

Enabling the synthesis of multi-payload thio-antibody conjugates through the use of pyridazinediones, *p*-anisidine derivatives and various click chemistries

Supplementary Information

General Experimental

Chemicals

All reagents were purchased from Sigma Aldrich, Alfa Aesar or ThermoFisher Scientific and were used as received unless otherwise stated. Solvents were used as received unless otherwise stated. GFP S147C was kindly supplied by Dr Ioanna Thanasi (UCL Chemistry), having been expressed according to literature.¹

Chromatography

All reactions were magnetically stirred and monitored by thin-layer chromatography (TLC) on pre-coated silica gel plates (254 μm). Silica gel plates were initially examined under UV light and then developed using aqueous potassium permanganate/ninhydrin/bromocresol green stains. Flash column chromatography was carried out with pre-loaded FlashPureTM cartridges on a Biotage[®] Isolera Spektra One flash chromatography system.

Spectroscopy

Nuclear magnetic resonance (NMR) spectra were obtained using Bruker Avance Neo 700, Avance III 600, DRX 500 or Avance III 400 NMR instruments. All samples were run at 25 °C. Chemical shifts (δ) are quoted on a parts per million (ppm) scale relative to tetramethylsilane, calibrated using residual signals of the solvent. In the case of amide rotamers and where possible, only the major rotamer has been assigned, and areas underneath all rotameric peaks have been considered for integration calculations. Coupling constants (J) are reported in Hertz (Hz) and are reported as $3J_{\text{H-H}}$ couplings between protons. Infrared (IR) spectra were obtained on a Perkin Elmer Spectrum 100 FTIR spectrometer operating in ATR mode. Small molecule mass spectra were obtained from the UCL mass spectroscopy service on an Orbitrap Q Exactive mass spectrometer.

LC-MS Method 1

Molecular masses of all protein conjugates were measured using an Agilent 6510 QTOF LC-MS system (Agilent, UK) (Method 1a) or an Agilent 6530 QTOF LC-MS system (Agilent, UK) (Method 1b).

Method 1a

The Agilent 1200 HPLC system was equipped with an Agilent PLRP-S, 1000A, 8 μM , 150 mm \times 2.1 120 mm column. 10 μL of a protein sample (diluted to 5 μM in LC-MS grade water) was separated on the column using mobile phase A (water-0.1% formic acid) and B (acetonitrile-0.1% formic acid) with an eluting gradient (Table S1) at a flow rate of 0.3 mL/min. The oven temperature was maintained at 60 °C. The column effluent was continuously electrosprayed into capillary ESI source of the Agilent 6510 QTOF mass spectrometer.

Time (min)	Solvent A (%)	Solvent B (%)
0	85	15

3	68	32
14	65	35
18	5	95
22	85	15
25	85	15

Table S1. LC-MS mobile phase gradient for A/B elution.

The Agilent 6510 QTOF mass spectrometer was operated in a positive polarity mode, coupled with an ESI ion source. The ion source parameters were set up with a VCap of 3500 V, a gas temperature at 350 °C, a dry gas flow rate at 10 L/min and a nebulizer of 30 psig. MS ToF was acquired under conditions of a fragmentor at 350 V, a skimmer at 65 V and an acquisition rate at 0.5 spectra/s in a profile mode, within a scan range between 700 and 4500 m/z. The data were analysed by deconvoluting a spectrum to a zero-charge mass spectrum using a maximum entropy deconvolution algorithm using MassHunter Qualitative Analysis version B.07.00.

Method 1a*

The Agilent 1200 HPLC system was equipped with an Agilent PLRP-S, 1000A, 8 µM, 150 mm × 2.1 120 mm column. 10 µL of a protein sample (diluted to 0.2 mg/mL in LC-MS grade H₂O) was separated on the column using mobile phase A (water-0.1% formic acid) and B (acetonitrile-0.1% formic acid) with an eluting gradient (Table S2) at a flow rate of 0.3 mL/min. The oven temperature was maintained at 60 °C. The column effluent was continuously electrosprayed into capillary ESI source of the Agilent 6510 QTOF mass spectrometer.

Time (min)	Solvent A (%)	Solvent B (%)
0	75	25
2	75	25
16	1	99
18	1	99
18.1	75	25
20	75	25

Table S2. LCMS mobile phase gradient for A/B elution.

The Agilent 6510 QTOF mass spectrometer was operated in a positive polarity mode, coupled with an ESI ion source. The ion source parameters were set up with a VCap of 4000 V, a gas temperature at 350 °C, a dry gas flow rate at 10 L/min and a nebuliser of 35 psig. MS ToF was acquired under conditions of a fragmentor at 175 V, a skimmer at 65 V and an acquisition rate at 1 spectra/s in a profile mode, within a scan range between 100 and 3100 m/z. The data were analysed by deconvoluting a spectrum to a zero-charge mass spectrum using a maximum entropy deconvolution algorithm using MassHunter Qualitative Analysis version B.07.00.

Method 1b

Agilent 1290 Infinity II UHPLC system was equipped with an Agilent PLRP-s, 1000 Å, 8 µm 50 mm × 2.1 mm column. 2 µL of protein sample (diluted to 5 µM in LC-MS grade water) was separated on the column using mobile phase A (water, 0.1% formic acid) and B (acetonitrile, 0.1% formic acid) with an eluting gradient (shown below) at a flow rate of 0.8 mL/min. Oven temperature was maintained at 60 °C. LC-MS mobile phase gradient for A/B elution:

Time (min)	Solvent A (%)	Solvent B (%)
0	80	20
1	80	20
6.5	40	60
7.5	40	60
7.6	80	20
8.5	80	20

Table S3. LC-MS mobile phase gradient for A/B elution.

Agilent 6530 QTOF mass spectrometer was operated in positive polarity mode, coupled with an ESI ion source. The ion source parameters were set up with a VCap of 4000 V, a gas temperature at 350 °C, a dry gas flow rate at 10 L/min and a nebulizer of 35 psig. MS TOF was acquired under conditions of a fragmentor at 175 V, a skimmer at 65 V and an acquisition rate at 1 spectra/s in a profile mode, within a scan range between 100 and 7000 m/z. The data were then analysed by deconvoluting a spectrum to a zero-charge mass spectrum using a maximum entropy deconvolution algorithm within the MassHunter software version B.07.00.

LC-MS analyses are shown as mentioned here: TIC LC-MS trace (top), non-deconvoluted LC-MS trace (upper middle), wide range deconvoluted MS data (lower middle) and zoomed in deconvoluted data (bottom) for each species. It is acknowledged that the signal intensity of the mass spectra of conjugates decreases as one goes through a reaction sequence - we believe this is due to the loss material over the sequence of steps.

Antibody samples (40 µL, 5 µM, in ammonium acetate buffer (0.2 M, pH 6.8)/water) were deglycosylated by incubating with PNGase F (0.67 µL, New England BioLabs) at 22 °C for 16 h.

LC-MS Method 2

LCMS was performed using a Waters Acquity UPLC connected to a Waters Acquity Single Quad Detector (SQD). Separations were performed on Column: XBridge OST C18 Column, 130Å, 2.5 µm, 2.1 mm X 50 mm at a flow rate of 0.6 mL/min; with UV detection wavelength set at 214 nm; with a mobile phase consisting of 0.1% formic acid in water (solvent A) and 0.1% formic acid in acetonitrile (solvent B). The eluting gradient is shown below (Table S4). Mass spectra were analysed using MassLynx software.

Time (min)	Solvent A (%)	Solvent B (%)
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0	95	5
0.5	95	5
3.5	5	95
4	5	95
4.5	95	5
5	95	5

Table S4. LCMS mobile phase gradient for A/B elution.

UV-Vis Spectroscopy

UV-Vis spectroscopy was used to determine protein concentrations and PARs (PDARs/FARs) using a NanoDrop™ One microvolume spectrophotometer operating at room temperature. Sample buffer was used as blank for baseline correction. Extinction coefficients for proteins (at A_{280}) and payloads (at $A_{\lambda_{Max}}$) are listed below. Where a value was not available (either from the literature or the chemical datasheet), one was calculated from the protein sequence using ProtParam. A correction factor was applied in the event that the conjugated payload had a competing absorption at A_{280} (Table S5). PAR values were calculated by comparing concentrations of the payloads and the protein (calculated with corrected A_{280} values).

Molecule	Extinction Coefficient $\epsilon_{\lambda_{max}}$ ($M^{-1} cm^{-1}$)	Correction Factor at A_{280}
Trastuzumab	215,000 (ϵ_{280}) ²	-
GFP S147C	20,500 (ϵ_{280}) ³	-
PD Scaffold	9,100 (ϵ_{335}) ²	0.25
Azide-fluor 488	74,000 (ϵ_{505})	0.11
BP Fluor 568	88,000 (ϵ_{578})	0.46
BP Fluor 647	270,000 (ϵ_{671})	0.03

Table S5. Extinction coefficients of proteins and payloads used.

Concentration (c) and PDARs of conjugates were calculated by UV-Vis using the following equations:

$$A_{280} = c(\epsilon_{280}(Ab) + 0.25 \cdot \epsilon_{335}(PD))$$

$$PDAR = \frac{\epsilon_{280}(Ab) \cdot A_{335}}{A_{280} \cdot \epsilon_{335}(PD) - 0.25 \cdot A_{335} \cdot \epsilon_{335}(PD)}$$

FARs were calculated using the following equation:

$$FAR = \frac{A_{\lambda_{max}}(\epsilon_{280}(Ab) + 0.25 \cdot n \cdot \epsilon_{335}(PD))}{\epsilon_{\lambda_{max}}(fluorophore)(A_{280} - 0.11 \cdot A_{\lambda_{max}})}$$

where n = PDAR as calculated directly after re-bridging and λ_{Max} = wavelength of max absorption for the fluorophore.

SDS-PAGE

Non-reducing glycine-SDS-PAGE 12% or 4–15% acrylamide gels were performed following standard lab procedures. A 6% stacking gel was used and a broad-range molecular weight marker (10–250 kDa, Prestained PageRuler Plus Protein Standards, ThermoScientific) was run alongside the samples to estimate protein weights. Samples (3 μ L at \sim 6 μ M) were mixed with loading buffer (3 μ L, composition for 5 \times SDS: 1 g SDS, 3 mL glycerol, 6 mL 0.5 M Tris buffer pH 6.8, 2 mg bromophenol blue in 10 mL), heated at 80 $^{\circ}$ C for 5 min, and centrifuged at 10,000 rcf for 5 min. Samples were subsequently loaded into the wells in a volume of 6 μ L. All gels were run at a constant voltage of 200 V for 60 min using 1 \times SDS running buffer. Gels were stained using a modified Coomassie stain (25 g ammonium sulfate, 250 mg Coomassie G-250, 8.8 mL 85% orthophosphoric acid, 50 mL ethanol, made up to a total of 250 mL with dH₂O) at 21 $^{\circ}$ C for 16 h.

All ladders are from the same gel in which samples were run. In some cases, lanes are cut out where they were occupied by samples from other reactions run in different lanes in the same gel and are between the sample in question and the ladder. In this case, there will be a space between the image of the ladder and the image of the sample.

Densitometry was performed using imageJ software. Background subtraction was achieved using the built-in plugin with a rolling ball radius of 30, sliding paraboloid, and smoothing. Brightness and contrast settings were auto-adjusted within the software.

Antibodies

Native trastuzumab refers to Ontruzant[®].

The HC S378C and LC S168C engineered cysteine mutants were kindly provided by Merck Sharp & Dohme Corp. UK (MSD UK). The HC S378C thio-trastuzumab mutant refers to Humanized x [ERBB2_H] mAb (Trastuzumab VH S378C/VL WT, often referred to herein as HC S378C) IgG₁/Kappa antibodies. This mutant was originally supplied in buffer solution (20 mM sodium acetate, pH 5.5, 9% sucrose buffer). The LC S168C thio-trastuzumab mutant refers to Humanized x [ERBB2_H] mAb (Trastuzumab VH WT/VL S168C, often referred to herein as LC S168C) IgG₁/Kappa antibodies. This mutant was originally supplied in buffer solution (60 mM sodium acetate, 14 mM Tris; 3% sucrose, pH 5.5 buffer).

In the case of thio-antibody mutants, +1445 peaks on LC-MS correspond to incomplete deglycosylation.

Where 4 $^{\circ}$ C is written, the reaction was incubated in a ThermoMixer[®] set to 4 $^{\circ}$ C, however, in many cases the actual temperature of the ThermoMixer[®] was 7–12 $^{\circ}$ C due to difficulty cooling down.

Antibody conjugate isomers

We note that the formation of half antibody conjugate species, owing to some non-native PD rebridging in the hinge region when reacting the reduced native disulfide bonds of thio-antibodies (or conjugates) thereof with BCN PD **1** and/or ArN₃ bisPD **2** or derivatives thereof (in all relevant experiments described below), is likely, but is typical when using disulfide rebridging reagents to rebridge antibody disulfide bonds.

Synthesis

Synthesis of BCN PD 1

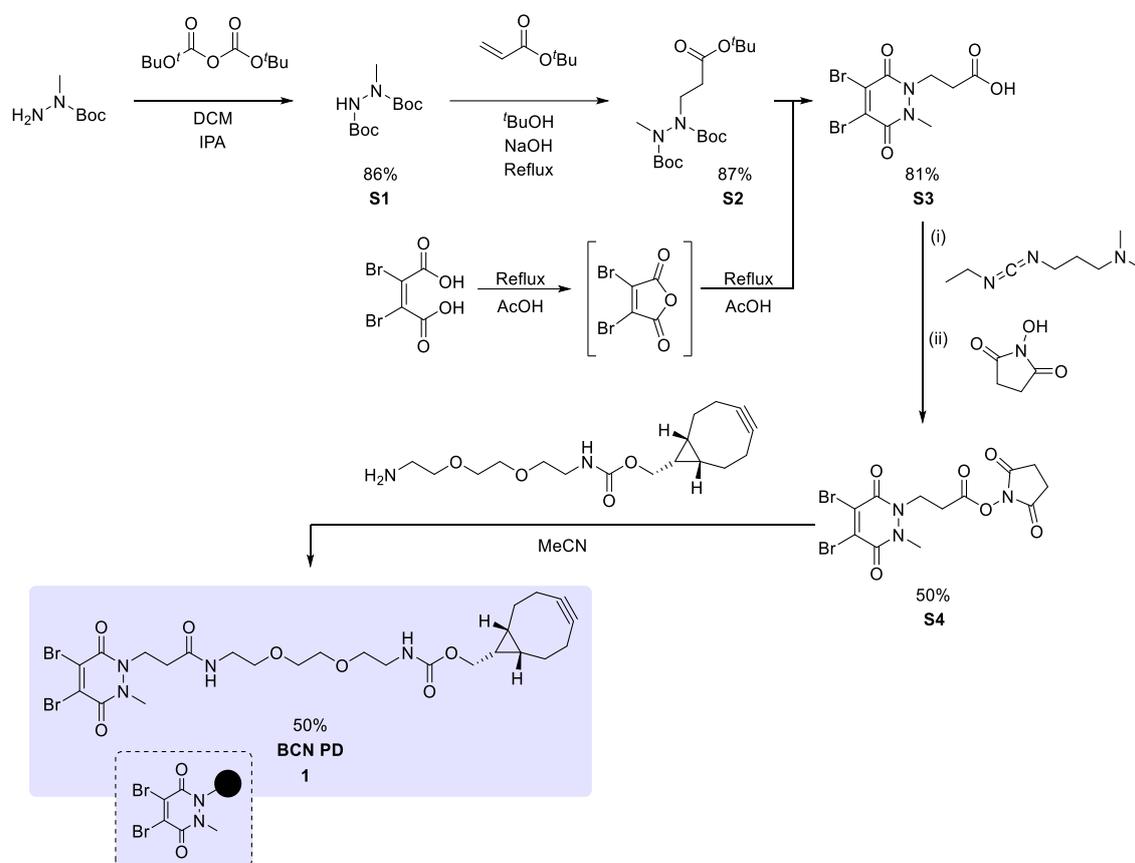
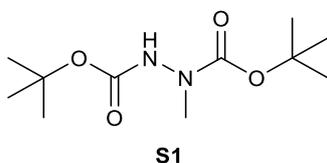


Figure S1. Synthetic Route to BCN PD, **1**. Firstly, 1-boc-1-methylhydrazine is protected using Boc₂O, to form protected hydrazine **S1**. *Tert*-butyl acrylate is then reacted with **S1** to form the acid hydrazine **S2**, which is refluxed with dibromomaleic anhydride to form MetPac **S3**. MetPac **S3** is activated with EDC then NHS and the methyl/NHS ester PD **S4** isolated. **S4** is subsequently reacted with BCN amine to form **1**.

Di-*tert*-butyl-1-methylhydrazine-1,2-dicarboxylate **S1**²

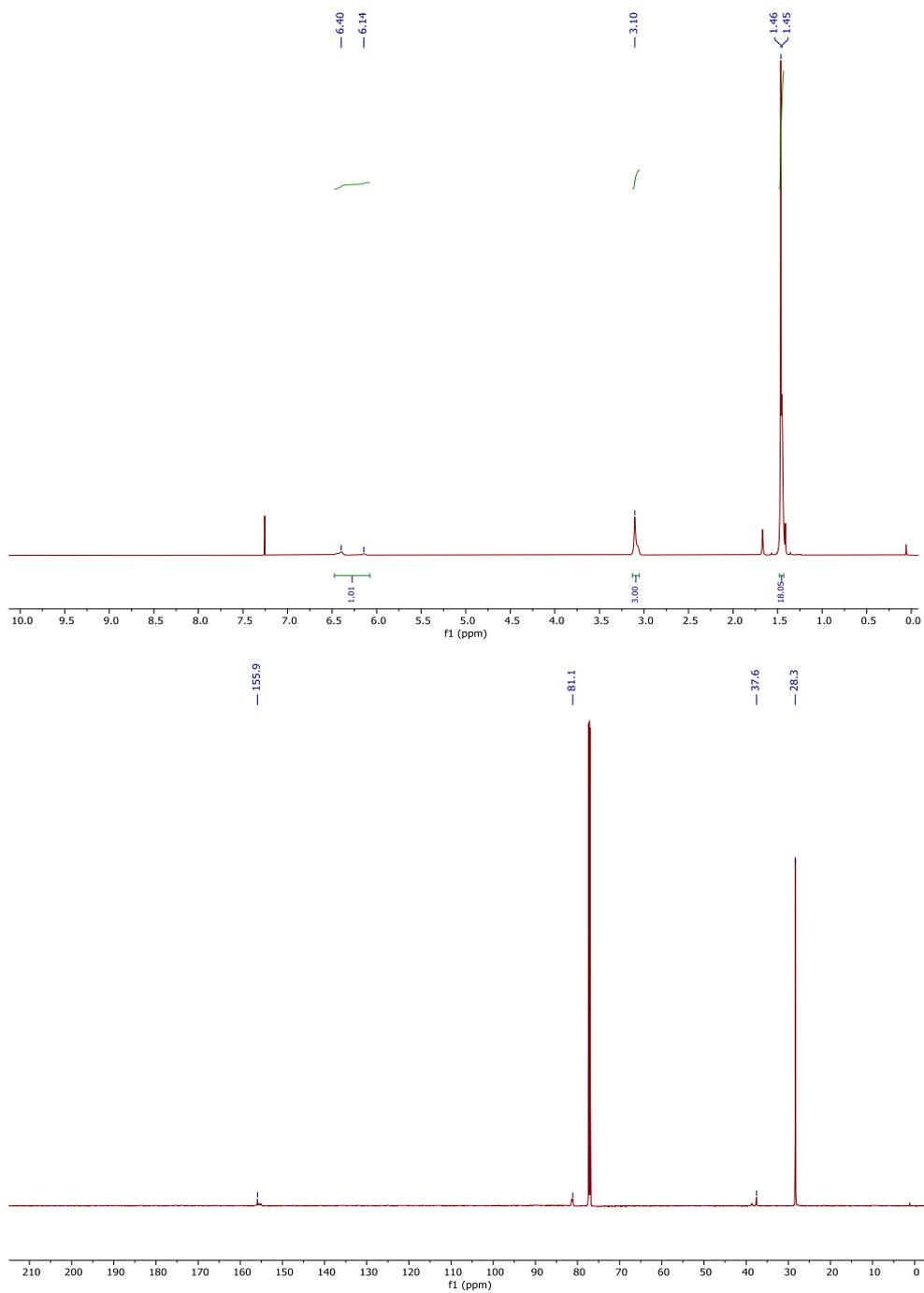


To a solution of 1-boc-1-methylhydrazine (3.00 mL, 20.2 mmol) in IPA (16 mL) was added dropwise over 30 min di-*tert*-butyl decarbonate (5.00 g, 22.9 mmol) pre-dissolved in DCM (12 mL). The reaction was then stirred at 21 °C for 18 h. Following this, the solvents were removed *in vacuo* and the crude residue purified by flash column chromatography (0% to 20% EtOAc/cyclohexane) to afford di-*tert*-butyl-1-methylhydrazine-1,2-dicarboxylate **S1** (4.29 g, 17.4 mmol, 86%) as a white solid.

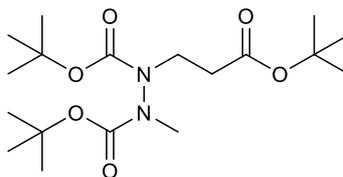
$^1\text{H NMR}$ (600 MHz, CDCl_3 , major rotamer) δ 6.40 (s, 1H) 3.10 (s, 3H), 1.46 (s, 18H).

$^{13}\text{C NMR}$ (150 MHz, CDCl_3 , major rotamer) δ 155.9 (C), 81.1 (C), 37.6 (CH_3), 28.3 (CH_3).

IR (solid) 3314, 2980, 2933, 1702 cm^{-1} .



Di-*tert*-butyl-1-(3-(*tert*-butoxy)-3-oxopropyl)-2-methylhydrazine-1,2-dicarboxylate **S2**²



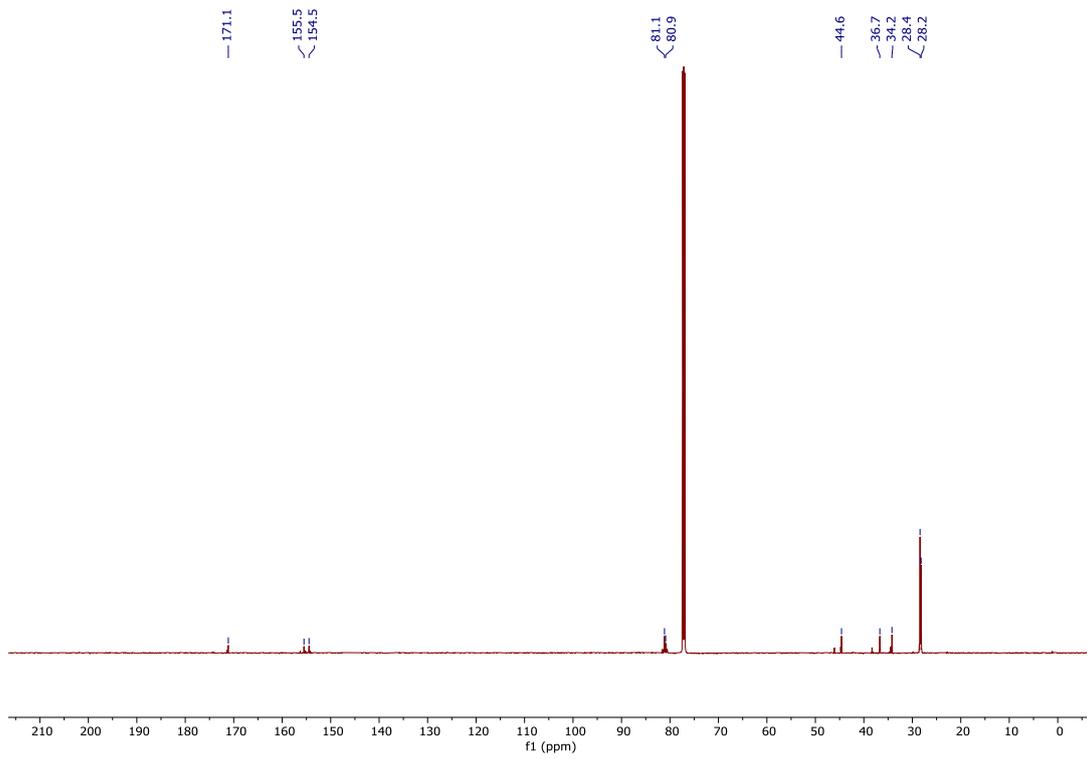
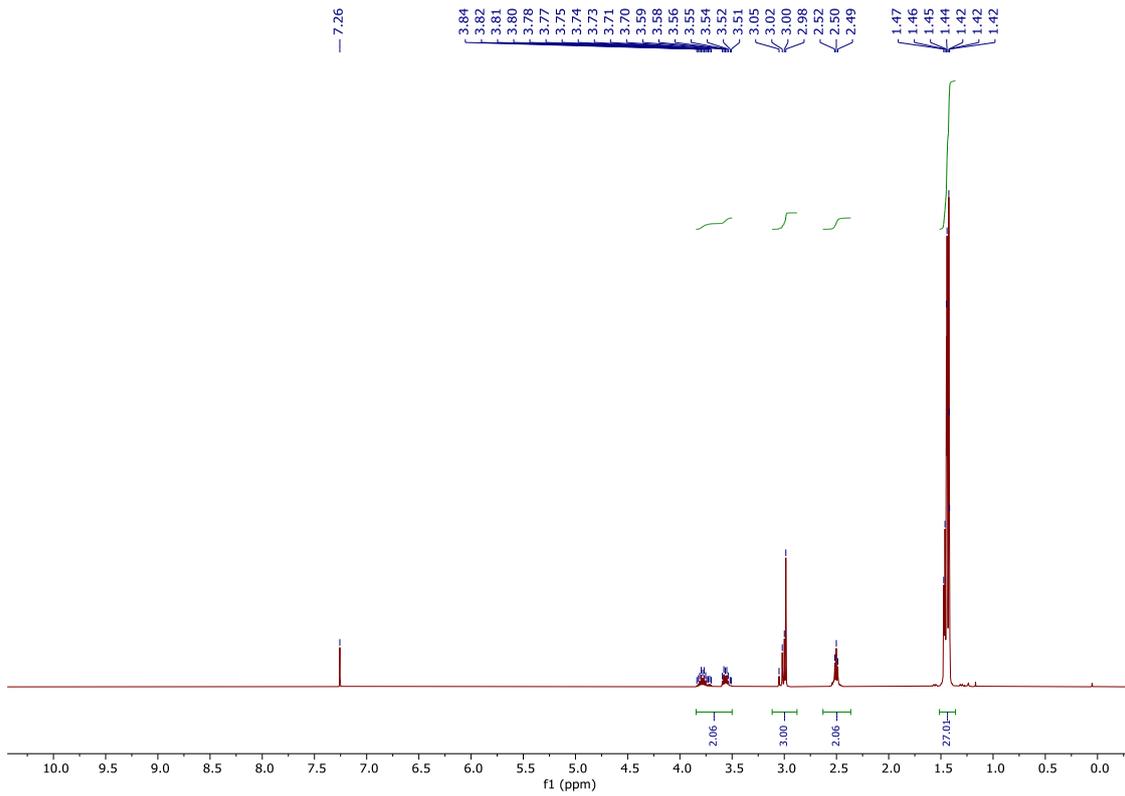
S2

To a solution of di-*tert*-butyl 1-methylhydrazine-1,2-dicarboxylate **S1** (3.75 g, 15.2 mmol) in *tert*-butanol (25 mL), was added 2 M NaOH (0.5 mL), and the reaction mixture stirred at 21 °C for 10 min. After this, *tert*-butyl acrylate (6.63 mL, 45.7 mmol) was added to the solution and the reaction mixture was heated under reflux for 68 h. The solvent was then removed *in vacuo* and the product suspended in water (50 mL). The product was then extracted into ethyl acetate (8 × 50 mL) and dried (MgSO₄) to give di-*tert*-butyl-1-(3-(*tert*-butoxy)-3-oxopropyl)-2-methylhydrazine-1,2-dicarboxylate **S2** as a clear oil (4.95 g, 13.2 mmol, 87%).

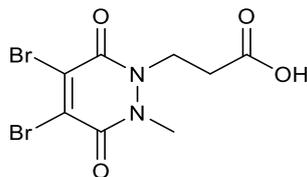
¹H NMR (600 MHz, CDCl₃, rotamers) δ 3.84–3.51 (m, 2H), 3.05–2.98 (m, 3H), 2.52–2.49 (m, 2H), 1.47–1.42 (m, 27H).

¹³C NMR (150 MHz, CDCl₃) δ 171.1 (C), 155.5 (C), 154.5 (C), 81.1 (C), 80.9 (C), 44.6 (CH₂), 36.7 (CH₃), 34.2 (CH₂), 28.4 (CH₃), 28.2 (CH₃).

IR (thin film) 2977, 2933, 1709 cm⁻¹.



3-(4,5-Dibromo-2-methyl-3,6-dioxo-3,6-dihydropyridazin-1(2H)-yl) propanoic acid **S3**²



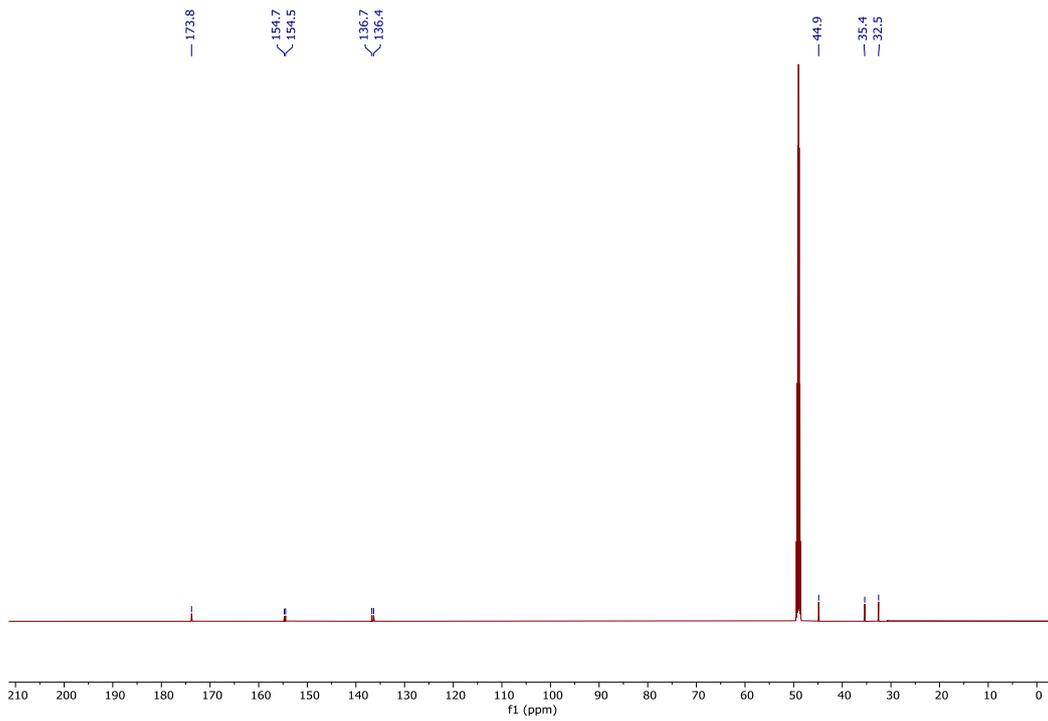
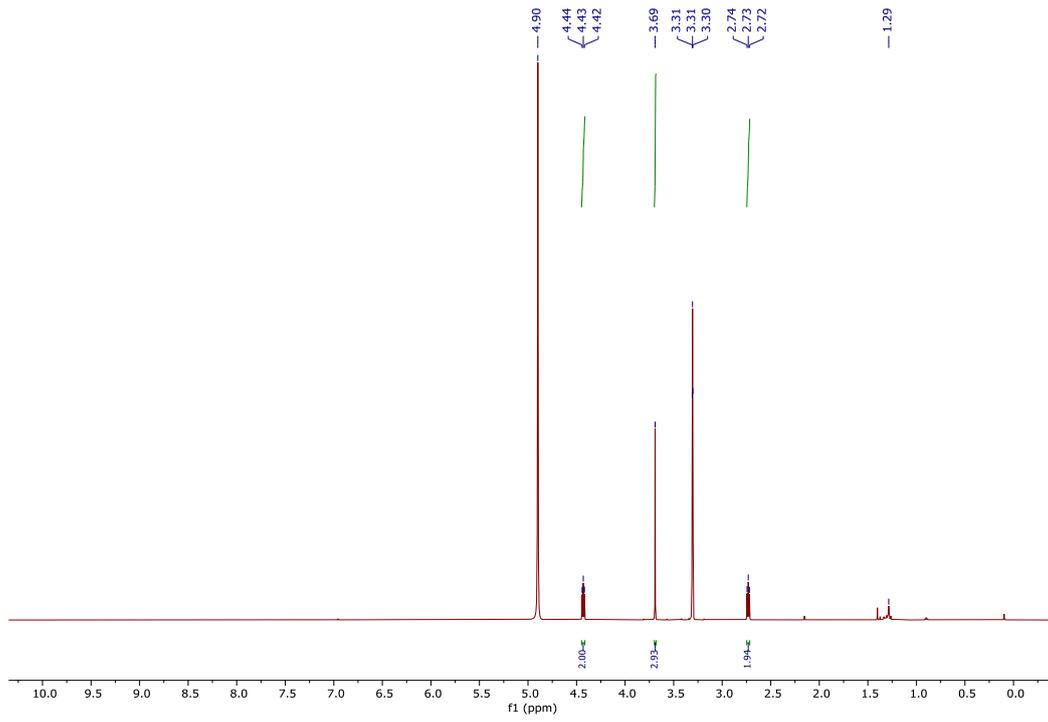
S3

Dibromomaleic acid (5.32 g, 19.4 mmol) was dissolved in AcOH (100 mL) and the solution heated under reflux for 30 min. To this solution was added di-*tert*-butyl-1-(3-(*tert*-butoxy)-3-oxopropyl)-2-methylhydrazine-1,2-dicarboxylate **S2** (6.08 g, 16.2 mmol) and the reaction heated under reflux for a further 6 h. After this time, the reaction mixture was concentrated *in vacuo* with toluene co-evaporation (3 × 30 mL, as an azeotrope) and chloroform co-evaporation (30 mL) and the crude residue purified by flash column chromatography (50% to 100% EtOAc/cyclohexane (1% AcOH)) to afford 3-(4,5-dibromo-2-methyl-3,6-dioxo-3,6-dihydropyridazin-1(2H)-yl) propanoic acid **S3** (4.66 g, 13.1 mmol, 81%) as a yellow solid.

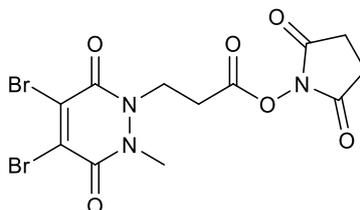
¹H NMR (600 MHz, MeOD) δ 4.43 (t, $J = 7.3$ Hz, 2H), 3.69 (s, 3H), 2.74 (t, $J = 7.3$ Hz, 2H).

¹³C NMR (150 MHz, MeOD) δ 173.8 (C), 154.7 (C), 154.5 (C), 136.7 (C), 136.4 (C), 44.9 (CH₃), 35.4 (CH₂), 32.5 (CH₂).

IR (thin film) 3226, 2945, 2835, 1731, 1660, 1572 cm⁻¹.



2,5-Dioxopyrrolidin-1-yl 3-(4,5-dibromo-2-methyl-3,6-dioxo-3,6-dihydropyridazin-1(2H)-yl) propanoate **S4**²



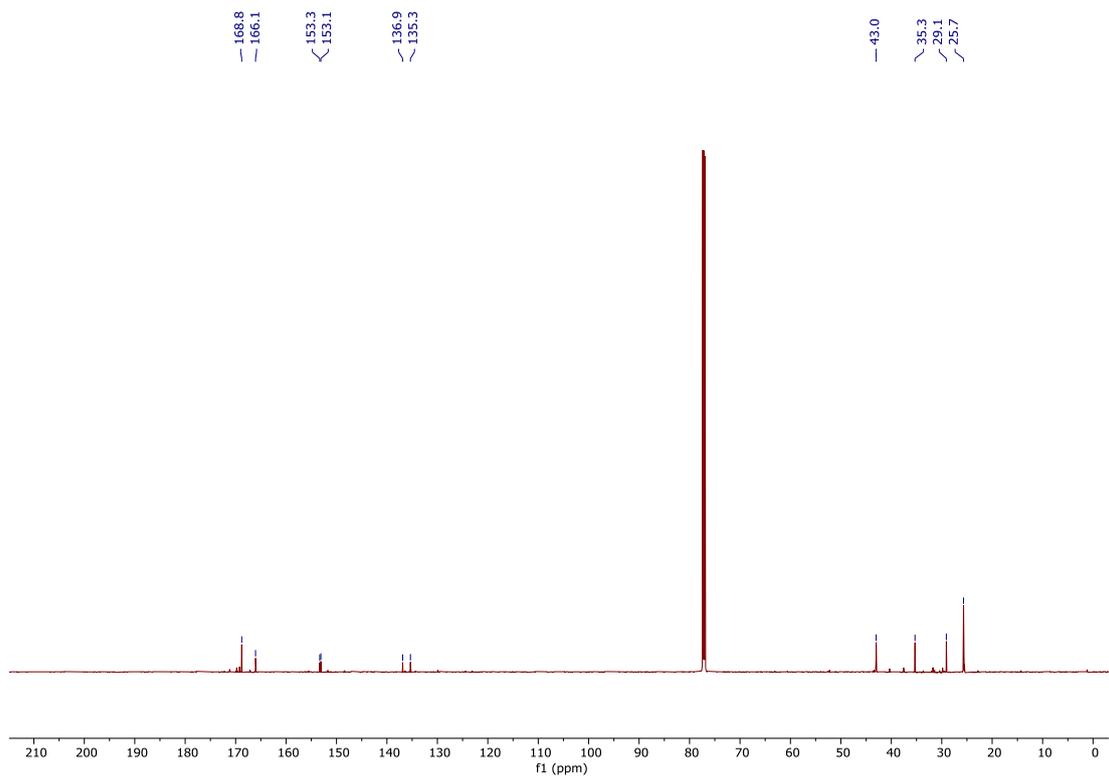
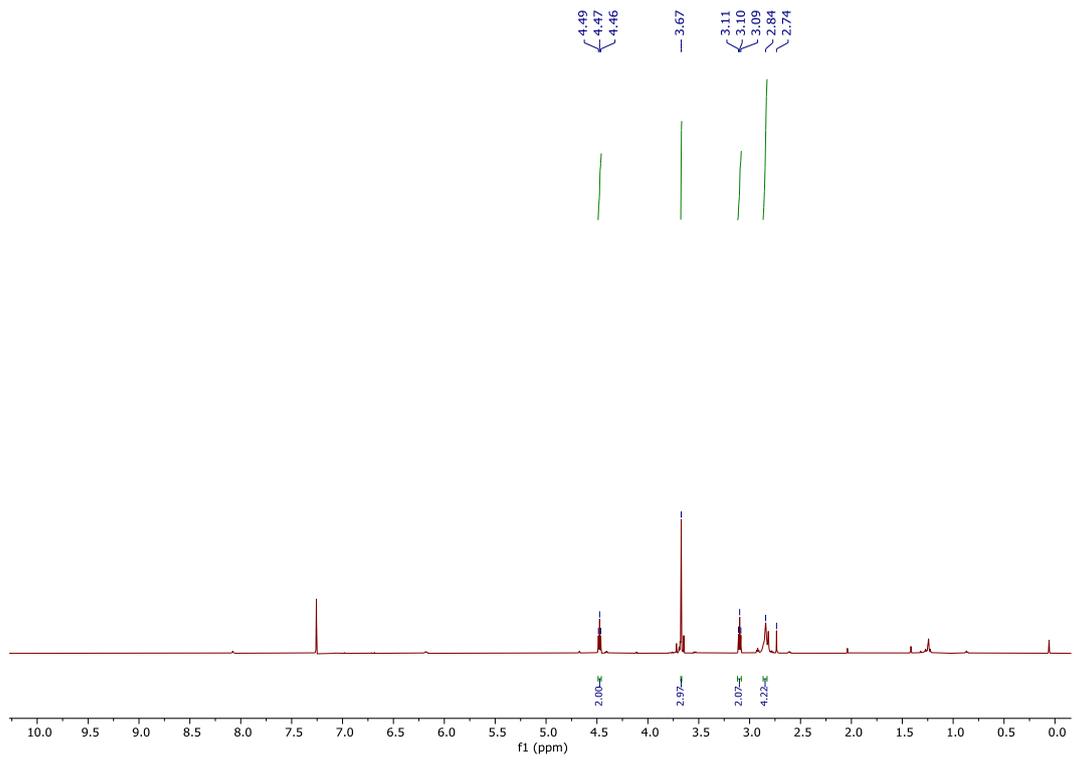
S4

To a solution of 3-(4,5-dibromo-2-methyl-3,6-dioxo-3,6-dihydropyridazin-1(2H)-yl) propanoic acid **S3** (704 mg, 1.98 mmol) in THF (20 mL), pre-cooled to 0 °C, was added *N*-(3-dimethylaminopropyl)-*N'*-ethylcarbodiimide (414 mg, 2.16 mmol). The homogeneous solution was then stirred at 0 °C for 30 min. Following this, was added *N*-hydroxysuccinimide (249 mg, 2.16 mmol) and the reaction stirred at 21 °C for a further 18 h. The solvent was then removed *in vacuo* and the crude residue dissolved in DCM. This was then filtered, and the filtrate concentrated *in vacuo*. Purification of the crude residue by flash column chromatography (50% to 100% EtOAc/cyclohexane) afforded 2,5-dioxopyrrolidin-1-yl 3-(4,5-dibromo-2-methyl-3,6-dioxo-3,6-dihydropyridazin-1(2H)-yl) propanoate **S4** (488 mg, 0.99 mmol, 50%) as a yellow solid.

