Electronic Supplementary Material (ESI) for Organic & Biomolecular Chemistry.

General dual functionalisation of biomacromolecules via a cysteine bridging strategy

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Supporting Information

1. Chemical Synthesis

All solvents and reagents were used as received unless otherwise stated.

LCMS was carried out using a Waters ACQUITY H-Class UPLC with an ESCi Multi-Mode Ionisation Waters SQ Detector 2 spectrometer using MassLynx 4.1 software; EI refers to the electrospray ionisation technique; LC system: solvent A: 2 mM NH_4OAc in H_2O/MeCN (95:5); solvent B: MeCN; solvent C: 2% formic acid; column: ACQUITY UPLC® CSH C18 (2.1 mm × 50 mm, 1.7 µm, 130 Å) at 40 °C; gradient: 5 – 95 % B with constant 5% C over 1 min at flow rate of 0.6 mL/min; detector: PDA eλ Detector 220 – 800 nm, interval 1.2 nm.

Analytical HPLC: Chromatographs were obtained on an Agilent 1260 Infinity® using a reversed-phase Supelcosil ABZ+PLUS column (150 mm x 4.6 mm, 3 µm) eluting with a linear gradient system (solvent A: 0.05% (v/v) TFA in water, solvent B: 0.05% (v/v) TFA in MeCN) over 20 min, unless otherwise stated, at a flow rate of 1 mL/min. HPLC was monitored by UV absorbance at 220 and 254 nm.

Preparative HPLC: Preparative HPLC was carried out on an Agilent 1260 Infinity® using a reversed-phase Supelcosil ABZ+PLUS column (250 mm x 21.2 mm, 5 µm) eluting with a linear gradient system (solvent A: 0.1% (v/v) TFA in water, solvent B: 0.05% (v/v) TFA in MeCN) over 15 min at a flow rate of 20 mL/min. HPLC was monitored by UV absorbance at 220 and 254 nm.

Proton and carbon nuclear magnetic resonance (NMR) were recorded using an internal deuterium lock on Bruker DPX-400 (400 MHz, 101 MHz), Bruker Avance 400 QNP (400 MHz, 101 MHz) and Bruker Avance 500 Cryo Ultrashield (500 MHz, 126 MHz). In proton NMR, chemical shifts (δ_H) are reported in parts per million (ppm), to the nearest 0.01 ppm and are referenced to the residual non-deuterated solvent peak ($CDCl_3$: 7.26, DMSO-d_6: 2.50, CD_3OD: 3.31, D_2O: 4.79). Coupling constants (J) are reported in Hertz (Hz) to the nearest 0.1 Hz. In carbon NMR, chemical shifts (δ_C) are quoted in ppm, to the nearest 0.1 ppm, and are referenced to the residual non-deuterated solvent peak ($CDCl_3$: 77.16, DMSO-d_6, 39.52, CD_3OD: 49.00). Multiplicity is reported as follows: s = singlet; d = doublet; t = triplet; q = quartet; qn = quintet; sx = sextet; sep = septet; m = multiplet; or as a combination of these, e.g. dd, dt etc.

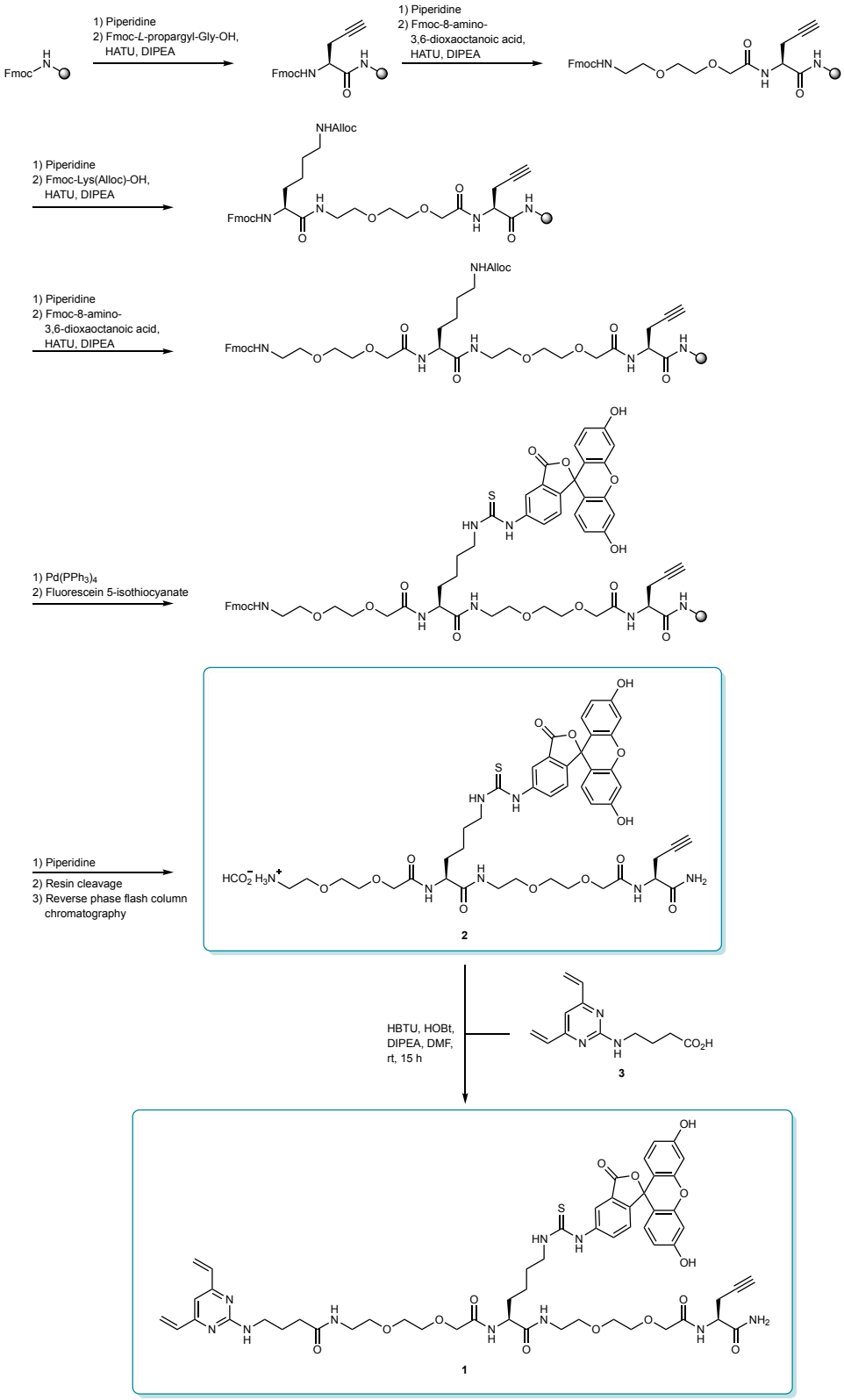
Protein LCMS was performed on a Xevo G2-S TOF mass spectrometer coupled to an Acquity UPLC system using an Acquity UPLC BEH300 C4 column (1.7 µm, 2.1 × 50 mm). H_2O with 0.1% formic acid (solvent A) and 95% MeCN and 5% water with 0.1% formic acid (solvent B), were used as the mobile phase at a flow rate of 0.2 mL/min. The gradient was programmed as follows: 95% A for 0.93 min, then a gradient to 100% B over 4.28 min, then 100% B for 1.04 minutes, then a gradient to 95% A over 1.04 min. The electrospray source was operated with a capillary voltage of 2.0 kV and a cone voltage of 40 or 150 V. Nitrogen was used as the desolvation gas at a total flow of 850 L/h. Total mass spectra were reconstructed from the ion series using the MaxEnt algorithm preinstalled on MassLynx software (v4.1 from Waters) according to the manufacturer's instructions. Trastuzumab samples were deglycosylated with PNGase F (New England Biolabs) prior to LC-MS analysis.

Reverse-phase flash column chromatography was carried out using a Combiflash Rf200 automated chromatography system with Redisep® reverse-phase C18-silica flash columns (20-40 µm).

Non-reducing Tris-Glycine SDS-PAGE with 12% acrylamide with 4% stacking gel was performed as standard. Broad range molecular weight marker (10-200 kDa, New England BioLabs) was run in all gels. Samples (10 µL of 0.4 mg/mL) were prepared with reducing loading dye (10 µL, containing β-mercaptoethanol) and heated to 70 °C for 2-5 min. Gels were run at constant voltage (160 V) for 60 min in 1 × Laemmli running buffer. All gels were stained with Coomassie brilliant blue dye and imaged on a Syngene gel imaging system.

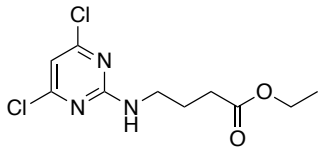
UV-visible (UV-vis) spectrums were obtained using a NanoDrop™ One spectrophotometer (ThermoFisher). Raw data was plotted using GraphPad Prism software (version 7).

Solid-phase synthesis of ***dfDVP 1***



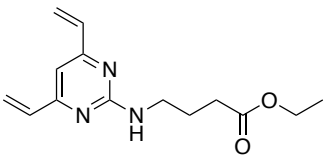
Scheme S1: Synthetic strategy used for the on-resin synthesis of amine **2**, and subsequent amide coupling to generate dfDVP **1**.

Ethyl 4-((4,6-dichloropyrimidin-2-yl)amino)butanoate (S1)



A solution of 2,4,6-trichloropyrimidine (1.00 g, 5.45 mmol), ethyl 4-aminobutyrate hydrochloride (1.10 g, 6.54 mmol) and triethylamine (1.90 mL, 13.6 mmol) in acetone (20 mL) was stirred at 0 °C for 2 h. Upon completion, the reaction was concentrated *in vacuo* and the residue redissolved in H_2O (20 mL) and CH_2Cl_2 (20 mL). The layers were separated and the aqueous phase was extracted with further CH_2Cl_2 (3 × 20 mL). The combined organic fractions were dried ($MgSO_4$), concentrated *in vacuo* and the crude residue purified by FCC (5-30% EtOAc/PE) to yield ester **S1** (420 mg, 1.51 mmol, 28%) as a white solid.
R_f 0.33 (SiO_2; 20% EtOAc/PE); **ν_{max}** (neat/cm^{-1}) 2981, 1735, 1569, 1513, 1450; **^{1}H NMR** (400 MHz, CD_3OD) δ 6.65 (s, 1H), 4.12 (q, 2H, *J* = 7.2 Hz), 3.40 (t, 2H, *J* = 7.0 Hz), 2.38 (t, 2H, *J* = 7.0 Hz), 1.89 (t, 2H, *J* = 7.0 Hz), 1.24 (t, 3H, *J* = 7.2 Hz); **^{13}C NMR** (101 MHz, CD_3OD) δ 175.1, 163.4, 108.8, 61.6, 41.6, 32.3, 25.5, 14.5; **HRMS** (ESI) *m/z* found $[M+H]^+$ 278.0448, $C_{10}H_{14}{}^{35}Cl_2N_3O_2^+$ required 278.0458.

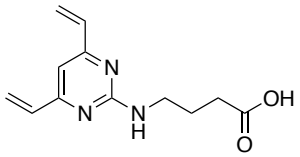
Ethyl 4-((4,6-divinylpyrimidin-2-yl)amino)butanoate (S2)



A solution of dichloropyrimidine **S2** (200 mg, 0.719 mmol), potassium vinyltrifluoroborate (482 mg, 3.60 mmol), $Pd(dppf)Cl_2 \cdot CH_2Cl_2$ (88.1 mg, 0.108 mmol) and potassium carbonate (596 mg, 4.31 mmol) in THF/H_2O (10:1, 5.5 mL) was heated to 70 °C for 15 h. Upon completion, the reaction mixture was filtered through Celite® and the solvent removed *in vacuo*. The resulting residue was purified by FCC (20% EtOAc/PE) to yield divinylpyrimidine **S2** (181 mg, 0.693 mmol, 96%) as a pale yellow oil.

R_f 0.18 (SiO_2; 20% EtOAc/PE); **ν_{max}** (neat/cm^{-1}) 2971, 1734, 1636, 1543, 1418, 1371; **^{1}H NMR** (400 MHz, CD_3OD) δ 6.69 (s, 1H), 6.61 (dd, 2H, *J* = 17.4, 10.7 Hz), 6.37 (d, 2H, *J* = 17.4 Hz), 5.57 (dd, 2H, *J* = 10.7, 1.5 Hz), 4.10 (q, 2H, *J* = 7.2 Hz), 3.48 (t, 2H, *J* = 7.1 Hz), 2.40 (t, 2H, *J* = 7.1 Hz), 1.92 (app qn, 2H, *J* = 7.1 Hz), 1.22 (t, 3H, *J* = 7.2 Hz); **^{13}C NMR** (101 MHz, CD_3OD) δ 175.4, 165.3, 164.1, 137.1, 122.0, 105.8, 61.5, 41.5, 32.6, 26.1, 14.5; **HRMS** (ESI) *m/z* found $[M+H]^+$ 262.1541, $C_{14}H_{20}N_3O_2^+$ required 262.1550.

