Supporting Information for

Site-Selective Antibody-Drug Conjugation by Proximity-driven *S* to *N* Acyl Transfer Reaction on a Therapeutic Antibody

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1. Materials and equipment

1.1. Chemicals and antibody

Chemicals were purchased from commercial suppliers and used without further purification. 5-Exo-norbornene carboxylic acid, ammonium sulfate ($(NH_4)_2SO_4$), *N*-Boc-ethylenediamine, triisopropylsilane (TIS), and 1,2-ethanedithiol (EDT) were purchased from Sigma-Aldrich (St. Louis, MO, USA). *N*-Methylmaleimide, *N*-ethylmaleimide, *N*-cyclohexylmaleimide, *N*-phenylmaleimide, *N*-benzylmaleimide, *N*-*tert*-butylmaleimide, and 3-maleimidopropionic acid were purchased from Tokyo Chemical Industry (Tokyo, Japan). *tert*-Butyl thioglycolate was obtained from Angene Chemical (Telangana, India), 4-methyltetrazine NHS ester was from Broadpharm Inc. (San Diego, CA, USA), Fmoc-PEG_8-OH was from Quanta Biodesign (Plain City, OH, USA), and DM1-SMCC from eNovation Chemicals LLC (Bridgewater Township, NJ, USA). All amino acids, rink amide resin, and coupling reagents were purchased from Aapptec (Louisville, KY, USA), GL Biochem Ltd. (Shanghai, China), and Combi-Blocks (San Diego, CA, USA). The solvents were used without distillation. Trifluoroacetic acid (TFA), *N*,*N*-diisopropylethylamine (DIPEA), ammonium hydroxide (ammonia solution), diethylamine, Na_2HPO_4, *N*,*N*-dimethylformamide (DMF), dichloromethane (DCM), methanol (MeOH), hexane (Hex), ethyl acetate (EA), and diethyl ether were obtained from Daejung (Siheung, Korea). High-performance liquid chromatography (HPLC)-grade acetonitrile (ACN), isopropanol, and water were obtained from Thermo Fisher Scientific (Waltham, MA, USA). Trastuzumab (trastuzumab) was purchased from Roche (Basel, Switzerland). To measure ^{1}H- and ^{13}C-NMR, $CDCl_3$ and DMSO-d6 were purchased from Cambridge Isotope Laboratories, Inc.

1.2. Characterization and purification of small molecules and peptides

Thin-layer chromatography was performed on pre-coated silica gel F_{254} plates (Merck, Billerica, MA, USA) and observed under ultraviolet light (254 nm) or by staining with $KMnO_4$ or ninhydrin solutions. All synthesized compounds were purified by Combi-flash purification using prepacked silica gel cartridges. All synthetic peptides were purified by preparative-HPLC (Waters, Milford, MA, USA; with XBridge^{TM} prep C18, 5 μm, 4.6 × 250 mm column).

1.3. Deglycosylation of antibodies for analysis of intact mass

Two microliters of samples (75 mg/mL) were combined with 8 μL of 6 M urea buffer and 50 mM Tris-HCl (pH 8.0) to a total reaction volume of 99 μL, to which 500 units (1 μL) of PNGaseF (glycerol-free) was added. The samples were mixed gently and incubated at 37 °C for 90 min at 400 W using the Rapid Enzyme Digestion System (ASTA, HST REDS, Suwon, Korea).

1.4. In-solution digestion for analysis of the antibody-linker binding site

The ADC sample (100 μg) was digested in a solution. Briefly, the samples were denatured with 8 M

urea and reduced with 10 mM DTT (30 min at 37 °C), followed by alkylation with 25 mM iodoacetamide (20 min at room temperature). We used chymotrypsin or IdeS enzyme, which was added at a 1:50 ratio of 1 µg chymotrypsin or IdeS: 50 µg protein, and the sample was incubated overnight at 37 °C. After incubation, the digests were desalted using a C18 SPE cartridge.

1.5. Instruments

NMR spectra were recorded with Bruker Avance III 700 MHz spectrometer.

Centrifugation was performed on a Fleta 4 centrifuge (Hanil, Korea).

All peptides and tetrazine-containing payloads were characterized using HPLC (Waters, XBridge®, C18, 4.6 × 250 mm, 5 µm).

All antibodies, including ADC precursor and ADC, were characterized by HIC-HPLC (Thermo Fisher Scientific, MAbPac™, HIC-butyl, 4.6 × 100 mm, 5 µm) and size-exclusion chromatography (SEC)-HPLC (Thermo Fisher Scientific, MAbPac™, SEC-1, 300 Å 4 × 300 mm, 5 µm).

Mass spectra of small molecules and peptides were obtained using electrospray ionization (ESI) and a time-of-flight (TOF) analyzer Quattro Premier XE mass spectrophotometer from Waters and a COSMOSIL COSMOCORE 2.6 C_{18} column (2.6 µm, 2.1 × 50 mm, Nacalai Tesque, Kyoto, Japan).

The masses of all antibodies were obtained using matrix-associated laser desorption ionization and a TOF analyzer (MALDI-TOF) with sinapinic acid (SA) as a matrix. MALDI-TOF mass spectra were acquired on an Ultraflex III mass spectrometer (Bruker Daltonics, Billerica, MA, USA) equipped with a 337-nm pulsed nitrogen laser operated in positive polarity and linear mode.

Ultra-performance liquid chromatography (UPLC) and MS were used for mass analysis. UPLC-MS analysis of antibodies was performed using an Orbitrap Fusion Tribrid (Thermo Fisher Scientific) coupled to a Waters Acquity UPLC and Waters BEH300 C4 column (1.7 µm, 2.1 × 100 mm).

To analyze the binding sites, the extracted peptides were analyzed with a quadrupole Orbitrap mass spectrometer (Q-Exactive, Thermo Fisher Scientific) coupled with an Easy-nLC system (Thermo Fisher Scientific). Peptides were reconstituted in 0.1% formic acid and separated on an analytical column (C18, 2 µm particle size, 50 µm id × 15 cm length, Thermo Fisher Scientific).

1.6. Instrumental parameters

Characterization of peptides and tetrazine-containing payloads using C18-HPLC

C18-HPLC was performed at a flow rate of 1 mL/min. All compounds were analyzed using the same elution conditions, that is, initial 80% mobile phase A (0.1% TFA in H_2O) for 1 min followed by a 20–80% gradient of mobile phase B (0.075% TFA in ACN) in A for 15 min. The chromatograms of all peptides were acquired at 280 nm, and the chromatograms of the payloads were acquired at 254 nm.

Characterization of antibodies using HIC-HPLC

HIC-HPLC was performed at a flow rate of 1 mL/min. All antibodies were analyzed under the same conditions, i.e., initial 100% mobile phase A (1.5 M $(NH_4)_2SO_4$, 50 mM Na_2HPO_4 at pH 7.0, 5% isopropanol) for 1 min followed by a 0–100% gradient of mobile phase B (50 mM sodium phosphate at pH 7.0, 20% isopropanol) in A for 15 min. All chromatograms were acquired at 280 nm.

Characterization of antibodies using SEC-HPLC

SEC-HPLC was performed at a flow rate of 0.2 mL/min. All antibodies were analyzed under the same conditions, that is, isocratic mobile phase D (1× PBS) for 20 min. All chromatograms were acquired at 280 nm.

Mass determination

***ESI-MS*:** The column temperature was set to 25 °C, and a flow rate of 0.3 mL/min was used. The compounds were injected into the column at a volume of 5 μL. Mobile phase A consisted of water with 0.05% TFA, whereas mobile phase B consisted of acetonitrile with 0.05% TFA. The initial gradient condition was 20% B for 0.5 min; next, B was increased gradually to 80% in 3.5 min, followed by a gradient decrease from 80% B to 20% B in 0.1 min.

***UPLC-MS*:** The column temperature was set to 65 °C with a flow rate of 0.4 mL/min. Ten microliters of each antibody were injected into the column. Mobile phase A consisted of water with 0.1% formic acid, whereas mobile phase B consisted of acetonitrile with 0.1% formic acid. The gradient started at 5% B for 0.5 min, and then increased gradually to 80% in 10 min. Next, the gradient was increased from 80% B to 100% B in 0.1 min. The column was washed with 100% B for 2 min, followed by equilibration at 2% B for 3 min.

***Orbitrap fusion Tribrid/data analysis*:** The intact mass of the antibodies was examined with an Orbitrap Fusion Tribrid mass spectrometer using a spray voltage of +3.2. The MS system was operated in the intact protein mode using an ion transfer tube at 275 °C and vaporizer at 350 °C. A full scan from m/z 600 to 6,000 at a resolution of 15,000 was acquired. The intact mass spectra were analyzed using the Intact Protein workflow in BioPharma Finder 3.1 integrated software, performing time-resolved deconvolution using the ReSpect algorithm in combination with sliding window integration. Deconvolution spectra were annotated by entering the amino acid sequence with a total of 16 disulfide bonds and variable modifications corresponding to six possible glycan combinations (G0/G0F, G0F/G0F, G0F/G1F, G1F/G1F, G1F/G2F, and G2F/G2F).

***MALDI-TOF*:** The antibodies were dissolved in H_2O at a concentration of 1 mg/mL, and a saturated solution of SA in 0.1% TFA in ACN:H_2O (1:1) was used as the matrix. Antibodies (2 μL) were spotted onto a ground steel 384 target plate, mixed with 2 μL of matrix solution, and vacuum dried. FlexControl software (version 3.0) was used for spectral acquisition under the following instrumental conditions: mass range at m/z 2,000–300,000, deflection suppressed up to m/z 5,000, shots accumulated to 1,000 replicates, laser frequency at 100 Hz, and 25 kV voltage on Ion Source I, 23 kV on Ion Source II, and 9

kV on Lens.

2. Synthetic methods

2.1. Synthesis of peptides

***tert-Butyl (5-exo-norbornene carbonylthio)acetate (1)*:** 5-Exo-norbornene carboxylic acid (200 mg, 1.45 mmol, 1.0 equiv.) was dissolved in 5 mL DMF. Hexafluorophosphate azabenzotriazole tetramethyl uronium (HATU) (660 mg, 1.74 mmol, 1.2 equiv.) and DIPEA (0.66 mL, 3.48 mmol, 2.4 equiv.) were added, and the mixture was stirred under argon atmosphere. *tert*-Butyl α-thioacetate (0.25 mL, 1.74 mmol, 1.2 equiv.) was added slowly to the reaction mixture. After 16 h, DMF was removed under reduced pressure, and compound **1** (350 mg, 90%, white solid) was purified via flash column chromatography using 2% ethyl acetate in hexane. R_F = 0.5 (10% ethyl acetate in hexane); ^{1}H NMR (700 MHz, $CDCl_3$) δ 6.17 (dd, J = 5.5, 2.9 Hz, 1H), 6.13 (dd, J = 5.4, 3.0 Hz, 1H), 3.62 (d, J = 1.6 Hz, 2H), 3.10-3.08 (m, 1H), 2.54-2.48 (m, 1H), 1.97 (dt, J = 11.8, 4.1 Hz, 1H), 1.56 (d, J = 8.4 Hz, 1H), 1.46 (s, 10H), 1.37 (dd, J = 9.4, 1.4 Hz, 2H). ^{13}C NMR (700 MHz, $CDCl_3$) δ 200.45, 167.91, 138.63, 135.56, 82.08, 52.29, 47.72 46.25, 41.76, 32.63, 30.76, 27.93. MS (ESI): m/z 269.34 $[M+H]^+$ HRMS: $[M+H]^+$ calcd. for $C_{14}H_{21}O_3S$ 269.1206 Found 269.1214

***(5-Exo-norbornene carbonylthio)acetic acid NHS ester (I)*:** To a solution of compound **1** (310 mg, 1.16 mmol, 1.0 equiv.) in 5 mL DCM in an ice-water bath, 5 mL TFA was added slowly. After 2 h, TFA was removed under reduced pressure, and the residue was redissolved in DCM (5 mL) and the volatiles were removed under reduced pressure three times. The residue was dried under high vacuum and re-dissolved in 10 mL DCM. DIPEA (0.6 mL, 3.48 mmol, and 3.0 equiv.) was added slowly to adjust the solution pH to 9.0 in an ice-water bath, and *N,N,N′,N′*-tetramethyl-*O*-(*N*-succinimidyl)uronium tetrafluoroborate (1.05 g, 3.48 mmol, 3.0 equiv.) was added. After 3 h, the reaction mixture was washed with 10% citric acid and brine and dried over Na_2SO_4. The solvent was then removed under reduced pressure. Compound **I** (310 mg, 87%) was purified by flash column chromatography using 10% ethyl acetate in hexane as a white solid. R_F = 0.3 (33% ethyl acetate in hexane); ^{1}H NMR (700 MHz, $CDCl_3$) δ 6.12 (dd, J = 5.6, 3.0 Hz, 1H), 6.06 (dd, J = 5.6, 3.1 Hz, 1H), 3.92 (d, J = 1.9 Hz, 2H), 3.06 (dd, J = 1.8, 1.0 Hz, 1H), 2.89 (s, 1H), 2.77 (s, 5H), 2.48-2.43 (m, 1H), 1.94-1.87 (m, 1H), 1.50 (d, J = 8.6 Hz, 1H), 1.34-1.29 (m, 2H). ^{13}C NMR (700 MHz, $CDCl_3$) δ 198.84, 168.60, 164.87, 138.80, 135.46, 52.43, 47.72, 46.32, 41.81, 30.85, 28.29, 25.57. MS (ESI): m/z 310.26 $[M+H]^+$ HRMS: $[M+H]^+$ calcd. for $C_{14}H_{16}NO_5S$ 310.0744, found 310.0756

***General method for the synthesis of peptides*:** The peptides were synthesized by solid-phase peptide synthesis (Supplementary Scheme 1) using Fmoc-protected amino acids. PEG_8 was attached to the *N*-terminus of the peptide. Acetylation of the *N*-terminus was performed in 20% Ac_2O in DMF with 10% pyridine. The synthesized peptide was cleaved (strong acidic condition, TFA:TIS:EDT:H_2O = 94:1:2.5:2.5), collected, purified, and cyclized as described previously.[1] The crude cyclized peptides

were purified by reverse-phase chromatography. Finally, 5-exo-norbornene thioester was introduced to the amine functional group (sixth position in the peptide) using compound **I** and 6 equiv. of DIPEA in DMF. The final peptides were purified by preparative HPLC, and their purity was verified by analytical HPLC.

***L6Dap (5-exo-norbornene thioester), AbClick-1*:** (sequence: Ac-PEG_8-DCAWHDapGELVWCT, Figure S2). 15 mg of L6Dap was synthesized using the general method described above. The final peptide (11 mg, 61%) was purified by preparative HPLC and obtained as a white solid by lyophilization; the purity was verified by analytical HPLC. MS (ESI): m/z 1082.34 $[M+2H]^{2+}$.

***L6Dab (5-exo-norbornene thioester), AbClick-2*:** (sequence: Ac-PEG_8-DCAWHDabGELVWCT, Figure S3). 15 mg of Dab was synthesized as described above. The final peptide (8 mg, 48%) was purified by preparative HPLC and obtained as a white solid by lyophilization; the purity was verified by analytical HPLC. MS (ESI): m/z 1089.94 $[M+2H]^{2+}$

L6Orn (5-exo-norbornene thioester), AbClick-3 (sequence: Ac-PEG_8-DCAWHOrnGELVWCT, Figure S4). 15 mg of L6Orn was synthesized as described above. The final peptide (10 mg, 61%) was purified by preparative HPLC and obtained as a white solid by lyophilization; the purity was verified by analytical HPLC. MS (ESI): m/z 1096.64 $[M+2H]^{2+}$.

L6Lys (5-exo-norbornene thioester), AbClick-4 (sequence: Ac-PEG_8-DCAWHLysGELVWCT, Figure S5). L6Lys was synthesized as described above. The final peptide (10 mg, 61%) was purified by preparative HPLC and obtained as a white solid by lyophilization; the purity was verified by analytical HPLC. MS (ESI): m/z 1103.64 $[M+2H]^{2+}$.

FcBP-biotin (bFcBP): (sequence: Biotin-PEG8-DCAWHKGELVWCT, Figure S5) This compound was synthesized using a previously reported procedure.[1] MS (ESI): m/z 1203.5 $[M+2H]^{2+}$

2.2. Synthesis of 4-methyltetrazine-PEG_8-SMCC-DM1 (non-cleavable DM1)

***4-methyltetrazine-PEG_8-Fmoc (2)*:** This compound was synthesized using a previously reported procedure.[1] R_f = 0.3 (5% MeOH in DCM); MS (ESI): m/z 847.52 $[M+H]^+$.

***4-methyltetrazine-PEG_8-amine (3)*:** This compound was synthesized using a previously reported procedure.[1] R_f = 0.1 (DCM:MeOH:NH_4OH = 74:23:3); MS (ESI): m/z 625.48 $[M+H]^+$.

***4-methyltetrazine-PEG_8-SMCC-DM1 (II)*:** This compound was synthesized using a previously reported procedure.[1] R_f = 0.5 (10% MeOH in DCM); ^{1}H NMR (700 MHz, DMSO-d_6) δ 8.52 (s, 1H), 8.43 (d, J = 8.0 Hz, 2H), 7.74 (s, 1H), 7.54 (d, J = 8.0 Hz, 2H), 7.18 (d, J = 17.7 Hz, 1H), 6.92 (s, 1H), 6.57 (dd, J = 14.8, 8.2 Hz, 3H), 5.95 (s, 1H), 5.77 (s, 1H), 5.57 (d, J = 9.5 Hz, 1H), 5.35-5.27 (m, 1H), 4.53 (d, J = 11.8 Hz, 1H), 4.42 (d, J = 5.7 Hz, 2H), 4.05 (dt, J = 14.2, 8.9 Hz, 3H), 3.95 (d, J = 21.7 Hz, 4H), 3.88 (d, J = 5.3 Hz, 1H), 3.67 (t, J = 6.1 Hz, 3H), 3.51 (d, J = 19.1 Hz, 38H), 3.41-3.31 (m, 15H), 3.26

(s, 4H), 3.17 (d, J = 13.2 Hz, 5H), 3.11 (s, 3H), 3.06-2.96 (m, 6H), 2.91 (d, J = 6.3 Hz, 1H), 2.86 (dd, J = 15.9, 7.6 Hz, 1H), 2.80 (d, J = 6.9 Hz, 2H), 2.71 (s, 4H), 2.44 (t, J = 6.1 Hz, 3H), 2.37-2.29 (m, 1H), 2.19 (d, J = 15.4 Hz, 1H), 2.09 (d, J = 6.5 Hz, 4H), 2.00 (s, 5H), 1.66 (s, 2H), 1.60 (s, 6H), 1.46 (d, J = 12.6 Hz, 4H), 1.24 (s, 5H), 1.18 (t, J = 6.9 Hz, 6H), 1.12 (t, J = 11.3 Hz, 5H), 0.84 (d, J = 25.5 Hz, 3H), 0.78 (d, J = 3.2 Hz, 3H). MS (ESI): m/z 1584.17 $[M+H]^+$.

2.3. Synthesis of 4-methyltetrazine-PEG$_8$-Val-Cit-EDA-SMCC-DM1 (cleavable DM1)

***General procedure for Fmoc deprotection*:** To a solution of Fmoc-protected compounds in DMF, diethylamine (2%, v/v%) was added slowly and stirred for 2 h. After the reaction, DMF was removed under reduced pressure and further concentrated with toluene three times. Fmoc-deprotected compounds were purified by flash chromatography.

***Fmoc-Cit-EDA-Boc (4)*:** To a solution of Fmoc-Cit-OH (9.2 g, 23.2 mmol, 1.0 equiv.) in DMF (200 mL), HATU (9.6 g, 25.2 mmol, 1.2 equiv.), and DIPEA (8.8 mL, 50.4 mmol, 2.4 equiv.) were added. The mixture was stirred for 10 min before adding *tert*-butyl (2-aminoethyl)carbamate (3.4 g, 21 mmol, 0.91 equiv.). The mixture was stirred for 26 h at 25 °C; upon completion of the reaction, the solvent was removed under reduced pressure. The residue was triturated using diethyl ether and stirred for 5 h at 25 °C. The crude product was obtained via filtration as a yellow solid (11 g, 96%) and used for the next step without further purification. R_F = 0.2 (10% MeOH in DCM); MS (ESI): m/z 540.01 $[M+H]^+$; HRMS: $[M+H]^+$ calcd. for $C_{28}H_{38}N_5O_6$ 540.2817, Found 540.2799.

***H_2N-Cit-EDA-Boc (5)*:** The Fmoc deprotection reaction was performed using the general procedure described above. Compound **3** was purified by flash column chromatography (DCM:MeOH:NH_4OH= 74:23:3). The product was obtained as a pale yellow oil (0.82 g, 69%). R_F = 0.1 (DCM:MeOH:NH_4OH= 74:23:3); MS (ESI): m/z 318.10 $[M+H]^+$, HRMS: $[M+H]^+$ calcd. for $C_{13}H_{28}N_5O_4$ 318.2136, found 318.2128.

***Fmoc-Val-Cit-EDA-Boc (6)*:** To a solution of Fmoc-Val-OH (0.98 g, 2.84 mmol, 1.1 equiv.) in 50 mL DMF, HATU (1.08 g, 2.84 mmol, 1.1 equiv.) and DIPEA (0.99 mL, 5.69 mmol, 2.2 equiv.) were added. The mixture was stirred for 10 min before adding **3** (0.82 g, 2.6 mmol, 1.0 equiv.) in 10 mL DMF. The mixture was stirred for 14 h at 25 °C, and the mixture was diluted with ethyl acetate. The organic layer was washed with 10% citric acid and brine and dried over Na_2SO_4. The solvent was removed under reduced pressure, and the residue was triturated using diethyl ether. The crude product (1.6 g, 97%) was obtained by filtration and used for the next step without further purification. R_F = 0.1 (10% MeOH in DCM); MS (ESI): m/z 639.04 $[M+H]^+$, HRMS: $[M+H]^+$ calcd. for $C_{33}H_{47}N_6O_7$ 639.3500, found 639.3507.

***H_2N-Val-Cit-EDA-Boc (7)*:** The Fmoc deprotection reaction was performed using the general procedure described above. Compound **5** was purified by flash column chromatography (DCM:MeOH:NH_4OH = 74:23:3). The product was obtained as a pale-yellow oil (100 mg, 93%). R_F = 0.4 (DCM:MeOH:NH_4OH= 74:23:3); MS (ESI): m/z 417.11 $[M+H]^+$, HRMS: $[M+H]^+$ calcd. for $C_{18}H_{37}N_6O_5$ 417.2820, found 417.2823.

***Fmoc-PEG$_8$-Val-Cit-EDA-Boc (8)*:** To a solution of Fmoc-PEG$_8$-OH (385 mg, 0.58 mmol, 1.2 equiv.) in 5 mL DMF, HATU (220 mg, 0.58 mmol, 1.2 equiv.) and DIPEA (0.2 mL, 1.15 mmol, 2.4 equiv.) were added. The mixture was stirred for 10 min, and then compound **5** (200 mg, 0.48 mmol, 1.0 equiv.) was added. The mixture was stirred for 3.5 h at 25 °C, after which the solvent was removed under reduced pressure and the product was purified via flash column chromatography (5% MeOH in DCM) as a pale-yellow oil (370 mg, 73%). RF = 0.2 (10% MeOH in DCM); MS (ESI): m/z 1062.88 [M+H$^+$], HRMS: [M+H]$^+$ calcd. for $C_{52}H_{84}N_7O_{16}$ 1062.5969, found 1062.5926.