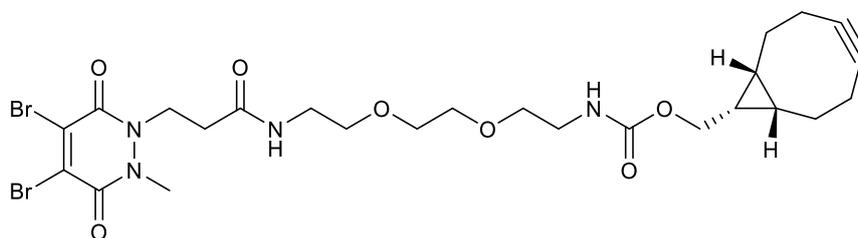
¹H NMR (600 MHz, CDCl₃) δ 4.47 (t, *J* = 6.9 Hz, 2H), 3.67 (s, 3H), 3.10 (t, *J* = 6.9 Hz, 2H), 2.84 (s, 4H).

¹³C NMR (151 MHz, CDCl₃) δ 168.8 (C), 166.1 (C), 153.3 (C), 153.1 (C), 136.9 (C), 135.3 (C), 43.0 (CH₂), 35.3 (CH₃), 29.1 (CH₂), 25.7 (CH₂).

IR (solid) 2919, 2852, 1810, 1780, 1732, 1624, 1577 cm⁻¹.



((1*R*,8*S*,9*S*)-Bicyclo[6.1.0]non-4-yn-9-yl)methyl (2-(2-(2-(3-(4,5-dibromo-2-methyl-3,6-dioxo-3,6-dihydropyridazin-1(2*H*)-yl)propanamido)ethoxy)ethoxy)ethyl)carbamate (BCN PD) 1²



1

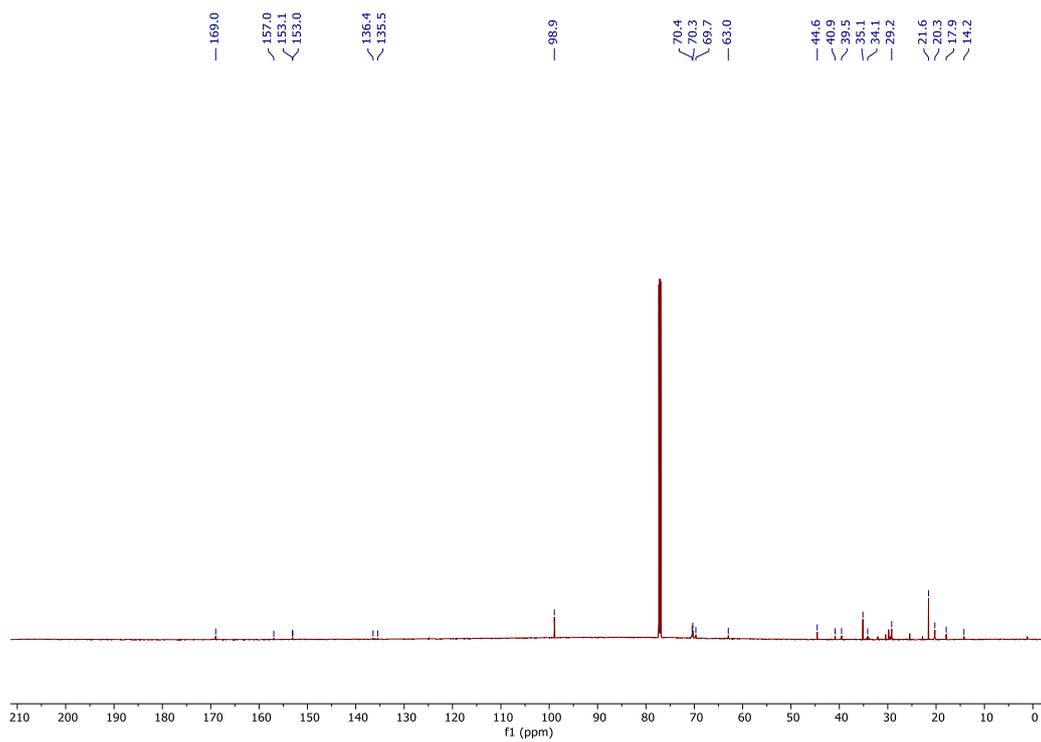
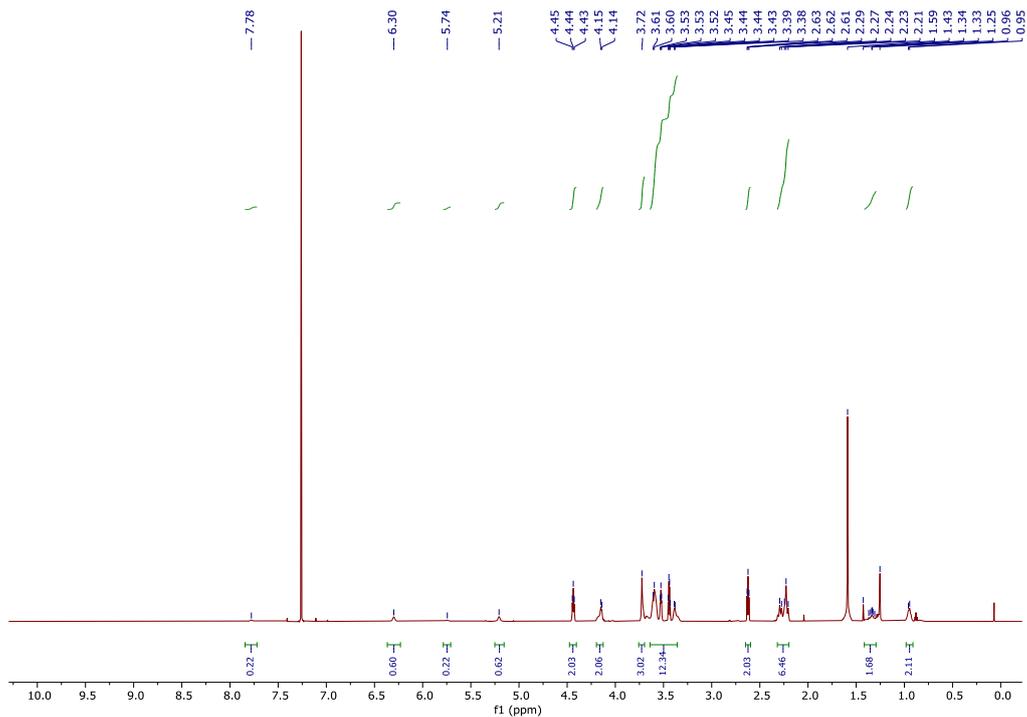
To a solution of 2,5-dioxopyrrolidin-1-yl 3-(4,5-dibromo-2-methyl-3,6-dioxo-3,6-dihydropyridazin-1(2*H*)-yl) propanoate **S4** (70 mg, 0.15 mmol) in dry MeCN (10 mL) was added *N*-[(1*R*,8*S*,9*S*)-bicyclo[6.1.0]non-4-yn-9-ylmethoxycarbonyl]-1,8-diamino-3,6-dioxaoctane (50 mg, 0.15 mmol), and the reaction stirred at 21 °C for 18 h. After this time, the solvent was removed *in vacuo* and the crude residue dissolved in CHCl₃ (50 mL). The organic solution was then washed with water (3 × 30 mL), followed by saturated aq. K₂CO₃ (30 mL). The organic layer was then dried (MgSO₄) and concentrated *in vacuo*. Purification of the crude residue by flash column chromatography (0% to 10% MeOH/EtOAc) afforded ((1*R*,8*S*,9*S*)-bicyclo[6.1.0]non-4-yn-9-yl)methyl (2-(2-(2-(3-(4,5-dibromo-2-methyl-3,6-dioxo-3,6-dihydropyridazin-1(2*H*)-yl)propanamido)ethoxy)ethoxy)ethyl) carbamate **1** (51.2 mg, 0.08 mmol, 51%) as a yellow gum.

¹H NMR (700 MHz, CDCl₃, rotamers) δ 7.78 (s, 0.2H), 6.30 (s, 0.6H), 5.74 (s, 0.2H), 5.21 (s, 0.6H), 4.44 (t, *J* = 7.0 Hz, 2H), 4.15–4.14 (m, 2H), 3.72 (s, 3H), 3.64–3.36 (m, 12H), 2.62 (t, *J* = 6.9 Hz, 2H), 2.33–2.19 (m, 6H), 1.61–1.57* (m, 2H), 1.43–1.26 (m, 3H), 0.95 (d, *J* = 9.0 Hz, 2H).

¹³C NMR (176 MHz, CDCl₃, rotamers) δ 169.0 (C), 157.0 (C), 153.1 (C), 153.0 (C), 136.4 (C), 135.5 (C), 98.9 (C), 70.4 (CH₂), 70.3 (CH₂), 69.7 (CH₂), 63.0 (CH₂), 44.6 (CH₂), 40.9 (CH₂), 39.5 (CH₂), 35.1 (CH₃), 34.1 (CH₂), 29.2 (CH₂), 21.6 (CH₂), 20.3 (CH₂), 17.9 (CH), 14.2 (CH).

IR (thin film) 3329, 2920, 2858, 1708, 1630, 1572, 1534 cm⁻¹.

*Behind water peak



Synthesis of ArN₃ bisPD 2

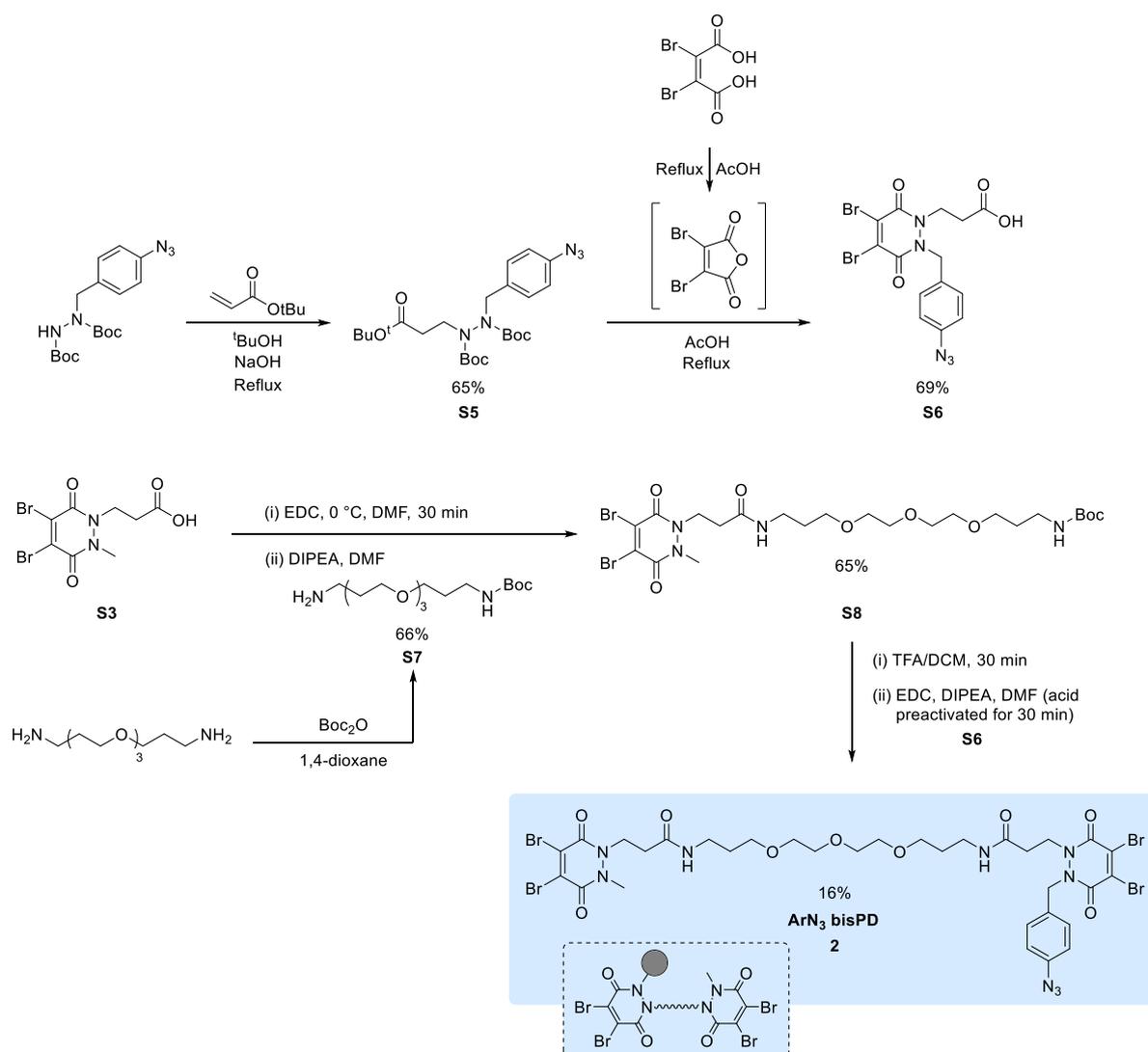
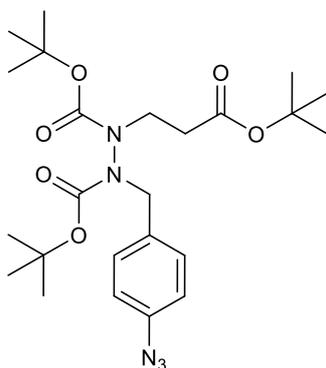


Figure S2. Synthetic Route to ArN₃ bisPD, **2**. Firstly, diBoc ArN₃ hydrazine (synthesised according to the literature)⁴ is reacted with *tert*-butyl acrylate to form acid/ArN₃ hydrazine **S5**, which is refluxed with dibromomaleic anhydride to form the ArN₃/Acid PD **S6**. 4,7,10-trioxa-1,13-tridecanediamine is then Boc-protected to form **S7**, which is coupled to **S3** to form MetPAC PEG₃ Boc amine **S8**. The Boc group is then removed and **S8** coupled to **S6** to form ArN₃ bisPD **2**.

Di-tert-butyl 1-(4-azidobenzyl)-2-(3-(tert-butoxy)-3-oxopropyl)hydrazine-1,2-dicarboxylate S5⁴



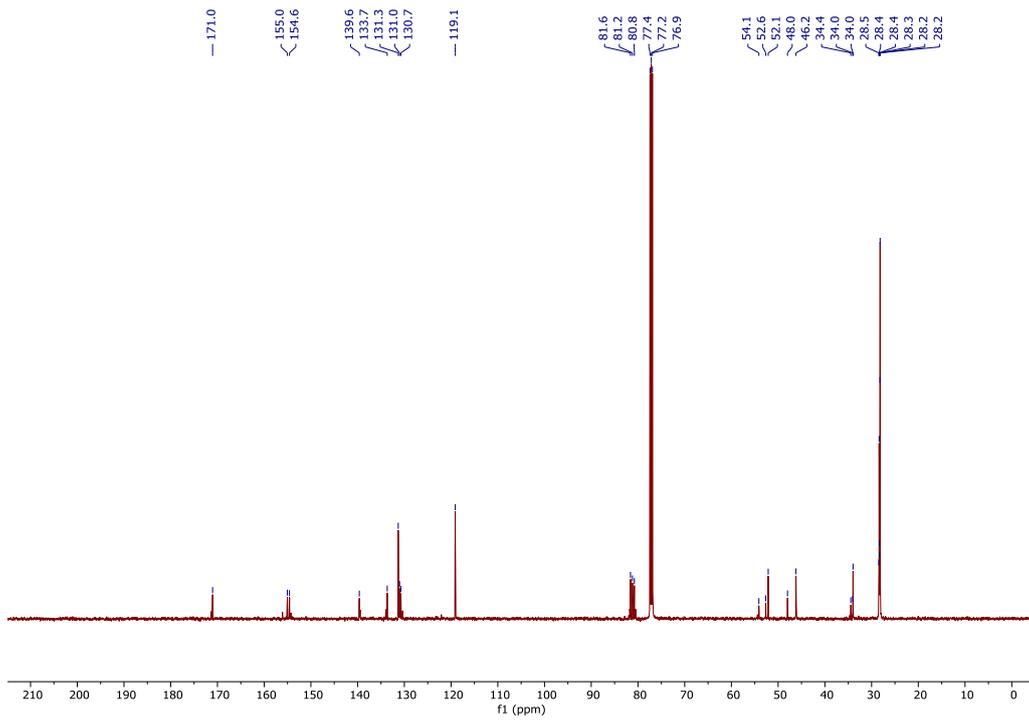
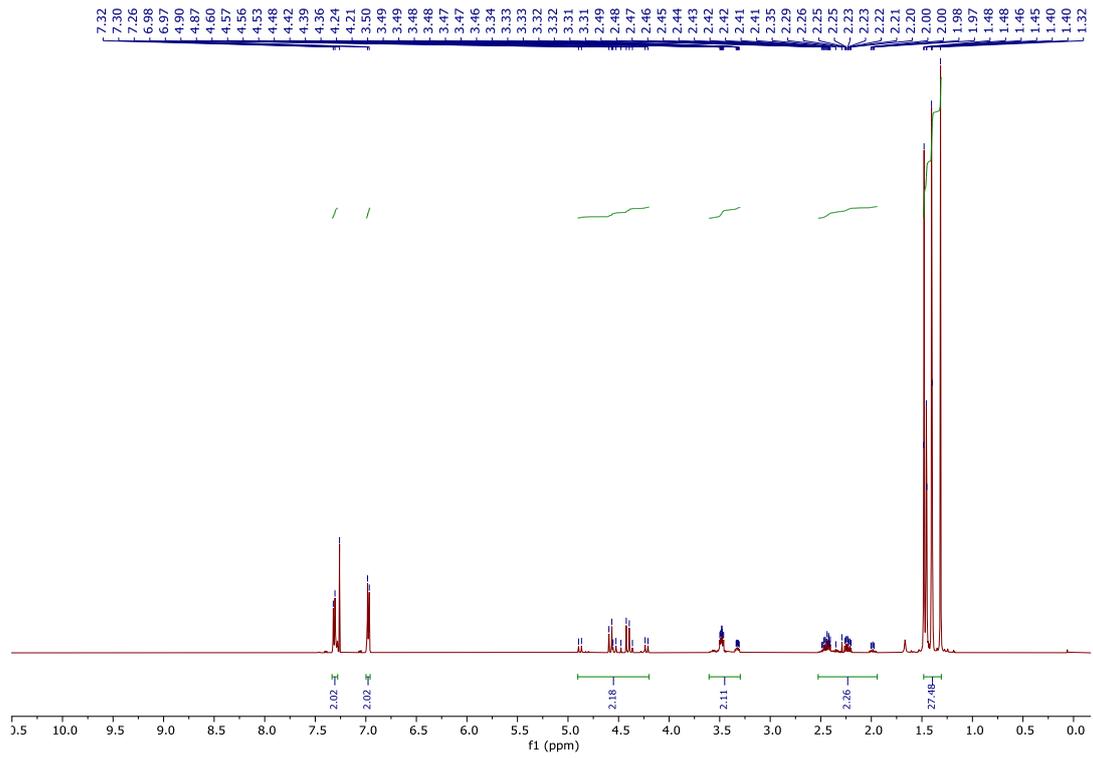
S5

To a solution of di-tert-butyl 1-(4-azidobenzyl)hydrazine-1,2-dicarboxylate (2 g, 5.51 mmol) in *t*BuOH (20 mL) was added 10% w/v NaOH (0.17 mL) and the solution stirred at 25 °C for 10 min. After this time, to the reaction mixture was added *tert*-butyl acrylate (1.60 mL, 11.02 mmol) and the solution stirred at 60 °C for 21.5 h. After this time, the solvent was removed *in vacuo* and the crude product dissolved in EtOAc (30 mL). The organic solution was then washed with water (5 × 100 mL) followed by brine (60 mL). The organic layer was then dried (MgSO₄) and concentrated *in vacuo*. The crude product was purified *via* flash column chromatography (0-40% EtOAc/cyclohexane) to afford di-tert-butyl 1-(4-azidobenzyl)-2-(3-(tert-butoxy)-3-oxopropyl)hydrazine-1,2-dicarboxylate **S5** (1.76 g, 3.58 mmol, 65%) as an orange oil.

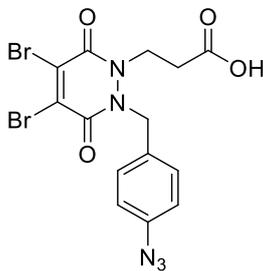
¹H NMR (500 MHz, CDCl₃, rotamers) δ 7.31 (d, *J* = 8.5 Hz, 2H), 6.97 (d, *J* = 8.4 Hz, 2H), 4.90–4.19 (m, 2H), 3.60–3.29 (m, 2H), 2.52–1.94 (m, 2H), 1.48–1.31 (m, 27H).

¹³C NMR (126 MHz, CDCl₃, rotamers) δ 171.0 (C), 155.0 (C), 154.6 (C), 139.6 (C), 133.7 (CH), 131.3 (CH), 131.0 (CH), 130.7 (CH), 119.1 (C), 81.6 (C), 81.2 (C), 80.8 (C), 54.1 (CH₂), 52.6 (CH₂), 52.1 (CH₂), 48.0 (CH₂), 46.2 (CH₂), 46.1 (CH₂), 34.5 (CH₃), 34.3 (CH₃), 34.0 (CH₃), 28.5 (CH₃), 28.4 (CH₃), 28.4 (CH₃), 28.3 (CH₃), 28.2 (CH₃), 28.2 (CH₃).

IR (thin film) 2977, 2934, 1705, 1518, 1453 cm⁻¹.



3-(2-(4-Azidobenzyl)-4,5-dibromo-3,6-dioxo-3,6-dihydropyridazin-1(2H)-yl)propanoic acid **S6**⁴



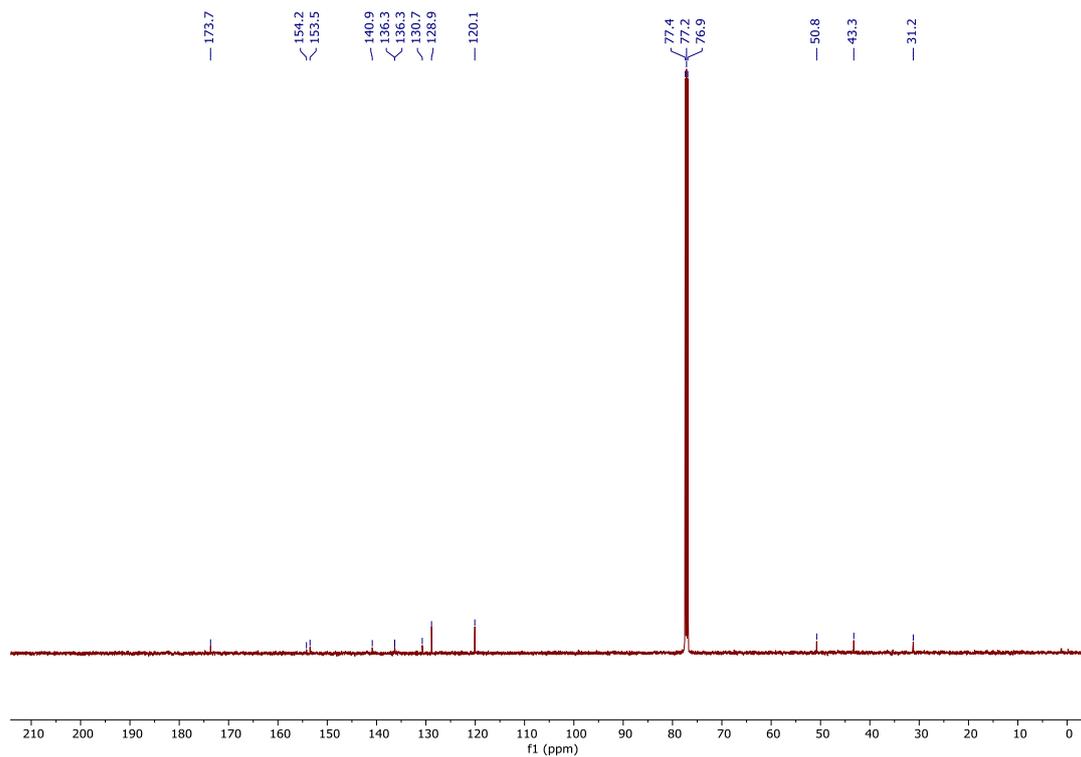
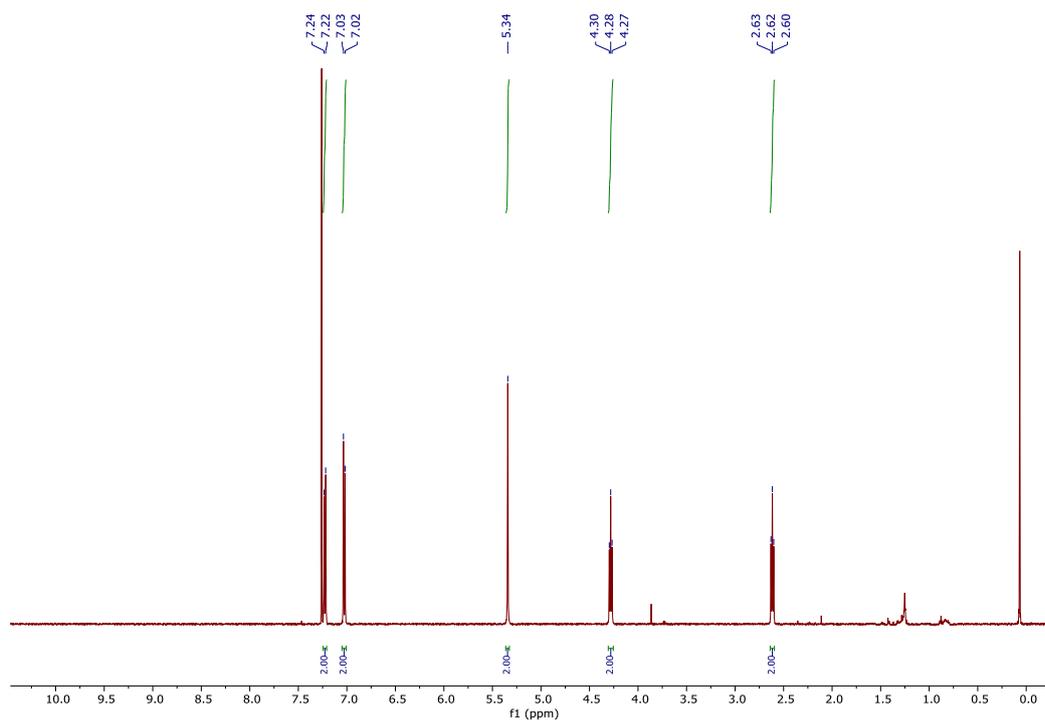
S6

Dibromomaleic acid (1.18 g, 4.30 mmol) in AcOH (35 mL) was heated under reflux for 30 min. After this time, to the reaction mixture, was added di-*tert*-butyl 1-(4-azidobenzyl)-2-(3-(*tert*-butoxy)-3-oxopropyl)hydrazine-1,2-dicarboxylate **S5** (1.76 g, 3.58 mmol), and the reaction stirred under reflux for 6 h. After this time, the solvent was removed *in vacuo* with toluene co-evaporation (3 × 30 mL, as an azeotrope) and then chloroform co-evaporation (3 × 30 mL). The crude residue was purified by flash column chromatography (20-80% EtOAc/cyclohexane, 1% AcOH) to give 3-(2-(4-azidobenzyl)-4,5-dibromo-3,6-dioxo-3,6-dihydropyridazin-1(2H)-yl)propanoic acid **S6** (1.17 g, 2.47 mmol, 69%) as a yellow solid.

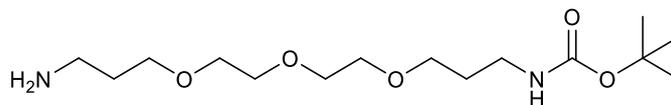
¹H NMR (500 MHz, CDCl₃) δ 7.23 (d, *J* = 8.5 Hz, 2H), 7.03 (d, *J* = 8.5 Hz, 2H), 5.34 (s, 2H), 4.28 (t, *J* = 7.1 Hz, 2H), 2.62 (t, *J* = 7.1 Hz, 2H).

¹³C NMR (126 MHz, CDCl₃) δ 173.7 (C), 154.2 (C), 153.5 (C), 140.9 (C), 136.3 (C), 136.3 (C), 130.7 (CH), 128.9 (CH), 120.1 (C), 50.8 (CH₂), 43.3 (CH₂), 31.2 (CH₂).

IR (solid) 3117, 2962, 2913, 2115, 1713, 1628, 1572, 1507 cm⁻¹.



Tert-Butyl (3-(2-(2-(3-aminopropoxy)ethoxy)ethoxy)propyl)carbamate **S7**



S7

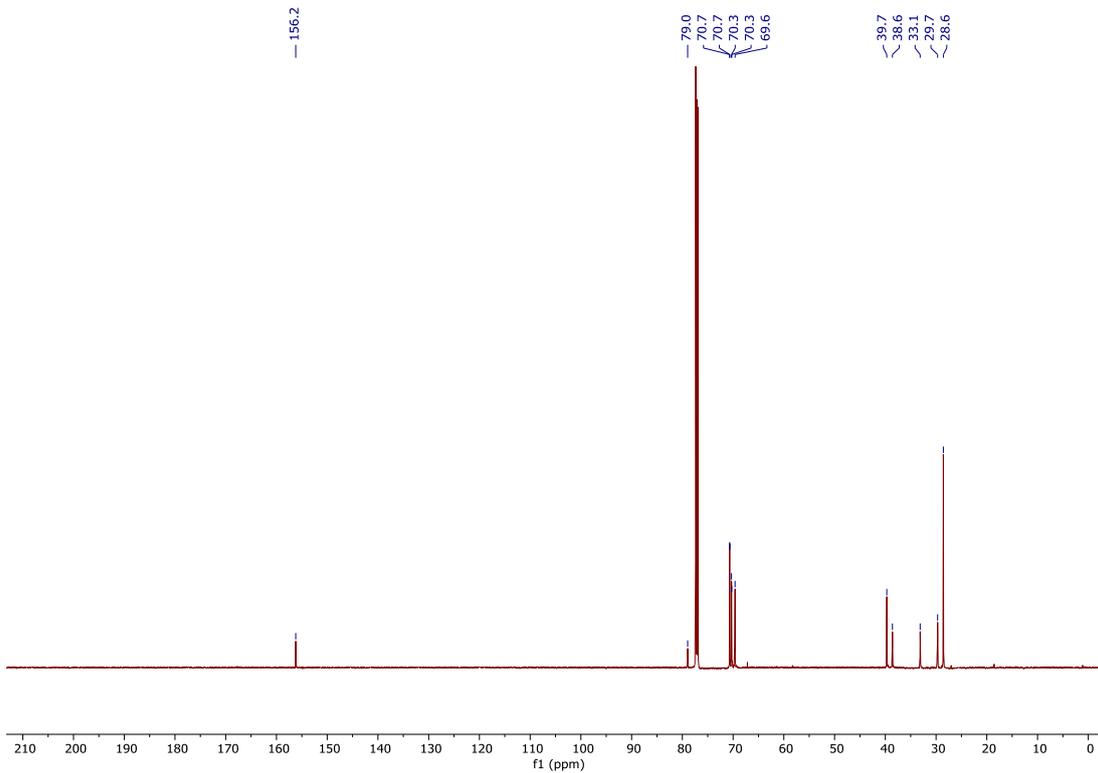
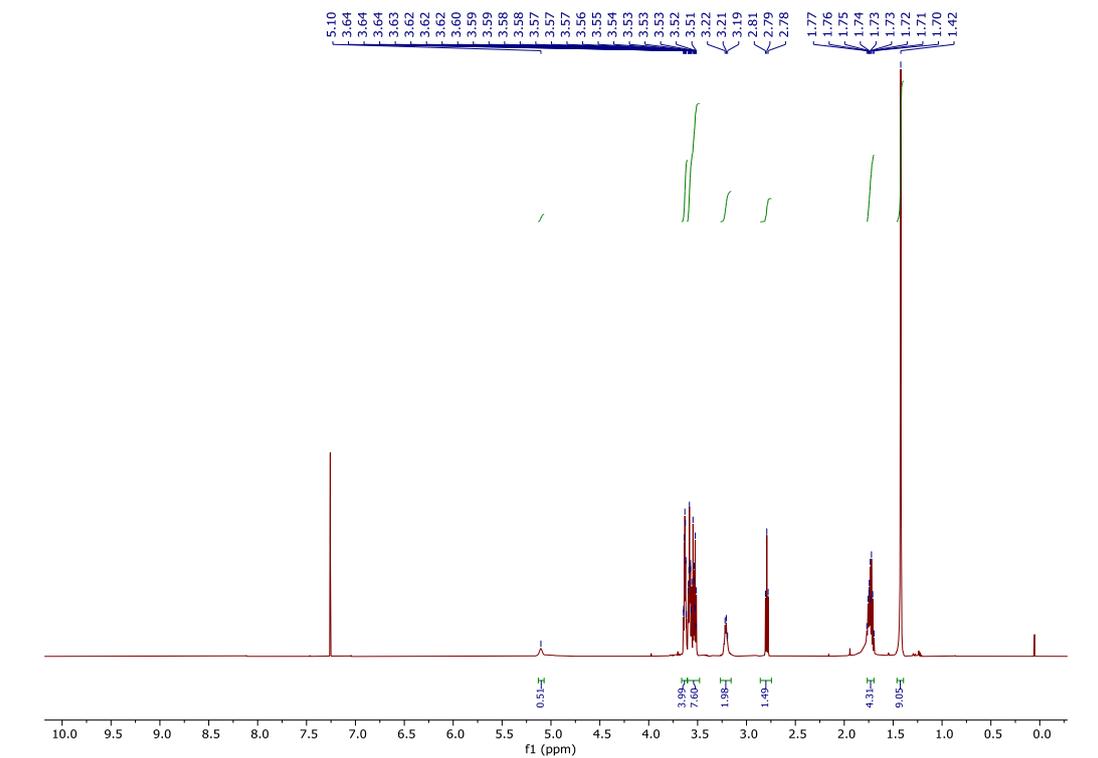
To a stirring solution of 3,3'-((oxybis(ethane-2,1-diyl))bis(oxy))bis(propan-1-amine) (4.05 g, 18.4 mmol) in 1,4-dioxane (30 mL) was added dropwise di-*tert*-butyl dicarbonate (0.5 g, 2.3 mmol, pre-dissolved in 1,4-dioxane (12.5 mL) over 2 h. After this time, the reaction mixture was stirred at 25 °C for a further 45 mins. Following this, the reaction mixture was concentrated *in vacuo*, the crude residue dissolved in water (30 mL), and the organics extracted into EtOAc (5 × 15 mL). The organics were combined, dried (MgSO₄) and concentrated *in vacuo* to give *tert*-butyl (3-(2-(2-(3-aminopropoxy)ethoxy)ethoxy)propyl)carbamate **S7** (502 mg, 2.36 mmol, 68%) as a clear yellow oil.

¹H NMR (500 MHz, CDCl₃) δ 5.10 (s, 0.5H*), 3.63 (dt, *J* = 5.2, 2.8 Hz, 4H), 3.60–3.48 (m, 8H), 3.21 (t, *J* = 6.3 Hz, 2H), 2.79 (t, *J* = 6.7 Hz, 1H), 1.73 (dt, *J* = 11.3, 6.4 Hz, 4H), 1.42 (s, 9H).

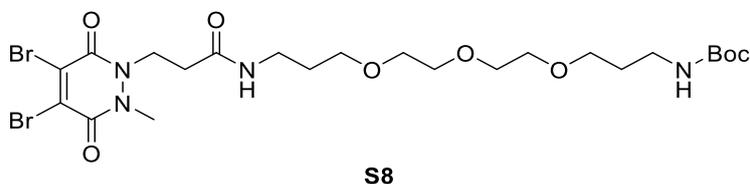
¹³C NMR (151 MHz, CDCl₃) δ 156.2 (C), 79.0 (C), 70.7 (CH₂), 70.7 (CH₂), 70.3 (CH₂), 70.3 (CH₂), 69.6 (CH₂), 39.7 (CH₂), 38.6 (CH₂), 33.1 (CH₂), 29.7 (CH₂), 28.6 (CH₃).

IR (thin film) 3353, 2923, 2865, 1694, 1563, 1516 cm⁻¹.

* Less than 1H potentially due to partial D-H exchange of the acidic NH amide proton in the NMR sample.



Tert-Butyl (17-(4,5-dibromo-2-methyl-3,6-dioxo-3,6-dihydropyridazin-1(2H)-yl)-15-oxo-4,7,10-trioxa-14-azaheptadecyl)carbamate **S8**²



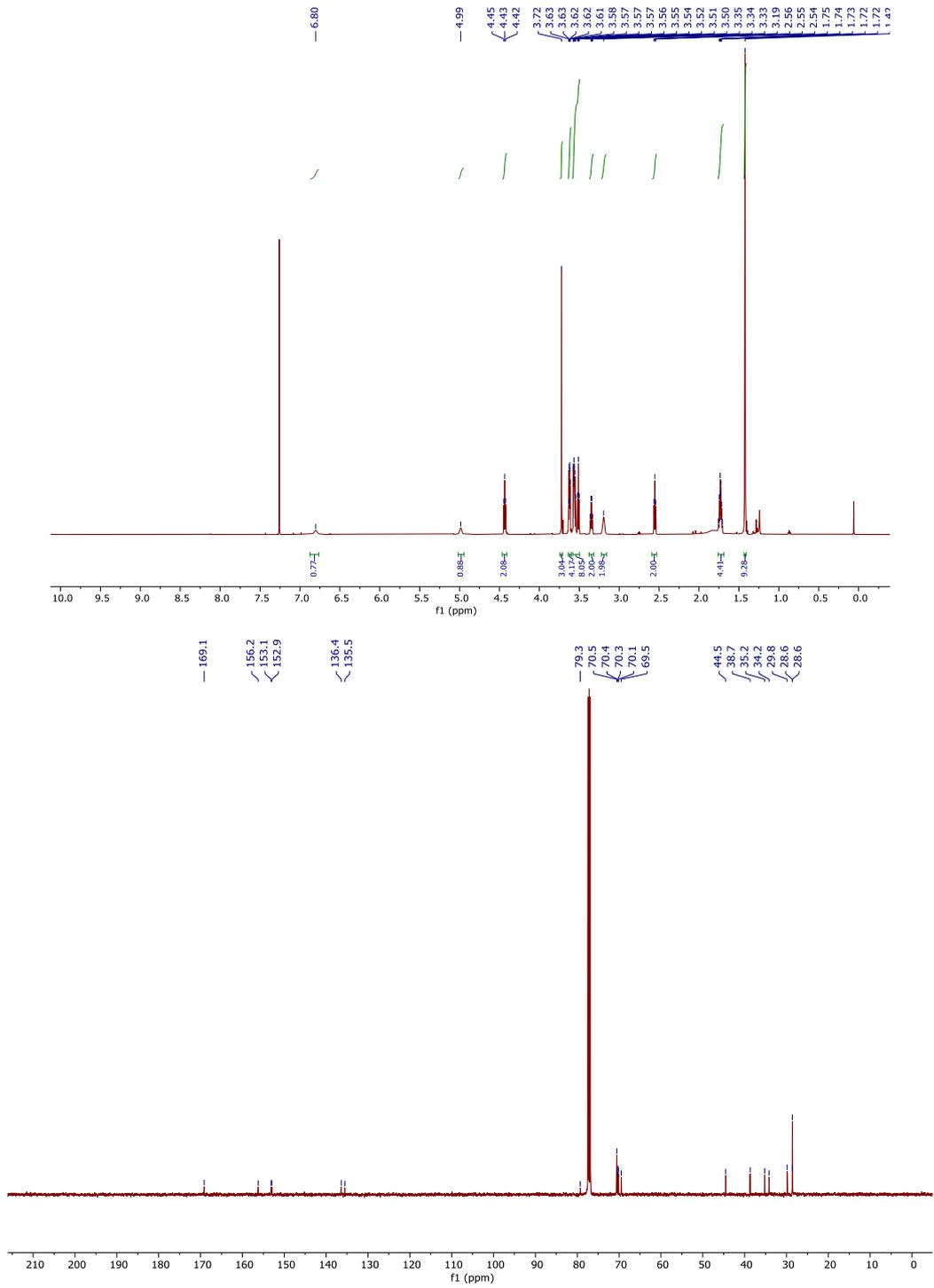
To a pre-cooled to 0 °C solution of 3-(4,5-dibromo-2-methyl-3,6-dioxo-3,6-dihydropyridazin-1(2H)-yl) propanoic acid **S3** (850 mg, 2.39 mmol) in anhydrous DMF (20 mL), was added EDC.HCl (458 mg, 2.39 mmol), and the reaction stirred under an inert atmosphere for 30 min. After this time, to the reaction mixture was added (3-(2-(2-(3-aminopropoxy)ethoxy)ethoxy)propyl)carbamate (696 mg, 2.17 mmol) **S7**, and the reaction allowed to warm to 25 °C and then stirred for 17 h. After this time, the solvent was removed *in vacuo* and the crude product diluted with water (100 mL). The organics were then extracted into EtOAc (4 × 50 mL). The organics were combined and washed with sat. aq. LiCl (100 mL), brine (70 mL), dried (MgSO₄) and then concentrated *in vacuo*. The crude product was purified *via* flash column chromatography (0-10% MeOH/EtOAc) to give *tert*-butyl (17-(4,5-dibromo-2-methyl-3,6-dioxo-3,6-dihydropyridazin-1(2H)-yl)-15-oxo-4,7,10-trioxa-14-azaheptadecyl)carbamate **S8** (926 mg, 1.41 mmol, 65%) as a yellow oil.