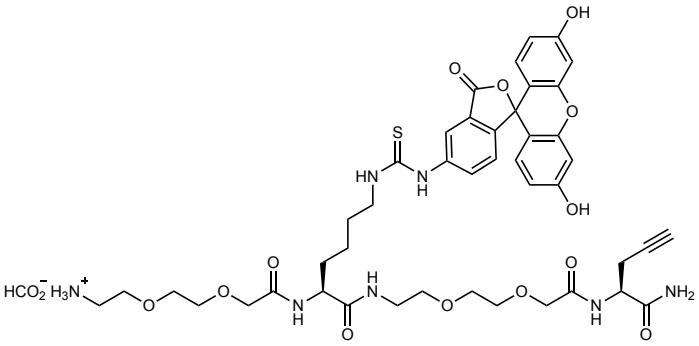
4-((4,6-Divinylpyrimidin-2-yl)amino)butanoic acid (3)



A solution of ester **S2** (160 mg, 0.612 mmol) and $LiOH \cdot H_2O$ (56.6 mg, 1.35 mmol) in THF/H_2O (6 mL, 1:1) was stirred at rt for 20 h. Upon completion, the reaction was diluted with H_2O (10 mL) and washed with Et_2O (15 mL) and concentrated *in vacuo*. The residue was suspended in MeOH, filtered and the filtrate concentrated *in vacuo* to yield DVP **3** (138 mg, 0.592 mmol, 97%) as a pale yellow solid.

ν_{max} (neat/cm^{-1}) 3311, 2935, 1711, 1636, 1542, 1418, 1348, 1320; **^{1}H NMR** (400 MHz, D_2O) δ 6.84 (s, 1H), 6.63 (dd, 2H, *J* = 17.5, 10.9 Hz), 6.31 (d, 2H, *J* = 17.5 Hz), 5.71 (d, 2H, *J* = 10.9 Hz), 3.40 (t, 2H, *J* = 7.3 Hz), 2.29 (t, 2H, *J* = 7.3 Hz), 1.88 (app qn, 2H, *J* = 7.3 Hz); **^{13}C NMR** (101 MHz, D_2O) δ 183.1, 164.7, 162.3, 134.8, 123.2, 104.7, 40.9, 35.0, 25.8; **HRMS** (ESI) *m/z* found $[M+H]^+$ 234.1225, $C_{12}H_{16}N_3O_2^+$ required 234.1237.

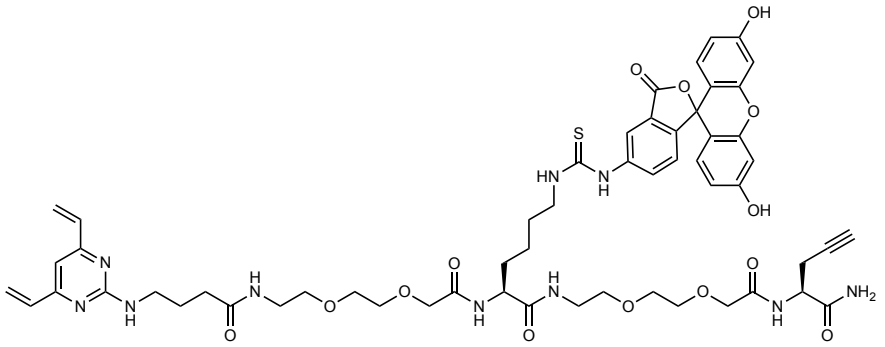
(*S*)-*N*-(2-(2-(2-(((*S*)-1-Amino-1-oxopent-4-yn-2-yl)amino)-2-oxoethoxy)ethoxy)ethyl)-2-(2-(2-(2-aminoethoxy)ethoxy)acetamido)-6-(3-(3',6'-dihydroxy-3-oxo-3*H*-spiro[isobenzofuran-1,9'-xanthen]-5-yl)thioureido)hexanamide (2)



On-resin synthesis of **2** was performed on Merck LL Rink amide resin (0.51 mmol/g). Couplings were carried out by adding HATU (4 equiv) to a solution of the Fmoc-protected amino acid (4 equiv) in DMF (~0.4 M). After 10 seconds, the mixture was added to $^{i}Pr_2EtN$ (8 equiv). This pre-activated mixture was then added to the resin in DMF and this mixture was agitated by a flow of N_2 for 20 minutes. Fmoc deprotection was carried out with 20% piperidine in DMF (2 × 10 minutes). On-resin attachment of FITC via Lys was achieved by orthogonal deprotection of the Lys(Alloc) with 3 equiv of $Pd(PPh_3)_4$ in a solution of $CHCl_3$/AcOH/*N*-methylmorpholine (37:2:1 (v/v)), followed by conjugation with FITC (2 equiv) in the presence of $^{i}Pr_2EtN$ (4 equiv) overnight. Cleavage from the resin was achieved with TFA/TIPS/CH_2Cl_2/H_2O (94:2:2:2 (v/v)) at rt for 3 hours. Following filtration and concentration of this mixture, the resulting residue was purified by reverse phase flash column chromatography (10 – 40% solvent B in solvent A. Solvent A: 2% aqueous formic acid. Solvent B: MeCN) and lyophilised to give **2** as an orange solid.

ν_{max} (neat/cm^{-1}) 3054, 1734, 1645, 1532; **^{1}H NMR** (CD_3OD, 500 MHz) δ 8.41 (1H, s), 8.12 (1H, d, *J* = 2.1 Hz), 7.71 (1H, d, *J* = 8.3 Hz), 7.17 (1H, d, *J* = 8.2 Hz), 6.74 (2H, d, *J* = 8.7 Hz), 6.68 (2H, d, *J* = 2.4 Hz), 6.56 (2H, dd, *J* = 8.8, 2.4 Hz), 4.62 (1H, app t, *J* = 6.3 Hz), 4.56 (1H, dd, *J* = 7.9, 5.6 Hz), 4.12 – 3.97 (4H, m), 3.77 – 3.54 (14H, m), 3.54 – 3.46 (1H, m), 3.41 – 3.32 (1H, m), 3.24 – 3.12 (2H, m), 2.76 (1H, ddd, *J* = 16.9, 5.9, 2.6 Hz), 2.68 (1H, ddd, *J* = 16.9, 6.7, 2.7 Hz), 2.42 (1H, app t, *J* = 2.6 Hz), 1.93 – 1.82 (1H, m), 1.79 – 1.66 (3H, m), 1.48 – 1.44 (2H, m); **^{13}C NMR** (CD_3OD, 126 MHz) δ 182.7, 174.3, 173.9, 172.6, 172.2, 171.4, 168.5, 163.3, 154.7, 146.4, 142.3, 134.4, 131.1, 130.6, 126.4, 120.6, 114.5, 112.0, 103.6, 80.1, 72.6, 72.2, 72.1, 71.3, 71.3, 71.1, 70.9, 70.7, 68.1, 53.8, 52.3, 45.3, 40.7, 40.4, 33.9, 29.7, 24.0, 23.3; **HRMS** (ESI) $[M+H]^+$ required for $C_{44}H_{54}N_7O_{13}{}^{32}S^+$ 920.3495, found 920.3505.

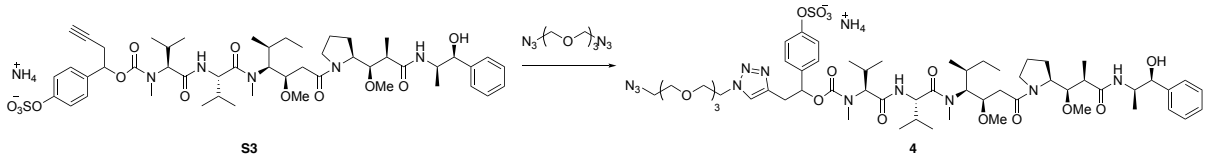
(*S*)-*N*-(2-(2-(2-(((*S*)-1-Amino-1-oxopent-4-yn-2-yl)amino)-2-oxoethoxy)ethoxy)ethyl)-6-(3-(3',6'-dihydroxy-3-oxo-3*H*-spiro[isobenzofuran-1,9'-xanthen]-5-yl)thioureido)-2-(2-(2-(2-(4-((4,6-divinylpyrimidin-2-yl)amino)butanamido)ethoxy)ethoxy)acetamido)hexanamide (1)



To a stirred solution of DVP-CO_2H **3** (11.4 mg, 49.0 μmol), $^{i}Pr_2EtN$ (23.0 μL, 130 μmol) HOBt (20% hydrate, 7.00 mg, 33.0 μmol) in DMF (2 mL), HBTU (12.0 mg, 33.0 μmol) was added. This resulting solution was stirred at rt for 20 min. To this mixture, amine **2** (15.0 mg, 16.0 μmol) in DMF (2 mL) was added, and the resulting solution was stirred at rt for 15 h. Purification by reverse phase flash column chromatography (0 – 30% solvent B in solvent A. Solvent A: 0.1 M aqueous ammonium hydroxide. Solvent B: MeCN) followed by lyophilisation gave dfDVP **1** (10.0 mg, 9.00 μmol, 56%) as an orange oil.

ν_{max} (neat/cm^{-1}) 3255, 1638, 1542; **^{1}H NMR** (CD_3OD, 500 MHz) δ 8.04 (1H, d, *J* = 2.1 Hz), 7.73 (1H, d, *J* = 7.8 Hz), 7.16 (1H, d, *J* = 8.2 Hz), 6.85 (2H, d, *J* = 8.9 Hz), 6.70 – 6.66 (3H, m), 6.64 – 6.55 (4H, m), 6.35 (2H, d, *J* = 17.4 Hz), 5.56 (2H, dd, *J* = 10.7, 1.5 Hz), 4.62 (1H, t, *J* = 6.3 Hz), 4.56 – 4.50 (1H, m), 4.11 – 3.95 (4H, m), 3.73 – 3.39 (19H, m), 3.37 – 3.32 (1H, m), 2.75 (1H, ddd, *J* = 17.0, 5.9, 2.7 Hz), 2.68 (1H, ddd, *J* = 17.0, 6.7, 2.8 Hz), 2.41 (1H, t, *J* = 2.6 Hz), 2.35 – 2.29 (2H, m), 1.97 – 1.81 (3H, m), 1.80 – 1.63 (3H, m), 1.50 – 1.39 (2H, m); **^{13}C NMR** (CD_3OD, 126 MHz) δ 182.6, 176.1, 175.5, 174.2, 173.9, 172.6, 172.5, 171.7, 165.3, 164.0, 155.6, 142.1, 137.1, 134.4, 131.1, 130.2, 127.5, 122.1, 121.7, 119.0, 105.8, 103.7, 80.1, 72.7, 72.3, 72.2, 71.2, 71.1, 71.1, 71.0, 70.8, 53.9, 52.3, 45.5, 41.7, 40.4, 40.4, 34.6, 33.7, 29.6, 27.0, 24.1, 23.3; **HRMS** (ESI) $[M+H]^+$ required for $C_{56}H_{67}N_{10}O_{14}{}^{32}S^+$ 1135.4553, found 1135.4377.

Azide-PEG$_3$-arylsulfate-MMAE (4)



To a degassed solution of sulfate **S3** (synthesised as described previously)[1] (1.60 mg, 1.60 µmol) and N_3-PEG_3-N_3 (1.67 µL, 7.98 µmol) in H_2O/^{t}BuOH (0.1 mL, 1:1) was added a degassed solution of $CuSO_4$·$5H_2O$ (0.200 mg, 0.800 µmol), THPTA (0.700 mg, 1.60 µmol) and sodium ascorbate (0.630 mg, 3.20 µmol) in H_2O/^{t}BuOH (0.2 mL, 1:1). The reaction mixture was stirred at rt for 30 min before being purified by reverse-phase flash column chromatography (25-90% solvent B in solvent A. Solvent A: 0.1 M NH_4OH (aq). Solvent B: MeCN) and lyophilised to yield azide **4** (1.05 mg, 0.843 µmol, 53%) as a white solid.

LRMS (ESI) *m/z* found $[M\text{-}H^+]^-$ 1228.9 $C_{58}H_{90}N_{11}O_{16}{}^{32}S^-$, required 1228.6; **HPLC** (5-95% MeCN/H_2O over 15 min) retention time 10.993 min.

2. Peptide Synthesis

Automated peptide synthesis was carried out on a CEM Liberty Blue® Automated Microwave Peptide Synthesiser using Merck LL MHBA Rink Amide resin (0.33 mmol/g, 1 equiv). All peptide couplings were performed with Fmoc-protected amino acids (5 equiv), Oxyma pure (10 equiv) and DIC (5 equiv) in DMF. Arg was coupled using double couplings for 15 min each without microwave irradiation. All other amino acids were coupled with 25 W power at 75 °C over 15 min.

Fmoc deprotection was achieved with a solution of 20% piperidine in DMF, using 45 W power at 75 °C over 3 min. *N*-terminal capping with Ac_2O (2 equiv) was achieved using DIPEA (4 equiv) in CH_2Cl_2 for 1 hour.

Side chain deprotection and cleavage from the resin was achieved with TFA containing 2.5% TIPS and 2.5% H_2O for 3 hours at rt or 1 hour at 42 °C. In case of cysteine-containing peptides, cleavage was achieved with TFA containing 1.25% EDT, 1.25% H_2O, 1.25% CH_2Cl_2 and 1.25% TIPS. After cleavage, the mixture was filtered through a sintered funnel, the beads washed with MeOH and the filtrate was concentrated under a stream of N_2. The crude residue was triturated with cold Et_2O before purification by preparative HPLC.

The side chain protecting groups used were: ^{t}Bu for Asp, Glu, Thr, Tyr; Boc for Trp; Pbf for Arg; Trt for Asn and Cys.

3. Peptide Stapling

The linear peptide **Pep-p53** (1 equiv.) was dissolved in 50 mM NaPi buffer pH 8.0 (containing 5% of DMF) to a final concentration of 2 mg/mL. dfDVP **1** (1.1 equiv) was added and the reaction shaken at rt for 1 h. Upon completion as monitored by analytical HPLC, the crude reaction was lyophilised and purified on a preparatory HPLC to yield the cyclised peptide, **Pep-FITC**. To obtain peptide **Pep-FITC-RRR**, the crude peptide **Pep-FITC** was used.