***H$_2$N-PEG$_8$-Val-Cit-EDA-Boc (9)*:** The Fmoc deprotection reaction was performed using the general procedure described above. Compound **7** was purified by flash column chromatography (DCM:MeOH:NH_4OH= 74:23:3). The product was obtained as a pale-yellow oil (695 mg, 80%). R_F = 0.5 (DCM:MeOH:NH_4OH= 74:23:3); MS (ESI): m/z 840.76 [M+H]$^+$, HRMS: [M+H]$^+$ calcd. for $C_{37}H_{74}N_7O_{14}$ 840.5289, found 840.5293.

***4-Methyltetrazine-PEG$_8$-Val-Cit-EDA-Boc (10)*:** A solution of compound **7** (500 mg, 0.6 mmol, 1.0 equiv.) in 10 mL DMF, 4-methyltetrazine NHS ester (234 mg, mmol, 1.2 equiv.), and DIPEA (0.13, 0.72 mmol, 1.2 equiv.) were added. The mixture was stirred for 1.5 h at 25 °C, after which the solvent was removed under reduced pressure and the product was purified via flash chromatography (5% MeOH in DCM) as a pink oil (490 mg, 78%). R_F = 0.4 (10% MeOH in DCM); MS (ESI): m/z 1052.94 [M+H]$^+$, HRMS: [M+H]$^+$ calcd. for $C_{48}H_{82}N_{11}O_{15}$ 1052.5987, found 1052.5937.

***4-Methyltetrazine-PEG$_8$-Val-Cit-EDA-SMCC-DM1 (III)*:** To a solution of compound **8** (350 mg, 0.33 mmol, 1.0 equiv.) in 5 mL DCM in an ice-water bath, TFA (5 mL) was added dropwise. After 2 h, TFA was removed under reduced pressure, and the resulting residual was dissolved in 5 mL DCM, and the volatiles were removed under reduced pressure three times. The residue was dried under high vacuum and re-dissolved in 10 mL DMF in an ice-water bath. DIPEA (0.29 mL, 1.65 mmol, 5.0 equiv.) was added slowly until the mixture attained a pH of 9.0, and DM1-SMCC (357 mg, 0.33 mmol, 1.0 equiv.) was added. After the reaction, the solvent was removed under reduced pressure. Compound **II** was purified via flash chromatography (10% MeOH in DCM) as a pink oil (200 mg, 61%). R_F = 0.1 (10% MeOH in DCM); ^{1}H NMR (700 MHz, DMSO-*d$_6$*) δ 8.43-8.38 (m, 1H), 8.27 (t, J = 5.5 Hz, 1H), 7.94-7.85 (m, 2H), 7.68 (d, J = 2.9 Hz, 1H), 7.55 (d, J = 8.4 Hz, 1H), 7.18 (dd, J = 17.8, 1.5 Hz, 1H), 6.92 (s, 1H), 6.61-6.52 (m, 2H), 5.94 (dd, J = 11.5, 5.7 Hz, 1H), 5.62-5.52 (m, 1H), 5.38 (s, 1H), 5.32 (t, J = 6.8 Hz, 1H), 4.53 (dd, J = 12.0, 2.6 Hz, 1H), 4.21-4.14 (m, 1H), 4.11 (q, J = 5.3 Hz, 1H), 4.09-4.00 (m, 1H), 3.98-3.92 (m, 2H), 3.64-3.56 (m, 2H), 3.54-3.46 (m, 17H), 3.43 (d, J = 5.7 Hz, 1H), 3.35 (s, 2H), 3.28-3.23 (m, 3H), 3.21-3.14 (m, 5H), 3.10 (t, J = 6.5 Hz, 2H), 3.05 (dd, J = 17.6, 9.3 Hz, 2H), 2.99 (d, J = 12.9 Hz, 2H), 2.95-2.83 (m, 2H), 2.82-2.77 (m, 1H), 2.71 (t, J = 10.7 Hz, 2H), 2.37 (dt, J = 14.0, 6.0 Hz, 1H), 2.09 (d, J = 6.8 Hz, 1H), 2.05 (dd, J = 11.6, 4.9 Hz, 1H), 2.00-1.93 (m, 1H), 1.69 (d, J = 9.3 Hz, 1H), 1.64-1.54 (m, 3H), 1.53-1.41 (m, 2H), 1.39-1.20 (m, 3H), 1.19-1.16 (m, 2H), 1.13 (d, J = 6.1 Hz, 2H), 0.91-0.76 (m, 6H). MS (ESI): m/z 1911.78 [M+H]$^+$, HRMS: [M+H]$^+$ calcd. for $C_{90}H_{135}ClN_{15}O_{26}S$ 1908.9107, found 1909.9053.

3. Stability and reactivity of the norbornene-thioester compared with those of the NHS ester

***Preparation of norbornene-NHS ester*:** This compound was prepared as previously reported[1].

***pH-Dependent manner*:** To test the reactivity of the thioester compared to that of the NHS ester in a pH-dependent manner, eight different pH buffers were prepared: pH 4.5, 5.0, 5.5, 6.0, 6.5, 7.0, 7.4, and 8.0. Buffers at pH 4.5–6.0, prepared using 20 mM histidine-HCl buffer, and pH 6.5–8.0 were prepared from 1× PBS. Ac-L-Lys-OH and 3 equiv. of each norbornene compound were incubated for 12 h at 25°C. The reaction was monitored by C18-HPLC, and acylated Ac-L-Lys (norbornene)-OH was detected.

***Time-dependent manner*:** To preliminarily evaluate the reactivity of the thioester compared to the NHS ester over time, Ac-L-Lys-OH and 3 equiv. of each norbornene compound were incubated for 12 h at 25 °C in 1× PBS, pH 7.4. Formation of Ac-L-Lys (norbornene)-OH was monitored by C18-HPLC.

***Temperature-dependent manner*:** To test the reactivity of thioester as compared to that of the NHS ester at different temperatures, Ac-L-Lys-OH and three equiv. of each norbornene compound were incubated for 12 h at 30°C, 40°C, 50°C, 60°C, and 70°C in 1× PBS pH 7.4. Their reaction and stability with respect to changes in temperature were monitored by C18-HPLC.

***Stability in aqueous buffer*:** To test the stability of thioester as compared to that of NHS ester, norbornene thioester and NHS ester were incubated for 12 h at 25 °C in 1× PBS pH 7.4. The stability of the thioester and NHS ester in aqueous buffer was monitored by C18-HPLC.

4. Docking study

To perform the molecular docking study, the Fc fragment of the human IgG1 crystal structure (PDB ID: 1DN2) was downloaded from the Protein Data Bank.[2] BIOVIA DISCOVERY STUDIO® 3.5 version was used for the docking study. The tertiary structure of the ligand was obtained using a ChemBio3D Ultra13.0. Before the docking study, BIOVIA DISCOVERY using STUDIO® 3.5 version was used for energy minimization (optimization) of the ligand. CHARMm Forcefield of DISCOVERY STUDIO® was used for the docking study, which was performed at a radius of 20 Å centered on K246 and K248 of the Fc fragment. All bound water molecules and heteroatoms were removed from the Fc fragment of the human IgG1 crystal structure. Thus, 50 conformers were identified, and pose analysis was performed on the conformer with the lowest binding energy.

5. Surface plasmon resonance assay

FcRn was covalently immobilized on the SPR sensor chip surface. FcRn was diluted to 1.25 μg/mL in 10 mM sodium acetate buffer, pH 5.0. The sensor chip surface was activated with a 1:1 mixture of 0.4 M EDC and 0.1 M NHS for 420 s, and then the diluted FcRn was immobilized on the sample channel. To deactivate the surface, 1 M ethanolamine was injected for 420 s. The reference channel was processed in the same manner as the sample channel, except for the FcRn immobilization step.

To determine the concentration range of the analyte, 10, 50, and 200 nM analyte were tested, and then the binding level was checked. Regeneration is the process of removing bound analyte from the surface after an analysis cycle without damaging the ligand in preparation for a new cycle. A 5 mM NaOH solution was injected into both the sample and reference channels at a flow rate of 30 μL/min for 30 s after the analyte binding step. The results confirmed that the baseline was restored without damaging the immobilized ligand.

All analytes were prepared at eight different concentrations (ranging from 3.125 to 400 nM) with running buffer (PBS, pH 6.0, 0.005% (v/v) Tween 20). The analyte was injected at a flow rate of 30 μL/min for 4 min, followed by dissociation for 6 min. After each cycle, regeneration was performed with 5 mM NaOH for 30 s at a flow rate of 30 μL/min. The resulting data were fitted to a 1:1 binding kinetic model using BiaEvaluation software v3.2.

6. Binding site analysis

***Mass spectrometric analysis and database search*:** Samples were eluted with a linear gradient of solvent B (100% ACN, 0.1% formic acid); 5–45% over 45 min, 45–80% over 2.5 min at a flow rate of 300 nL/min. The separated peptide ions were introduced into the mass spectrometer at an electrospray voltage of 2.3 kV. All MS/MS spectra were obtained in data-dependent mode for fragmentation of the ten most abundant peaks from the full MS scan with 25% normalized collision energy. The dynamic exclusion duration was set to 30 s and isolation mass width was 1.2 m/z. MS spectra were acquired with a mass range of 350–2000 m/z and 70,000 resolution at an m/z 200. MS/MS resolution was acquired at a resolution of 17,500 (Fig. 2e and Supplementary Fig. 24–26).

***Data analysis*:** To identify the antibody-linker binding site, the acquired MS/MS spectra were searched against the provided antibody database using the Sequest algorithm in Proteome Discoverer 1.4 (Thermo Fisher Scientific). The search parameters were as follows: chymotrypsin specificity with up to two missed cleavage sites, mass tolerance for fragment ions was set to 10 ppm for higher collisional dissociation, variable modification for methionine oxidation, carbamidomethylation (57.02 Da), and toxin fragmentation residue (adding modification: 120.06 Da).

7. Antigen binding assay

HER2/v (10004-H08H100, Sinobio) was coated on an enzyme-linked immunosorbent assay plate (100 ng per well) and incubated at 4 °C for 24 h. The plate was washed three times with TBS-T. Bovine serum albumin (3%) was incubated for 1.5 h at 25 °C. After blocking, the buffer was discarded, and serially diluted samples were added and incubated for 1.5 h at 25°C. The plate was washed three times with 100 μL of TBS-T. Next, each well was incubated with F(ab')2-goat anti-human IgG (H+L)-conjugated horseradish peroxidase (PA5-33290, Thermo Fisher Scientific) at 25°C for 1.5 h. The plate was washed and incubated with TMB solution (T0440, Sigma) for 3 min, and 0.5 M H_2SO_4 (stop solution) was added. Absorbance was measured at 450 nm using a plate reader (Multiskan GO, Thermo Fisher Scientific).

8. Cell viability assay

Cancer cells were seeded into a 96-well optical-bottom plate (5,000 cells per well, 165306, Thermo Fisher Scientific) and incubated at 37 °C under 5% CO_2 for 24 h. After the medium was discarded, serially diluted antibodies were added and incubated at 37 °C for 120 h (NCI-N87) or 96 h (MDA-MB-231). Cell viability was assessed using the CellTiter-Glo (G7570, Promega, Madison, WI, USA).

9. NCI-N87 human gastric cancer xenograft model

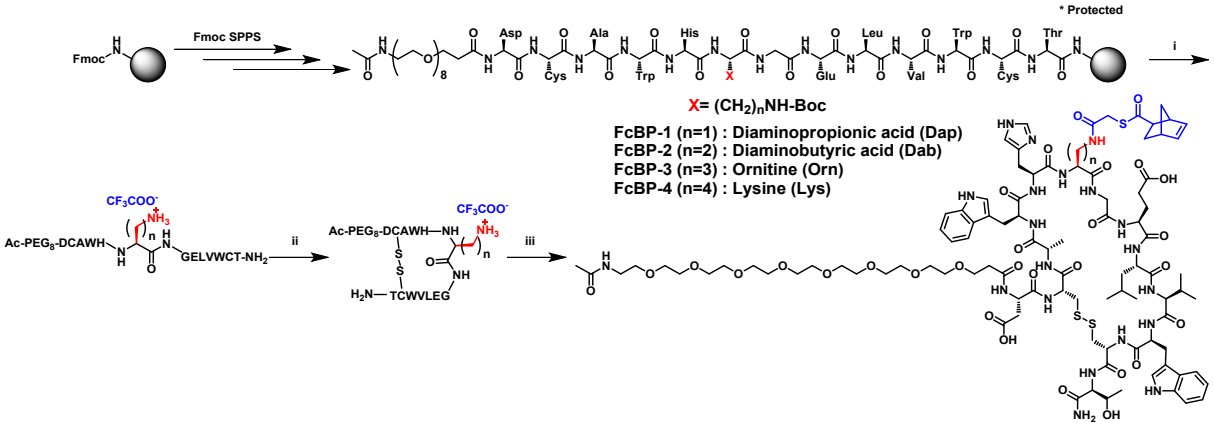
We evaluated the potential therapeutic effects and tolerability of test articles for the treatment of NCI-N87 human gastric cancer xenograft model in BALB/c nude mice (CAnN.Cg-Foxn1nu/CrljOri). All procedures related to animal handling, care, and treatment were performed according to guidelines approved by the Institutional Animal Care and Use Committee (Permit #: SKKUIACUC2018-01-05-1) of Sungkyunkwan University.

Xenograft models were established by inoculation of the NCI-N87 cell line. NCI-N87 cells (2.5×10^6) were inoculated into the right flank of 6-week-old female BALB/c nude mice. The mice were divided into four groups each containing 5 mice, and treatments were started when the mean tumor volume reached approximately 100–150 mm^3. Based on the tumor volume, the mice were assigned to different groups such that the average starting tumor size was the same in each treatment group.

Each group of tumor-bearing mice was intravenously administered PBS, trastuzumab, T-nc-DM1, and T-VC-DM1 once every three days. The tumor size and body weight were measured twice weekly using a caliper and recorded. The tumor volume (mm^3, TV) was estimated using the formula: $TV = a \times b^2/2$, where a and b are long and short diameters of a tumor[3], respectively. At the end of the study, the tumors were collected, images were acquired, and the tumor weight was measured.

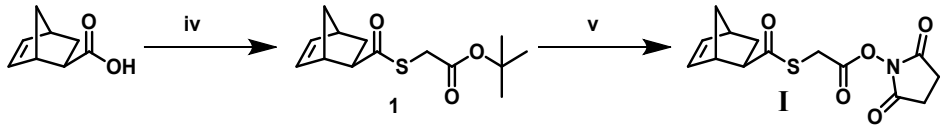
10. Supplementary schemes

10.1. Synthesis of AbClick 1-4



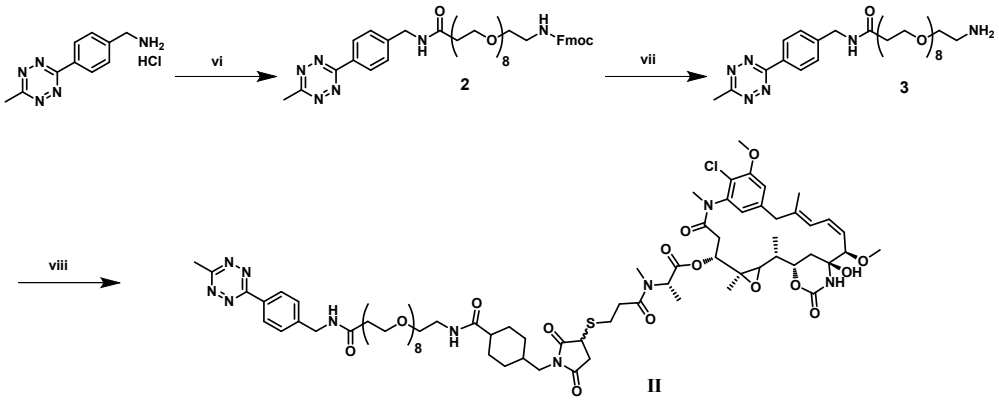
Scheme S1. Synthesis of AbClick 1-4. (i) TFA:TIS:EDT:H_2O = 94:1:2.5:2.5; **(ii)** 1% DMSO in ACN:H_2O (1:4), pH 6.0; **(iii)** compound **I**, DIPEA, DMF.

10.2. Synthesis of bio-orthogonal handle



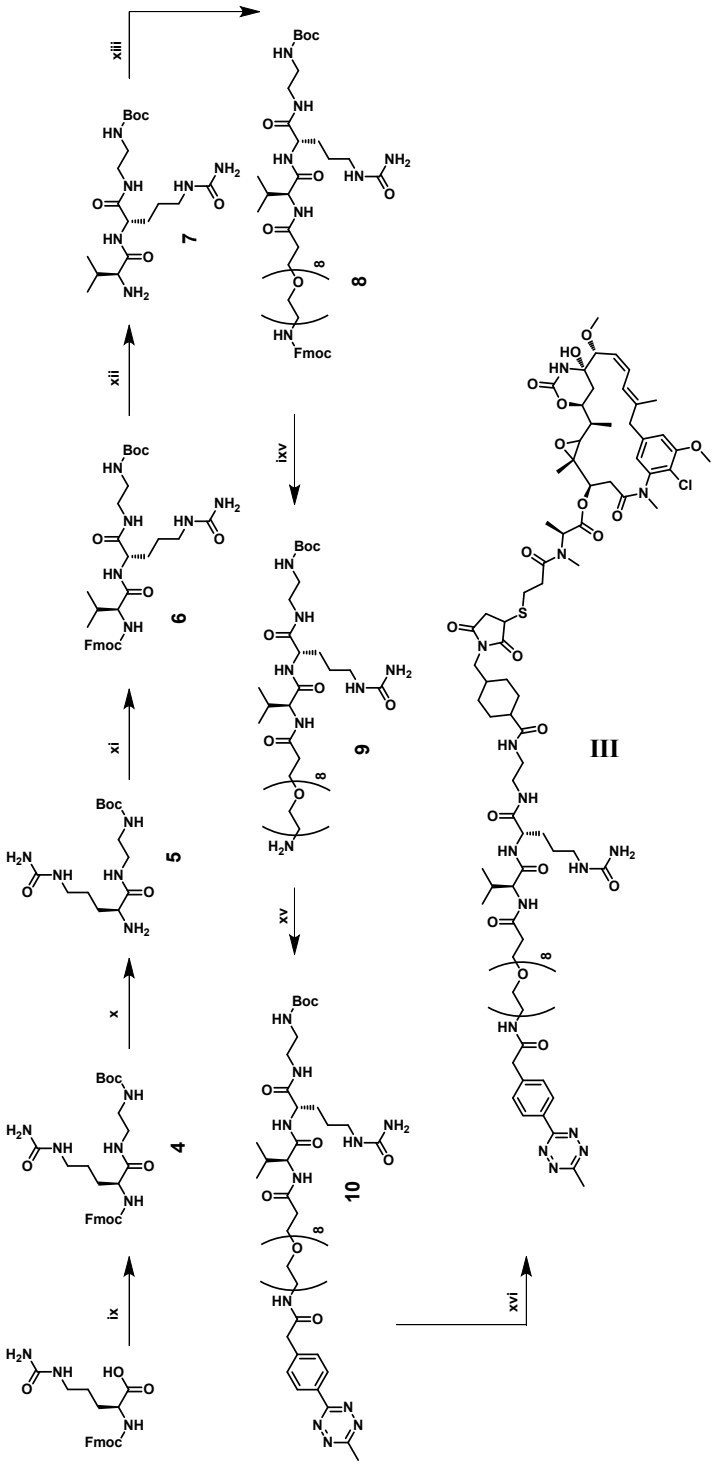
Scheme S2. Synthesis of bio-orthogonal handles (iv) HATU, DIPEA, *tert*-butyl thioglycolate, DMF, 16 h; **(v)** a) DCM:TFA = 1:1 2 h; b) NHS, EDCi, DMAP, DIPEA, DMF, 3 h.

10.3. Synthesis of 4-methyltetrazine-PEG$_8$-SMCC-DM1 (non-cleavable DM1)[1]



Scheme S3. Synthesis of 4-methyltetrazine-PEG$_8$-SMCC-DM1. h; (vi) Fmoc-PEG$_8$-OH, DIPEA, DMF, 4 h; **(vii)** dimethylamine, DMF, 2.5 h; **(viii)** DM1-SMCC, DIPEA, DMF, 2 h.

10.4. Synthesis of 4-methyltetrazine-PEG$_8$-Val-Cit-EDA-SMCC-DM1 (cleavable DM1)



Scheme S4. Synthesis of 4-methyltetrazine-PEG$_8$-Val-Cit-EDA-SMCC-DM1 (ix) *tert*-butyl (2-aminoethyl)carbamate, HATU, DIPEA, DMF, 4 h; **(x)** diethylamine, DMF, 2.5 h; **(xi)** Fmoc-Val-OH, HATU, DIPEA, DMF, 2 h; **(xii)** diethylamine, DMF, 2.5 h; **(xiii)** Fmoc-PEG$_8$-OH, HATU, DIPEA, DMF, 2 h; **(ixv)** diethylamine, DMF, 2.5 h; **(xv)** methyltetrazine-OSu, DIPEA, 2 h; **(xvi)** DCM:TFA=1:1, 2 h, DM1-SMCC, DIPEA, DMF, 2 h;

11. Supplementary Figures

11.1. Overview of conventional and recent ADC development technologies

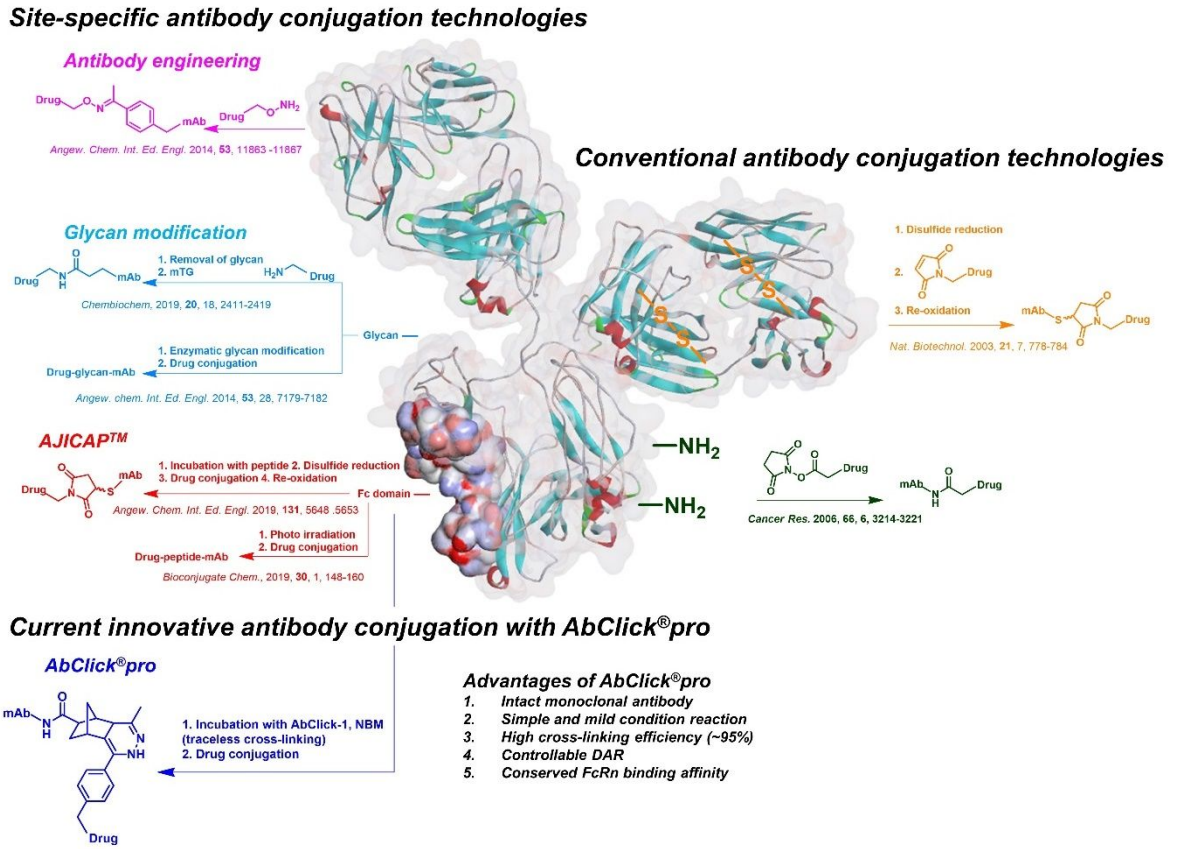
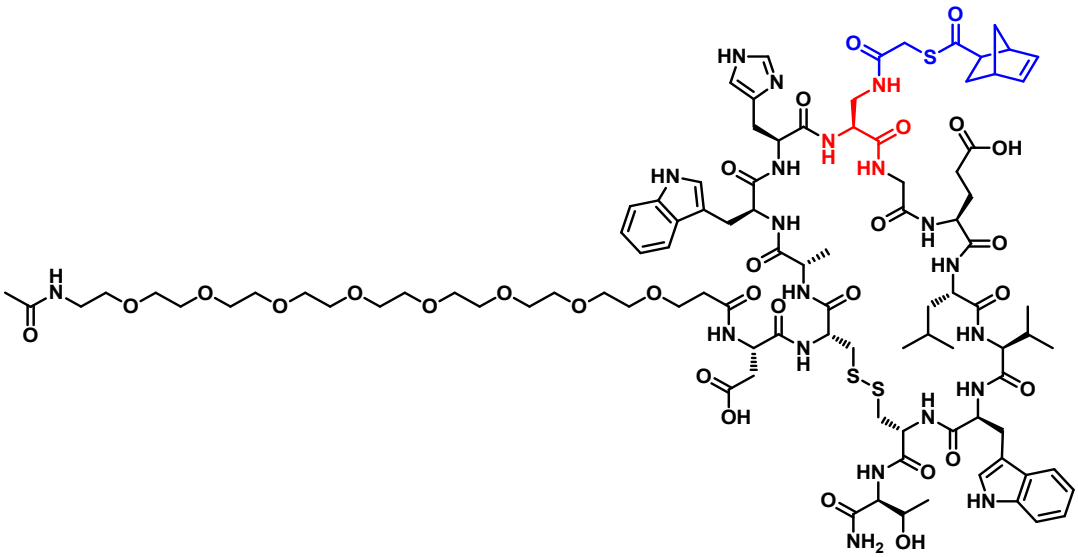


Figure S1. Overview of previous conjugation technologies and our latest AbClick®Pro technology for generating ADCs. Conventional technologies mostly include reaction of lysine or cysteine residues by reducing the disulfide for antibody conjugation. Site-specific antibody conjugation includes antibody engineering, glycan modification, exploiting Fc domain peptides as in AJICAP™, and our current traceless AbClick®Pro technology.