¹H NMR (600 MHz, CDCl₃) δ 6.80 (s, 0.8H*), 4.99 (s, 0.9H*), 4.43 (t, *J* = 6.8 Hz, 2H), 3.72 (s, 3H), 3.64–3.60 (m, 4H), 3.58–3.50 (m, 8H), 3.35 (q, *J* = 5.8 Hz, 2H), 3.19 (s, 2H), 2.55 (t, *J* = 6.9 Hz, 2H), 1.76–1.69 (m, 4H), 1.42 (s, 9H).

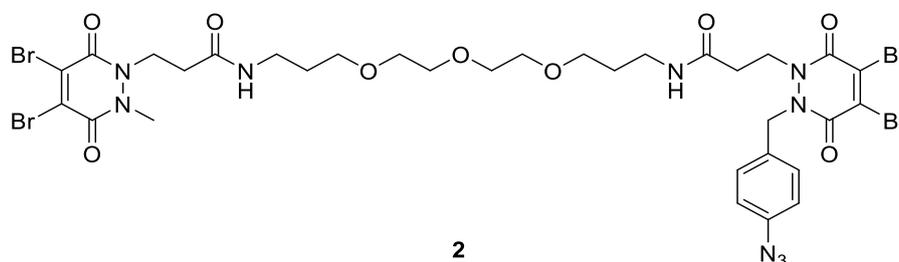
¹³C NMR (126 MHz, CDCl₃) δ 169.1 (C), 156.3 (C), 153.2 (C), 152.9 (C), 136.4 (C), 135.5 (C), 79.3 (C) 70.6 (CH₂), 70.4 (CH₂), 70.3 (CH₂), 70.1 (CH₂), 69.5 (CH₂), 44.5 (CH₂), 38.7 (CH₂), 35.2 (CH₂), 34.2 (CH₂), 29.8 (CH₃), 28.6 (CH₃), 28.6 (CH₃).

IR (thin film) 3507, 3497, 3334, 2921, 2869, 1692, 1627, 1571, 1529 cm⁻¹.

* Less than 1H potentially due to partial D-H exchange of the acidic NH amide proton in the NMR sample.



3-(2-(4-Azidobenzyl)-4,5-dibromo-3,6-dioxo-3,6-dihydropyridazin-1(2H)-yl)-N-(17-(4,5-dibromo-2-methyl-3,6-dioxo-3,6-dihydropyridazin-1(2H)-yl)-15-oxo-4,7,10-trioxa-14-azaheptadecyl)propanamide (ArN₃ bisPD) 2



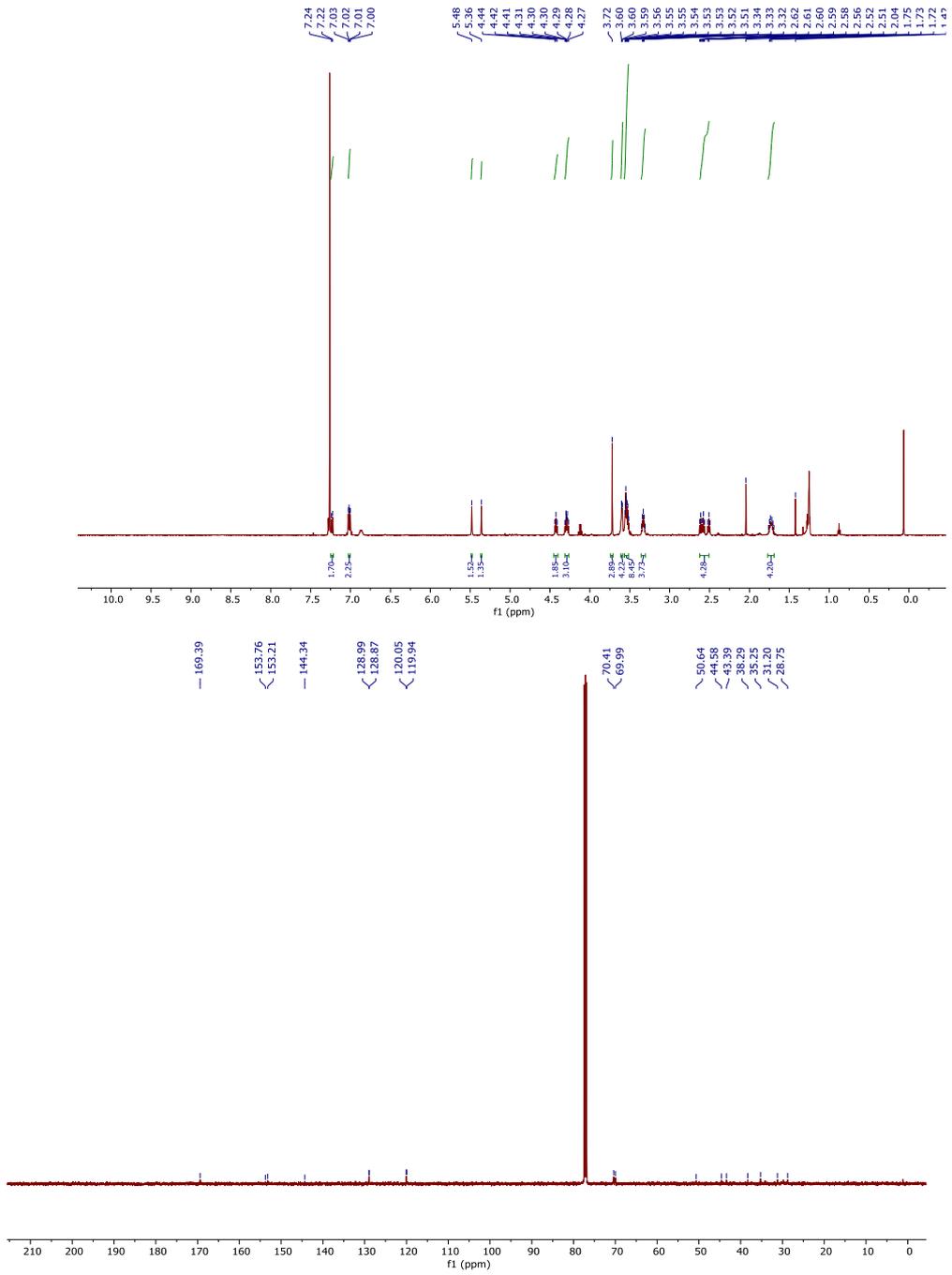
To a solution of *tert*-butyl (17-(4,5-dibromo-2-methyl-3,6-dioxo-3,6-dihydropyridazin-1(2H)-yl)-15-oxo-4,7,10-trioxa-14-azaheptadecyl)carbamate (305 mg, 0.46 mmol) in DCM (5 mL) was added TFA (5 mL) and the reaction stirred at 25 °C for 30 min. After this time, the solvent was removed *in vacuo* with DCM co-evaporation (5 × 5 mL). The deprotected amine was dissolved in DMF (3 mL) and stirred with DIPEA (200 μL). This was added dropwise to a solution of 3-(2-(4-azidobenzyl)-4,5-dibromo-3,6-dioxo-3,6-dihydropyridazin-1(2H)-yl)propanoic acid (240 mg, 0.51 mmol), pre-cooled to 0 °C, which had been stirred at 0 °C with EDC.HCl (98 mg, 0.51 mmol) under an inert atmosphere in dry DMF (6 mL) for 30 min. The reaction was allowed to warm to room temperature and stirred for 30 h. After this time, the reaction mix was diluted with water (100 mL) and the product extracted with DCM (4 × 50 mL). The organic layers were combined, washed with sat. aq. LiCl (100 mL) and dried (MgSO₄). The solvent was removed *in vacuo*. The crude product was purified by flash column chromatography (0-30% MeOH/EtOAc, 1% AcOH) to give 3-(2-(4-azidobenzyl)-4,5-dibromo-3,6-dioxo-3,6-dihydropyridazin-1(2H)-yl)-N-(17-(4,5-dibromo-2-methyl-3,6-dioxo-3,6-dihydropyridazin-1(2H)-yl)-15-oxo-4,7,10-trioxa-14-azaheptadecyl)propanamide **2** (76 mg, 0.07 mmol, 16%) as a yellow solid.

¹H NMR (500 MHz, CDCl₃) δ 7.27 (d, *J* = 8.2 Hz, 2H), 7.01 (d, *J* = 8.2 Hz, 2H), 5.48 (s, 2H), 4.42 (t, *J* = 6.9 Hz, 2H), 4.30 (t, *J* = 6.8 Hz, 2H), 3.72 (s, 3H), 3.60 (dd, *J* = 6.2, 3.4 Hz, 4H), 3.56–3.49 (m, 8H), 3.37–3.26 (m, 4H), 2.54 (dt, *J* = 33.4, 6.9 Hz, 4H), 1.74 (m, 4H).

¹³C NMR (126 MHz, CDCl₃) δ 169.3 (C), 169.2 (C), 153.8 (C), 153.2 (C), 153.1 (C), 153.0 (C), 140.5 (C), 136.5 (C), 136.3 (C), 136.1 (C), 131.1 (CH), 129.0 (CH), 119.9 (C), 70.4 (CH₂), 70.1 (CH₂), 70.0 (CH₂), 70.0 (CH₂), 49.9 (CH₂), 44.6 (CH₂), 44.3 (CH₂), 38.3 (CH₂), 38.2 (CH₂), 35.2 (CH₂), 34.2 (CH₂), 34.1 (CH₂), 28.8 (CH₃).

IR (solid) 3326, 2923, 2856, 2110, 1626, 1573, 1548, 1506 cm⁻¹.

LRMS (ESI) 1018 (20, [M⁸¹Br₄+H]⁺), 1016 (70, [M⁸¹Br₃⁷⁹Br+H]⁺), 1014 (100, [M⁸¹Br₂⁷⁹Br₂+H]⁺), 1012 (70, [M⁸¹Br⁷⁹Br₃+H]⁺), 1010 (20, [M⁷⁹Br₃+H]⁺); **HRMS (ESI)** calcd for C₃₂H₄₀O₉N₉Br₄ [M⁸¹Br₂⁷⁹Br₂+H]⁺ 1014.3309; observed 1013.9654.



Synthesis of N₃ Aniline 3

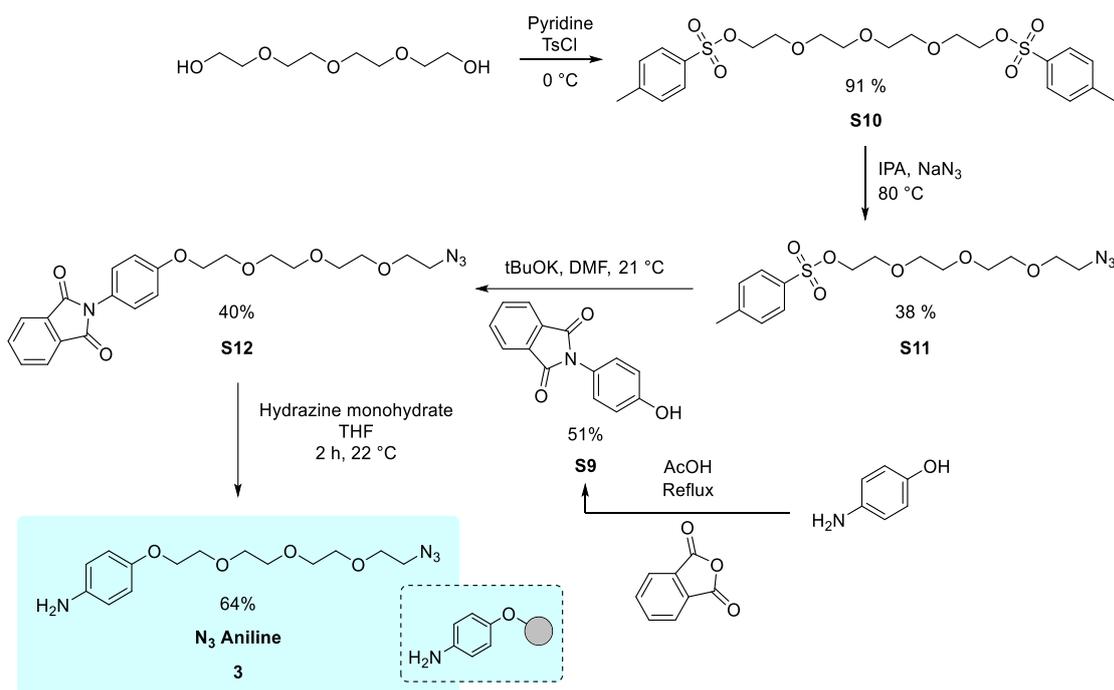
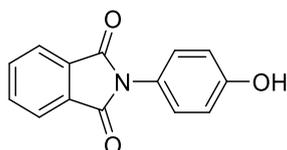


Figure S3. Synthetic route to N₃ aniline 3. *para*-Aminophenol is protected with phthalic anhydride to form protected *p*-aminophenol S9. Tetraethylene glycol is tosylated to form bis-tosyl tetraethylene glycol S10. One of the tosyl groups of S10 is then displaced with NaN₃ to form tosyl-PEG₃-N₃ S11. S11 is then reacted with S9 to form phthalimide-protected PEG₃ N₃ S12, which is deprotected to form N₃ aniline 3.

2-(4-Hydroxyphenyl)isoindoline-1,3-dione S9³



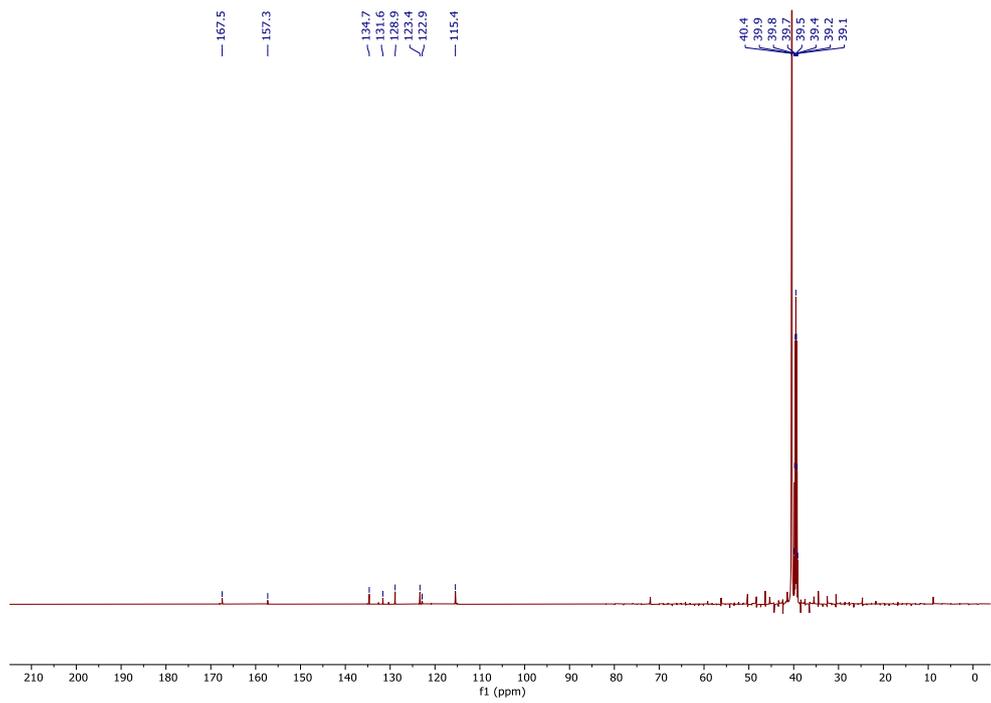
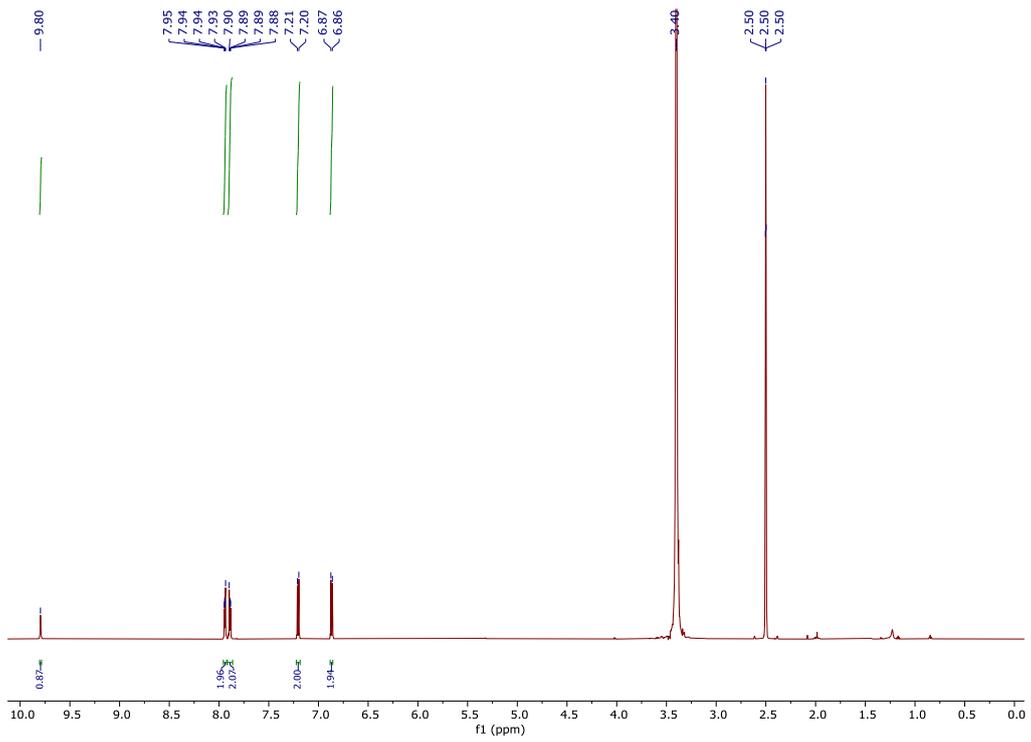
S9

To a solution of phthalic anhydride (1 g, 6.75 mmol), pre-dissolved in AcOH (40 mL) was added 4-aminophenol (737 mg, 6.75 mmol). The mixture was heated under reflux for 16 h. After cooling to room temperature, the precipitate was filtered and washed with MeOH (50 mL). The solvent was removed *in vacuo* with toluene (3 × 30 mL, as an azeotrope) then chloroform (3 × 30 mL) co-evaporation to afford 2-(4-hydroxyphenyl)isoindoline-1,3-dione S9 (826 mg, 3.45 mmol, 51%) as a white solid.

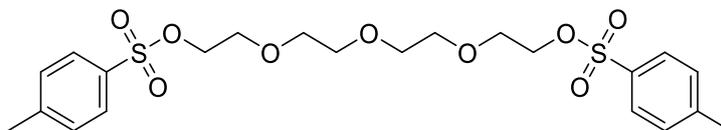
¹H NMR (600 MHz, DMSO) δ 9.80 (br s, 1H), 7.94 (dd, *J* = 5.4, 3.0 Hz, 2H), 7.89 (dd, *J* = 5.4, 3.1 Hz, 2H), 7.20 (d, *J* = 8.7 Hz, 2H), 6.87 (d, *J* = 8.7 Hz, 2H).

¹³C NMR (151 MHz, DMSO) δ 167.5 (C), 157.3 (C), 134.7 (CH), 131.6 (C), 128.9 (CH), 123.4 (CH), 122.9 (C), 115.3 (C).

IR (solid) 3410, 3035, 2923, 2852, 1707, 1610, 1595, 1580, 1514 cm⁻¹.



((Oxybis(ethane-2,1-diyl))bis(oxy))bis(ethane-2,1-diyl) bis(4-methylbenzenesulfonate) **S10³**



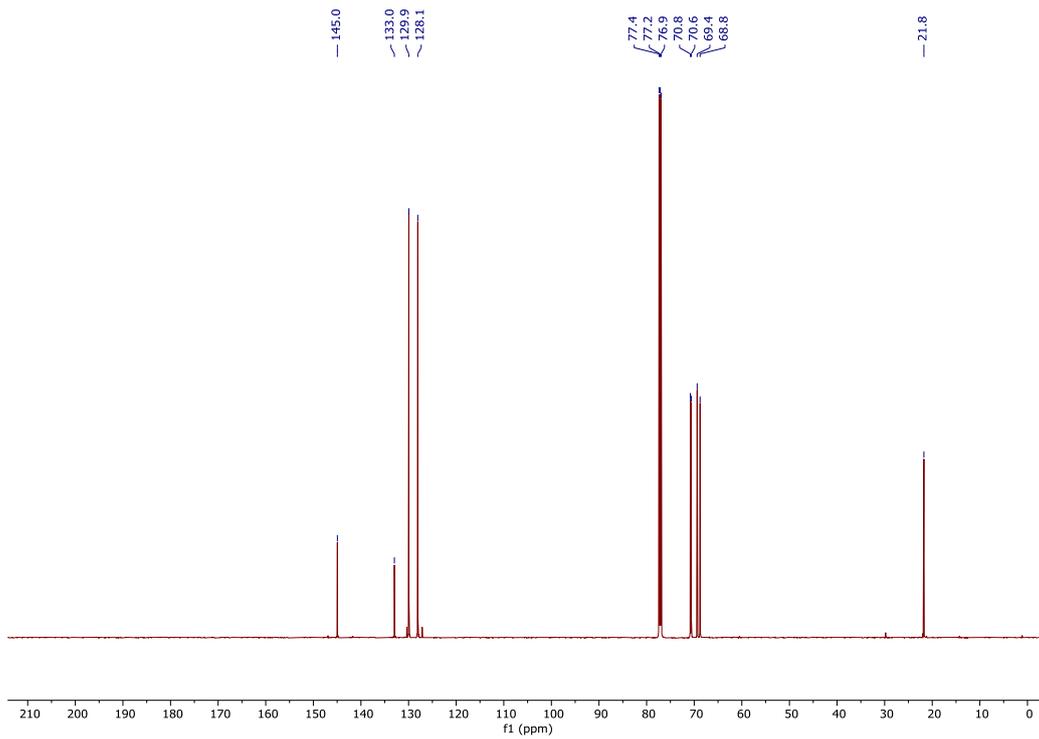
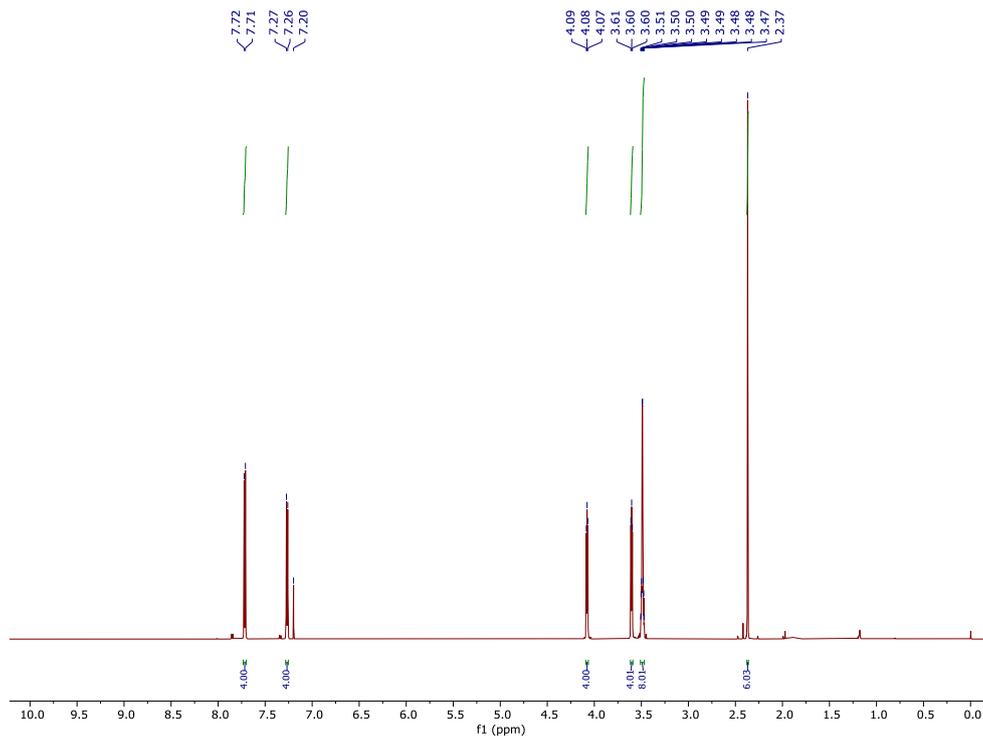
S10

To a stirred solution of tetraethylene glycol (1.08 g, 5.56 mmol) in pyridine (3.6 mL) at 0 °C was added dropwise over 30 min tosyl chloride (2.33 g, 12.23 mmol) pre-dissolved in DCM (5 mL). The mixture was stirred at 0 °C for 4 h. After this time, the reaction was incomplete by TLC so more tosyl chloride (1.17 g, 6.12 mmol), pre-dissolved in DCM (2 mL) was added and the reaction stirred at 0 °C for a further 2 h. Ice water (30 mL) was added to the reaction mixture and the crude product extracted with EtOAc (3 × 30 mL). The organic layer was washed with 2 M HCl (2 × 13 mL) and dried (MgSO₄). The solvent was removed *in vacuo* to afford ((oxybis(ethane-2,1-diyl))bis(oxy))bis(ethane-2,1-diyl) bis(4-methylbenzenesulfonate) **S10** (2.54 g, 5.05 mmol, 91%) as a clear pink oil.

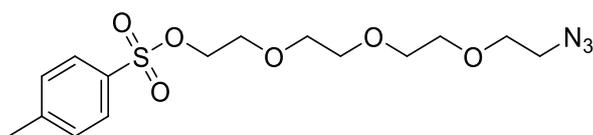
¹H NMR (600 MHz, CDCl₃) δ 7.72 (d, *J* = 8.3 Hz, 4H), 7.27 (d, *J* = 8.1 Hz, 4H), 4.08 (t, *J* = 4.7 Hz, 4H), 3.60 (t, *J* = 5.0 Hz, 4H), 3.51–3.47 (m, 8H), 2.37 (s, 6H).

¹³C NMR (151 MHz, CDCl₃) δ 145.0 (C), 133.0 (C), 129.9 (CH), 128.1 (CH), 70.8 (CH₂), 70.6 (CH₂), 69.4 (CH₂), 68.8 (CH₂), 21.8 (CH₃).

IR (thin film) 2947, 2872, 1597 cm⁻¹.



2-(2-(2-(2-Azidoethoxy)ethoxy)ethoxy)ethyl 4-methylbenzenesulfonate **S11**³



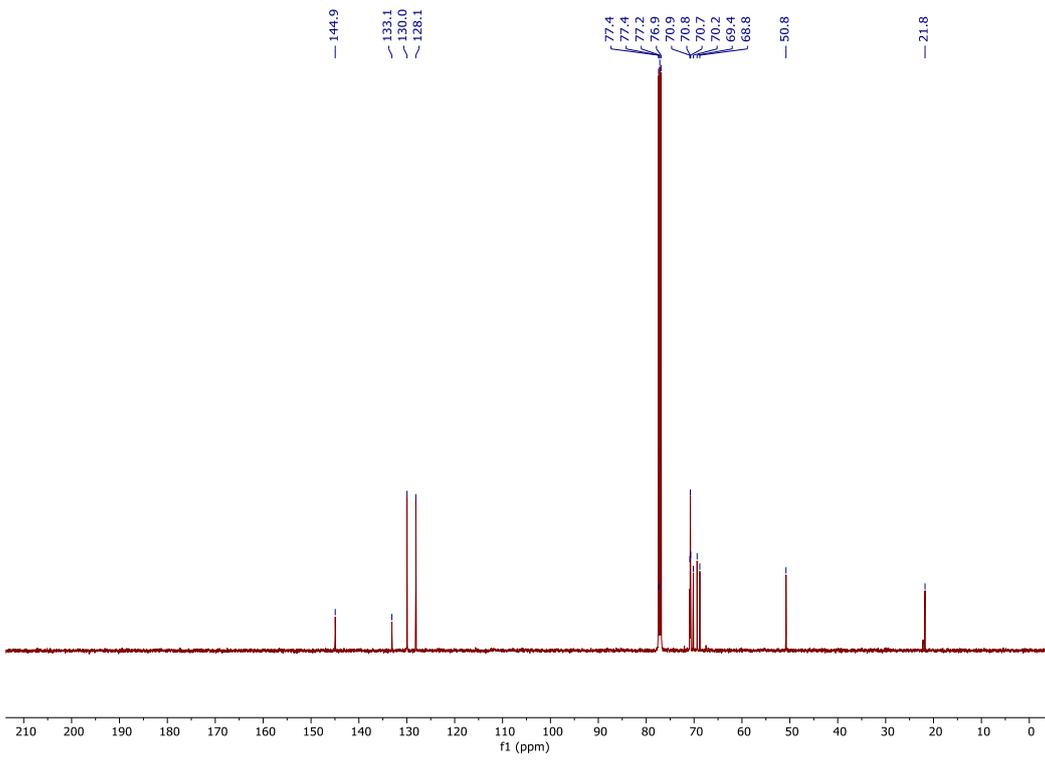
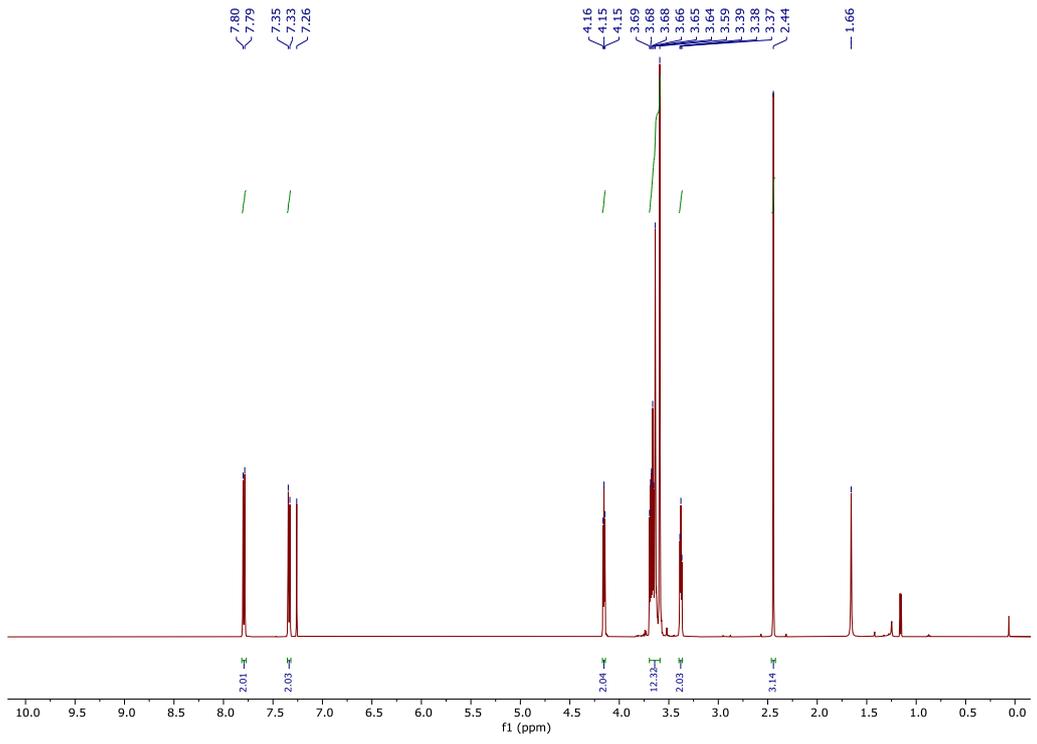
S11

To a solution of ((oxybis(ethane-2,1-diyl))bis(oxy))bis(ethane-2,1-diyl) bis(4-methylbenzenesulfonate) **S10** (1 g, 1.99 mmol) in IPA (20 mL) was added sodium azide (142 mg, 2.19 mmol). The resulting solution was heated to 60 °C for 20 h and then to 80 °C for a further 10 h. After this time, the reaction was poured into ice water (50 mL) and the product extracted with EtOAc (3 × 50 mL). The organic extracts were combined and washed with water (50 mL), then brine (50 mL), dried (MgSO₄) and the solvent removed *in vacuo*. The crude product was purified *via* flash column chromatography (30-80% EtOAc/cyclohexane) to give 2-(2-(2-(2-azidoethoxy)ethoxy)ethoxy)ethyl 4-methylbenzenesulfonate **S11** (282 mg, 0.76 mmol, 38%) as a clear oil.

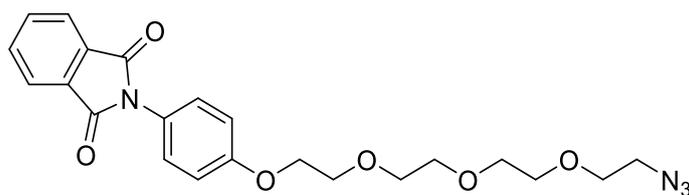
¹H NMR (500 MHz, CDCl₃) δ 7.79 (d, *J* = 8.3 Hz, 2H), 7.34 (d, *J* = 8.0 Hz, 2H), 4.17–4.14 (m, 2H), 3.70–3.59 (m, 12H), 3.38 (t, *J* = 5.0 Hz, 2H), 2.44 (s, 3H).

¹³C NMR (126 MHz, CDCl₃) δ 144.9, 133.1, 130.0, 128.1, 77.4, 77.4, 77.2, 76.9, 70.9, 70.8, 70.7, 70.2, 69.4, 68.8, 50.8, 21.8.

IR (thin film) 2915, 2869, 2099, 1598 cm⁻¹.



2-(4-(2-(2-(2-(2-Azidoethoxy)ethoxy)ethoxy)ethoxy)phenyl)isoindoline-1,3-dione **S12**³



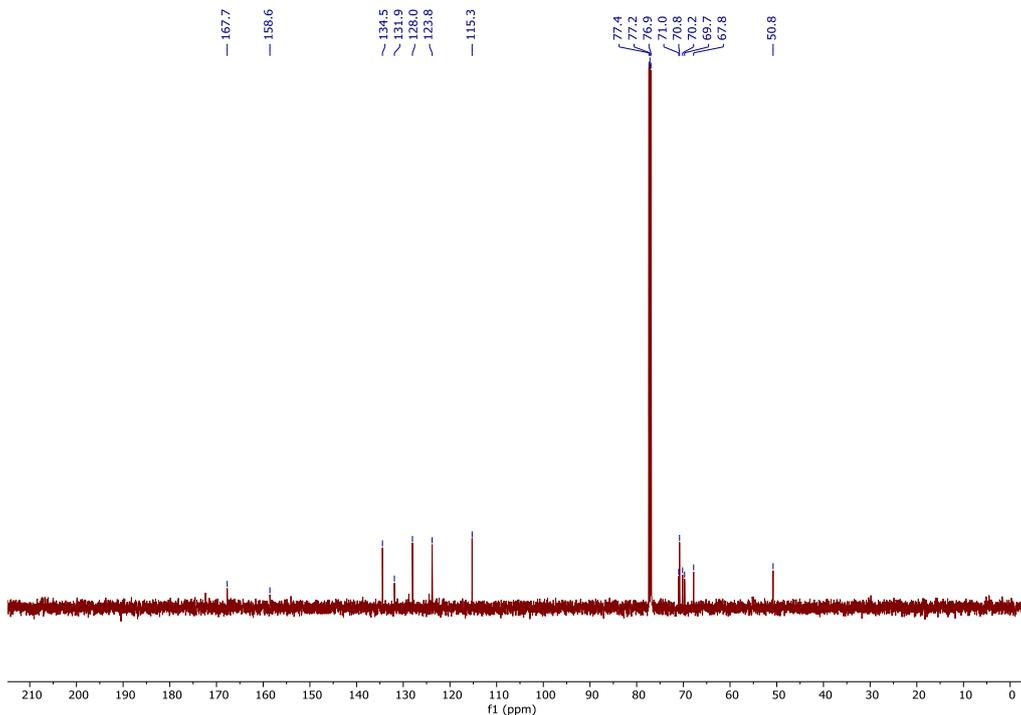
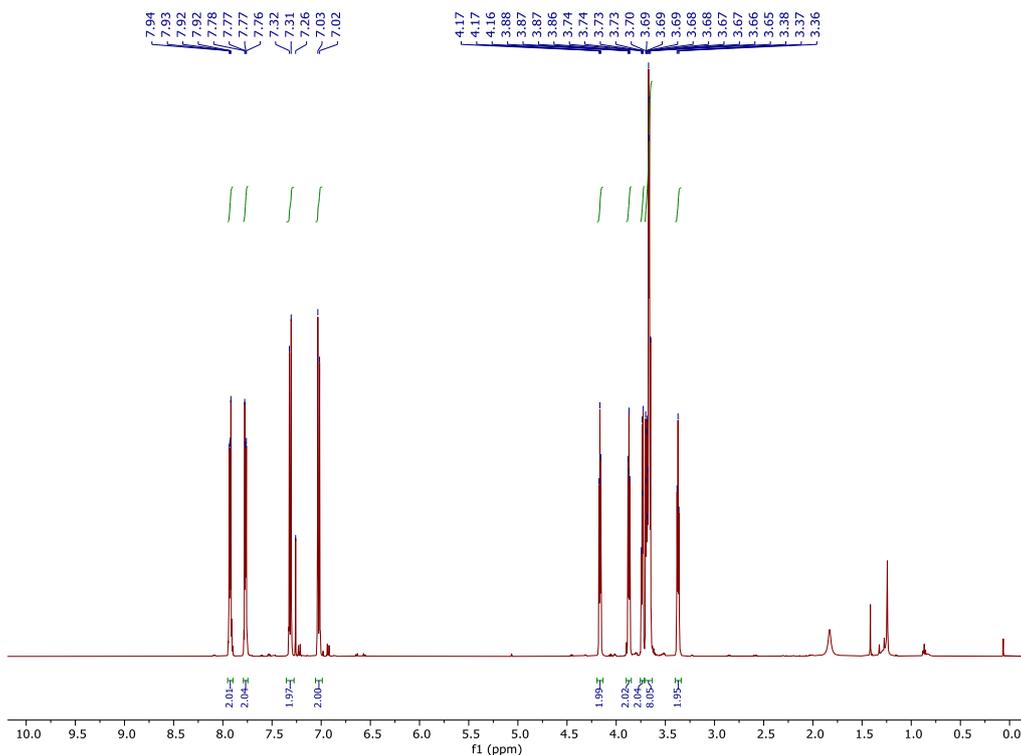
S12

To a solution of 2-(4-hydroxyphenyl)isoindoline-1,3-dione **S9** (271 mg, 1.14 mmol) in anhydrous DMF (20 mL) was added *t*BuOK (128 mg, 1.14 mmol) and the solution stirred at 21 °C for 10 min. After this time was added 2-(2-(2-(2-azidoethoxy)ethoxy)ethyl 4-methylbenzenesulfonate **S11** (282 mg, 0.76 mmol) and the reaction mix stirred at 90 °C for 20 h. After this, the reaction was diluted with water (100 mL) and the product extracted with EtOAc (4 × 50 mL). The organic extracts were washed with sat. aq. LiCl (2 × 30 mL), dried (MgSO₄) and the solvent removed *in vacuo*. The crude product was purified *via* flash column chromatography (20-80% EtOAc/cyclohexane) to give the product 2-(4-(2-(2-(2-(2-azidoethoxy)ethoxy)ethoxy)ethoxy)phenyl)isoindoline-1,3-dione **S12** (135 mg, 0.31 mmol, 40%) as a yellow oil.