4. Peptide CuAAC

A solution of crude **Pep-FITC** (1 equiv) and azide **N_3-RRR** (1.1 equiv) in 1:1 ^{t}BuOH/H_2O (0.8 mL/mg peptide) was degassed with N_2 for 15 min, followed by the addition of $CuSO_4 \cdot 5H_2O$ (3 equiv), THPTA (3 equiv), and sodium ascorbate (6 equiv). The reaction was stirred under N_2 overnight. After this time, the reaction mixture was diluted with H_2O and lyophilised prior to purification via preparative HPLC.

5. Peptide Characterisation

Peptide	Mass	*m/z* found	*m/z* calcul.	Species	Purity	Rt* (min)
Pep-p53	1681.8	1683.9	1682.8	M+H	99%	10.2
N_3-RRR	610.4	611.8	611.4	M+H	99%	4.7
Pep-FITC	2816.2	1409.0	1409.6	M+2H	99%	9.7
Pep-FITC-RRR	3426.6	1715.3	1714.8	M+2H	99%	8.9

* 5% to 95% ACN in water (0.05% TFA) gradient over 15 minutes

6. Trastuzumab Fab Synthesis

Immobilised pepsin (0.15 mL, Pierce) was washed with Digest Buffer 1 (20 mM sodium acetate trihydrate, pH 3.1) four times and trastuzumab (0.5 mL, 6 mg/mL in Digest Buffer 1) was added. The reaction was mixed (1100 rpm) at 37 °C for 15 h. The digestion mixture was filtered from the pepsin using a filter column and washed with Digest Buffer 2 (50 mM sodium phosphate, 1 mM EDTA, 150 mM NaCl, pH 6.8) three times (0.4 mL per wash). The

volume was adjusted to 0.5 mL using an Amicon-Ultra centrifugal filter (10,000 MWCO, Merck Millipore).

Immobilised papain (0.5 mL, Pierce) was activated with DTT (10 mM) in Digest Buffer 2 for 1 h at 37 °C (1100 rpm). The resin was then washed with Digest Buffer 2 four times and the trastuzumab-F(ab')$_2$ (0.5 mL) from the pepsin digestion was added to the resin. The reaction was mixed (1100 rpm) at 37 °C for 15 h. The digestion mixture was filtered from the pepsin using a filter column and washed with TBS (25 mM Tris HCl, 25 mM NaCl, 0.5 mM EDTA, pH 8) three times (0.4 mL per wash). The digest and washes were combined, and buffer exchanged into TBS using an Amicon-Ultra centrifugal filter (10,000 MWCO, Merck Millipore).[2]

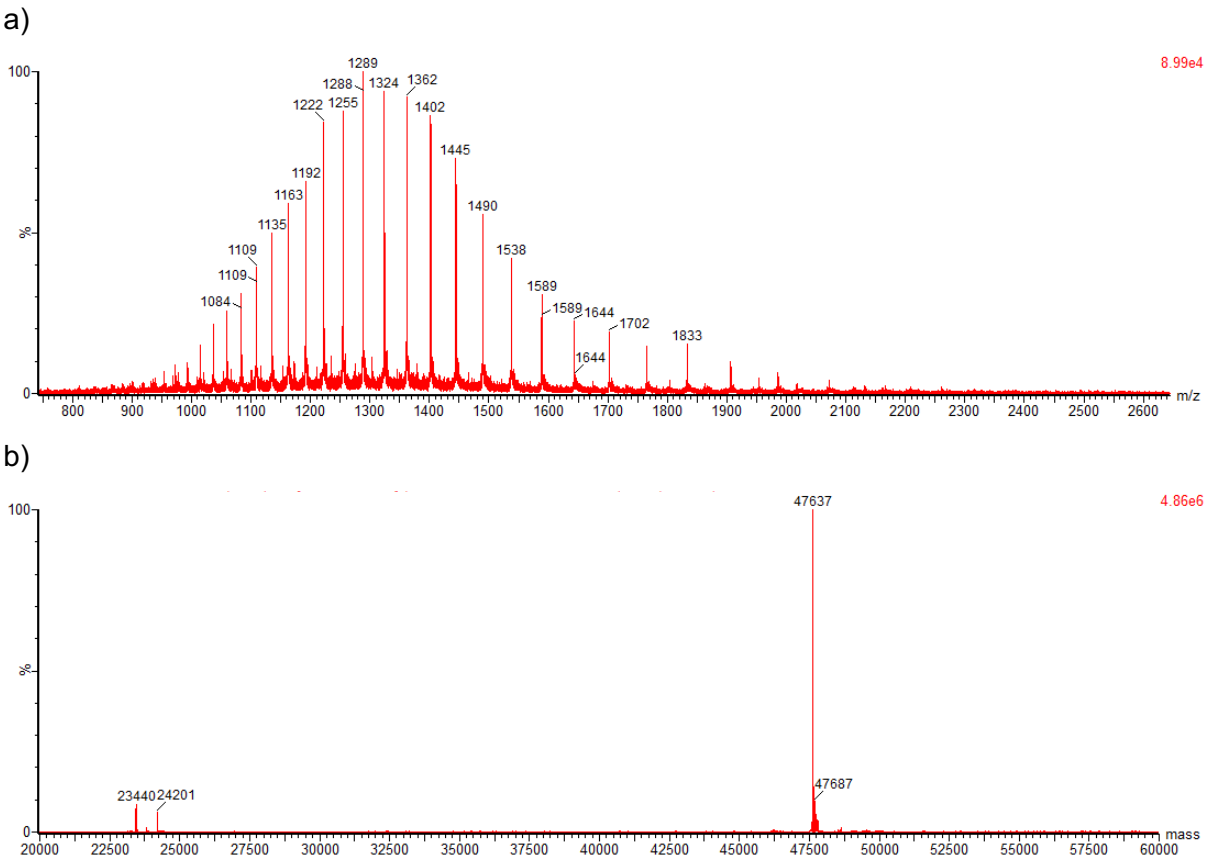
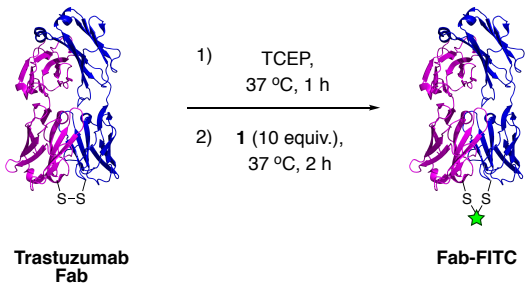
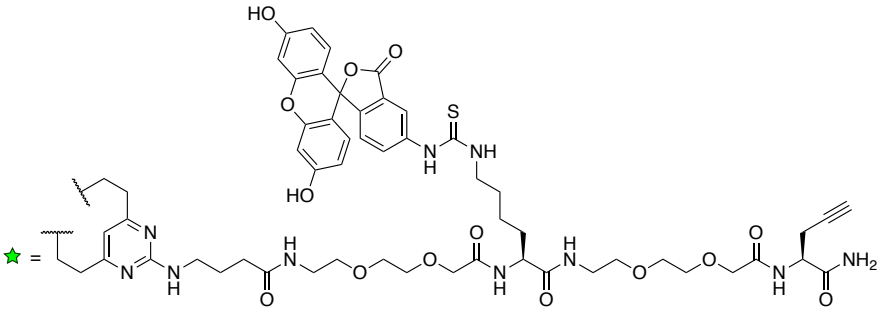


Figure S1: LCMS analysis of trastuzumab Fab. **a)** Non-deconvoluted mass spectrum and **b)** deconvoluted mass spectrum. Expected mass 47,637 Da, observed mass 47,637 Da.

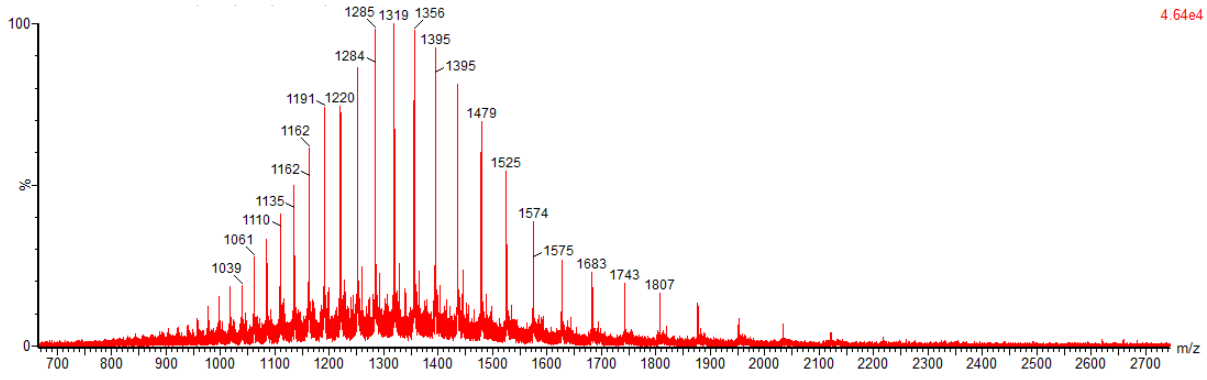
7. Trastuzumab Fab Rebridging



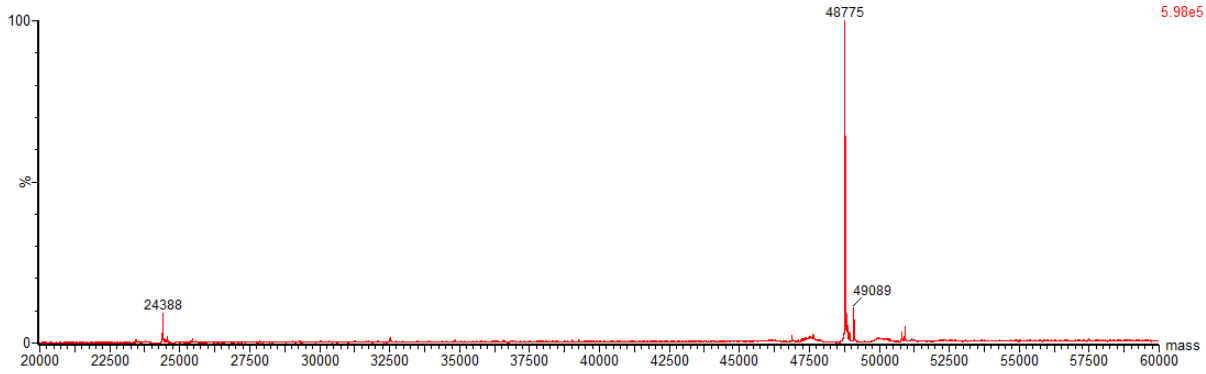


To a solution of trastuzumab Fab (50 µL, 44.9 µM, 2.11 mg/mL) in TBS (25 mM Tris HCl pH 8, 25 mM NaCl, 0.5 mM EDTA) was added TCEP.HCl (from here-on referred to as TCEP, 5 equiv.). The mixture was vortexed and incubated at 37 °C for 1 h. A solution of dfDVP **1** (20 mM in DMF) was added (final concentration of 449 µM, 10 equiv., 10% v/v DMF) and the reaction mixture incubated at 37 °C for 2 h. The excess reagents were removed using a Zeba™ Spin desalting column (40,000 MWCO, 0.5 mL, ThermoFisher) pre-equilibrated with PBS. The reaction product was completely buffer exchanged into PBS by repeated diafiltration using an Amicon-Ultra centrifugal filter (10,000 MWCO, Merck Millipore). LCMS, UV-Vis and SDS-PAGE analysis demonstrated >95% conversion to the desired conjugate, **Fab-FITC**.

a)



b)



c)

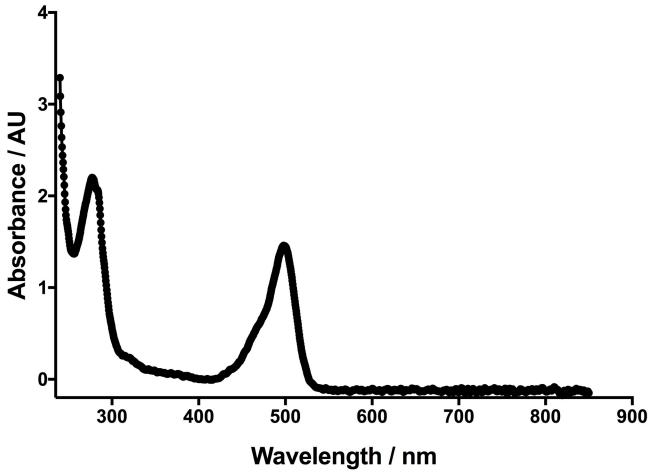
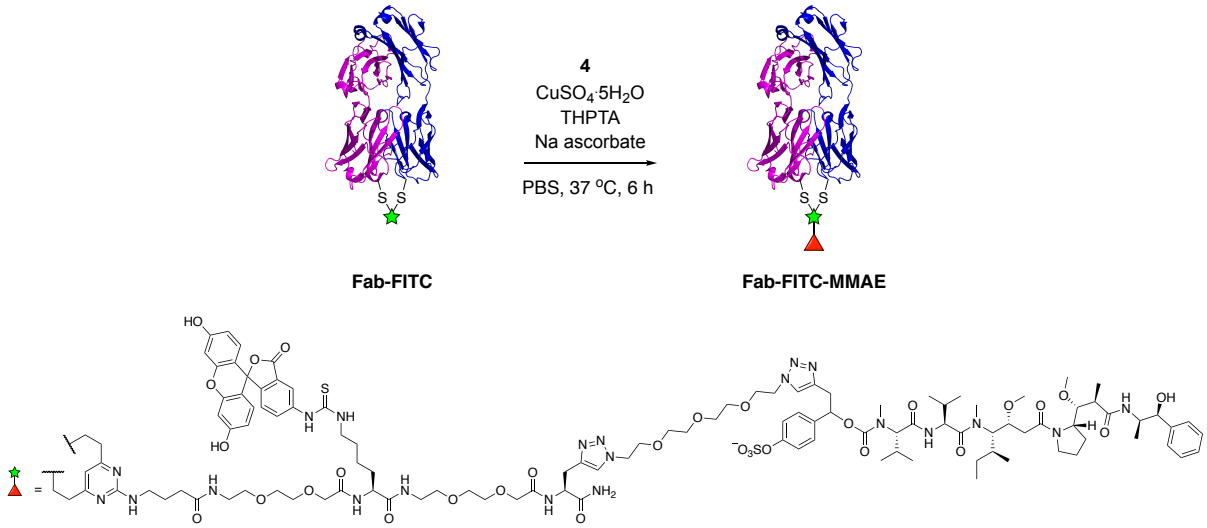