11.2. Structures and HPLC chromatograms of purified FcBP derivatives and payloads

L6Dap (5-exo-norbornene-thioester), AbClick-1



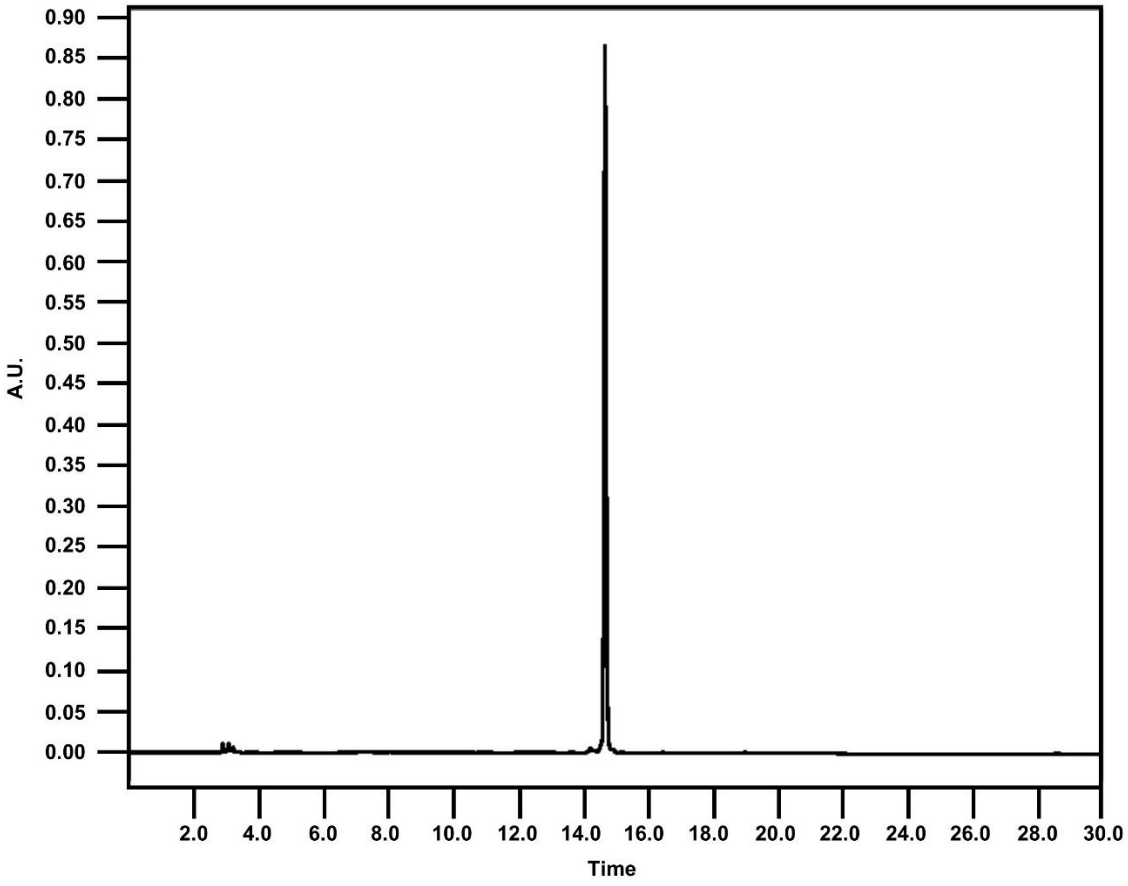


Figure S2. Chemical structure and HPLC chromatogram (RT = 14.7 min) of L6Dap (5-exo-norbornene-thioester).

L6Dab (5-exo-norbornene-thioester), AbClick-2

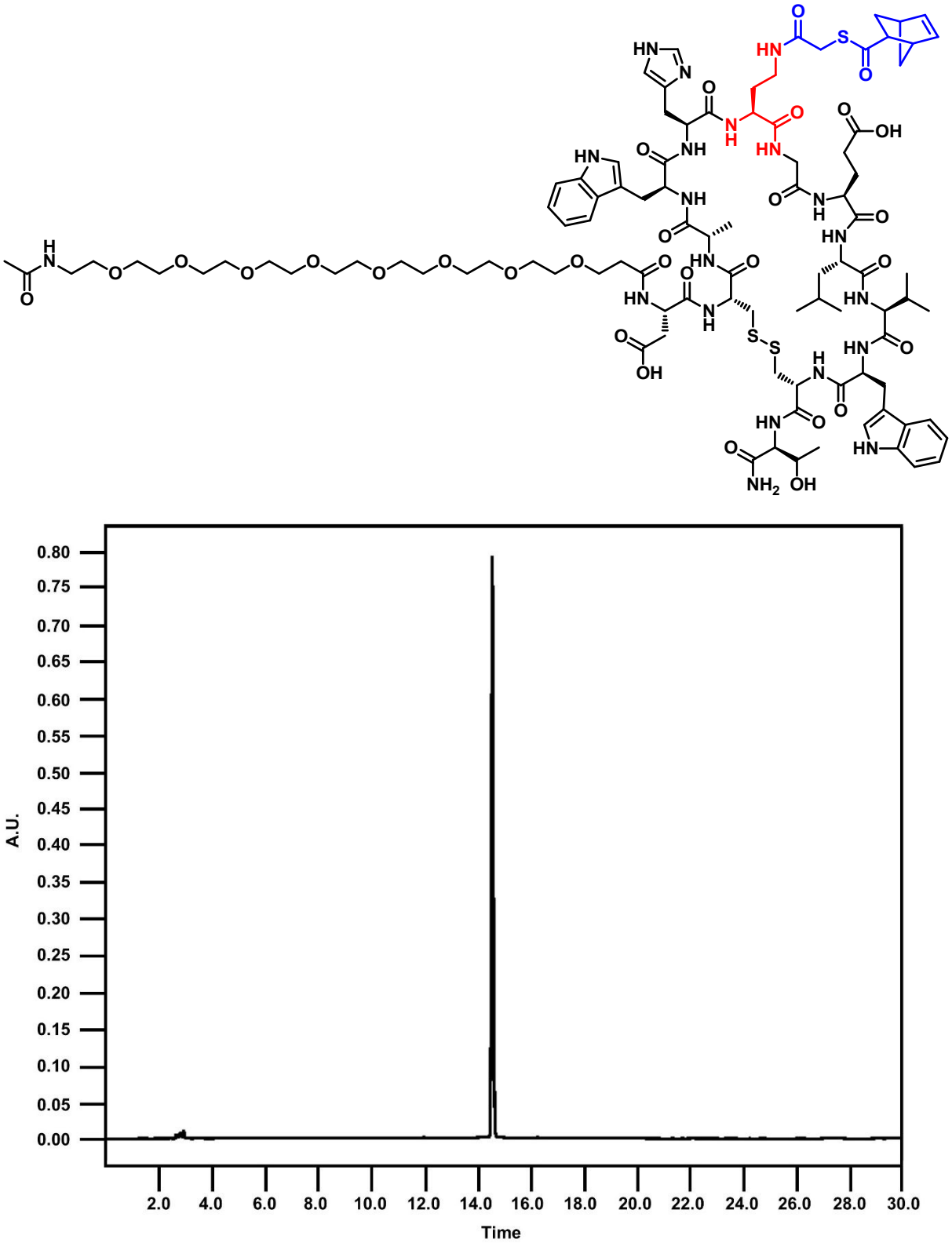
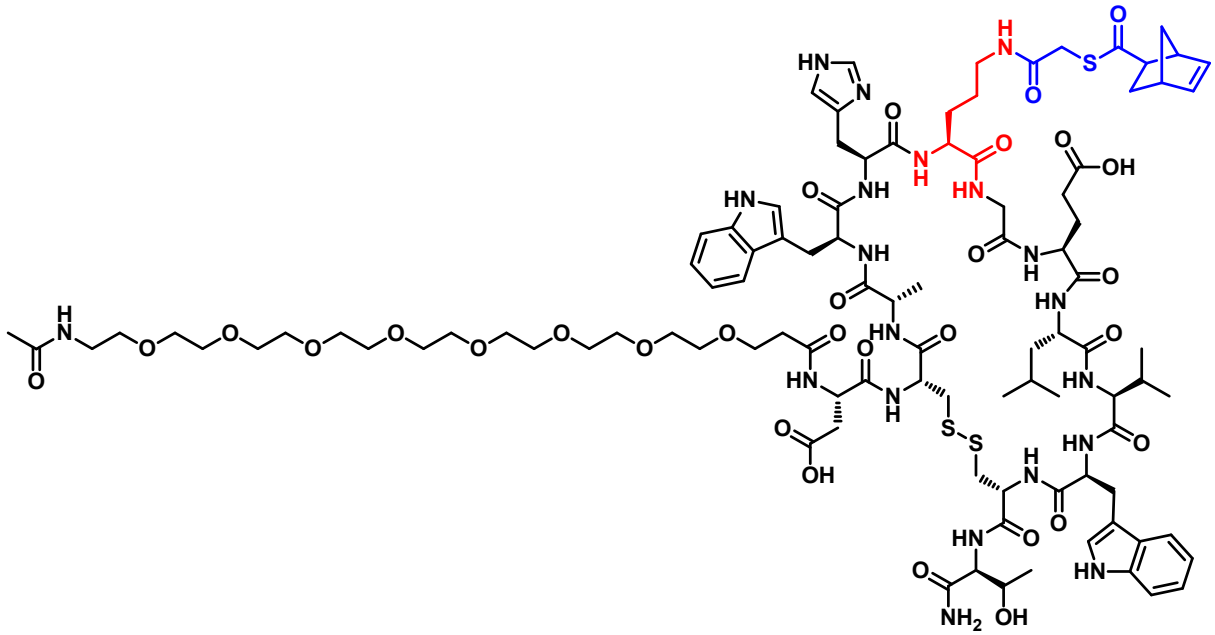


Figure S3. Chemical structure and HPLC chromatogram (RT = 14.6 min) of L6Dab (5-exo-norbornene-thioester).

L6Orn (5-exo-norbornene-thioester), AbClick-3



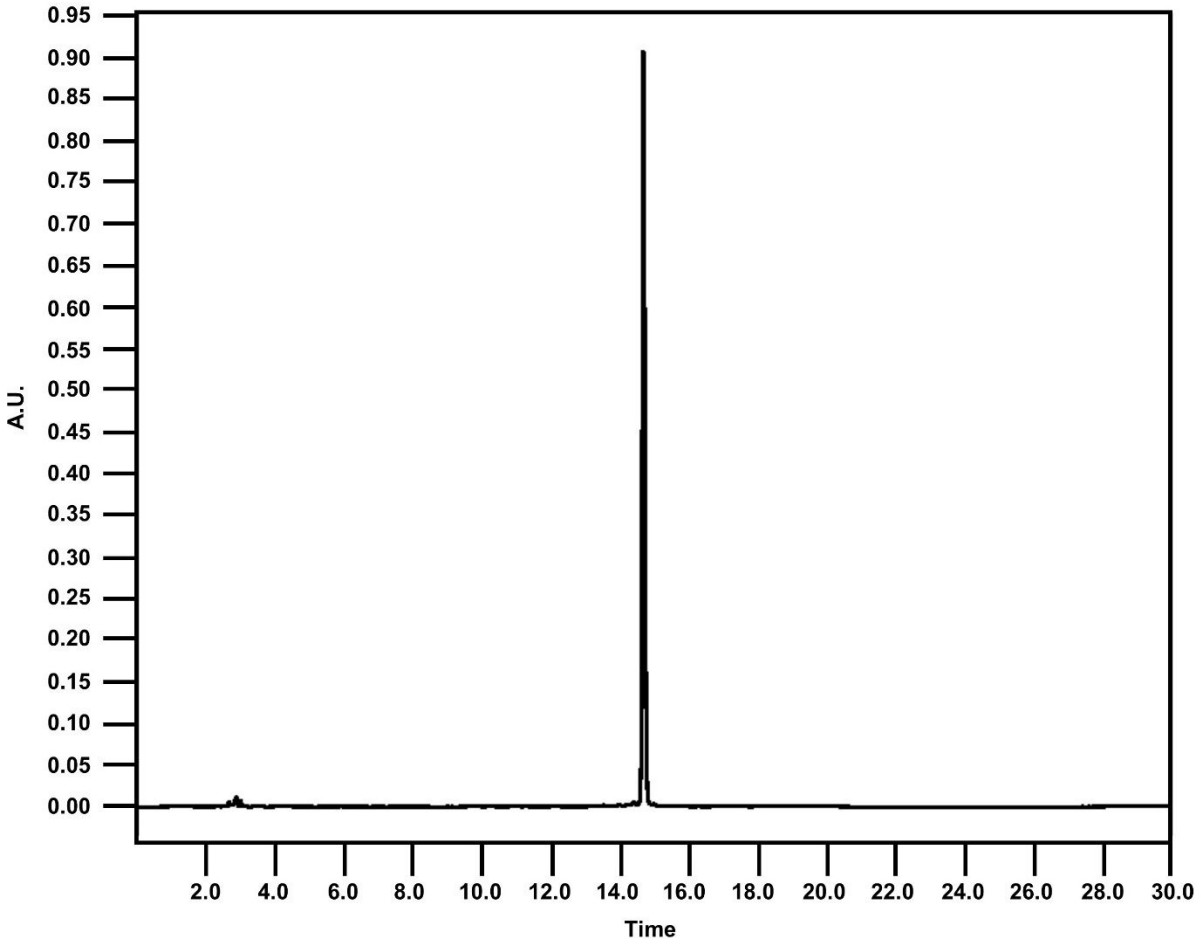
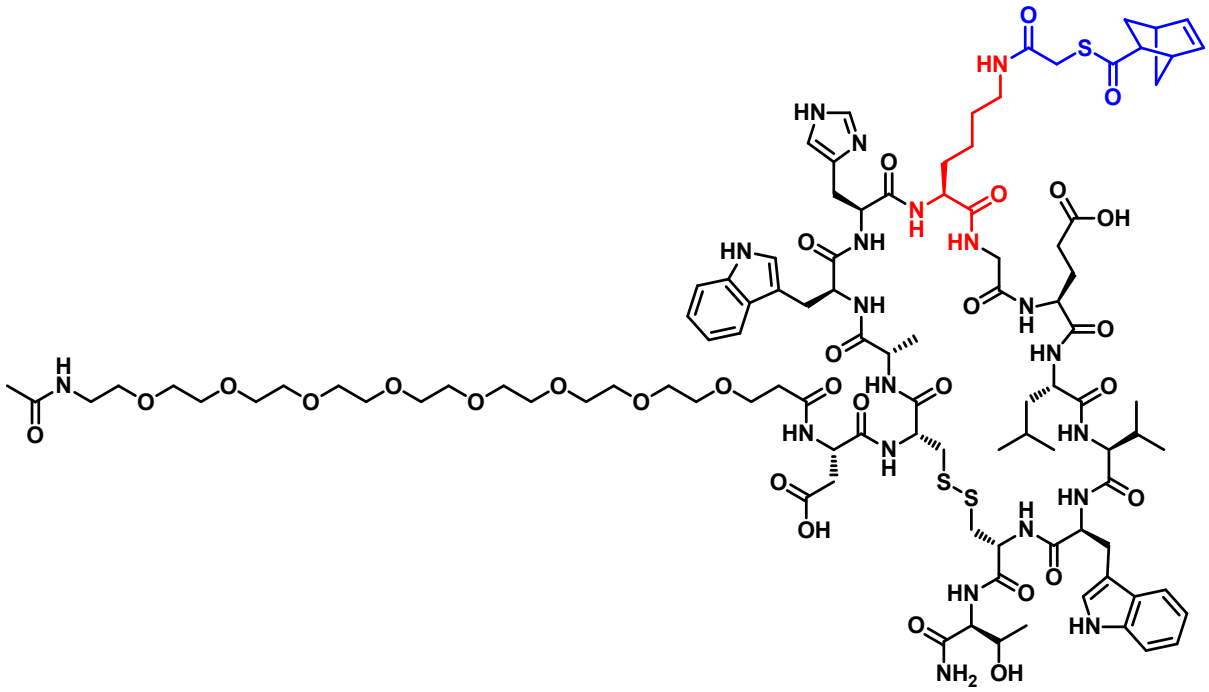


Figure S4. Chemical structure and HPLC chromatogram (RT = 14.7 min) of L6Orn (5-exo-norbornene-thioester).

L6Lys (5-exo-norbornene-thioester), AbClick-4



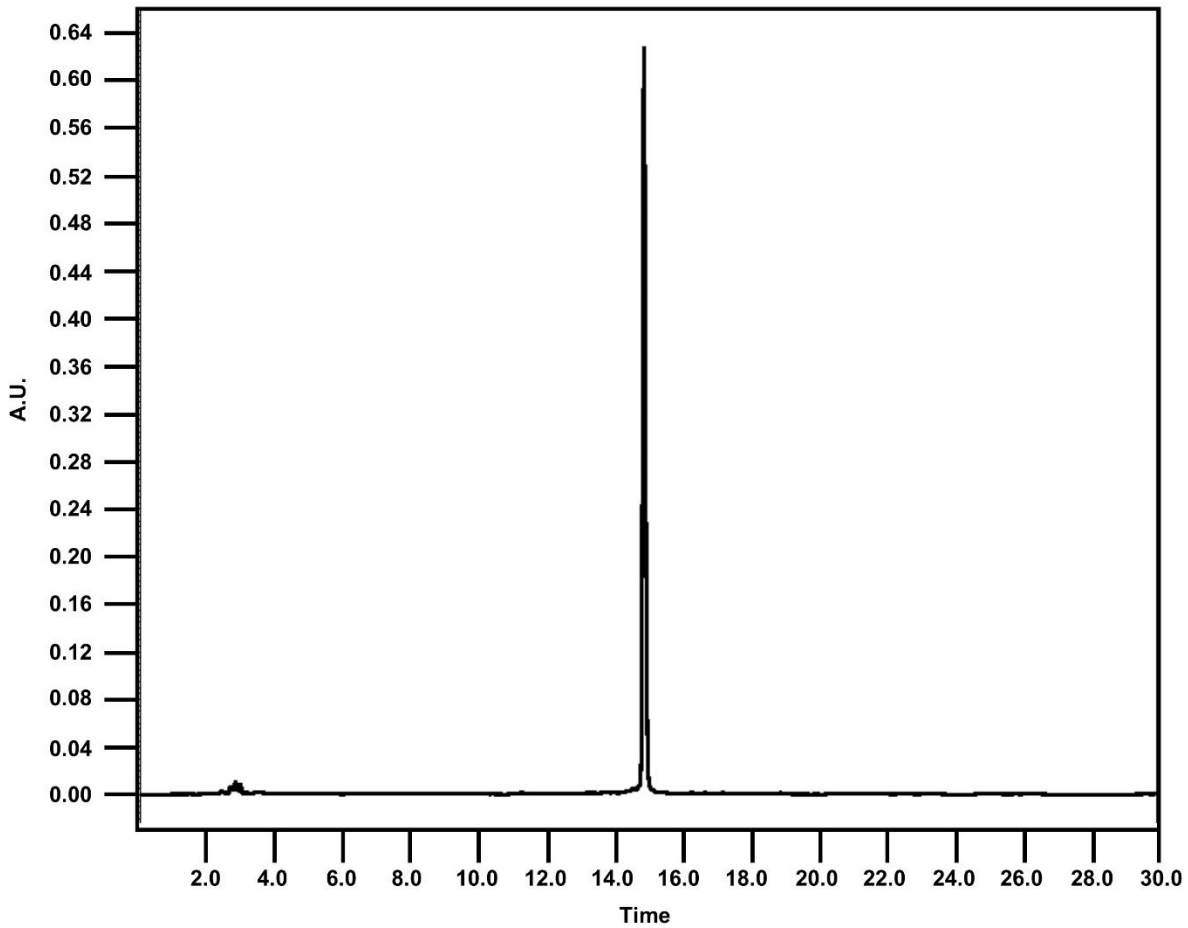
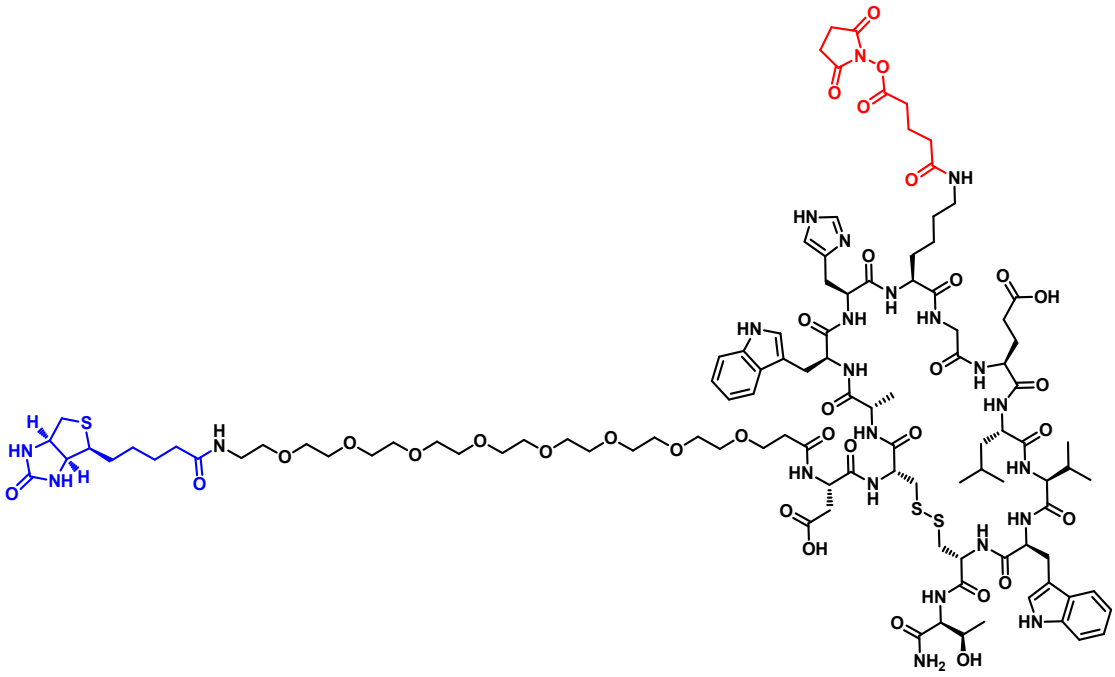


Figure S5. Chemical structure and HPLC chromatogram (RT = 14.9 min) of L6Lys (5-exo-norbornene-thioester).

