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Use of Pyridazinediones as Extracellular Cleavable Linkers Through Reversible Cysteine Conjugation

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General Experimental:

All chemical reagents were purchased from Sigma Aldrich, Alfa Aesar and Acros. Compounds and solvents were used as received. Petrol refers to petroleum ether (b.p. 40–60 °C). All reactions were carried out under positive pressure of argon, unless stated otherwise, and were monitored using thin layer chromatography (TLC) on pre-coated silica gel plates (254 µm). Flash column chromatography was carried out with pre-loaded GraceResolv™ Silica Flash cartridges (Grace™) or FlashPure EcoFlex catridges (Büchi) on a Biotage® Isolera Spektra One flash chromatography system (Biotage®). ^{1}H NMR spectra were obtained at 300 MHz, 400 MHz, 500 MHz, 600 MHz or 700 MHz. ^{13}C NMR spectra were obtained at 125 MHz, 150 MHz, or 175 MHz. All results were obtained using Bruker NMR instruments, the models are as follows: Avance Neo 700, Avance III 600, Avance 500, Avance III 400, Avance 300. Unless otherwise specified, all samples were run at 25 °C. Chemical shifts (δ) for ^{1}H NMR and ^{13}C NMR are quoted on a parts per million (ppm) scale relative to tetramethylsilane (TMS), calibrated using residual signals of the solvent. Where amide rotamers are the case, and when possible, only the chemical shifts of the major rotamer has been assigned and areas underneath all rotameric peaks have been considered for the integral intensity calculations. Coupling constants (*J* values) are reported in Hertz (Hz) and are reported as $J_{H\text{-}H}$ couplings between protons. Infrared spectra were obtained on a Perkin Elmer Spectrum 100 FTIR spectrometer operating in ATR mode. Mass spectra were obtained, for synthetic products, from the UCL mass spectroscopy service on a Waters LCT Premier XE (ES) mass spectrometer. Melting points were measured with Gallenkamp apparatus and are uncorrected.

Small molecule NMR deconjugation and transfer studies

^{1}H NMR spectra for the deconjugation and transfer experiments were obtained using a Bruker Avance III 400 instrument with 32 scans using CD_3CN as the reference solvent and water suppression. Data was processed using MestreNova software.

UV-Vis spectroscopy

UV-Vis spectroscopy was used to determine 2-nitro-5-thiobenzoate (TNB) **13**, protein, and protein conjugate concentrations using either a Jenway 7305 spectrophotomer (Jenway) or a NanoDrop One^{C} spectrophotometer (ThermoScientific) operating at 21 °C unless stated otherwise. Sample buffer was used as a blank for baseline correction with extinction coefficient ε_{412} = 14,150 M^{-1} cm^{-1} for TNB **13**, ε_{280} = 68,590 M^{-1} cm^{-1} for trastuzumab Fab **15**, ε_{280} = 20,500 M^{-1} cm^{-1} and ε_{490} = 55,000 M^{-1} cm^{-1} for GFPS147C **14**.

LCMS analysis – Method 1

LCMS was performed on protein samples (<50 kDa) using a Waters Acquity uPLC connected to Waters Acquity Single Quad Detector (SQD). All samples were diluted to a final concentration of 1 mg/mL in deionised water and run with the following parameters. Column: Hypersil Gold C4, 1.9 µm, 2.1 µm × 50 µm. Wavelength: 254 nm. Mobile Phase: 95:5 Water (0.1% Formic Acid): MeCN (0.1% Formic Acid) Gradient over 4 min (to 5:95 Water (0.1% Formic Acid): MeCN (0.1% Formic Acid). Flow Rate: 0.6 mL/min. MS Mode: ES+. Scan Range: *m/z* = 250 – 2000 (*m/z* = 100 – 1000 for glutathione

experiments). Scan time: 0.25 s. Data obtained in continuum mode. The electrospray source of the MS was operated with a capillary voltage of 3.5 kV and a cone voltage of 50 V. Nitrogen was used as the nebulizer and desolvation gas at a total flow of 600 L/h. Ion series were generated by integration of the total ion chromatogram (TIC) over the appropriate range. Total mass spectra for protein samples were reconstructed from the ion series using the MaxEnt 1 algorithm pre-installed on MassLynx software.

