# Supporting Information

## One-Pot Conversion of Free Sialoglycans to Functionalized Glycan Oxazolines and Efficient Synthesis of Homogeneous Antibody-Drug Conjugates Through Site-Specific Chemoenzymatic Glycan Remodeling

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## Experimental Procedures

### 1. Materials and Methods.

Chemicals, reagents, and solvents were purchased from Sigma−Aldrich and/or TCI, and used as received unless otherwise specified. Monoclonal antibody Hercptin was purchased from Premium Health Services Inc. (Columbia, MD). All moisture sensitive reactions were carried out under argon atmosphere, using standard Schlenk techniques. All dry solvents were prepared according to standard procedures. Thin-layer chromatography was performed on silica gel 60-$F_{254}$ on glass plates (Merck) and revealed with *p*-anisaldehyde stain. Silica gel (200−425 mesh) used in flash chromatography for large-scale reactions was purchased from Sigma-Aldrich. Columns for flash chromatography for small-scale reactions were performed on Isolera One system with ZIP KP-Sil columns (Biotage) with elution condition specified for each target compound. Solvent gradients were given refer to stepped gradients and concentrations are reported as % v/v. Preparative HPLC was performed with Waters 1525 Binary HPLC pump coupled with 2489 UV/Vis Detector under UV 214 nm and 280 nm with a Waters Symmetry C18 column (7 μm, 19 × 300 mm) using water containing 0.1% trifluoracetic acid as phase A, MeCN containing 0.1% trifluoracetic acid as phase B. Semi-preparative HPLC for the toxic payloads was performed on the same instrument with an Aglient Eclipse XDB-C18 column (5 μm, 9.4× 250 mm) using water containing 0.1% formic acid as phase A, MeCN containing 0.1% formic acid as phase B .

### Purification of sialoglycans and proteins using AKTA prime plus FPLC system.

The FPLC system (GE Healthcare) was used for purification of the SCT, mono-functionalized SCT equipped with HiTrap Q XL 2 × 5 mL (GE Heatlthcare), antibodies with HiTrap Protein A HP 1 mL (GE Healthcare), and Endo-S2 WT with Histrap HP histidine-tagged protein purification columns 5 mL (GE Healthcare). Concentration of antibodies and enzymes was determined by NanoDrap 200c (Thermo Scientific).

### LC-ESI-MS analysis of glycans and MMAE derivatives

LC-MS for glycans, glycopeptides and payload derivatives were performed on HPLC-SQ2 detector (Waters) with a Waters XBridge C18 column (3.5 μm, 2.1× 50 mm) using water containing 0.1% formic acid as phase A, MeCN containing 0.1% formic acid as phase B. Analytical HPLC for modified N-glycans was performed on the same instrument equipped with a Waters XBridge BEH130 C18 column (3.5 μm, 4.6 × 250 mm) for modified glycans with a linear gradient of acetonitrile (0−60%, v/v) with water containing FA (0.1%) over 30 min at a flow rate of 0.5 mL/min under UV 214 nm. The analytical HPLC for payload derivatives was analyzed with an Agilent Eclipse SDB-C18 column (5 μm, 3.0× 250 mm) under UV 214 nm and 280 nm with methods specialized for each compound.

#### LC-ESI-MS analysis of intact antibody derivatives.

LC-ESI-MS analysis of intact tagged antibodies and antibody-drug conjugates was performed with Exactive Plus Orbitrap Mass Spectrometer (Thermo Scientific) equipped with a Waters XBridge

BEH300 C-4 column (3.5 μm, 2.1 × 50 mm) with gradient elution of water containing 0.1% formic acid as phase A, MeCN containing 0.1% formic acid as phase B. Mass spectra were deconvoluted using MagTran (ver 1.03 b2). For the antibody Fc analysis, the antibody samples in PBS were incubated with Ide-S at 37 °C for 2 hours. The samples were analyzed by with Exactive Plus Orbitrap Mass Spectrometer (Thermo Scientific) equipped with an Agilent Poroshell 300SB C8 column (5 μm, 1.0 × 75 mm) with gradient elution of water containing 0.1% formic acid as phase A, MeCN containing 0.1% formic acid as phase B. Mass spectra were deconvoluted using MagTran (ver 1.03 b2).

**LC-ESI-MS analysis of Fc domains released by IdeS treatment.**

The antibody samples in PBS were incubated with IdeS at 37 °C for 2 h. The samples were analyzed by with Exactive Plus Orbitrap Mass Spectrometer (Thermo Scientific) equipped with an Agilent Poroshell 300SB C8 column (5 μm, 1.0 × 75 mm) with gradient elution of water containing 0.1% formic acid as phase A, MeCN containing 0.1% formic acid as phase B. Mass spectra were deconvoluted using MagTran (ver 1.03 b2).

**Deglycosylation of the antibody-drug conjugates with PNGase F.**

To the solution of the antibody-drug conjugates (**10a-c**) (3 ug in 10 μL PBS) was added PNGase F (0.3 μg). The reaction mixture was incubated at 30 °C for 3 h then it was centrifuged. The solution was subjected to LC-ESI-MS analysis to verify the antibody backbone.

**NMR analysis.**

$^{1}H$, $^{13}C$, and $^{1}H–^{1}H$ COSY NMR spectra were recorded on 400 MHz or 600 MHz spectrometer (Bruker) with $CDCl_3$, MeOD-$d_4$, $D_2O$ or DMSO-$d_6$ as the solvent (solvent residue peak 7.26, 3.31, 4.79, 2.50 ppm). All $^{13}C$ NMR spectra were performed with proton decoupling, and all chemical shifts are reported in part per million (ppm) and referenced to residual solvent. $^{1}H$-NMR chemical shifts were recorded relative to the solvent residual peak ($CDCl_3$ at 7.26 ppm, MeOD-$d_4$ at 3.31ppm, $D_2O$ at 4.79 ppm, DMSO-$d_6$ at 2.50 ppm). $^{13}C$ NMR chemical shifts are reported relative to the solvent residual peak ($CDCl_3$ at 77.00 ppm, MeOD-$d_4$ at 49.00 ppm, DMSO-$d_6$ at 39.51 ppm). The number of protons (*n*) corresponding to a resonance signal was indicated by *n*H and spin-spin coupling constants (*J* value) recorded in Hz.

## 2. Preparation of free sialoglycan (SCT) from the sialoglycopeptide (SGP) isolated from chicken egg yolks.

***2.1. Isolation of the sialoglycopeptide (SGP) from chicken egg yolks.*** SGP was isolated from chicken egg yolk powder according to the previously reported procedure.[1] ESI-MS: calcd. for $C_{112}H_{189}N_{15}O_{70}$, M = 2865.17 Da; found (*m/z*), 1433.81 $[M + 2H]^{2+}$, 956.27 $[M + 3H]^{3+}$.

**2.2.** ***Enzymatic cleavage of SGP to produce the sialoglycan (1).*** SGP (150 mg) was dissolved in 3 mL pH 7.4 PBS buffer, 500 μg of Endo-S2 wild type was added to the solution (substrate to enzyme ratio, 300 to 1). The reaction mixture was incubated at 37 °C overnight, the digestion can

be checked with LC-SQ2. After the digestion was complete, the reaction mixture was purified by G-15 column to remove salts and most of the peptide. The elutes were concentrated and loaded to a HiTrap Q XL 2 × 5 mL column. The column was eluted with a linear gradient of 200 mM NaCl (0−40%, v/v) with water over 60 min. The fractions were checked with *p*-anisaldehyde stain, the glycan containing fractions were collect and lyophilized. The solid was dissolved in 1 mL water and desalt with G-10 column. The fractions containing the free glycans were pooled and lyophilized to give the N-glycan (**1**) as a white powder (86 mg, 81 % yield). ESI-MS: calcd for $C_{76}H_{125}N_5O_{57}$, M = 2019.70 Da; found (*m/z*), 1011.03 $[M + 2H]^{2+}$, 674.30 $[M + 3H]^{3+}$.

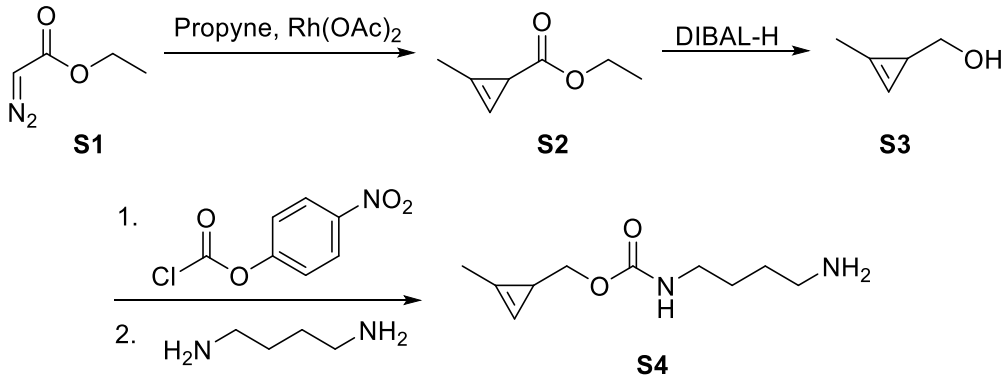
## 3. Endo-S2 overexpression, purification and immobilization

**3.1.** ***Endo-S2 WT overexpression***. Endo-S2 with $His_{10}$-tag was overexpressed and purified following the previously reported procedure [2]. From 800 mL of cell culture, 32 mg of the Endo-S2 WT was isolated. The purity was checked by SDS-PAGE. The activity was tested with SGP cleavage reaction.

**3.2.** ***Immobilization of Endo-S2 WT on agarose resin.*** The immobilization was carried out following the manufacturer's instructions. A mixture of NHS-activated agarose resin (150 mg) and Endo-S2 WT (2.5 mL, 14 mg) was incubated at r.t. in a column for 2 h. Then the column was washed and the flow-through was collected to measure the free enzyme that was not immobilized, which was used to determine the loading ratio with Bradford protein assay. The resin was washed with 3 mL PBS buffer twice. And then 3 mL of the Quenching Buffer (1 M Tris) was added. The mixture was mixed end-over-end for 20 min at room temperature. The resin was washed with 3 mL PBS buffer, and kept in pH 7.4 PBS buffer with 0.5% $NaN_3$ at 4 °C. About 7 mg of the enzyme was immobilized, and the suspension was aliquoted into 7 tubes. The activity of the immobilized enzyme was test by an enzymatic deglycosylation reaction of IVIG, with 100:1 substrate to enzyme ratio.

## 4. Synthesis of the functionalized linkers

### 4.1 Synthesis of alkene-based linkers

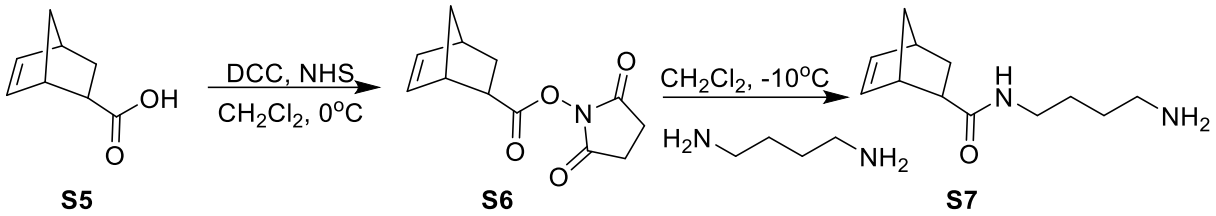



**Scheme S1.** Synthesis of the cyclopropene-functionalized amine

**Synthesis of ethyl-2-methylcycloprop-2-ene-1-carboxylate (S2).** A 100 mL 2-neck round bottom flask with $Rh(OAc)_2$ (442 mg, 1 mmol, 5 mol %) powder was fitted with a dry ice condenser and exchanged to argon. Dry $CH_2Cl_2$ was added to the flask, then the cold trap was cooled by dry ice/acetone. Propyne (about 10 mL) was condensed into the flask. The round bottom flask was then lowered into a water bath (20 °C). Ethyl diazoactate (2.4 mL, 23 mmol) was added to the mixture dropwise over 1 hour with rapid stirring. The reaction was stirred at RT for another hour, and TLC analysis suggested the reaction was completed. The product was purified by flash silica gel column chromatography by pentane with a linear gradient of diethyl ether (0-15%, v/v) over 15 CV. After careful evaporation, compound **S2** was isolated as a colorless oil (2.3 g, 79%) [3]. $^1$H NMR ($CDCl_3$, 600 MHz) $\delta$ = 1.24 (3H, t, $J$ = 7.1 Hz, $CH_2CH_3$), 2.11 (1H, s, C*H*CO), 2.16 (3H, s, $CH_3C$=), 4.12–4.14 (2H, m, $CH_2CH_3$), 6.34 (1H, d, $J$ = 0.6 Hz, C*H*=C). $^{13}$C NMR (150 MHz, $CDCl_3$) $\delta$ = 10.0, 13.9, 19.6, 59.7, 94.2, 111.2, 176.0. DART-TOF MS: $[M + H]^+$ calcd for $C_7H_{11}O_2^+$, 127.08; found (*m/z*), 127.05.

**Synthesis of (2-methylcycloprop-2-en-1-yl) methanol (S3).** In a 250 mL 2-neck round bottom flask, **S2** (3.8 g, 30 mmol, 1 eq) was dissolved in 40 mL $CH_2Cl_2$ under argon. With fast stirring, DIBAL-H (45 mL of 1M solution in $CH_2Cl_2$, 45 mmol, 1.5 eq) was added dropwise to the above solution over 30 min at -10 °C. The mixture was stirred for another 30 min when the TLC showed the reaction was complete. Then the reaction mixture was quenched carefully by $H_2O$ (2 mL), NaOH (2 mL, 1 M solution in $H_2O$), and $H_2O$ (4.6 mL). The mixture was stirred for a further 2 hours at room temperature before it was diluted with 400 mL $CH_2Cl_2$ and dried over $MgSO_4$. The organic solution was carefully concentrated to 10 mL then purified by flash silica gel column chromatography by pentane with a linear gradient of diethyl ether (0-40%, v/v) over 15 CV. The desired cyclopropene alcohol **S3** was isolated as a colorless oil (1.5 g, 56%). $^1$H NMR ($CDCl_3$, 600 MHz) $\delta$ = 1.67 (1H, td, C*H*CO), 2.15 (3H, s, $CH_3C$=), 3.47–3.58 (2H, m, $CH_2CH_3$), 6.63 (1H, s, C*H*=C). DART-TOF MS: $[M + H]^+$ calcd for $C_5H_9O^+$, 85.06; found (*m/z*), 845.02.

**Synthesis of (2-methylcycloprop-2-en-1-yl)methyl (4-aminobutyl)carbamate (Linker b).** To the solution of **S3 (**1.5 g, 17.8 mmol, 1 eq) in 40 mL $CH_2Cl_2$, DIPEA (13.8 g, 110 mmol, 6 eq) was added, followed by the addition of 4-nitrophenyl chloroformate (10.7 g, 54 mmol, 3 eq) at room temperature. After the reaction was stirred for 8 hours, TLC shows the complete consumption of **S3**. The mixture was added dropwise to a solution of 1,4-diaminobutane (4.7 g, 54 mmol, 3 eq) in 100 mL $CH_2Cl_2$ over 30 min at 0 °C. The reaction was completed after stirred at room temperature for 6 hours. After TLC confirmed the complete consumption of the starting material, the reaction mixture was washed with brine (50 mL) three times. Then it was concentrated purified by flash silica gel column chromatography by ethyl acetate and methanol (6:1 – 3:1, v/v, with 1% TEA). The product **S4** was isolated as a slightly yellow oil (1.5 g, 42% over two steps). $^1$H NMR (MeOD-$d_4$, 600 MHz) $\delta$ = 1.51-1.53 (4H, m, $NH_2CH_2CH_2CH_2$), 1.63 (1H, td, C*H*$CH_2O$), 2.15 (3H, s, $CH_3C$=), 2.68 (2H, t, $NH_2CH_2$), 3.12 (2H, t, CPNHC*H*$_2$), 3.80–3.97 (2H, m, $CH_2CH_3$), 6.65 (1H, s, C*H*=C). $^{13}$C NMR (150 MHz, MeOD-$d_4$ ) $\delta$ = 11.59, 18.36, 28.32, 30.53, 41.44, 42.07, 73.02, 102.90, 122.24, 159.44. HR-ESI-MS: $[M + H]^+$ calcd for $C_{10}H_{19}N_2O_2^+$, 199.1441; found (*m/z*), 199.1436 $[M + H]^+$.




**Scheme S2**. Synthesis of the norbornene functionalized amine

**Synthesis of norbornenyl *N*-hydroxysuccinimidyl ester (S6).** To the solution of **S5 (**1.26 g, 9.1 mmol, 1 eq) and *N*-hydroxysuccinimide (1.15 g, 10.0 mmol, 1.1 eq) in 20 mL $CH_2Cl_2$, EDC (2.1 g, 10.9 mmol, 1.2 eq) was added at 0 °C. The reaction was stirred at room temperature for 3 hours before TLC confirmed its completion. The reaction mixture was washed with brine (50 mL) three times and dried over $MgSO_4$. Then the product was concentrated and used without further purification. The $^1$H NMR spectra agreed with the reported data. $^1$H NMR ($CDCl_3$, 400 MHz) $\delta$ = 1.43-1.45 (2H, m), 1.52-1.55 (1H, m), 2.05 (1H, m), 2.49 (1H, m,), 2.83 (4H, s, COC$H_2$), 3.00 (1H, s, =CHC*H*), 3.12 (1H, s, =CHC*H*), 3.80–3.97 (2H, m, C$H_2$CH$_3$), 6.12-6.21 (2H, m, C*H*=C).

**Synthesis of norbornenyl-amine (Linker c).** The solution of **S6** (1 eq) in $CH_2Cl_2$ (40 mL) was added dropwise to a solution of 1,4-diaminobutane (2.4 g, 27.3 mmol, 3 eq) and DIPEA (3.5 g, 27.3 mmol, 3 eq) over 30 min at 0 °C. The reaction was completed after stirred at room temperature for 6 hours. After TLC confirmed the complete consumption of the starting material, the reaction mixture was washed with brine (50 mL) three times. Then it was concentrated purified by flash silica gel column chromatography by ethyl acetate and methanol (6:1 – 3:1, v/v, with 1% TEA). The product **S7** was isolated as a slightly yellow oil (835 mg, 44% over two steps). $^1$H NMR (MeOD-$d_4$, 600 MHz) $\delta$ = 1.28 (2H, m), 1.55 (4H, m, $NH_2CH_2CH_2CH_2$), 1.69 (1H, m), 1.84 (1H, m), 2.10 (1H, m), 2.76 (2H, t, $NH_2CH_2$), 2.78 (1H, s, =CHC*H*), 2.84 (1H, s, =CHC*H*), 3.19 (2H, t, NBNHC$H_2$), 6.14 (2H, s, C*H*=C). $^{13}$C NMR (150 MHz, MeOD-$d_4$) $\delta$ = 27.77, 29.09, 31.23, 39.99, 41.48, 42.76, 45.24, 47.08, 137.31, 139.00, 178.45. HR-ESI-MS: $[M + H]^+$ calcd for $C_{10}H_{19}N_2O_2^+$, 199.1441; found (*m/z*), 199.1436 $[M + H]^+$.

### 4.2 Synthesis of the azide-, cyclopropene- and norbornene-modified N-glycans and N-glycan oxazolines

**Synthesis of the Di-$N_3$-SCT-DMT (2).** To a mixture of **1** (10 mg, 20 mg/mL) and N3-linker-NH2 (30 mg, 20 eq), DMTMM (55 mg, 40 eq) was added into a buffer (PBS, pH7.4). The reaction mixture was incubated at 37 °C for 16 h and monitored with LC-MS. After the completion of reaction, the product was purified with size-exclusion column (G15, Bio-Rad) to give white power (12.6 mg, 93%). $^1$H NMR ($D_2O$, 400 MHz) $\delta$ = 1.77 (2H, dd, H3f$_{ax}$, H3f'$_{ax}$), 1.92, 1.94, 1.95 (15H, 3s, 5 × CH$_3$), 2.602 (2H, dd, H3f$_{eq}$, H3f'$_{eq}$), 3.37–3.47 (12H, m, H4c, H4c', H2e, H2e', 2 × $CH_2NH$, 2 × $CH_2N_3$), 3.48–3.63 (59H, m, H4a, H5a(β), H5b, H5c', H6c, H6c', H4d, H4d', H5d, H5d', H3e, H3e', H6e, H6e', H4f, H4f', H7f, H7f', H9f, H9f', 10 × $CH_2OCH_2$), 3.61–3.75 (10H, m, H3a, H6a, H3b, H5c, H2d, H2d', H3d, H3d', H6f, H6f'), 3.76–3.89 (25H, m, H2a, H6'a, H4b, H6b, H6'b, H3c, H3c', H6'c, H6'c', H6d, H6d', H6'd, H6'd',

H4e, H4e', H5e, H5e', H6'e, H6'e', H5f, H5f', H8f, H8f', H9'f, H9'f'), 4.03 (1H, br s, H2c'), 4.11 (1H, br s, H2c), 3.90 (6H, s, 2 × $OCH_3$ on DMT), 4.18 (1H, br s, H2b), 4.34 (2H, d, $J_{1,2}$ = 8.0 Hz, H1e, H1e'), 4.50 (2H, br s, H1d, H1d'),, 4.70 (1H, d, H1b), 4.86 (1H, s, H1c'), 5.05 (1H, s, H1c), 6.41 (1H, d, $J_{1,2}$ = 3.2 Hz, H1a(α)). $^{13}$C NMR (100 MHz, $D_2O$) $\delta$ =21.62, 21.98, 37.77, 38.30, 46.22, 49.71, 49.73, 51.26, 54.17, 55.42, 59.74, 61.19, 62.21, 62.59, 66.56, 67.38, 67.90, 68.05, 68.70, 68.77, 69.11, 69.15, 70.23, 70.64, 72.03, 73.07, 73.94, 80.03, 80.37, 98.95, 103.21, 168.66, 172.86, 174.25, 174.56. ESI-MS: $[M + 2H]^{2+}$ calcd for $C_{105}H_{180}N_{16}O_{67}^{2+}$, 1369.0596; found (*m/z*), 1369.0471$[M + 2H]^{2+}$.