FcBP-biotin (bFcBP)



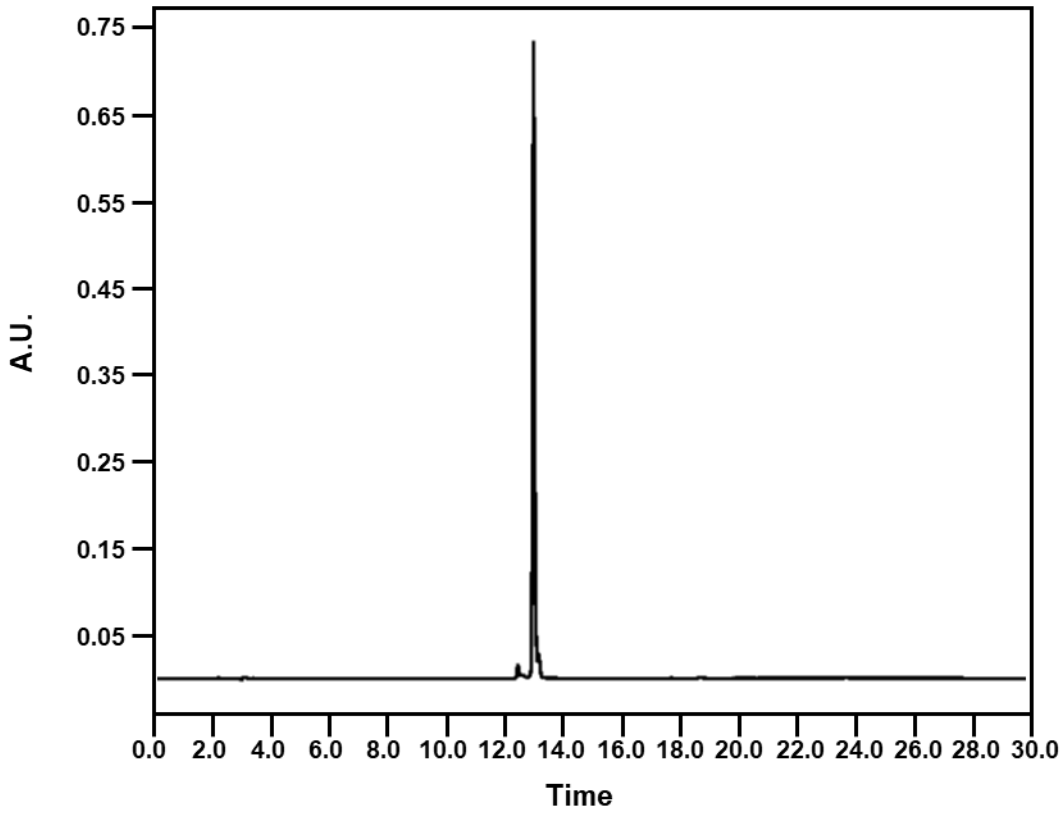
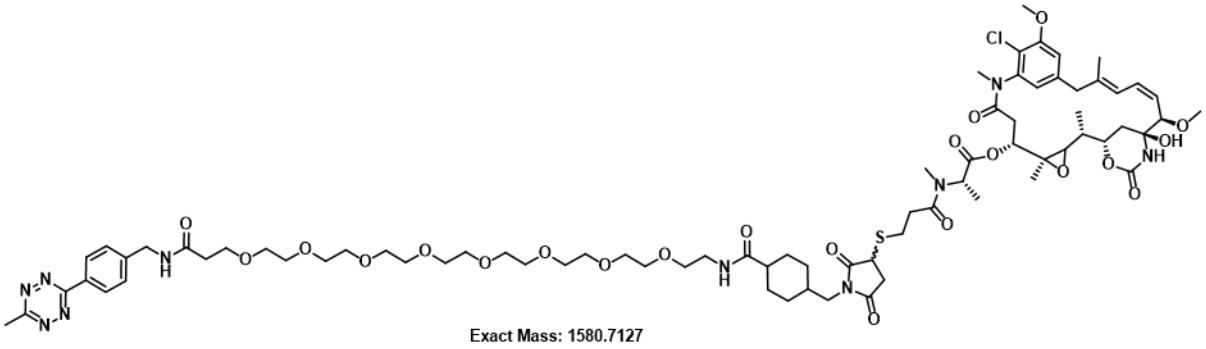


Figure S6. The chemical structure and HPLC chromatogram of FcBP-biotin (bFcBP).

4-methyltetrazine-PEG$_8$-SMCC-DM1



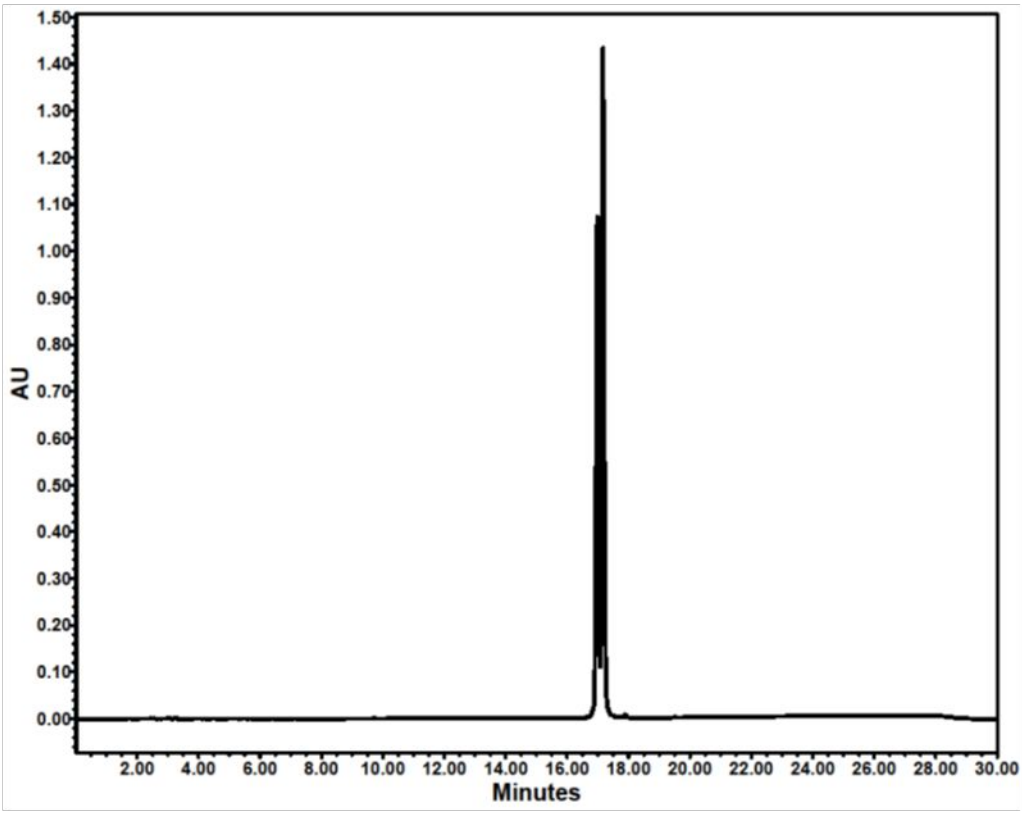


Figure S7. The chemical structure and HPLC chromatogram of 4-methyltetrazine-PEG$_8$-SMCC-DM1.

4-methyltetrazine-PEG$_8$-Val-Cit-EDA-SMCC-DM1

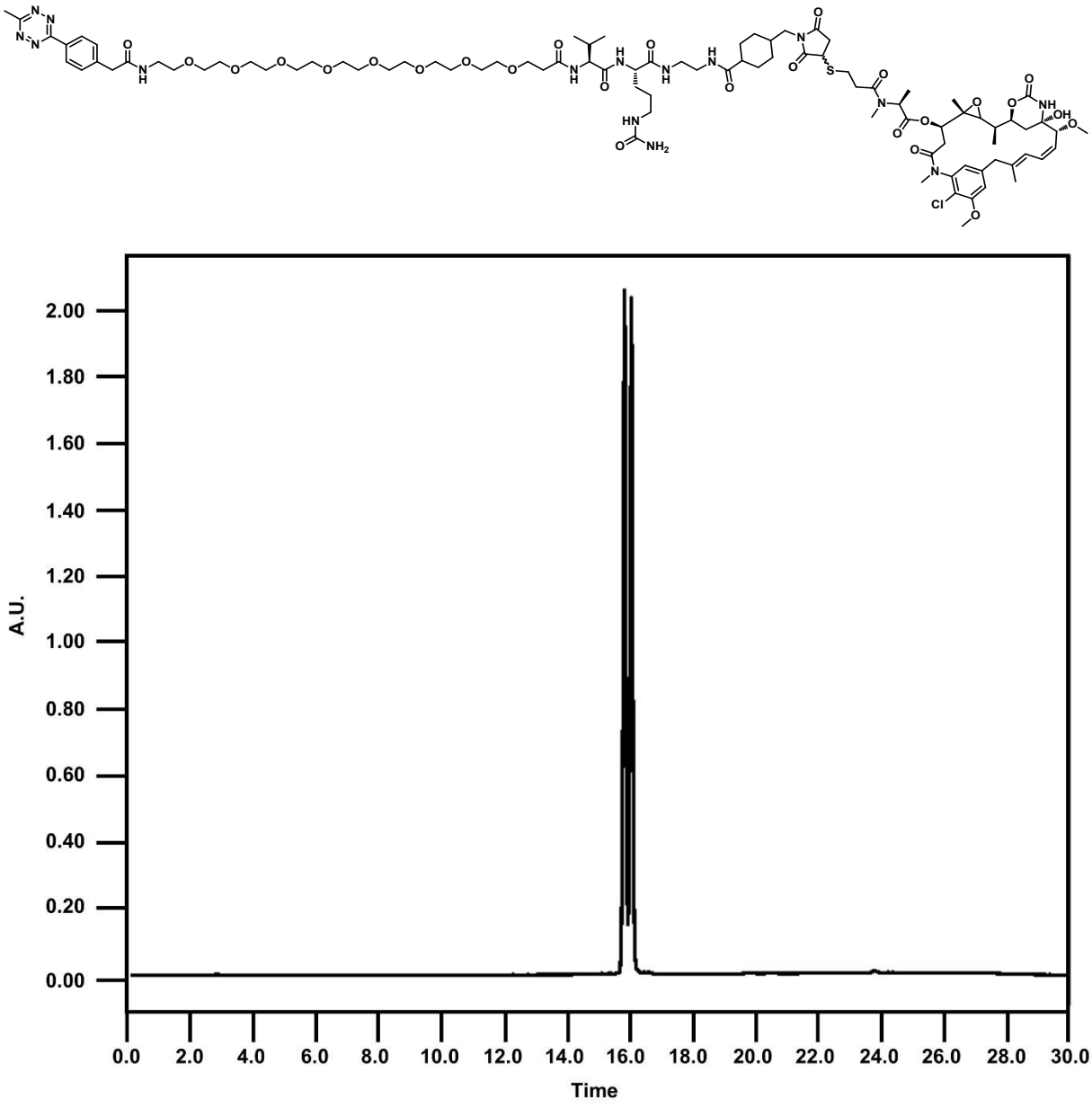


Figure S8. Chemical structure and HPLC chromatogram (RT = 15.9 min) of 4-methyltetrazine-PEG$_8$-Val-Cit-EDA-SMCC-DM1.

11.3. ^{1}H- and ^{13}C-NMR spectra

^{1}H-NMR spectrum of compound 1

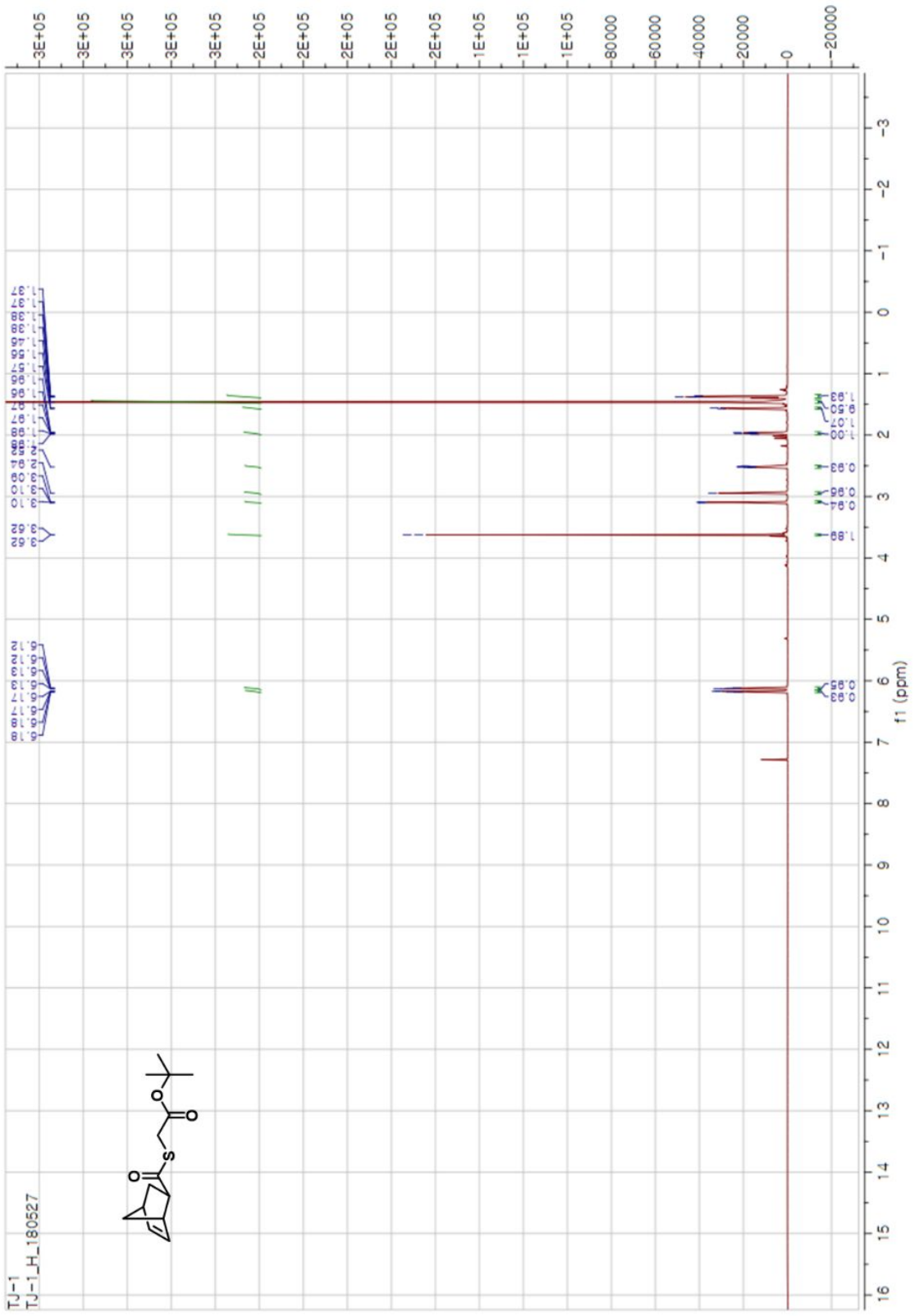


Figure S9. ^{1}H NMR spectrum of compound 1.

^{13}C-NMR spectrum of compound 1

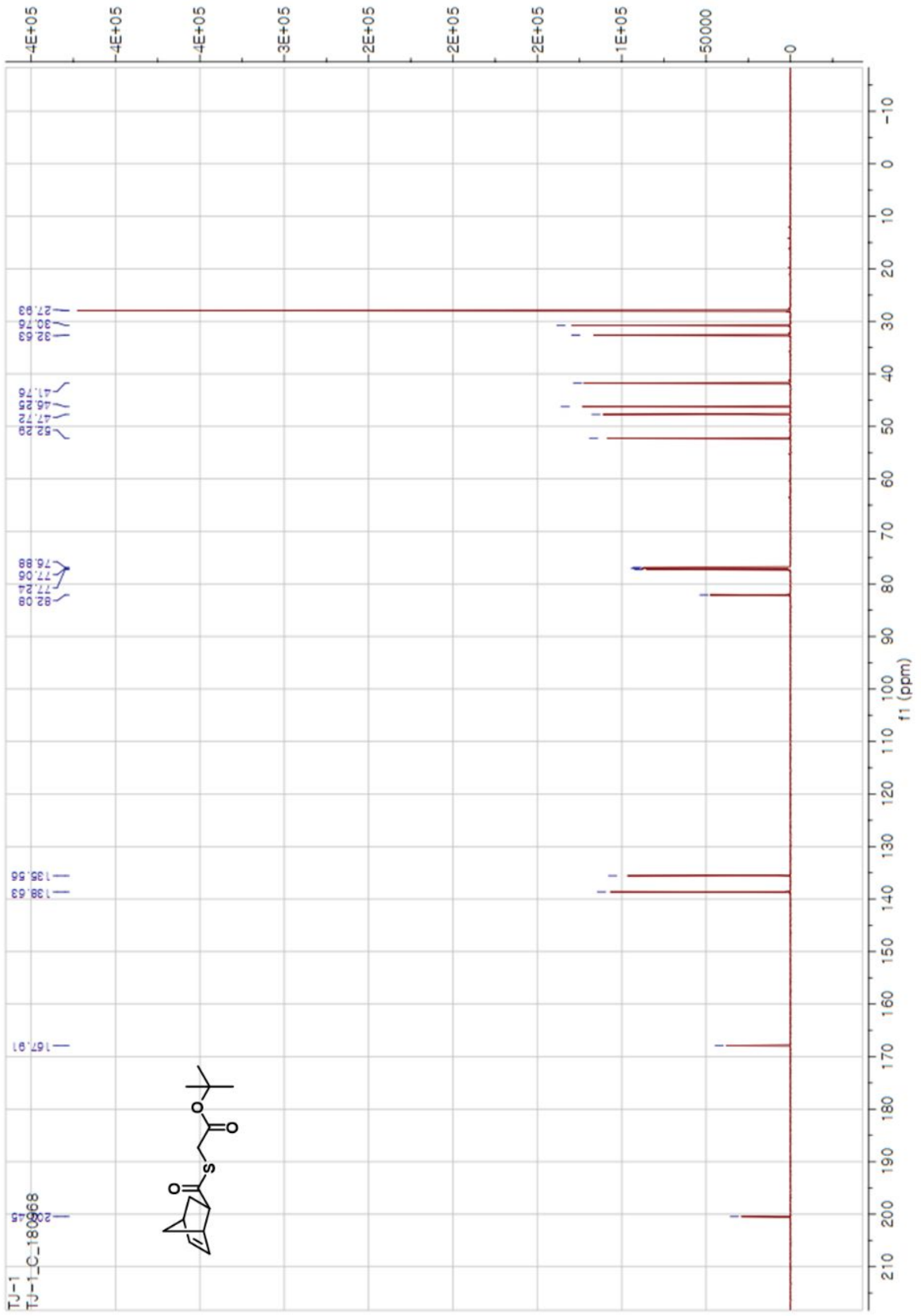


Figure S10. ^{13}C NMR spectrum of compound 1.

[1]H-NMR spectrum of compound I

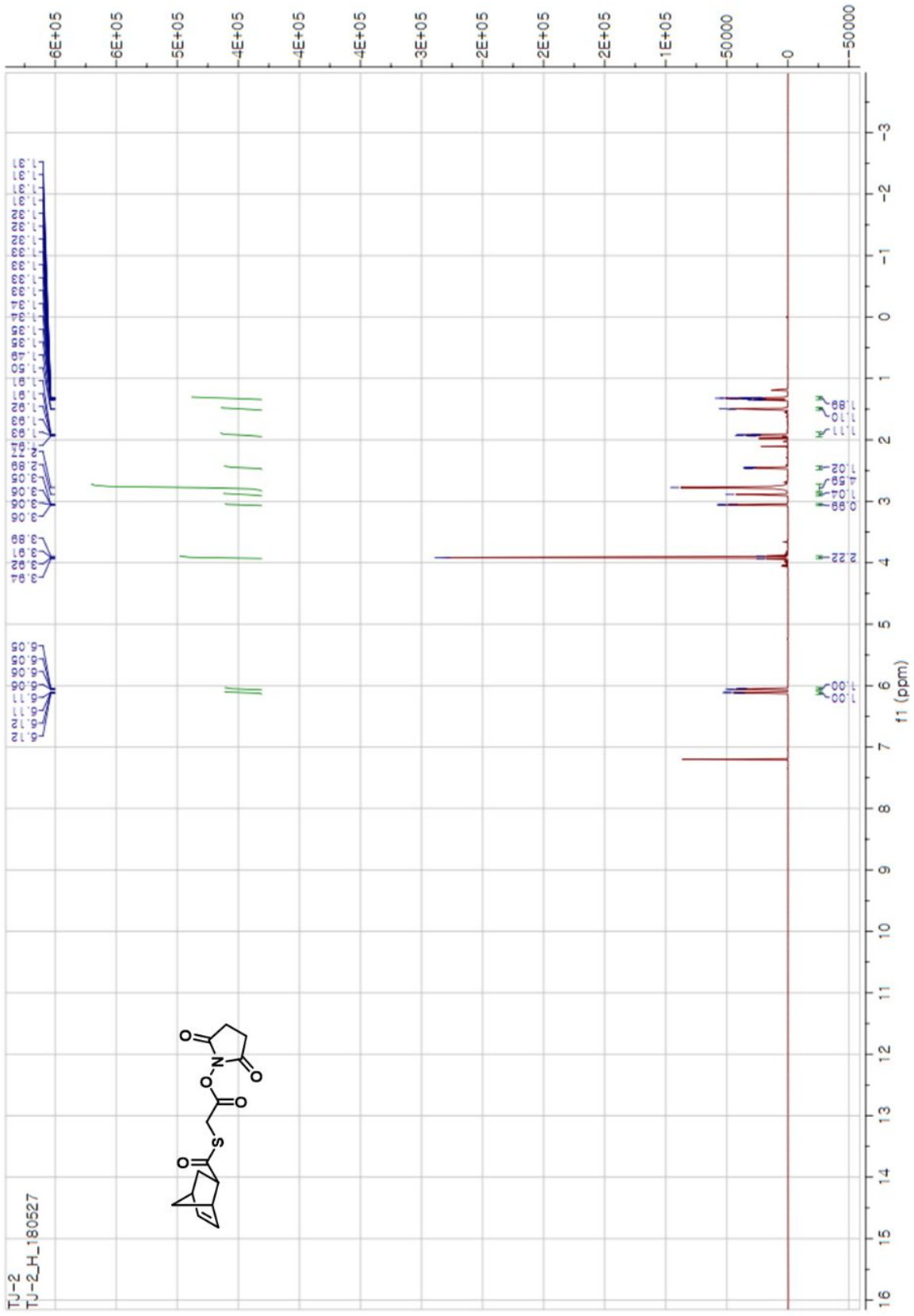


Figure S11. [1]H NMR spectrum of compound **I**.

