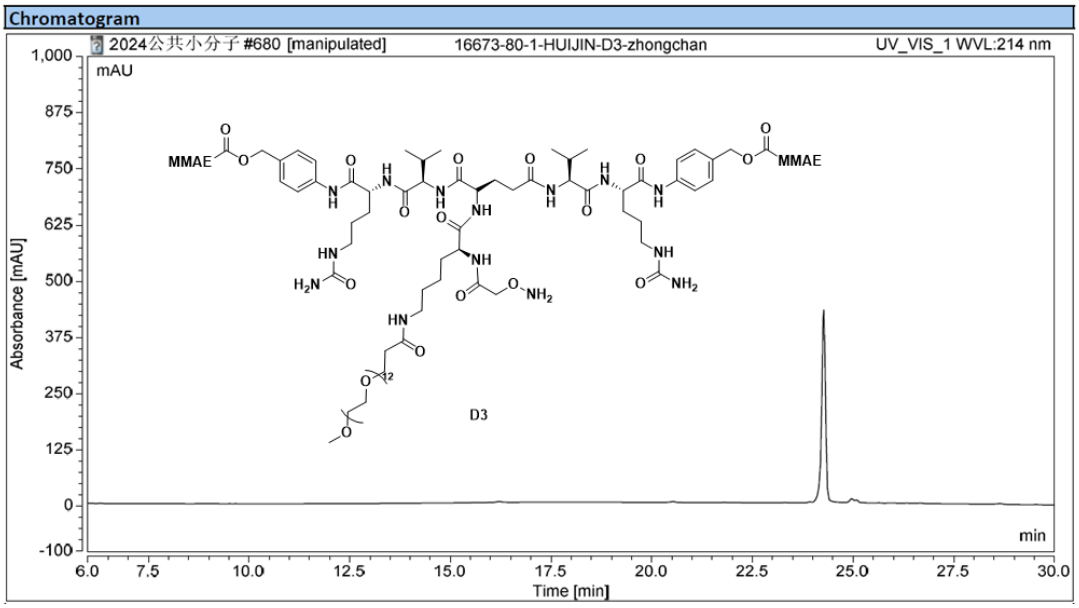
S7
MMAE
MMAE
min
Absorbance [mAU]
Time [min]


Chromatogram
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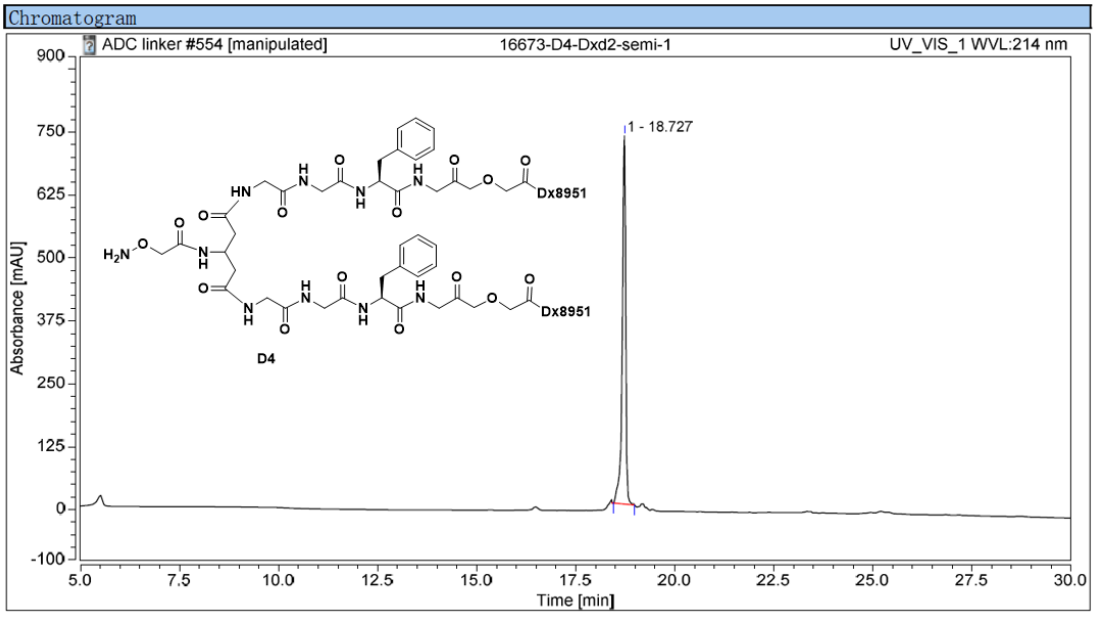
MMAE
MMAE
D3
Absorbance [mAU]
Time [min]
min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UV_VIS_1 WVL:214 nm
1 - 18.727
Dx8951
Dx8951
D4
Absorbance [mAU]
Time [min]

7. Supplementary References

1. Li, T.; Tong, X.; Yang, Q.; Giddens, J. P.; Wang, L. X, Glycosynthase Mutants of Endoglycosidase S2 Show Potent Transglycosylation Activity and Remarkably Relaxed Substrate Specificity for Antibody Glycosylation Remodeling. *J Biol Chem*. **2016,** 291 (32), 16508-18.

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3. Shi, W.; Li, W.; Zhang, J.; Li, T.; Song, Y.; Zeng, Y.; Dong, Q.; Lin, Z.; Gong, L.; Fan, S. et al. One-step synthesis of site-specific antibody-drug conjugates by reprograming IgG glycoengineering with LacNAc-based substrates. *Acta Pharm Sin B*, **2022,** 12 (5), 2417-2428.

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