LCMS analysis – Method 2

Molecular masses of proteins (>50 kDa) were measured using an Agilent 6510 QTOF LCMS system (Agilent, UK). Agilent 1200 HPLC system was equipped with an Agilent PLRP-S, 1000A, 8 µM, 150 mm x 2.1 mm column. 10 µL of a protein sample (diluted to 0.2 mg/mL in d.d. H_2O) 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 (as shown below) at a flow rate of 300 µL/min. The oven temperature was maintained at 60 °C.

LCMS mobile phase gradient for A/B elution

Time (min)	Solvent A (%)	Solvent B (%)
0	85	15
2	85	15
3	68	32
4	68	32
14	65	35
18	5	95
20	5	95
22	85	15
25	85	15

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 3500V, 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 was 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. Deconvoluted spectra were avoided where possible in the quantification of conjugates due to differing ionisation tendencies between species with significantly different masses.

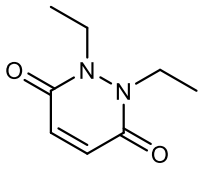
SDS-PAGE

Non-reducing glycine-SDS-PAGE at 12% acrylamide running were performed following standard lab procedures. A 4% stacking gel was used and a broad-range MW marker (10–250 kDa, Prestained PageRuler Plus Protein Standards, ThermoScientific) was co-run to estimate protein weights. Samples (10 µL at 7 µM) were mixed with loading buffer (2 µL, composition for 5 × 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 75 °C for 5 minutes, and centrifuged at 16,000 RPM for 5 minutes. Samples were subsequently loaded into the wells in a

volume of 5 µL. All gels were run at constant 10 mA for 15 minutes, then constant 30 mA until complete. Gels were stained using a Coomassie stain/

Synthesis of small molecules

1,2-Diethyl-1,2-dihydropyridazine-3,6-dione ***11***[1]



To a solution of maleic anhydride (0.51 g, 5.20 mmol) in glacial AcOH (20 mL) was added di-*tert*-butyl 1,2-diethylhydrazine-1,2-dicarboxylate[2] (1.00 g, 3.46 mmol). The reaction mixture was then heated under reflux with stirring overnight. After this time, the reaction mixture was allowed to cool to 21 °C, and the solvent was removed *in vacuo* with toluene co-evaporation (3 x 20 mL, as an azeotrope). Residual toluene was subsequently removed *in vacuo* with chloroform co-evaporation (3 x 20 mL, as an azeotrope). The crude residue was then purified by flash column chromatography (20% to 80% EtOAc/petrol), yielding 1,2-diethyl-1,2-dihydropyridazine-3,6-dione **11** (0.32 g, 1.90 mmol, 55%) as a white solid. ^{1}H NMR (600 MHz, $CDCl_3$) δ 6.87 (s, 2H), 4.13 (q, *J* = 7.1 Hz, 4H), 1.26 (t, *J* = 7.1 Hz, 6H); ^{13}C NMR (150 MHz, $CDCl_3$) δ 157.6 (C), 134.7 (CH), 40.3 (CH_2), 13.3 (CH_3); IR (solid) 2981, 1620, 1452 cm^{-1}.