**Synthesis of the Di-$N_3$-SCT (3).** SCT **1** (58.2 mg, 28.8 µmol, 1 eq) was dissolved in 200 µL 50 mM Phosphate Buffer in a 1 mL glass vial with a stirring bar. $N_3$-$PEG_5$-$NH_2$ (**S8**, 26.4 mg, 86.4 µmol, 3 eq) was added to the solution, and the pH was adjusted to 5 by adding 2 M HCl solution. DMTMM (39.7 mg, 144 µmol, 5 eq) was added. The reaction was stirred at 50 °C for 3 hours. Another portion of DMTMM (39.7 mg, 144 µmol, 5 eq) was added, and the reaction was heated for another 3 hours. HPLC was used to confirm the completion of the reaction. If there is still some DMT remains, TFA solution was added, the solution was stirred at room temperature for 3 hours. Once its completed, the mixture was centrifuged at 14000 rpm for 5 min, the supernatant was purified by G-15 size exclusion column. The glycan containing fractions were collected and lyophilized to afford **2** (70.6 mg, 92 %). $^1$H NMR ($D_2O$, 600 MHz) $\delta$ = 1.76 (2H, dd, $J$ = 12.3 Hz, $H3f_{ax}$, $H3f'_{ax}$), 1.90, 1.95, 1.97 (15H, 3s, 5 × $CH_3$), 2.62 (2H, t, $J$ = 12.7 Hz, $H3f_{eq}$, $H3f'_{eq}$), 3.39–3.47 (12H, m, H4c, H4c', H2e, H2e', 2 × $CH_2NH$, 2 × $CH_2N_3$), 3.48–3.63 (59H, m, H4a, H5a(β), H5b, H5c', H6c, H6c', H4d, H4d', H5d, H5d', H3e, H3e', H6e, H6e', H4f, H4f', H7f, H7f', H9f, H9f', 10 × $CH_2OCH_2$), 3.61–3.75 (10H, m, H3a, H6a, H3b, H5c, H2d, H2d', H3d, H3d', H6f, H6f'), 3.76–3.93 (25H, m, H2a, H6'a, H4b, H6b, H6'b, H3c, H3c', H6'c, H6'c', H6d, H6d', H6'd, H6'd', H4e, H4e', H5e, H5e', H6'e, H6'e', H5f, H5f', H8f, H8f', H9'f, H9'f'), 4.03 (1H, br s, H2c'), 4.11 (1H, br s, H2c), 4.18 (1H, br s, H2b), 4.36 (2H, d, $J_{1,2}$ = 7.8 Hz, H1e, H1e'), 4.51 (2H, d, $J_{1,2}$ = 6.3 Hz, H1d, H1d'), 4.53 (0.3H, d, H1a(β)), 4.70 (1H, d, H1b), 4.86 (1H, s, H1c'), 5.05 (1H, s, H1c), 5.13 (0.7H, d, $J_{1,2}$ = 2.7 Hz, H1a(α)). $^{13}$C NMR (150 MHz, $D_2O$) $\delta$ = 21.85, 21.97, 37.76, 38.29, 49.71, 51.25, 53.13, 54.17, 55.61, 59.60, 59.72, 59.92, 61.18, 61.25, 62.20, 62.59, 65.21, 65.28, 65.50, 66.54, 66.85, 66.90, 67.37, 67.88, 68.03, 68.67, 68.76, 69.08, 69.10, 69.14, 69.45, 69.80, 70.21, 70.63, 71.25, 71.61, 71.68,71.90, 72.01, 72.42, 73.06, 73.92, 74.00, 75.81, 75.98, 79.39, 79.76, 80.03, 80.29, 80.37, 90.04, 94.46, 96.53, 98.84, 98.92, 98.95, 99.09, 99.99, 103.19, 168.63, 174.05, 174,15, 174.30, 174.53. HR-ESI-MS: $[M + 2H]^{2+}$ calcd for $C_{100}H_{175}N_{13}O_{65}^{2+}$, 1299.5405; found (*m/z*), 1299.5355 $[M + 2H]^{2+}$.

**One-Pot Synthesis of Azide Functionalized SCT-oxazoline (4a).** SCT **1** (60.0 mg, 29.7 µmol, 1 eq) was dissolved in 200 µL 50 mM Phosphate Buffer in a 1 mL glass vial with a stirring bar. $N_3$-$PEG_5$-$NH_2$ (**S8**, 27.4 mg, 89.1 µmol, 3 eq) was added to the solution, and the pH was adjusted to 5 by adding 2 M HCl solution. DMTMM (40.1 mg, 148 µmol, dry powder, 5 eq) was added. The reaction was stirred at 50 °C for 3 hours. Another portion of DMTMM (40.1 mg, 148 µmol) was added, and the reaction was heated for another 3 hours. HPLC was used to confirm the completion of the reaction. If there is still some DMT remains on the reducing end, TFA solution was added, the solution was stirred at room temperature for 3 hours. The reaction

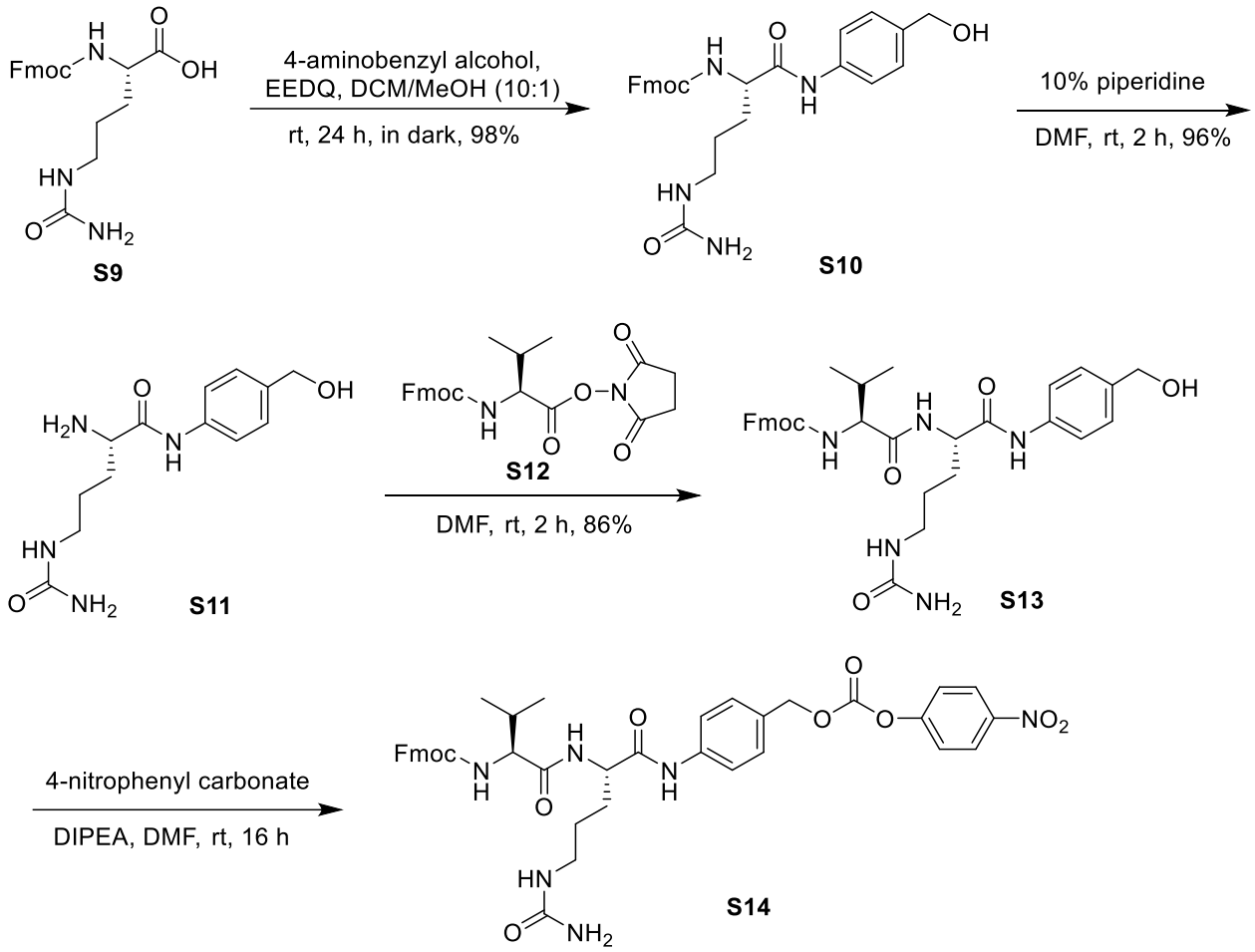
mixture was neutralized cooled on ice for 30 min. Triethyalamine (TEA, 212 mg, 2.08 mmol, 70 eq) was added to the solution, followed by 2-chloro-1,3-dimethylimidazolinium chloride (DMC, 150 mg, 0.891 mmol, 30 eq). The mixture was allowed to react on ice for 30 min. The mixture was centrifuged at 14000 rpm for 3 min before it was purified by P-2 size exclusion column with 0.1% TEA as the eluent. The glycan containing fractions were lyophilized with addition of 5 µL 1M NaOH to yield **4a** as a white powder (62.6 mg, 82%). $^{1}$H NMR ($D_2O$, 600 MHz) $\delta$ = 1.76 (2H, t, $J$ = 12.3 Hz, H3f$_{ax}$, H3f'$_{ax}$), 1.90, 1.95, 1.97 (15H, 3 s, 5 × $CH_3$), 2.62 (2H, t, $J$ = 12.7 Hz, H3f$_{eq}$, H3f'$_{eq}$), 3.39–3.47 (12H, m, H4c, H4c', H2e, H2e', 2 × $CH_2NH$, 2 × $CH_2N_3$), 3.48-3.63 (59H, m, H4a, H5a(β), H5b, H5c', H6c, H6c', H4d, H4d', H5d, H5d', H3e, H3e', H6e, H6e', H4f, H4f', H7f, H7f', H9f, H9f', 10 x $CH_2OCH_2$), 3.61–3.75 (10H, m, H3a, H6a, H3b, H5c, H2d, H2d', H3d, H3d', H6f, H6f'), 3.76–3.93 (25H, m, H2a, H6'a, H4b, H6b, H6'b, H3c, H3c', H6'c, H6'c', H6d, H6d', H6'd, H6'd', H4e, H4e', H5e, H5e', H6'e, H6'e', H5f, H5f', H8f, H8f', H9'f, H9'f'), 4.03 (1H, br s, H2c'), 4.11 (1H, br s, H2c), 4.18 (1H, br s, H2b), 4.36 (2H, d, $J_{1,2}$ = 7.8 Hz, H1e, H1e'), 4.51 (2H, d, $J_{1,2}$ = 6.3 Hz, H1d, H1d'), 4.70 (1H, d, H1b), 4.86 (1H, s, H1c'), 5.05 (1H, s, H1c), 6.00 (1H, d, $J_{1,2}$ = 7.2 Hz, H1a). $^{13}$C NMR (150 MHz, $D_2O$) $\delta$ = 12.53, 21.62, 21.97, 37.77, 38.30, 49.71, 51.26, 54.16, 59.73, 61.19, 62.20, 62.59, 64.78, 65.30, 65.38, 66.53, 66.86, 67.38, 67.88, 68.03, 68.68, 68.76, 68.96, 69.09, 69.14, 69.81, 70.21 70.44, 70.63, 71.62, 71.67, 72.01, 72.42, 73.05, 73.84, 73.93, 75.62, 76.01, 77.46, 80.00, 80.37, 96.13, 98.77, 98.93, 99.01, 99.52, 100.88, 103.20, 168.08, 168.63, 174.17, 174.54. HR-ESI-MS: $[M + 2H]^{2+}$ calcd for $C_{100}H_{173}N_{13}O_{64}^{2+}$, 1290.5352; found (*m/z*), 1290.5300.

**One-pot synthesis of cyclopropene functionalized SCT-oxa (4b).** SCT **1** (20.0 mg, 9.9 µmol, 1 eq) was dissolved in 50 µL water and 30 µL THF in a 1 mL glass vial with a stirring bar. CP-$NH_2$ (**S4**, 9.8 mg, 49.6 µmol, 5 eq); NB-$NH_2$ (**S7**, 10.3 mg, 49.6 µmol, 5 eq) was added to the solution, and the pH was adjusted to 5 by adding 2 M HCl solution. DMTMM (13.7 mg, 49.6 µmol, dry powder, 5 eq) was added. The reaction was stirred at 50 °C for 3 hours. Another portion of DMTMM (13.7 mg, 49.6 µmol, 5 eq) was added, and the reaction was heated for another 3 hours. If there is still some DMT remains on the reducing end, TFA solution was added, the solution was stirred at room temperature for 3 hours. The reaction mixture was neutralized cooled on ice for 30 min Triethyalamine (TEA, 70.7 mg, 0.69 mmol, 70 eq) was added to the solution, followed by 2-chloro-1,3-dimethylimidazolinium chloride (DMC, 50.2 mg, 0.297 mmol, 30 eq). The mixture was allowed to react on ice for 30 min. The mixture was centrifuged at 14000 rpm for 3 min before it was purified by P-2 size exclusion column with 0.1% TEA as the eluent. The glycan containing fractions were lyophilized with addition of 2 µL 1M NaOH to give the Di-CP-SCT-oxa (**4b**) as a white solid (17.8 mg, 76%). $^{1}$H NMR ($D_2O$, 600 MHz) $\delta$ 1.52–1.55 (8H, m, 4 × $NHCH_2C\mathit{H}_2$), 1.61 (2H, s, 4 × OCH2C*H*), 1.82 (2H, dd, $J$ = 12.3 Hz, H3f$_{ax}$, H3f'$_{ax}$), 2.03, 2.05, 2.06 (15H, 3s, 5 × $CH_3$), 2.11 (6H, s, 2 × CH3 on CP) 2.70 (2H, t, $J$ = 12.7 Hz, H3f$_{eq}$, H3f'$_{eq}$), 2.98–3.12 (8H, m, 2 × C$\mathit{H}_2$ NH-NB, 2 × C$\mathit{H}_2$NH-SCT), 3.39–3.47 (4H, m, H4c, H4c', H2e, H2e'), 3.48–3.63 (19H, m, H4a, H5a(β), H5b, H5c', H6c, H6c', H4d, H4d', H5d, H5d', H3e, H3e', H6e, H6e', H4f, H4f', H7f, H7f', H9f, H9f'), 3.61–3.75 (10H, m, H3a, H6a, H3b, H5c, H2d, H2d', H3d, H3d', H6f, H6f'), 3.76–3.93 (29H, m, H2a, H6'a, H4b, H6b, H6'b, H3c, H3c', H6'c, H6'c', H6d, H6d', H6'd, H6'd', H4e, H4e', H5e, H5e', H6'e, H6'e', H5f, H5f', H8f, H8f', H9'f, H9'f', 2 × CHC$\mathit{H}_2$O), 4.15 (2H, br s), 4.19 (2H, br s, H2c, H2c'), 4.39 (1H, br s, H2b), 4.43 (2H, d, $J_{1,2}$ = 7.8 Hz, H1e, H1e'), 4.63 (2H, d, $J_{1,2}$ = 6.3 Hz, H1d, H1d'), 4.74 (1H, d, H1b), 4.94 (1H, s, H1c'), 5.12 (1H, s, H1c), 6.10 (1H, d, $J_{1,2}$ = 7.2 Hz,

H1a), 6.64 (2H, m, 2 × C*H*=C). $^{13}$C NMR (150 MHz, $D_2O$) $\delta$ = 10.35, 12.53, 16.13, 21.65, 21.98, 25.13, 26.15, 33.48, 37.67, 38.75, 39.51, 48.64, 51.34, 54.20, 59.75, 61.19, 62.61, 64.81, 65.31, 65.40, 66.50, 66.88, 67.32, 67.85, 68.84, 68.97, 69.14, 69.82, 70.23, 70.45, 70.68, 71.63, 71.68, 72.01, 72.44, 73.05, 73.86, 73.96, 75.65, 76.04, 77.47, 79.99, 80.30, 96.14, 98.80, 98.95, 99.01, 99.54, 100.73, 100.89, 103.16, 120.08, 158.51, 168.08, 168.25, 174.13, 174.58. HR-ESI-MS: [M + 2H]$^{2+}$ calcd for $C_{96}H_{157}N_9O_{58}^{2+}$, 1182.4818; found (*m/z*), 1182.4755 [M + 2H]$^{2+}$.

**One-pot synthesis of norbornene-modified glycan oxazoline Di-NB-SCT-oxa (4c**). The oxazoline **4c** was synthesized starting with SCT (20 mg) in the same way as described for the synthesis of **4b**. The product was purified by gel filtration as described above to give **4c** as a white solid (16.5 mg, 70%). $^1$H NMR ($D_2O$, 600 MHz) $\delta$ = 1.22–1.35 (4H, m, Hs on NB), 1.42–1.52 (8H, m, 4 × NHCH$_2$C*H*$_2$), 1.64–1.67 (4H, m, Hs on NB), 1.76 (2H, dd, *J* = 12.3 Hz, H3f$_{ax}$, H3f'$_{ax}$), 1.90, 1.95, 1.97 (15H, 3s, 5 × CH$_3$), 2.10–2.12 (2H, m, Hs on NB), 2.62 (2H, t, *J* = 12.7 Hz, H3f$_{eq}$, H3f'$_{eq}$), 2.87 (4H, m, 2 × C*H*$_2$ NH-NB), 3.39–3.47 (8H, m, H4c, H4c', H2e, H2e', 2 × C*H*$_2$NH-SCT), 3.48–3.63 (19H, m, H4a, H5a(β), H5b, H5c', H6c, H6c', H4d, H4d', H5d, H5d', H3e, H3e', H6e, H6e', H4f, H4f', H7f, H7f', H9f, H9f'), 3.61–3.75 (10H, m, H3a, H6a, H3b, H5c, H2d, H2d', H3d, H3d', H6f, H6f'), 3.76–3.93 (25H, m, H2a, H6'a, H4b, H6b, H6'b, H3c, H3c', H6'c, H6'c', H6d, H6d', H6'd, H6'd', H4e, H4e', H5e, H5e', H6'e, H6'e', H5f, H5f', H8f, H8f', H9'f, H9'f'), 4.03 (1H, br s, H2c'), 4.11 (1H, br s, H2c), 4.18 (1H, br s, H2b), 4.36 (2H, d, $J_{1,2}$ = 7.8 Hz, H1e, H1e'), 4.51 (2H, d, $J_{1,2}$ = 6.3 Hz, H1d, H1d'), 4.70 (1H, d, H1b), 4.86 (1H, s, H1c'), 5.05 (1H, s, H1c), 6.00 (1H, d, $J_{1,2}$ = 7.2 Hz, H1a), 6.13 (4H, m, 4 × C*H*=CH). $^{13}$C NMR (150 MHz, $D_2O$) $\delta$ = 12.54, 21.65, 21.99, 25.26, 25.74, 29.70, 37.68, 38.42, 38.78, 40.87, 43.63, 45.57, 45.87, 51.33, 54.18, 59.74, 61.19, 62.16, 62.63, 64.80, 65.31, 65.40, 66.49, 66.88, 67.33, 67.84, 68.83, 68.96, 69.13, 69.82, 70.22, 70.45, 70.68, 71.63, 71.68, 72.01, 72.43, 73.06, 73.85, 73.95, 75.64, 76.05, 77.47, 79.98, 80.30, 96.14, 98.80, 98.95, 99.02, 99.54, 100.88, 103.17, 135.79, 137.83, 168.08, 168.24, 174.14, 174.58, 178.68. HR-ESI-MS: [M + 2H]$^{2+}$ calcd for $C_{100}H_{161}N_9O_{56}^{2+}$, 1192.5025; found (*m/z*), 1192.4965 [M + 2H]$^{2+}$.