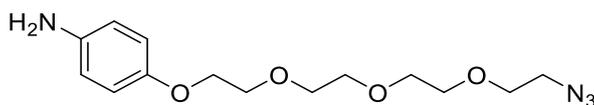
¹H NMR (500 MHz, CDCl₃) δ 7.93 (dd, *J* = 5.5, 3.0 Hz, 2H), 7.77 (dd, *J* = 5.5, 3.0 Hz, 2H), 7.31 (d, *J* = 8.9 Hz, 2H), 7.03 (d, *J* = 8.9 Hz, 2H), 4.17 (t, *J* = 4.8 Hz, 2H), 3.87 (dd, *J* = 5.7, 4.1 Hz, 2H), 3.73 (dd, *J* = 6.2, 3.5 Hz, 2H), 3.71–3.63 (m, 8H), 3.37 (t, *J* = 5.0 Hz, 2H).

¹³C NMR (151 MHz, CDCl₃) δ 167.7 (C), 158.6 (C), 134.5 (CH), 131.9 (C), 128.0 (CH), 123.8 (CH), 115.3 (CH), 77.4 (CH₂), 77.2 (CH₂), 76.9 (CH₂), 71.0 (CH₂), 70.8 (CH₂), 70.2 (CH₂), 69.7 (CH₂), 67.8 (CH₂), 50.8 (CH₂).

IR (thin film) 2921, 2858, 2114, 1784, 1705, 1612, 1588, 1516 cm⁻¹.



4-(2-(2-(2-(2-Azidoethoxy)ethoxy)ethoxy)ethoxy)ethoxy)aniline (**3**)



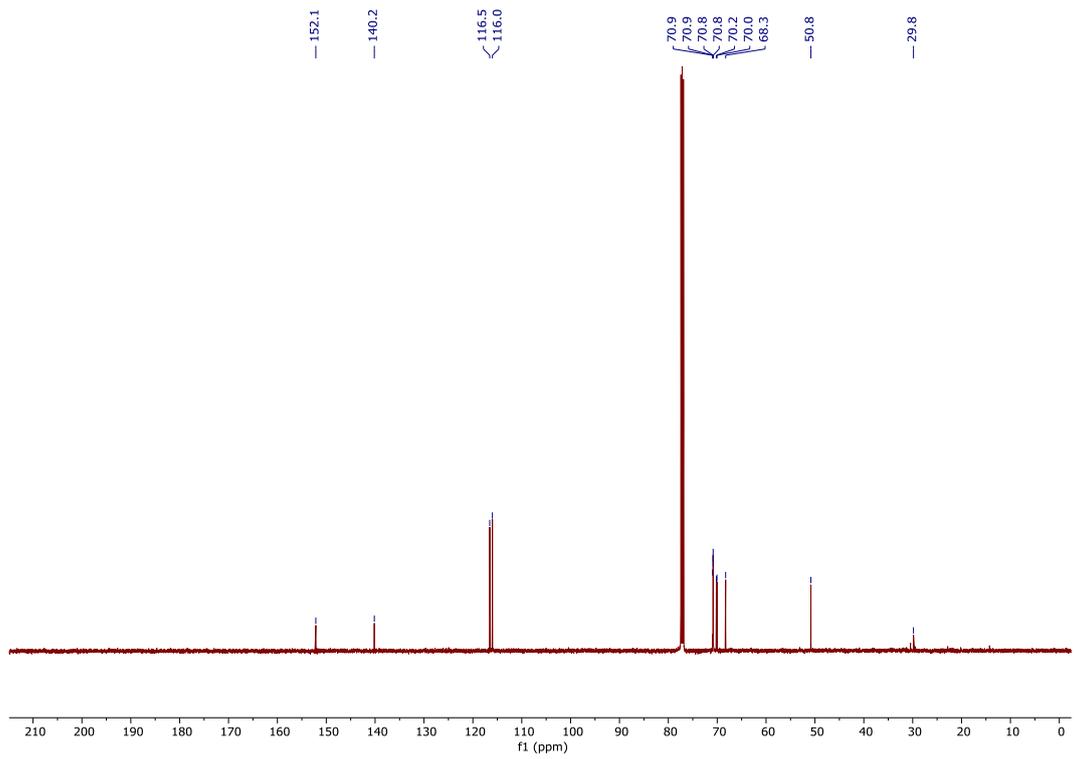
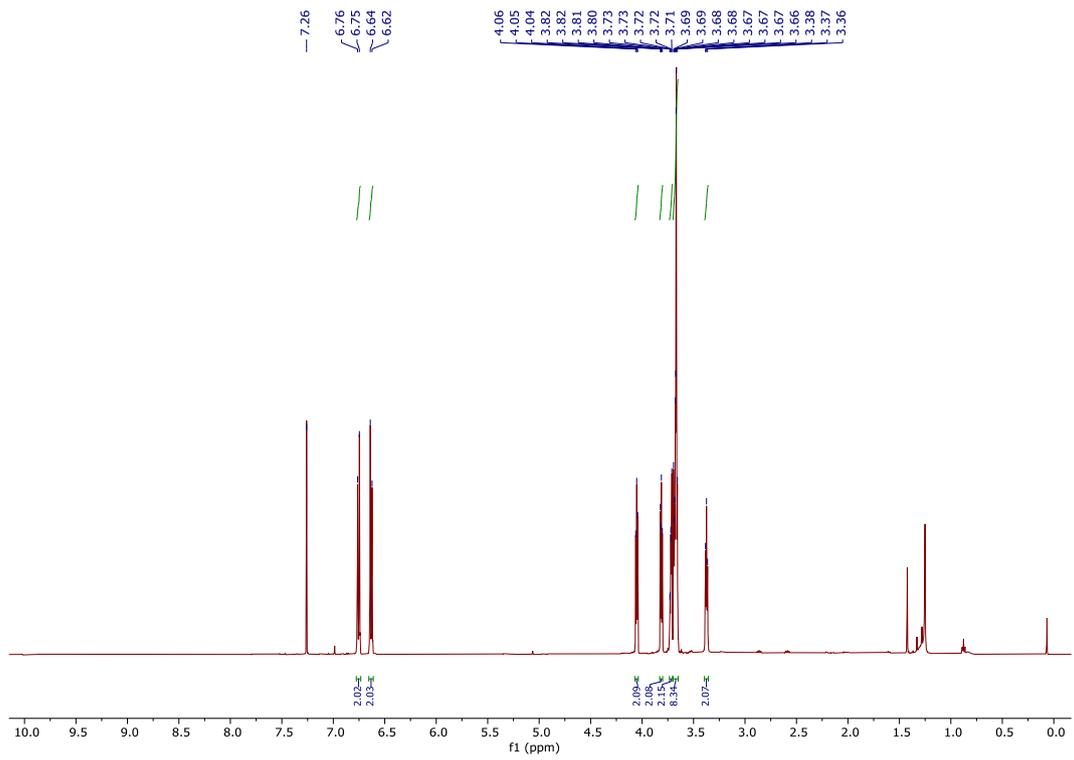
3

To a solution of 2-(4-(2-(2-(2-(2-azidoethoxy)ethoxy)ethoxy)ethoxy)phenyl)isoindoline-1,3-dione **S12** (135 mg, 0.31 mmol) in THF (20 mL) was added hydrazine monohydrate (307 μ L, 3.72 mmol, 60% solution). The reaction was then stirred at 22 °C for 2 h. After this time the reaction mixture was diluted with water (100 mL) and the product extracted with EtOAc (3 \times 30 mL). The organic layers were combined, washed with brine (30 mL) and dried (MgSO_4). The solvent was removed *in vacuo* and the crude product purified *via* flash column chromatography (20-100% EtOAc/cyclohexane) to give 4-(2-(2-(2-(2-azidoethoxy)ethoxy)ethoxy)ethoxy)aniline **3** (61.8 mg, 0.2 mmol, 64%) as a brown oil.

$^1\text{H NMR}$ (500 MHz, CDCl_3) δ 6.76 (d, $J = 8.7$ Hz, 2H), 6.63 (d, $J = 8.7$ Hz, 2H), 4.05 (t, $J = 4.9$ Hz, 2H), 3.81 (dd, $J = 5.6, 4.2$ Hz, 2H), 3.73–3.70 (m, 2H), 3.70–3.65 (m, 8H), 3.37 (t, $J = 5.1$ Hz, 2H).

$^{13}\text{C NMR}$ (126 MHz, CDCl_3) δ 152.1 (C), 140.2 (C), 116.5 (CH), 116.0 (CH), 70.9 (CH_2), 70.9 (CH_2), 70.8 (CH_2), 70.8 (CH_2), 70.2 (CH_2), 70.0 (CH_2), 68.3 (CH_2), 50.8 (CH_2), 29.8 (CH_2).

IR (thin film) 3384 3358, 2920, 2874, 2104, 1631, 1511 cm^{-1} .



Synthesis of diEt PD **4**

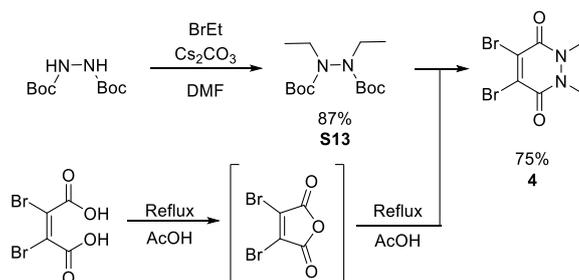
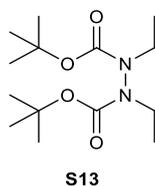


Figure S4. Synthetic Route to diEt PD, **4**. Diboc hydrazine is ethylated to form **S13**, which is subsequently refluxed with dibromomaleic anhydride to form the product.

Di-tert-butyl 1,2-diethylhydrazine-1,2-dicarboxylate **S13**³



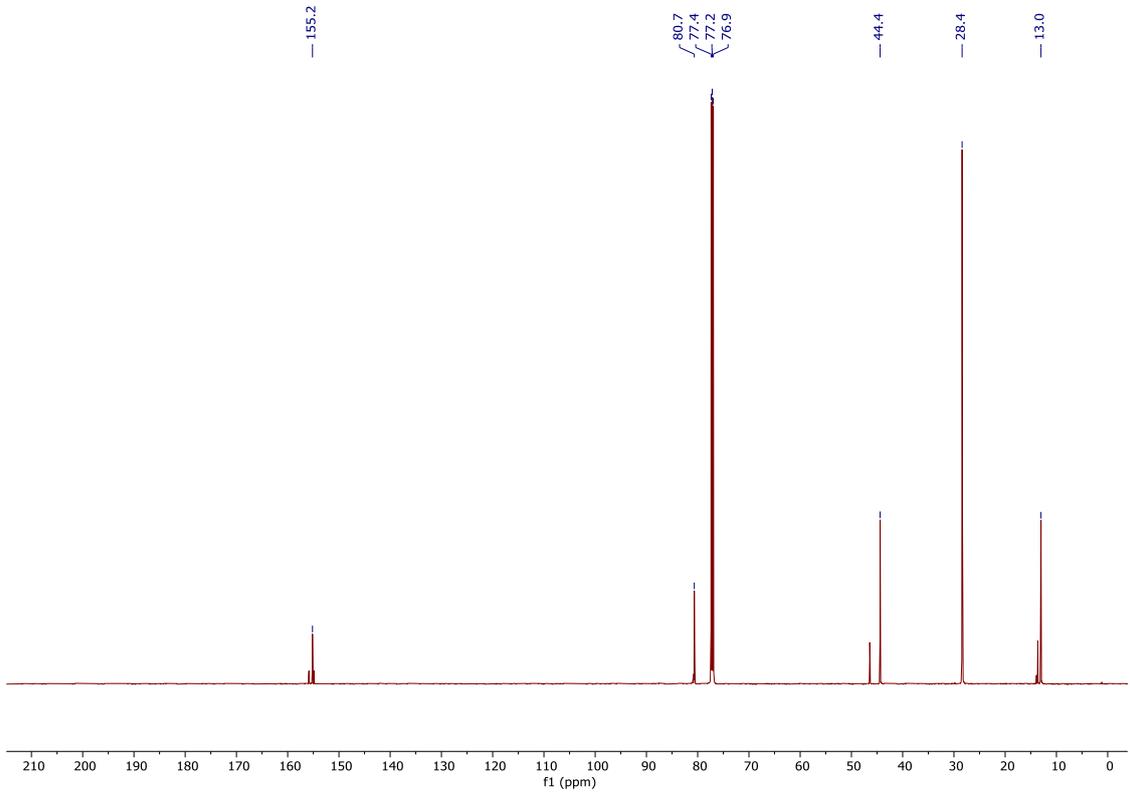
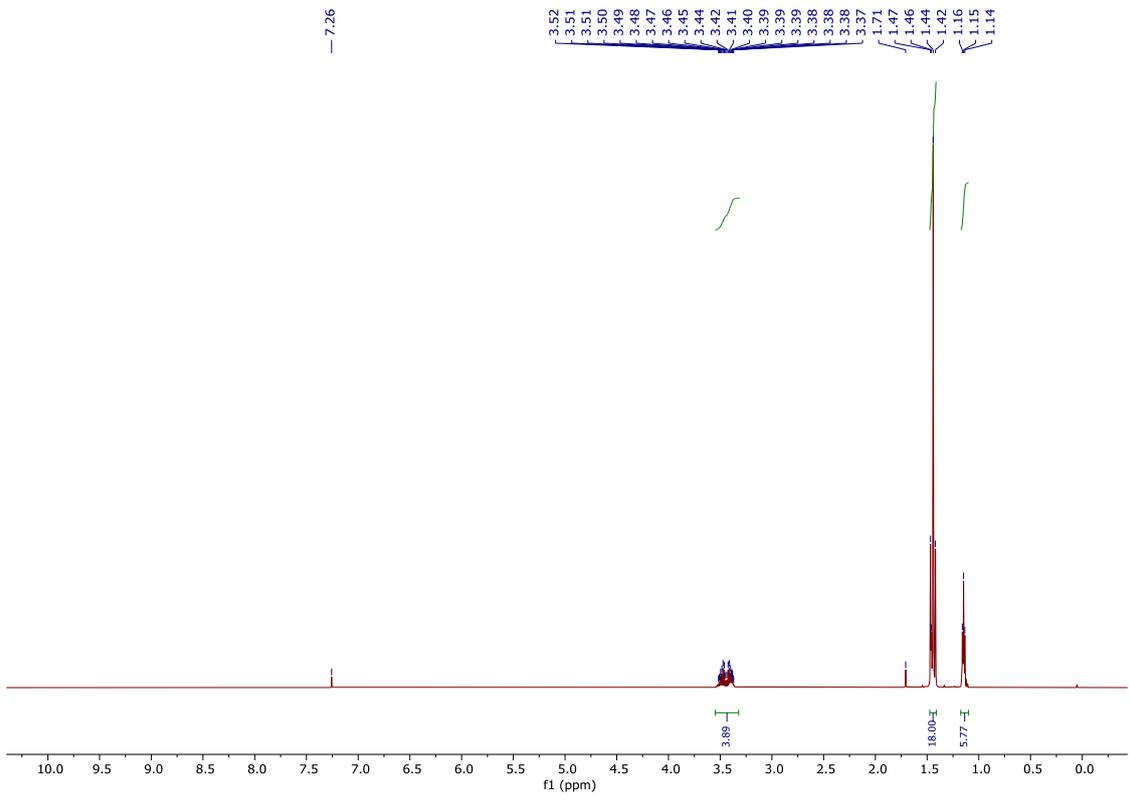
To a solution of di-tert-butyl hydrazine-1,2-dicarboxylate (1.16 g, 5.0 mmol) in DMF (20 mL) was added caesium carbonate (3.26 g, 10.0 mmol) and bromoethane (0.7 mL, 14.7 mmol). The mixture was then stirred at 21 °C for 24 h, after this time was added another 0.5 eq. of caesium carbonate (0.81 g, 2.5 mmol). The reaction was stirred for a further 4 h. After this time the solvent was removed *in vacuo* with toluene co-evaporation (3 × 50 mL as an azeotrope). The crude reaction mixture was then dissolved in diethyl ether (100 mL) and washed with water (2 × 30 mL), followed by saturated aq. LiCl solution (2 × 30 mL). The organic layer was dried (MgSO₄) and concentrated *in vacuo* to give di-tert-butyl 1,2-diethylhydrazine-1,2-dicarboxylate **S13** (1.25 g, 4.3 mmol, 87%).

¹H NMR (600 MHz, CDCl₃, rotamers) δ 3.53-3.37 (m, 4H), 1.47-1.42 (m, 18H), 1.15 (t, *J* = 7.2 Hz, 6H).

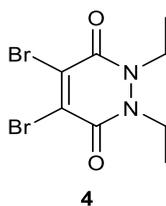
¹³C NMR (151 MHz, CDCl₃, rotamers*) δ 155.2 (C), 80.7 (C), 44.4 (CH₂), 28.4 (CH₃), 13.0 (CH₃).

IR (thin film) 2976, 2935, 1703 cm⁻¹.

*Only one peak per carbon is assigned



4,5-Dibromo-1,2-diethyl-1,2-dihydropyridazine-3,6-dione (diEt PD) **4**³

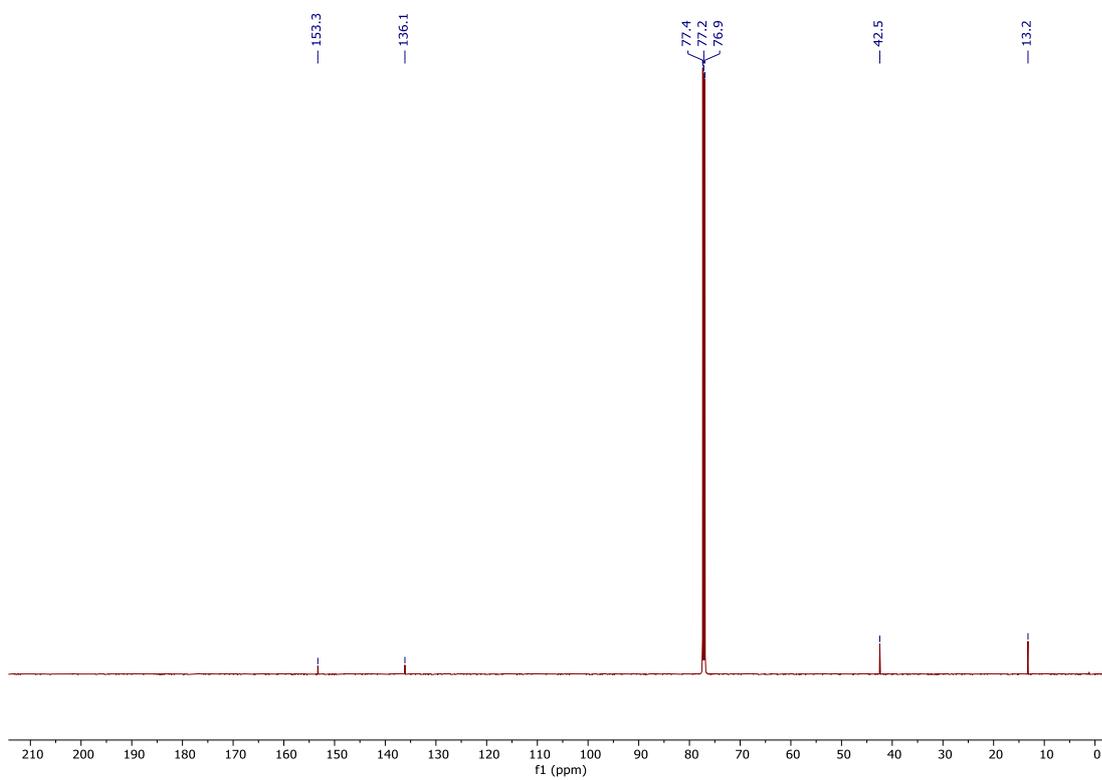
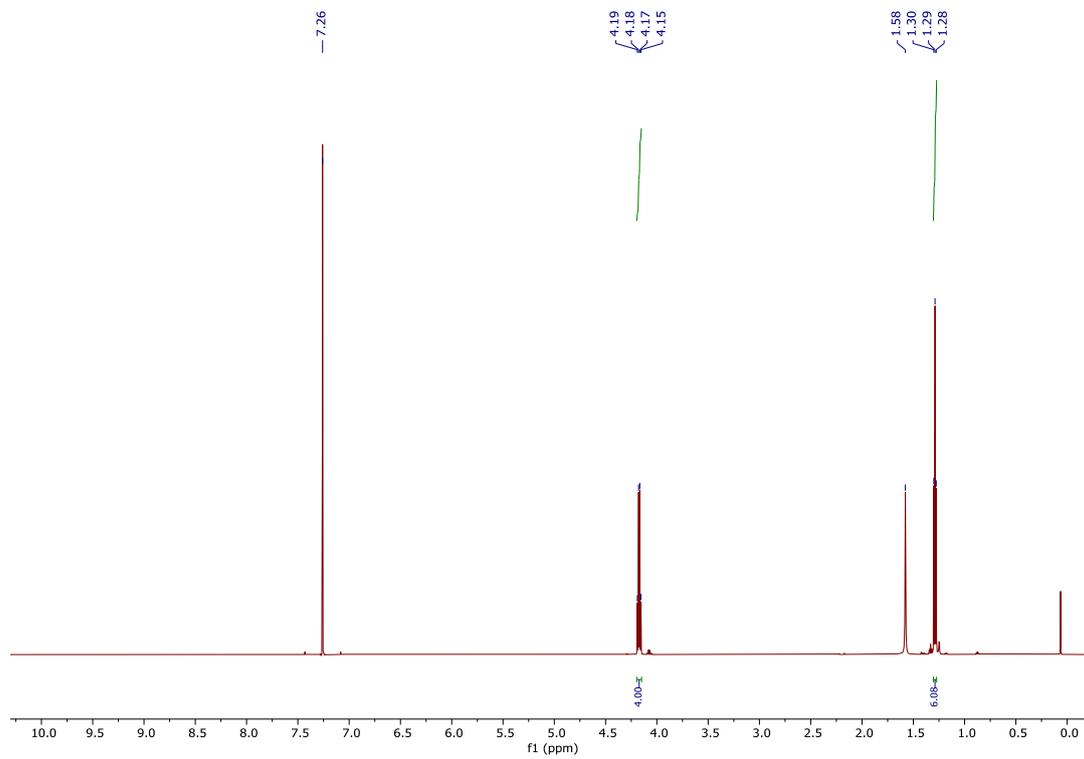


Dibromomaleic acid (219 mg, 0.8 mmol) was dissolved in AcOH (10 mL) and heated under reflux for 2 h. After this time, di-*tert*-butyl 1,2-diethylhydrazine-1,2-dicarbonate **S13** (288 mg, 1.0 mmol) was added and the mixture heated under reflux for a further 24 h. After this time, the solvent was removed *in vacuo* with toluene co-evaporation (3 × 30 mL, as an azeotrope). The crude product was then purified *via* flash column chromatography (20-80% EtOAc/cyclohexane) to give 4,5-dibromo-1,2-diethyl-1,2-dihydropyridazine-3,6-dione **4** (196 mg, 0.6 mmol, 75%) as a yellow solid.

¹H NMR (600 MHz, CDCl₃) δ 4.17 (q, *J* = 7.1 Hz, 4H), 1.29 (t, *J* = 7.1 Hz, 6H).

¹³C NMR (151 MHz, CDCl₃) δ 153.3 (C), 136.1 (C), 42.5 (CH₂), 13.2 (CH₃).

IR (solid) 2979, 2937, 2873, 1629, 1574 cm⁻¹



Synthesis of diMe bisPD 5

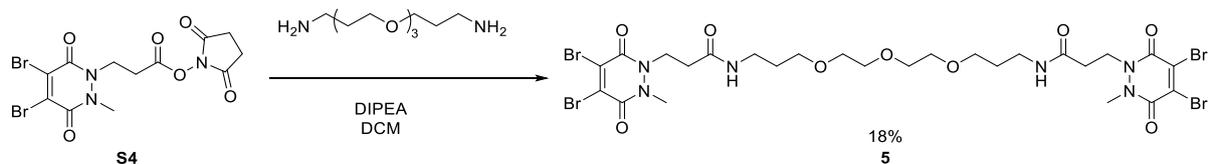
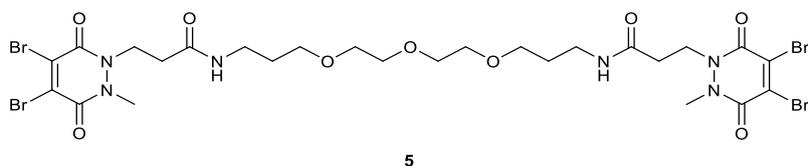


Figure S5. Synthetic Route to diMe bisPD, **5**. **S4** is reacted with a (PEG)₃ amine to form the product.

N,N'-(((Oxybis(ethane-2,1-diyl))bis(oxy))bis(propane-3,1-diyl))bis(3-(4,5-dibromo-2-methyl-3,6-dioxo-3,6-dihydropyridazin-1(2H)-yl)propanamide) (diMe bisPD) **5**



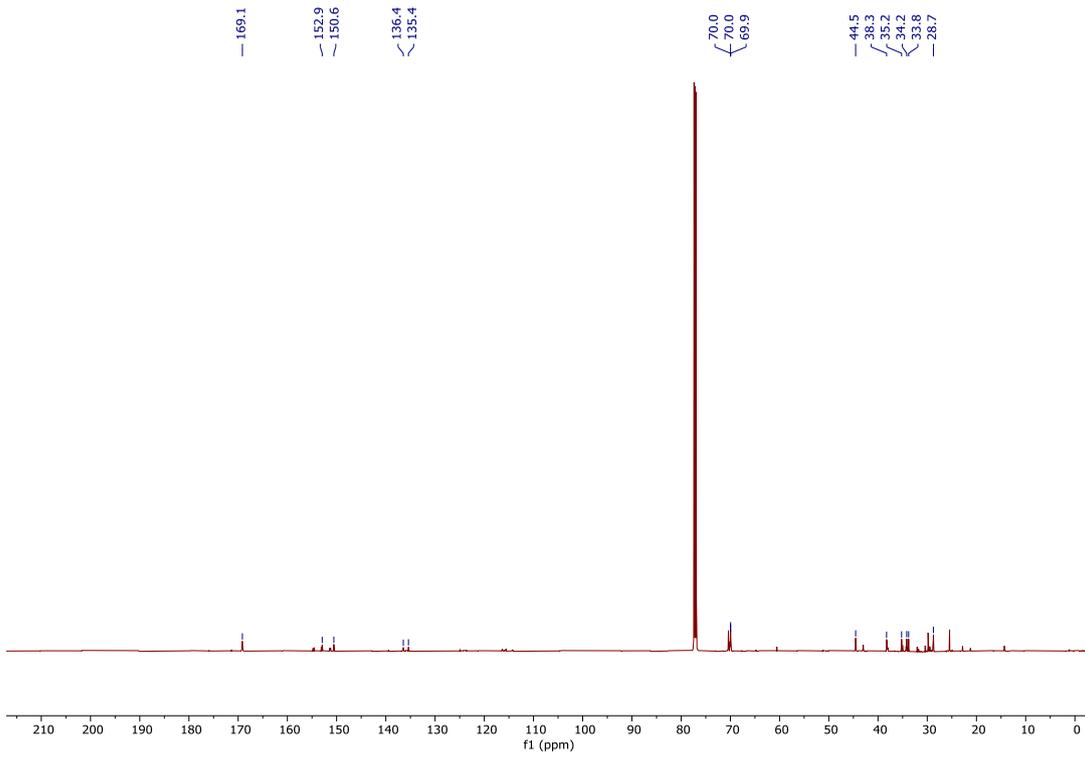
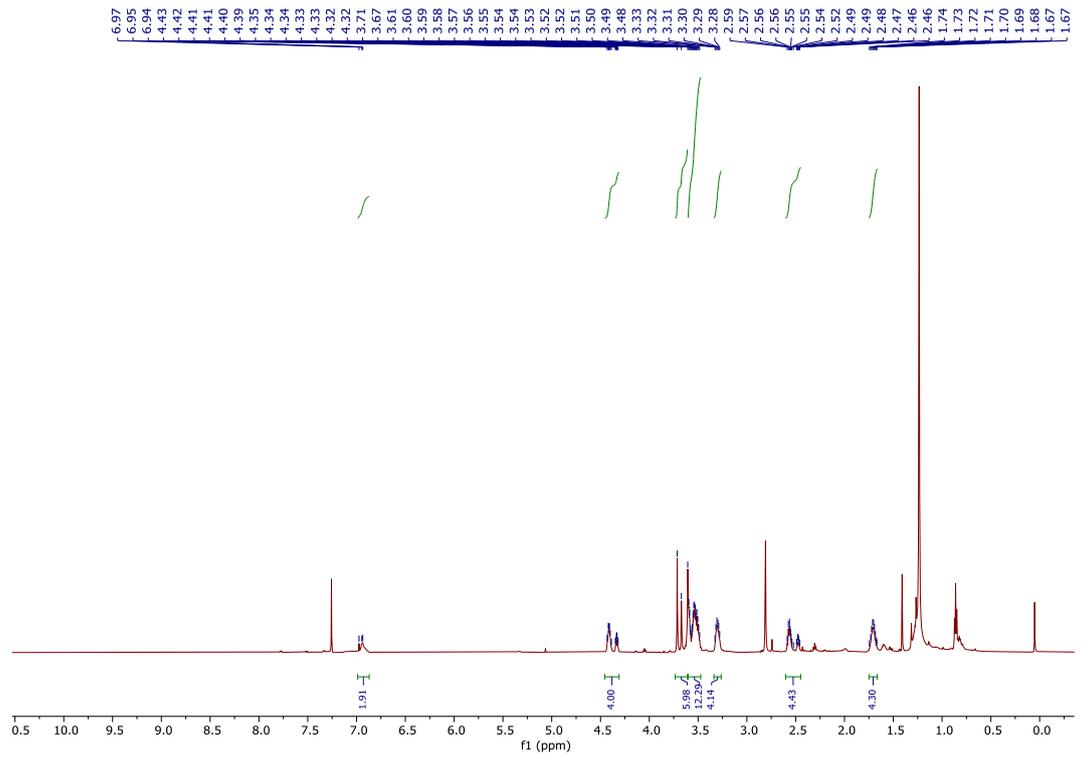
To a solution of 4,7,10-trioxa-1,13-tridecanediamine (24.3 mg, 0.11 mmol) in DCM (10 mL) was added DIPEA (38.3 μ L, 0.22 mmol) and the solution stirred at 21 $^{\circ}$ C for 30 min. After this time, was added dropwise 2,5-dioxopyrrolidin-1-yl 3-(4,5-dibromo-2-methyl-3,6-dioxo-3,6-dihydropyridazin-1(2H)-yl)propanoate **S4** (150 mg, 0.33 mmol) pre-dissolved in DCM (5 mL), and the solution stirred at 21 $^{\circ}$ C for 24 h. After this time the reaction mixture was washed with water (2 \times 30 mL) then brine (30 mL). The aqueous phases were combined and the product extracted with DCM (60 mL). The organic phases were combined, dried (MgSO₄) and the solvent removed *in vacuo*. The crude product was purified *via* flash column chromatography (0-30% MeOH/EtOAc) to give *N,N'*-(((oxybis(ethane-2,1-diyl))bis(oxy))bis(propane-3,1-diyl))bis(3-(4,5-dibromo-2-methyl-3,6-dioxo-3,6-dihydropyridazin-1(2H)-yl)propanamide) **5** (18 mg, 0.02 mmol, 18%) as a yellow oil.

¹H NMR (600 MHz, CDCl₃, rotamers) δ 6.99–6.87 (m, 2H), 4.46–4.31 (m, 4H), 3.69 (d, 6H), 3.60–3.47 (m, 12H), 3.30 (m, 4H), 2.60–2.45 (m, 4H), 1.70 (m, 4H).

¹³C NMR (151 MHz, CDCl₃, rotamers) δ 169.1 (C), 152.9 (C), 150.6 (C), 136.4 (C), 135.4 (C), 70.0 (CH₂), 70.0 (CH₂), 60.9 (CH₂), 44.5 (CH₂), 38.3 (CH₂), 35.2 (CH₂), 34.2 (CH₂), 28.7 (CH₃).

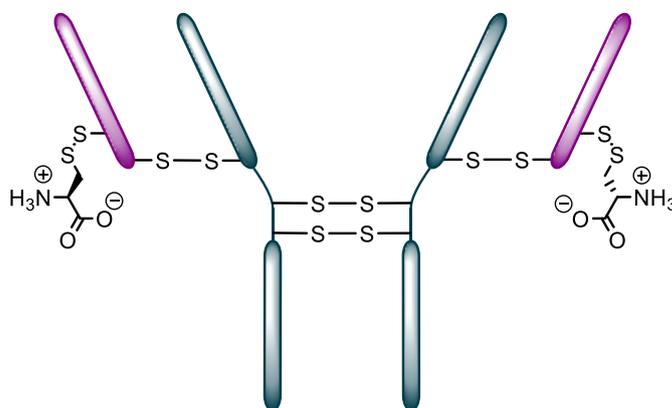
*Some impurities are present in this sample

LRMS (ESI) 893 (20, [M⁷⁹Br₄+H]⁺), 895 (70, [M⁷⁹Br₃⁸¹Br+H]⁺) 897 (100, [M⁷⁹Br₂⁸¹Br₂+H]⁺), 899 (70, [M⁷⁹Br⁸¹Br₃+H]⁺), 901 (20, [M⁸¹Br₄+H]⁺); **HRMS (ESI)** calcd for C₂₆H₃₇O₉N₆Br₄ [M⁸¹Br₂⁷⁹Br₂+H]⁺ 896.9309; observed 896.9241.



Chemical Biology

Capped LC S168C Thio-Trastuzumab Mutant 6



6

LC S168C thio-trastuzumab was obtained capped and analysed unmodified by LC-MS (method 1a).

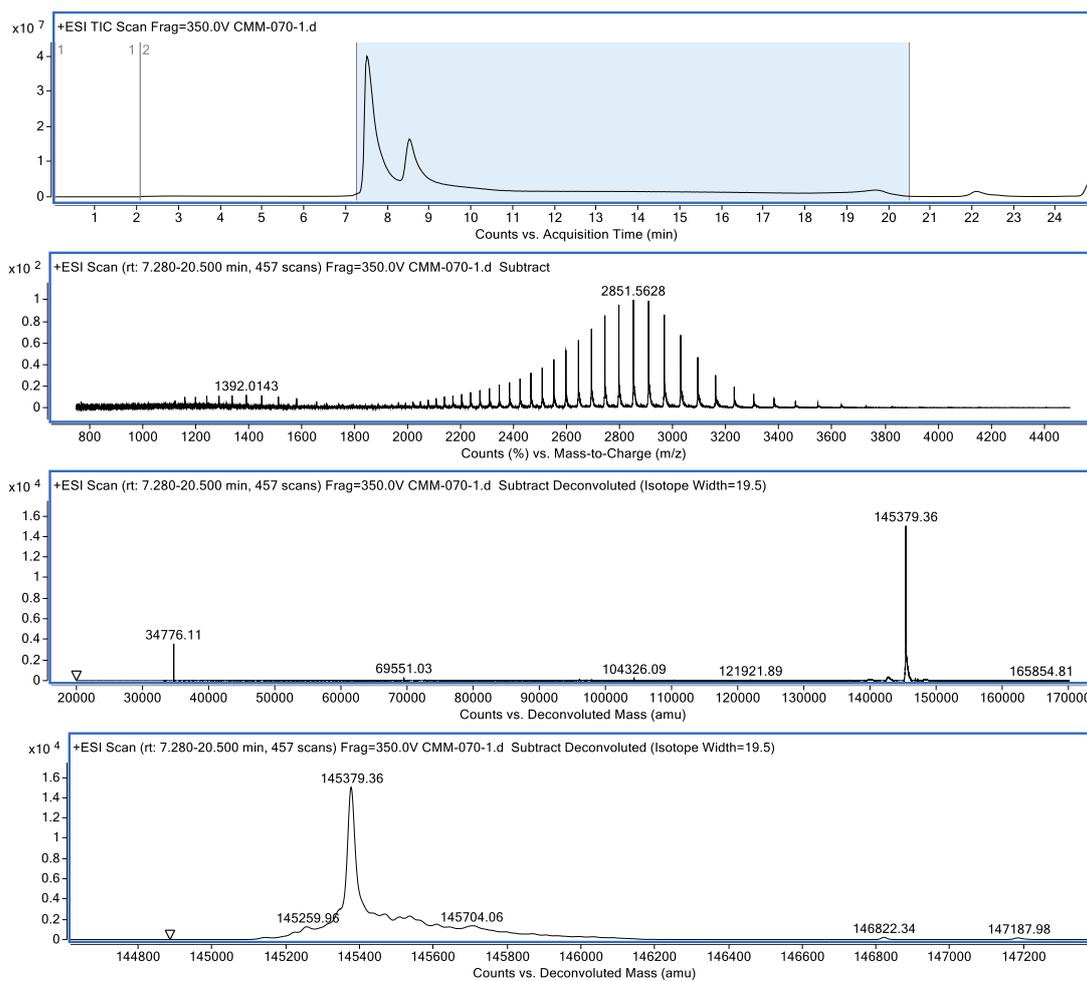
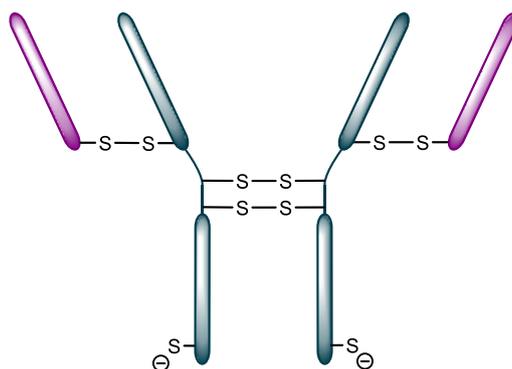


Figure S6: (i) TIC LC-MS trace (top), (ii) non-deconvoluted LC-MS trace (upper middle), (iii) deconvoluted MS data (lower middle, wide range), (iv) deconvoluted MS data (bottom, zoom in range).

Uncapped HC S378C Thio-Trastuzumab Mutant 7



7

HC S378C thio-trastuzumab was obtained uncapped and analysed unmodified by LC-MS (method 1a).

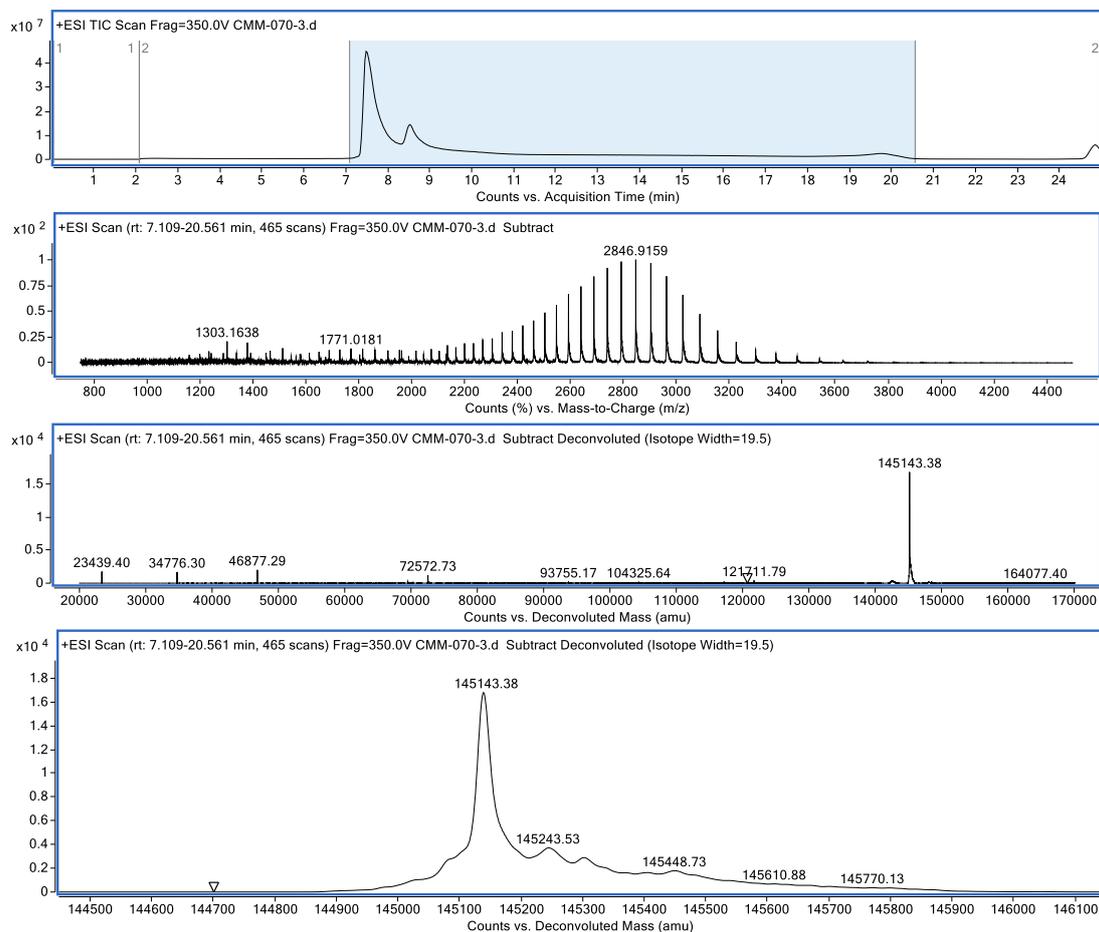
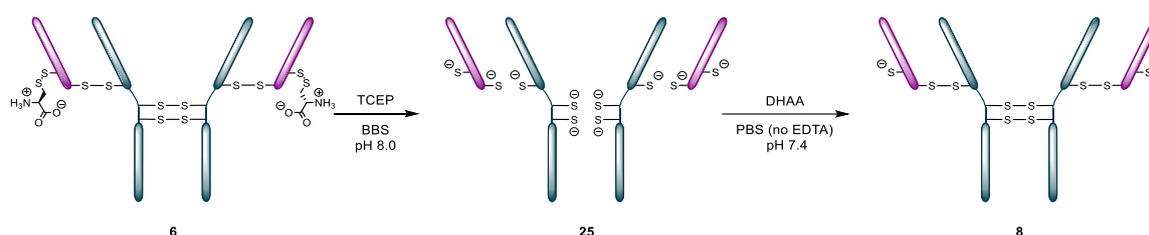


Figure S7: (i) TIC LC-MS trace (top), (ii) non-deconvoluted LC-MS trace (upper middle), (iii) deconvoluted MS data (lower middle, wide range), (iv) deconvoluted MS data (bottom, zoom in range).