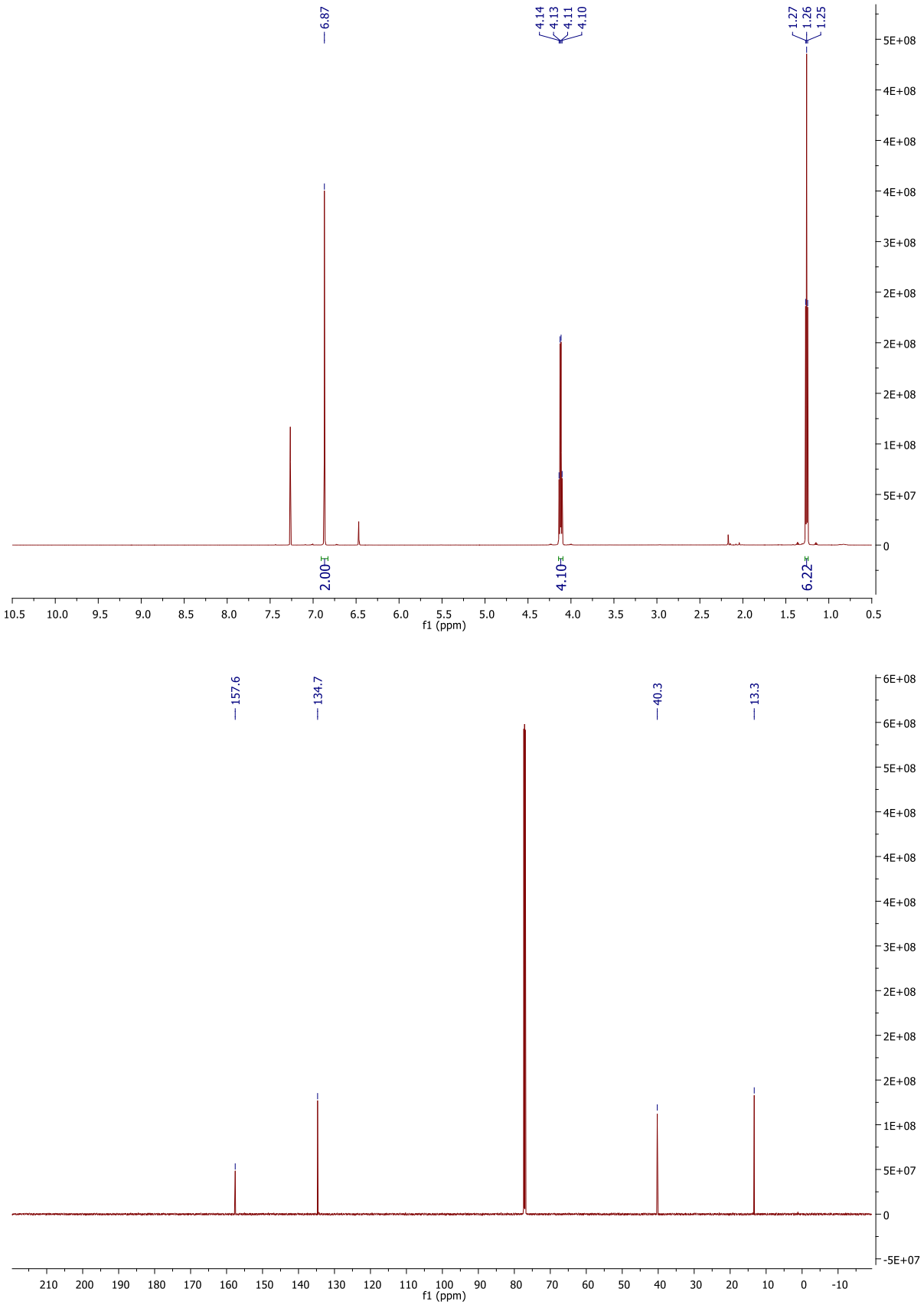
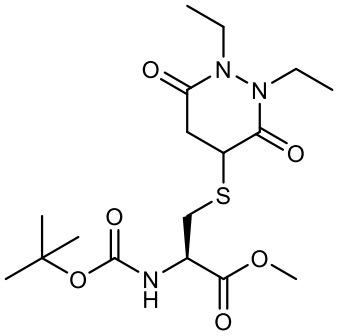


Figure S1: ^{1}H and ^{13}C NMR data of **11**

Methyl N-*(tert-butoxycarbonyl)-(1,2-diethyl-3,6-dioxohexahydropyridazin-4-yl)-L-cysteinate* ***9***