### 4.3 Synthesis of the cytotoxic payloads



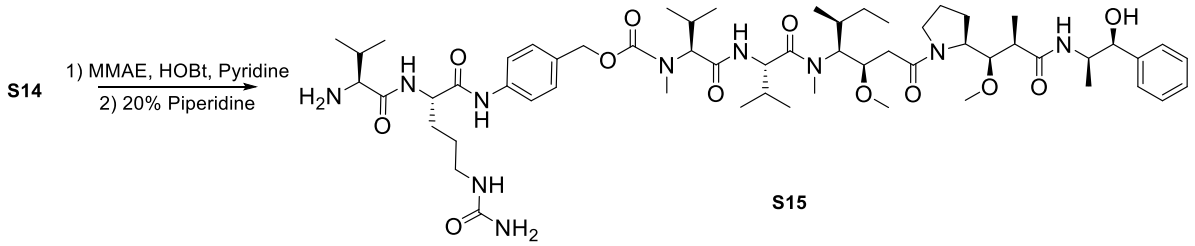

**Scheme S3**. Synthesis of the activated dipeptide linker **S14**

**Synthesis of Fmoc-Cit-PABOH (S10).** To a stirred solution of compound **S9** (2.40 g, 6.04 mmol, 1 eq) and 4-aminobenzyl alcohol (1.12 g, 9.06 mmol, 1.5 eq) in DCM/MeOH (40 mL, 10:1, v/v) was added EEDQ (2.98 g, 12.1 mmol, 2 eq) at 25 °C. The mixture was stirred at the same temperature for 24 hours in dark under argon atmosphere. Then, the solvents were removed in vacuo and the solid was filtered, washed with ethyl ether (30 mL) three times. The filter cake was collected and dried over oil pump to afford **S10** (2.98 g, 98%) as a white solid, which was used in the next step without further purification. $^1$H NMR (400 MHz, $CDCl_3$) $\delta$ = 1.32– 1.79 (4H, m), 2.90– 3.12 (2H, m), 3.18 (3H, d, J = 4.9 Hz), 4.10 – 4.16 (1H, m), 4.23 (4H, m), 4.44 (2H, d, J = 5.2 Hz), 5.13 (1H, t, J = 5.6 Hz), 5.45 (2H, s), 6.02 (1H, t, J = 5.4 Hz), 7.30 (4H, m), 7.41 (2H, t, J = 7.4 Hz), 7.57 (2H, d, J = 8.3 Hz), 7.71– 7.79 (m, 2H), 7.88 (2H, d, J = 7.5 Hz), 9.99 (1H, br).

**Synthesis of Cit-PABOH (S11).** To a stirred solution of **S10** （2.0 g, 3.98 mmol, 1 eq） in DMF (16 mL) was added piperidine (1.7 mL, 17.2 mmol, 4.3 eq) dropwise at 25 °C. The mixture was stirred at 25 °C for another 2 hours. The reaction was concentrated in vacuo and the residue was washed with ethyl ether (40 mL) twice and dried over oil pump to afford **S11** (1.07 g, 96%). The crude product was used directly in the following step.

**Synthesis of Fmoc-Val-Cit-PABOH (S13).** Fmoc-L-valine *N*-hydroxysuccinimide ester **S12** (2.1 g, 4.78 mmol, 1.25 eq) in DMF (10 mL) was added to a solution of **S11** (1.07 g, 3.82 mmol, 1 eq) in DMF (10 mL) was at 25 °C. The solution was stirred for another 2 hours under argon atmosphere. TLC showed **S11** was fully consumed. The reaction mixture was concentrated to dryness, filtered and washed with MeOH (20 mL) and ethyl ether (50 mL). The filter cake was collected to afford the dipeptide **S13** (2.06 g, 86%) as a white solid. $^{1}$H NMR (400 MHz, DMSO-$d_6$) $\delta$ = 0.87 (6H, dd), 1.28 – 1.53 (2H, m), 1.53 – 1.80 (2H, m), 1.87– 2.10 (1H, m), 2.86– 3.11 (2H, m), 3.94 (1H, t, *J* = 7.8 Hz), 4.15– 4.35 (3H, m), 4.44 (3H, m), 5.12 (1H, br), 5.42 (2H, br), 5.99 (1H, br), 7.24 (2H, d, *J* = 8.1 Hz), 7.49 – 7.28 (5H, m), 7.55 (2H, d, *J* = 8.1 Hz), 7.74 (2H, t, *J* = 7.9 Hz), 7.88 (2H, d, *J* = 7.3 Hz), 8.12 (1H, d, *J* = 7.3 Hz), 9.99 (1H, s). $^{13}$C NMR (101 MHz, DMSO-$d_6$) $\delta$ = 17.76, 18.72, 24.71, 26.28, 29.04, 29.95, 46.19, 52.57, 59.62, 62.10, 65.19, 118.39, 119.59, 124.85, 126.43, 126.57, 127.14, 136.95, 137.01, 140.21, 143.27, 143.41, 155.63, 158.41, 169.89, 170.75, 172.40. ESI-MS: $[M + H]^+$ calcd for $C_{33}H_{40}N_5O_6^+$, 602.30; found (*m/z*), 602.50 $[M + H]^+$.

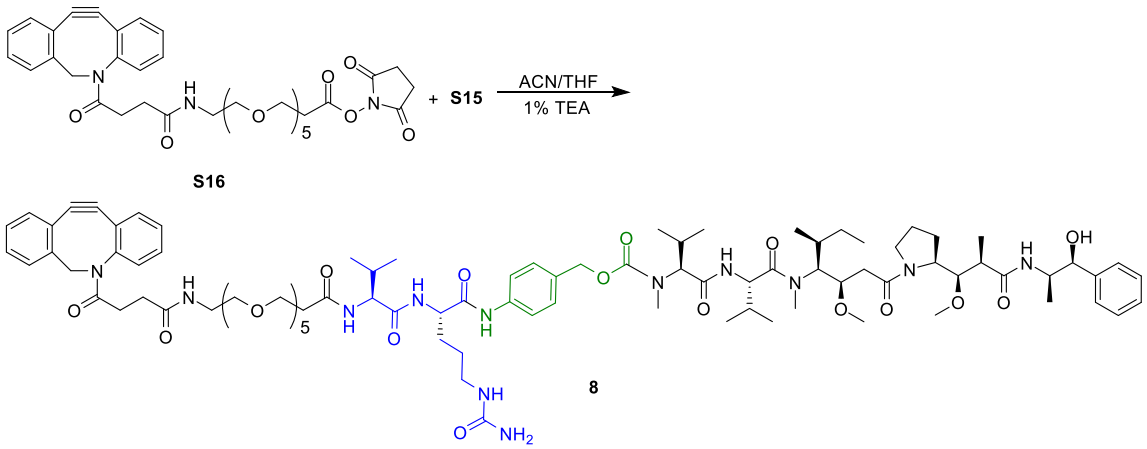
**Synthesis of Fmoc-Val-Cit-PABC-PNP (S14).** To a stirred solution of **S13** (60 mg, 0.1 mmol, 1.0 eq) and 4-nitrophenyl carbonate in anhydrous DMF (5 mL) was added DIPEA (69 µL, 0.4 mmol, 4 eq) dropwise at 25 °C. The mixture was stirred at room temperature for 16 hours. The reaction was quenched by adding 15% citric acid (30 mL), extracted with ethyl acetate (50 mL) twice. The organic phase was combined and washed with brine. The solvents were concentrated in vacuo and the residue was purified by prep-HPLC to afford **S14** (44 mg, 57%) as a white solid. $^{1}$H NMR (600 MHz, DMSO-$d_6$) $\delta$ = 0.87 (6H, dd), 1.34 – 1.49 (2H, m), 1.57 – 1.80 (2H, m), 1.95 – 2.05 (1H, m), 2.90 – 3.08 (2H, m), 3.94 (1H, dd), 4.20– 4.35 (1H, m), 4.44 (1H, m), 5.24 (2H, br), 5.42 (2H, br), 5.99 (1H, br), 7.32 (2H, d, *J* = 8.1 Hz), 7.39 – 7.45 (5H, m), 7.55 (2H, m), 7.64 (2H, d), 7.72-7.77 (2H, dd), 7.88 (2H, d, *J* = 7.3 Hz), 8.12 (1H, d, *J* = 7.3 Hz),8.30-8.33 (2H, m), 10.14 (1H, s). $^{13}$C NMR (150 MHz, DMSO) $\delta$ = 18.21, 19.16, 26.72, 29.38, 30.40, 46.66, 53.09, 60.03, 65.64, 70.21, 119.03, 120.03, 122.55, 125.30, 125.34, 127.58, 129.41, 139.32, 140.66, 143.73, 143.86, 145.15, 151.90, 155.26, 156.06, 158.85, 170.67, 171.23. ESI-MS: $[M + H]^+$ calcd for $C_{40}H_{43}N_6O_{10}^+$, 767.30; found (*m/z*), 767.58 $[M + H]^+$.




**Scheme S4**. Synthesis of **S15**

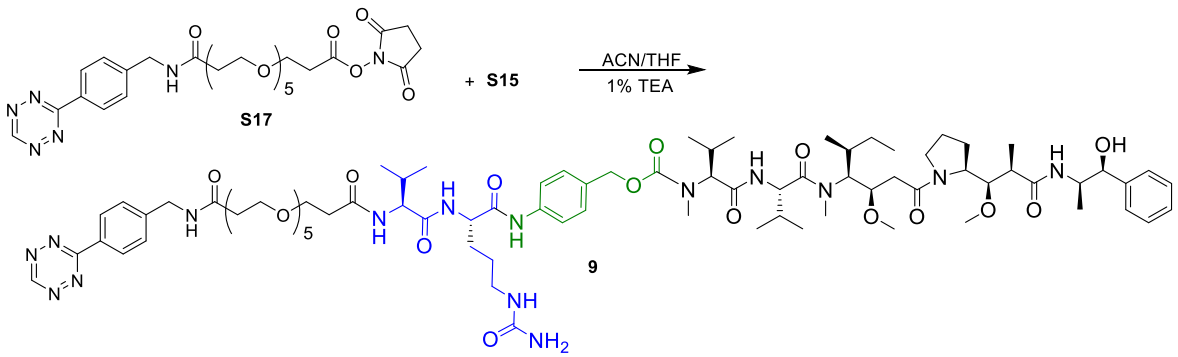
**Synthesis of Val-Cit-PABC-MMAE (S15).** In a 1.5 mL centrifuge tube, **S14** (20 mg, 26 µmol, 1.3 eq), MMAE (14.4 mg, 20 µmol, 1.0 eq), and HOBt (3.5 mg, 26 µmol, 1.3 eq) was dissolved in dry DMF (400 µL) and pyridine (200 µL). The mixture reacted at room temperature under argon for 9 hours before HPLC-SQ2 analysis showed complete consumption of the reactant. Then the reaction mixture was quenched by sat. $NaHCO_3$ (25 mL), the product was collected as a white solid after high-speed centrifugation. Without further purification, the solid was dissolved

in ACN and THF (v/v, 1/1, 4 mL). After high-speed centrifugation, piperidine (1 mL) was added to the supernatant at room temperature. The HPLC-SQ2 confirmed the reaction was finished after 30 min, the mixture was diluted with water (5 mL) and then concentrated in vacuo and the residue was purified by semi prep-HPLC to afford **S15** (12.6 mg, 56% over two steps) [4]. $^1$H NMR (600 MHz, DMSO + 5% $D_2O$) $\delta$ = 0.68 – 1.07 (31H, m), 1.26 (1H, s), 1.34 – 1.84 (10H, m), 1.87 – 1.98 (1H, m), 2.00 – 2.12 (3H, m), 2.20 – 2.29 (1H, m), 2.37 (1H, d), 2.79 –2.89 (3H, m), 2.91 –3.32 (14H, m), 3.52 (1H, m), 3.73 (1H, d), 3.90 – 4.02 (2H, m), 4.21 (1H, m), 4.39 (1H, d), 4.43 – 4.50 (3H, m), 4.97 –5.10 (2H, m), 7.10 – 7.18 (1H, m), 7.20-7.37 (6H, m), 7.54 (2H, br). ESI-MS: [M + H]$^+$ calcd for $C_{58}H_{95}N_{10}O_{12}^+$, 1123.71; found (*m/z*), 1123.85 [M + H]$^+$.




**Scheme S5**. Synthesis of the DBCO-tagged MMAE (**8**).

**Synthesis of DBCO-PEG-Val-Cit-PABC-MMAE (8) {Dubowchik, 2002 #1053}.** In a 1.5 mL centrifuge tube, **S16** (1.4 mg, 1.65 µmol, 1.5 eq) and **S15** (1.2 mg, 1.1 µmol) were dissolved in ACN and THF (v/v, 1/1, 300 µL) with TEA (3 µL) at room temperature. The reaction was finished after 4 hours, confirmed by HPLC-SQ2. The mixture was diluted with 50% ACN with 0.1% TFA (2 mL) and into semi prep-HPLC directly to afford **11** (1.5 mg, 80%) as a white powder. HR-ESI-MS: [M + H]$^+$ calcd for $C_{90}H_{133}N_{12}O_{20}^+$, 1701.9754; found (*m/z*), 1701.9694 [M + H]$^+$.




**Scheme S6**. Synthesis of the tetrazine-tagged MMAE (**9**).

**Synthesis of the Tz-PEG-Val-Cit-PABC-MMAE (9).** In a 1.5 mL centrifuge tube, **S17** (2.9 mg, 4.8 µmol, 1.5 eq) and **S15** (3.6 mg, 3.2 µmol) were dissolved in ACN and THF (v/v, 1/1, 800 µL) with TEA (8 µL) at room temperature. The reaction completed within 4 hours, confirmed by HPLC-SQ2. The mixture was diluted with 50% ACN with 0.1% TFA (3 mL) and into semi prep-

HPLC directly to afford **12** (4.3 mg, 84%) as a pink powder. HR-ESI-MS: $[M + H]^+$calcd for $C_{81}H_{126}N_{15}O_{19}^+$, M = 1612.9349; found (*m/z*), 1612.9407 $[M + H]^+$.

## 5. Chemoenzymatic synthesis of the azide-, cyclopropene- and norbornene-tagged antibodies.

**Preparation of GNF-Her (6) with immobilized Endo-S2 WT.** The suspension of the immobilized Endo-S2 WT (100 µL, 2 mg/mL) was centrifuged at 14000 rpm for 5 min, the supernatant was removed and discarded. Intact Herceptin **5** solution (4 mL, 5.5 mg/mL) was added to the resin, and mixed end to end at 30 °C. The progress was checked by LC-MS and compared with reported data. The cleavage reaction was completed within 3 hours, then the mixture was centrifuged at 14000 rpm for 5 min. The immobilized enzyme was recovered and resuspend in 1 x PBS buffer with 0.5% $NaN_3$. The GNF-Her **6** in the solution was purified with protein A chromatography, exchanged to Tris buffer (100 mM, pH 7.2) and concentrated to 25 mg/mL with a spin filter to yield deglycosylated Herceptin **6** (20 mg, 91%). ESI-MS: calcd for GNF-Her, M = 145865 Da; found (*m/z*), 2702.16 $[M + 54H]^{54+}$, 2753.11 $[M + 53H]^{53+}$, 2806.10 $[M + 52H]^{52+}$,2861.00 $[M + 51H]^{51+}$, 2977.90 $[M + 49H]^{49+}$, deconvolution of the ESI-MS, M = 145865 Da.

**General Procedure for transglycosylation reactions.** To a solution of antibody **6** (25 mg/ml) in a Tris buffer (100 mM, pH 7.2) was added glycan oxazoline **4a**, **4b**, or **4c** (30 mol equiv. per antibody). Then the Endo-S2 D184M mutant (0.4% of the antibody, w/w) was added to the solution and the mixture was incubated at 30 °C. LC-ESI-MS monitoring indicated the complete glycosylation within 20 min for all the three reactions. The functionalized Herceptin **7a**, **7b**, **7c** were purified by protein A chromatography. With a spin filter, the buffer was exchanged to phosphate buffer (50 mM pH 7.2), and the product was concentrated to 5 mg/mL.

**Synthesis of compound 7a.** Prepared from **6** (6.5 mg, 45 nmol, 1 eq) and **5** (3.4 mg, 1.4 µmol, 30 eq) with General Procedure B to yield azide functionalized Herceptin **7a** (6 mg, 92%). ESI-MS: calcd for Her-(Di-$N_3$-SCT)$_2$, M = 151022 Da; found (*m/z*), 2797.64 $[M + 54H]^{54+}$, 2850.5 $[M + 53H]^{53+}$, 2905.20 $[M + 52H]^{52+}$,2962.20 $[M + 51H]^{51+}$, 3021.40 $[M + 50H]^{50+}$, 3083.00 $[M + 49H]^{49+}$, deconvolution of the ESI-MS, M = 151024 Da.

**Synthesis of compound 7b** Prepared from **6** (5.0 mg, 35 nmol, 1 eq) and **4b** (2.4 mg, 1.0 µmol, 30 eq) with General Procedure B to yield cyclopropene functionalized Herceptin **7b** (5.0 mg, 96%). ESI-MS: calcd for Her-(Di-Cp-SCT)$_2$, M = 150589 Da; found (*m/z*), 2789.61 $[M + 54H]^{54+}$, 2842.21 $[M + 53H]^{53+}$, 2953.64 $[M + 51H]^{51+}$, 3012.69 $[M + 50H]^{50+}$, deconvolution of the ESI-MS, M = 150586 Da.

**Synthesis of compound 7c.** Prepared from **6** (8.0 mg, 55 nmol, 1 eq) and **4c** (4.0 mg, 1.6 µmol, 30 eq) with General Procedure B to yield cyclopropene functionalized Herceptin **7c** (6.5 mg, 81%). ESI-MS: calcd for Her-(Di-NB-SCT)$_2$, M = 150629 Da; found (*m/z*), 2739.82 $[M + 55H]^{55+}$, 2843.07 $[M + 53H]^{53+}$, 2954.55 $[M + 51H]^{51+}$, 3013.63 $[M + 50H]^{50+}$, deconvolution of the ESI-MS, M = 150632 Da.

**Comparison of the transglycosylation reaction catalyzed by Endo-S2 D184M and Endo-S D233Q mutant.** To the solution of **6** (1 mg, 6.7 nmol, 25 mg/mL in 100 mM Tris buffer, pH 7.2) was added **4a** (0.35 mg, 133 nmol, 20 mol. equiv. per antibody, 3.5 µL, 100 mg/mL). The pH of the mixture was confirmed to be around 7.2 by a pH paper. Endo-S2 D184M (4.4 µg, 0.34 µL) or Endo-S D233Q (4.4 µg, 0.34 µL) was then added (final concentration of the mutant enzyme, 0.4% of the antibody, w/w) and the mixture was incubated at 30 °C. A sample was taken every 20 min from each vial until the reaction was completed. (Figure S2') The Endo-S2 D184M catalyzed reaction was complete within 20 min. On the other hand, the Endo-S D233Q required 80 min.

## 6. Preparation of antibody-drug conjugates by click reactions.

**Preparation of 10a**. A solution of azide-tagged antibody **7a** (2 mg, 13 nmol) and the DBCO-modified MMAE (**8**) (459 µg, 0.27 µmol, 20 eq) in a phosphate buffer (50 mM, pH 7.2) containing 30% dimethyl sulfoxide (DMSO) (final volume, 1 mL) was incubated at ambient temperature (23 $^{o}$C). The reaction mixture was shielded from light and gently vortexed. The reaction was monitored by LC-ESI-MS analysis. After 8 h, the click reaction was complete as indicated by LC-ESI-MS. The mixture was then diluted with phosphate buffer (5 mL, 50 mM, pH 7.2) and filtered by 0.22 µm syringe filter. The conjugate product in the filtrate was purified by protein A chromatography to give **10a** (1.7 mg, 83%). ESI-MS of **10a**: calcd. M = 157830 Da; found (*m/z*), 2870.74 $[M + 55H]^{55+}$, 2923.86 $[M + 54H]^{54+}$, 2978.93 $[M + 53H]^{53+}$, 3036.20 $[M + 52H]^{52+}$, 3095.80 $[M + 51H]^{51+}$, deconvolution of the ESI-MS, M = 157834 Da. Fc analysis: calcd, M = 30118 Da; found (m/z), 1772.74 $[M + 17H]^{17+}$, 1883.39 $[M + 16H]^{16+}$, 2008.92 $[M + 15H]^{15+}$, deconvolution data, M = 30118 Da.