^{13}C-NMR spectrum of compound I

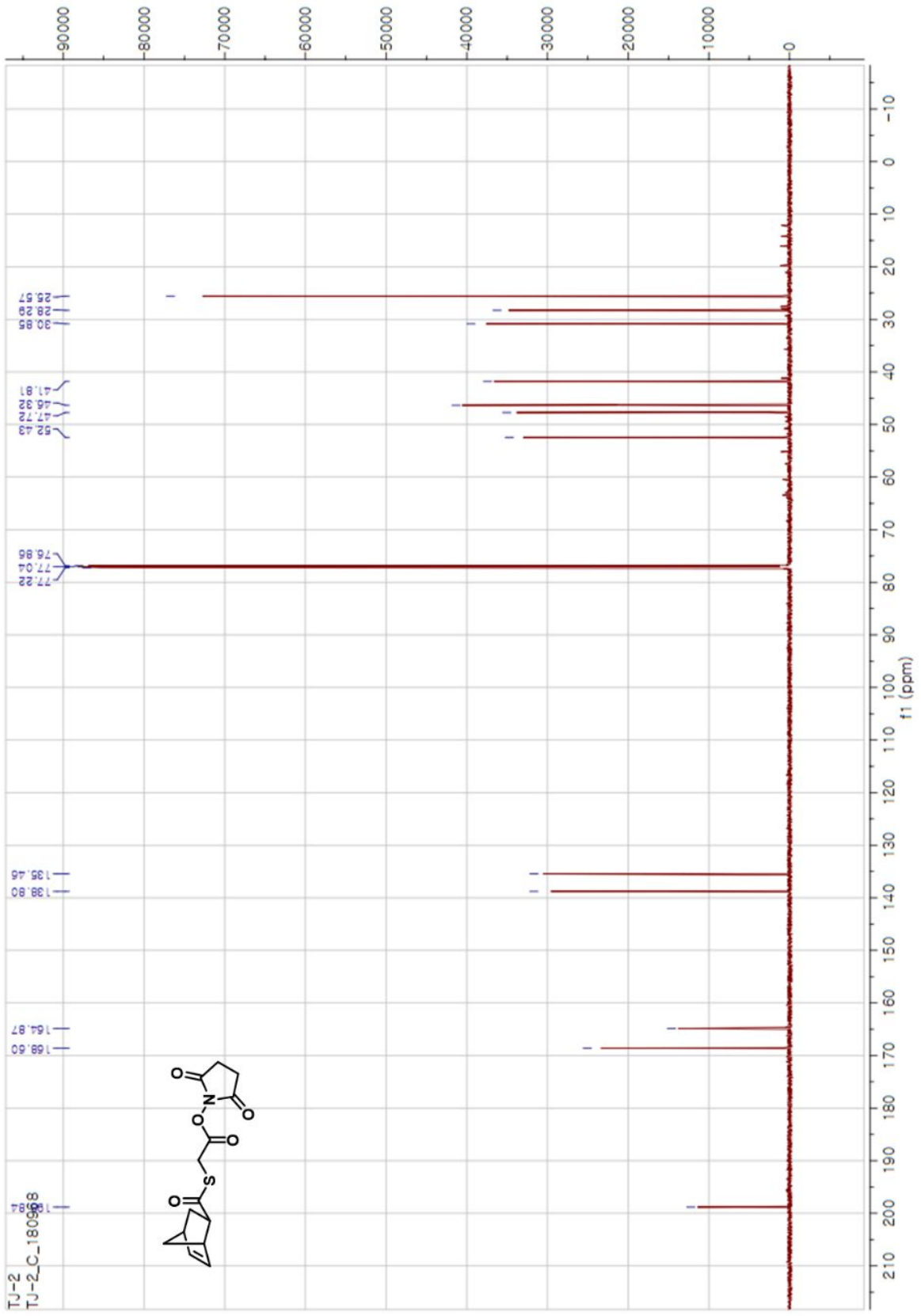


Figure S12. ^{13}C NMR spectrum of compound **I**.

[1]H-NMR spectrum of compound II

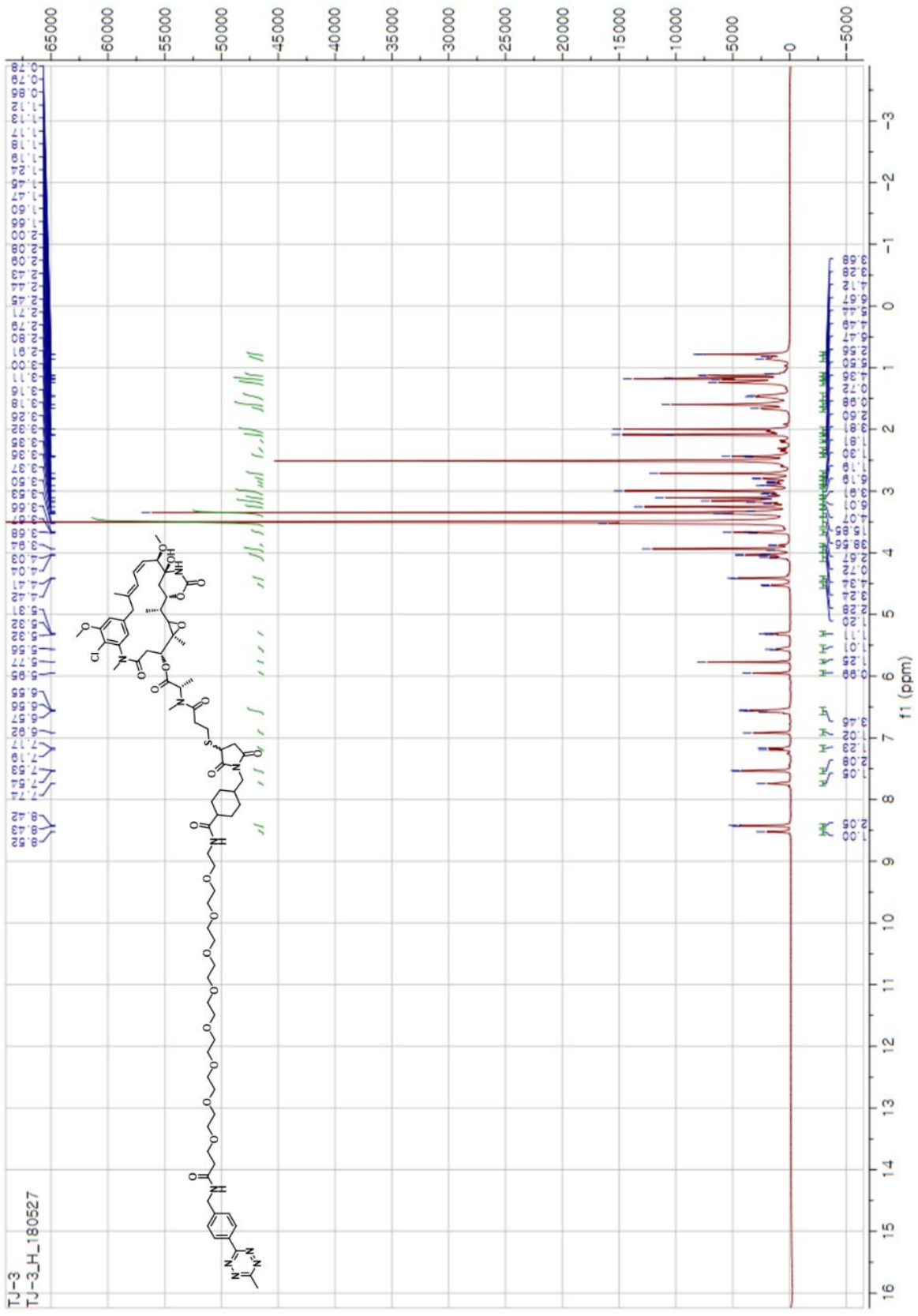


Figure S13. [1]H NMR spectrum of compound **II**.

[1]H-NMR spectrum of compound III

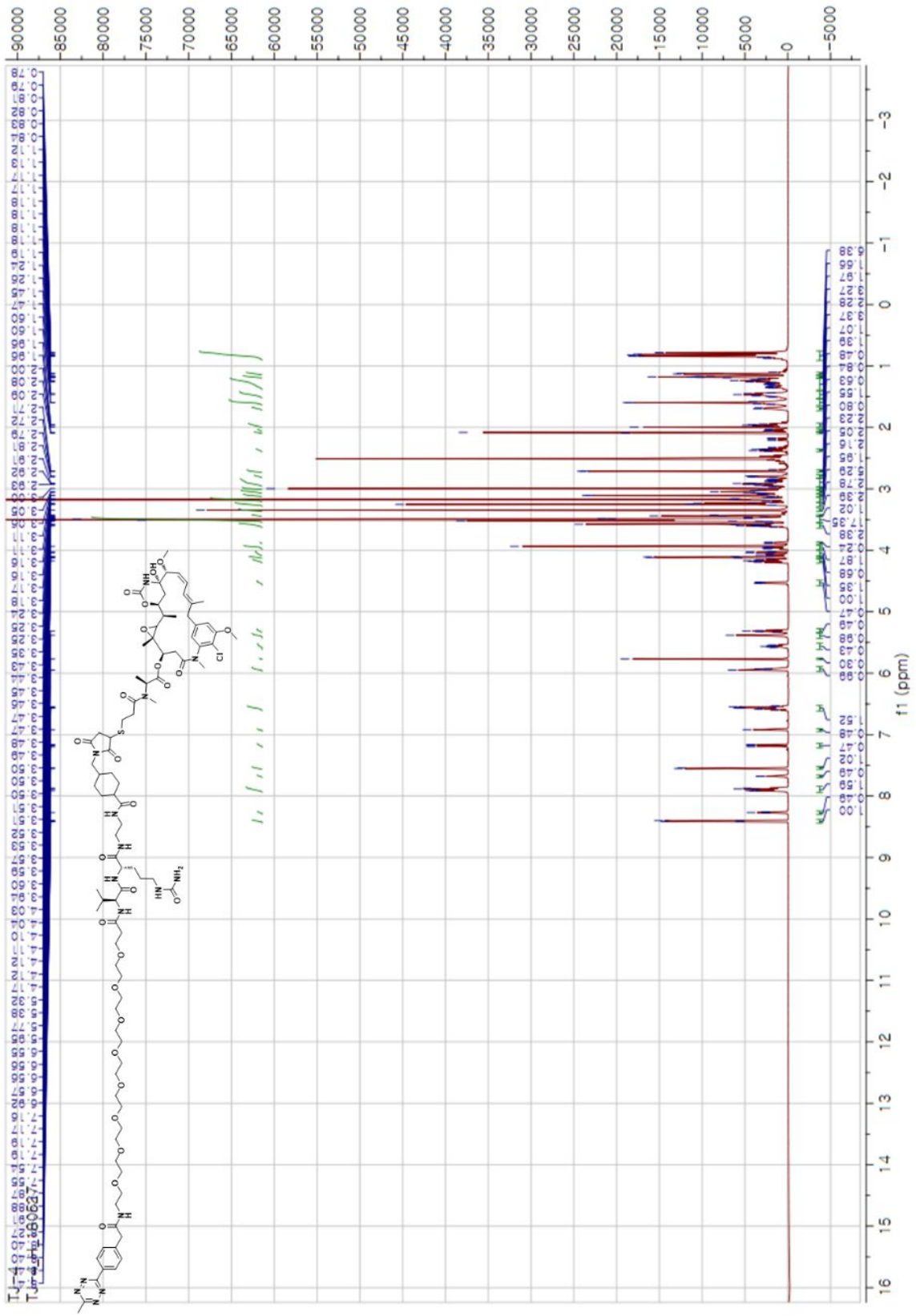


Figure S14. [1]H NMR spectrum of compound **III**.

11.4. HIC chromatograms for discovering an ideal scavenger

N-methylmaleimide

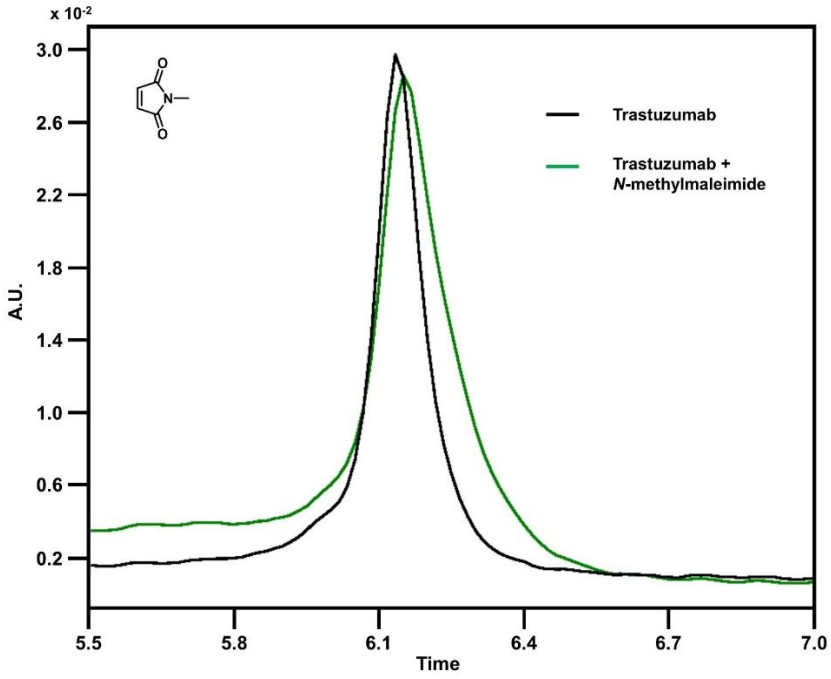


Figure S15. HIC-HPLC chromatogram of SEC-purified trastuzumab with *N*-methylmaleimide.

N-ethylmaleimide

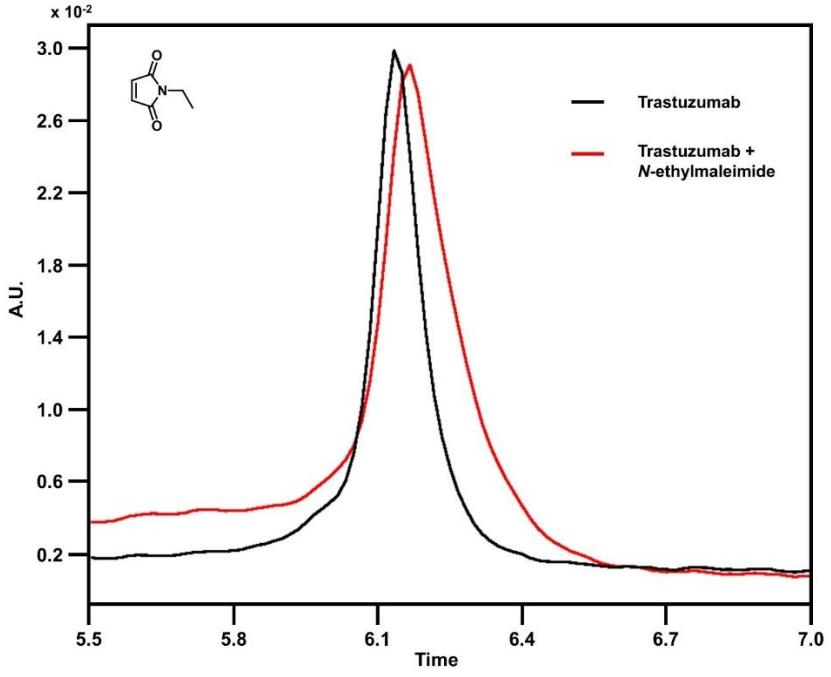


Figure S16. HIC-HPLC chromatogram of the SEC-purified trastuzumab with *N*-ethylmaleimide.

***N*-cyclohexylmaleimide**

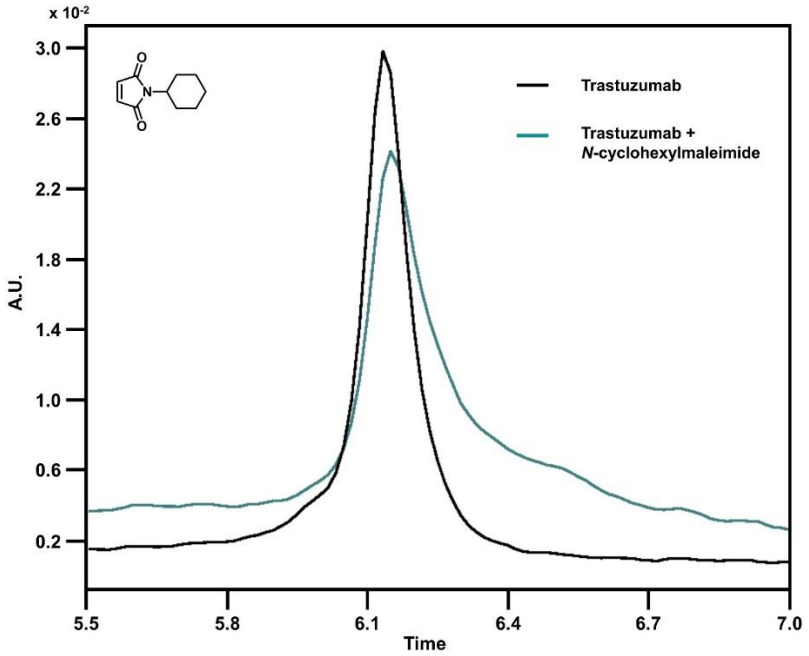


Figure S17. HIC-HPLC chromatogram of the SEC-purified trastuzumab with *N*-cyclohexylmaleimide.

***N*-phenylmaleimide**

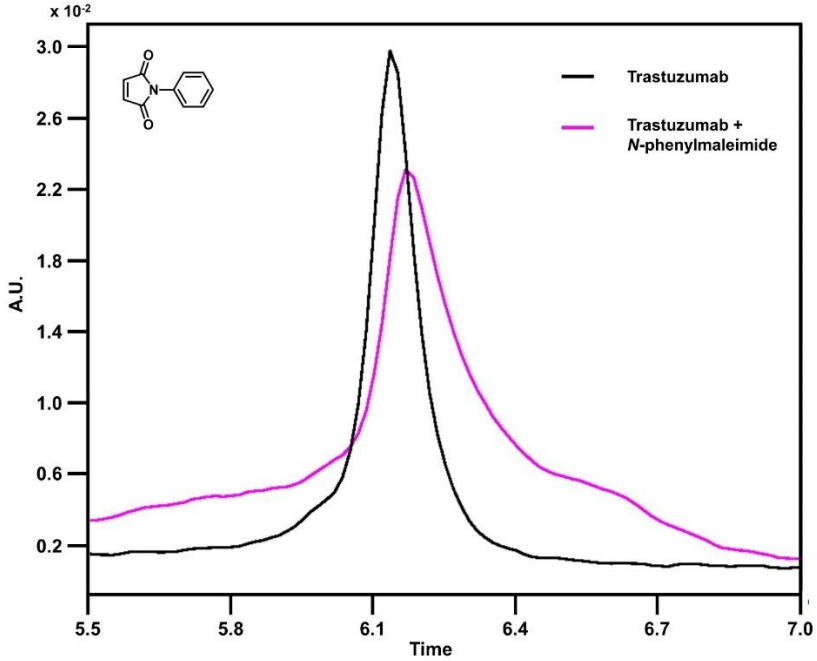


Figure S18. HIC-HPLC chromatogram of the SEC-purified trastuzumab with *N*-phenylmaleimide.

N-benzylmaleimide

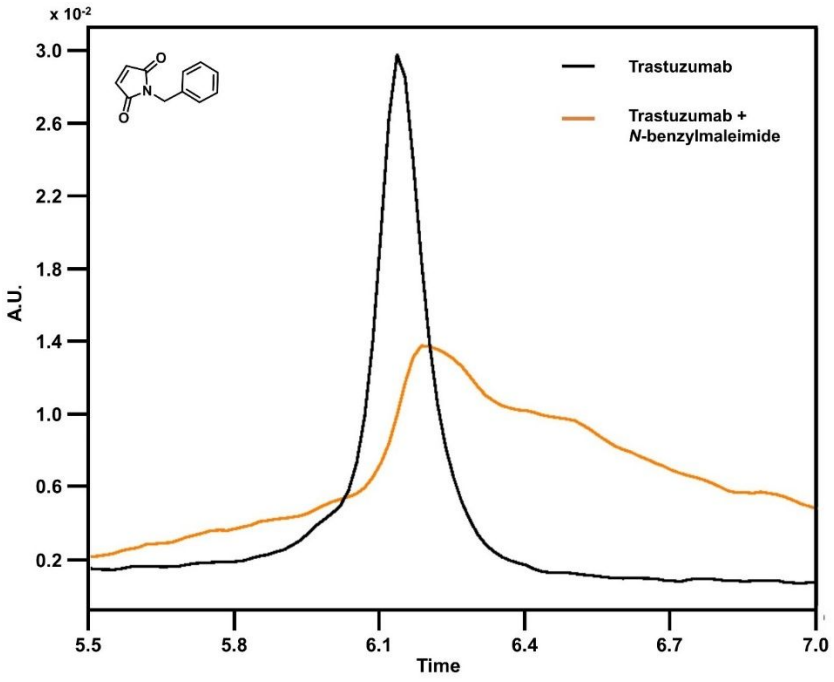


Figure S19. HIC-HPLC chromatogram of the SEC-purified trastuzumab with *N*-benzylmaleimide.

3-maleimido propionic acid

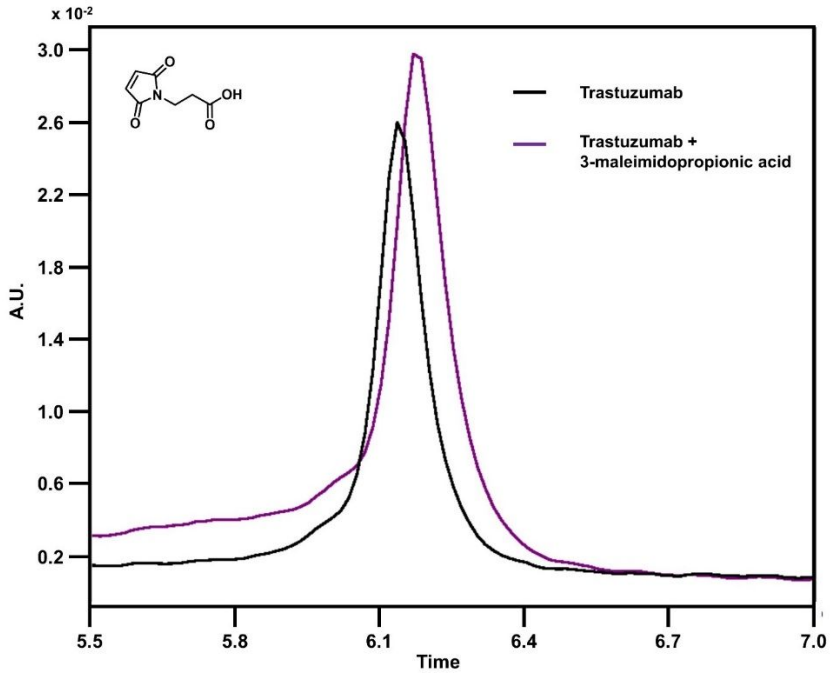


Figure S20. HIC-HPLC chromatogram of the SEC-purified trastuzumab with 3-maleimido propionic acid.