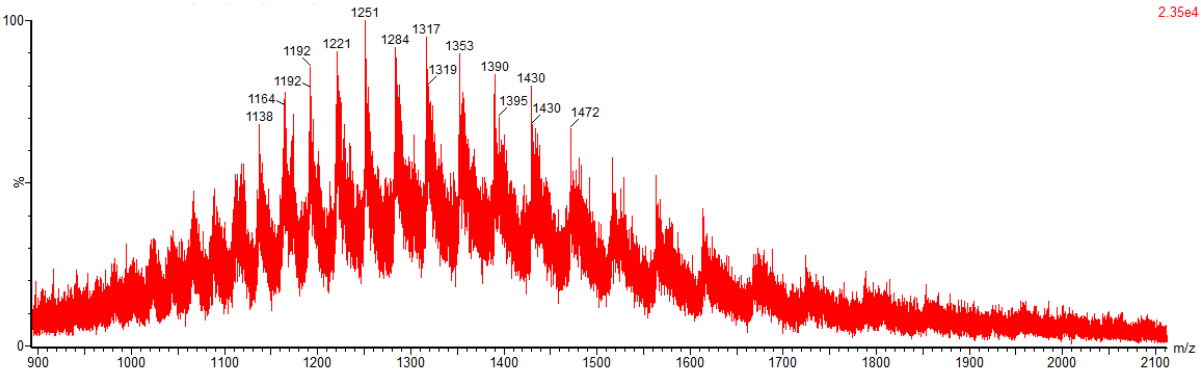
Figure S2: LCMS analysis of **Fab-FITC**. **a)** Non-deconvoluted mass spectrum and **b)** deconvoluted mass spectrum. Expected mass 48,772 Da, observed mass 48,775 Da. **c)** UV-vis spectrum of **Fab-FITC**.

8. Trastuzumab Fab CuAAC

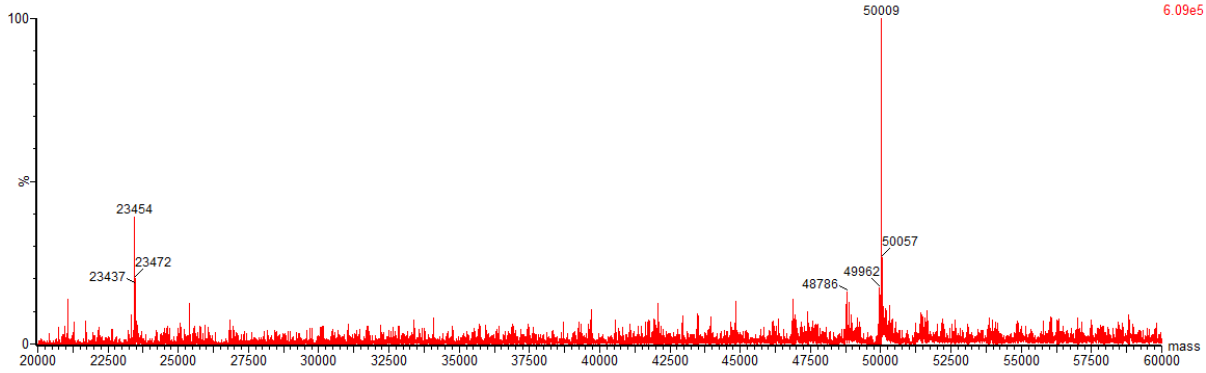


To a solution of trastuzumab **Fab-FITC** (10 µL, 20.5 µM, 1 mg/mL) in PBS was added azide **4** (20 mM in DMS, to 0.82 mM), $CuSO_4 \bullet 5H_2O$ (to 0.82 mM), THPTA (to 2.46 mM) and sodium ascorbate (to 4.1 mM). The mixture was vortexed and incubated at 37 °C for 6 h. The excess reagents were removed using a Zeba™ Spin desalting column (40,000 MWCO, 0.5 mL, ThermoFisher) pre-equilibrated with PBS. LCMS analysis revealed conversion to the desired conjugate, **Fab-FITC-MMAE**.

a)



b)



c)

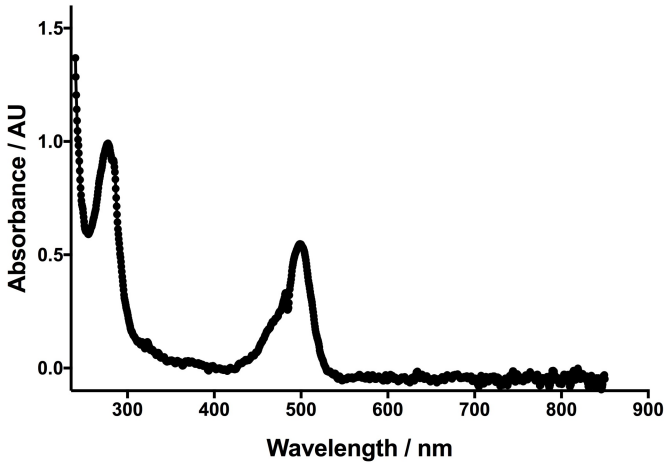


Figure S3: LCMS analysis of **Fab-FITC-MMAE**. **a)** Non-deconvoluted mass spectrum and **b)** deconvoluted mass spectrum. Expected mass 50,002 Da, observed mass 50,009 Da. **c)** UV-vis spectrum of **Fab-FITC-MMAE**.

The concentration of the modified Fab (**Fab-FITC** and **Fab-FITC-MMAE**) was calculated via UV-vis spectroscopy using the following formula:

$$Fab\ (mg/mL) = \frac{Abs_{280} - (0.61\ \times\ Abs_{298}) - (0.35\ \times\ Abs_{495}) + (0.1\ \times\ Abs_{280})}{1.43}$$

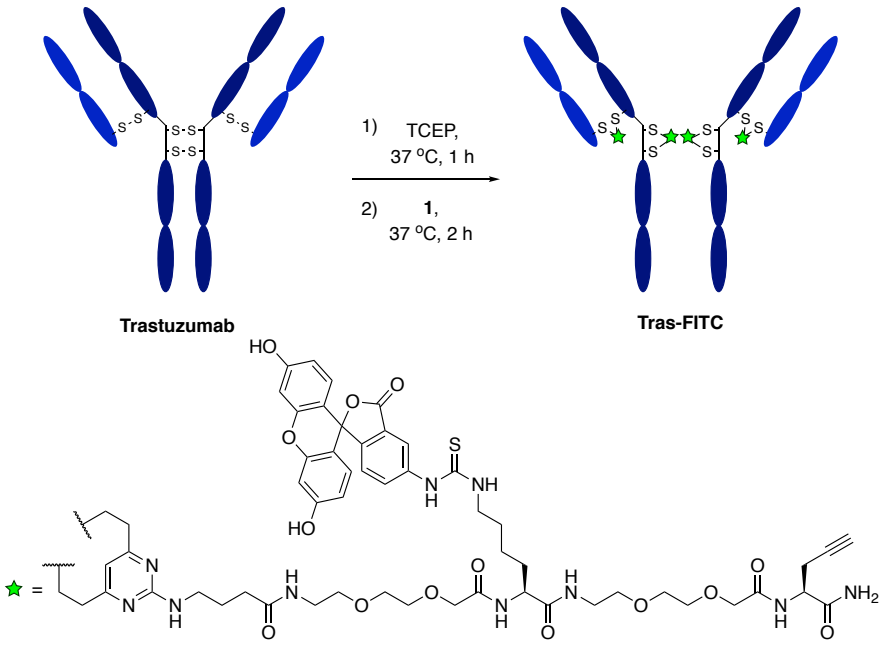
where:

0.61 is a correction factor for dfDVP absorbance at 280 nm,

0.35 is a correction factor for FITC absorbance at 280 nm,

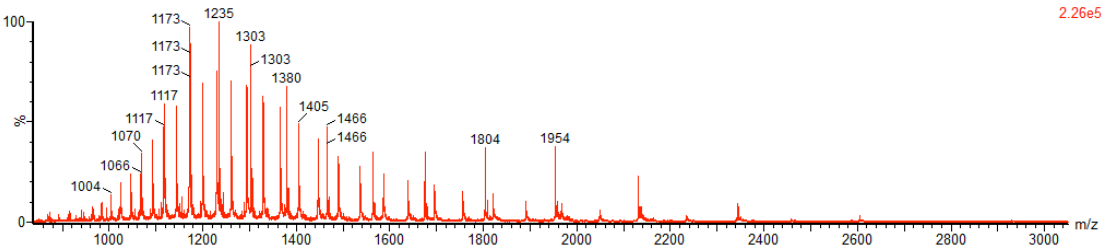
0.1 is a correction factor for Fab absorbance at 298 nm.

9. Trastuzumab IgG Rebridging

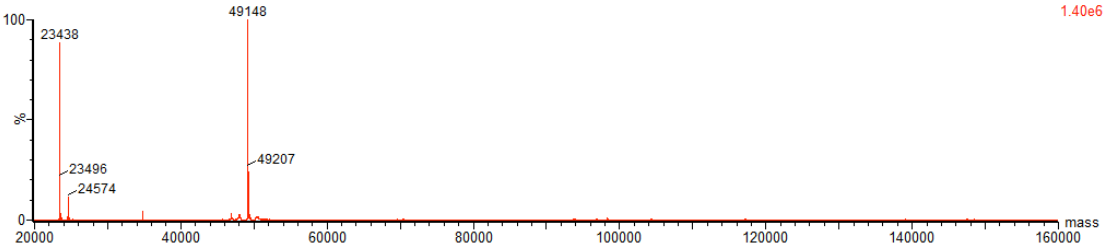


To a solution of trastuzumab (50 µL, 17 µM, 2.5 mg/mL) in TBS (25 mM Tris HCl pH 8, 25 mM NaCl, 0.5 mM EDTA) was added TCEP (10 equiv.). The mixture was vortexed and incubated at 37 °C for 1 h. A solution of dfDVP **1** (20 mM in DMF) was added (final concentration of 680 µM, 40 equiv.) and the reaction mixture incubated at 37 °C for 2 h. The excess reagents were removed using a Zeba™ Spin desalting column (40,000 MWCO, 0.5 mL, ThermoFisher) pre-equilibrated with PBS. The reaction product was completely buffer exchanged into PBS by repeated diafiltration using an Amicon-Ultra centrifugal filter (10,000 MWCO, Merck Millipore). LCMS, UV-Vis and SDS-PAGE analysis demonstrated >95% conversion to the desired conjugate.

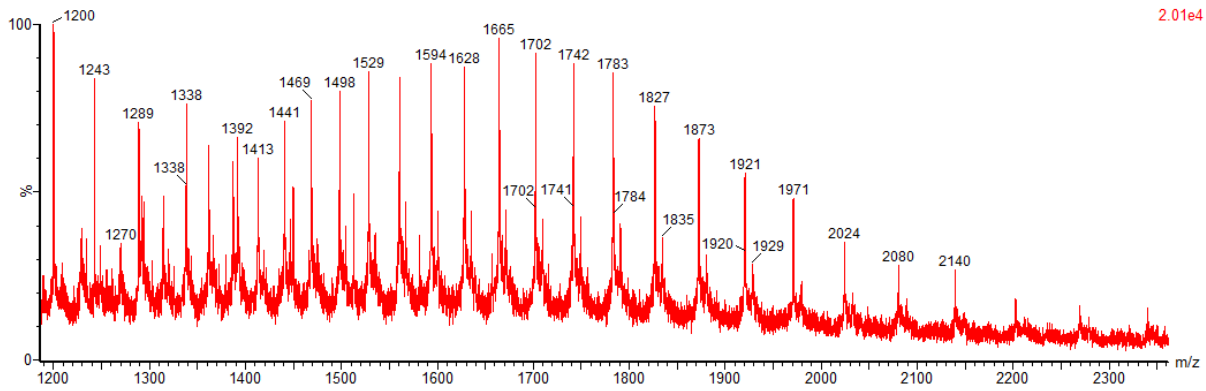
a)



b)



c)



d)