**Preparation of 10b.** The click reaction between the cyclopropene-modified antibody (**7b**) (2.0 mg, 13 nmol, 1 eq) and the tetrazine-modified MMAE (**9) (**419 µg, 0.26 µmol, 20 eq) was performed in the same way as described for the synthesis of **10a**. The reaction was complete within 4 h and subsequent affinity column purification gave **10b** (1.7 mg, 83%). ESI-MS of **10b**: calcd M = 157129 Da; found (*m/z*), 2854.21 $[M + 55H]^{55+}$, 2907.16 $[M + 54H]^{54+}$, 2961.90 $[M + 53H]^{53+}$, 3018.84 $[M + 52H]^{52+}$, 3078.08 $[M + 51H]^{51+}$, deconvolution of the ESI-MS, M = 157132 Da. Fc analysis: calcd M = 29697 Da; found (m/z), 1746.21 $[M + 17H]^{17+}$, 1855.26 $[M + 16H]^{16+}$, 1978.86 $[M + 15H]^{15+}$, deconvolution of the ESI-MS, M = 29698 Da.

**Preparation of 10c.** The click reaction between the norbornene-modified antibody (**7c**) (2.0 mg, 13 nmol, 1 eq) and the tetrazine-modified MMAE (**9) (**430 µg, 0.27 µmol, 20 eq) was performed in the same way as described for the synthesis of **10b**. The reaction was complete within 16 h and subsequent affinity column purification gave **10c** (1.4 mg, 68%). ESI-MS of **10c**: calcd M = 156969 Da; found (*m/z*), 2854.90 $[M + 55H]^{55+}$, 2907.88 $[M + 54H]^{54+}$, 2962.64 $[M + 53H]^{53+}$, 3019.60 $[M + 52H]^{52+}$, 3078.71 $[M + 51H]^{51+}$, deconvolution of the ESI-MS, M = 156971 Da. Fc analysis: calcd M = 29688 Da; found (m/z), 1747.26 $[M + 17H]^{17+}$, 1856.41 $[M + 16H]^{16+}$, 1980.13 $[M + 15H]^{15+}$, deconvolution of the ESI-MS, M = 29687 Da.

## 7. *In Vitro* Cytotoxicity Assay

T47D and SKBR3 (ATCC) cells were cultured in DMEM supplemented with 10% FBS, 50 U/ml penicillin, and 50 µg/mL streptomycin (ThermoFisher) and seeded at 200,000 cells/mL into a 96

well plate (20,000 cells/well) and grown overnight until 60% confluent. Cells were then washed with PBS and incubated with fresh media containing the ADCs starting at a concentration of 1 μg/mL and serially diluted 1:2. Each compound was assessed in duplicate wells, and cells without compound served as control. Plates were incubated for 72 hours and cell viability was analyzed by CellTiter-Glo Luminescent Cell Viability Assay (Promega) as per manufacturer's instructions. Briefly, equal volume kit reagent to media was added to each well. Cells were then lysed and incubated for 10 mins before luminescence was recorded.

## 8. Size Exclusion Chromatography

The size exclusion chromatography was carried out by a ÄKTA pure system (Cytiva), equipped with a Superdex® 200 Increase 10/300 GL column (Cytiva). PBS was used as the mobile phase. The flow rate was 0.75 mL/min, over 48 min. 50 μg of the conjugates **10a-c** was injected for the analysis. No aggregation was found for **10a**, while ca. 8% aggregation was found for conjugates **10b** and **10c** (Figure S18)

## 9. Serum Stability Assay of the ADCs

In a 1.5 mL centrifuge tube, the respective antibody-drug conjugate (**10a**, **10b**, or **10c**) (50 μL, 2.0 mg/ml) and rat serum (200 μL, Sigma Aldrich, United States) were mixed in a 1.5 mL vial. Then the solution was filtered with UFC30GV0S centrifugal filter units (Merck, Germany) and the filtrate was incubated at 37°C for 3 days. The conjugates were enriched by 50 μL anti-human-IgG (Fc-Specific) agarose slurry (Sigma Aldrich, United States) according to the reported procedures.[5] The concentration of the conjugates was measured, then analyzed by LC-MS. No apparent degradation of the conjugates (**10a**, **10b**, and **10c**) was found (Figure S19)

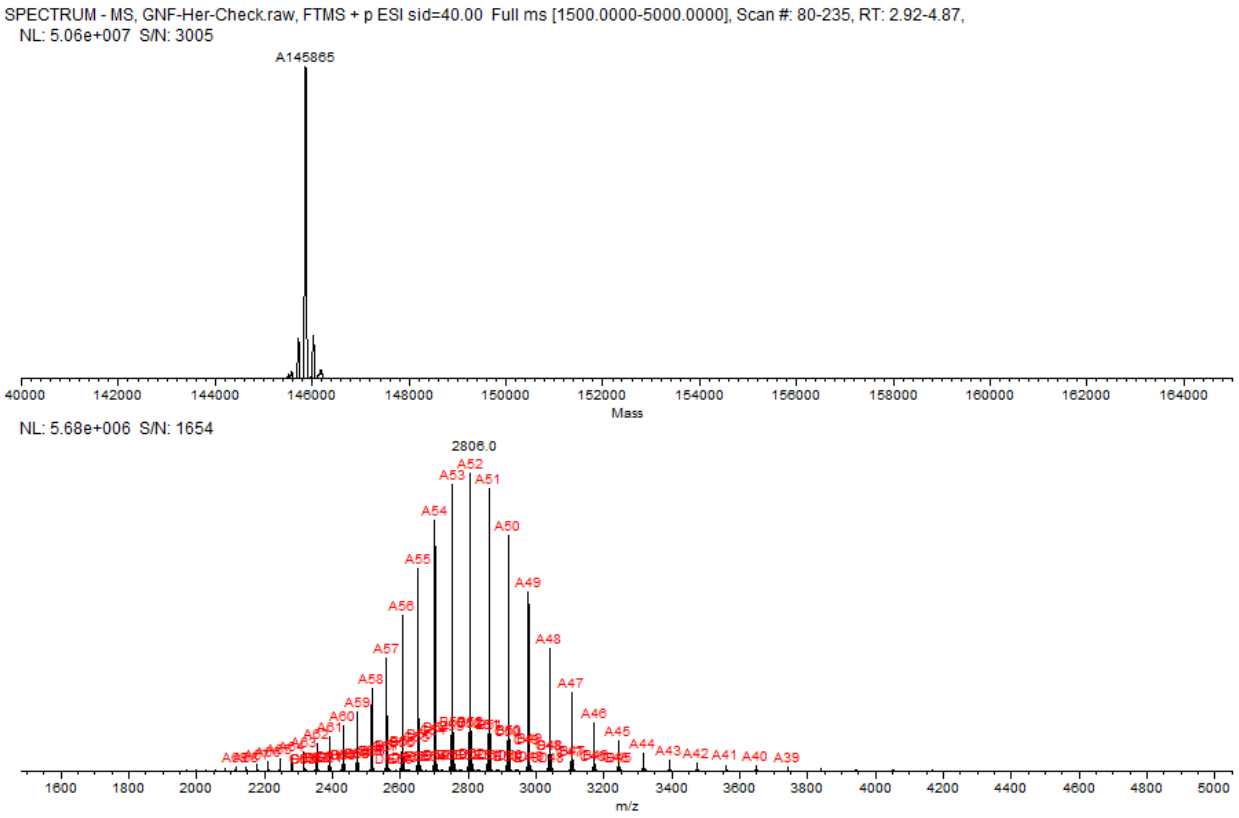



**Figure S1.** LC-MS analysis of the deglycosylated trastuzumab (Fucα1,6GlcNAc-trastuzumab, **6**).

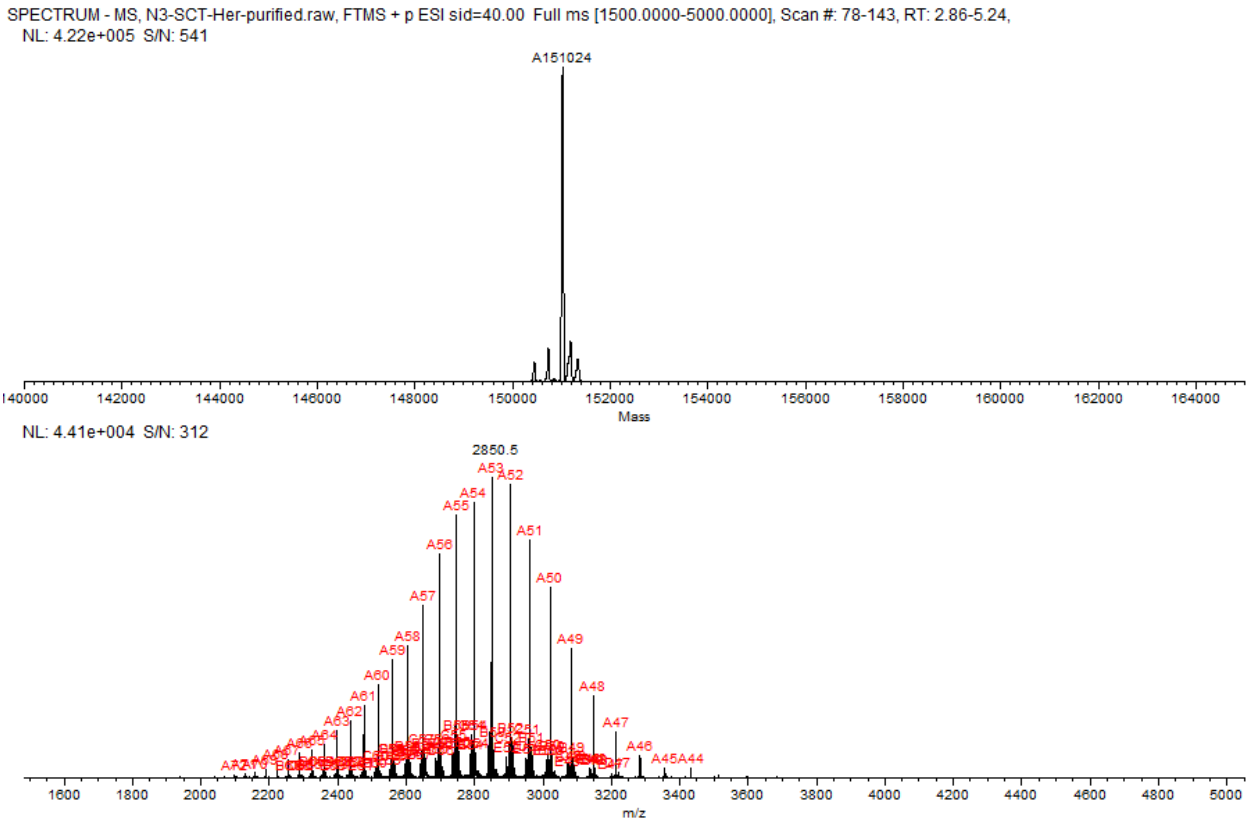



**Figure S2**. LC-ESI-MS analysis of the intact azide-tagged antibody (**7a**).

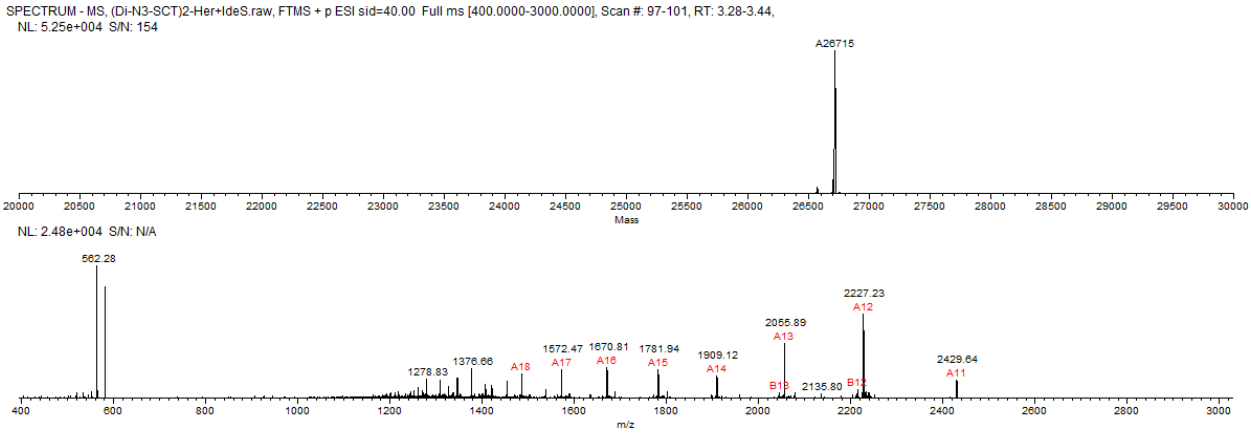



**Figure S3**. LC-ESI-MS analysis of the Fc domains released by IdeS treatment of the azide-tagged antibody (**7a**).

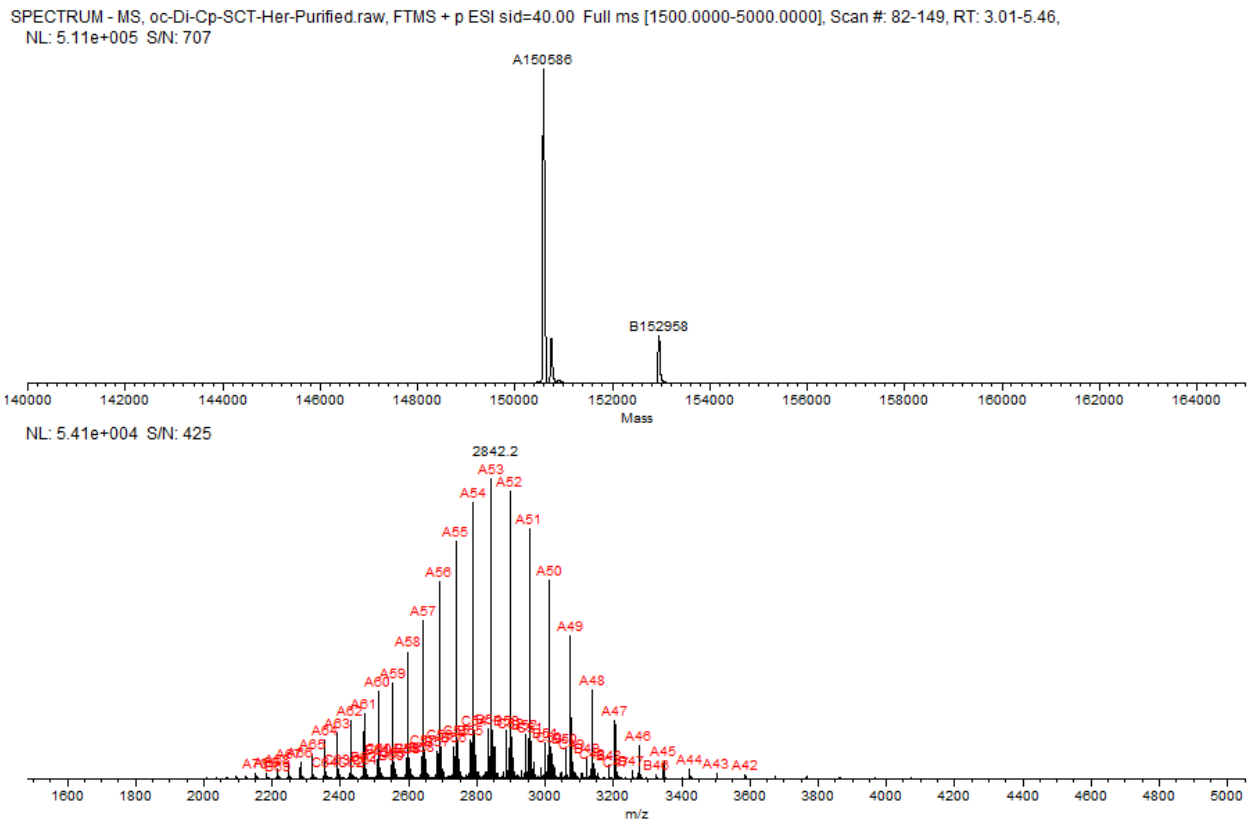



**Figure S4**. LC-ESI-MS analysis of the intact cyclopropene-tagged antibody (**7b**).

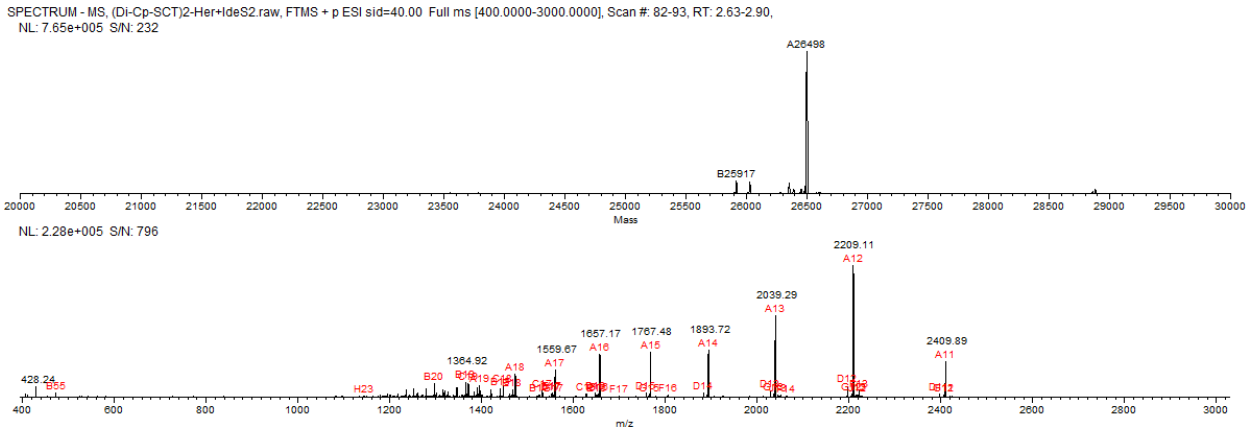



**Figure S5**. LC-ESI-MS analysis of the Fc domains released by IdeS treatment of the cyclopropene-tagged antibody (**7b**).

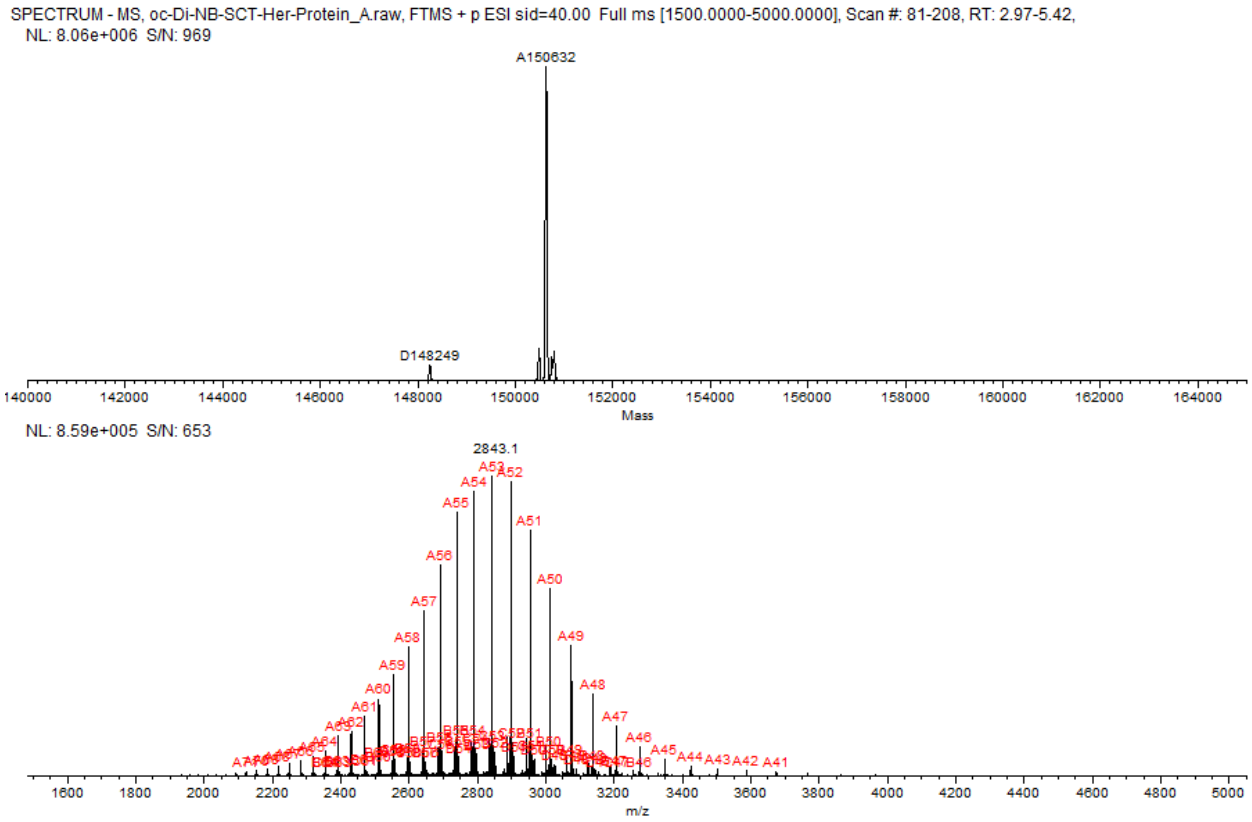



**Figure S6**. LC-ESI-MS analysis of the intact norbornene-tagged antibody (**7c**).

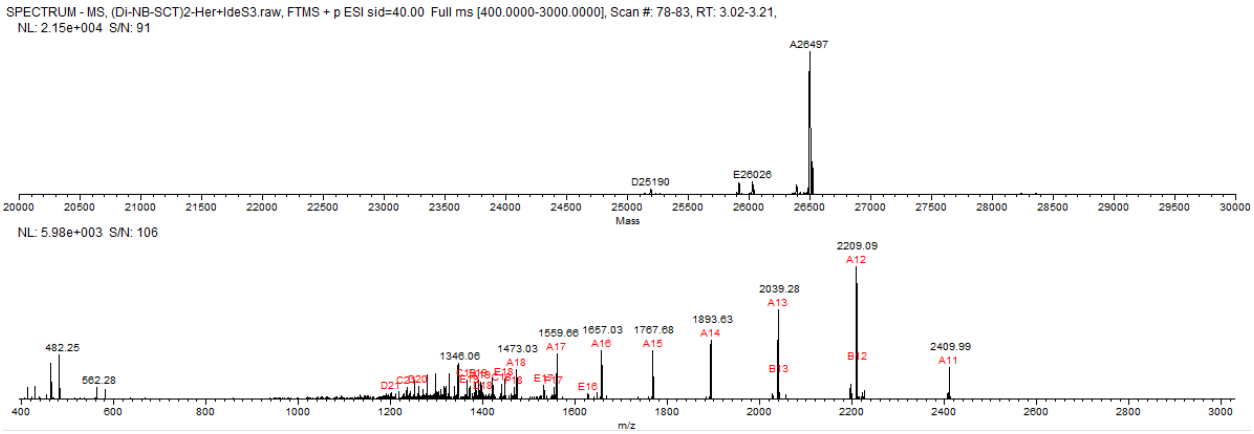



**Figure S7**. LC-ESI-MS analysis of the Fc domains released by IdeS treatment of the norbornene-tagged antibody (**7c**).