N-tert-butylmaleimide

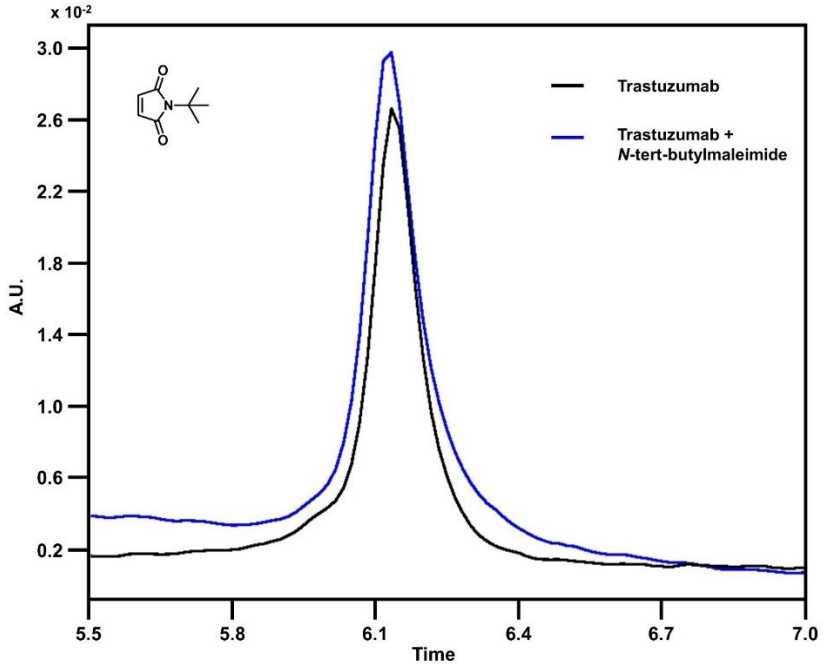


Figure S21. HIC-HPLC chromatogram of the SEC-purified trastuzumab with *N*-tert-butylmaleimide.

11.5. MALDI-TOF analysis

Trastuzumab

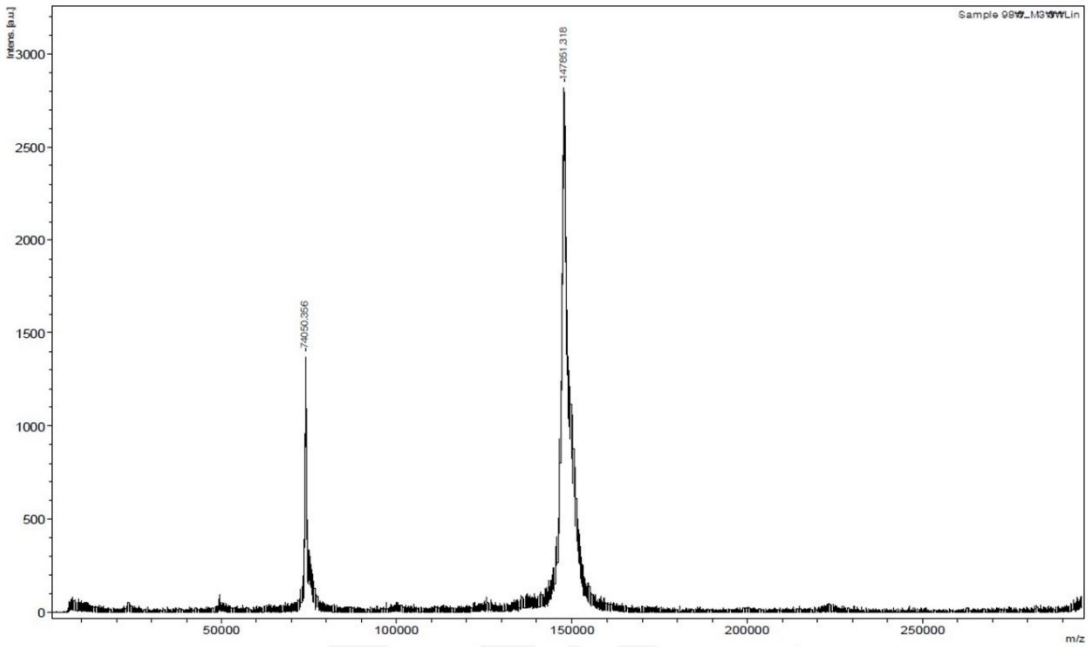


Figure S22. MALDI showing the observed molecular weight of trastuzumab.

Obtained mass: 147,851 Da

N-methylmaleimide

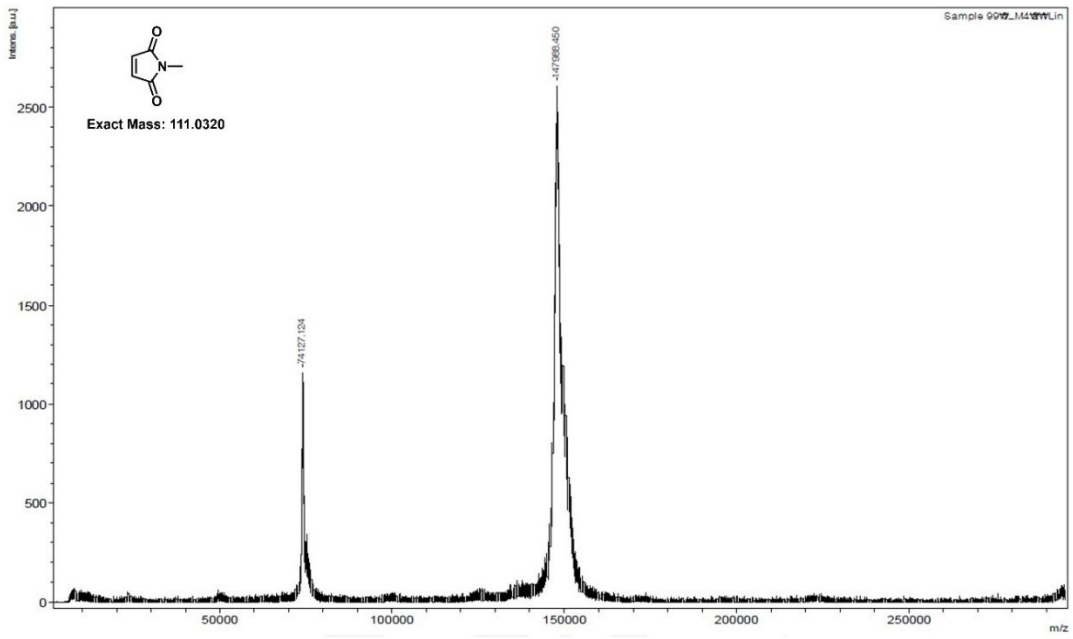


Figure S23. MALDI showing the observed molecular weight of trastuzumab reacted with *N*-methylmaleimide.

Mass: 147,981 Da (147,851 Da (trastuzumab) + 137 Da (*N*-methylmaleimide))

N-ethylmaleimide

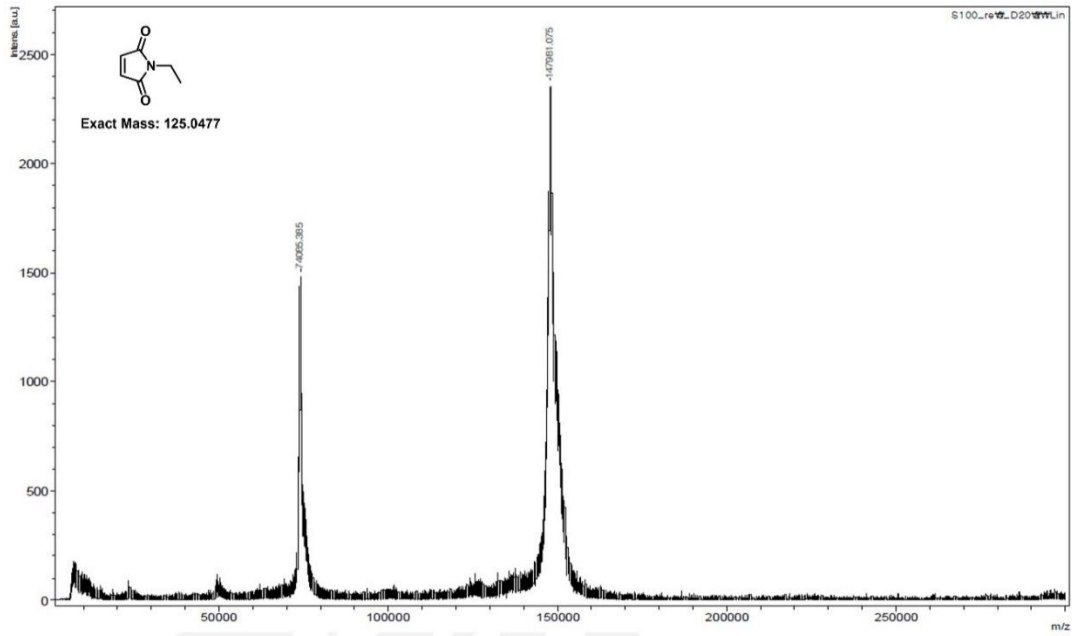


Figure S24. MALDI showing the observed molecular weight of trastuzumab reacted with *N*-ethylmaleimide.

Mass: 147,981 Da (147,851 Da (trastuzumab) + 130 Da (*N*-ethylmaleimide))

N-phenylmaleimide

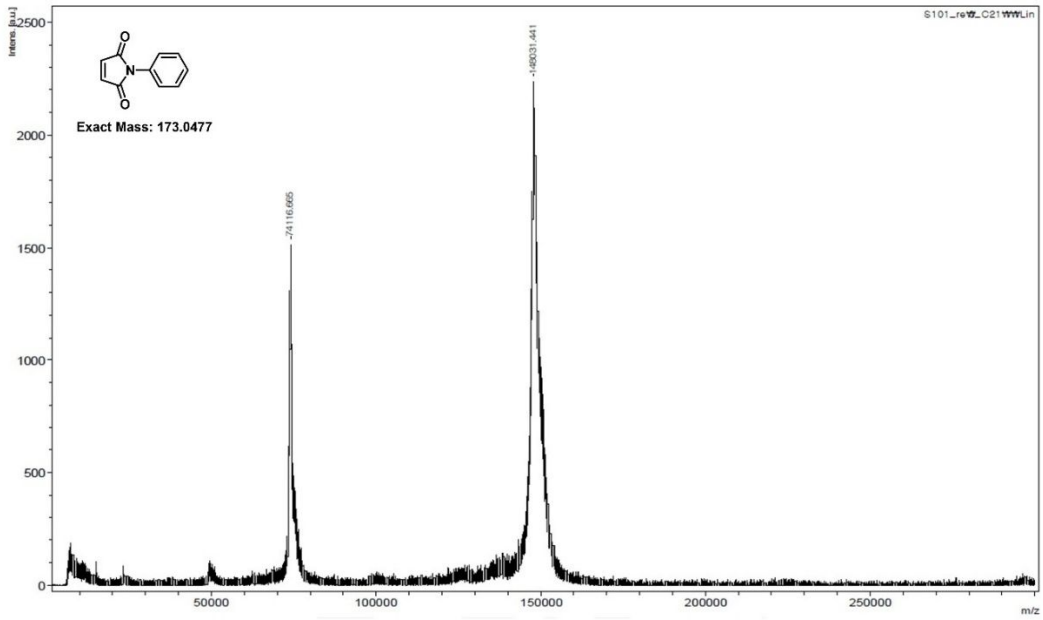


Figure S25. MALDI showing the observed molecular weight of trastuzumab reacted with *N*-phenylmaleimide.

Obtained mass: 148,031 Da (147,851 Da (trastuzumab) + 180 Da (*N*-phenylmaleimide))

N-benzylmaleimide

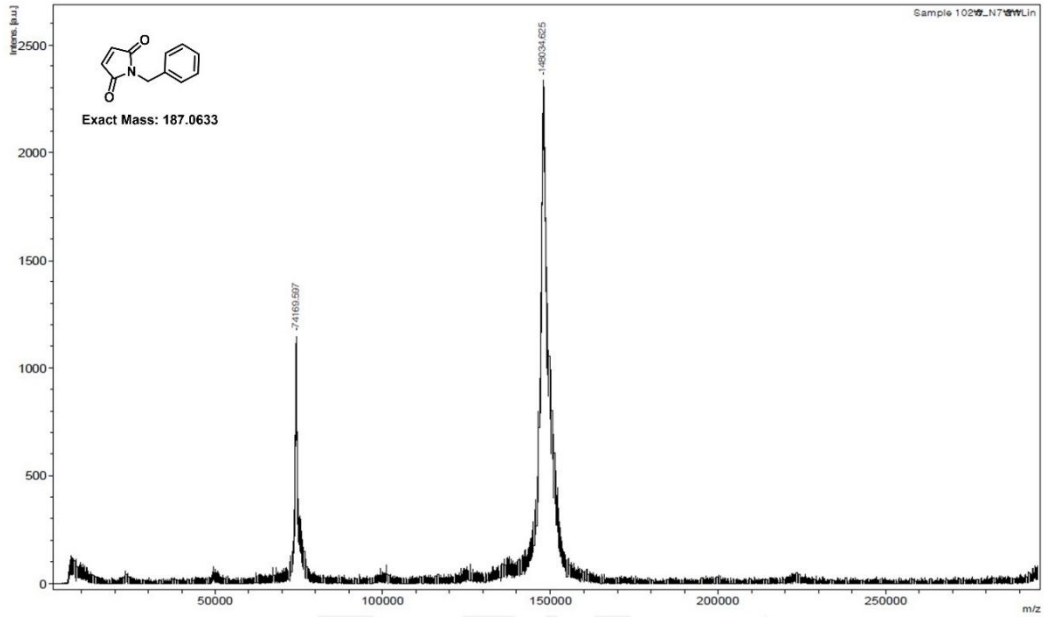


Figure S26. MALDI showing the observed molecular weight of trastuzumab reacted with *N*-benzylmaleimide.

Obtained mass: 148,034 Da (147,851 Da (trastuzumab) + 183 Da (*N*-benzylmaleimide))

N-cyclohexylmaleimide

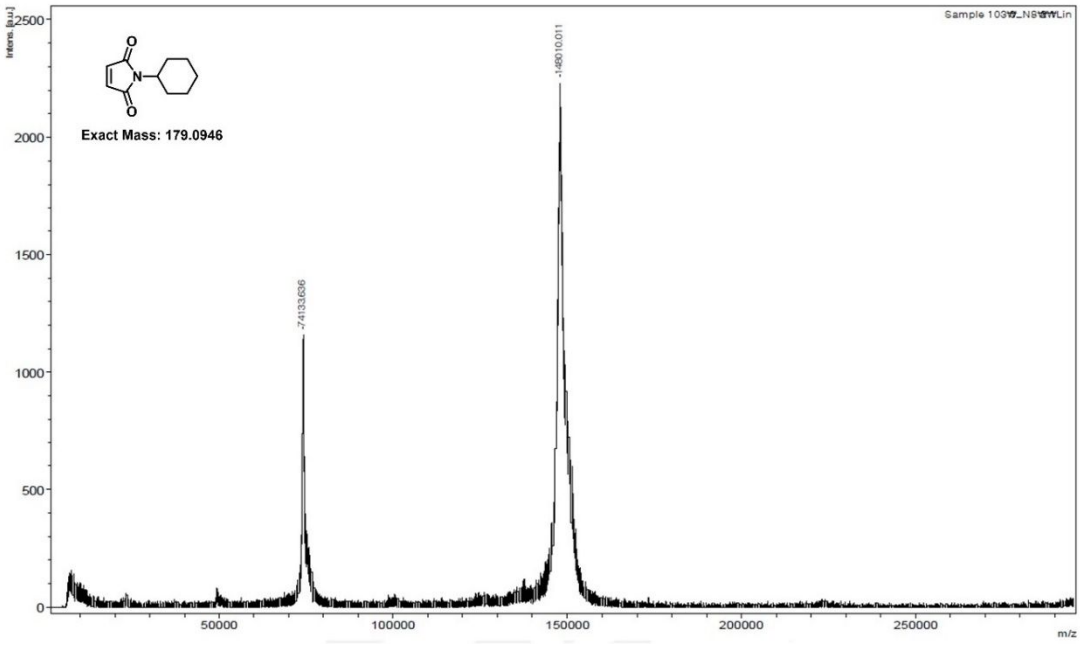


Figure S27. MALDI showing the observed molecular weight of trastuzumab reacted with *N*-cyclohexylmaleimide.

Obtained mass: 148,010 Da (147,851 Da (trastuzumab) + 159 Da (*N*-cyclohexylmaleimide))

3-maleimidopropionic acid

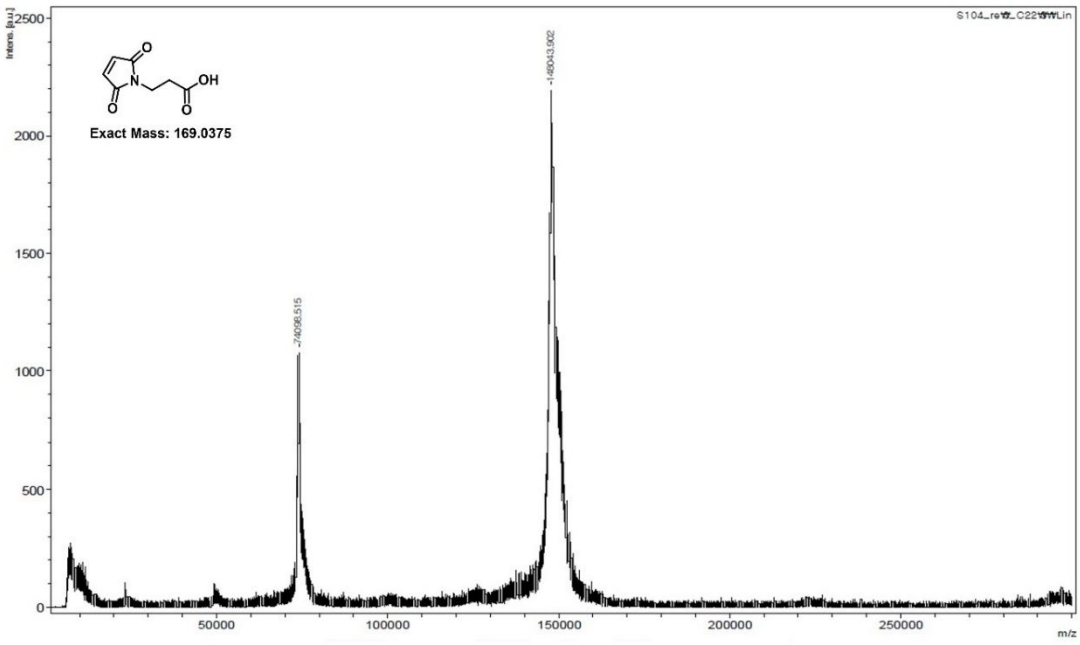


Figure S28. MALDI showing the observed molecular weight of trastuzumab reacted with 3-maleimidopropionic acid.

Obtained mass: 148,043 Da (147,851 Da (trastuzumab) + 192 Da (3-maleimidopropionic acid))

***N*-tert-butylmaleimide**

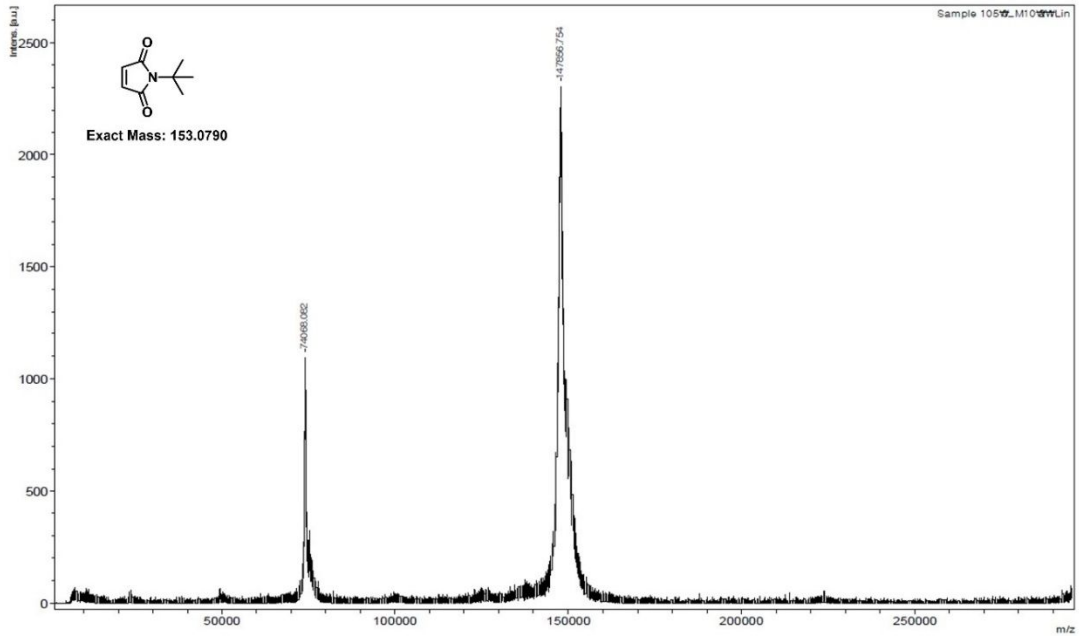


Figure S29. MALDI showing the observed molecular weight of trastuzumab reacted with *N*-tert-butylmaleimide.

Obtained mass: 147,856 Da (147,851 Da (trastuzumab) + 0 × 153.08 Da (*N*-tert-butylmaleimide))

11.6. HIC-HPLC chromatograms of cross-linking between trastuzumab and AbClicks in the absence of a scavenger

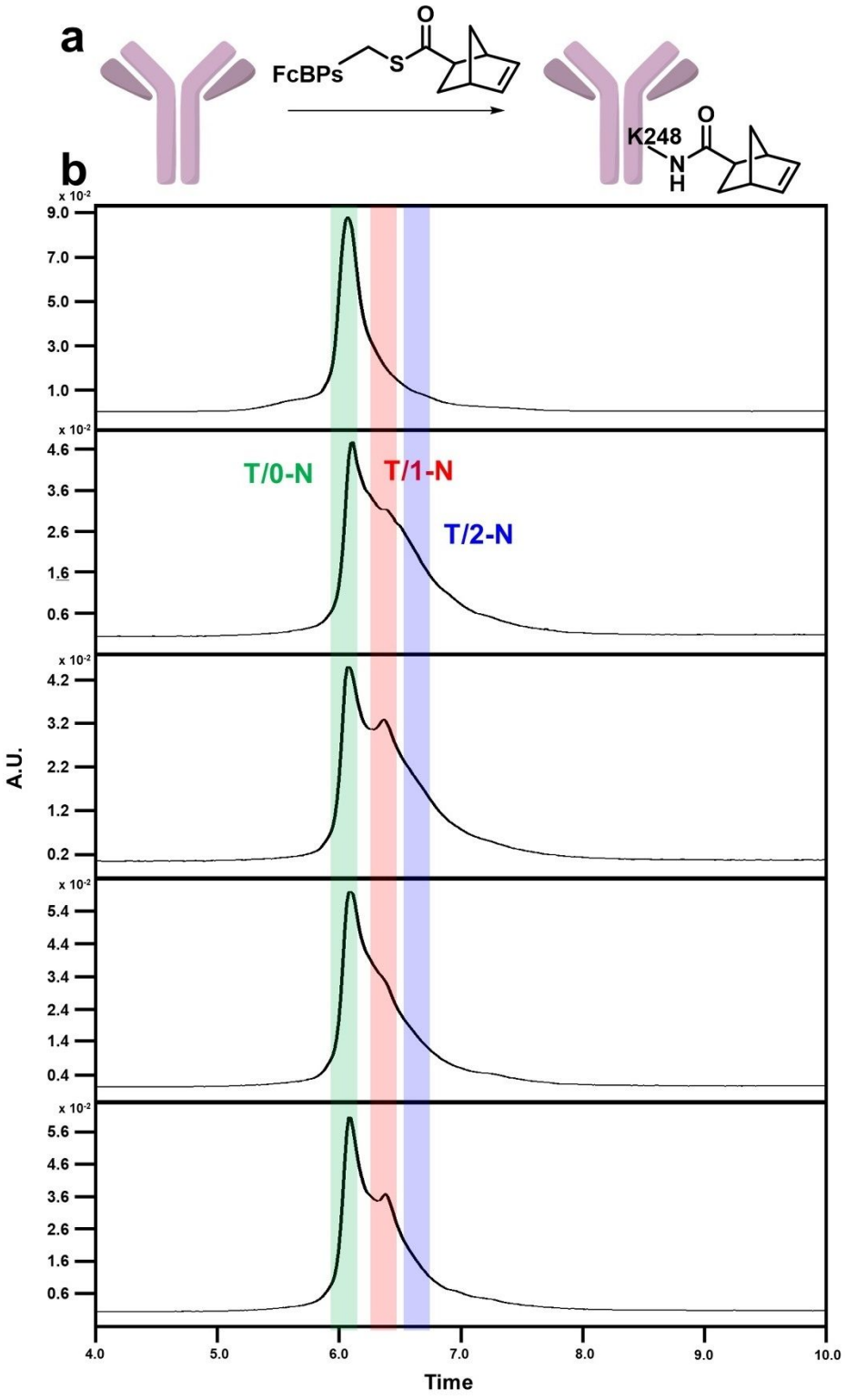


Figure S30. (a) Schematic procedure of cross-linking in the absence of a scavenger. **(b)** HIC-HPLC chromatograms for reaction of AbClicks with trastuzumab in the absence of a scavenger.