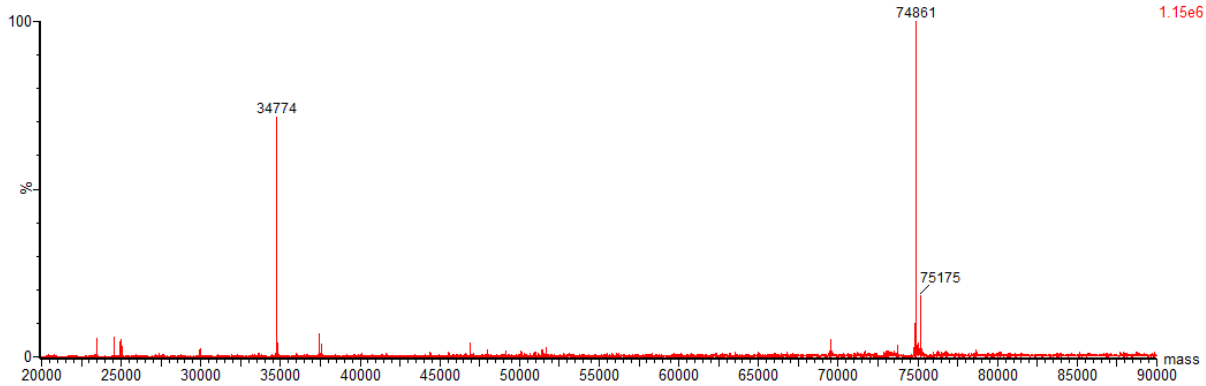
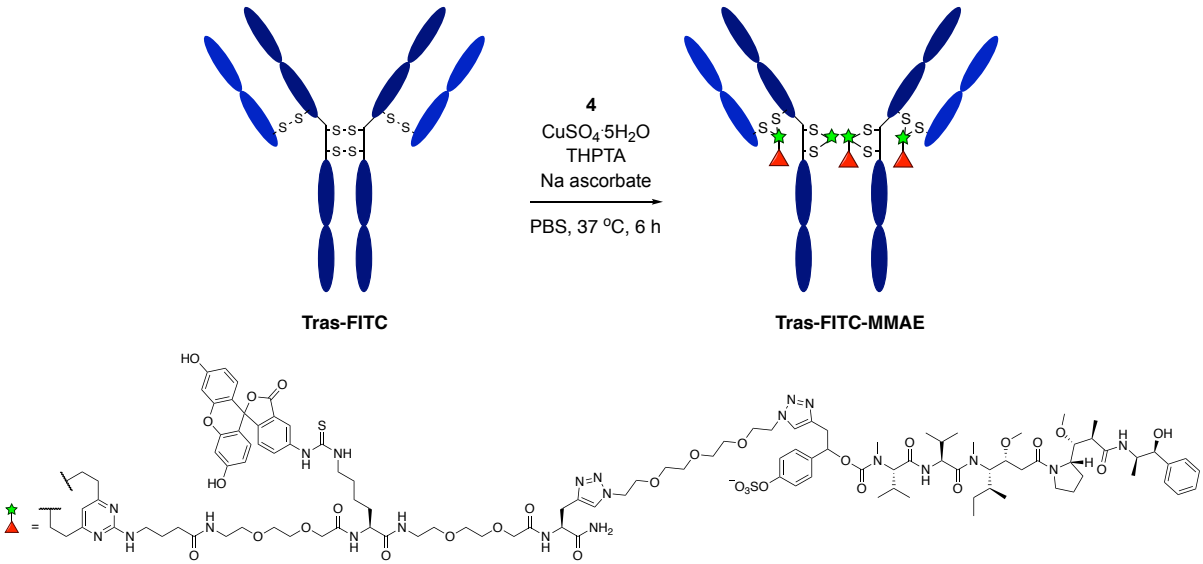


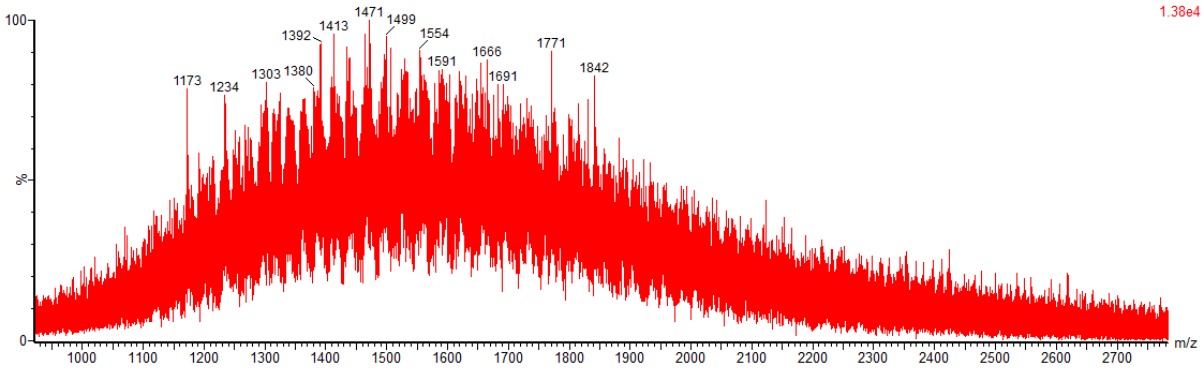
Figure S4: LCMS analysis of reduced trastuzumab and **Tras-FITC**. **a)** Non-deconvoluted mass spectrum and **b)** deconvoluted mass spectrum of reduced trastuzumab. **c)** Non-deconvoluted mass spectrum and **d)** deconvoluted mass spectrum of **Tras-FITC**. Expected mass 74,858 Da, observed mass 74,861 Da. The mass at 34,774 Da corresponds to PNGase F.

10. Trastuzumab IgG CuAAC

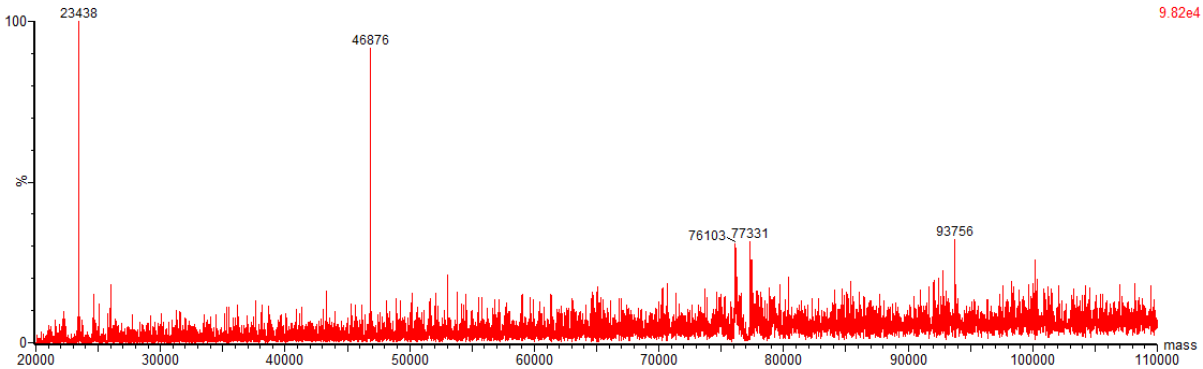


To a solution of **Tras-FITC** (30 µL, 8.4 µM, 1.27 mg/mL) in PBS was added azide **4** (20 mM in DMSO, to 0.42 mM), $CuSO_4 \bullet 5H_2O$ (to 0.84 mM), THPTA (to 2.52 mM) and sodium ascorbate (to 4.2 mM). The mixture was vortexed and incubated at 37 °C for 6 h. The excess reagents were removed using a Zeba™ Spin desalting column (40,000 MWCO, 0.5 mL, ThermoFisher) pre-equilibrated with PBS. LCMS analysis revealed conversion to the desired conjugate, **Tras-FITC-MMAE**. Increasing reagent stoichiometry, reaction concentration or reaction time had no effect on the DAR. Resubmission of the reaction product to further rounds of CuAAC also had no effect.

a)



b)



c)

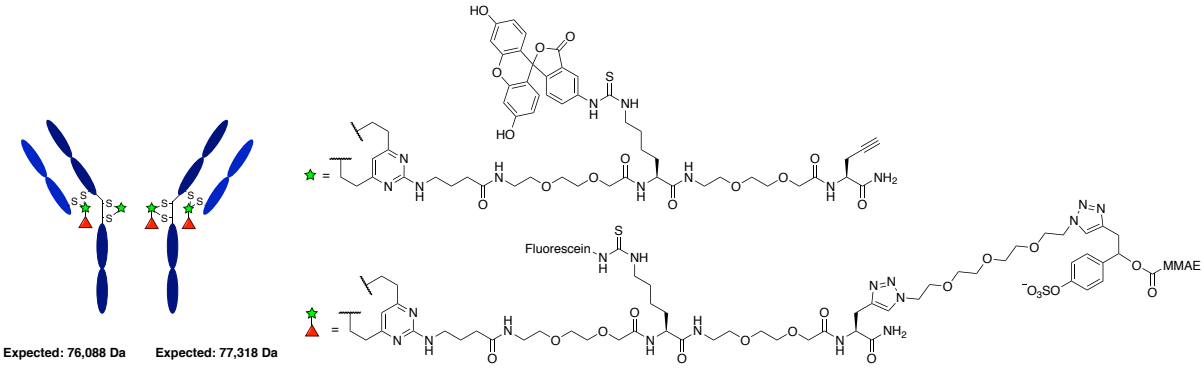


Figure S5: LCMS analysis of **Tras-FITC-MMAE**. **a)** Non-deconvoluted mass spectrum and **b)** deconvoluted mass spectrum of **Tras-FITC-MMAE**, **c)** graphical representation of the two ion series observed via LCMS analysis. Due to the poor ionisation of **Tras-FITC-MMAE**, it was purified from PNGase F prior to LCMS analysis using a Protein A HP SpinTrap™ (GE Healthcare), to maximise the signal intensity. Masses of 23,438 and 46,876 Da correspond to the light chain and light chain dimer of trastuzumab, respectively.

The concentration of the modified trastuzumab (**Tras-FITC** and **Tras-FITC-MMAE**) was calculated via UV-vis spectroscopy using the following formula:

$$Tras\ (mg/mL) = \frac{Abs_{280} - (0.61 \times Abs_{298}) - (0.35 \times Abs_{495}) + (0.1 \times Abs_{280})}{1.46}$$

where:

0.61 is a correction factor for dfDVP absorbance at 280 nm,

0.35 is a correction factor for FITC absorbance at 280 nm,

0.1 is a correction factor for Tras absorbance at 298 nm.

11. Cell Lines

SKBR3 cells were obtained from the American Type Culture Collection (ATCC) and MCF7 cells were obtained from the European Collection of Authenticated Cell Cultures (ECACC). U2OS cells were obtained from the AstraZeneca cell bank. SKBR3 cells were maintained in high glucose McCoy's 5A medium, supplemented with 10% heat-inactivated foetal-bovine serum (FBS), 50 U/mL penicillin and 50 µg/mL streptomycin. MCF7 cells were maintained in Dulbecco's Modified Eagle Medium (DMEM) supplemented with 10% FBS, 2 mM *L*-glutamine, 50 U/mL penicillin and 50 µg/mL streptomycin. U2OS cells were maintained in RPMI-1640 medium supplemented with 10% FBS and 2 mM *L*-glutamine. All cell lines were incubated at 37 °C with 5% CO_2.

12. Peptide Internalisation

Confocal imaging of fixed U2OS cells was conducted using a Yokogawa Cell Voyager CV8000™ confocal microscope with a 20X dry objective. Images were acquired with excitation at 488 and 405 nm and emissions detected with a 525/50 and 445/460 nm BP filter for the green and blue channel respectively.

U2OS cells were seeded at 2500 cell per well into a flat-bottomed tissue 384-well plate (PerkinElmer CellCarrier Ultra™) in a volume of 40 µL of growth at 37°C / 5% CO_2 in a humidified environment. After 24 hours, FITC-labelled compounds dissolved in DMSO were added to the cells using HP Tecan™ dispenser to a final concentration of 25 µM and DMSO normalised to a final concentration of 1% (v/v). Cells were fixed after 3, 1, 0.5-hour incubation using 40 µL of 8% PFA (final PFA concentration 4%). Cells were fixed for 20 minutes and washed with PBS (3x). 40 µL of Hoechst nuclei stain (1:5000 in PBS) was added to the plate. After 1 hour at 4 °C the plate was washed with PBS (3x) and imaged as described above. Images were analysed using Fiji (ImageJ) software.

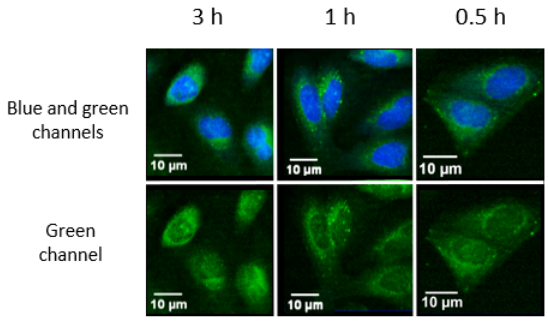


Figure S6: Confocal images of **Pep-FITC-RRR** showing localisation in the cytosol and in the nucleus.

13. ADC Cytotoxicity

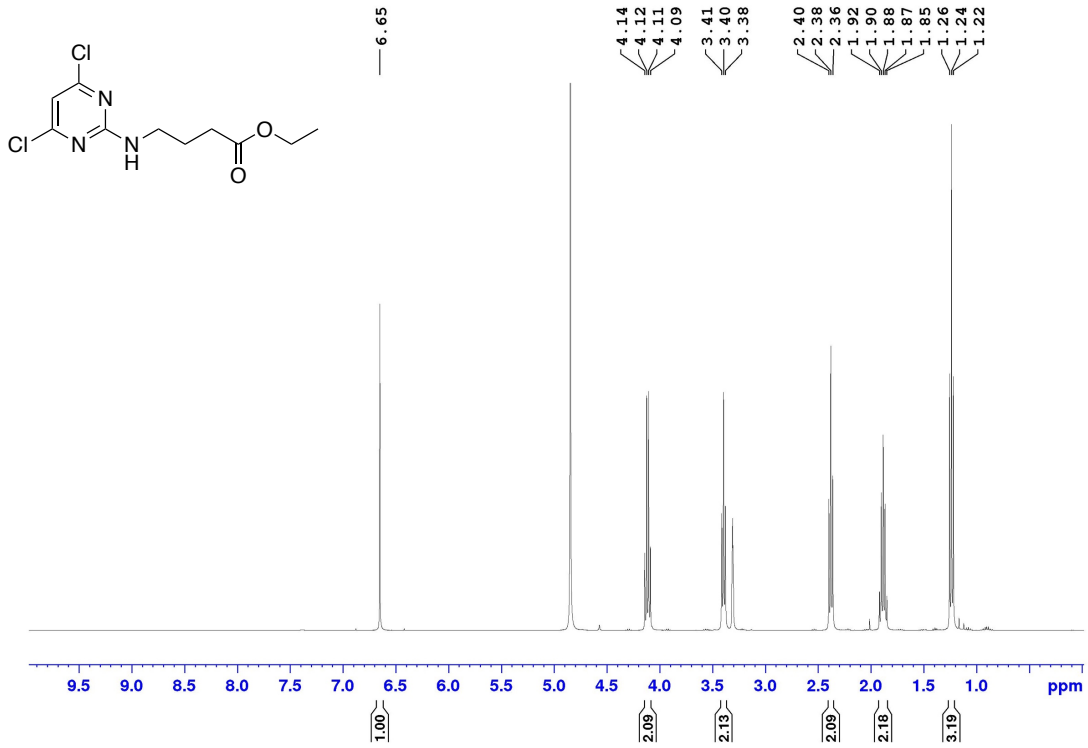
Cells were seeded in 96-well plates for 24 h at 37 °C with 5% CO_2. SKBR3 cells were seeded at 20,000 cells/well and MCF7 cells seeded at 7,500 cells/well. Serial dilutions of **Tras-FITC**, **Tras-FITC-MMAE** and trastuzumab were added to the cells in complete growth medium and incubated at 37 °C with 5% CO_2 for 96 h. Cell viability was measured using CellTiter-Glo viability assay (Promega) according to the manufacturer's instructions. Cell viability was plotted as a percentage of untreated cells. Each measurement was taken in triplicate and three independent repeats were performed.