To a solution of *N*-(*tert*-butoxycarbonyl)-L-cysteine methyl ester **10** (122 µL, 140 mg, 0.594 mmol) in MeOH (6 mL) was added 1,2-diethyl-1,2-dihydropyridazine-3,6-dione **11** (100 mg, 0.594 mmol) and sodium acetate (146 mg, 1.78 mmol). The reaction mixture was then stirred at 21 °C for 1 h. After this time, the solvent was removed *in vacuo*, and the crude residue was purified by flash column chromatography (20% to 80% EtOAc/petrol) to give methyl *N*-(*tert*-butoxycarbonyl)-(1,2-diethyl-3,6-dioxohexahydropyridazin-4-yl)-L-cysteinate **9** as a colourless oil (128 mg, 0.317 mmol, 54%) as a mixture of diastereoisomers. ^{1}H NMR (700 MHz, $CDCl_3$, diastereomers, rotamers) δ 5.57-5.44 (m, 1H), 4.62-4.55 (m, 1H), 4.10-4.09 (m, 2H), 3.77-3.75 (m, 3H), 3.74-3.73 (m, 0.5 H), 3.71-3.69 (m, 0.5 H) 3.40-3.39 (m, 2H), 3.21-3.18 (m, 0.5 H), 3.14-3.07, (m, 1H), 2.98-2.96, (m, 0.5 H), 2.93-2.89 (m, 1H), 2.62-2.58 (m, 1H), 1.45-1.44 (m, 9H), 1.16-1.15 (m, 3H), 1.13-1.11 (m, 3H); ^{13}C NMR (175 MHz, $CDCl_3$, diastereomers, rotamers) δ 171.4 (C), 171.3 (C), 167.5 (C), 167.3 (C), 166.4 (C), 166.3 (C), 155.4 (C), 155.3 (C), 80.4 (C), 53.7 (CH), 52.9 (CH), 52.9 (CH_3), 41.4 (CH), 41.3 (CH), 38.7 (CH_2), 38.7 (CH_2), 38.6 (CH_2), 35.0 (CH_2), 35.0 (CH_2), 34.2 (CH_2), 33.8 (CH_2), 28.4 (CH_3), 12.2 (CH_3), 11.7 (CH_3), 11.7 (CH_3); IR (thin film) 3018, 2979, 2937, 1743 cm^{-1}; LRMS (ESI) 404 (100, M+H]$^{+}$); HRMS (ESI) calcd for $C_{17}H_{29}NaN_3O_6S$ [M+Na]$^{+}$ 426.1659, observed 426.1687.