Uncapped LC S168C Thio-Trastuzumab Mutant 8



LC S168C thio-trastuzumab was obtained with the mutant thiols capped (see **6**), therefore an uncapping procedure was required.

To a solution of LC S168C thio-trastuzumab **6** (100 μ L, 20 μ M) in BBS (25 mM borate, 25 mM NaCl, 2 mM EDTA, 2% DMSO, pH 8.0) was added TCEP.HCl (20 mM in dH₂O, 10 eq.) and the resulting solution incubated for 3 h at 37 $^{\circ}$ C. After this time, the TCEP was removed *via* ultrafiltration into PBS (10 mM phosphates, 2.7 mM KCl, 137 mM NaCl, pH 7.4) and (in the case of conjugates **8a-e**) DHAA (20 mM in PBS, A eq.) was added. In the case of conjugates **8f-k**, no further reagents were added. The reaction was then incubated at B $^{\circ}$ C for C h/min. After this time, the DHAA was removed *via* ultrafiltration into BBS and the product analysed by LC-MS (method 1b).

As LC-MS analysis at this point does not show whether over oxidation has occurred, to assess the extent of conjugation, the uncapped conjugates were immediately reacted with *N*-methylmaleimide (NMM) or BCN PD (see conjugates **S14** and **9**, respectively).

	Oxidant + Eq. (A)	Re-oxidation Temperature ($^{\circ}$ C) (B)	Re-oxidation Time (C)
8a	DHAA, 20	22	1.5 h
8b	DHAA, 10	22	1.5 h
8c	DHAA, 10	22	30 min
8d	DHAA, 10	22	20 min
8e	DHAA, 10	22	10 min
8f	Air	22	16 h
8g	Air	22	2.5 h
8h	Air	22	2 h
8i	Air	22	1.5 h
8j	Air	37	1 h
8k	Air	37	30 min

Table S6. Conditions used to form **8**.

8a

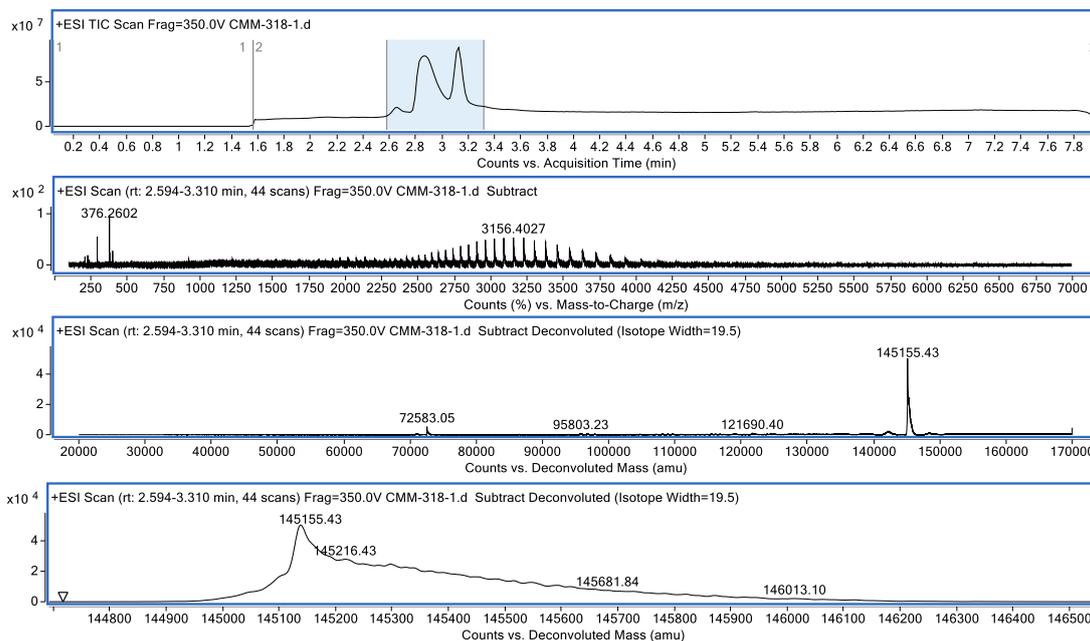


Figure S8: (i) TIC LC-MS trace (top), (ii) non-deconvoluted LC-MS trace (upper middle), (iii) deconvoluted MS data (lower middle, wide range), (iv) deconvoluted MS data (bottom, zoom in range).

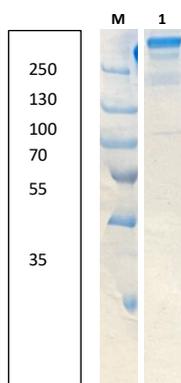
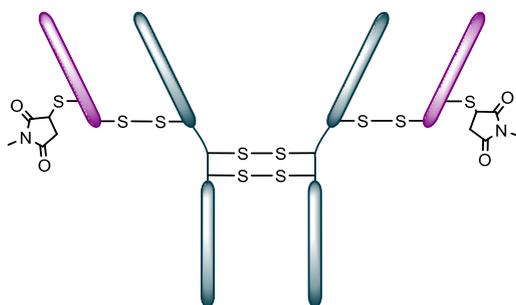


Figure S9: SDS-PAGE gel: M. Ladder, 1. Conjugate 8

No LC-MS is available for **8b** - **8k** as they were immediately reacted with NMM or Mepstra PD **1** to assess uncapping, for post-conjugation MS see conjugates **S14** and **9** (respectively).

LC S168C NMM conjugate **S14** (LC S168C Thio-Trastuzumab Conjugated to *N*-Methylmaleimide)



S14

To a solution of uncapped LC S168C thio-trastuzumab **8c** (30 μ L, 20 μ M) in BBS (25 mM borate, 25 mM NaCl, 2 mM EDTA, 2% DMSO, pH 8.0) was added NMM (0.12 μ L, 20 mM in DMSO, 4 eq.) and the reaction incubated at 22 $^{\circ}$ C for 3 h under constant agitation (300 rpm). After this time, excess reagents were removed to give conjugate **S14** (expected mass 145,365 Da, observed mass 145,366 Da) which was analysed by LC-MS (method 1a), UV-Vis spectroscopy and SDS-PAGE.

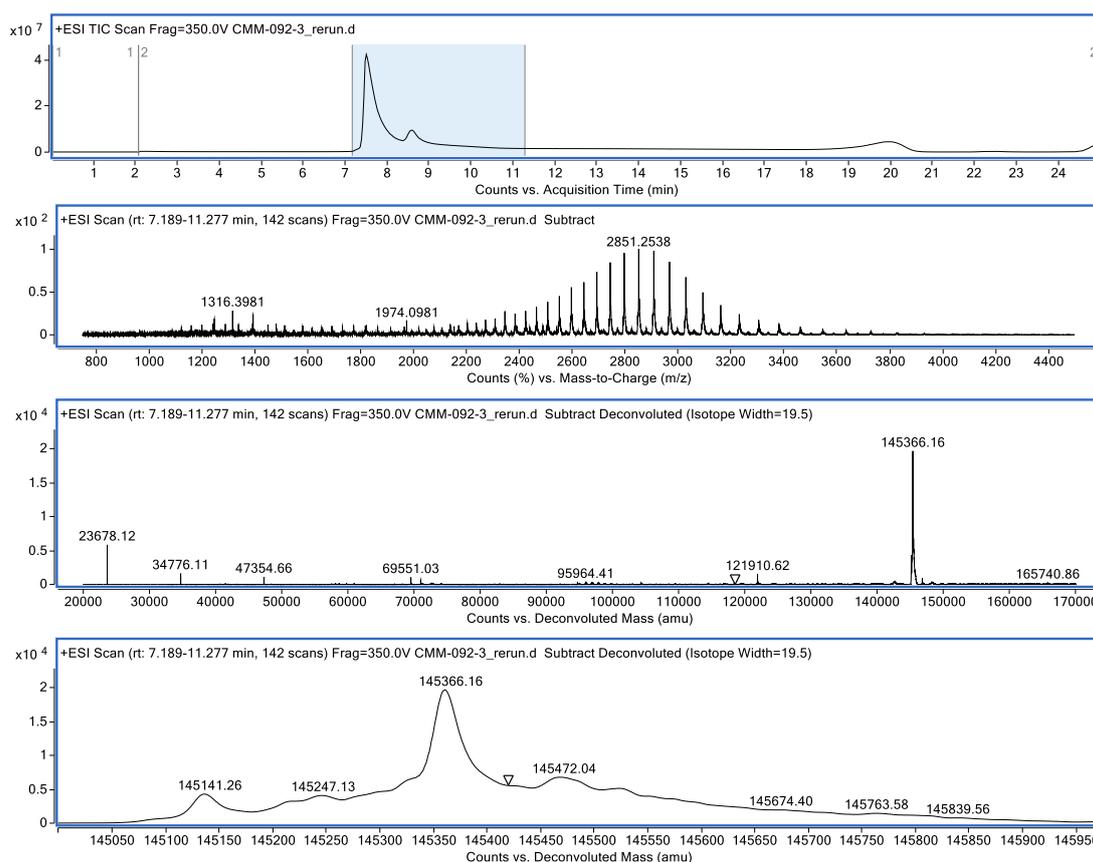


Figure S10: (i) TIC LC-MS trace (top), (ii) non-deconvoluted LC-MS trace (upper middle), (iii) deconvoluted MS data (lower middle, wide range), (iv) deconvoluted MS data (bottom, zoom in range).

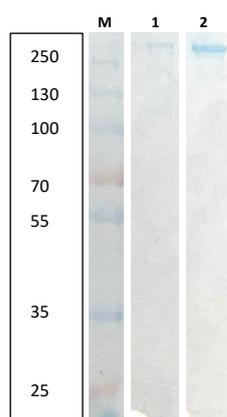
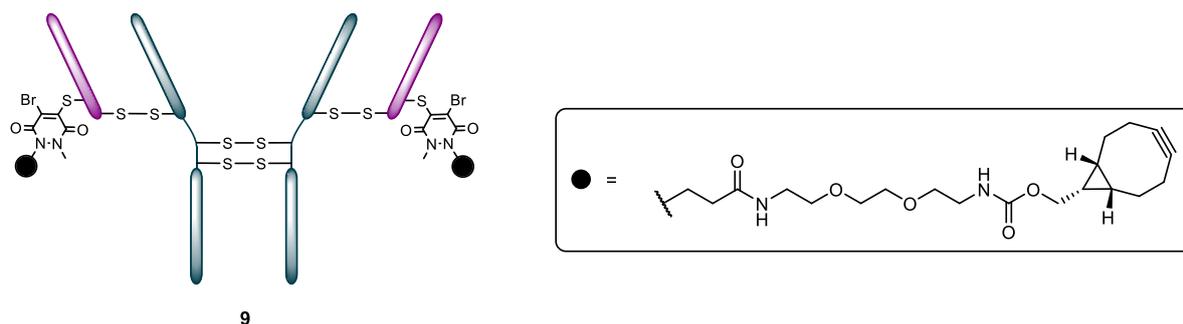


Figure S11: SDS-PAGE gel: M. Ladder, 1. Conjugate **8c**, 2. Conjugate **S14**

LC S168C PD conjugate 9 (LC S168C Thio-Trastuzumab Conjugated to BCN PD)



Uncapping Tests

To a solution of uncapped LC S168C thio-trastuzumab **8A** (100 μ L, 20 μ M) in BBS (25 mM borate, 25 mM NaCl, 2 mM EDTA, 2% DMSO, pH C) was added BCN PD **1** (20 mM in DMSO, 4 eq.) and the reaction incubated at 37 $^{\circ}$ C for 3 h under constant agitation (300 rpm). After this time, excess reagents were removed to give conjugate **9** (expected mass 146,307 Da, observed mass 146,307 Da) which was analysed by LC-MS (method 1b), UV-Vis spectroscopy and SDS-PAGE.

Uncapping Protocol (A)	
9a	8a
9b	8b
9c	8c
9d	8d
9e	8e
9f	8f
9g	8g
9h	8h
9i	8i
9j	8j
9k	8k

Table S7. Conditions used to form **9**.

Optimisation on Uncapped Ab

To a solution of uncapped LC S168C thio-trastuzumab **8** (100 μ L, B μ M) in BBS (25 mM borate, 25 mM NaCl, 2 mM EDTA, 2% DMSO, pH C) was added BCN PD **1** (20 mM in DMSO, D eq.) and the reaction incubated at 37 $^{\circ}$ C for E h under constant agitation (300 rpm). After this time, excess reagents were removed to give conjugate **9** (expected mass 146,309 Da, observed mass 146,308 Da) which was analysed by LC-MS (method 1b), UV-Vis spectroscopy and SDS-PAGE.

	Antibody Concentration (B)	Buffer pH (C)	BCN PD Eq. (D)	Reaction Time (E)
9l	20	8.0	8	3 h
9m	20	8.0	5	16 h
9n	20	8.0	4	1 h
9o	20	8.0	4	2 h
9p	20	8.0	4	3 h
9q	20	8.0	4	4 h
9r	20	8.5	4	3 h
9s	10	8.0	4	3 h

Table S8. Conditions used to form 9.

9a

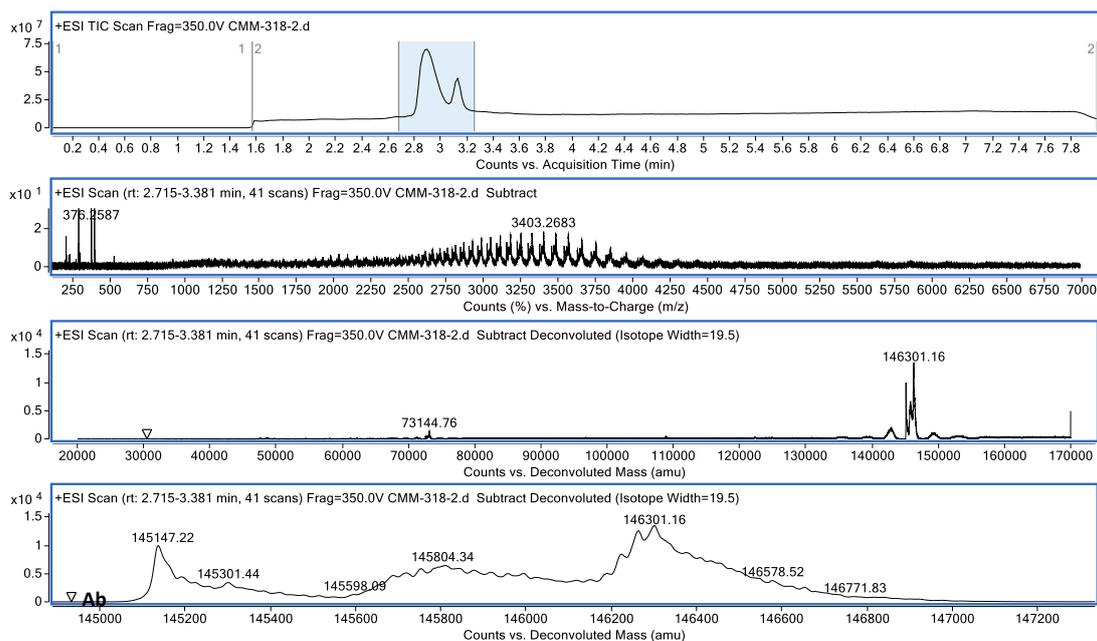


Figure S12: (i) TIC LC-MS trace (top), (ii) non-deconvoluted LC-MS trace (upper middle), (iii) deconvoluted MS data (lower middle, wide range), (iv) deconvoluted MS data (bottom, zoom in range).

9b

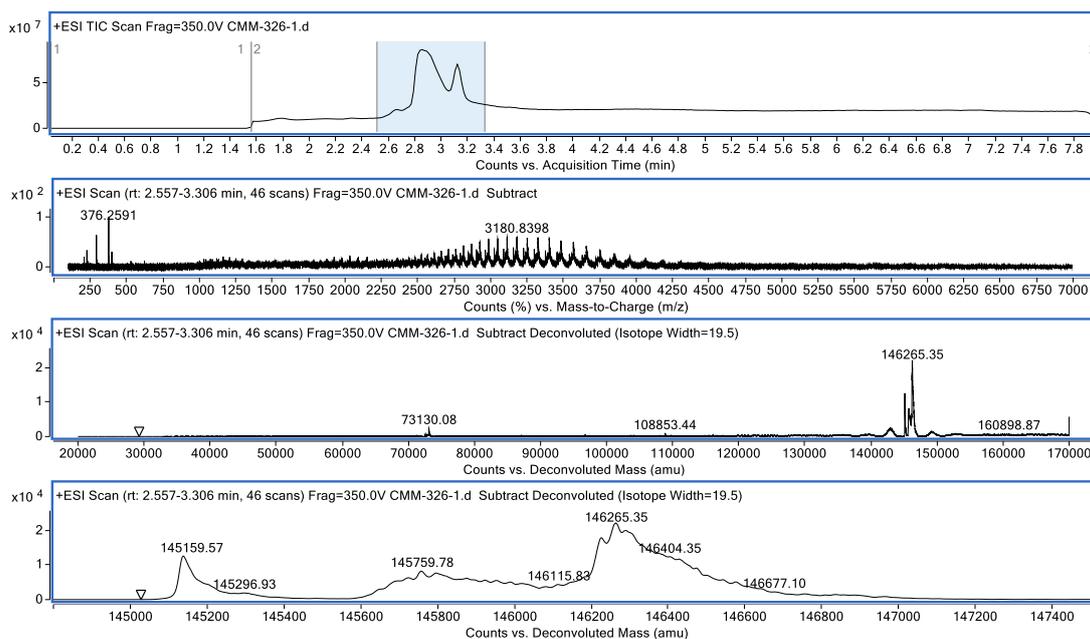


Figure S13: (i) TIC LC-MS trace (top), (ii) non-deconvoluted LC-MS trace (upper middle), (iii) deconvoluted MS data (lower middle, wide range), (iv) deconvoluted MS data (bottom, zoom in range).

9c

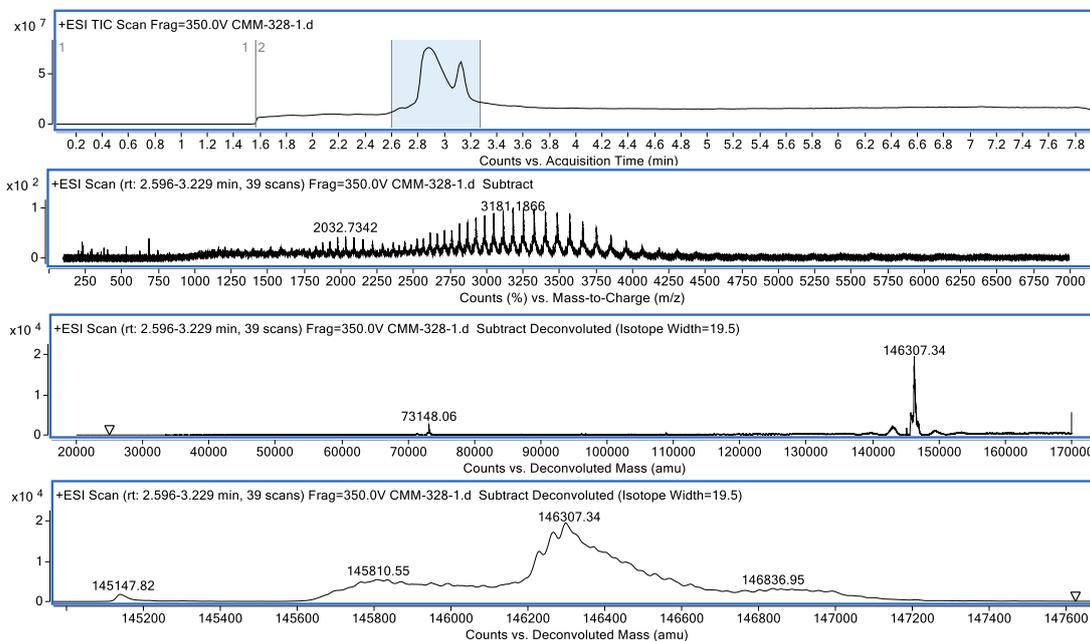


Figure S14: (i) TIC LC-MS trace (top), (ii) non-deconvoluted LC-MS trace (upper middle), (iii) deconvoluted MS data (lower middle, wide range), (iv) deconvoluted MS data (bottom, zoom in range).

9d

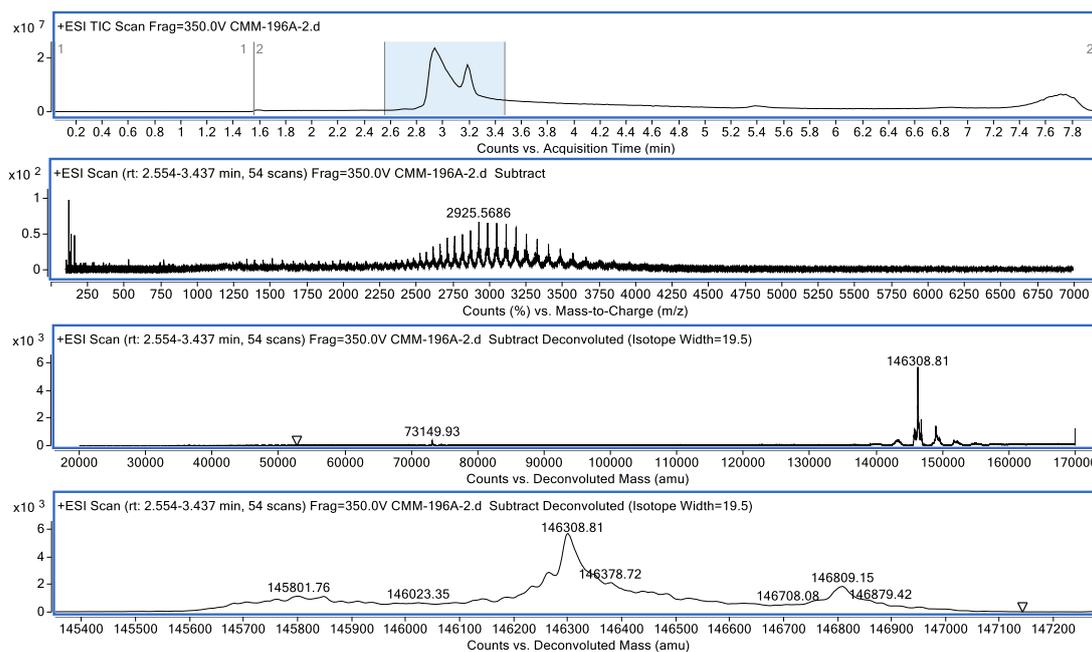


Figure S15: (i) TIC LC-MS trace (top), (ii) non-deconvoluted LC-MS trace (upper middle), (iii) deconvoluted MS data (lower middle, wide range), (iv) deconvoluted MS data (bottom, zoom in range).

9e

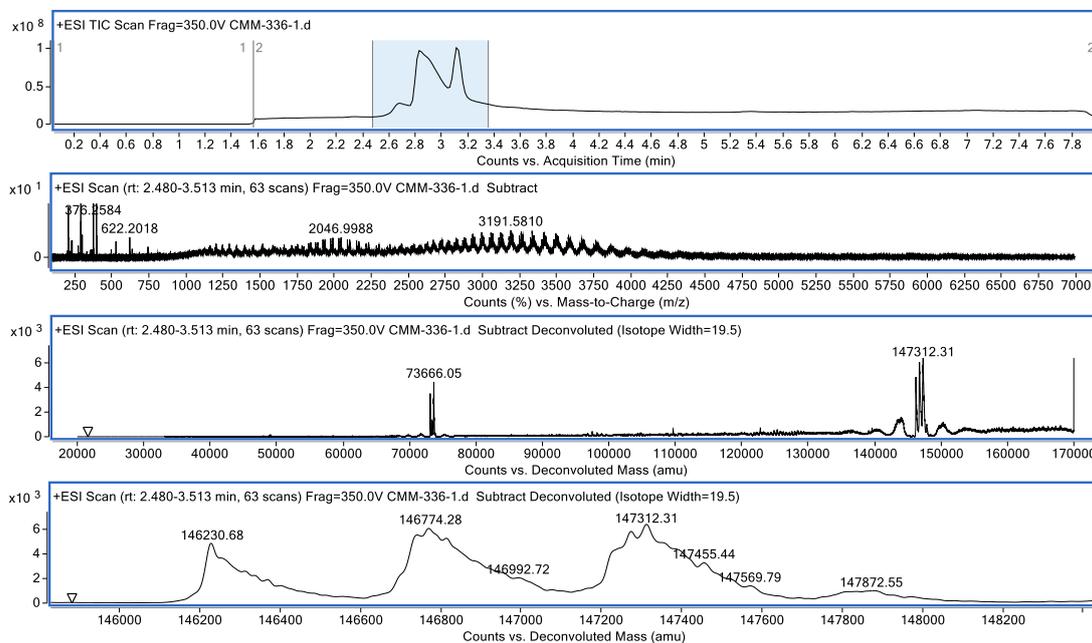


Figure S16: (i) TIC LC-MS trace (top), (ii) non-deconvoluted LC-MS trace (upper middle), (iii) deconvoluted MS data (lower middle, wide range), (iv) deconvoluted MS data (bottom, zoom in range).

9f

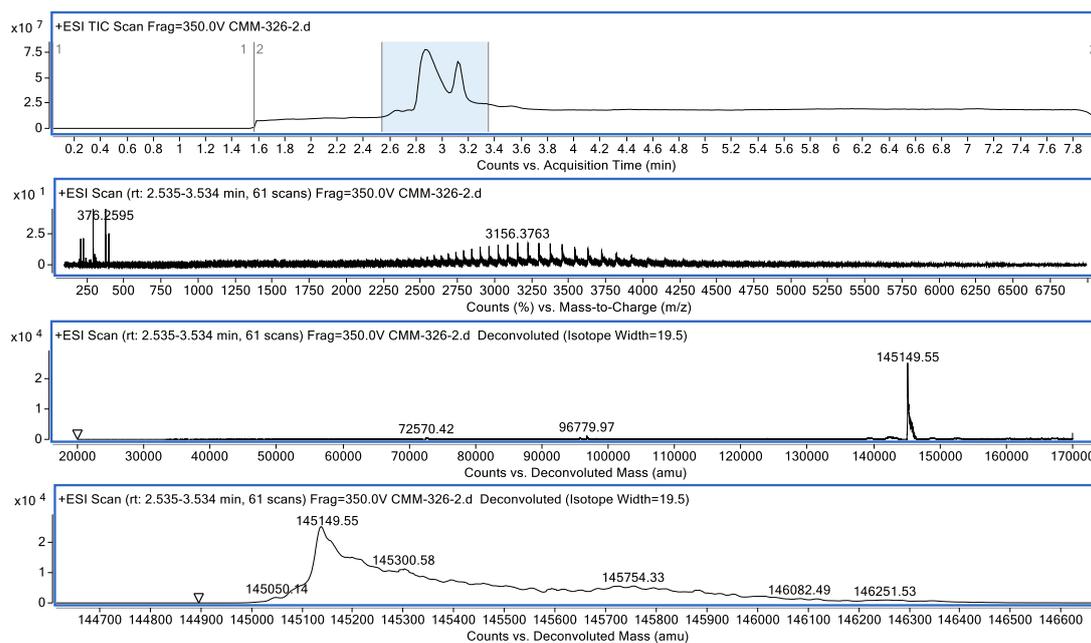


Figure S17: (i) TIC LC-MS trace (top), (ii) non-deconvoluted LC-MS trace (upper middle), (iii) deconvoluted MS data (lower middle, wide range), (iv) deconvoluted MS data (bottom, zoom in range).

9g

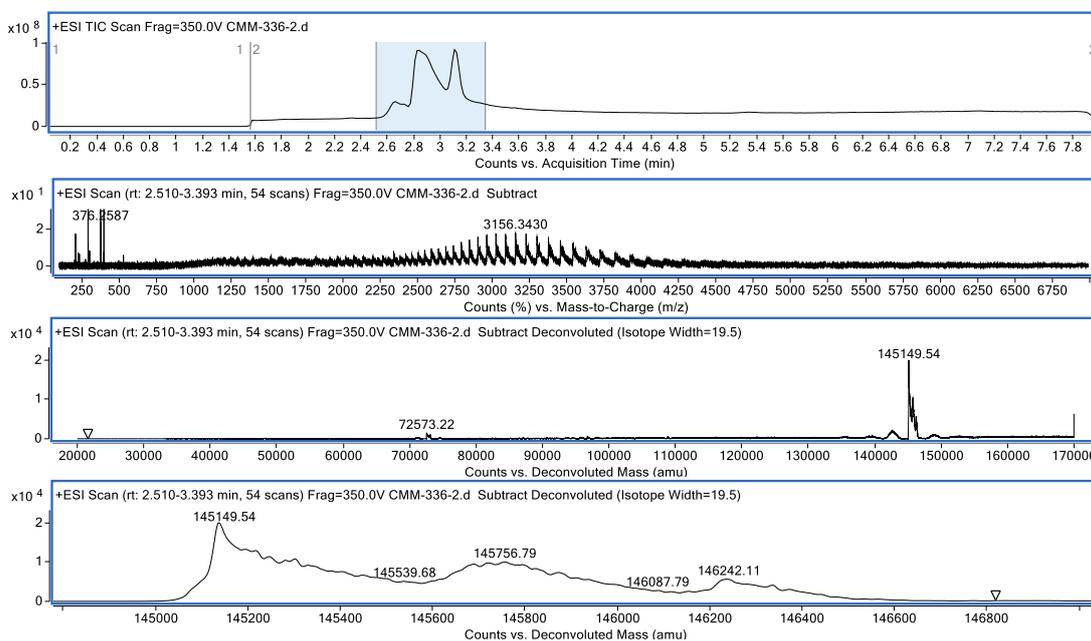


Figure S18: (i) TIC LC-MS trace (top), (ii) non-deconvoluted LC-MS trace (upper middle), (iii) deconvoluted MS data (lower middle, wide range), (iv) deconvoluted MS data (bottom, zoom in range).

9h

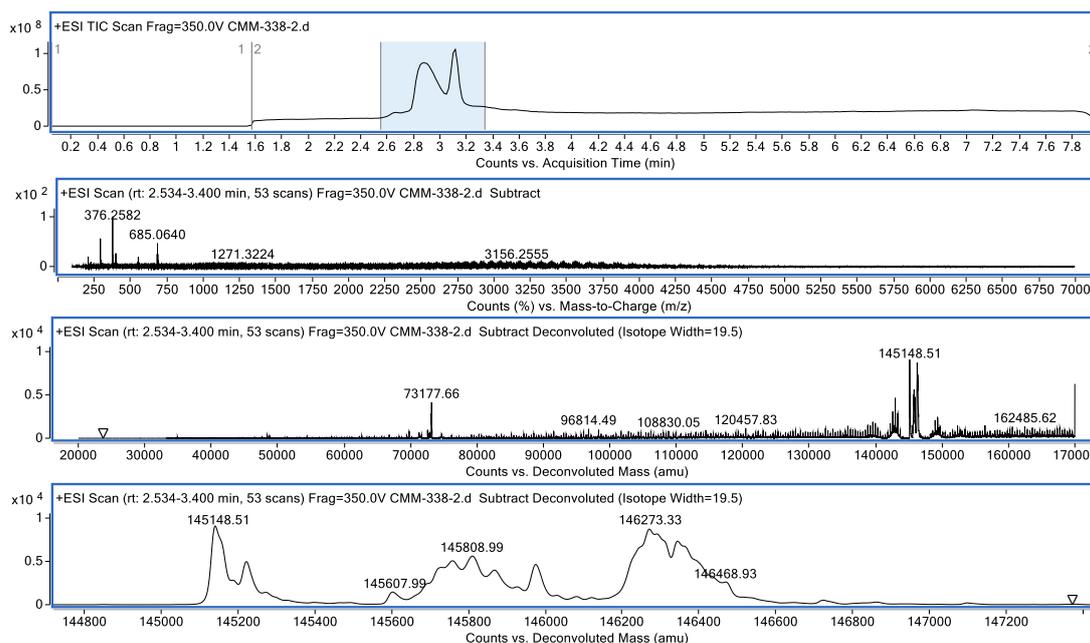


Figure S19: (i) TIC LC-MS trace (top), (ii) non-deconvoluted LC-MS trace (upper middle), (iii) deconvoluted MS data (lower middle, wide range), (iv) deconvoluted MS data (bottom, zoom in range).

9i

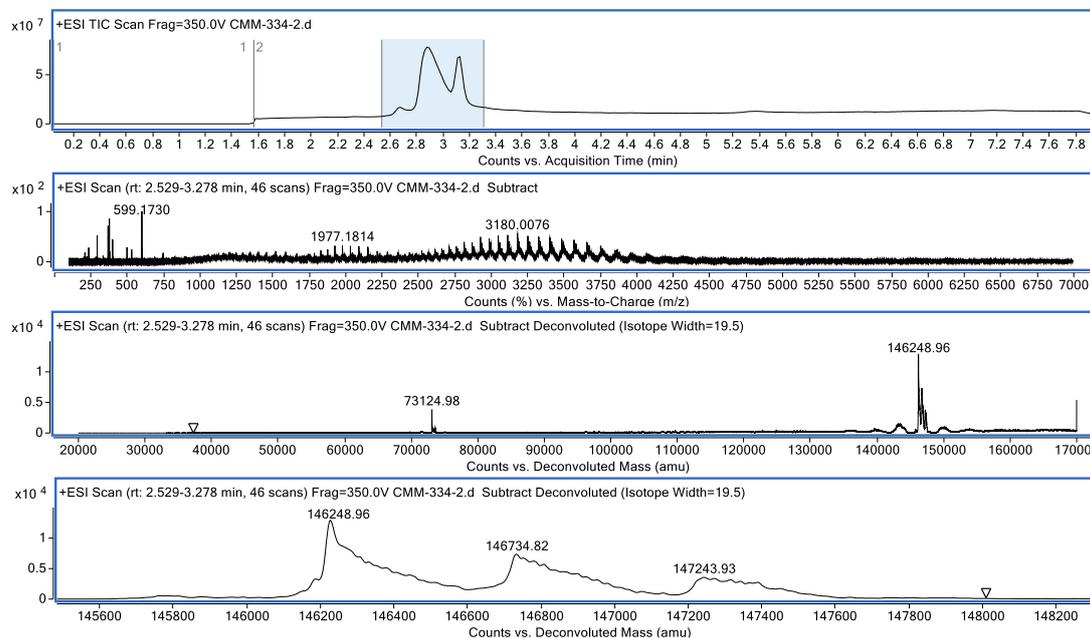


Figure S20: (i) TIC LC-MS trace (top), (ii) non-deconvoluted LC-MS trace (upper middle), (iii) deconvoluted MS data (lower middle, wide range), (iv) deconvoluted MS data (bottom, zoom in range).

9j

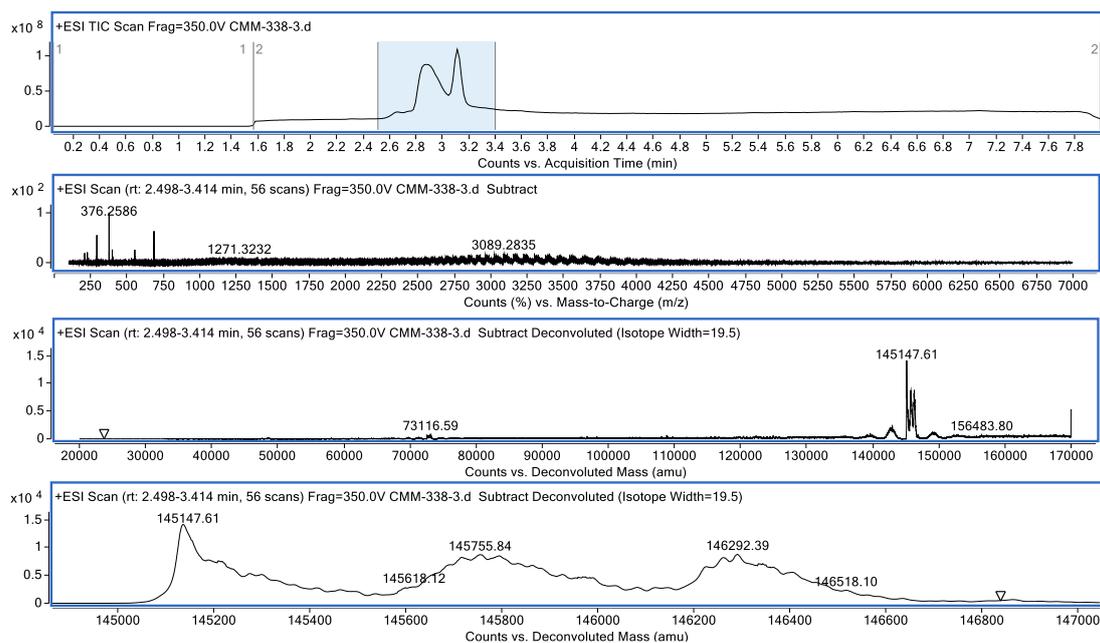


Figure S21: (i) TIC LC-MS trace (top), (ii) non-deconvoluted LC-MS trace (upper middle), (iii) deconvoluted MS data (lower middle, wide range), (iv) deconvoluted MS data (bottom, zoom in range).

9k

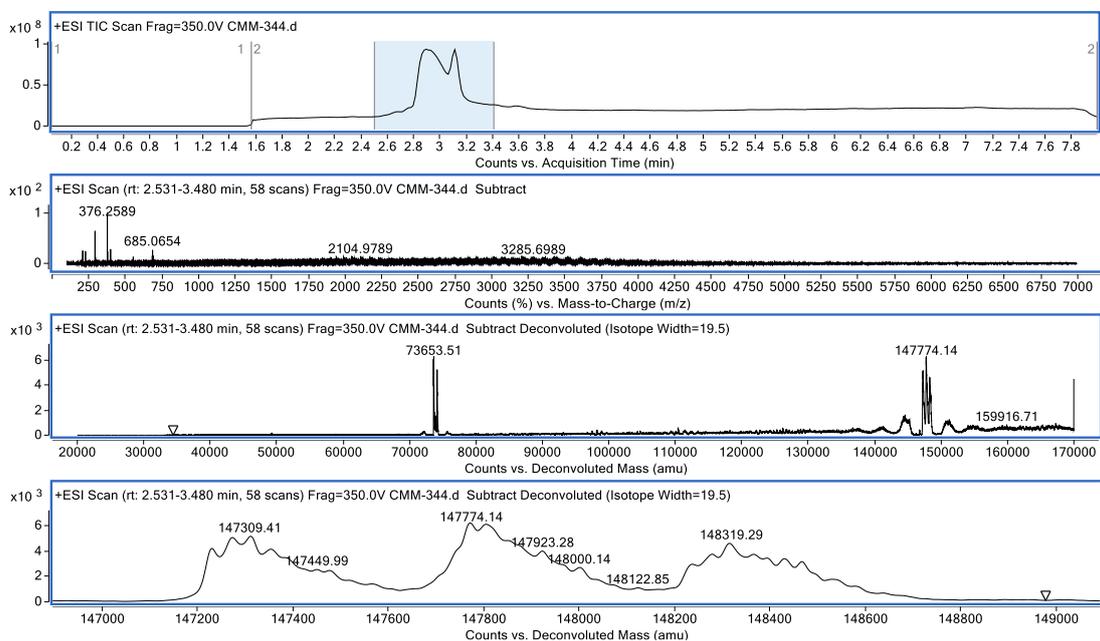


Figure S22: (i) TIC LC-MS trace (top), (ii) non-deconvoluted LC-MS trace (upper middle), (iii) deconvoluted MS data (lower middle, wide range), (iv) deconvoluted MS data (bottom, zoom in range).