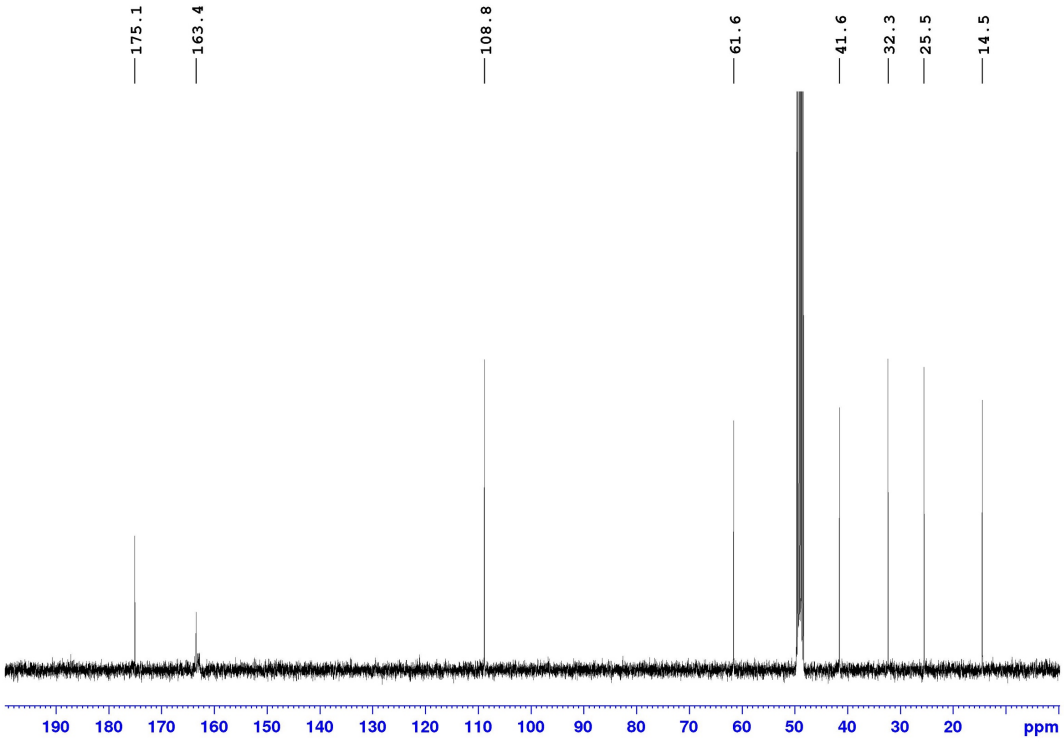
14. ADC Internalisation

SKBR3 or MCF7 cells were seeded at 20,000 cells/well in 8-well chambered µ-slide (Ibidi, 80826) for 48 h at 37 °C with 5% CO_2. Slides were placed on ice and antibody conjugates (50 nM) or untreated vehicle control (PBS) were added to the cells in complete growth medium at 4 °C in the dark for 1 h. Cells were then washed with PBS (3 × 200 µL). For cell surface binding analysis, cells were fixed in cold PFA (4% in PBS) for 20 min at 4 °C. Cells were then washed with PBS (3 × 200 µL) and blocked with normal goat serum (5%, 200 µL) containing 0.3% Triton-X100 for 30 min at 4 °C. Cells were again washed with PBS (3 × 200 µL) and DAPI (1 µg/mL) and Phalloidin-iFluor 594 (Abcam, ab176757, 1:1000) for 25 min at rt. Cells were again washed with PBS (3 × 200 µL) and stored in the dark at 4 °C. For internalisation analysis, after incubation at 4 °C for 1 h, cells were washed with PBS (3 × 200 µL) and incubated in full growth medium at 37 °C, 5% CO_2 for 1 h. Fixing, blocking and nucleus/cytoplasm staining was conducted as before. Images were obtained on a Zeiss Cell Observer wide-field microscope with a 40X oil objective. Images were analysed using Fiji (ImageJ) software. One of two replicates is shown.

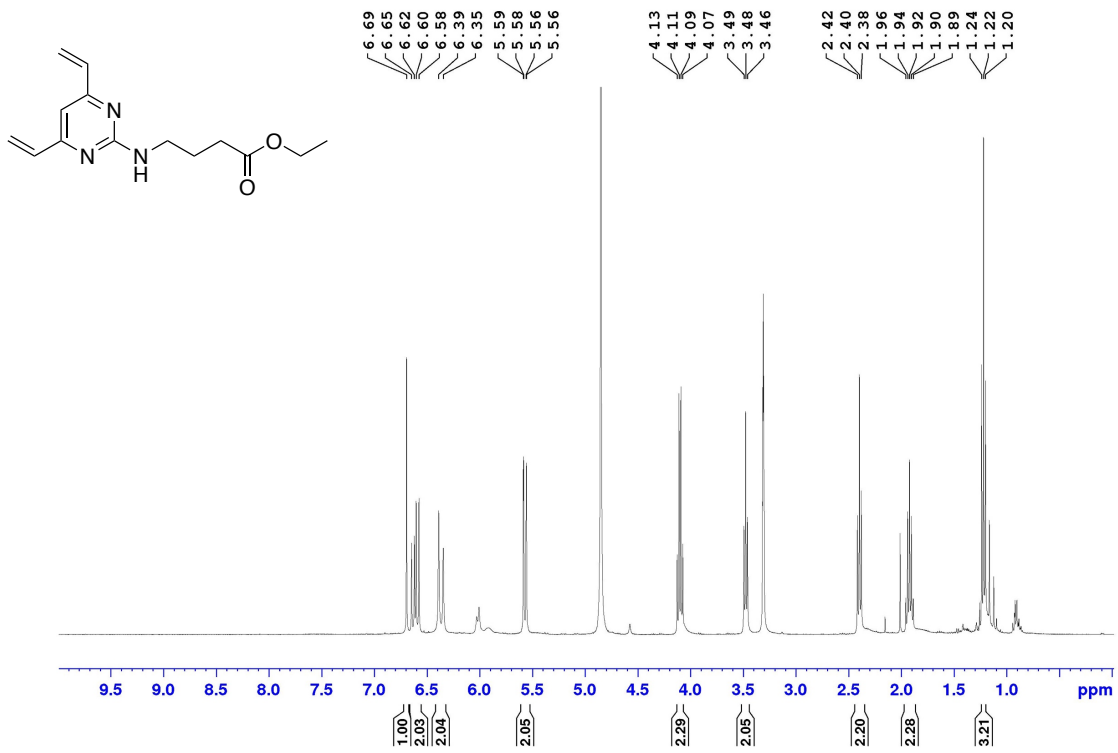
15. NMR Spectra

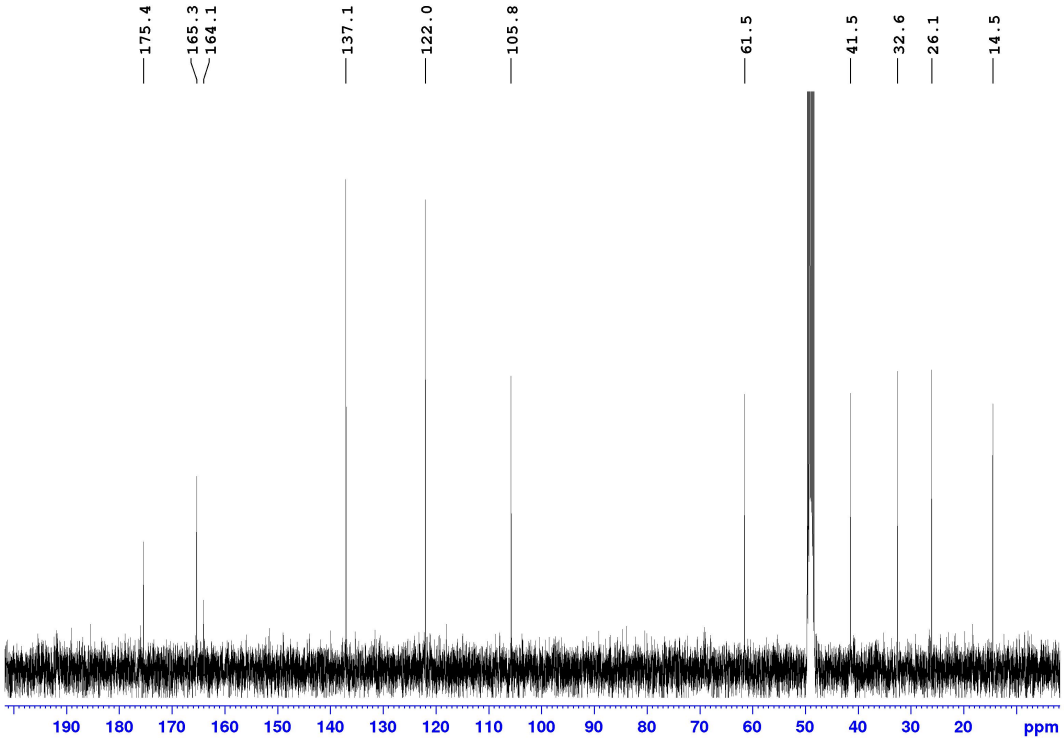
Ethyl 4-((4,6-dichloropyrimidin-2-yl)amino)butanoate (S1)