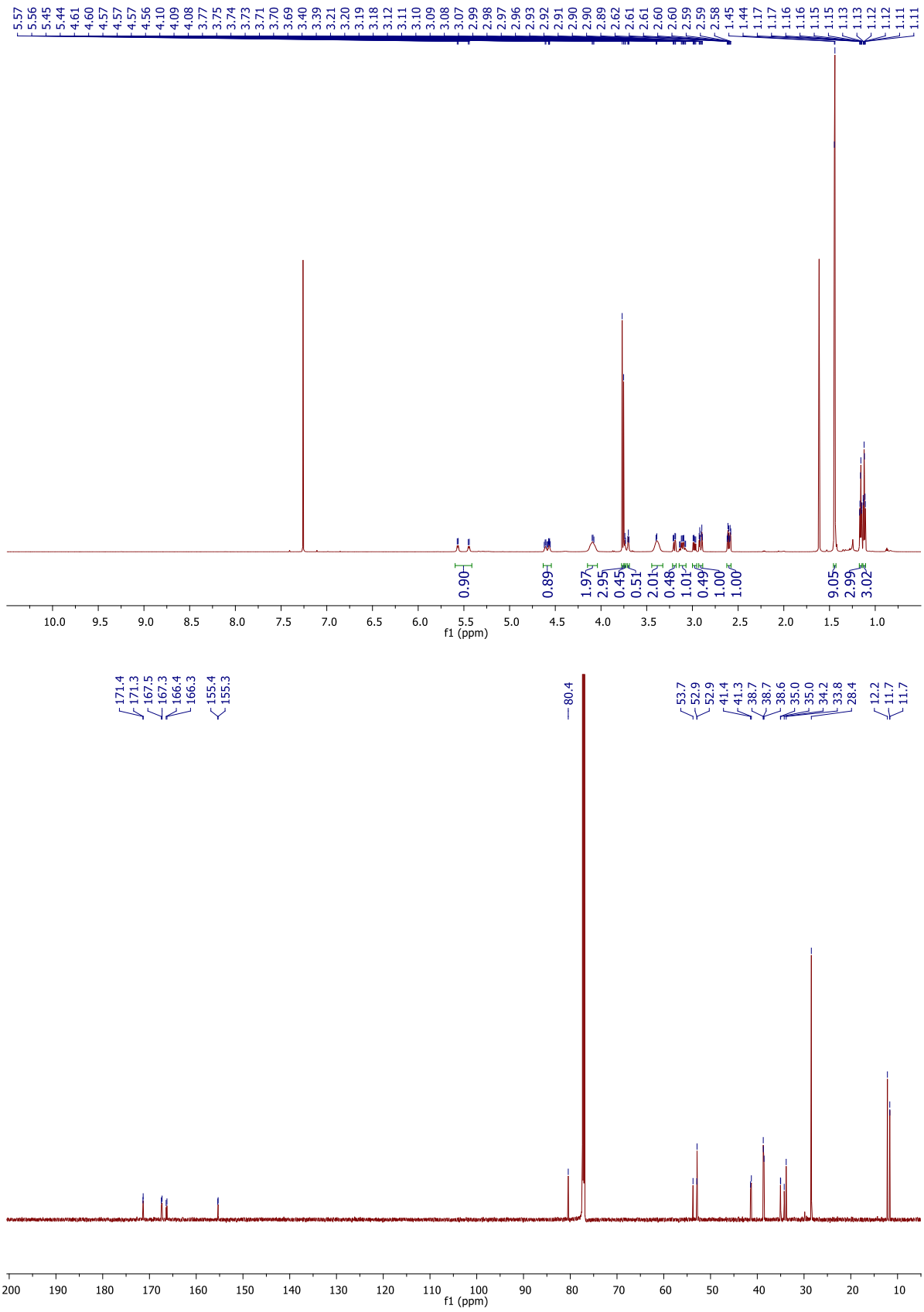
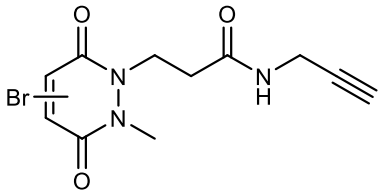


Figure S2: ^{1}H and ^{13}C NMR data of **9**

*3-(4,5-Dibromo-2-methyl-3,6-dioxo-3,6-dihydropyridazin-1(2*H*)-yl)*-N-*(prop-2-yn-1-yl)propenamide* (***monoBr PD model species***)



To a solution of 2,5-dioxopyrrolidin-1-yl 3-(bromo-2-methyl-3,6-dioxo-3,6-dihydropyridazin-1(2*H*)yl) propanoate[3] (100 mg, 0.221 mmol, pre-dissolved in MeCN (10 mL)), was added propargyl amine (10% in MeCN) (150 µL, 0.243 mmol) and the reaction mixture was stirred at 21 °C for 16 h. After this time, the reaction was concentrated *in vacuo* and the crude residue dissolved in $CHCl_3$ (50 mL), and washed with water (2 × 30 mL) and saturated aq. K_2CO_3 (30 mL). The organic layer was then dried ($MgSO_4$) and concentrated *in vacuo.* Purification of the crude residue by flash column chromatography (0% to 10% MeOH/EtOAc) afforded an inseparable 1:1 mixture of regioisomers 