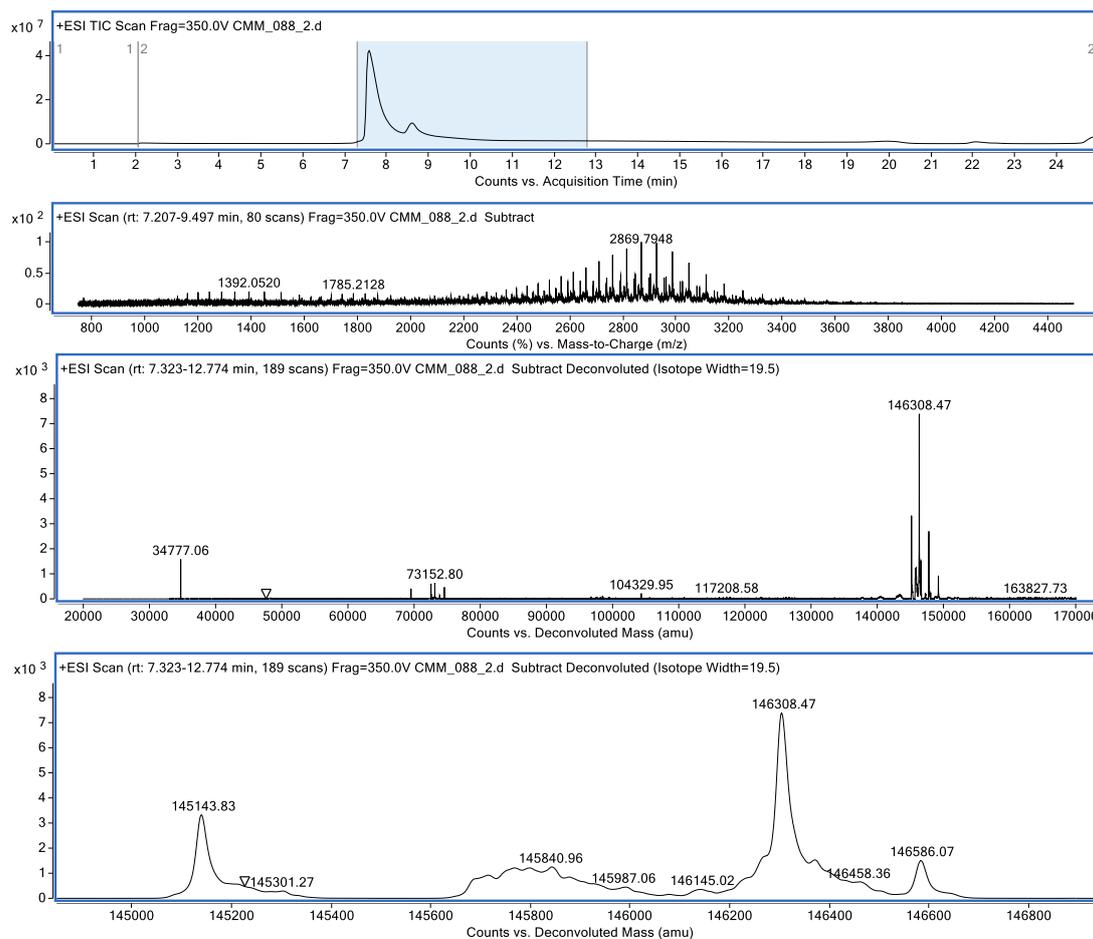


Figure S23: (i) TIC LC-MS trace (top), (ii) non-deconvoluted LC-MS trace (upper middle), (iii) deconvoluted MS data (lower middle, wide range), (iv) deconvoluted MS data (bottom, zoom in range).

9m

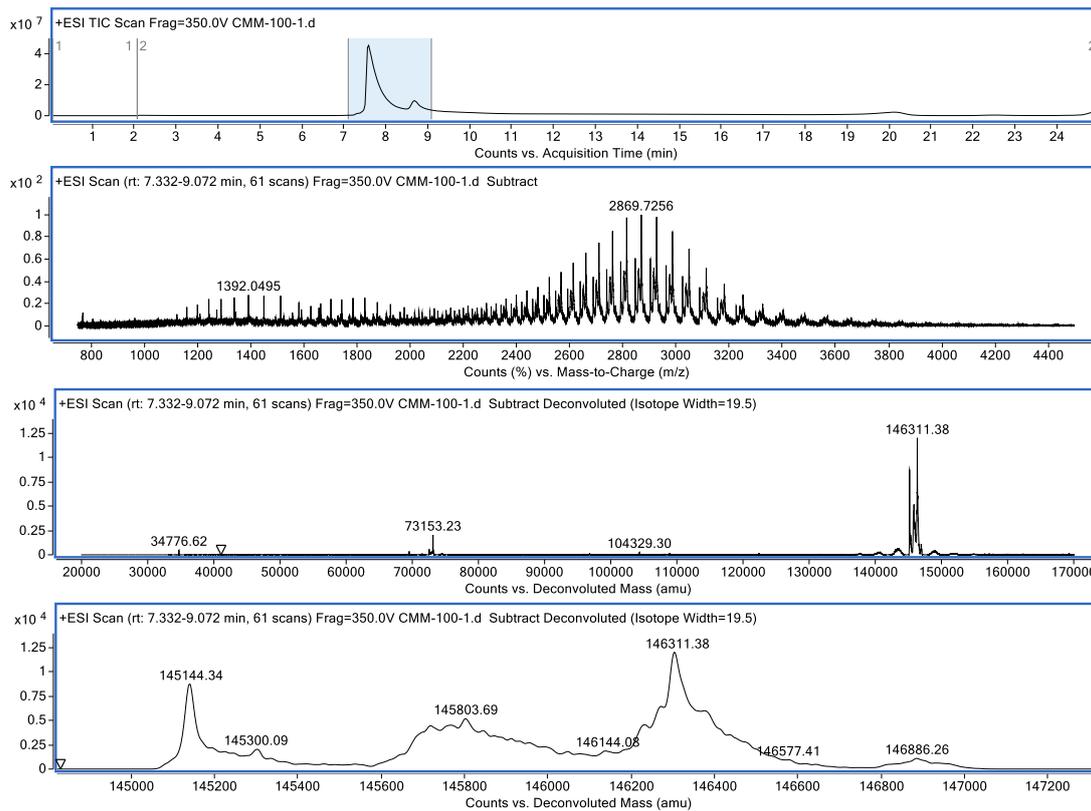


Figure S24: (i) TIC LC-MS trace (top), (ii) non-deconvoluted LC-MS trace (upper middle), (iii) deconvoluted MS data (lower middle, wide range), (iv) deconvoluted MS data (bottom, zoom in range).

9n

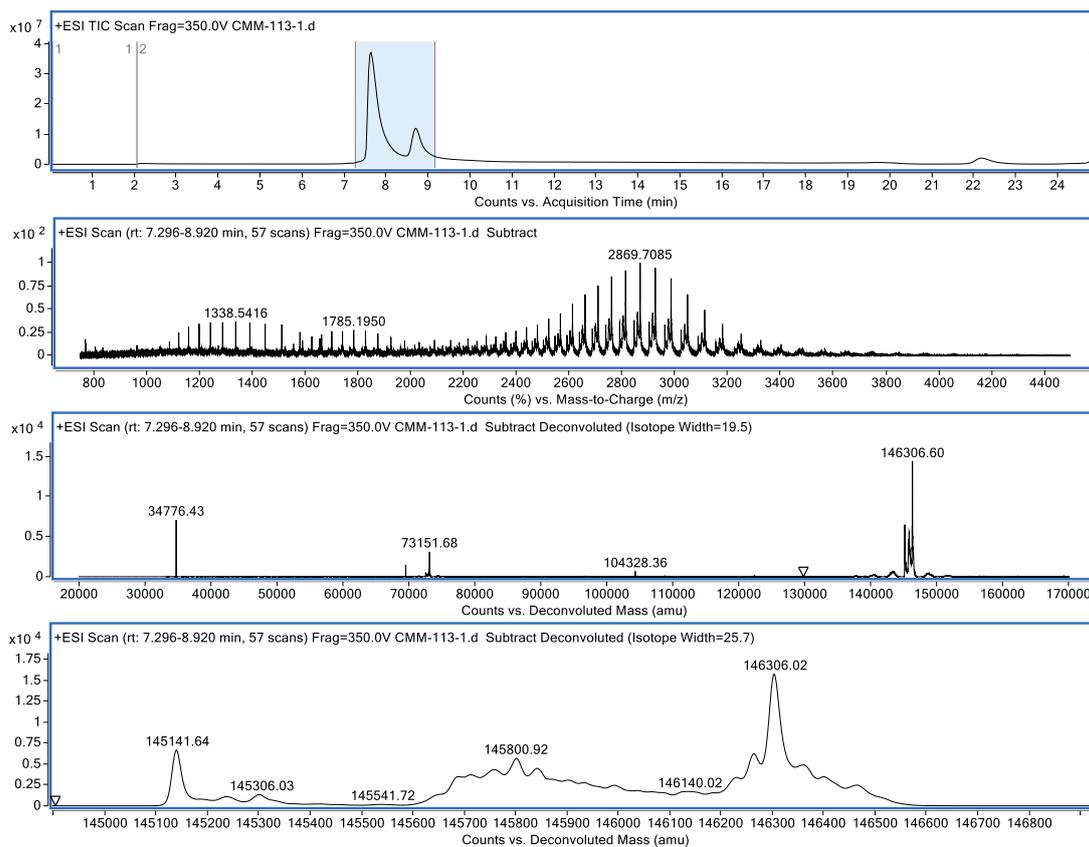


Figure S25: (i) TIC LC-MS trace (top), (ii) non-deconvoluted LC-MS trace (upper middle), (iii) deconvoluted MS data (lower middle, wide range), (iv) deconvoluted MS data (bottom, zoom in range).

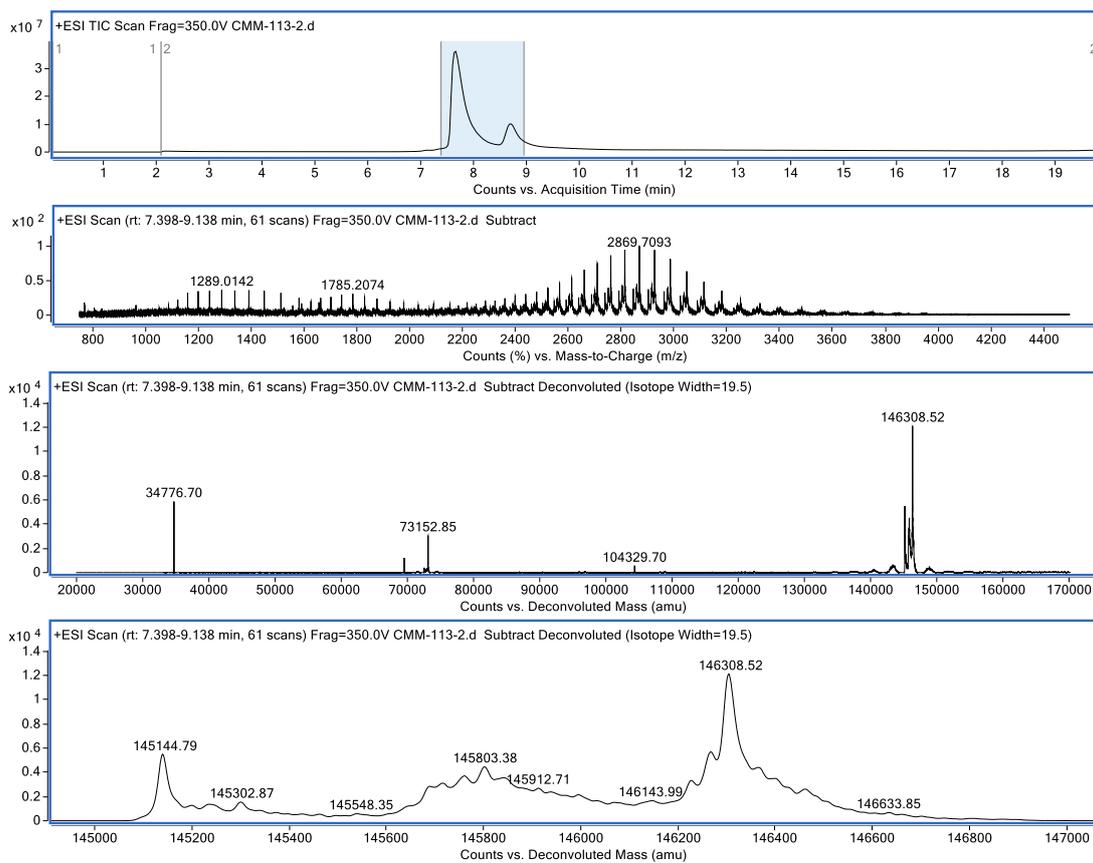


Figure S26: (i) TIC LC-MS trace (top), (ii) non-deconvoluted LC-MS trace (upper middle), (iii) deconvoluted MS data (lower middle, wide range), (iv) deconvoluted MS data (bottom, zoom in range).

9p

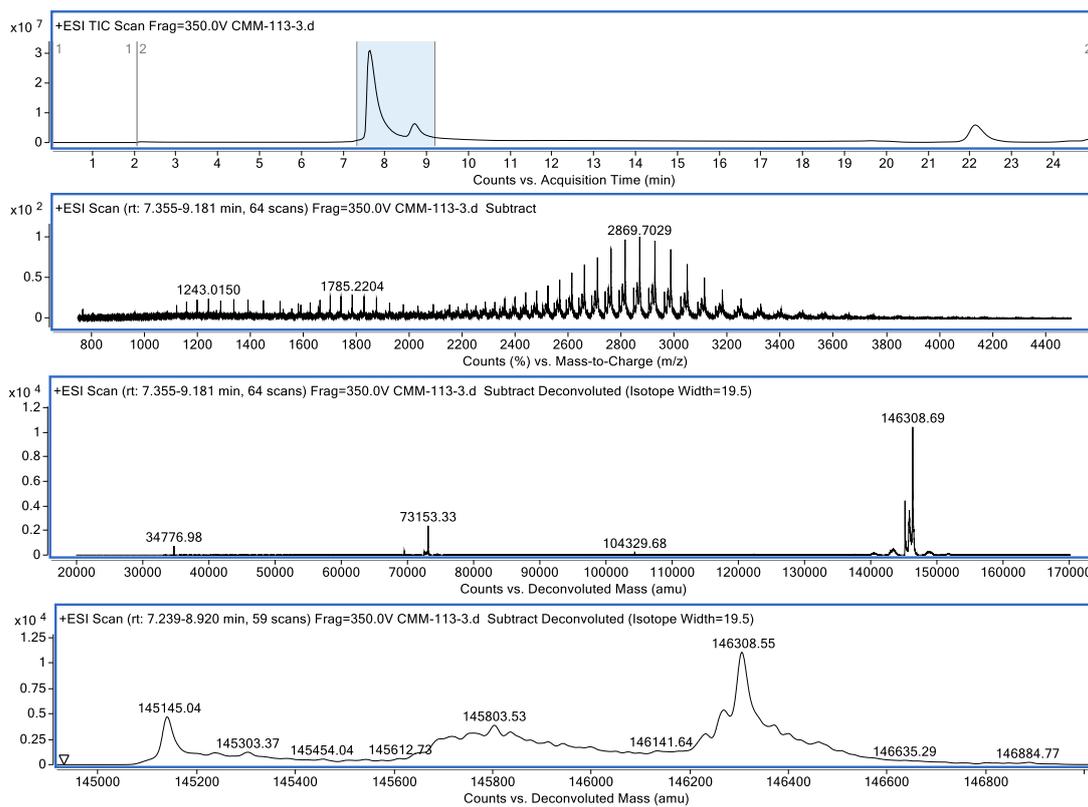


Figure S27: (i) TIC LC-MS trace (top), (ii) non-deconvoluted LC-MS trace (upper middle), (iii) deconvoluted MS data (lower middle, wide range), (iv) deconvoluted MS data (bottom, zoom in range).

9q

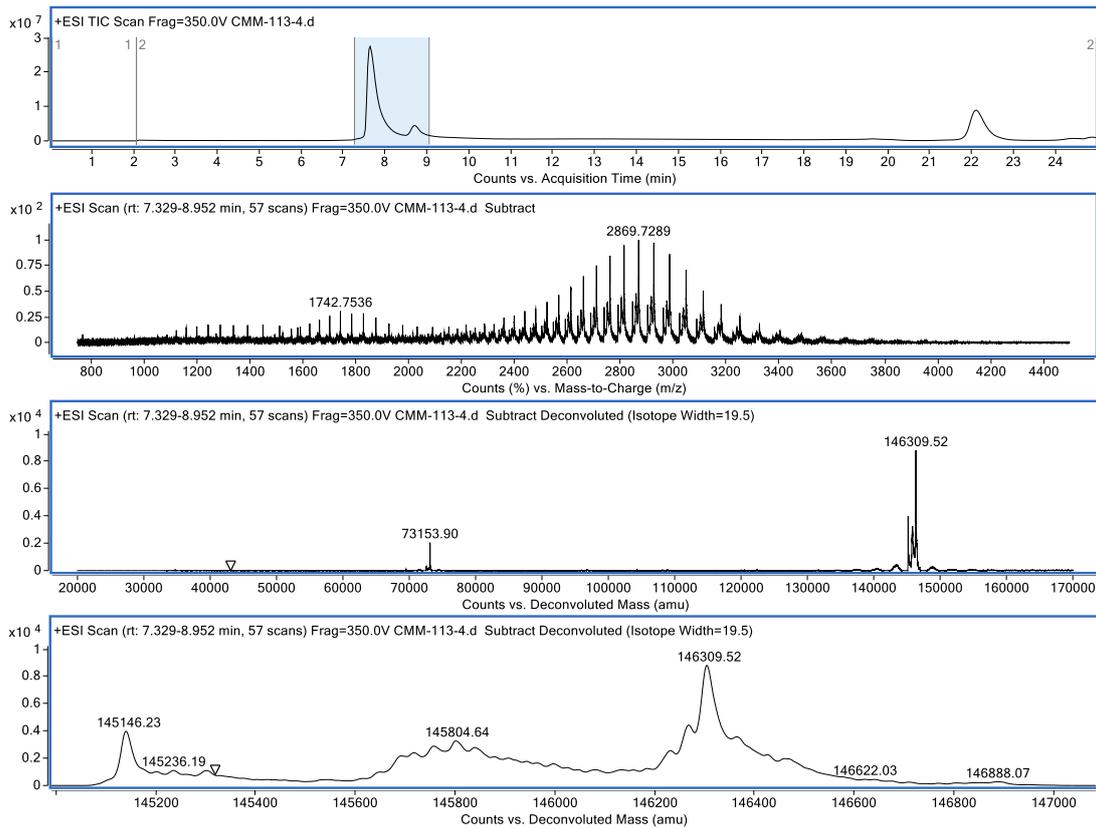


Figure S28: (i) TIC LC-MS trace (top), (ii) non-deconvoluted LC-MS trace (upper middle), (iii) deconvoluted MS data (lower middle, wide range), (iv) deconvoluted MS data (bottom, zoom in range).

9r

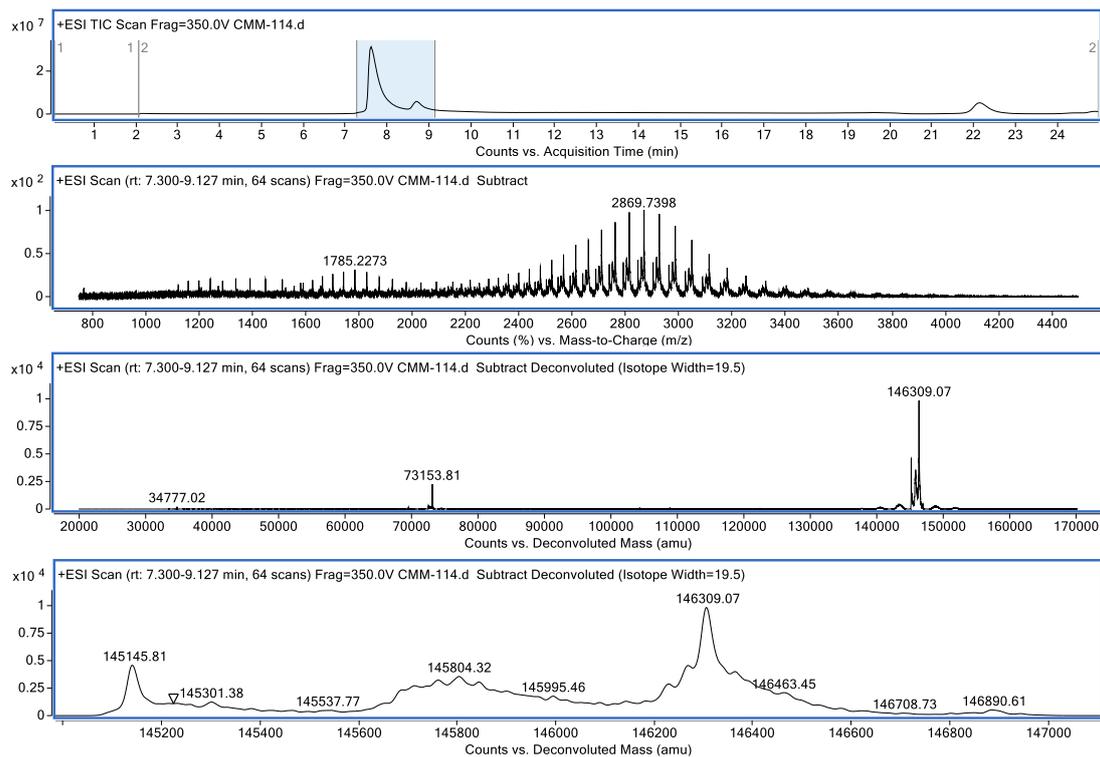


Figure S29: (i) TIC LC-MS trace (top), (ii) non-deconvoluted LC-MS trace (upper middle), (iii) deconvoluted MS data (lower middle, wide range), (iv) deconvoluted MS data (bottom, zoom in range).

9s

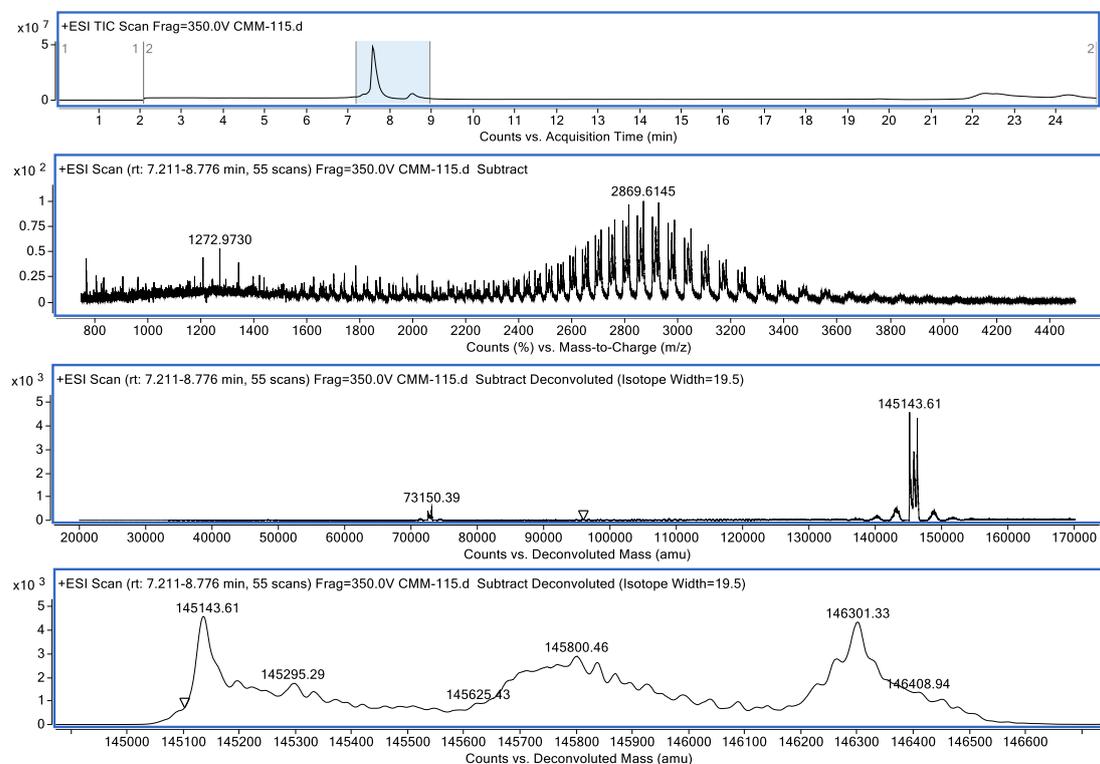


Figure S30: (i) TIC LC-MS trace (top), (ii) non-deconvoluted LC-MS trace (upper middle), (iii) deconvoluted MS data (lower middle, wide range), (iv) deconvoluted MS data (bottom, zoom in range).

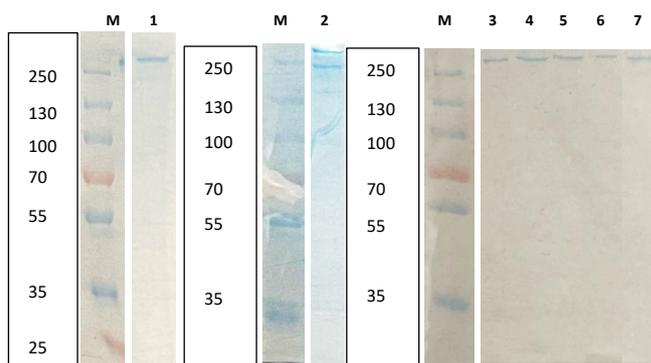
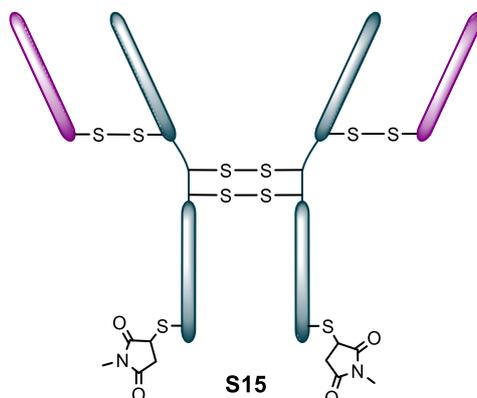


Figure S31: SDS-PAGE gel: M. Ladder, 1. Conjugate **9l**, 2. Conjugate **9m**, 3. Conjugate **9n**, 4. Conjugate **9o**, 5. Conjugate **9p**, 6. Conjugate **9q**, 7. Conjugate **9s**.

HC S378C NMM conjugate **S15** (HC S378C Thio-Trastuzumab Conjugated to *N*-methylmaleimide)



To a solution of uncapped HC S378C thio-trastuzumab **7** (85 μL , 13.9 μM) in BBS (25 mM borate, 25 mM NaCl, 2 mM EDTA, 2% DMSO, pH 8.0) was added NMM (0.236 μL , 20 mM in DMSO, 4 eq.) and the reaction incubated at 22 $^{\circ}\text{C}$ for 3 h under constant agitation (300 rpm). After this time, excess reagents were removed to give conjugate **S15** (expected mass 145,365 Da, observed mass 145,367 Da) which was analysed by LC-MS (method 1b), UV-Vis spectroscopy and SDS-PAGE.

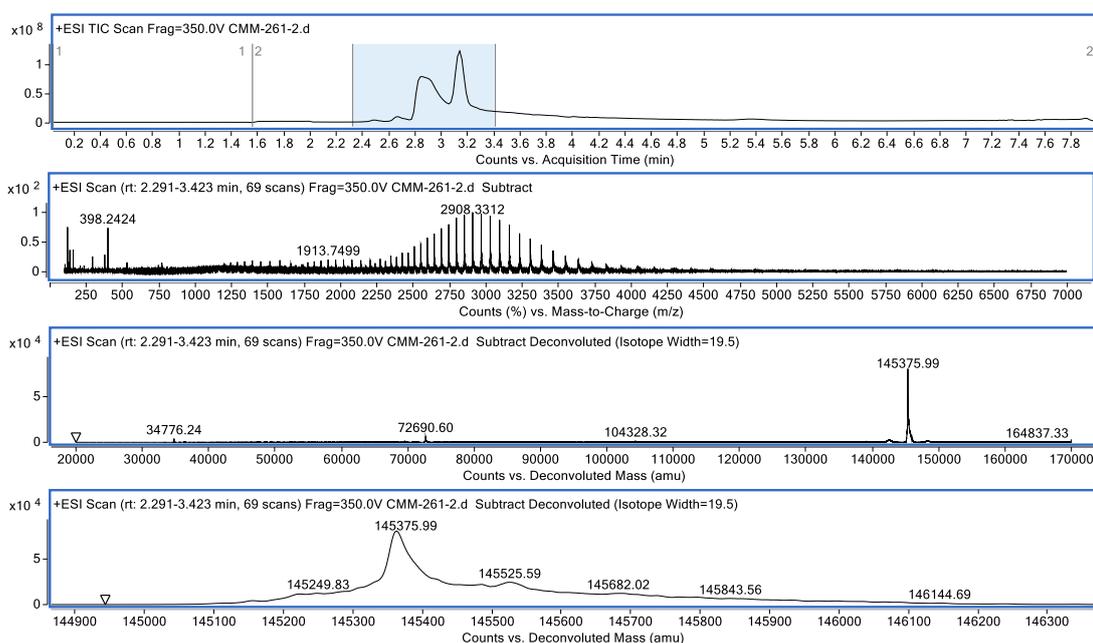


Figure S32: (i) TIC LC-MS trace (top), (ii) non-deconvoluted LC-MS trace (upper middle), (iii) deconvoluted MS data (lower middle, wide range), (iv) deconvoluted MS data (bottom, zoom in range).

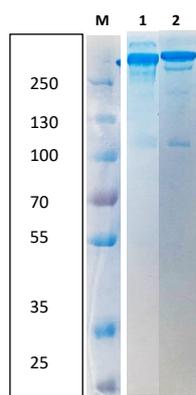
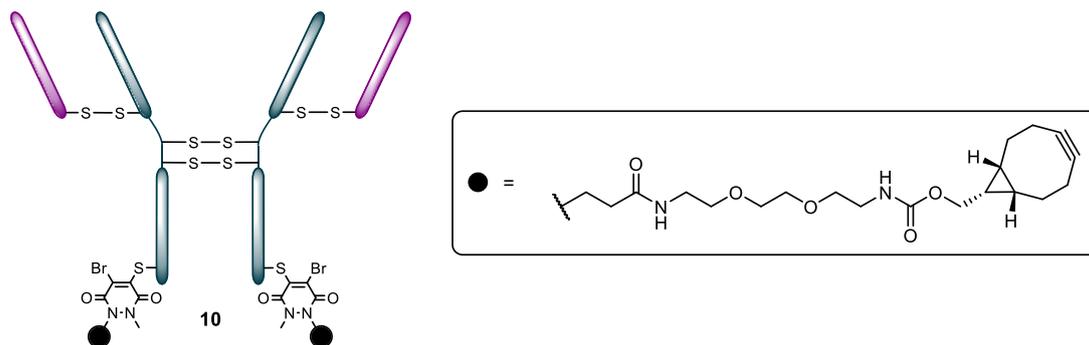


Figure S33: SDS-PAGE gel: M. Ladder, 1. Conjugate **7**, 2. Conjugate **S15**.

HC S378C PD conjugate **10** (HC S378C Thio-trastuzumab conjugated to BCN PD **1**)



To a solution of uncapped HC S378C thio-trastuzumab **7** (100 μ L, 20 μ M) in A was added BCN PD **1** (20 mM in DMSO, B eq.) and the reaction incubated at C $^{\circ}$ C for D h under constant agitation (300 rpm). After this time, excess reagents were removed to give conjugate **10** (expected mass 146,309 Da, observed mass 146,302 Da) which was analysed by LC-MS (method 1a), UV-Vis spectroscopy and SDS-PAGE.

	Buffer (A)	BCN PD Eq. (B)	Incubation Temperature (C)	Reaction Time
10a	BBS, pH 8	4	22	3 h
10b	BBS, pH 8	5	37	3 h
10c	BBS, pH 8	5	37	16 h
10d	BBS, pH 8.5	5	37	3 h

Table S9. Conditions used to form **10**.

10a

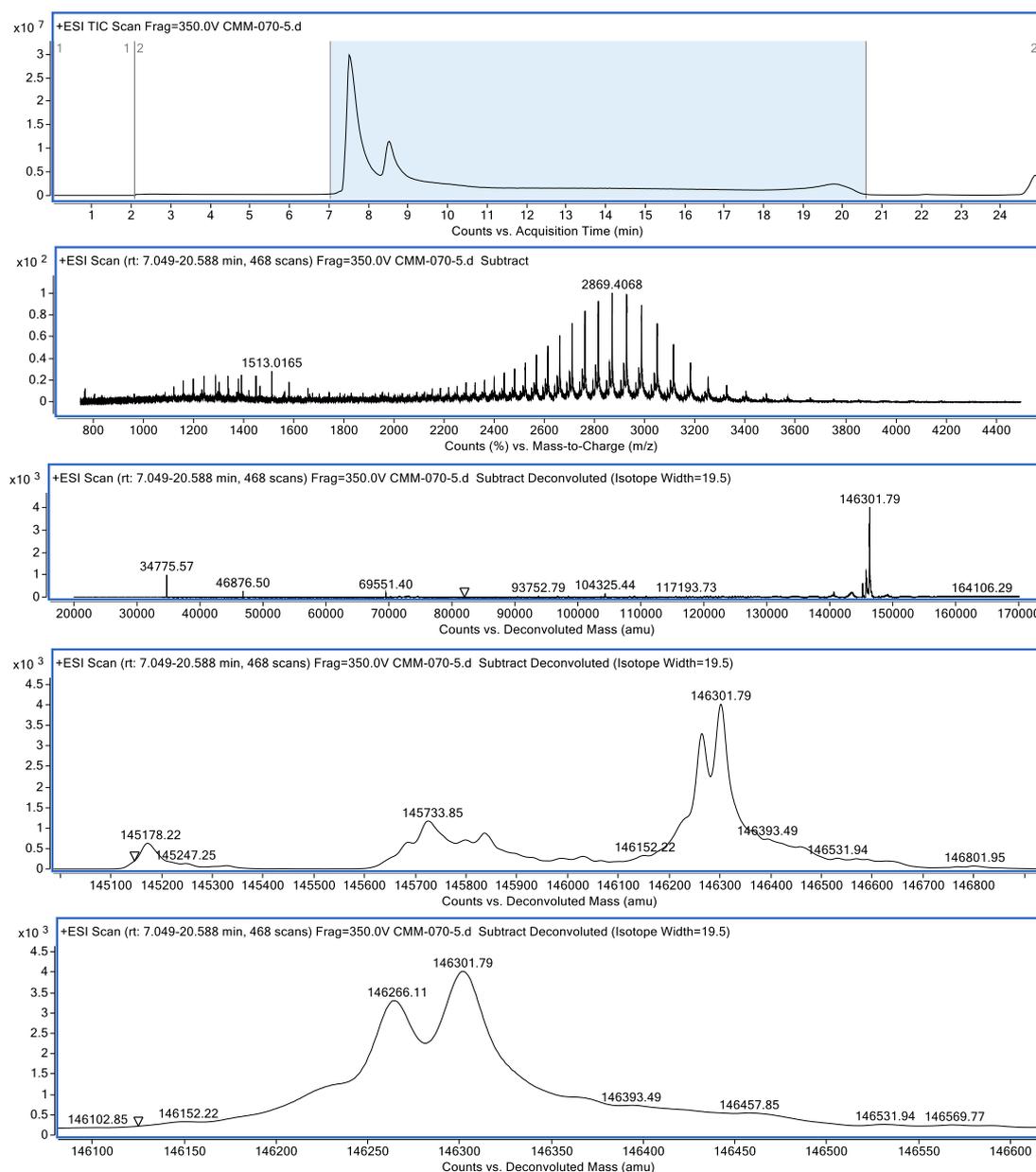


Figure S34: (i) TIC LC-MS trace (top), (ii) non-deconvoluted LC-MS trace (upper middle), (iii) deconvoluted MS data (lower middle, wide range), (iv) and (v) deconvoluted MS data (bottom, zoom in range).

10b

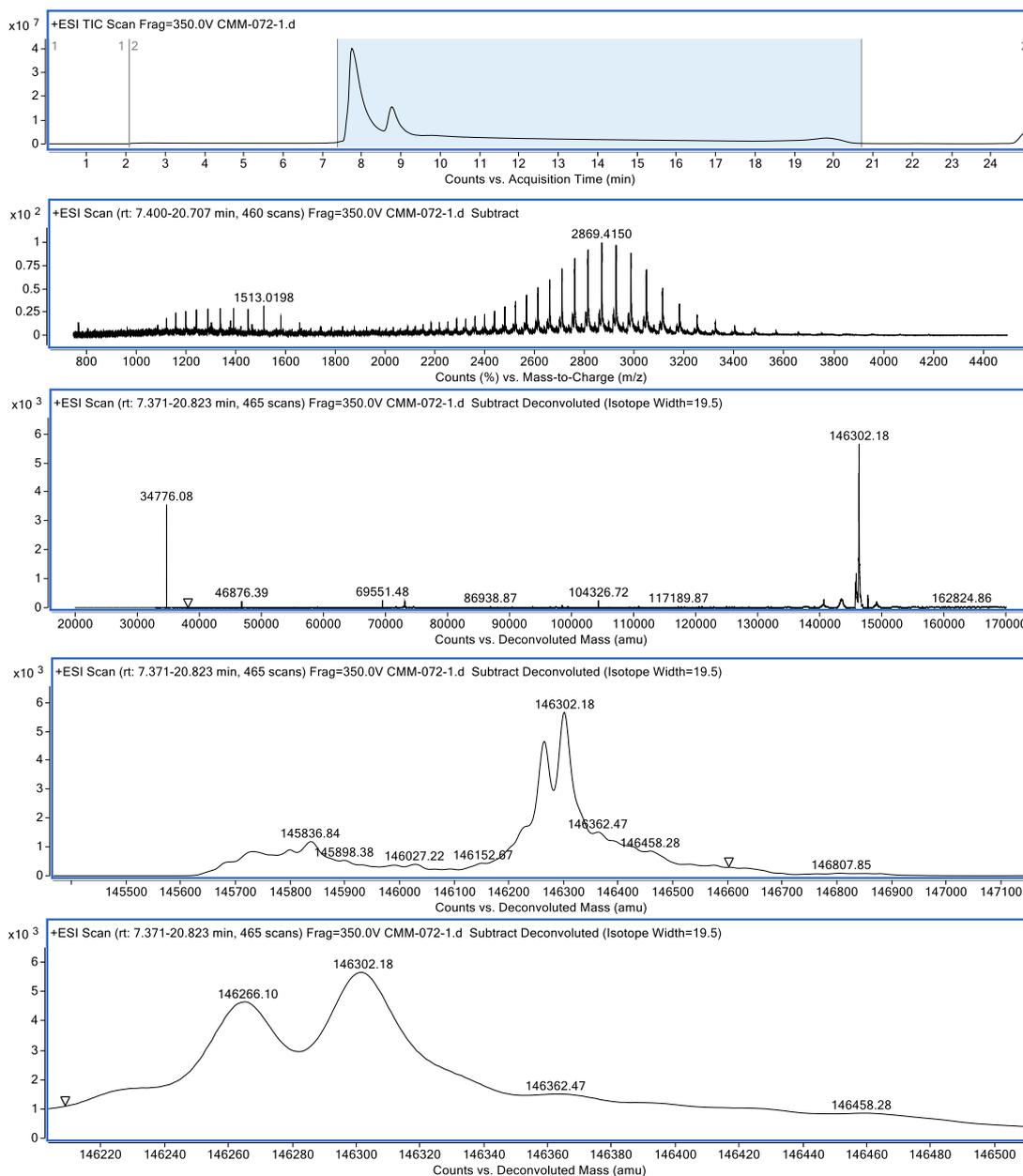


Figure S35: (i) TIC LC-MS trace (top), (ii) non-deconvoluted LC-MS trace (upper middle), (iii) deconvoluted MS data (lower middle, wide range), (iv) and (v) deconvoluted MS data (bottom, zoom in range).