In the presence of scavenger

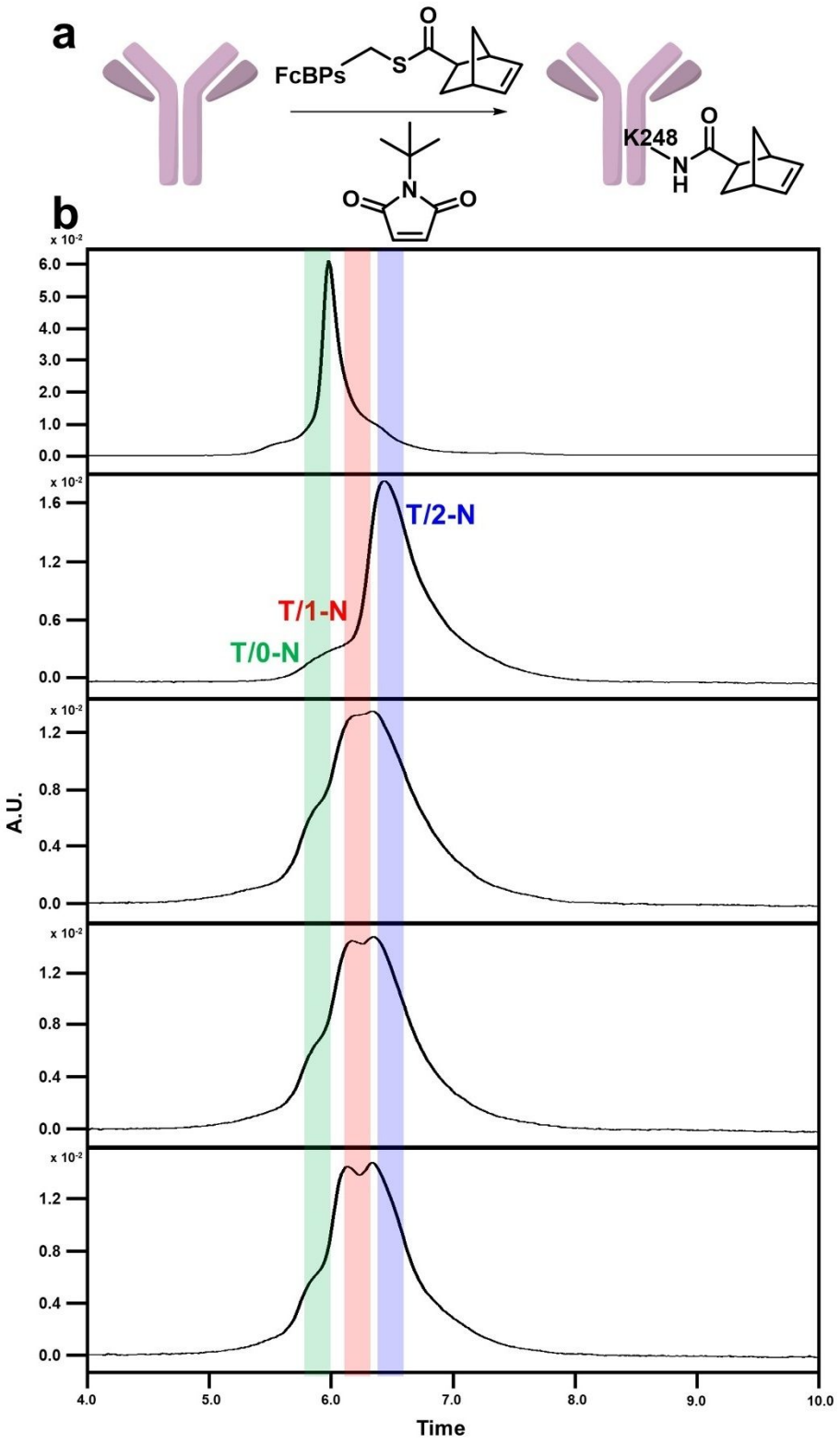


Figure S31. (a) Schematic procedure of cross-linking in the presence of a scavenger. **(b)** HIC-HPLC chromatograms of reaction of AbClicks with trastuzumab in the presence of a scavenger.

Time-dependent cross-linking reaction

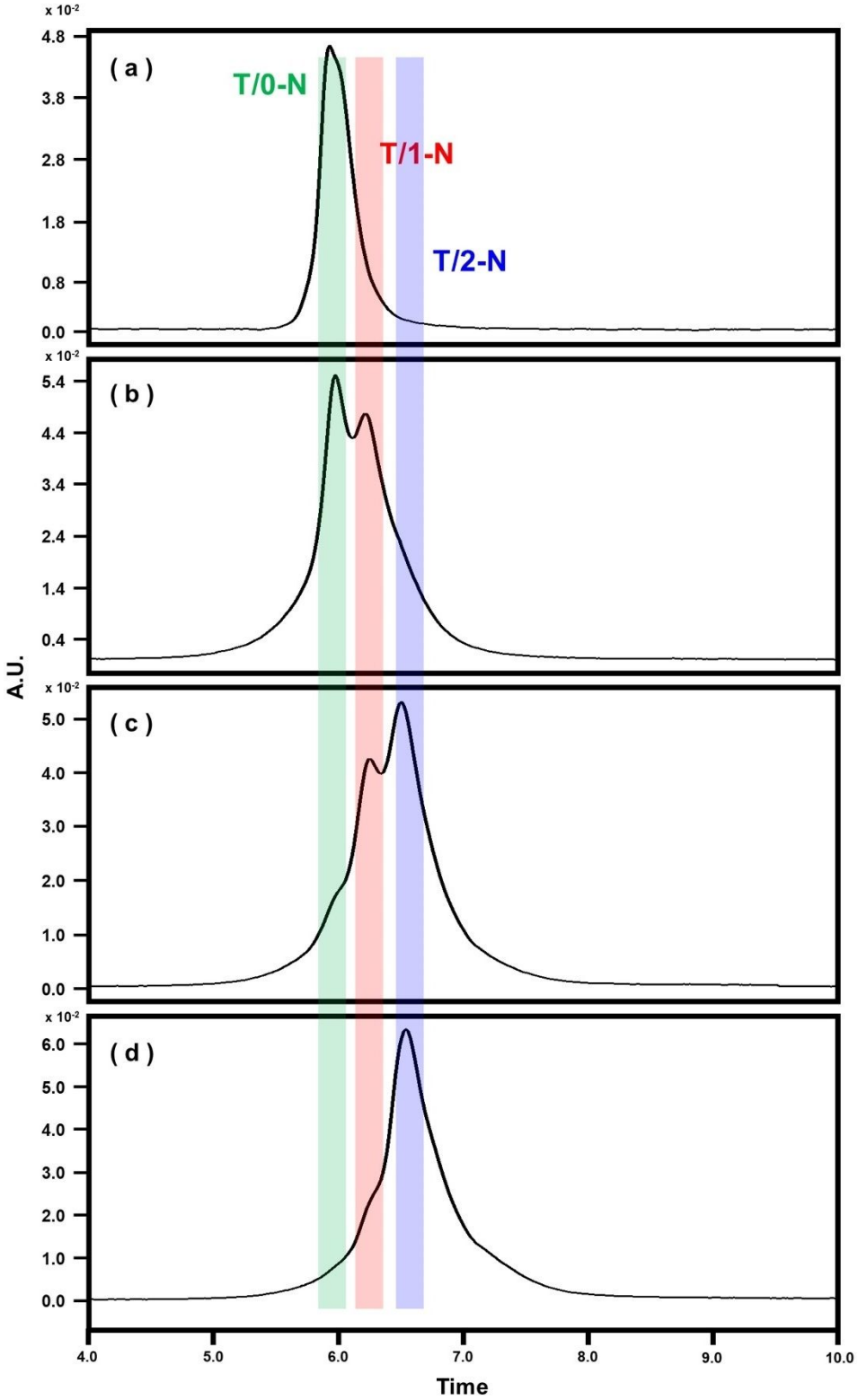


Figure S32. HIC-HPLC chromatograms of proximity-mediated covalent cross-linking with respect to time. (a) trastuzumab (5.9 m), (b) incubation 2 h, (c) incubation 4 h, (d) incubation 6 h.

11.7. Docking study

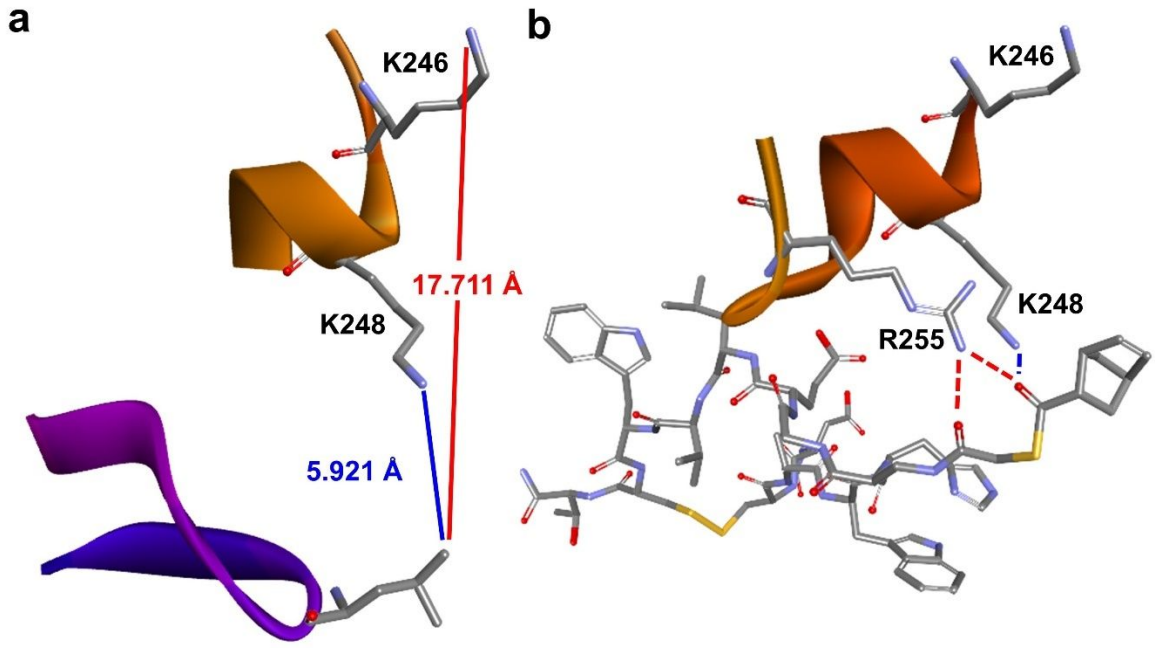


Figure S33. X-ray crystal structure analysis of Fc fragment of human IgG1 in complex with an engineered 13 residue peptide DCAWHLGELVWCT-NH_2 (PDB ID: 1DN2) and docking study of the Fc domain with AbClick-1. a. Distance of L6 on the Fc binding peptide from two proximal nucleophiles (K246 and K248). **b.** Hydrogen bonding between K248 and R255 on trastuzumab with AbClick-1.

11.8. MS/MS analysis

Trastuzumab

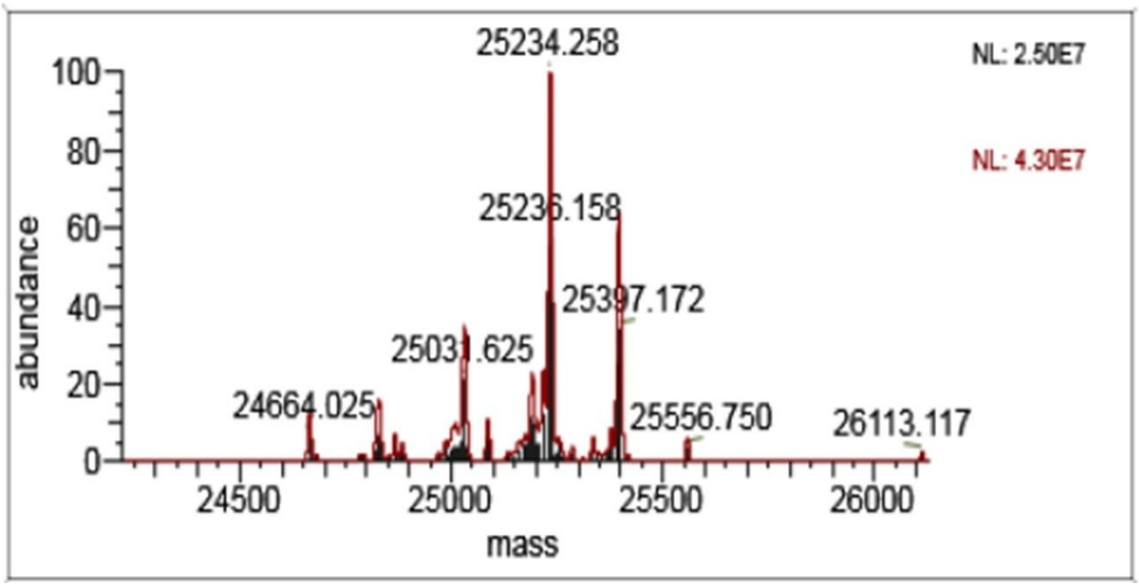


Figure S34. Mass data of Fc domain after IdeS digestion of trastuzumab.

Trastuzumab/2-norbornene conjugate (ADC precursor)

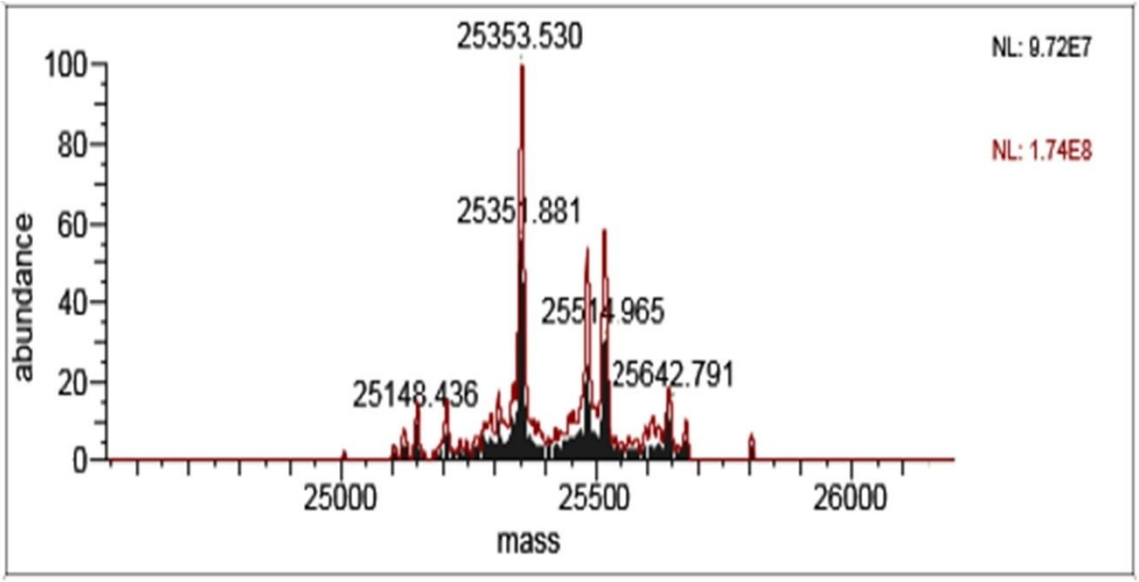


Figure S35. Mass data of Fc domain after IdeS digestion of ADC (T/2-N).

Potential binding site

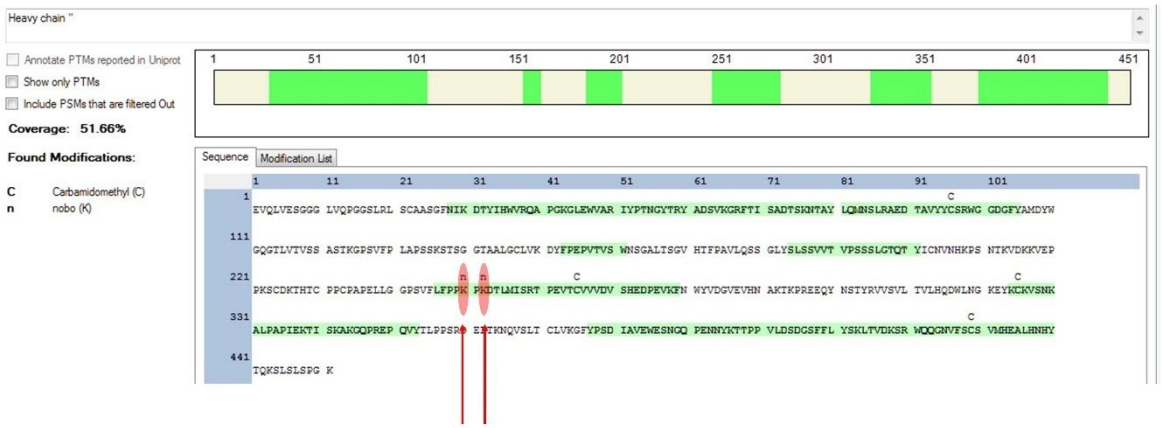


Figure S36. Potential binding site of norbornene toward Fc domain. Two Lys are shown as the closest nucleophile from the Fc domain.

Chemical structure and theoretical mass of peptide fragment in the case of K246 conjugation

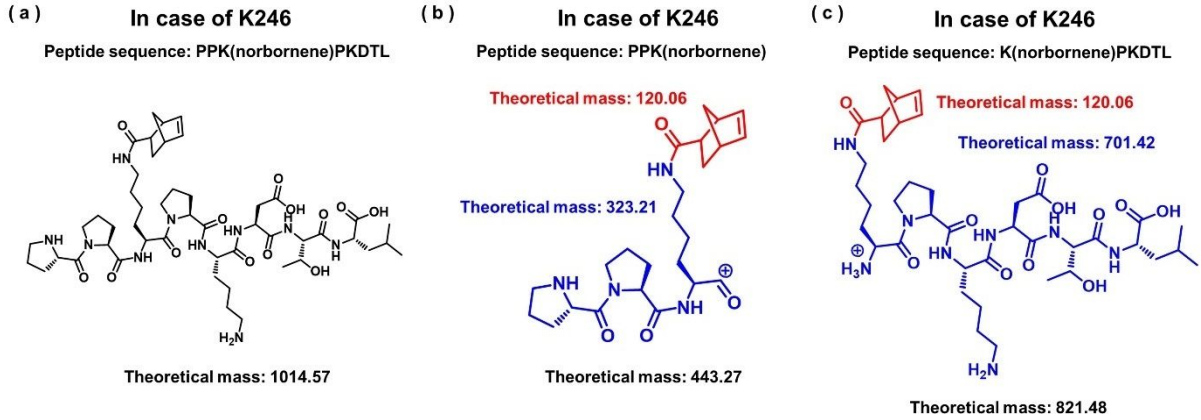


Figure S37. Chemical structure and theoretical mass of peptide fragment in the case of K246 conjugation and y_6^+ of K248 conjugate. (a) Peptide fragment in the case of K246 conjugation. **(b)** Chemical structure and theoretical mass of peptide fragment by b^+ analysis. **(c)** Chemical structure and theoretical mass of peptide fragment by y^+ analysis.

11.9. Size exclusion chromatography

Trastuzumab

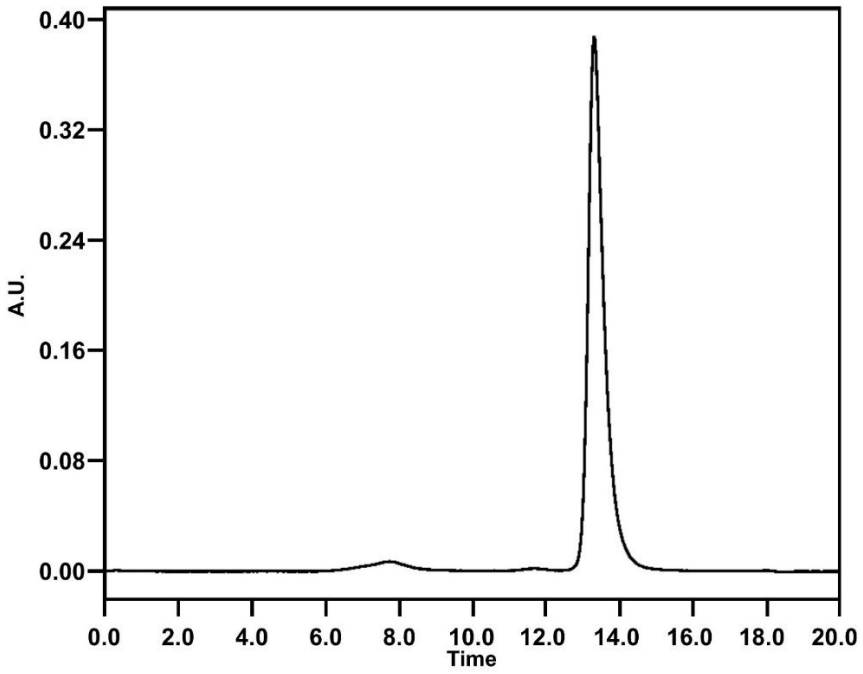


Figure S38. SEC-HPLC chromatogram of trastuzumab (RT = 13.3 m).