3-(4-bromo-2-methyl-3,6-dioxo-3,6-dihydropyridazin-1(2*H*)-yl)-*N*-(prop-2-yn-1-yl)propanamide and 3-(5-bromo-2-methyl-3,6-dioxo-3,6-dihydropyridazin-1(2*H*)-yl)-*N*-(prop-2-yn-1-yl)propanamide (80 mg, 0.255 mmol, 95%) as a light-yellow oil. ^{1}H NMR (600 MHz, $CDCl_3$, regioisomers) δ 7.39 (s, 1H), 7.35 (s, 1H), 6.25–6.03 (m, 2H), 4.44 (t, *J* = 7.0 Hz, 2H), 4.37 (t, *J* = 7.0 Hz, 2H), 4.04–4.03 (m, 4H), 3.73 (s, 3H), 3.65 (s, 3H), 2.67–2.62 (m, 4H), 2.25–2.24 (m, 2H); ^{13}C NMR (150 MHz, $CDCl_3$, regioisomers) δ 168.8 (C), 156.0 (C), 155.8 (C), 154.1 (C), 154.1 (C), 136.4 (CH), 135.4 (CH), 134.4 (C), 133.0 (C), 79.1 (C), 79.0 (C), 72.1 (CH), 44.1 (CH_2), 43.0 (CH_2), 34.7 (CH_2), 34.1 (CH_3), 34.0 (CH_2), 33.5 (CH_3), 29.5 (CH_2); IR (thin film) 3290, 3058, 2924, 2849, 1623, 1540 cm^{-1}; LRMS (ESI) 314 (100, $[M^{79}Br+H]^+$), 316 (99, $[M^{81}Br+H]^+$), 336 (30, $[M^{79}Br+Na]^+$); HRMS (ESI) calcd for $C_{11}H_{13}Br_1N_3O_3$ $[M^{79}Br+H]^+$ 314.0135; observed 314.0137.