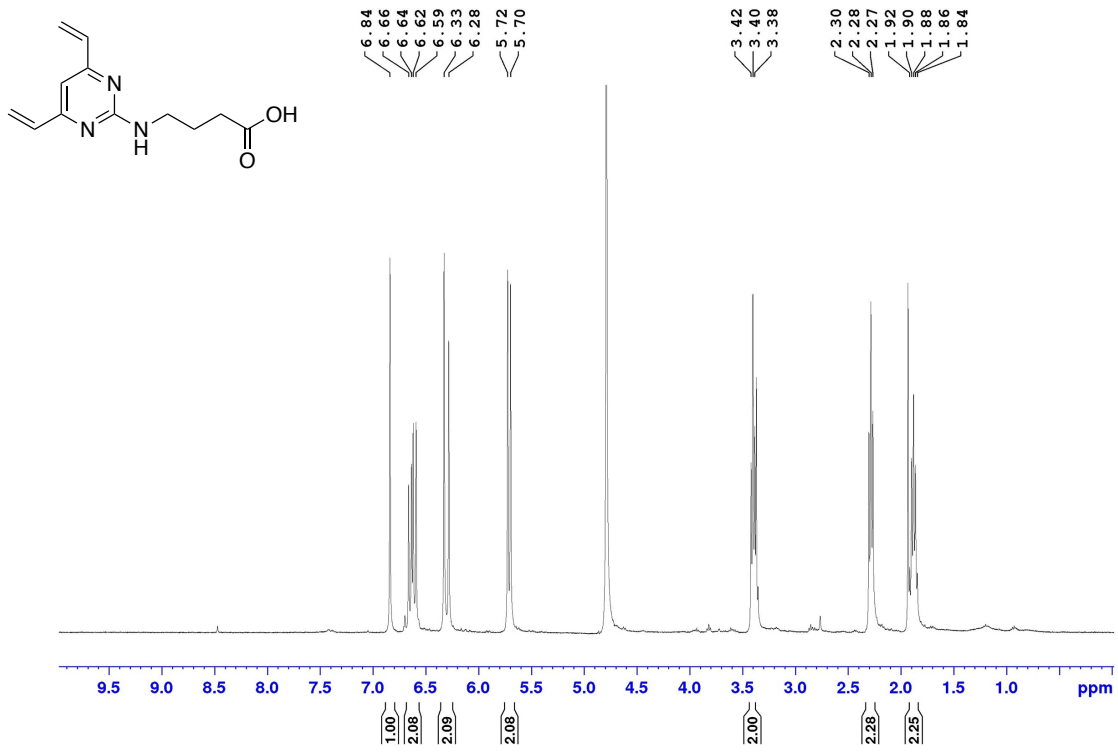


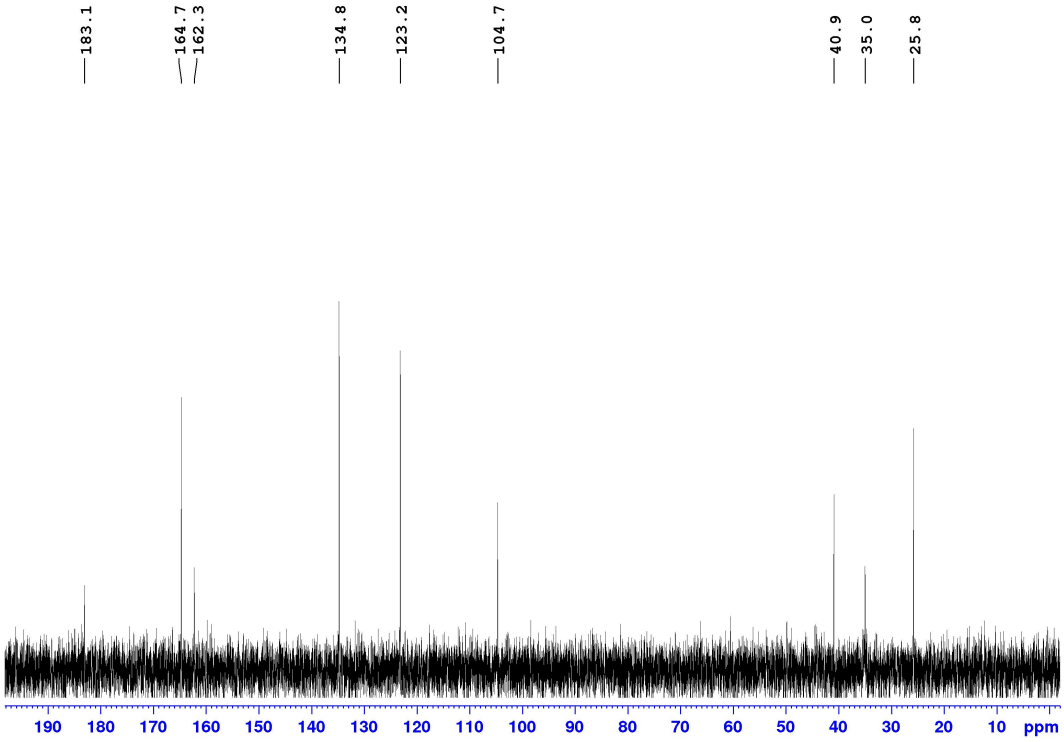
Ethyl 4-((4,6-divinylpyrimidin-2-yl)amino)butanoate (S2)



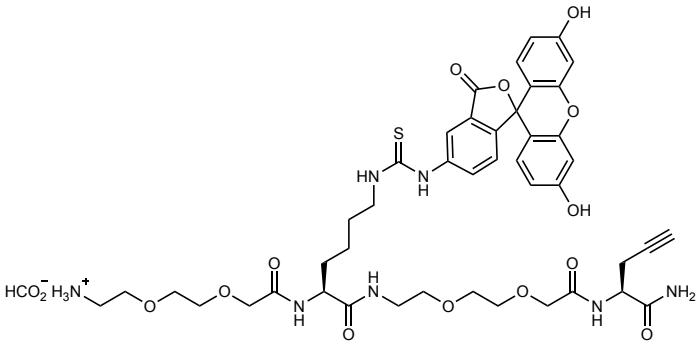


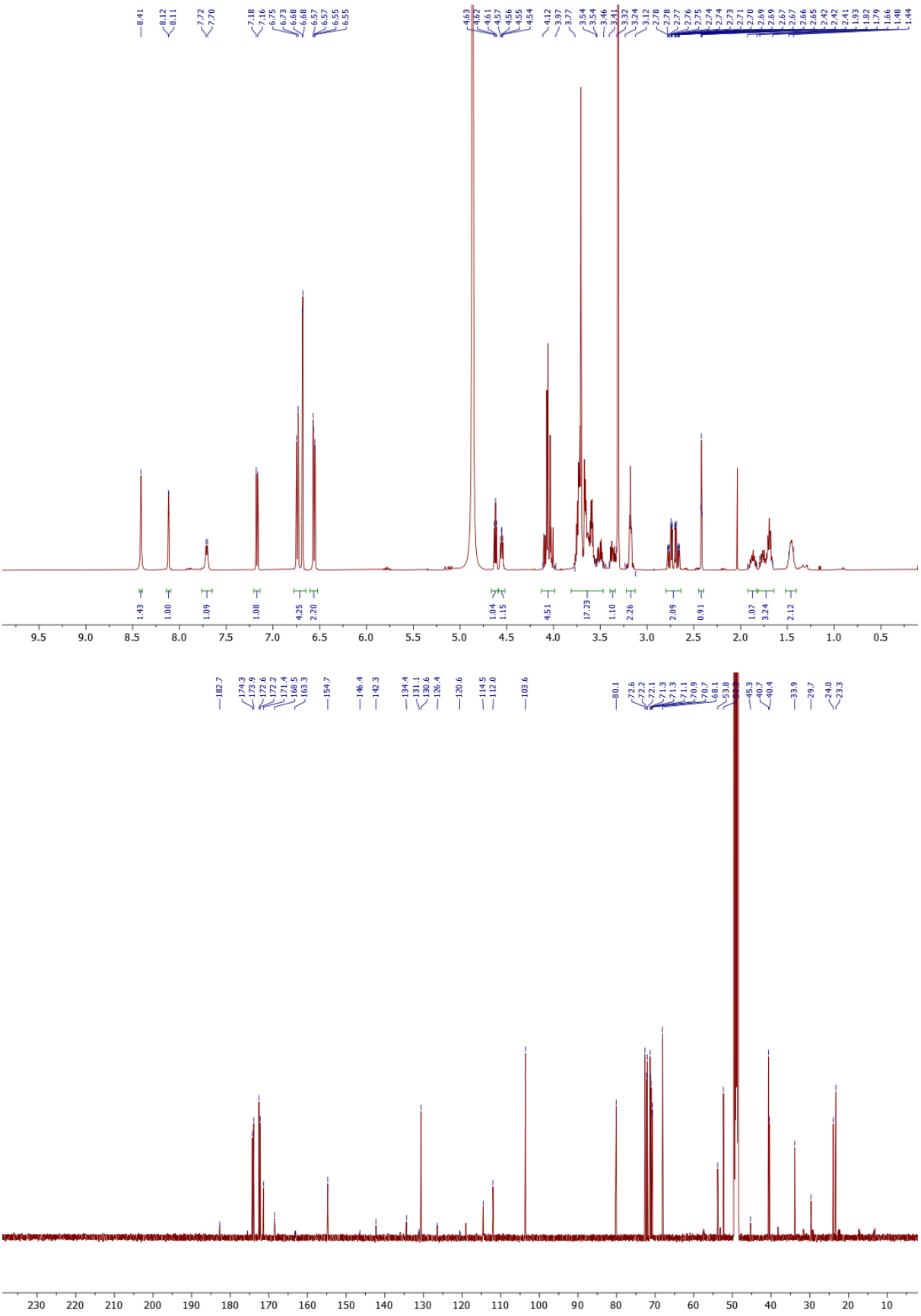
4-((4,6-Divinylpyrimidin-2-yl)amino)butanoic acid (3)



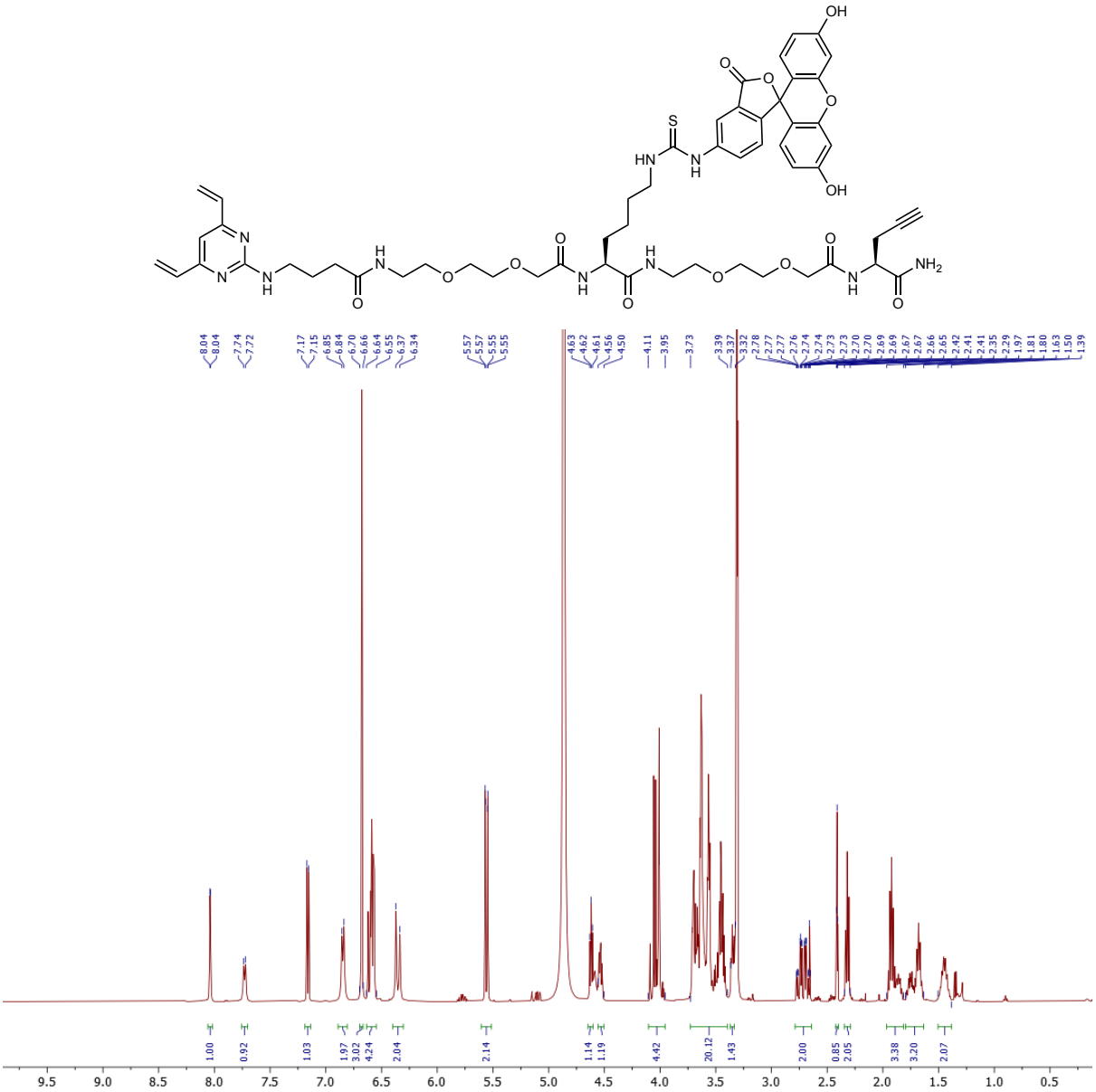


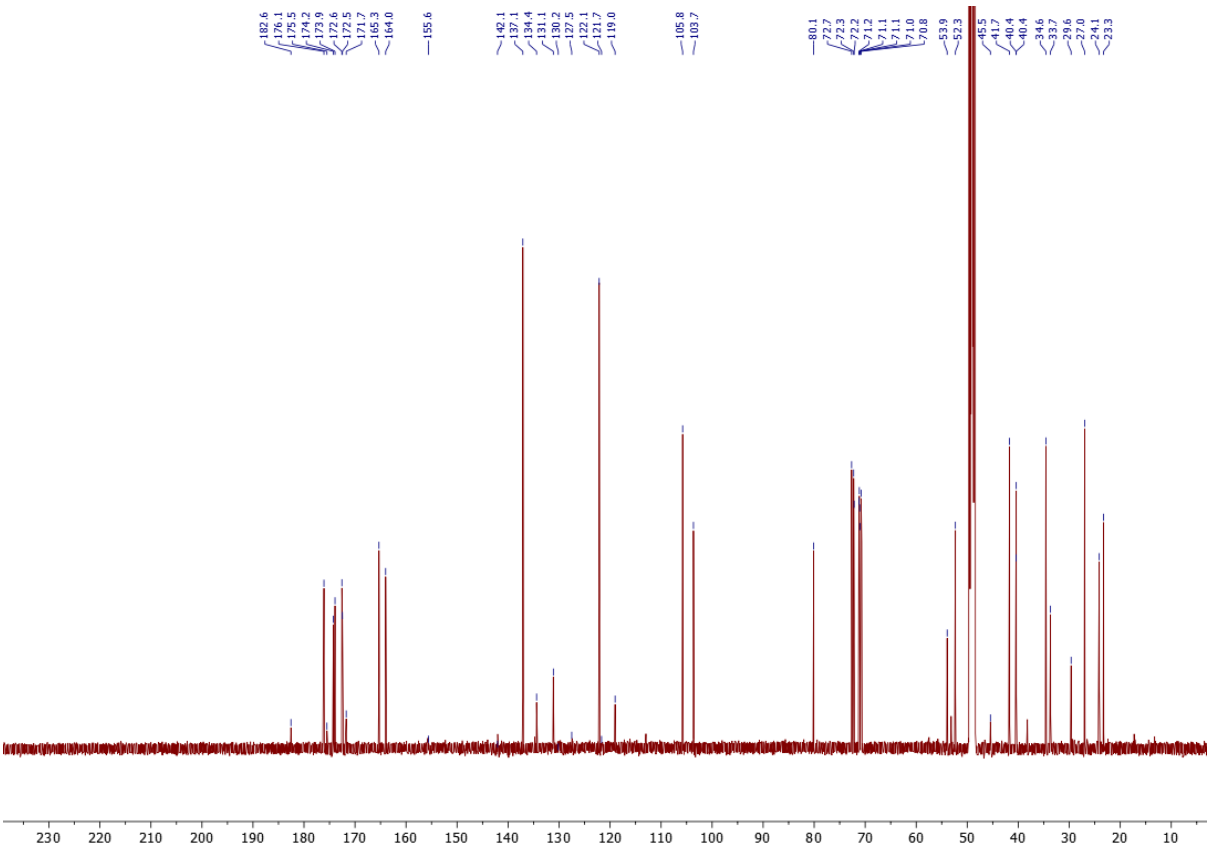
(*S*)-*N*-(2-(2-(2-(((*S*)-1-Amino-1-oxopent-4-yn-2-yl)amino)-2-oxoethoxy)ethoxy)ethyl)-2-(2-(2-(2-aminoethoxy)ethoxy)acetamido)-6-(3-(3',6'-dihydroxy-3-oxo-3*H*-spiro[isobenzofuran-1,9'-xanthen]-5-yl)thioureido)hexanamide (2)