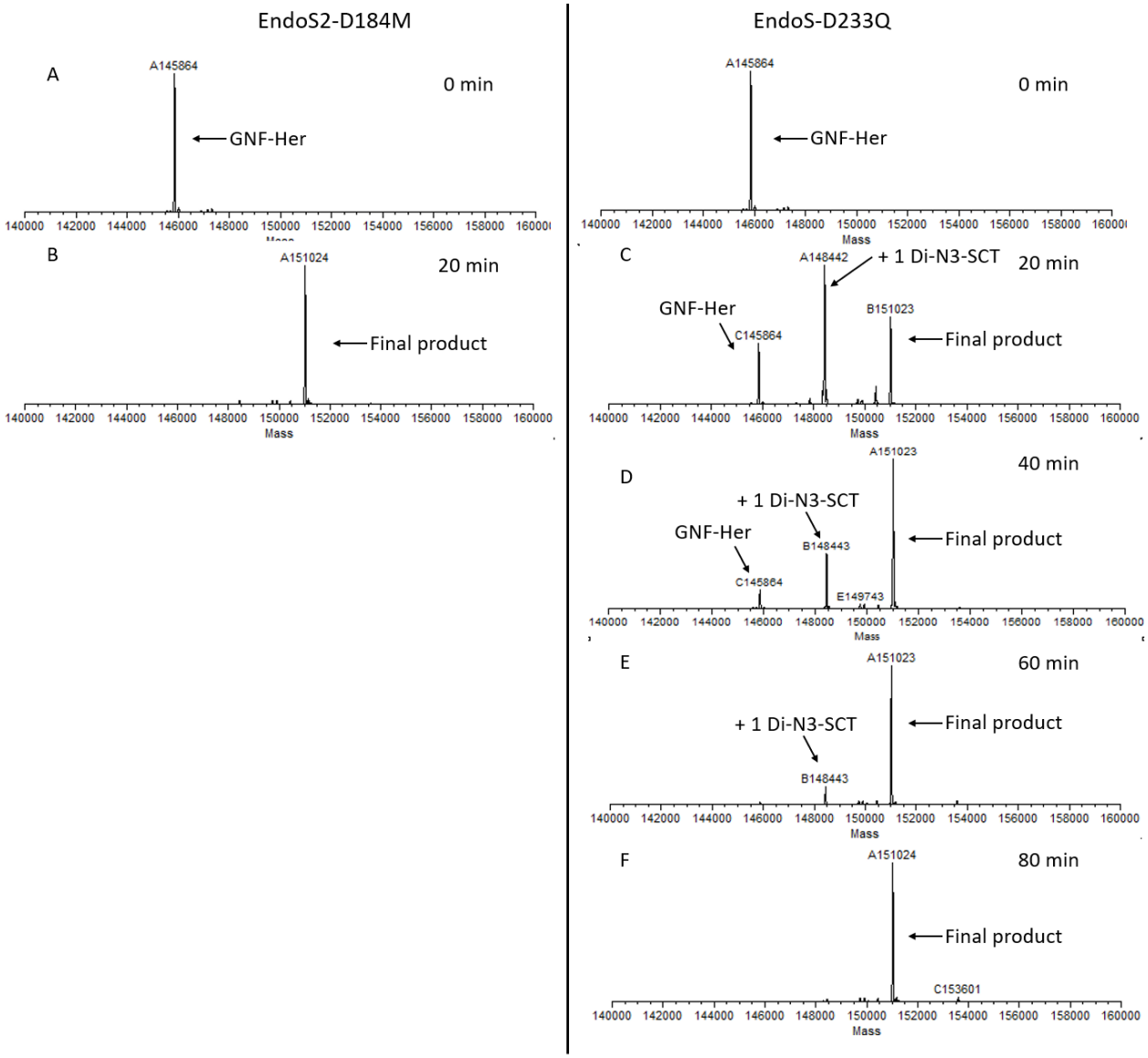



**Figure S8.** The LC-ESI-MS monitoring of the glycosylation reactions between azide-glycan oxazoline **4a** and antibody **6** catalyzed by Endo-S2 D184M and Endo-S D233Q mutant. A mixture of the deglycosylated antibody **6** (1 mg, 6.7 nmol, 25 mg/mL), azide-tagged glycan oxazoline **4a** (0.35 mg, 133 nmol, 20 *mol. equiv.* of the antibody), and the mutant enzyme (0.4 mg/mL) in a Tris buffer (100 mM, pH 7.2) was incubated at 30 $^{o}$C and the reaction was monitored by LC-ESI-MS analysis of the intact antibodies at 20 min intervals. A) the deconvoluted mass of **6**; B) the deconvoluted mass of the reaction mixture catalyzed by Endo-S2 D184M at 20 min; C) the deconvoluted mass of the reaction mixture catalyzed by Endo-S D233Q at 20 min; D) the deconvoluted mass of the reaction mixture catalyzed by Endo-S D233Q at 40 min; E) the deconvoluted mass of the reaction mixture catalyzed by Endo-S D233Q at 60 min; F) the deconvoluted mass of the reaction mixture catalyzed by Endo-S D233Q at 80 min. Under the same conditions, the reaction catalyzed by Endo-S2 D184M mutant was completed within 20 min with essential 100% conversion without formation of by-products (panel B), while the reaction catalyzed by Endo-S D233Q mutant was completed at 80 min, with trace amount of formation of non-enzymatic addition of an addition glycan (panel F, MW 153601).

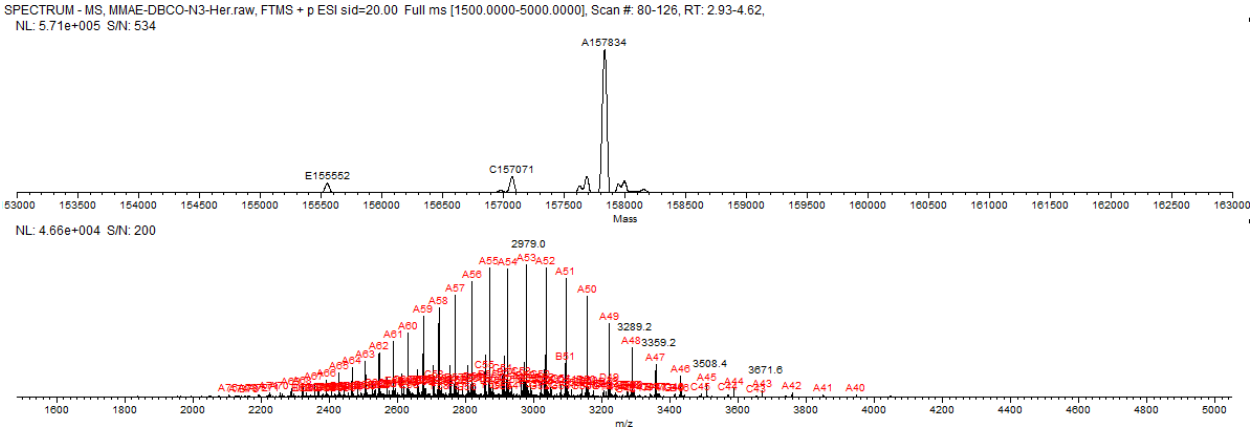



**Figure S9**. LC-ESI-MS analysis of the intact antibody-drug conjugate (**10a**).

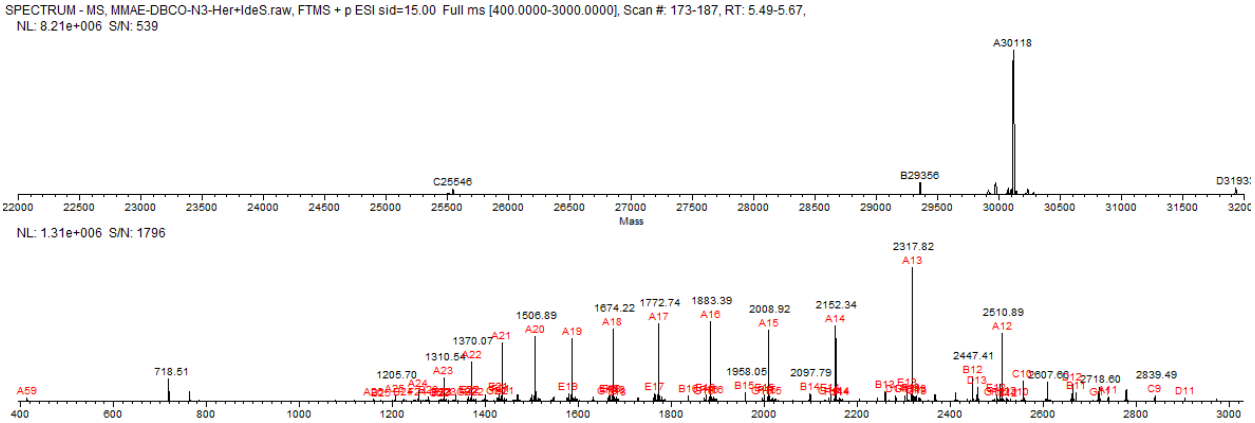



**Figure S10**. LC-ESI-MS analysis of the Fc domain released by IdeS treatment of the antibody-drug conjugate (**10a**).

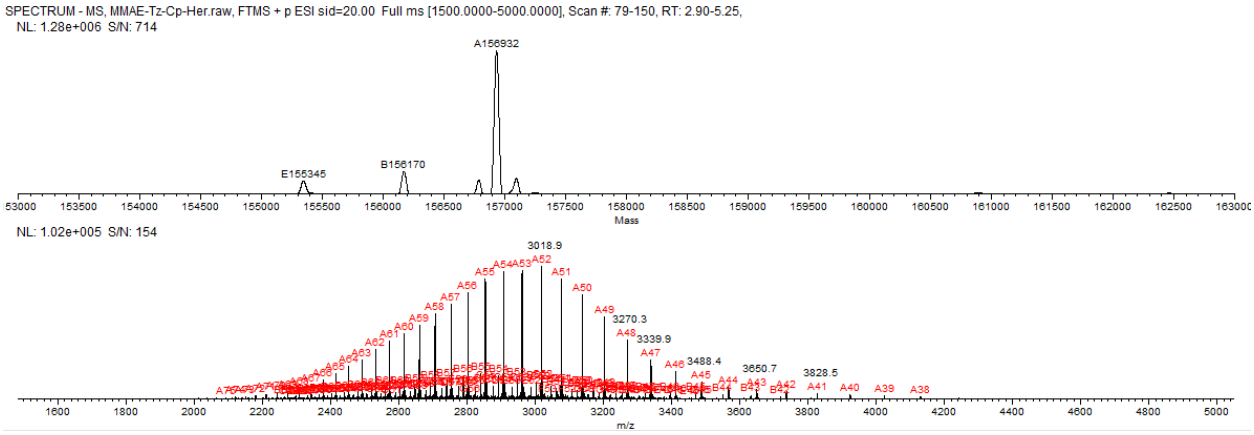



**Figure S11**. LC-ESI-MS analysis of the intact antibody-drug conjugate (**10b**).

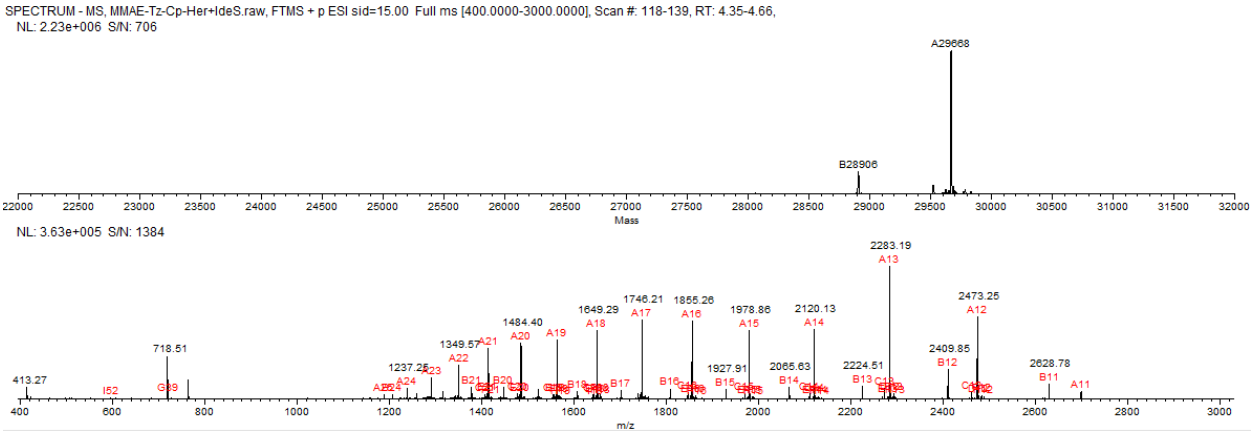



**Figure S12**. LC-ESI-MS analysis of the Fc domain released by IdeS treatment of the antibody-drug conjugate (**10b**).

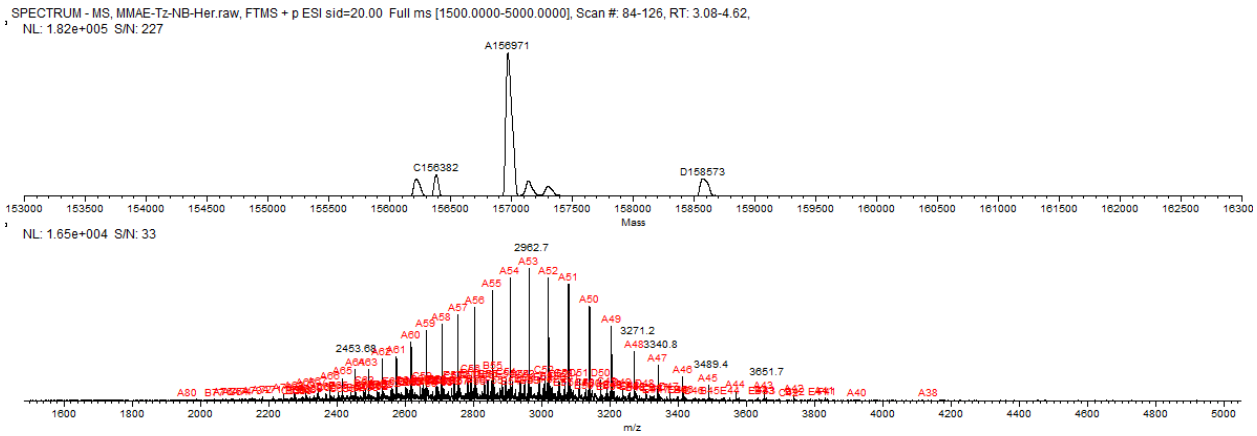



**Figure S13**. LC-ESI-MS analysis of the intact antibody-drug conjugate (**10c**).

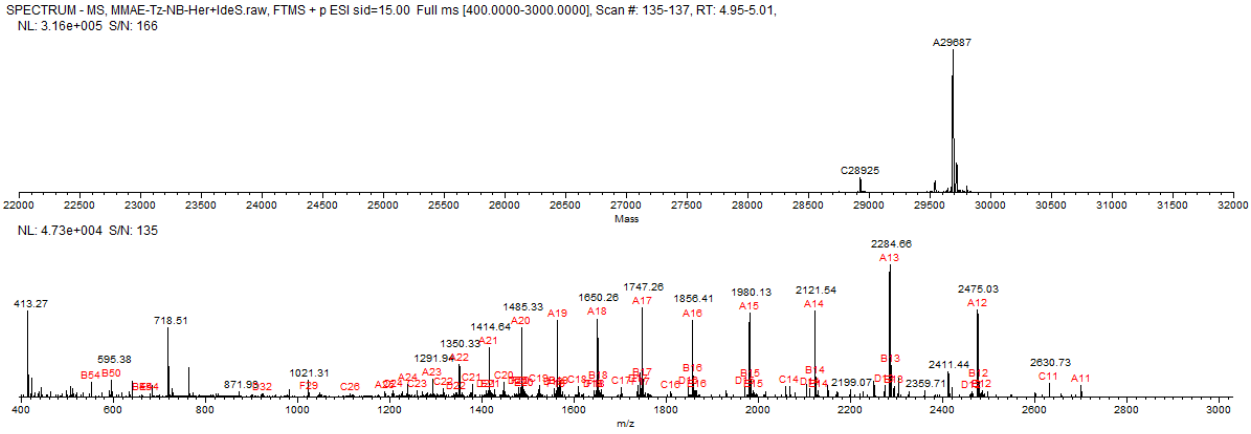



**Figure S14**. LC-ESI-MS analysis of the Fc domain released by IdeS treatment of the antibody-drug conjugate (**10c**).

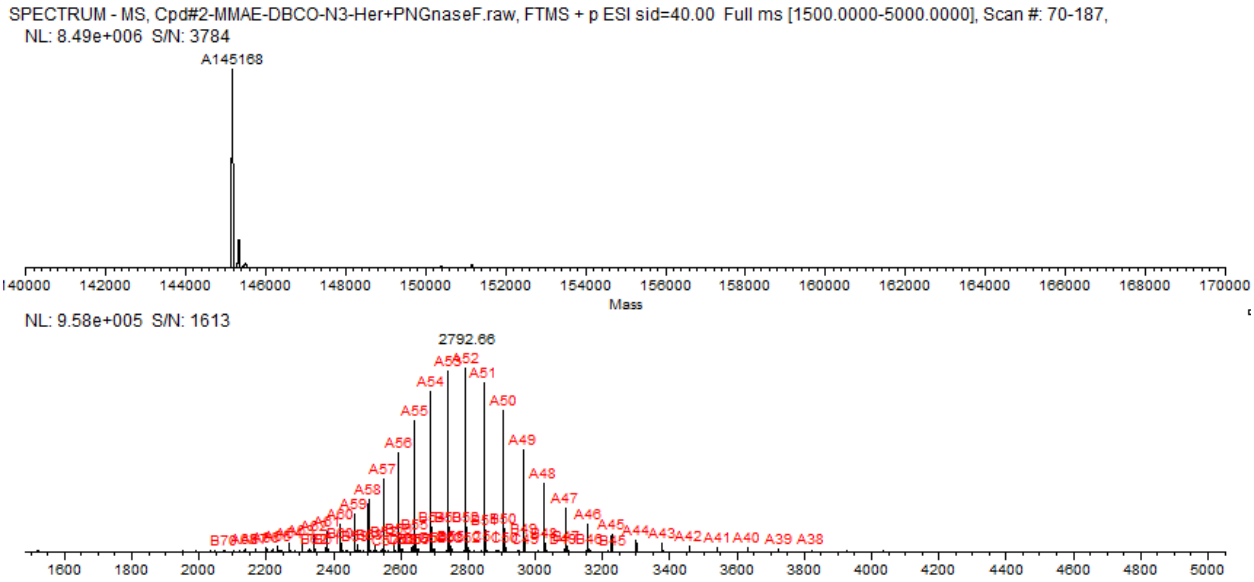



**Figure S15**. LC-ESI-MS analysis of the intact antibody backbone after PNGase F treatment of the antibody-drug conjugate (**10a**).