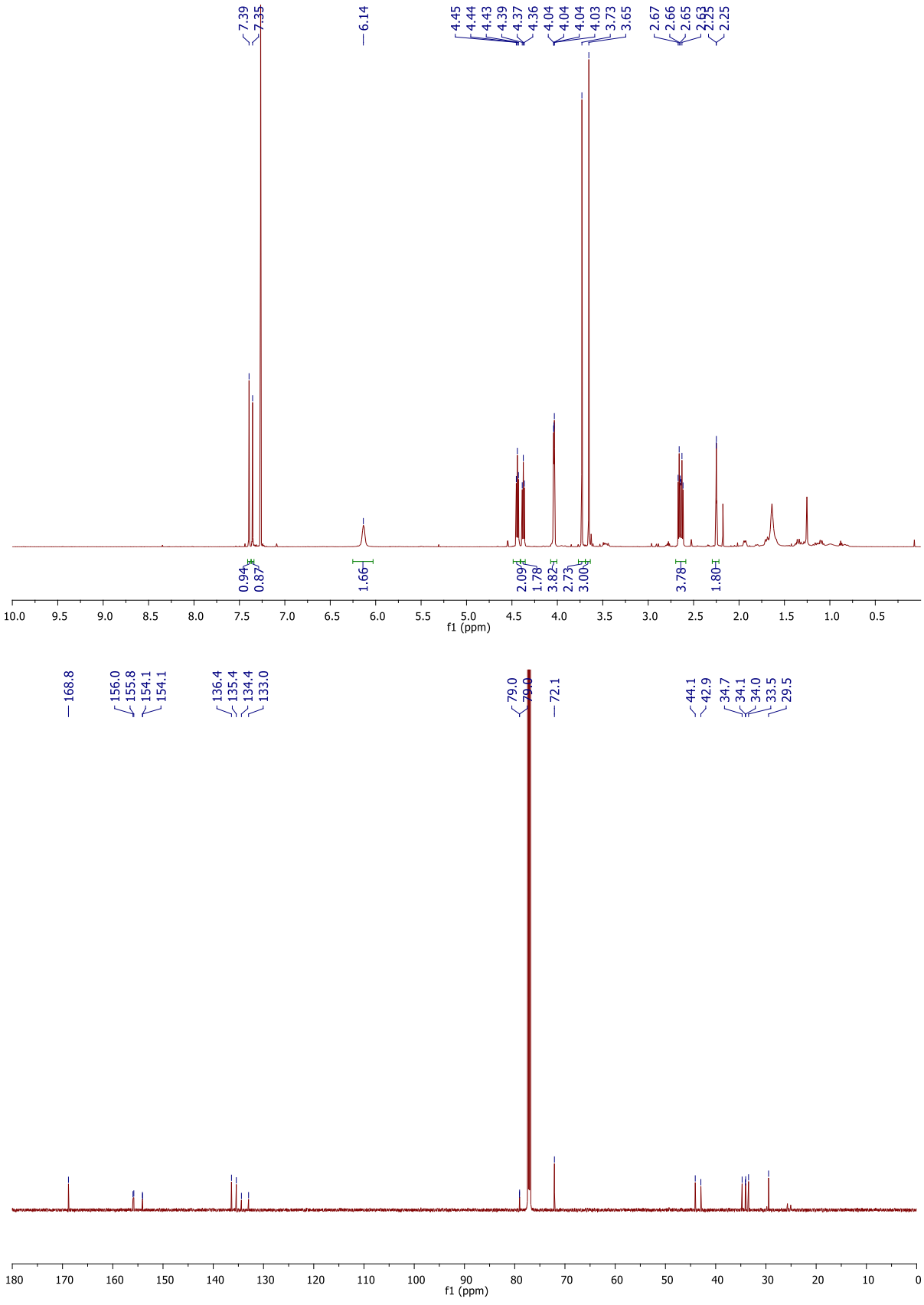
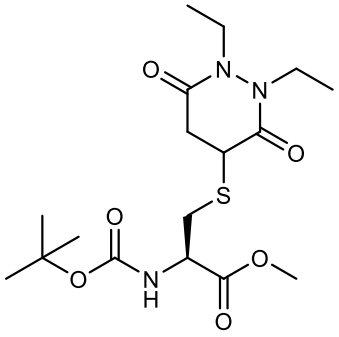