10c

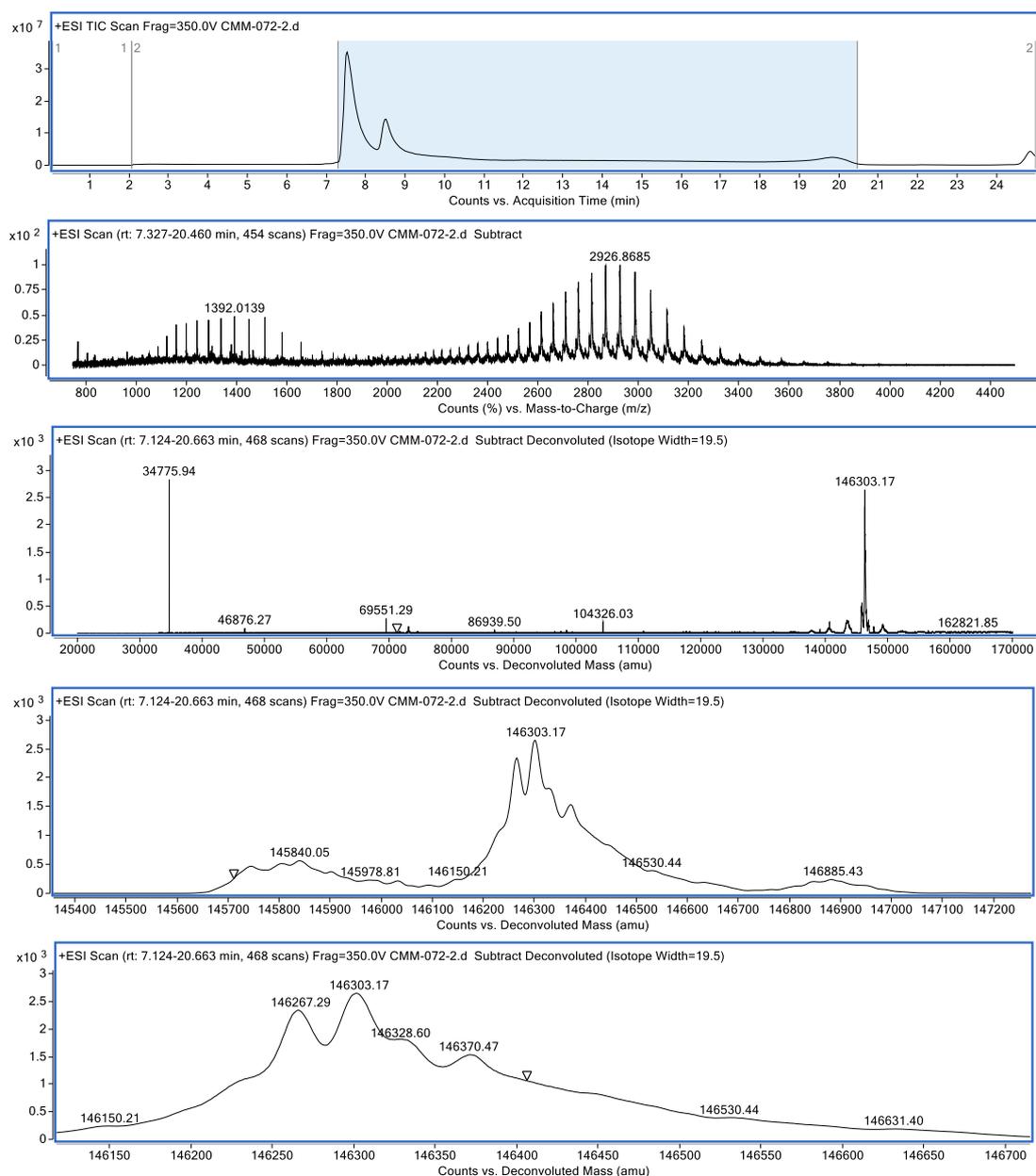


Figure S36: (i) TIC LC-MS trace (top), (ii) non-deconvoluted LC-MS trace (upper middle), (iii) deconvoluted MS data (lower middle, wide range), (iv) and (v) deconvoluted MS data (bottom, zoom in range).

10d

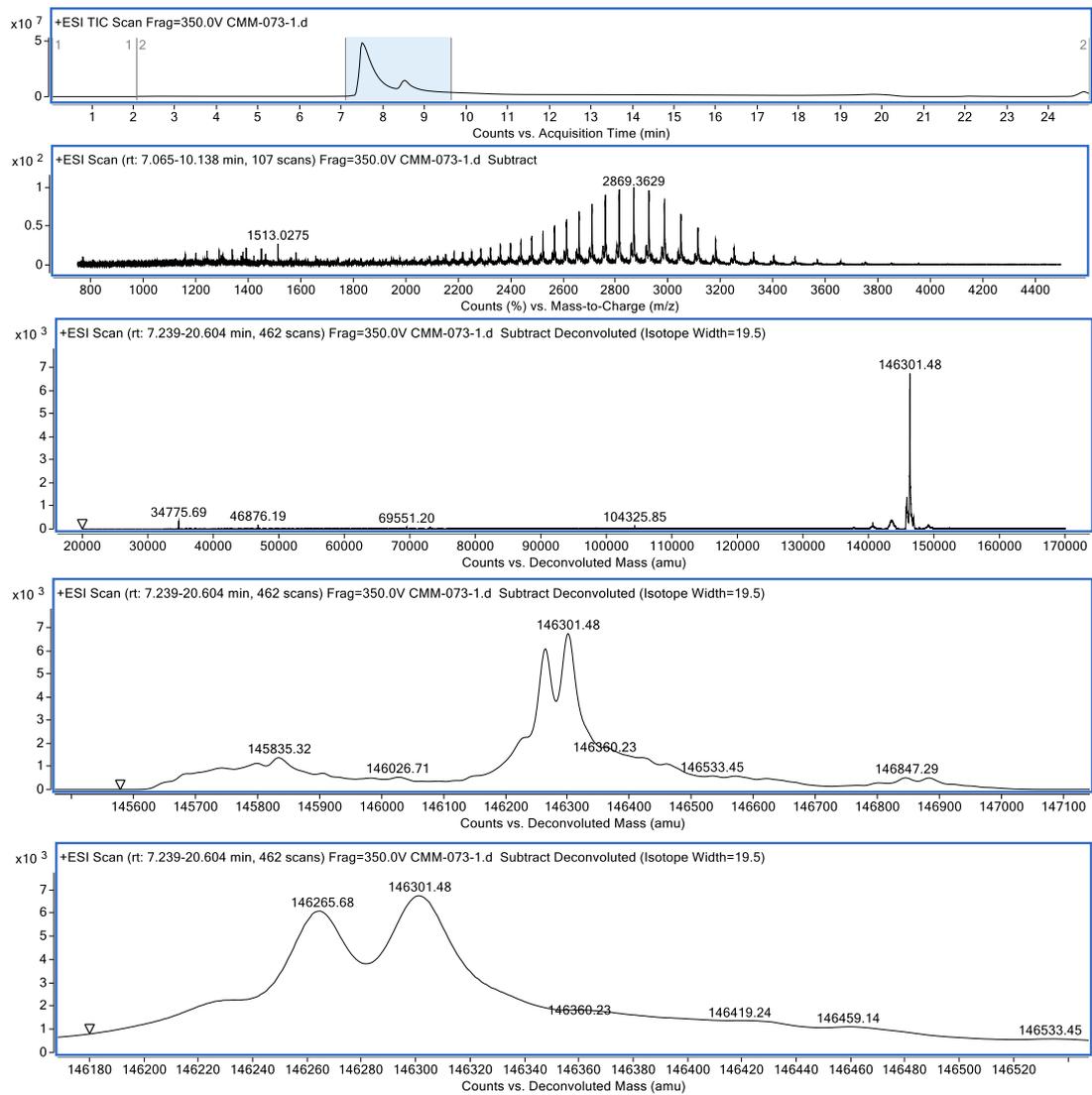


Figure S37: (i) TIC LC-MS trace (top), (ii) non-deconvoluted LC-MS trace (upper middle), (iii) deconvoluted MS data (lower middle, wide range), (iv) and (v) deconvoluted MS data (bottom, zoom in range).

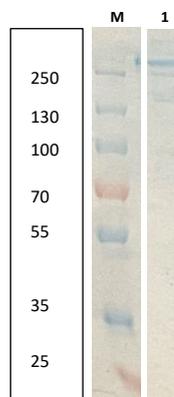


Figure S38: SDS-PAGE gel: M. Ladder, 1. Conjugate 10

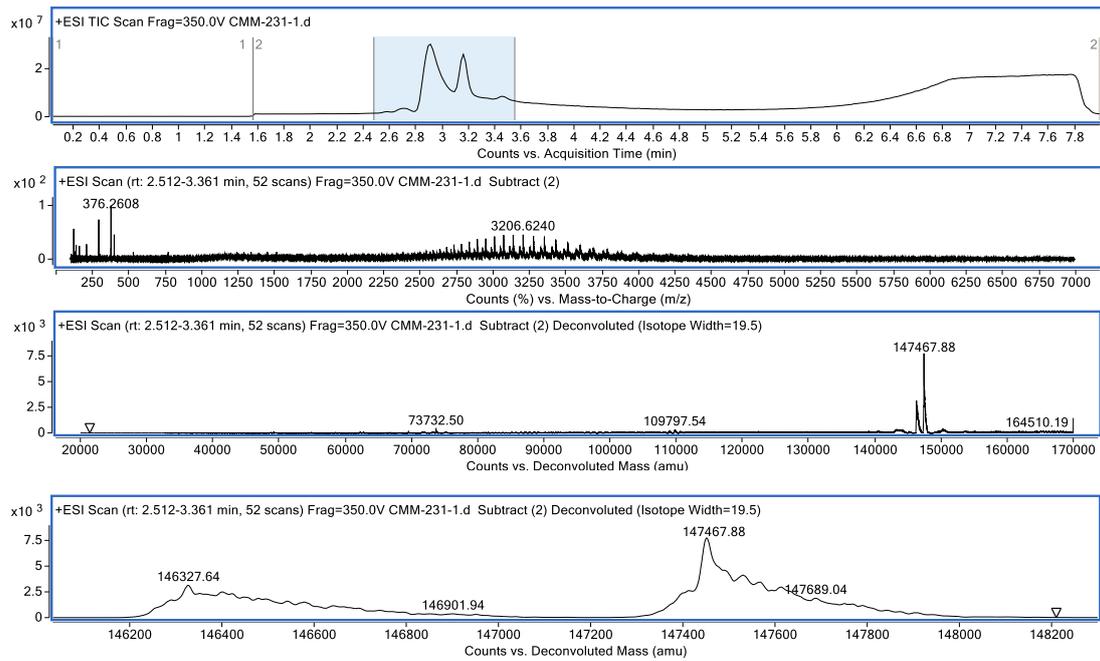


Figure S39: (i) TIC LC-MS trace (top), (ii) non-deconvoluted LC-MS trace (upper middle), (iii) deconvoluted MS data (lower middle, wide range), (iv) deconvoluted MS data (bottom, zoom in range).

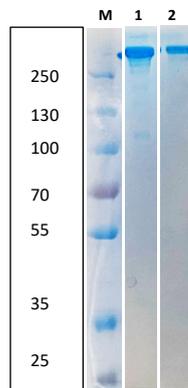
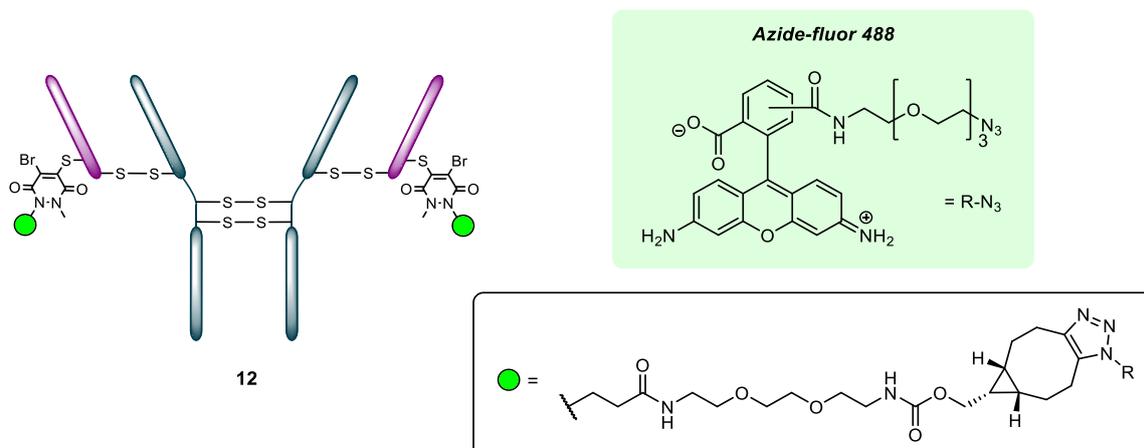


Figure S40: SDS-PAGE gel: M. Ladder, 1. Conjugate 10, 2. Conjugate 11

LC S168C PD fluorophore conjugate **12** (LC S168C thio-trastuzumab conjugated to BCN PD **1**, Clicked with Azide-fluor 488)



Method 1: Post Click

To a solution of uncapped LC S168C thio-trastuzumab conjugated to BCN PD **9a** (50 μ L, 13.2 μ M) in BBS (25 mM borate, 25 mM NaCl, 2 mM EDTA, 2% DMSO, pH 8.0) was added Azide-fluor 488 (0.66 μ L, 10 mM in DMSO, 10 eq.) and the reaction incubated at 37 $^{\circ}$ C for 16 h under constant agitation (300 rpm). After this time, excess reagents were removed to give conjugate **12** (expected mass 147,459 Da, observed mass 147,459 Da) which was analysed by LC-MS (method 1b), UV-Vis spectroscopy and SDS-PAGE.

Method 2: Pre Click

To BBS (22.4 μ L, 25 mM borate, 25 mM NaCl, 2 mM EDTA, 2% DMSO, pH 8.0) was added BCN PD (1.6 μ L, 10 mM DMSO) and Azide-fluor 488 (16 μ L, 10 mM DMSO, 10x excess) and the reaction incubated at 37 $^{\circ}$ C for 20 h under constant agitation (300 rpm) under argon.

To a solution of uncapped LC S168C thio-trastuzumab **8** (160 μ L, 25 μ M) in BBS was added the BCN PD pre-clicked with Azide-fluor 488 (40 μ L, 4 eq.) and the reaction incubated at 37 $^{\circ}$ C for 5 h under constant agitation (300 rpm). After this time, excess reagents were removed to give conjugate **12** (expected mass 147,459 Da, observed mass 147,459 Da) which was analysed by LC-MS (method 1b), UV-Vis spectroscopy and SDS-PAGE.

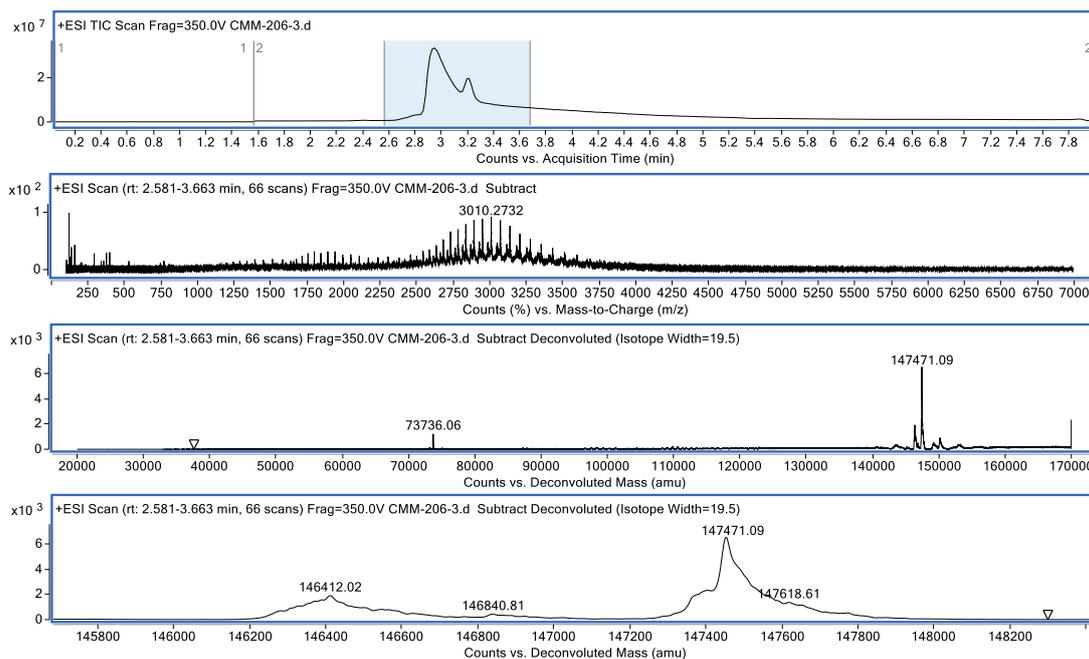


Figure S41: (i) TIC LC-MS trace (top), (ii) non-deconvoluted LC-MS trace (upper middle), (iii) deconvoluted MS data (lower middle, wide range), (iv) deconvoluted MS data (bottom, zoom in range).

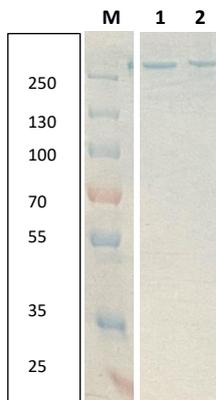
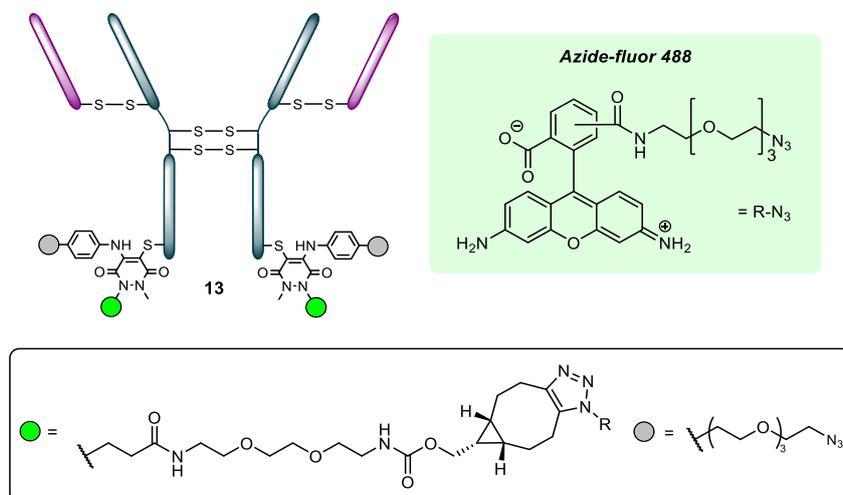


Figure S42: SDS-PAGE gel: M. Ladder, 1. Conjugate 9, 2. Conjugate 12.

HC S378C PD aniline fluorophore conjugate **13** (HC S378C Thio-trastuzumab conjugated to BCN PD 1, Azide-fluor 488 and N₃ Aniline **3**)



To a solution of conjugated HC S378C thio-trastuzumab **11** (40 μ L, 20 μ M) in BBS (25 mM borate, 25 mM NaCl, 2 mM EDTA, 2% DMSO, pH 8.0) was added N₃ Aniline **3** (1.6 μ L, 0.5 M in DMSO, 1000 eq.) and the reaction incubated at 37 $^{\circ}$ C for 16 h under constant agitation (300 rpm). After this time, excess reagents were removed to give conjugate **13** (expected mass 147,917 Da, observed mass 147,915 Da) which was analysed by LC-MS (method 1b), UV-Vis spectroscopy and SDS-PAGE.

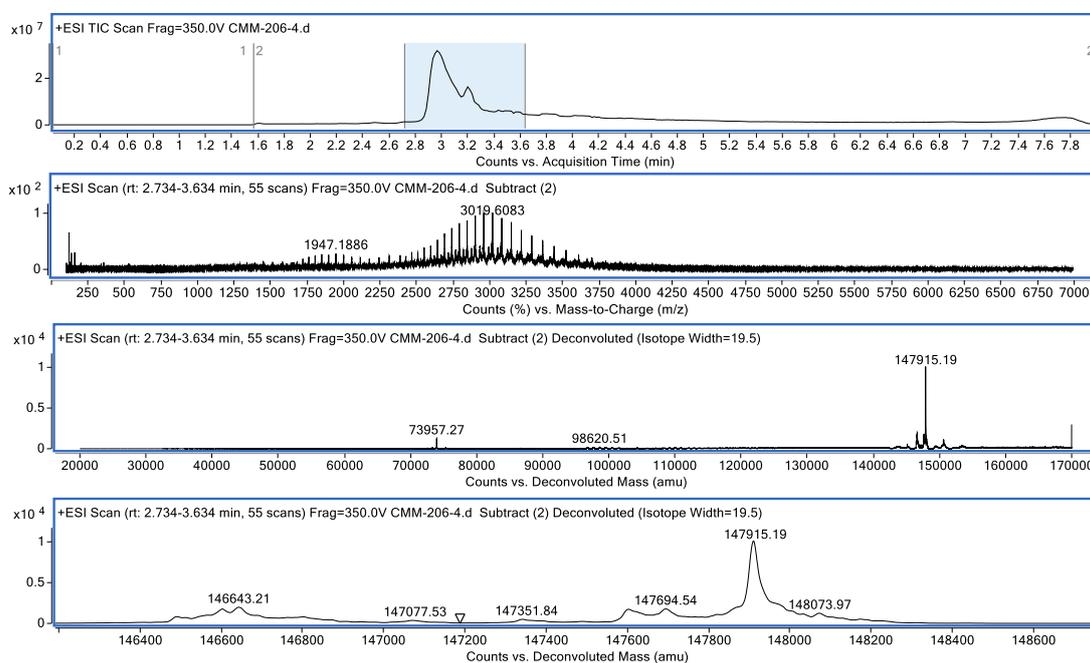


Figure S43: (i) TIC LC-MS trace (top), (ii) non-deconvoluted LC-MS trace (upper middle), (iii) deconvoluted MS data (lower middle, wide range), (iv) deconvoluted MS data (bottom, zoom in range).

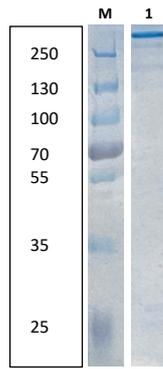
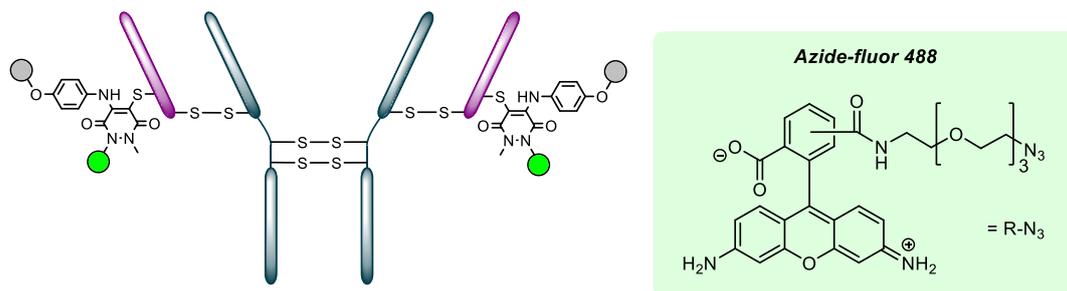
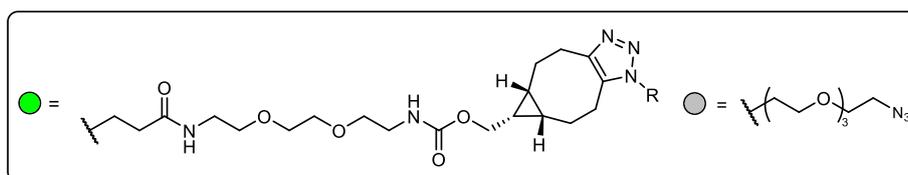


Figure S44: SDS-PAGE gel: M. Ladder, 1. Conjugate **13**.

LC S168C PD aniline fluorophore conjugate **14 (LC S168C thio-trastuzumab conjugated to BCN PD 1, Azide-fluor 488 and N₃ Aniline)**



14



To a solution of conjugated LC S168C thio-trastuzumab **12** (190 μ L, 11.9 μ M) in BBS (25 mM borate, 25 mM NaCl, 2 mM EDTA, 2% DMSO, pH 8.0) was added N₃ Aniline (6.8 μ L, 0.5 M in DMSO, 1500 eq.) and the reaction incubated at 37 °C for 16 h under constant agitation (300 rpm). After this time, excess reagents were removed to give conjugate **14** (expected mass 147,917 Da, observed mass 147,920 Da) which was analysed by LC-MS (method 1b), UV-Vis spectroscopy and SDS-PAGE.

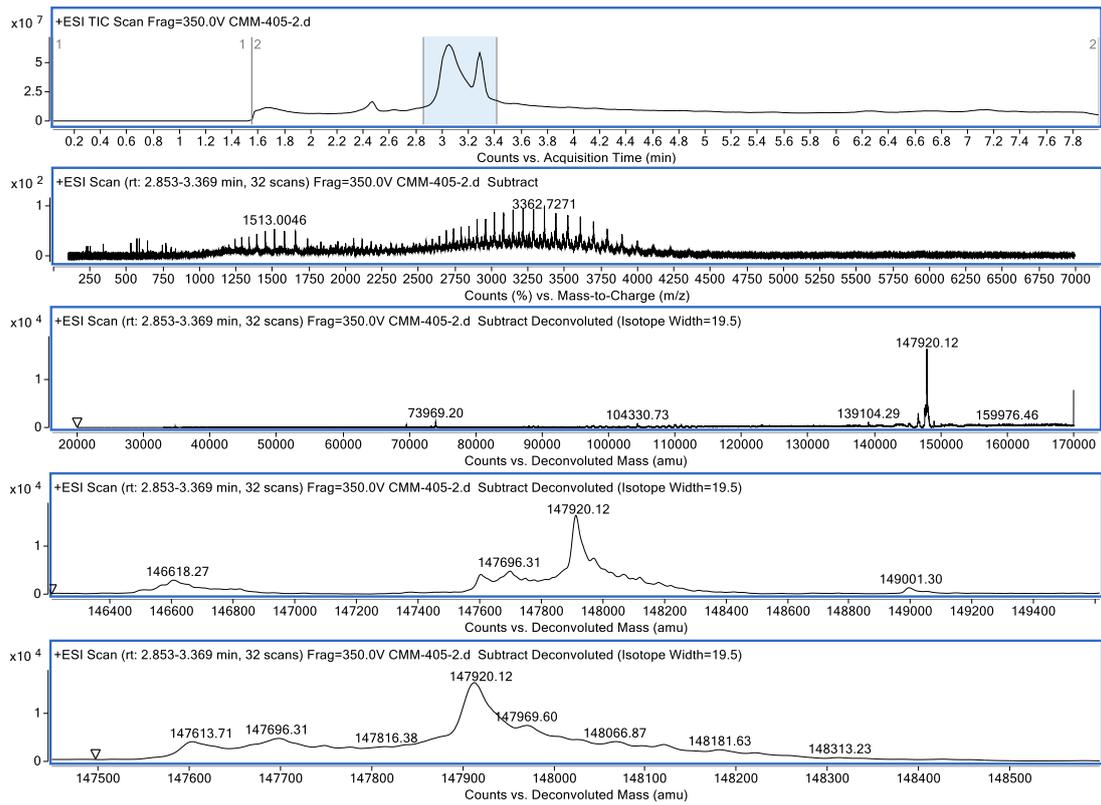


Figure S45: (i) TIC LC-MS trace (top), (ii) non-deconvoluted LC-MS trace (upper middle), (iii) deconvoluted MS data (lower middle, wide range), (iv) deconvoluted MS data (bottom, zoom in range).

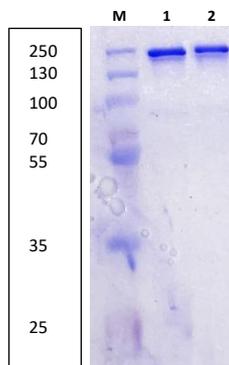


Figure S46: SDS-PAGE gel: M. Ladder, 1. Conjugate **12**, 2. Conjugate **14**.

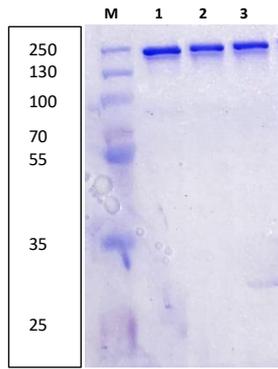
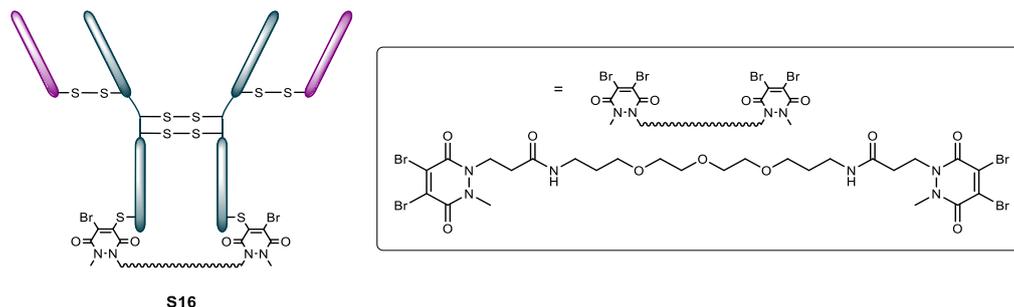


Figure S48: SDS-PAGE gel: M. Ladder, 1. Conjugate **12**, 2. Conjugate **14**, 3. Conjugate **15**.

HC S378C bisPD conjugate S16 (HC S378C thio-trastuzumab conjugated to diMe bisPD 5)



To a solution of HC S378C thio-trastuzumab **7** (30 μ L, 20 μ M) in BBS (25 mM borate, 25 mM NaCl, 2 mM EDTA, 2% DMSO, pH 8.0) was added diMe bisPD **5** (0.37 μ L, 8.2 mM in MeCN, 5 eq.) and the reaction incubated at 37 $^{\circ}$ C for 3 h under constant agitation (300 rpm). After this, excess reagents were removed to give conjugate **S16** (expected mass 145,879 Da, observed mass 145,880 Da) which was analysed by LC-MS (method 1a), UV-Vis spectroscopy and SDS-PAGE.

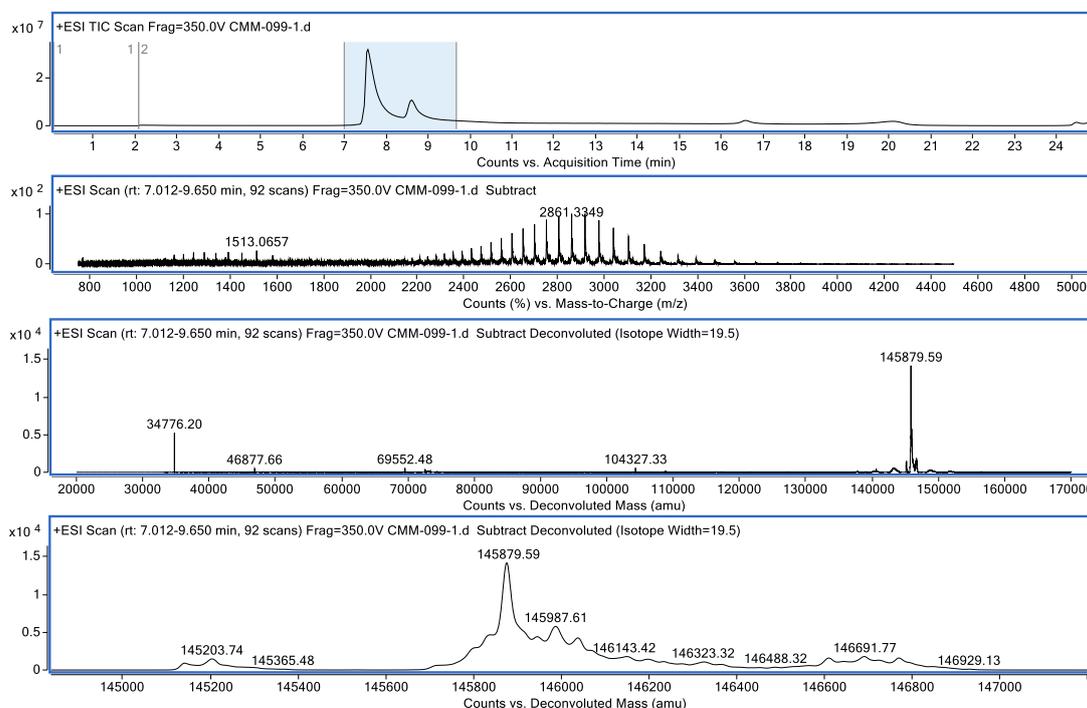


Figure S49: (i) TIC LC-MS trace (top), (ii) non-deconvoluted LC-MS trace (upper middle), (iii) deconvoluted MS data (lower middle, wide range), (iv) deconvoluted MS data (bottom, zoom in range).

Residue Distance Analysis

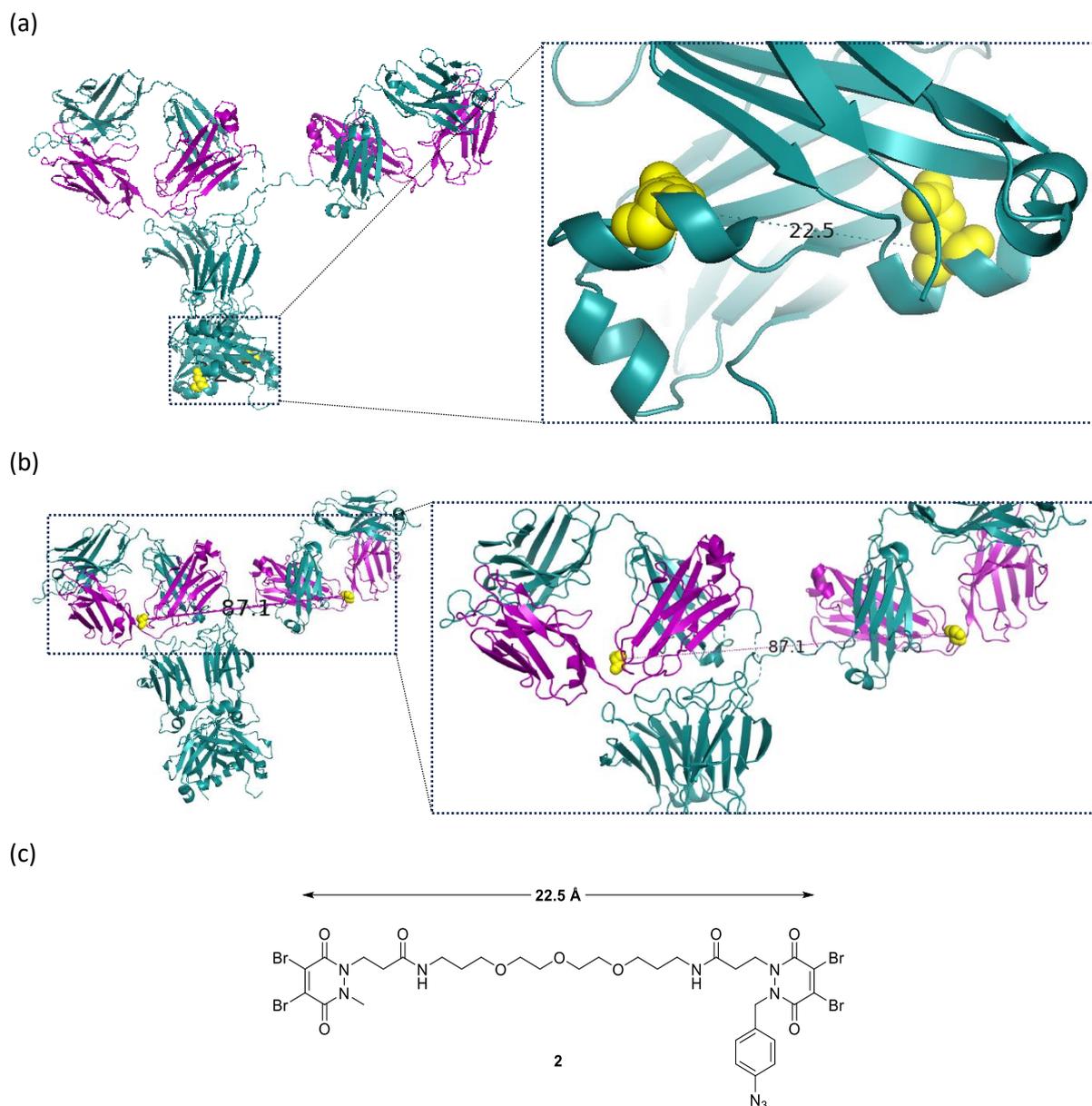
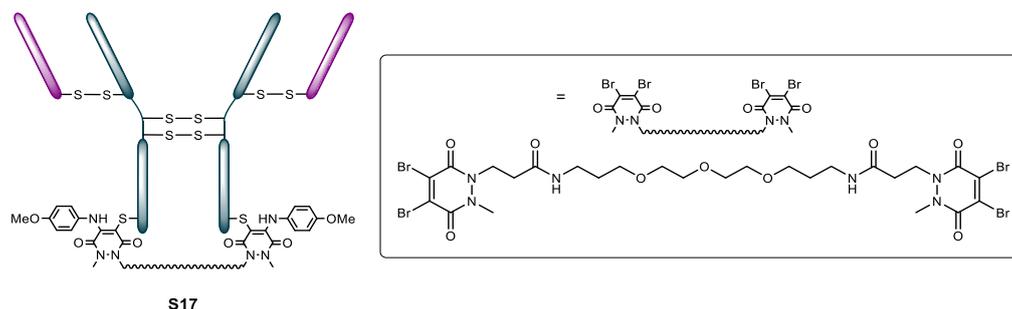


Figure S50. (a) The distance between the two HC residue 378s. Measurement was done on PyMol using the 1HZH file (human IgG1 b12) as there is no available crystal structure for full trastuzumab.

(b) The distance between the two LC residue 168s. Measurement was done on PyMol using the 1HZH file (human IgG1 b12) as there is no available crystal structure for full trastuzumab.

(c) PhN₃ bisPD **2** length, measured from carbon to carbon using MolView.

HC S378C bisPD aniline conjugate **S17** (HC S378C thio-trastuzumab conjugated to diMe bisPD 5, reacted with *p*-Anisidine)



To a solution of conjugated HC S378C thio-trastuzumab **S16** (21.4 μ L, 20 μ M) in BBS (25 mM borate, 25 mM NaCl, 2 mM EDTA, 2% DMSO, pH 8.0) was added *p*-anisidine (0.43 μ L, 1 M in DMSO, 1000 eq.) and the reaction incubated at 37 $^{\circ}$ C for 16 h under constant agitation (300 rpm). After this, excess reagents were removed to give conjugate **S17** (expected mass 145,964 Da, observed mass 145,968 Da) which was analysed by LC-MS (method 1a), UV-Vis spectroscopy and SDS-PAGE.

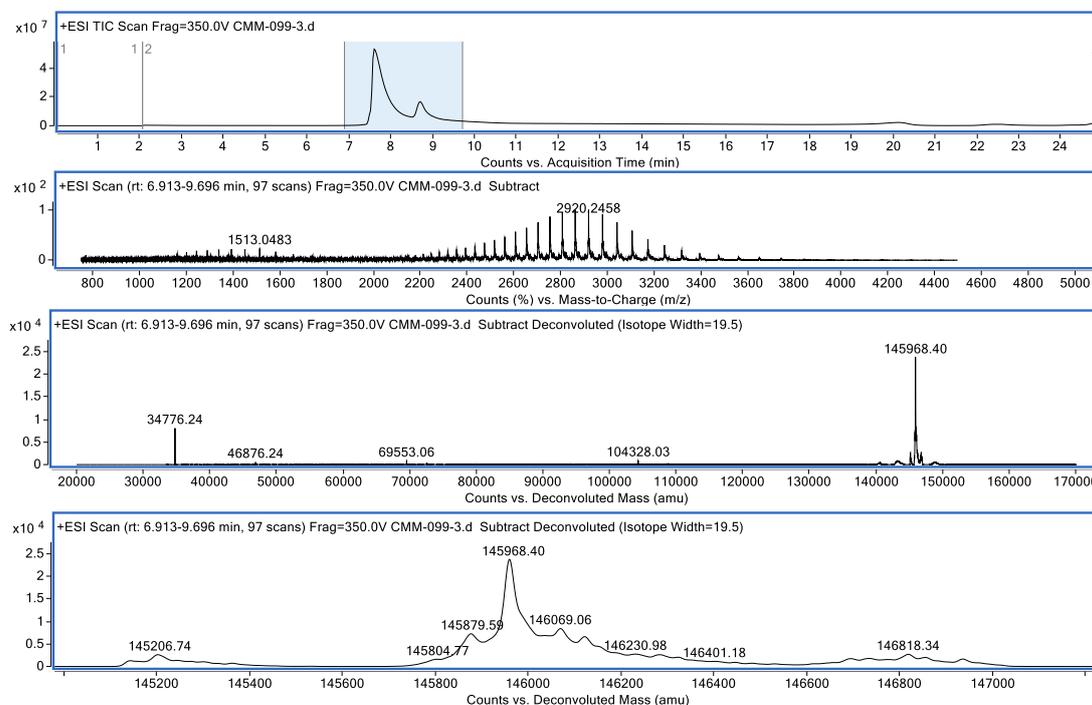


Figure S51: (i) TIC LC-MS trace (top), (ii) non-deconvoluted LC-MS trace (upper middle), (iii) deconvoluted MS data (lower middle, wide range), (iv) deconvoluted MS data (bottom, zoom in range).