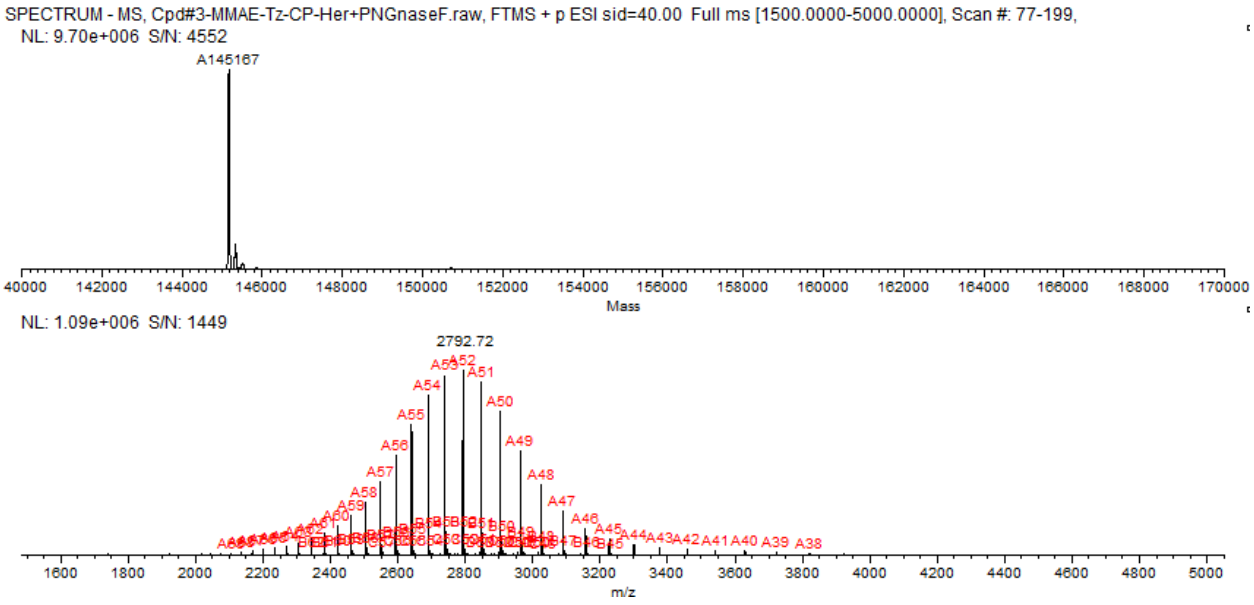



**Figure S16**. LC-ESI-MS analysis of the intact antibody backbone after PNGase F treatment of the antibody-drug conjugate (**10b**).

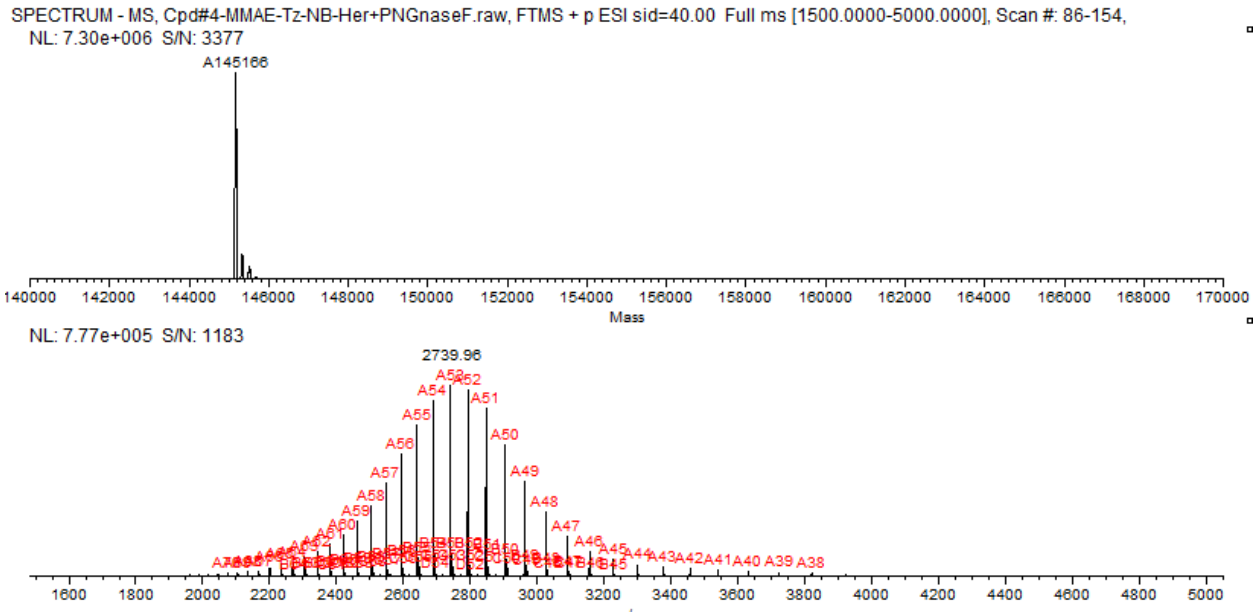



**Figure S17**. LC-ESI-MS analysis of the intact antibody backbone after PNGase F treatment of the antibody-drug conjugate (**10c**).

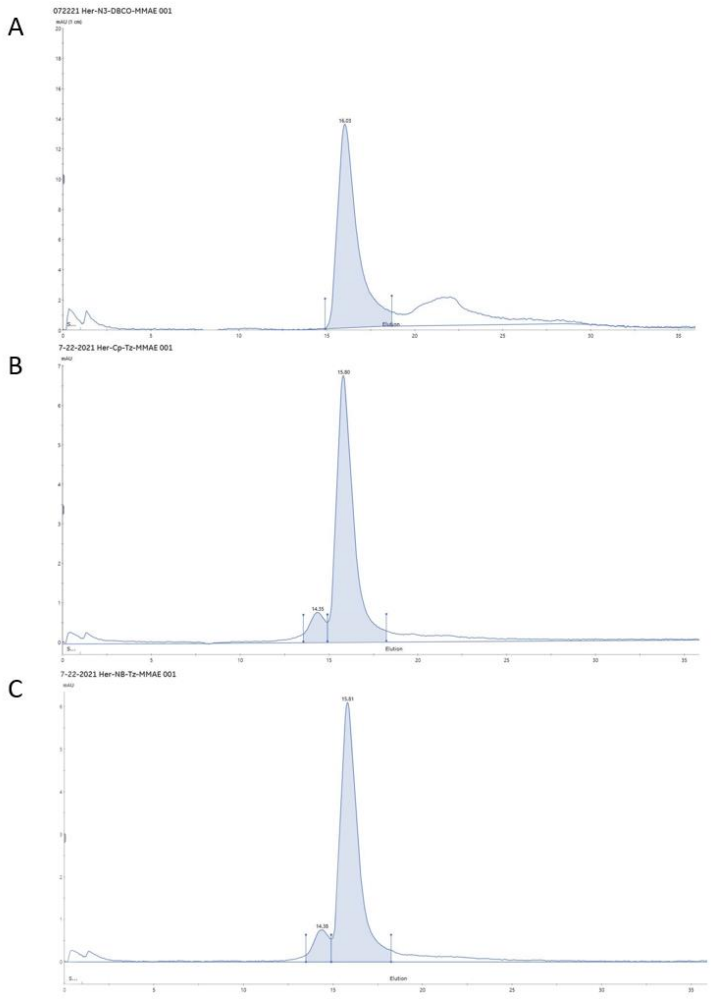



**Figure S18.** Size Exclusion for the ADCs (**10a-10c)**. A) size exclusion chromatography for **10a**; B) size exclusion chromatography for **10b**; C) size exclusion chromatography for **10c**.

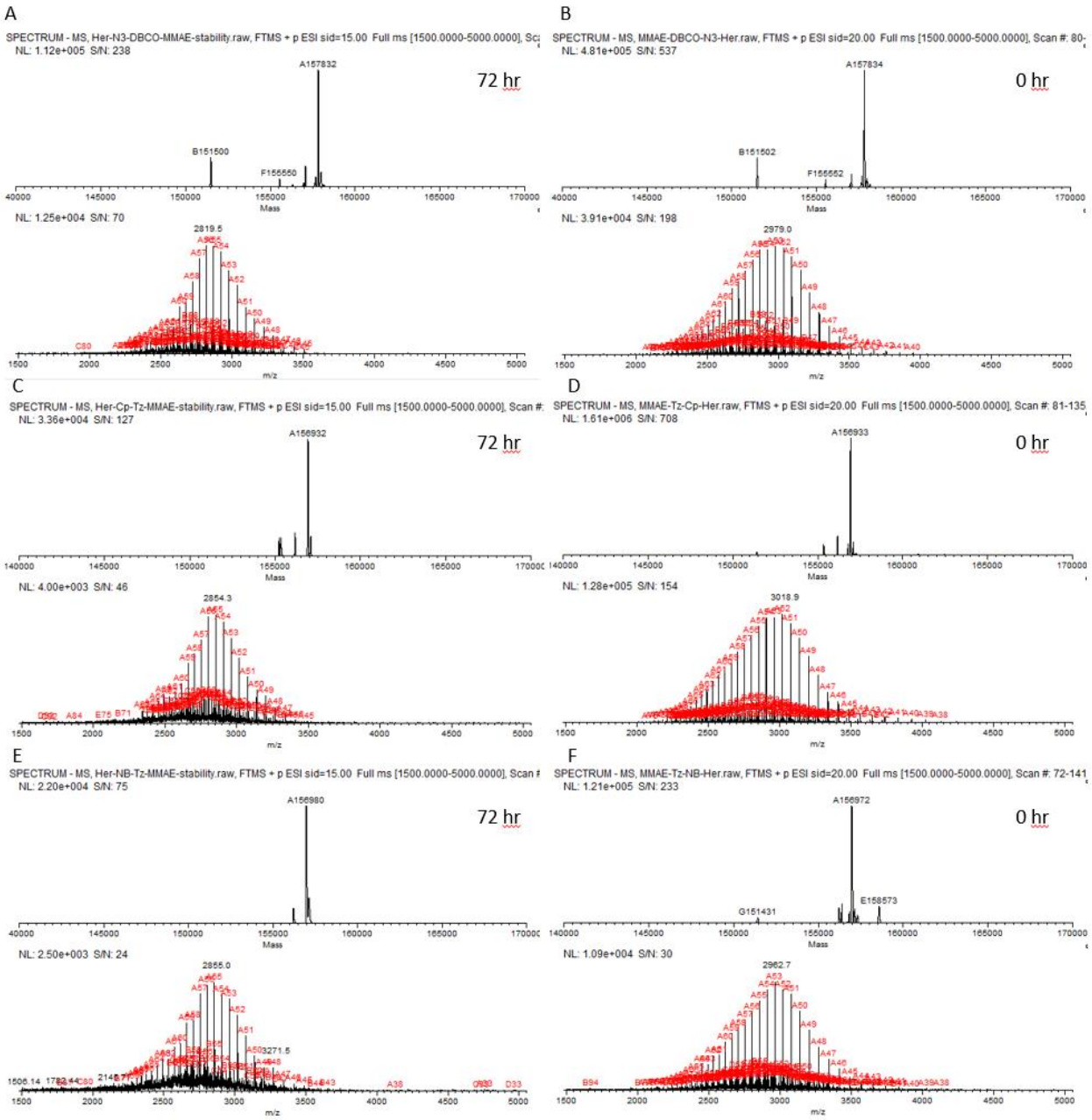
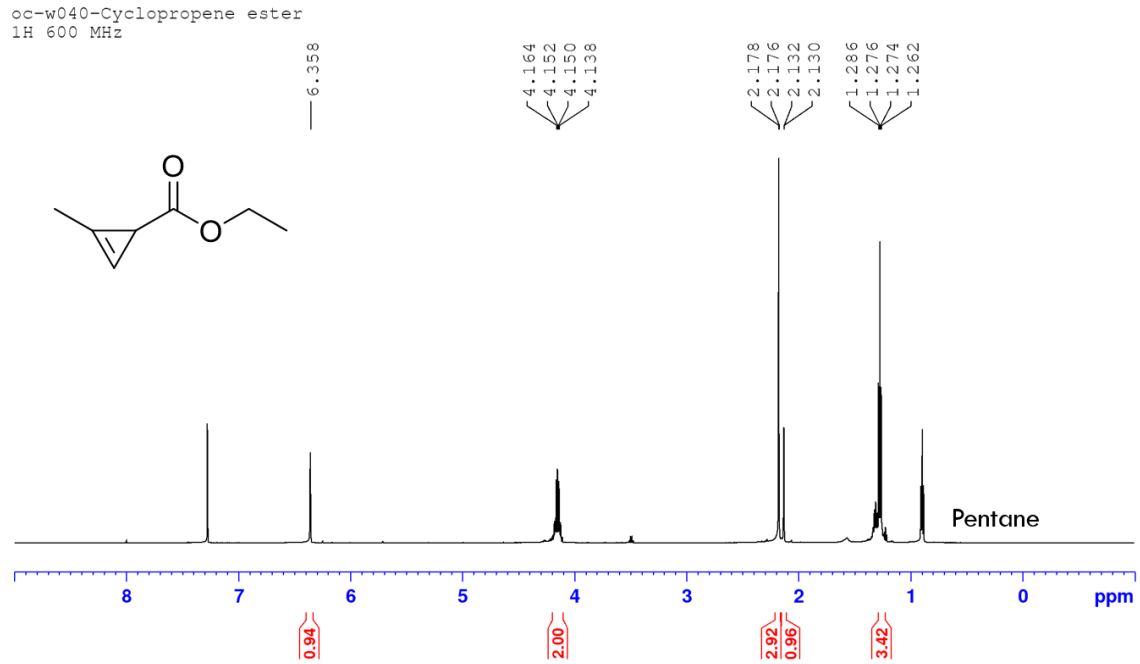
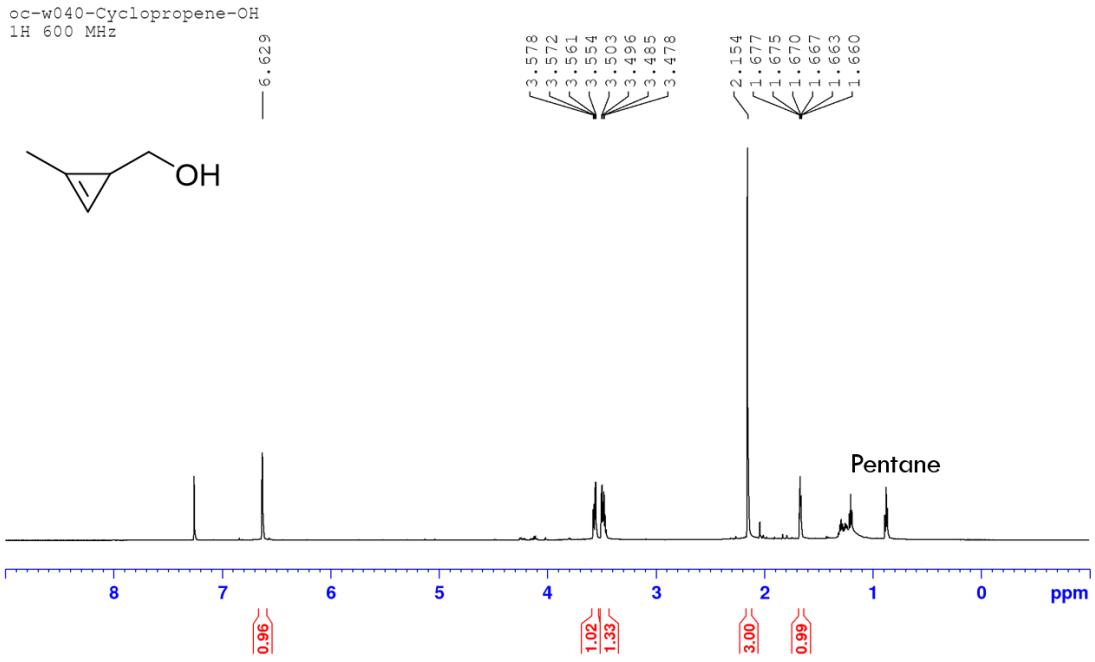



**Figure S19**. Serum Stability results. A) the deconvoluted mass of **10a** incubated in rat serum for 72 hours; B) the deconvoluted mass of **10a** at 0 hour; C) the deconvoluted mass of **10b** incubated in rat serum for 72 hours; D) the deconvoluted mass of **10b** at 0 hour; E) the deconvoluted mass of **10c** incubated in rat serum for 72 hours; F) the deconvoluted mass of **10c** at 0 hour.