Trastuzumab/2-norbornene conjugate T2/N (ADC precursor)

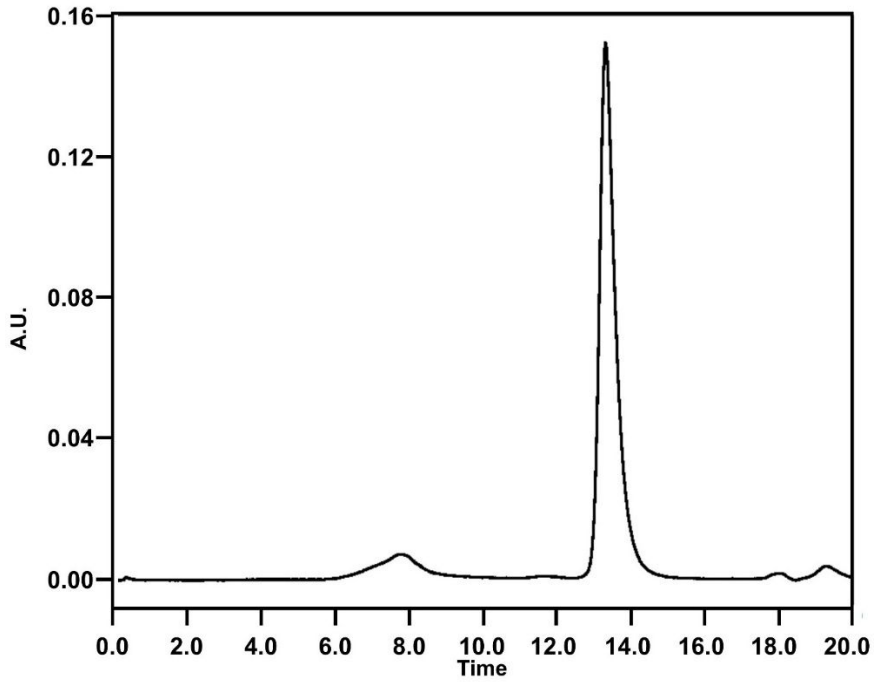


Figure S39. SEC-HPLC chromatogram of trastuzumab/2-norbornene conjugate (RT = 13.3 m).

T-nc-DM1

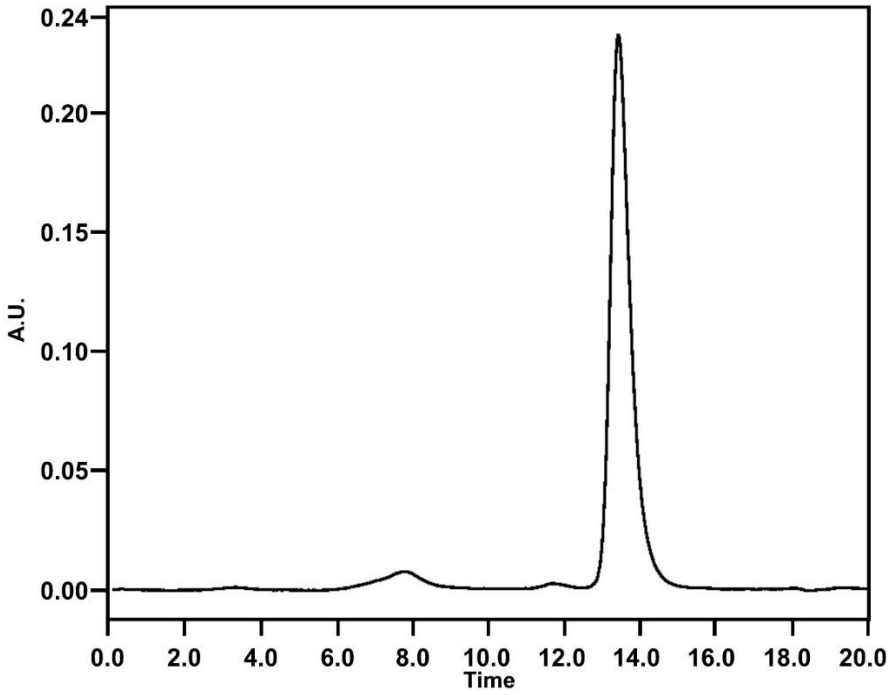


Figure S40. SEC-HPLC chromatogram of T-nc-DM1 (RT = 13.3 m).

T-VC-DM1

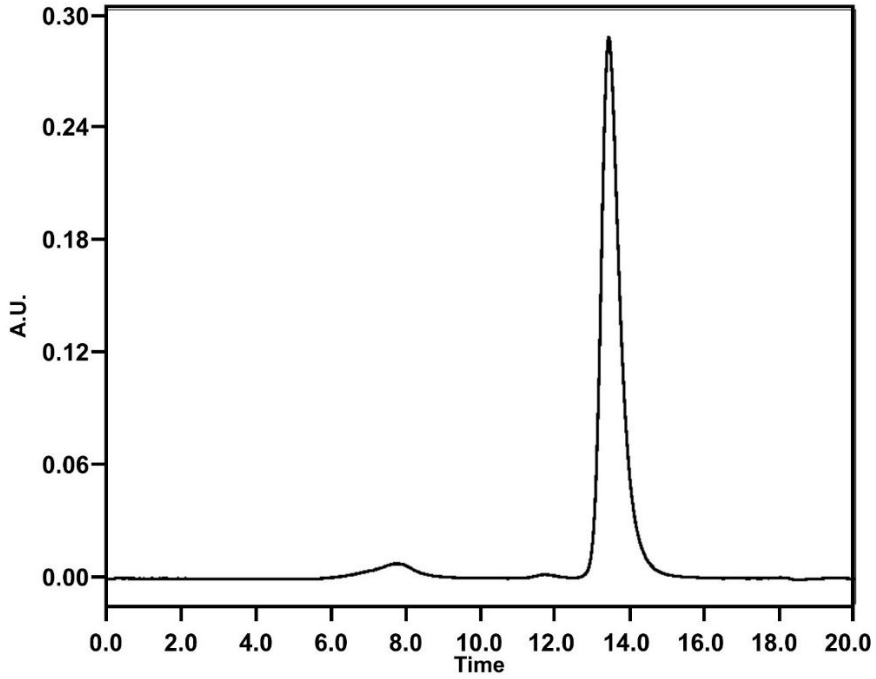


Figure S41. SEC-HPLC chromatogram of T-VC-DM1 (RT = 13.3 m).

11.10. Validation of FcRn binding using SPR assay

Trastuzumab

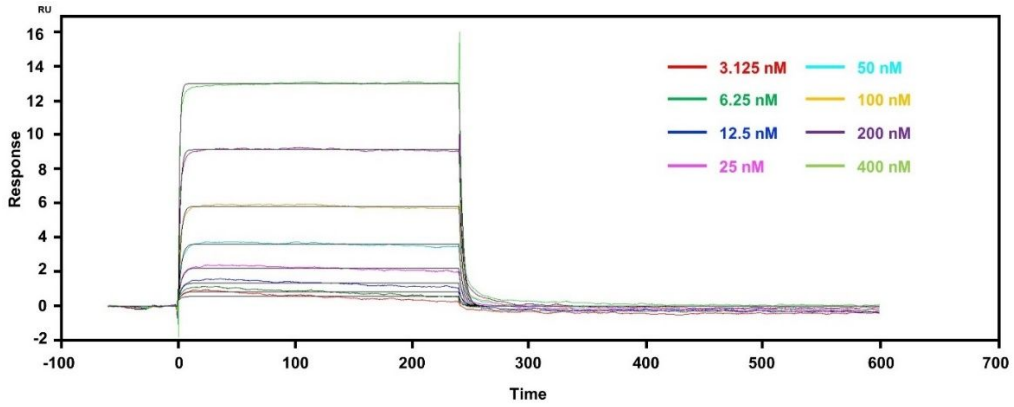


Figure S42. FcRn binding assay of trastuzumab.

Trastuzumab/2-norbornene conjugate (T/2-N, ADC precursor)

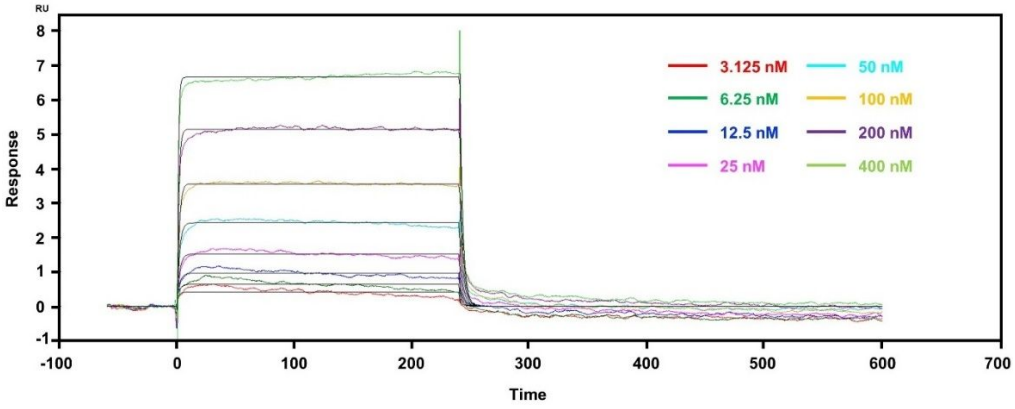


Figure S43. FcRn binding assay of T/2-N.

T-nc-DM1

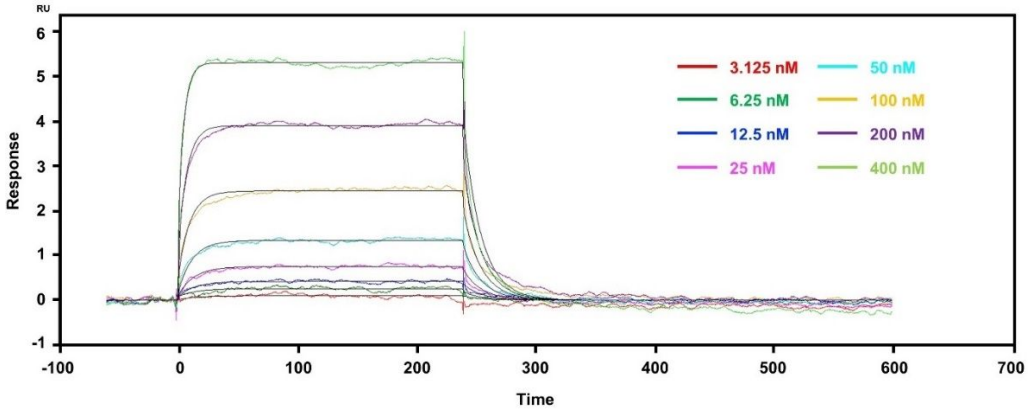


Figure S44. FcRn binding assay of T-nc-DM1.

T-VC-DM1

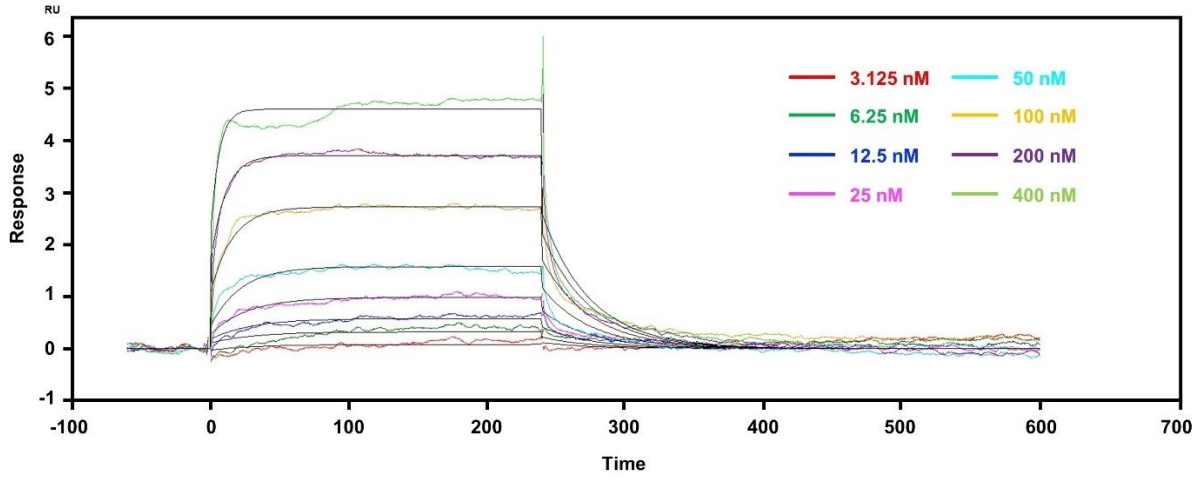


Figure S45. FcRn binding assay of T-VC-DM1.

Trastuzumab-FcBP conjugate produced by photo-conjugation

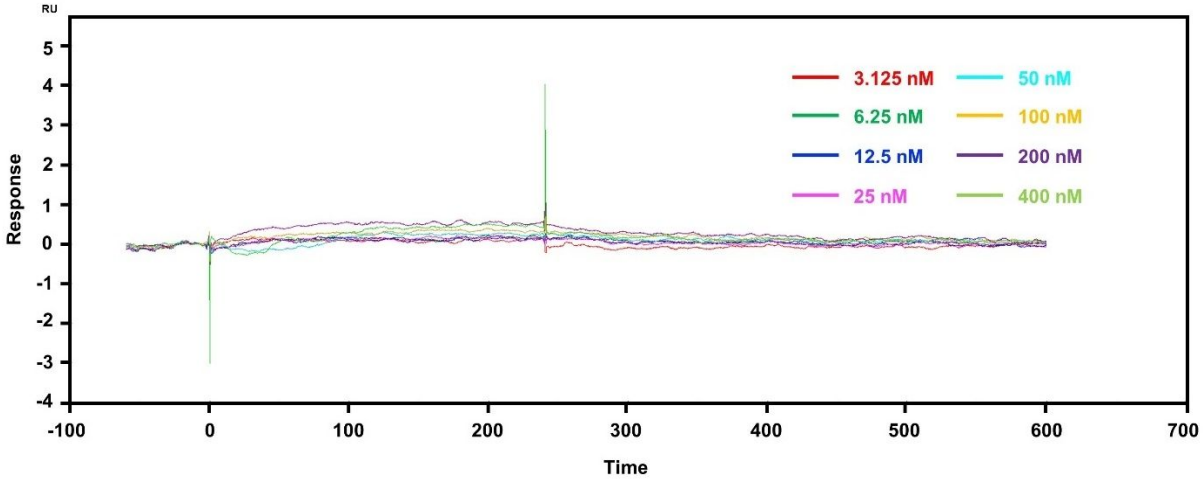


Figure S46. FcRn binding assay of trastuzumab-FcBP conjugate by photo-conjugation.[1]

11.11. in vitro *assay: Antigen binding assay*

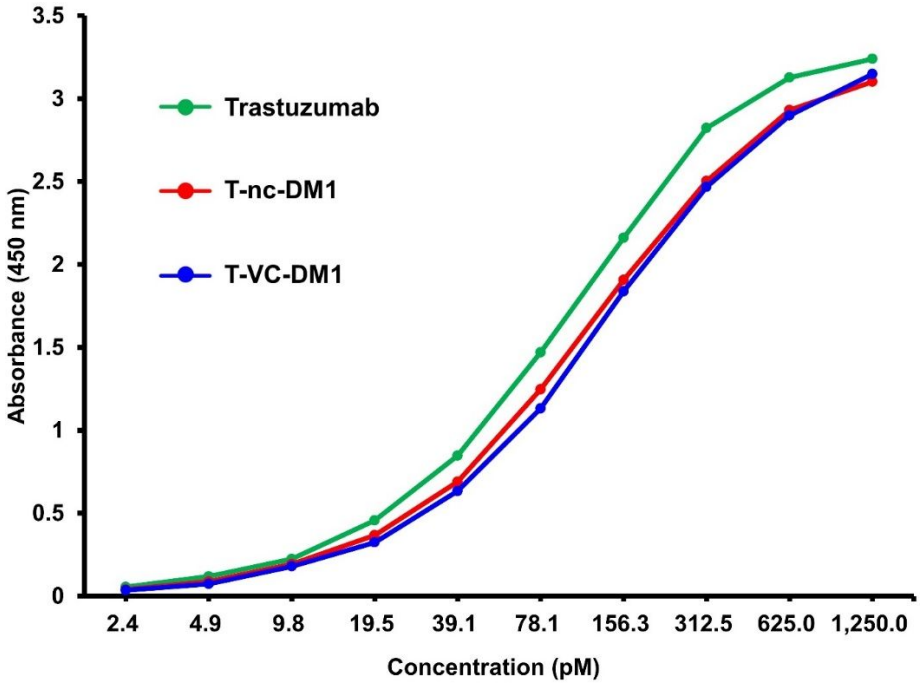


Figure S47. HER2 antigen binding assay. The binding activities of the antibodies were analyzed by enzyme-linked immunosorbent assay using recombinant HER2 proteins. ADCs (T-VC-DM1 and T-nc-DM1) were compared to the control antibody (trastuzumab), revealing no change in binding affinity. Data represent the mean value ± SD (n = 3).

11.12. in vivo *assay: Body weight profiles*

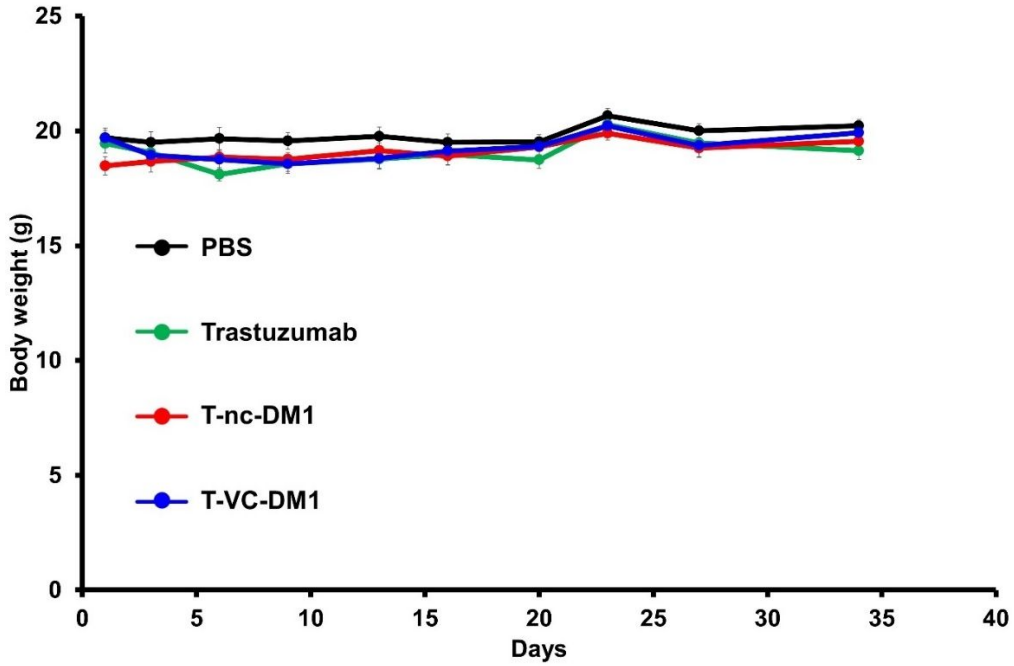
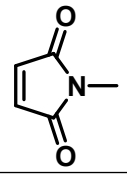
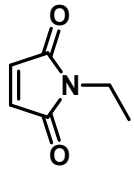
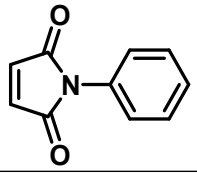
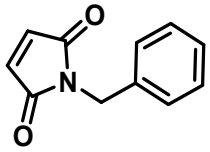
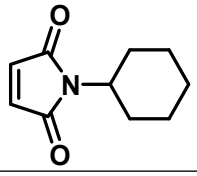
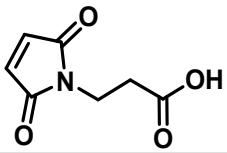
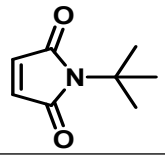


Figure S48. Body weight gain/loss profiles of mice following treatments. Data represents the mean value ± SE (n = 4 or 5 mice/group).

12. Supplementary Tables

12.1. Screening of an ideal scavenger

Table S1. Reaction of maleimide derivatives with trastuzumab to investigate the competitive reactivity of plausible scavengers with lysine residues of native antibody.

Scavenger Candidates	Molecular weight	Observed intact mass	Increased mass	Number of reacted Lys
	111 Da	147,988 Da	+ 137 Da	1
	125 Da	147,981 Da	+ 130 Da	1
	173 Da	148,031 Da	+ 180 Da	1
	187 Da	148,034 Da	+ 183 Da	1
	179 Da	148,010 Da	+ 159 Da	1
	169 Da	148,043 Da	+ 192 Da	1
	153 Da	147,856 Da	+ 5 Da	0

12.2. Cross-linking in the absence of a scavenger

Table S2. Cross-linking ratio of norbornene in the absence of a scavenger

	T/0-N (5.9 min)	T/-1N (6.2 min)	T/2-N (6.5 min)
AbClick-1 (L6Dap)	46%	33%	21%
AbClick-2 (L6Dab)	41%	39%	20%
AbClick-3 (L6Orn)	53%	31%	15%
AbClick-4 (L6Lys)	53%	33%	14%

12.3. Cross-linking in the presence of a scavenger

Table S3. Cross-linking ratio of norbornene in the presence of a scavenger

	T/0-N (5.9 min)	T/-1N (6.2 min)	T/2-N (6.5 min)
AbClick-1 (L6Dap)	1%	4%	95%
AbClick-2 (L6Dab)	14%	29%	57%
AbClick-3 (L6Orn)	13%	31%	56%
AbClick-4 (L6Lys)	12%	31%	57%

12.4. Binding constants of the antibodies through SPR assay

Table S4. Binding constants of Trastuzumab at pH 6.0.

Concentration (nM)	K_{on} (1/Ms)	K_{off} (1/s)	K_D (M)
3.125–400	1.434×10^{6}	3.398×10^{-1}	2.369×10^{-7}

Table S5. Binding constants of T/2-N.

Concentration (nM)	K_{on} (1/Ms)	K_{off} (1/s)	K_D (M)
3.125–400	2.057×10^{6}	4.057×10^{-1}	1.973×10^{-7}

Table S6. Binding constants of T-nc-DM1.

Concentration (nM)	K_{on} (1/Ms)	K_{off} (1/s)	K_D (M)
3.125–400	1.434×10^{6}	3.398×10^{-1}	2.369×10^{-7}

Table S7. Binding constants of T-VC-DM1.

Concentration (nM)	K_{on} (1/Ms)	K_{off} (1/s)	K_D (M)
3.125–400	3.475×10^{5}	2.887×10^{-2}	8.309×10^{-8}

Table S8. Binding constants of Trastuzumab-FcBP conjugate.

Concentration (nM)	K_{on} (1/Ms)	K_{off} (1/s)	K_D (M)
3.125–400	N.D.	N.D.	N.D.

12.5. Antigen binding assay

Table S9. Dissociation constants of trastuzumab and our ADCs (T-VC-DM1 and T-nc-DM1). Data represent the mean value ± SD (n = 3).

Antibodies	Dissociation constant (K_D, pM)
Trastuzumab	105.7
T-nc-DM1	122.9
T-VC-DM1	139.0

12.6. in vitro *cytotoxicity*

Table S10. IC_{50} values of the antibodies on HER2/v positive and negative cells.

	IC_{50} (nM)		
Antibodies	**SKBR3**	**NCI-N87**	**MDA-MB-231**
Trastuzumab	-	-	-
T-nc-DM1	0.046	7.3	-
T-VC-DM1	0.027	1.0	-

References

1. Lee T, Kim JH, Kwon SJ, Park SH, Kim J, Kang HJ*, et al.* Photoconjugation of an Fc-Specific Peptide Enables Efficient DAR 2 Antibody–Drug Conjugate Formation. *Org. Lett.* 2020, **22,** 21, 8419-8423.

2. DeLano WL, Ultsch MH, de Vos AM, Wells JA. Convergent solutions to binding at a protein-protein interface. *Science* 2000, **287**(5456)**:** 1279-1283.

3. Ackerman SE, Pearson CI, Gregorio JD, Gonzalez JC, Kenkel JA, Hartmann FJ*, et al.* Immune-stimulating antibody conjugates elicit robust myeloid activation and durable antitumor immunity. *Nat. Cancer* 2021, **2**(1)**:** 18-33.