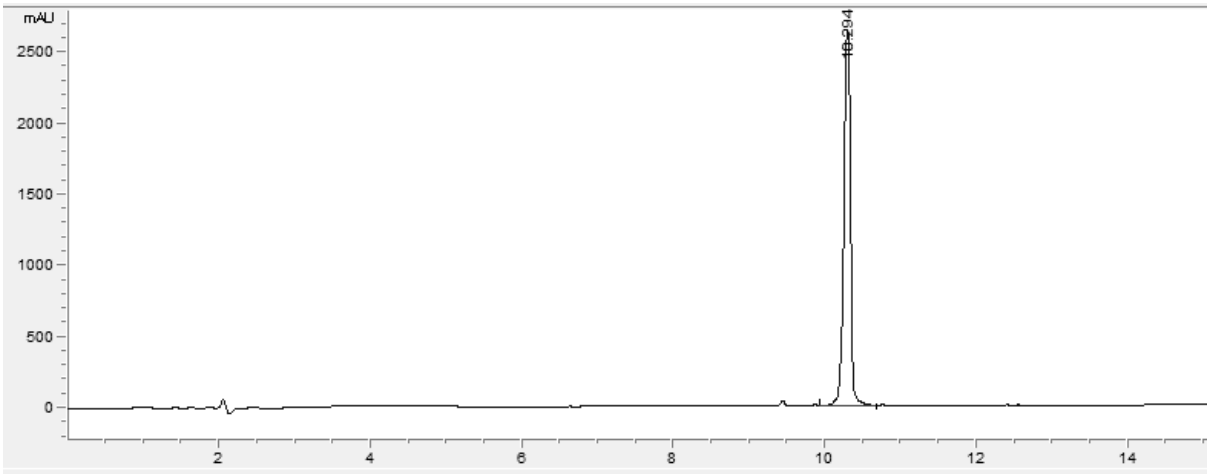
(*S*)-*N*-(2-(2-(2-(((*S*)-1-Amino-1-oxopent-4-yn-2-yl)amino)-2-oxoethoxy)ethoxy)ethyl)-6-(3-(3',6'-dihydroxy-3-oxo-3*H*-spiro[isobenzofuran-1,9'-xanthen]-5-yl)thioureido)-2-(2-(2-(2-(4-((4,6-divinylpyrimidin-2-yl)amino)butanamido)ethoxy)ethoxy)acetamido)hexanamide (1)



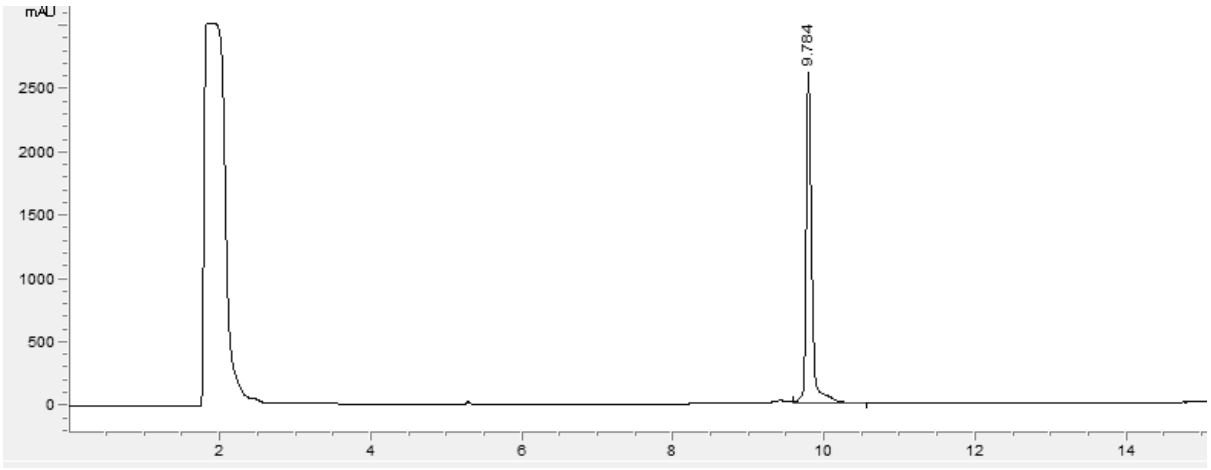


16. HPLC Traces

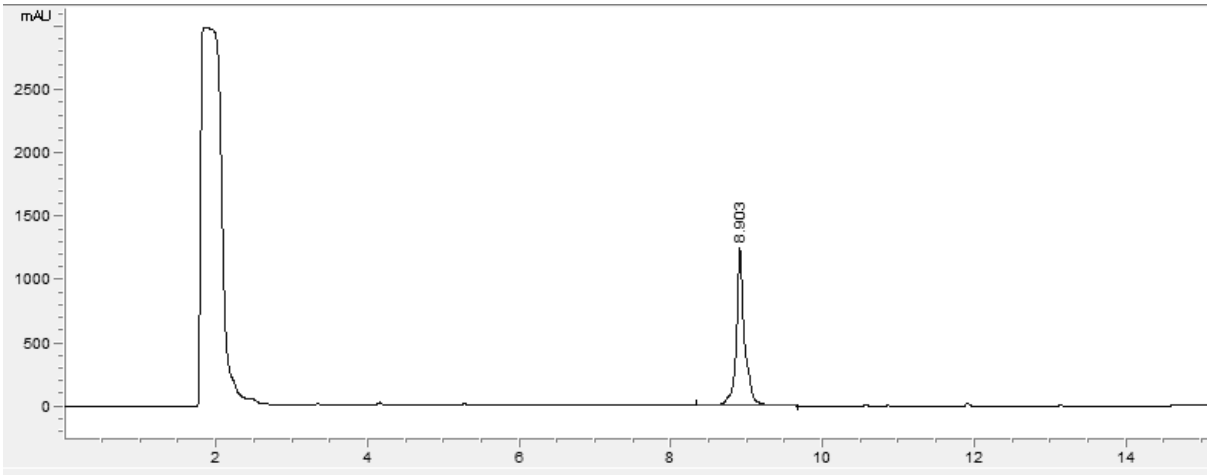
Pep-p53



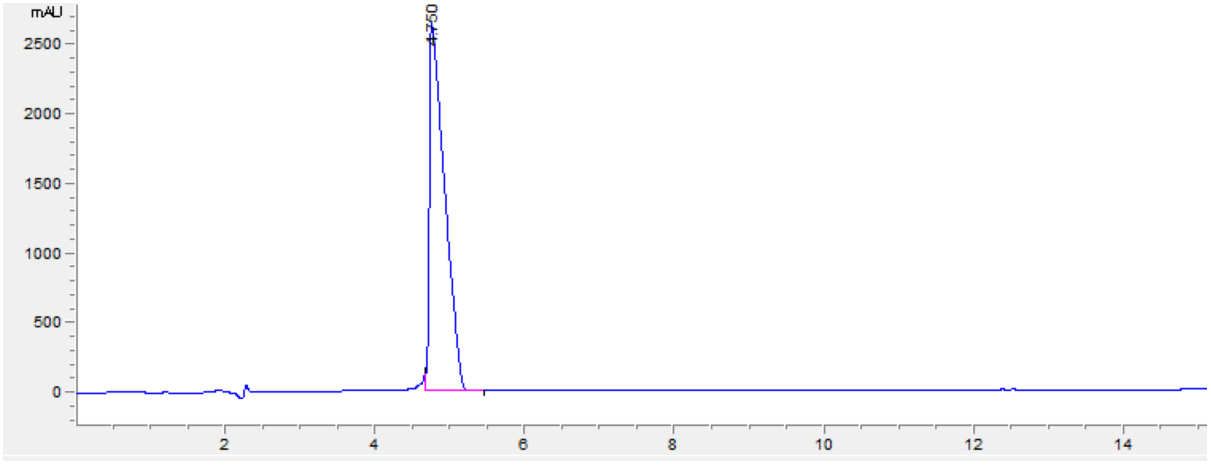
Pep-FITC



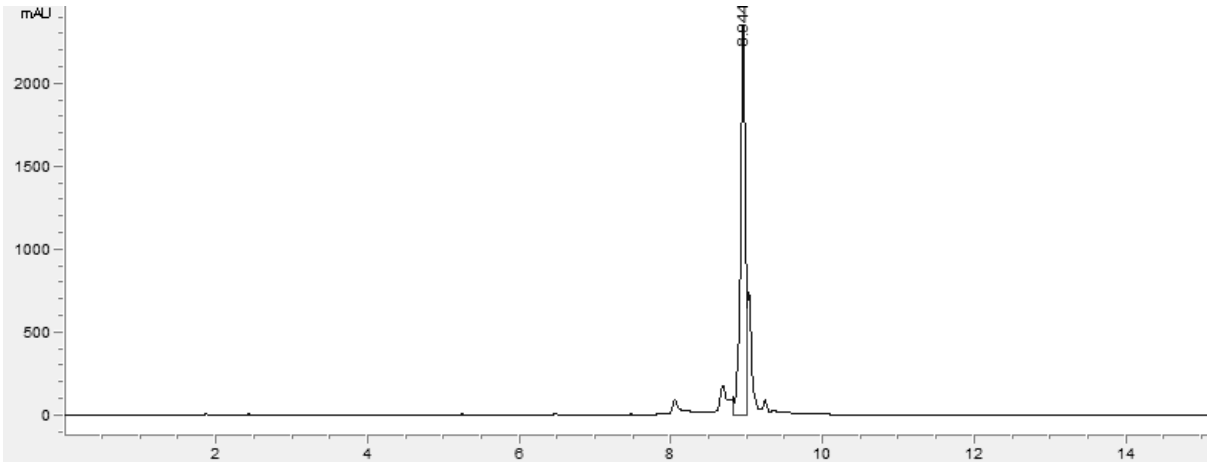
Pep-FITC-RRR



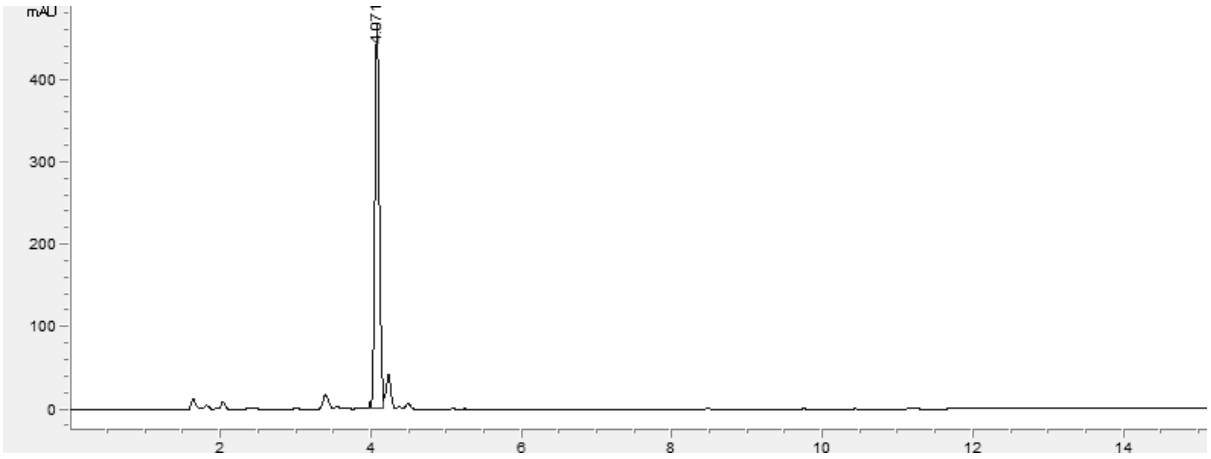
N_3-RRR



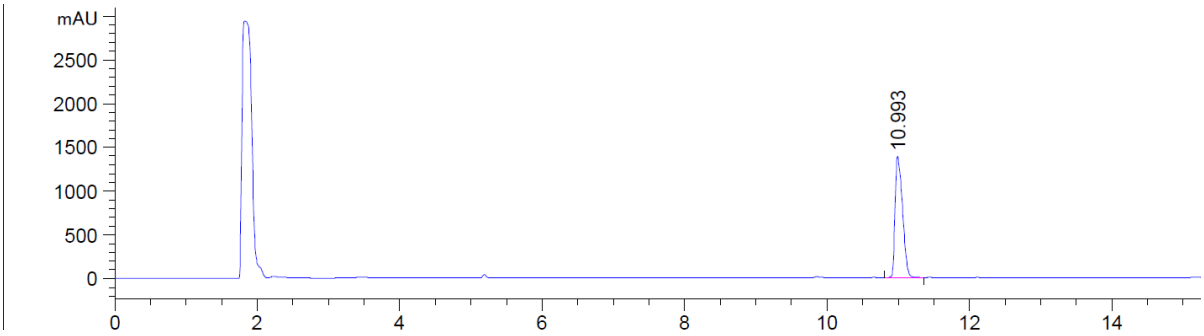
dfDVP **1**



Amine **2**



Azide-sulfate-MMAE **(4)**



*Peak at 2 min in HPLC traces of **Pep-FITC**, **Pep-FITC-MMAE** and **4** is DMSO.

17. References

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