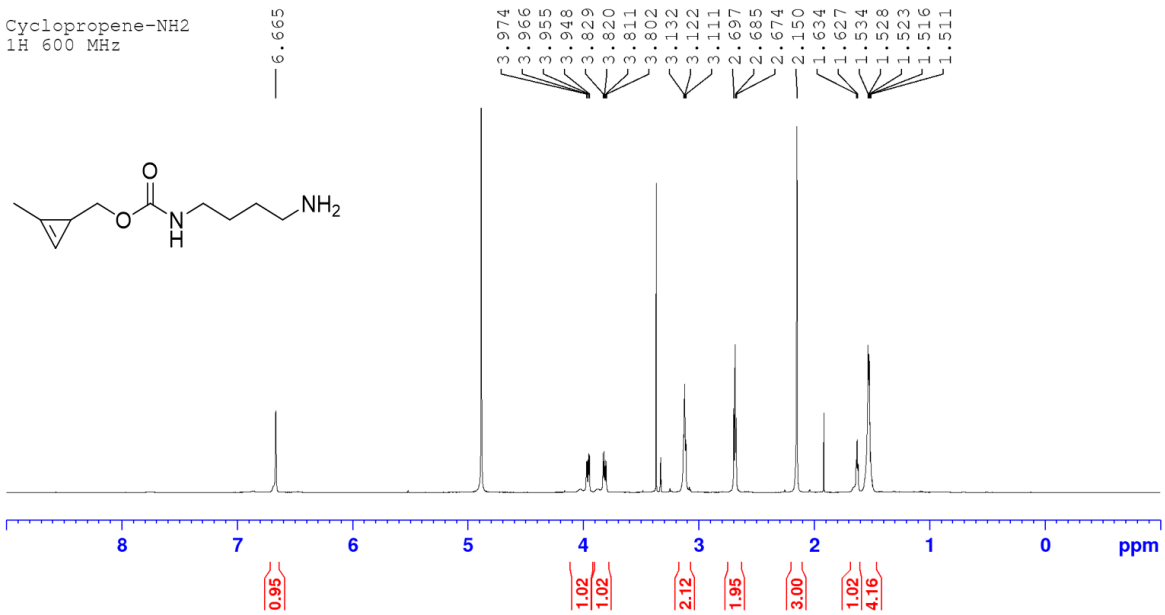
## NMR spectra



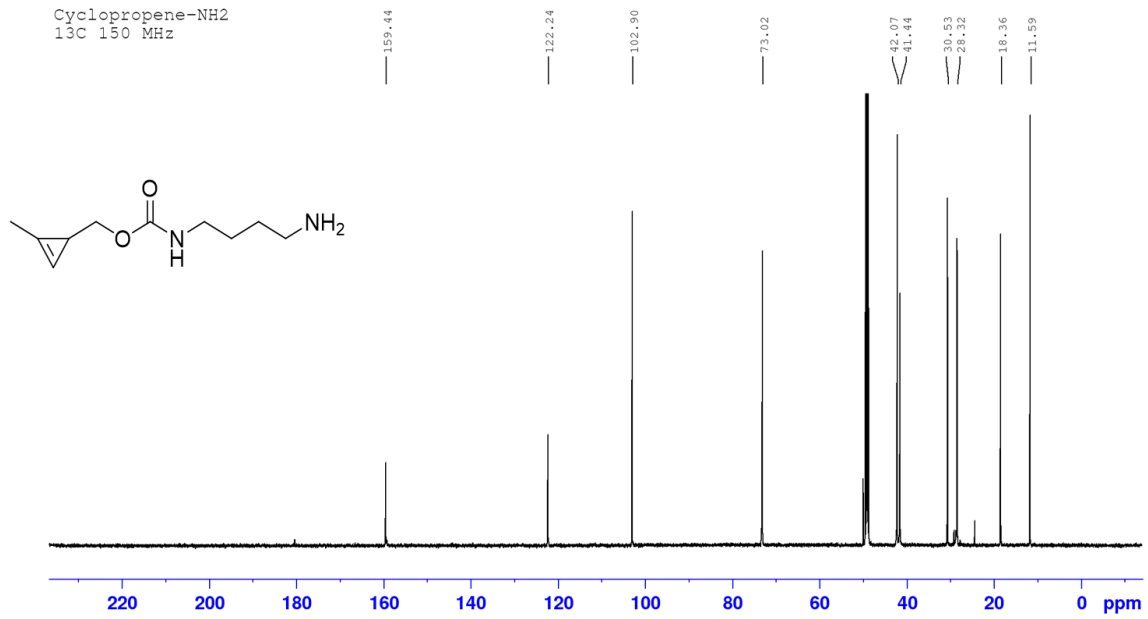

$^{1}$H NMR (600MHz, $CDCl_3$): compound **S2**



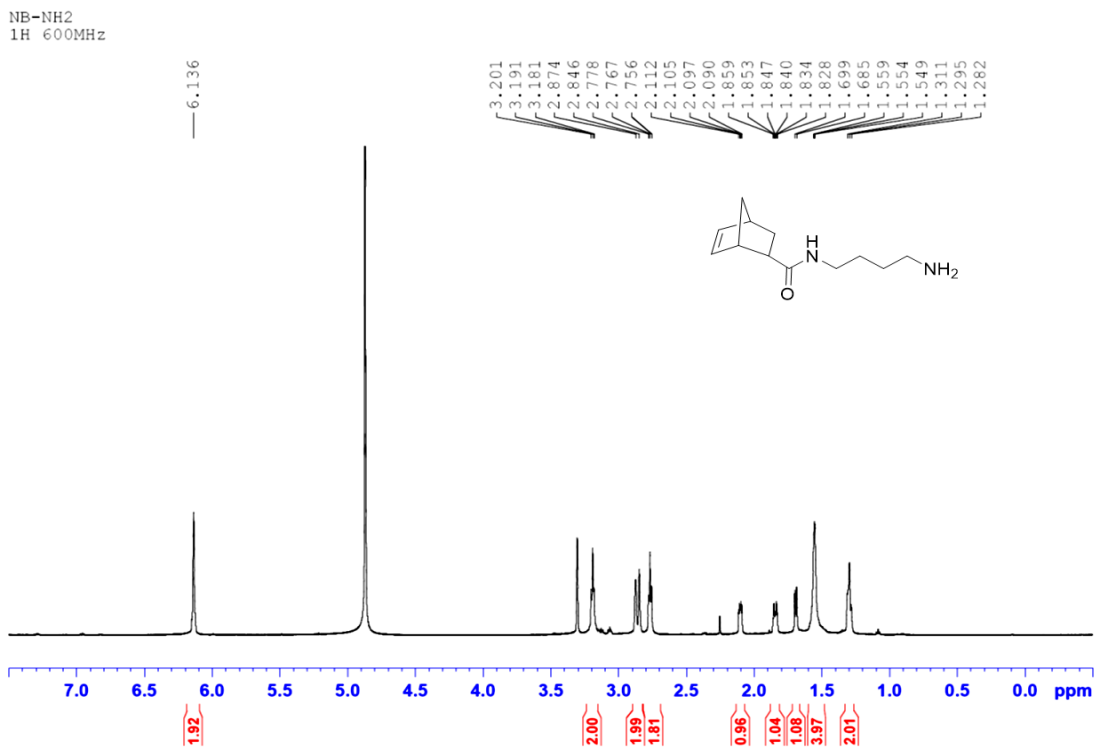

$^{1}$H NMR (600MHz, $CDCl_3$): compound **S3**



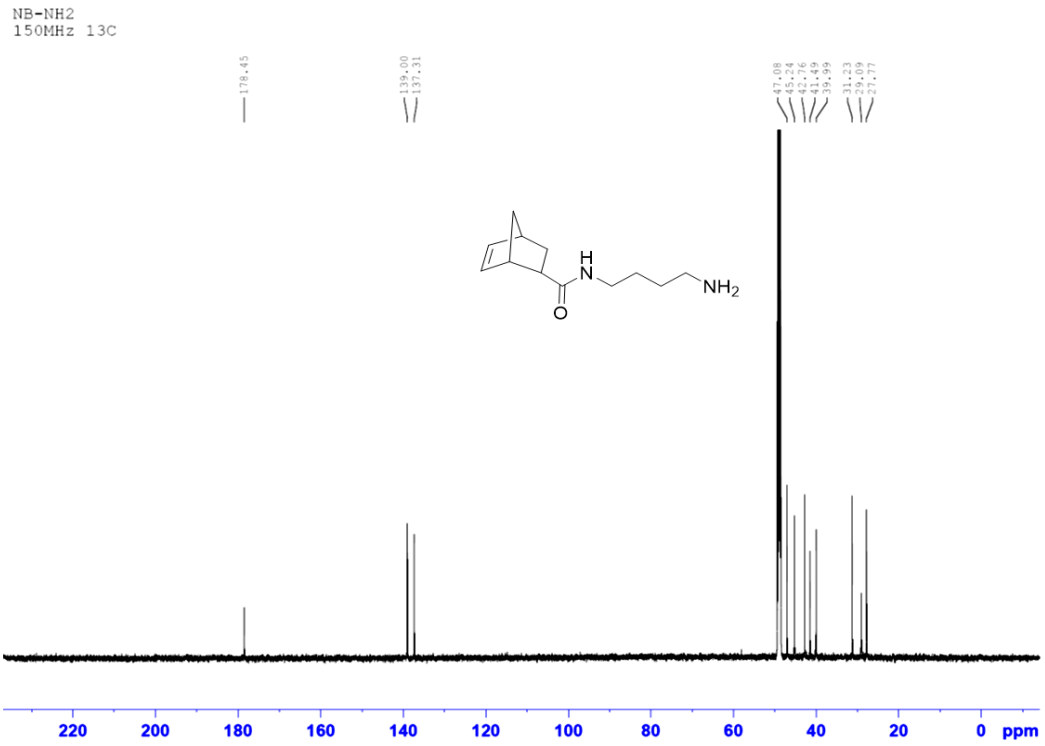

$^{1}$H NMR (600MHz, $CDCl_3$): compound **S4**



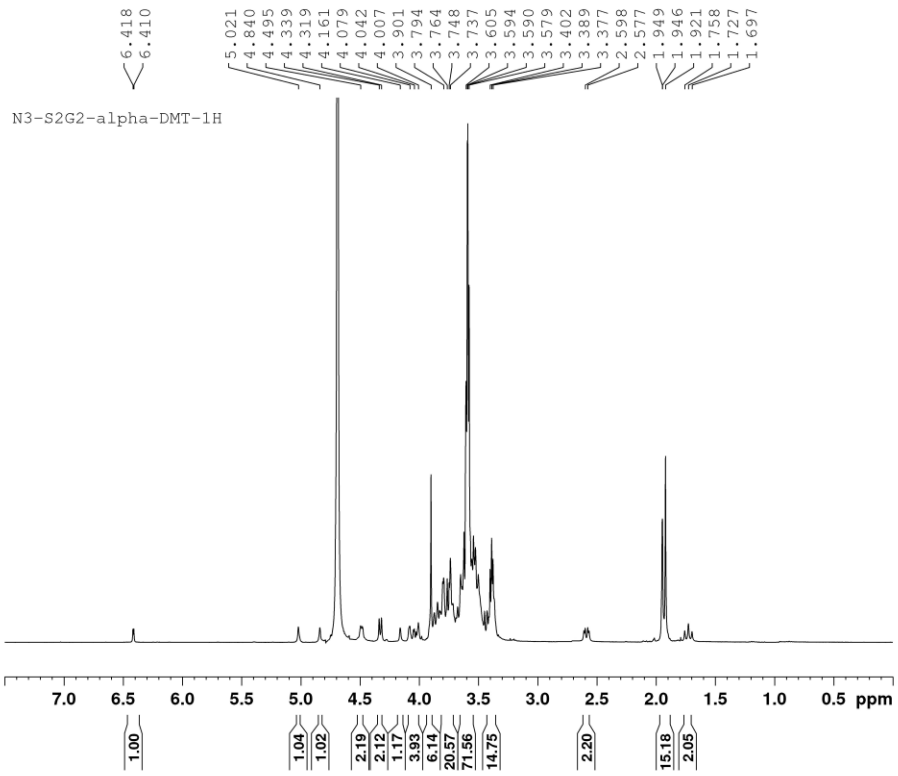

$^{13}$C NMR (150MHz, $CDCl_3$): compound **S4**



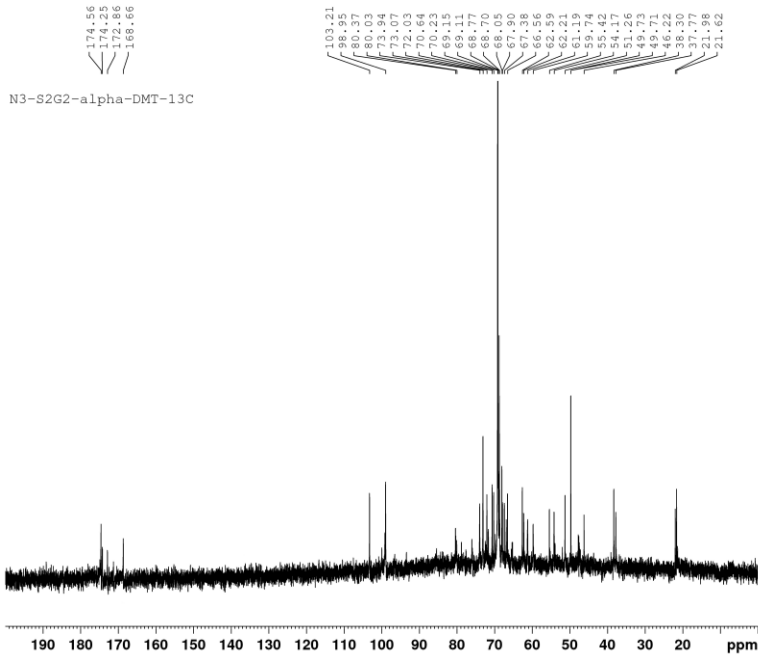

$^{1}$H NMR (600MHz, $CDCl_3$): compound **S7**



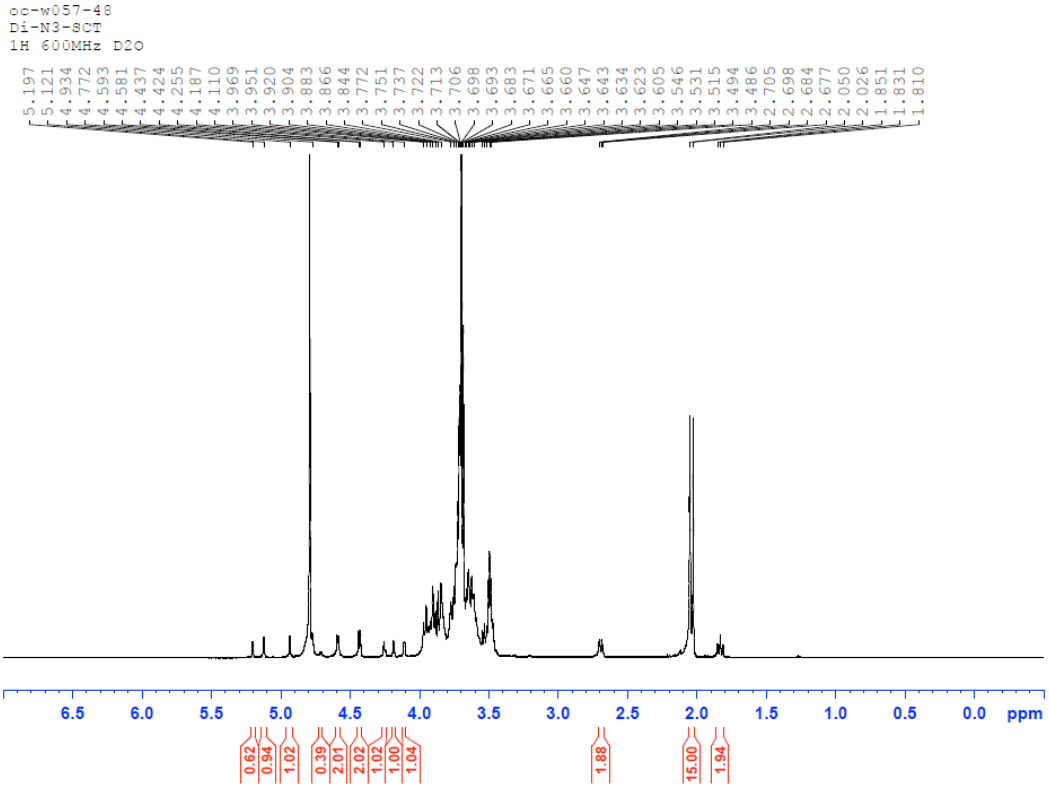

$^{13}$C NMR (150MHz, $CDCl_3$): compound **S7**



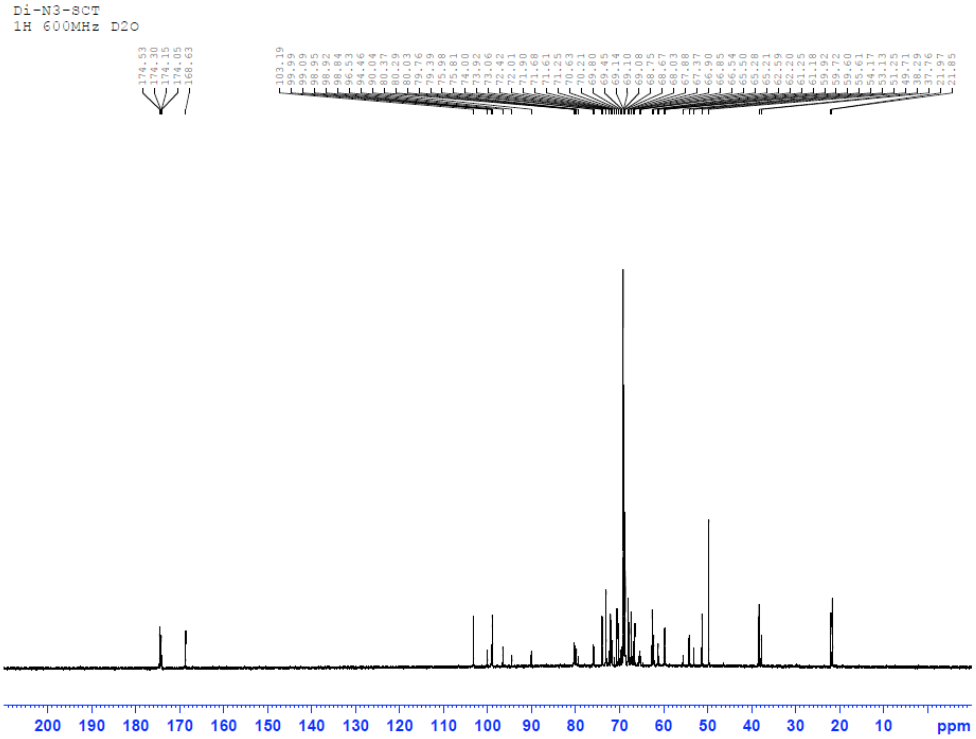

$^{1}$H NMR (400MHz, $D_2O$): compound **2**



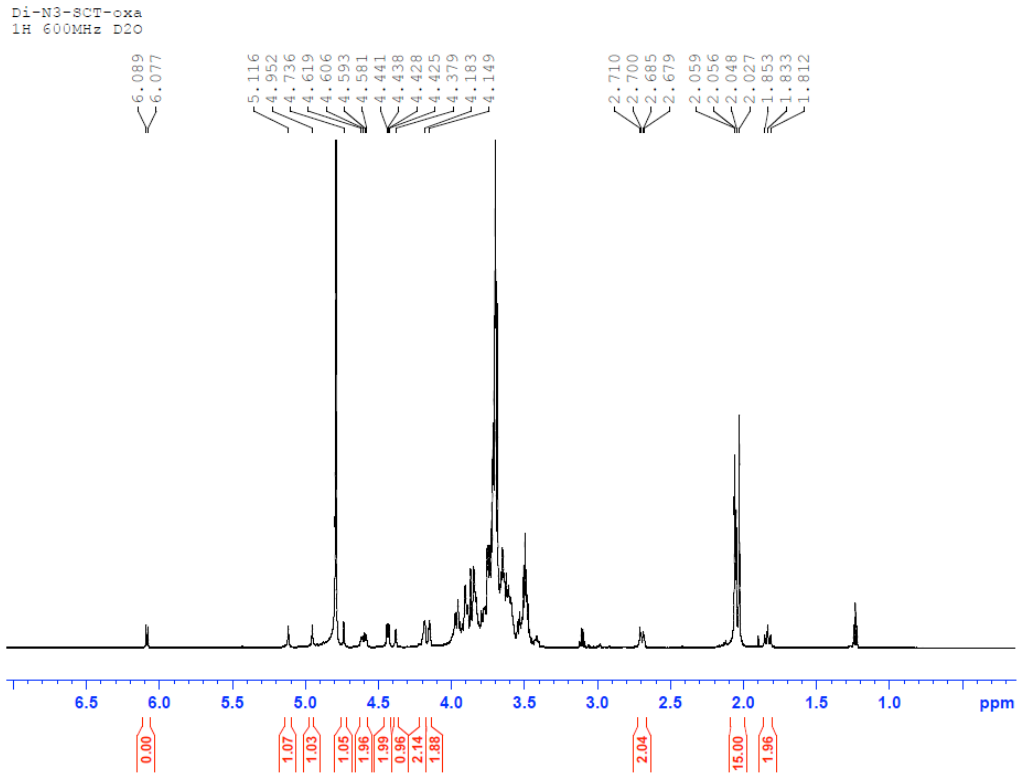

$^{13}$C NMR (100MHz, $CDCl_3$): compound **2**



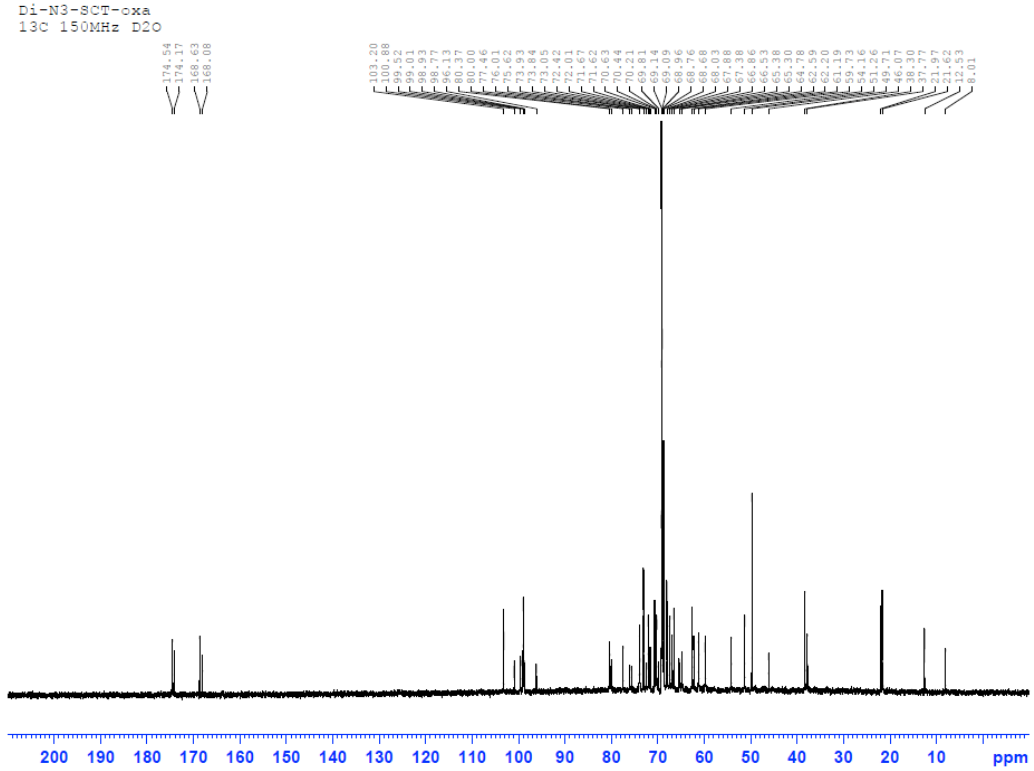

$^{1}$H NMR (600MHz, $D_2O$): compound **3**



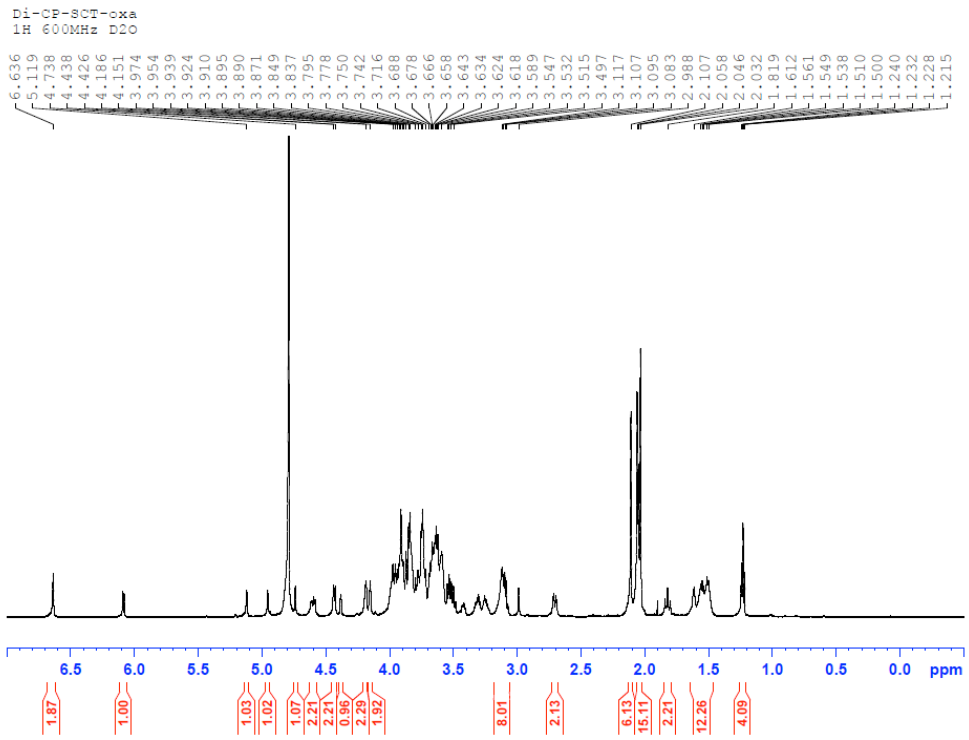

$^{13}$C NMR spectrum (150 MHz, $D_2O$): compound **3**



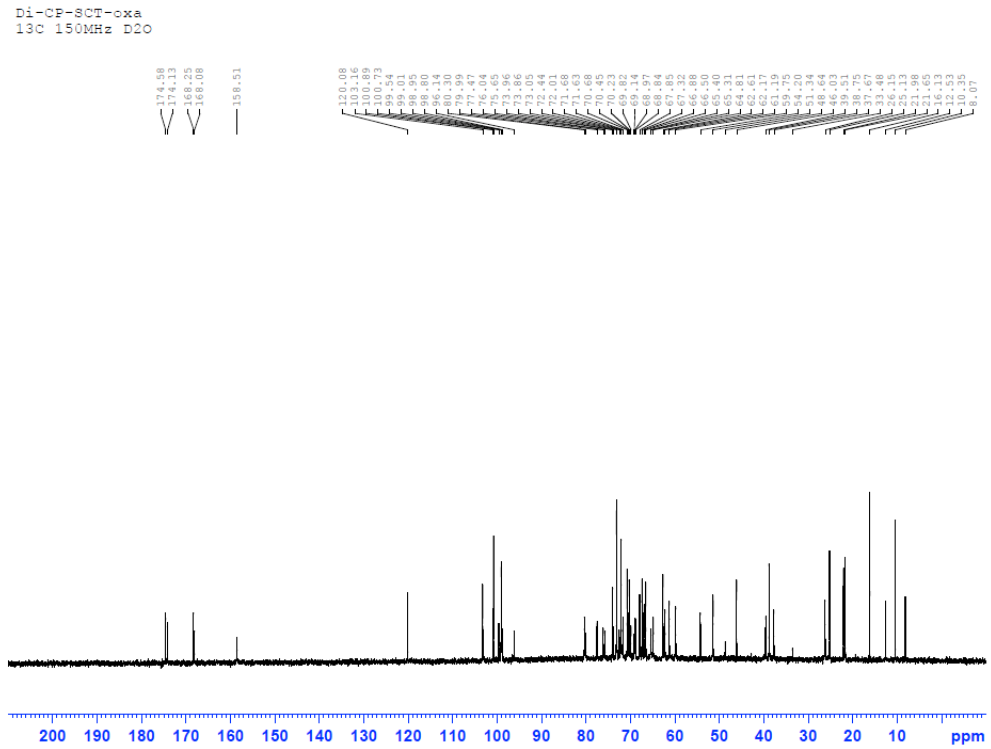