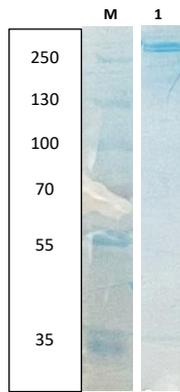
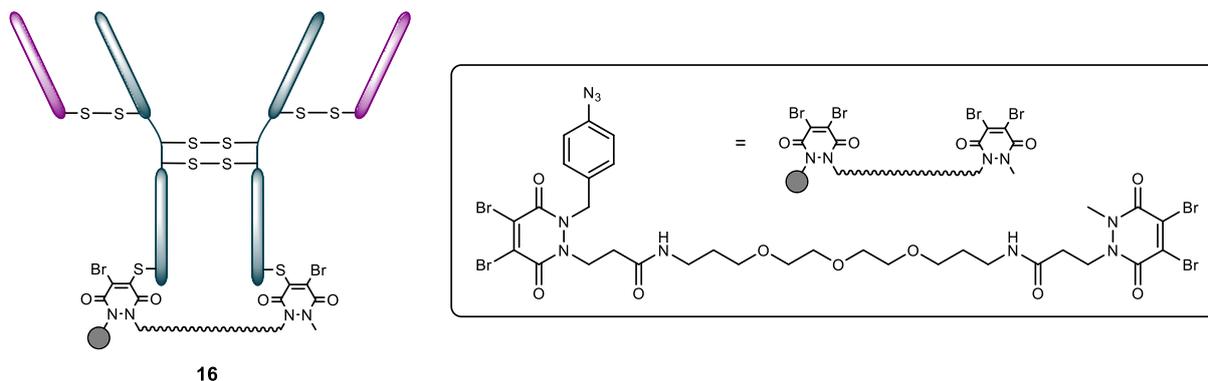


Figure S52: SDS-PAGE gel: M. Ladder, 1. Conjugate **S17**.

HC S378C bisPD conjugate **16** (HC S378C thio-trastuzumab conjugated to ArN₃ bisPD **2**)



To a solution of HC S378C thio-trastuzumab **7** (200 μ L, 20 μ M) in BBS (25 mM borate, 25 mM NaCl, 2 mM EDTA, 2% DMSO, pH 8.0) was added ArN₃ bisPD **2** (1 μ L, 20 mM in DMSO, 5 eq.) and the reaction incubated at 37 $^{\circ}$ C for 3 h under constant agitation (300 rpm). After this time, excess reagents were removed to give conjugate **16** (expected mass 145,997 Da, observed mass 146,007 Da) which was analysed by LC-MS (method 1b), UV-Vis spectroscopy and SDS-PAGE.

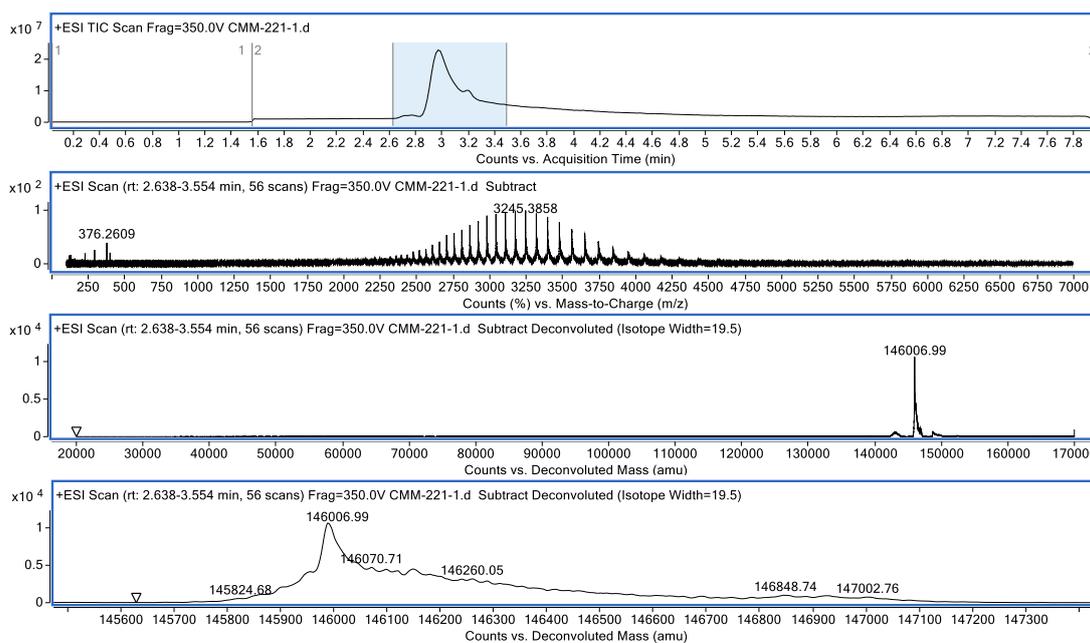


Figure S53: (i) TIC LC-MS trace (top), (ii) non-deconvoluted LC-MS trace (upper middle), (iii) deconvoluted MS data (lower middle, wide range), (iv) deconvoluted MS data (bottom, zoom in range).

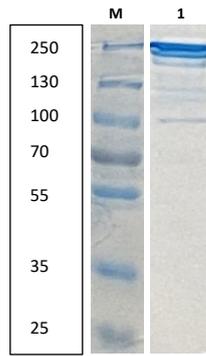
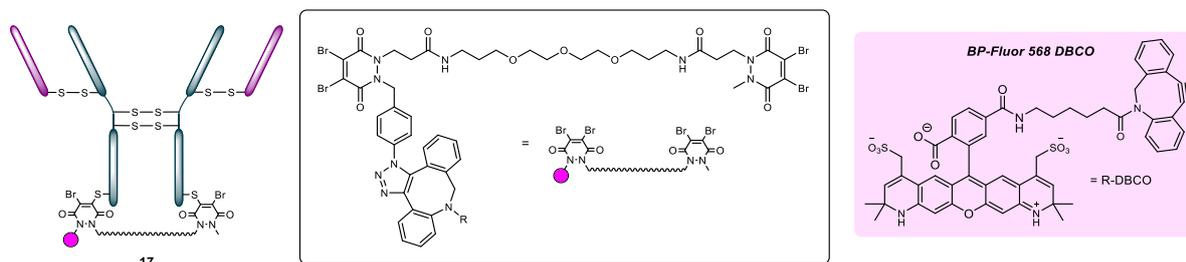


Figure S54: SDS-PAGE gel: M. Ladder, 1. Conjugate **16**.

HC S378C bisPD fluorophore conjugate **17** (HC S378C thio-trastuzumab conjugated to ArN₃ bisPD **2** and BP Fluor 568)



Pre-Click Method

To BBS (9 μ L, 25 mM borate, 25 mM NaCl, 2 mM EDTA, 2% DMSO, pH 8.0) was added ArN₃ bisPD **2** (1 μ L, 20 mM DMSO) and BP Fluor 568 DBCO (10 μ L, 20 mM DMSO, 10x excess) and the reaction incubated at 37 °C for 16 h under constant agitation (300 rpm) under argon.

To a solution of uncapped HC S378C thio-trastuzumab **7** (180 μ L, 22.2 μ M) in BBS was added the ArN₃ bisPD clicked with BP Fluor 568 (20 μ L, 5 eq.) and the reaction incubated at 37 °C for 5 h under constant agitation (300 rpm). After this time, excess reagents were removed to give conjugate **17** (expected mass 146,950 Da, observed mass 146,962 Da) which was analysed by LC-MS (method 1b), UV-Vis spectroscopy and SDS-PAGE.

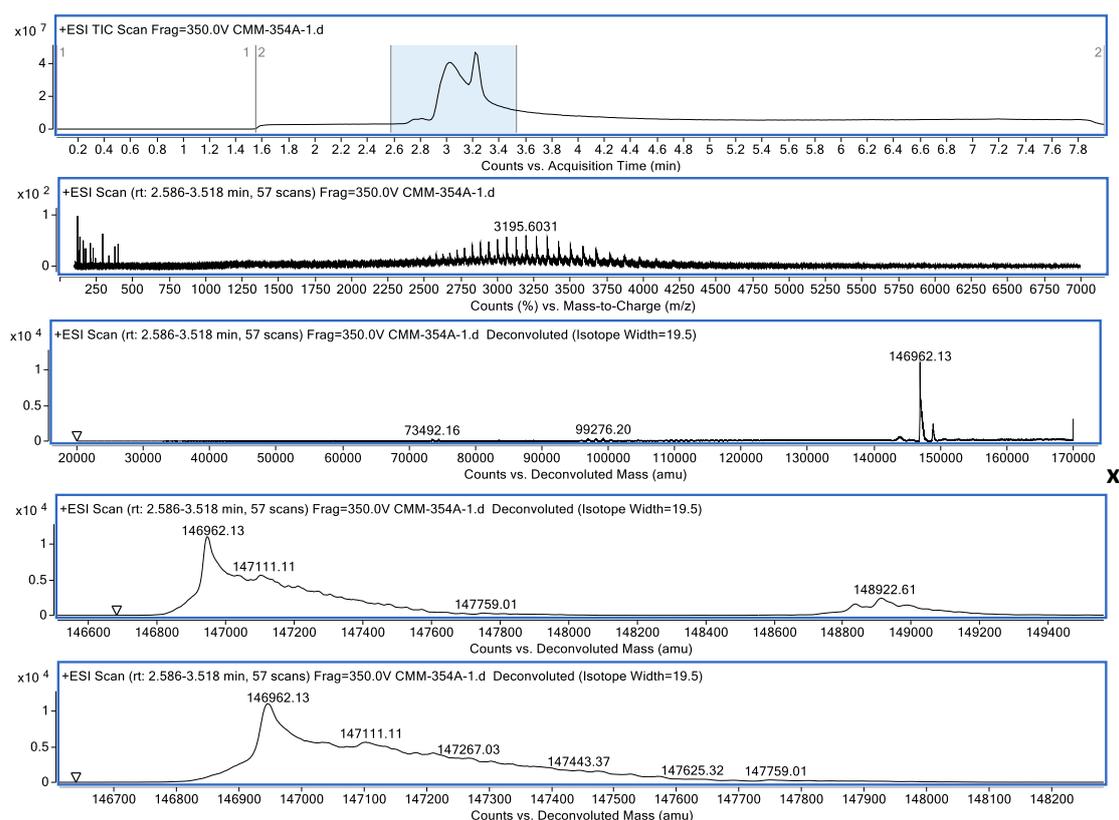


Figure S55: (i) TIC LC-MS trace (top), (ii) non-deconvoluted LC-MS trace (upper middle), (iii) deconvoluted MS data (lower middle, wide range), (iv) deconvoluted MS data (bottom, zoom in range).

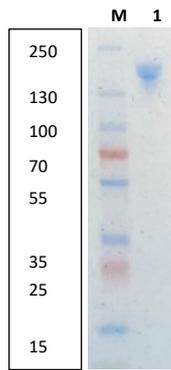
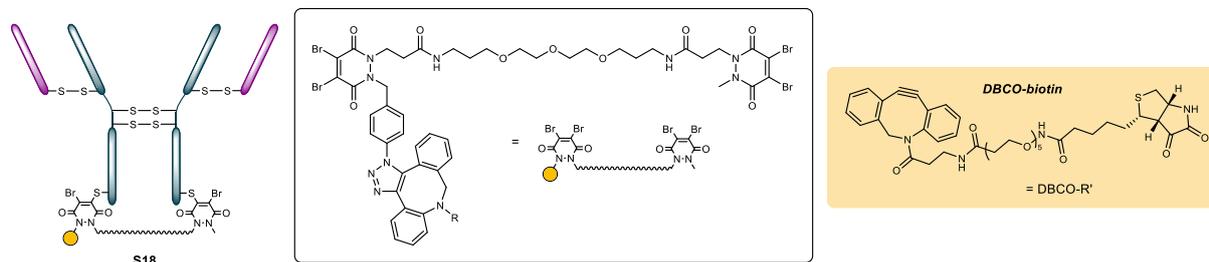


Figure S56: SDS-PAGE gel: M. Ladder, 1. Conjugate **17**.

HC S378C bisPD fluorophore conjugate **S18** (HC S378C Thio-trastuzumab conjugated to ArN₃ bisPD **2** and DBCO biotin)



Pre-Click Method

To BBS (9 μ L, 25 mM borate, 25 mM NaCl, 2 mM EDTA, 2% DMSO, pH 8.0) was added ArN₃ bisPD **2** (1 μ L, 20 mM DMSO) and DBCO biotin (10 μ L, 20 mM DMSO, 10x excess) and the reaction incubated at 37 $^{\circ}$ C for 16 h under constant agitation (300 rpm) under argon.

To a solution of uncapped HC S378C thio-trastuzumab **7** (180 μ L, 22.2 μ M) in BBS was added the ArN₃ bisPD clicked with DBCO biotin (20 μ L, 5 eq.) and the reaction incubated at 37 $^{\circ}$ C for 5 h under constant agitation (300 rpm). After this time, excess reagents were removed to give conjugate **S18** (expected mass 146,746 Da, observed mass 146,760 Da) which was analysed by LC-MS (method 1b), UV-Vis spectroscopy and SDS-PAGE.

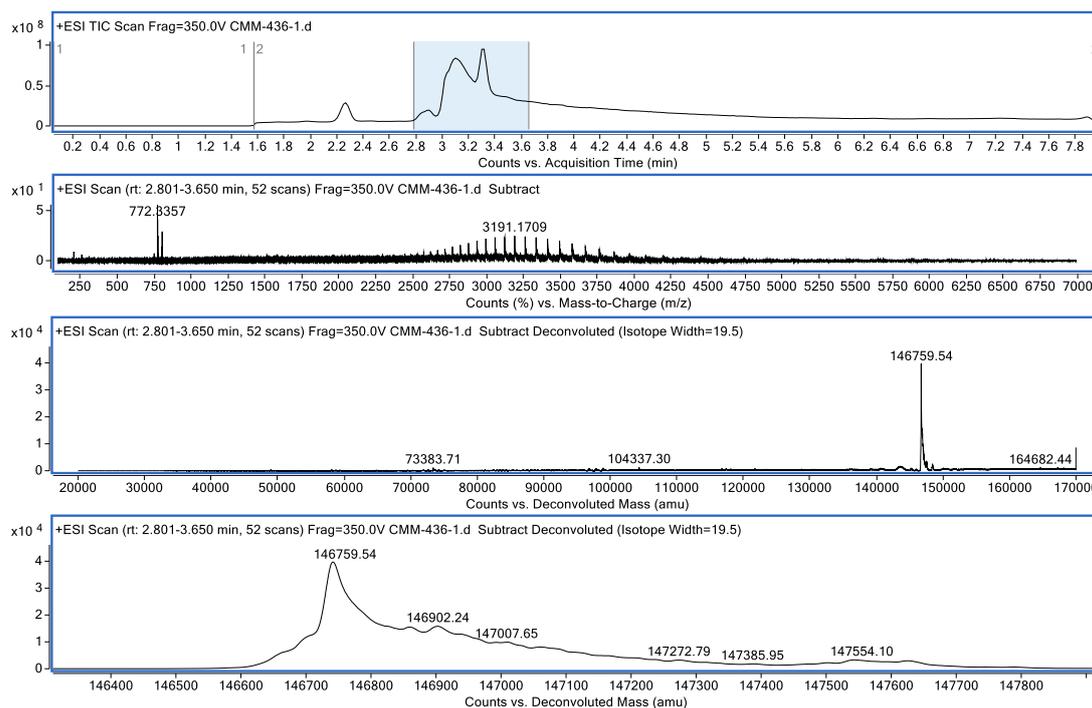


Figure S57: (i) TIC LC-MS trace (top), (ii) non-deconvoluted LC-MS trace (upper middle), (iii) deconvoluted MS data (lower middle, wide range), (iv) deconvoluted MS data (bottom, zoomed range).

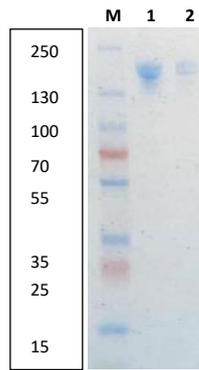
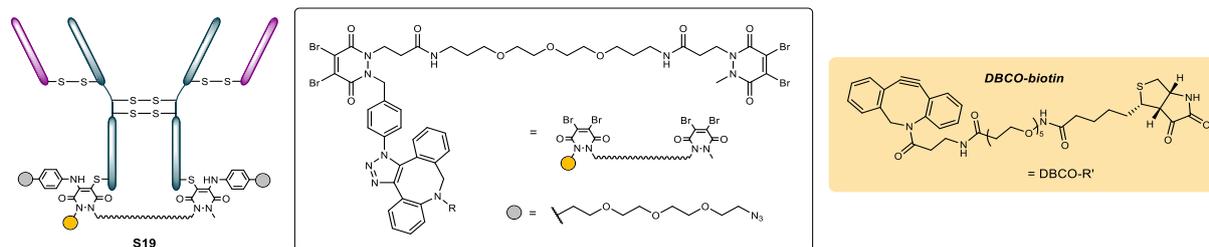


Figure S59: SDS-PAGE gel: M. Ladder, 1. Conjugate **17**, 2. Conjugate **18**.

HC S378C bisPD aniline fluorophore conjugate **S19** (HC S378C thio-trastuzumab conjugated to ArN₃ bisPD **2**, DBCO biotin and N₃ Aniline **3**)



To a solution of conjugated HC S378C thio-trastuzumab **S18** (180 μ L, 20 μ M) in BBS (25 mM borate, 25 mM NaCl, 2 mM EDTA, 2% DMSO, pH 8.0) was added N₃ aniline **3** (7.2 μ L, 0.5 M in DMSO, 1000 eq.) and the reaction incubated at 37 $^{\circ}$ C for 16 h under constant agitation (300 rpm). After this, excess reagents were removed to give conjugate **S19** (expected mass 147,207 Da, observed mass 147,212 Da) which was analysed by LC-MS (method 1b), UV-Vis spectroscopy and SDS-PAGE.

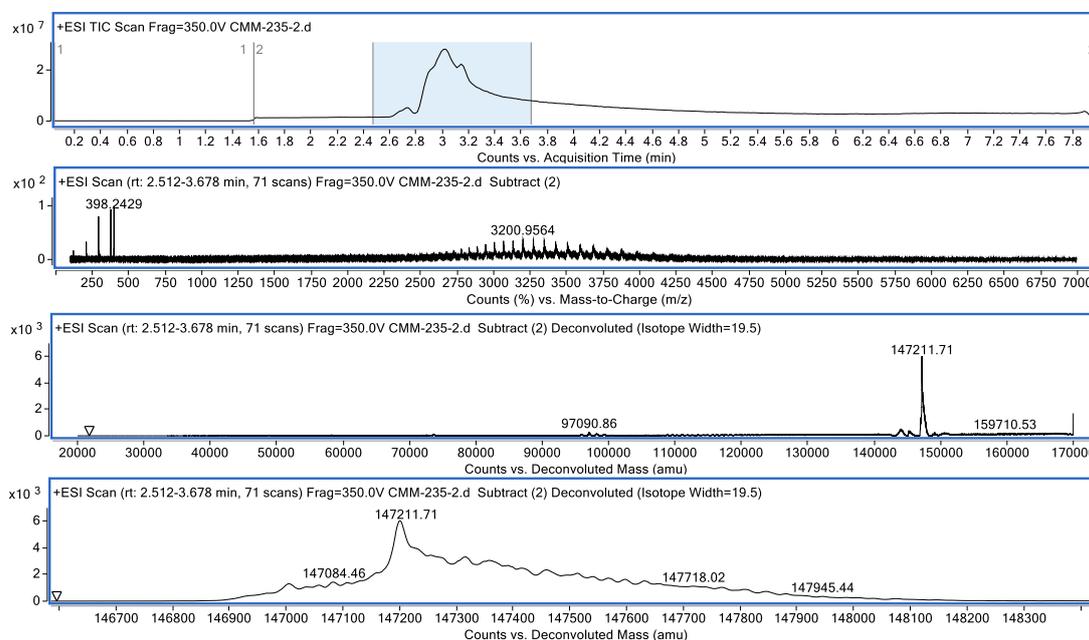
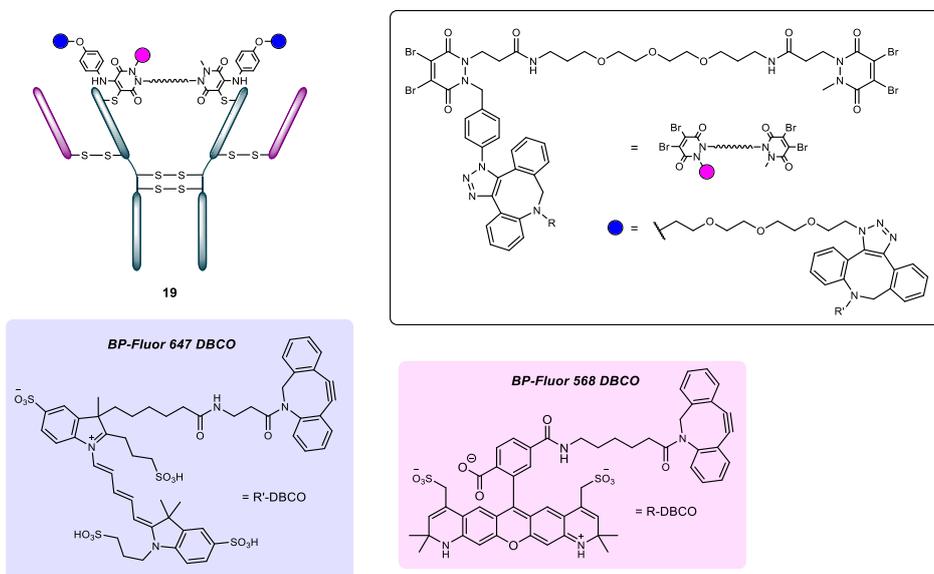


Figure S60: (i) TIC LC-MS trace (top), (ii) non-deconvoluted LC-MS trace (upper middle), (iii) deconvoluted MS data (lower middle, wide range), (iv) and (v) deconvoluted MS data (bottom, zoom in range).

HC S378C bisPD aniline fluorophore conjugate **19** (HC S378C thio-trastuzumab conjugated to ArN₃ bisPD **2**, BP Fluor 568, N₃ Aniline and BP Fluor 647)



To a solution of conjugated HC S378C thio-trastuzumab **18** (175 μ L, 18.8 μ M) in BBS (25 mM borate, 25 mM NaCl, 2 mM EDTA, 2% DMSO, pH 8.0) was added BP Fluor 647 (9.9 μ L, 20 mM in DMSO, 60 eq.) and the reaction incubated at 37 $^{\circ}$ C for 16 h under constant agitation (300 rpm). After this, excess reagents were removed to give conjugate **19** (expected mass 149,673 Da, observed mass 149,655 Da) which was analysed by LC-MS (method 1b), UV-Vis spectroscopy and SDS-PAGE.

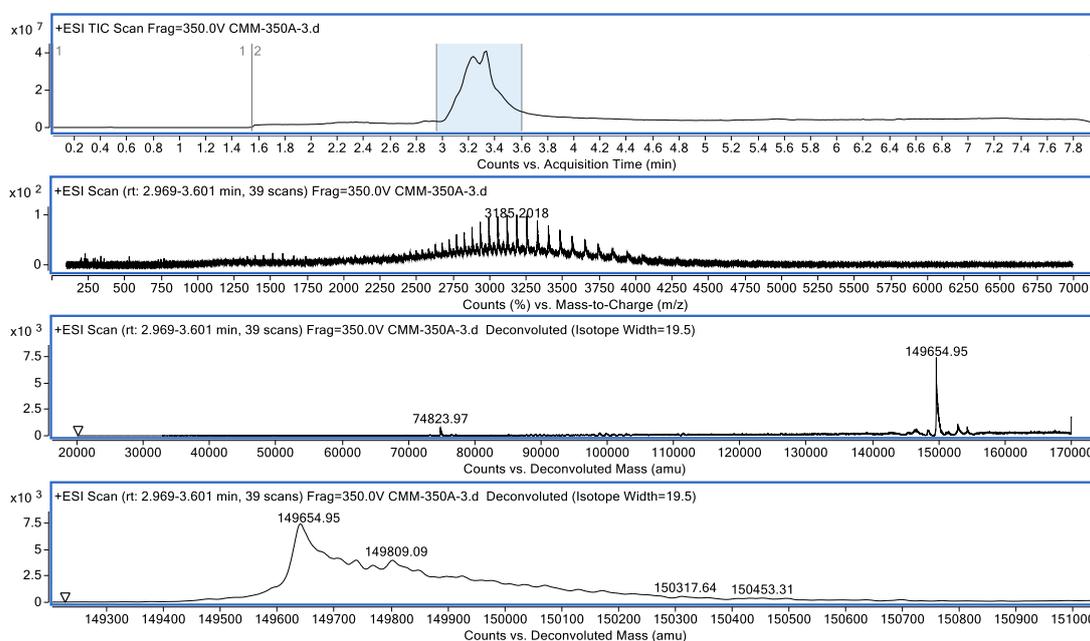


Figure S61: (i) TIC LC-MS trace (top), (ii) non-deconvoluted LC-MS trace (upper middle), (iii) deconvoluted MS data (lower middle, wide range), (iv) deconvoluted MS data (bottom, zoom in range)

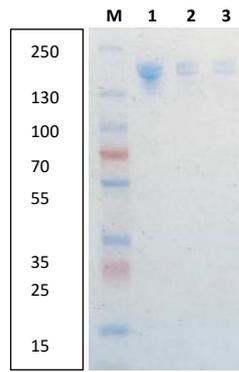
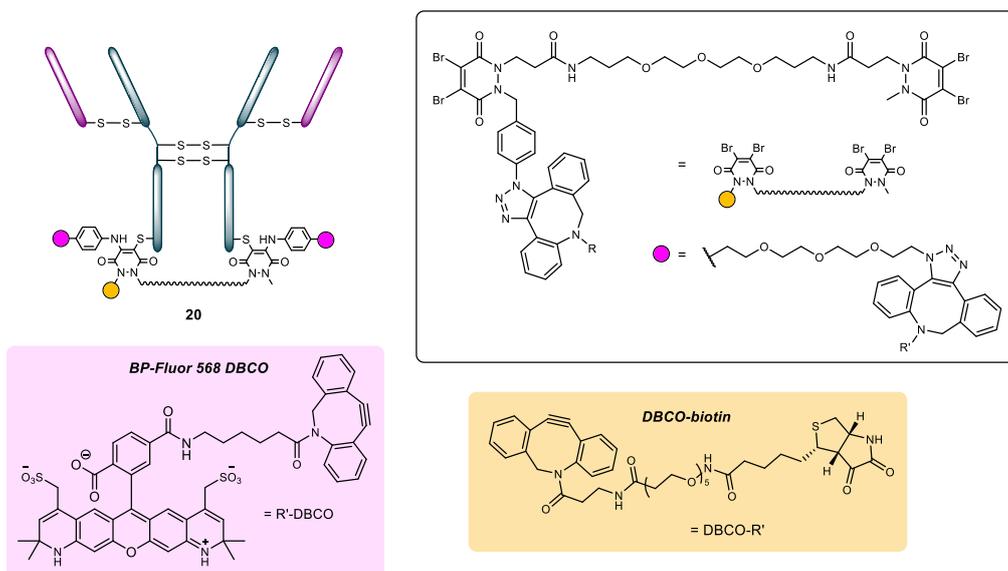


Figure S62: SDS-PAGE gel: M. Ladder, 1. Conjugate **17**, 2. Conjugate **18**, 3. Conjugate **19**.

HC S378C bisPD aniline fluorophore conjugate 20 (HC S378C thio-trastuzumab conjugated to ArN₃ bisPD 2, DBCO biotin, N₃ Aniline 3 and BP Fluor 568)



To a solution of conjugated HC S378C thio-trastuzumab **19** (100 μ L, 18.7 μ M) in BBS (25 mM borate, 25 mM NaCl, 2 mM EDTA, 2% DMSO, pH 8.0) was added BP Fluor 568 (1.9 μ L, 20 mM in DMSO, 20 eq.) and the reaction incubated at 37 $^{\circ}$ C for 16 h under constant agitation (300 rpm). After this, excess reagents were removed to give conjugate **20** (expected mass 149,114 Da, observed mass 149,121 Da) which was analysed by LC-MS (method 1b), UV-Vis spectroscopy and SDS-PAGE.

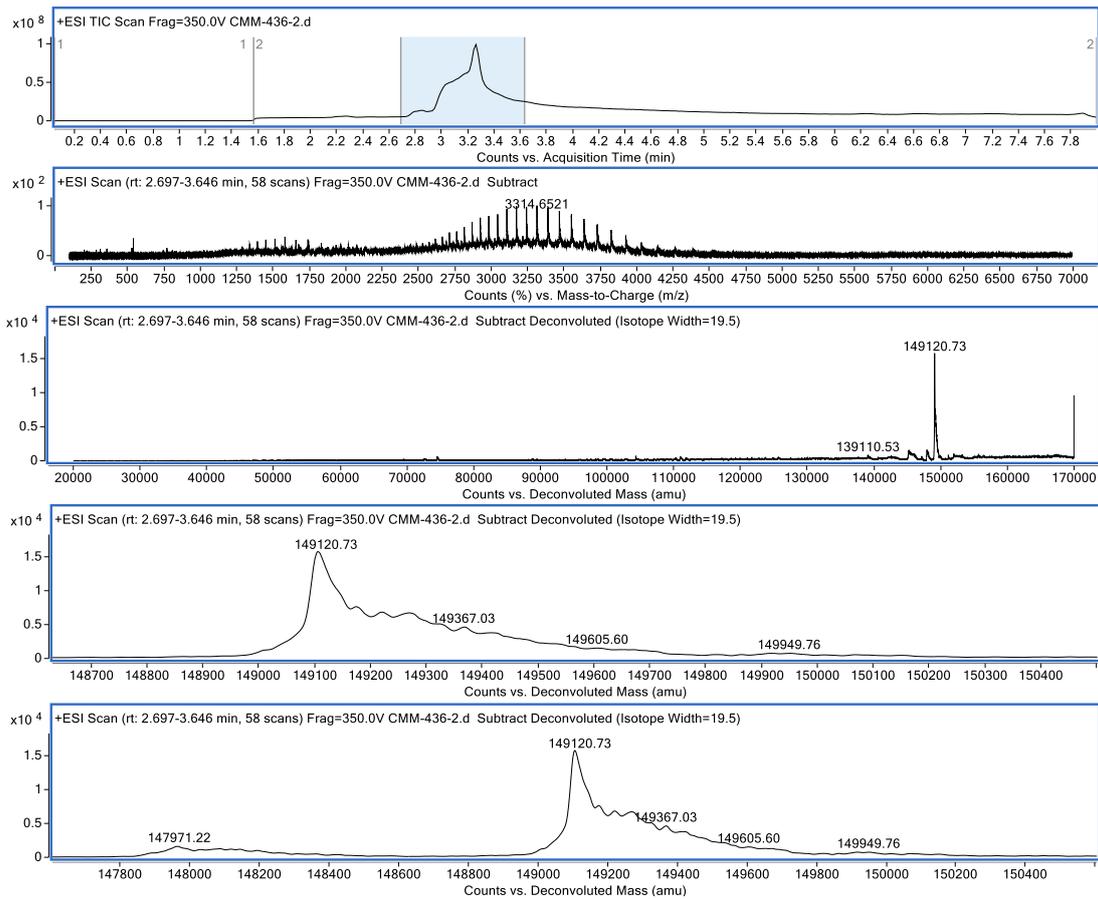
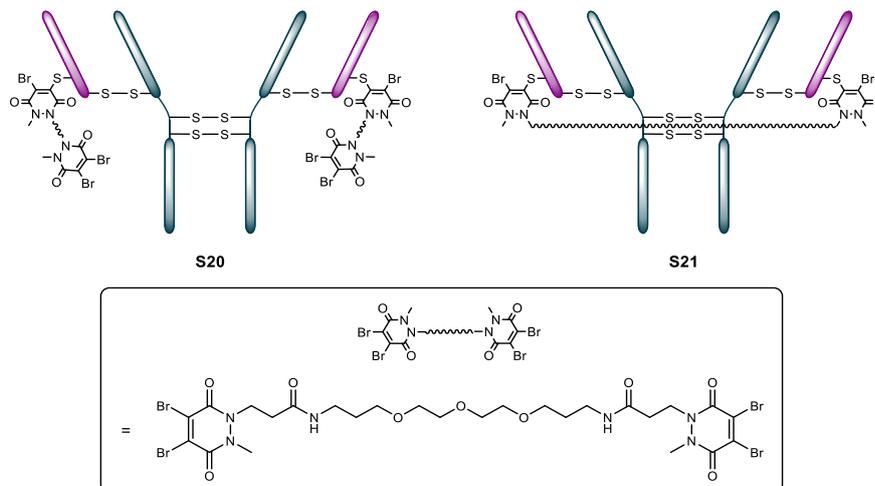


Figure S63: (i) TIC LC-MS trace (top), (ii) non-deconvoluted LC-MS trace (upper middle), (iii) deconvoluted MS data (lower middle, wide range), (iv) and (v) deconvoluted MS data (bottom, zoom in range).

LC S168C bisPD conjugates **S20** and **S21** (Reaction of HC S378C thio-trastuzumab with diMe bisPD 5)



To a solution of HC S378C thio-trastuzumab **7** (25 μ L, 20 μ M) in BBS (25 mM borate, 25 mM NaCl, 2 mM EDTA, 2% DMSO, pH 8.0) was added diMe bisPD **5** (0.24 μ L, 8.2 mM in MeCN, 4 eq.) and the reaction incubated at 37 $^{\circ}$ C for 3 h under constant agitation (300 rpm). After this, excess reagents were removed to give conjugates **S20** and **S21** and the sample analysed by LC-MS (method 1a), UV-Vis spectroscopy and SDS-PAGE.

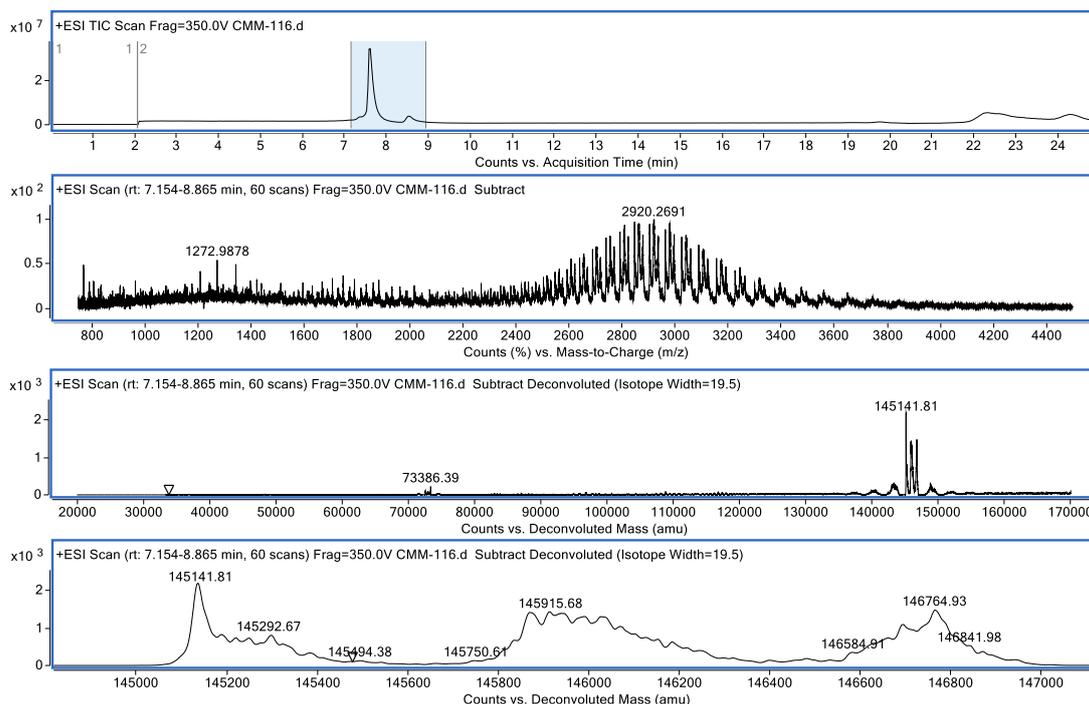


Figure S64: (i) TIC LC-MS trace (top), (ii) non-deconvoluted LC-MS trace (upper middle), (iii) deconvoluted MS data (lower middle, wide range), (iv) deconvoluted MS data (bottom, zoom in range).

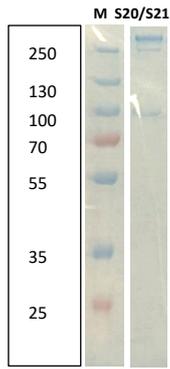
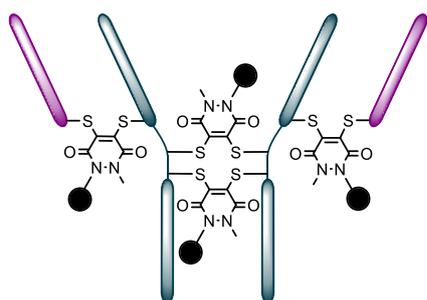
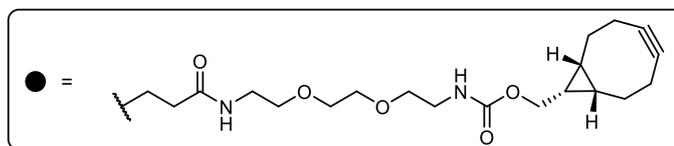


Figure S65: SDS-PAGE gel: M. Ladder, 1. Conjugates **S20** and **S21**.

Trastuzumab PD conjugate S22 (Trastuzumab re-bridged with BCN PD 1)



S22



To a solution of trastuzumab (Ontruzant[®], A μM , 100 μL) in BBS (25 mM borate, 25 mM NaCl, pH 8.0, 2 mM EDTA, pH 8.0, 2% DMSO (unless otherwise specified below), was added TCEP.HCl (20 mM in dH_2O , B eq.). The mixture was incubated for C h at D $^\circ\text{C}$ under constant agitation (300 rpm). After this time, excess TCEP was removed *via* ultrafiltration into BBS and BCN PD 1 (20 mM in DMSO, E eq.) was added. The reaction was incubated at F $^\circ\text{C}$ for D h under constant agitation (300 rpm). After this time, excess reagents were removed *via* ultrafiltration into BBS and the concentration of the sample and PDAR were assessed by UV-Vis spectroscopy and LC-MS (method 1a).

PDAR 4: Expected mass = 147,189 Da; PDAR 3: Expected mass = 146686 Da; PDAR 2: Expected mass = 146,183 Da; Native Ontruzant[®]: Expected mass = 145,176 Da.

	Antibody Concentration (μ M) (A)	TCEP eq. (B)	Reduction Time (C)	Reduction temperature ($^{\circ}$ C) (D)	PD eq. (E)	Reaction Time and Temperature ($^{\circ}$ C) (F, G)	pH
S22a	40	10	1.5 h	4	20	16 h, 4	8.0
S22b	40	10	1.5 h	4	40	16 h, 4	8.0
S22c	40	10	1.5 h	4	200	16 h, 4	8.0
S22d	40	10	1.5 h	4	20	16 h, 4	7.4
S22e	40	20	1.5 h	4	20	16 h, 4	8.0
S22f	40	80	1.5 h	4	20	16 h, 4	8.0
S22g	40	10	1.5 h	22	20	16 h, 4	8.0
S22h	40	10	1.5 h	37	20	16 h, 4	8.0
S22i	40	10	8 h	4	20	16 h, 4	8.0
S22j	40	10	4 h	4	20	16 h, 4	8.0
S22k	40	10	1.5 h	37	15	16 h, 4	8.0
S22l	40	10	1.5 h	37	10	16 h, 4	8.0
S22m	40	10	1.5 h	37	8	16 h, 4	8.0
S22n	40	10	1.5 h	37	5	16 h, 4	8.0
S22o	10	10	1.5 h	37	8	16 h, 4	8.0
S22p	20	10	1.5 h	37	8	16 h, 4	8.0
S22q	60	10	1.5 h	37	8	16 h, 4	8.0
S22r	20	10	1.5 h	37	10	3 h, 37	8.0
S22s	20	10	1.5 h	37	10	16 h, 4	8.0
S22t	20	10	1 h	37	10	3 h, 37	8.0

Table S10. Stepwise trials at forming conjugate **S22**.