$^{1}$H NMR spectrum (600 MHz, $D_2O$): compound **4a**



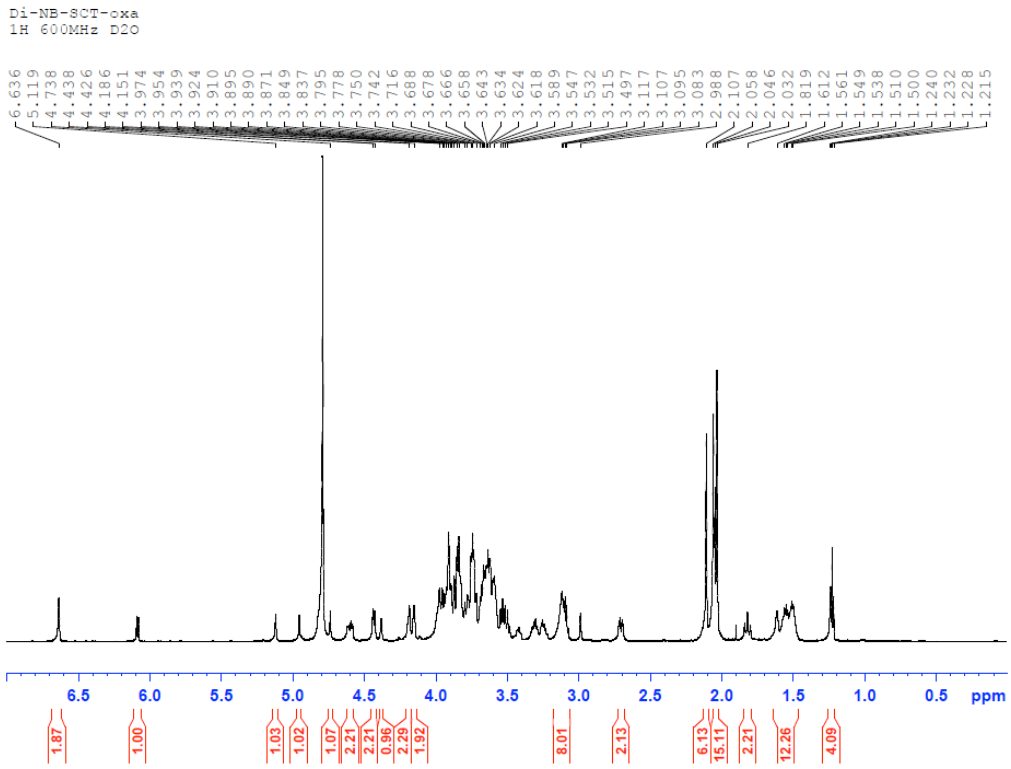

$^{13}$C NMR spectrum (150 MHz, $D_2O$): compound **4a**



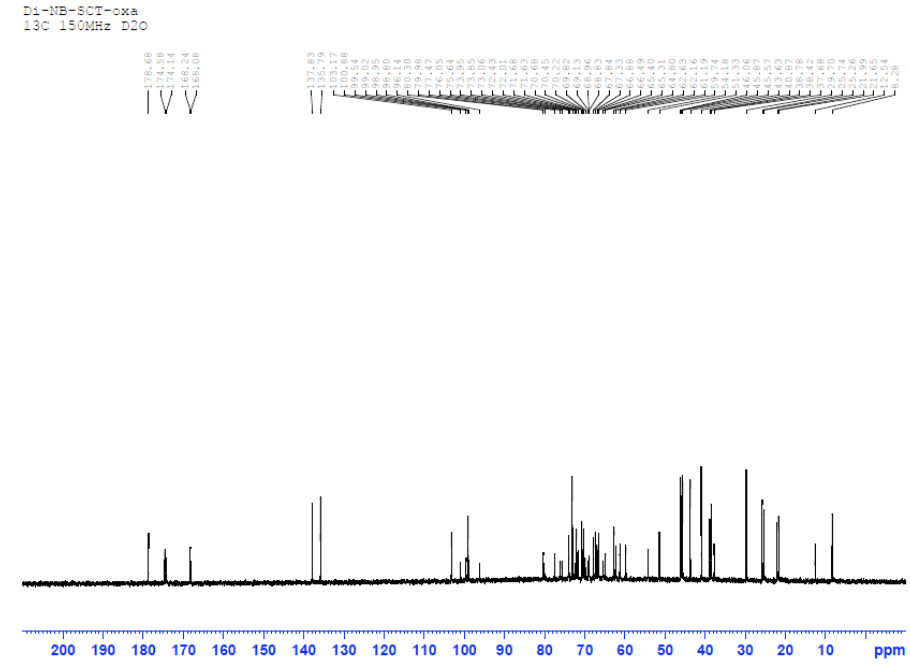

$^{1}$H NMR spectrum (600 MHz, $D_2O$): compound **4b**



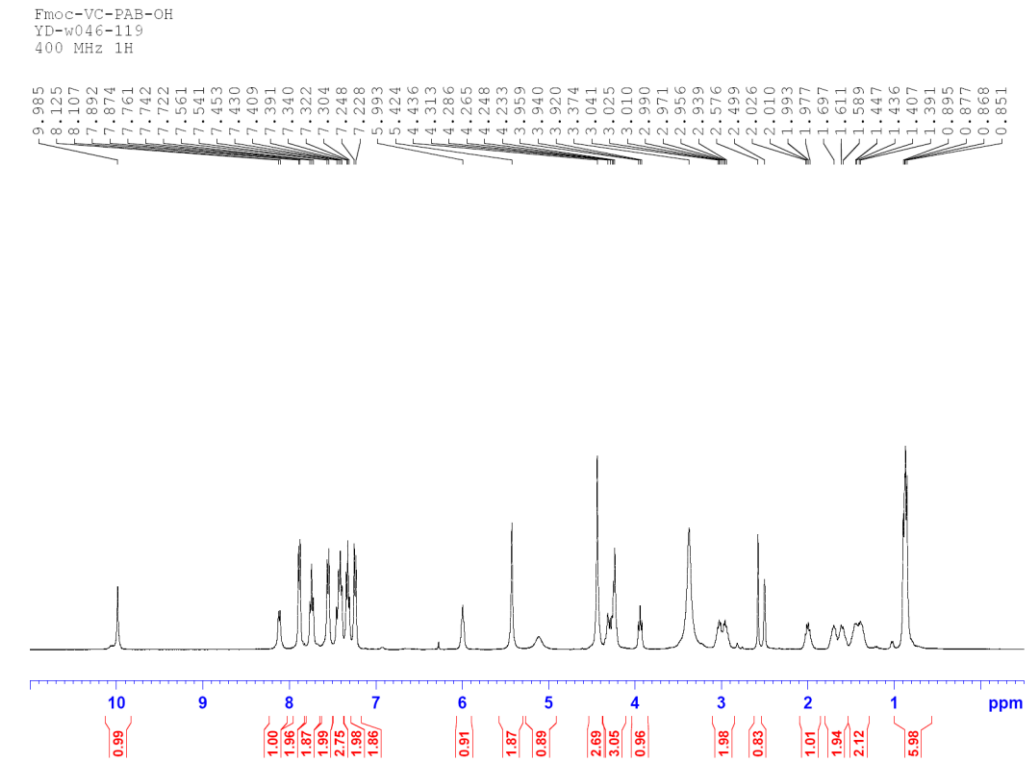

$^{13}$C NMR spectrum (150 MHz, $D_2O$): compound **4b**



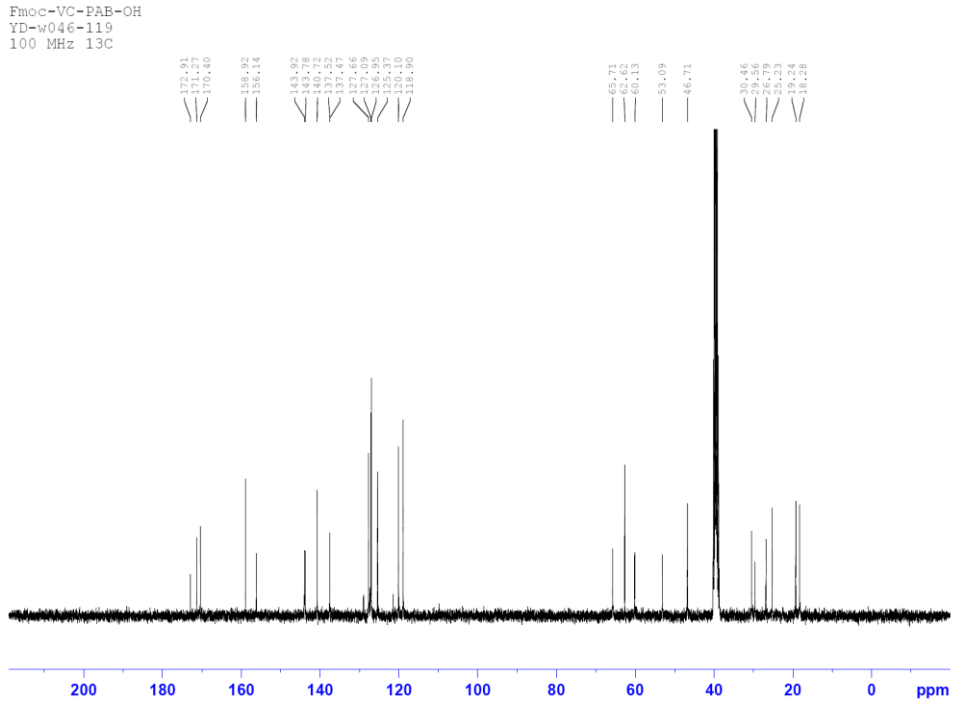

$^{1}$H NMR spectrum (600 MHz, $D_2O$): compound **4c**



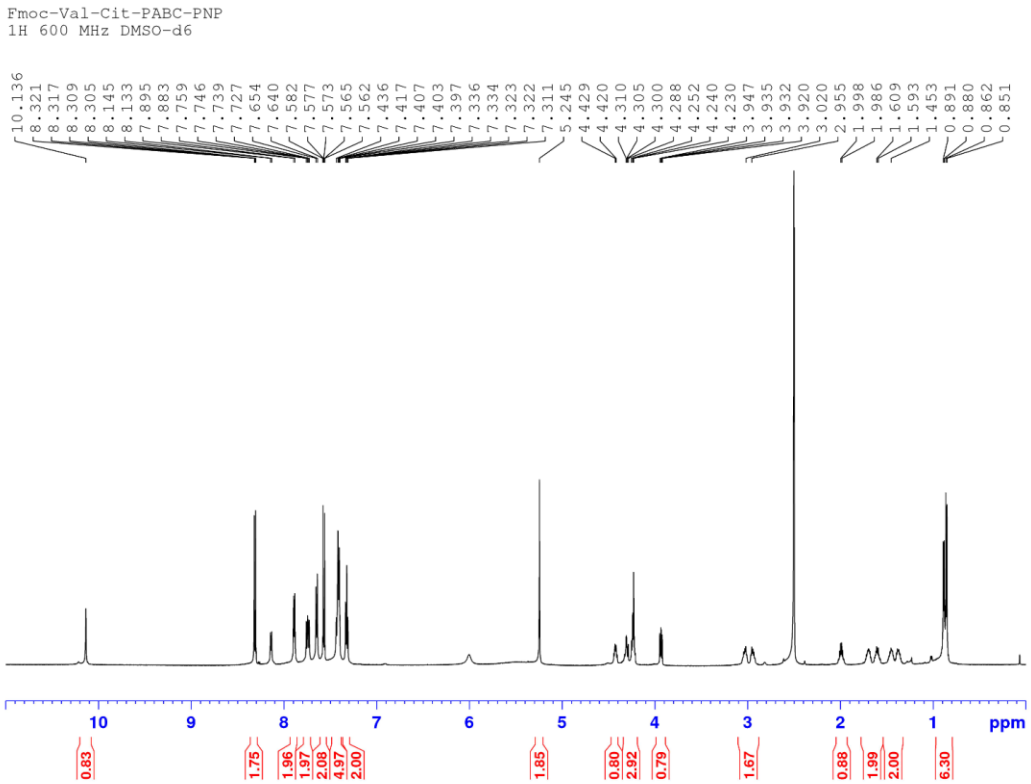

$^{1}$C NMR (150MHz, $D_2O$): compound **4c**



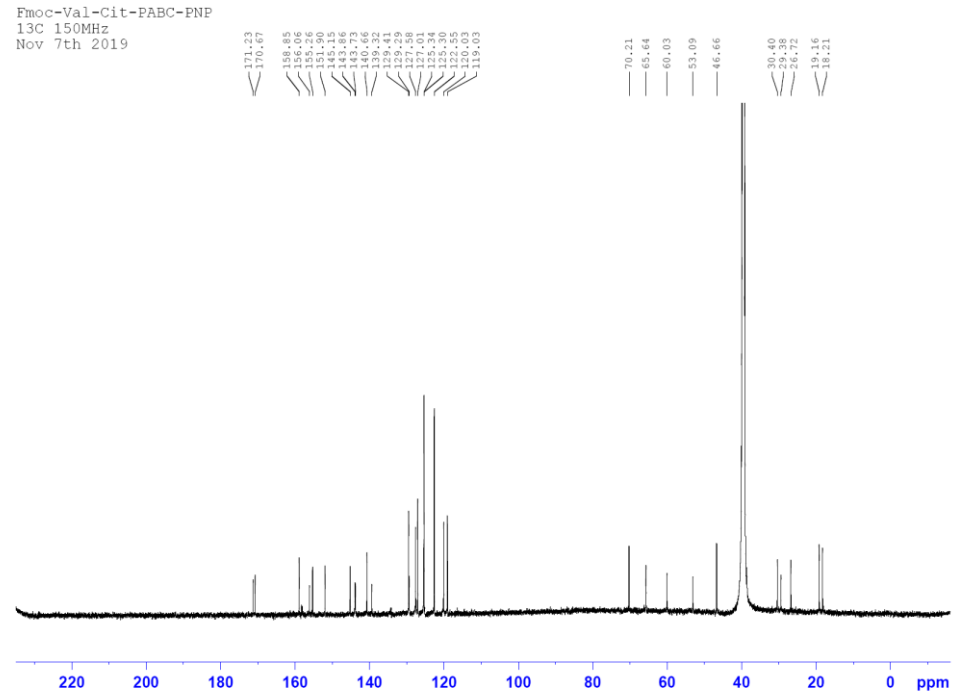

$^{1}$H NMR spectrum (400 MHz, DMSO-d6): compound **S13**



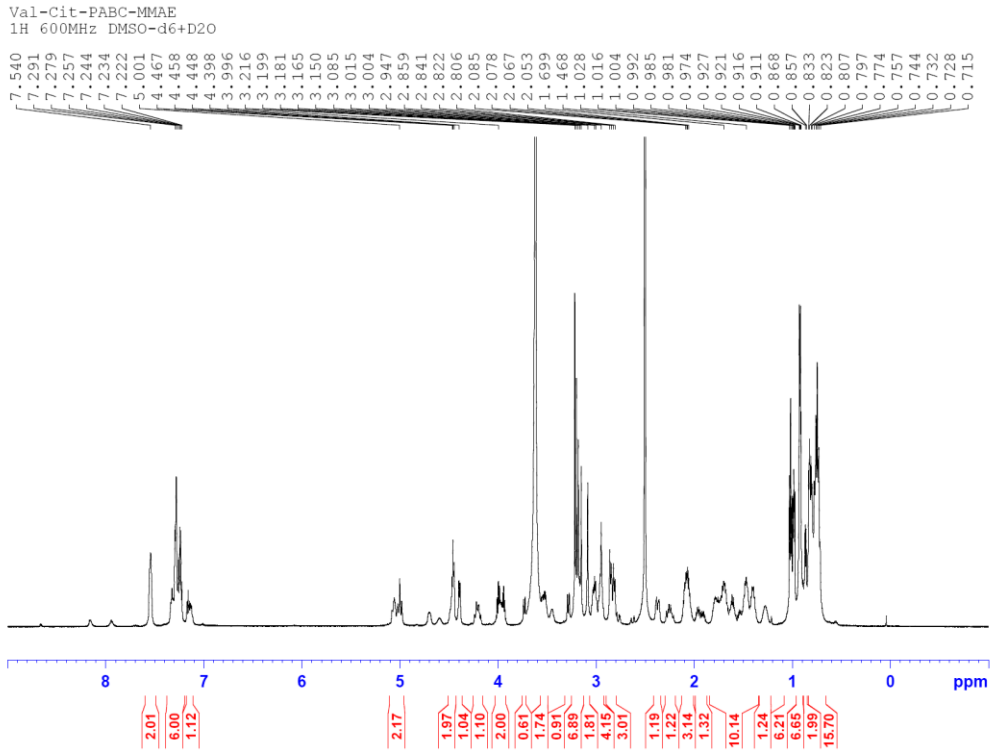

$^{13}$C NMR spectrum (100 MHz, DMSO-d6): compound **S13**




$^{1}$H NMR spectrum (600 MHz, DMSO-d6): compound **S14**




$^{13}$C NMR spectrum (150 MHz, DMSO-d6): compound **S14**




$^{1}$H NMR spectrum (600 MHz, DMSO-d6): compound **S15** (Commercial product).

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