Figure S4: ^{1}H and ^{13}C NMR data of monoBr PD

Conformational analysis of *Methyl* N-*(tert-butoxycarbonyl)-(1,2-diethyl-3,6-dioxohexahydropyridazin-4-yl)-L-cysteinate* 9



In order to estimate conformational populations of **9** conformers with pseudoaxial and pseudoequatorial orientations of the sulfur atom, molecular mechanics calculations were carried out using the MMX force field.[4,5] The optimised geometries of the two conformers are shown in Figure S5).

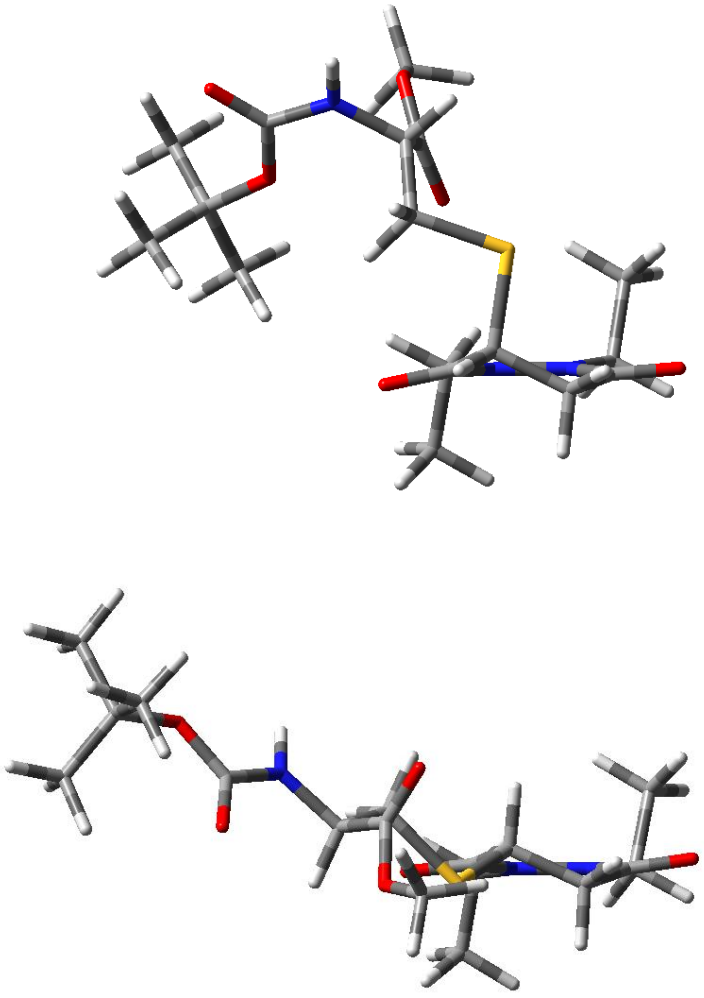
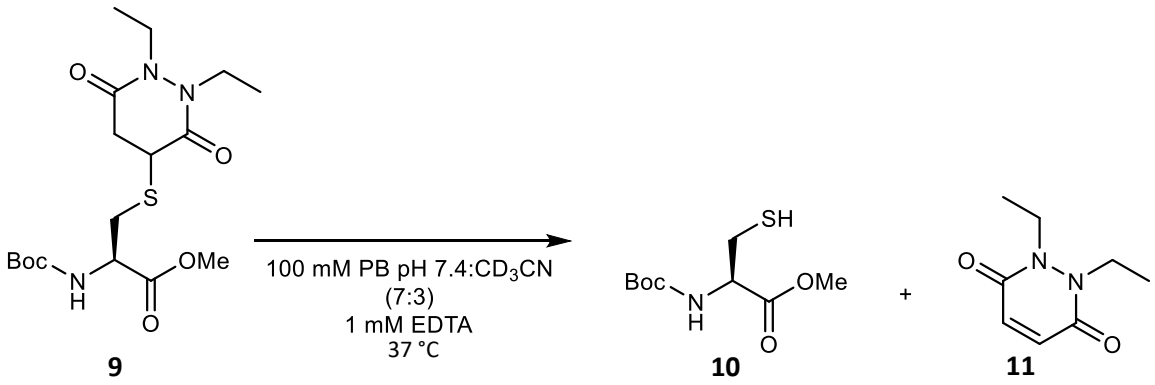


Figure S5: Optimised **9** geometries of the conformers with S-pseudoaxial (top) and S-pseudoequatorial (bottom) orientations using the MMX force field[4,5] for a single molecule *in vacuo.*

The exchange between two half-chair conformers is very fast in the NMR timescale and cannot be frozen at practically achievable low temperatures by NMR spectroscopy due to the fact that the half-chair interconversion energy barrier is very small. An alternative approach was therefore used, which relies on conformer populations calculated using the observed averaged coupling constants. In the case of two-site equilibrium, the populations of conformers with S-pseudoaxial [p_{Sax}, in %] and S-pseudoequatorial [p_{Seq} = 100 - p_{Sax}] orientations are calculated using $\overline{J} = J^{aa}p_{Seq} + J^{ee}p_{Sax}$, where $\overline{J}$ is the measured averaged coupling constant, whereas J^{aa} and J^{ee} are the boundary values of corresponding trans coupling constants between pseudoaxial and pseudoequatorial cyclic protons in S-pseudoequatorial and S-pseudoaxial conformers, respectively. In order to determine J^{aa} and J^{ee}, we used the above optimised geometries of the two conformers and the modified Karplus equation by Haasnoot *et al*.,[6] which has been shown to perform with a satisfactory accuracy in predicting vicinal ^3J couplings, comparable to that of DFT calculations.[7] Boundary $^3J_{HH}$ values determined in this manner were J^{aa} = 12.18 and J^{ee} =2.20 Hz. The experimentally measured value of the trans ^3J couplings were 4.7 Hz for both diastereomers of **9** in $CDCl_3$, as well as in $CD_3CN + H_2O$, thus the calculated population of the preferred conformer with the S-pseudoaxial orientation was p_{Sax} = 75%. Assuming that the uncertainty in the predicted boundary values of *J* couplings is ±0.5 Hz, the corresponding uncertainty in the predicted populations is estimated to be within ±3 %. In DMSO-d_6, the experimentally measured value of the trans ^3J couplings were 4.8 Hz and 4.9 Hz for two diastereomers of **9** at 298 K, corresponding to the calculated populations of the preferred conformer with the S-pseudoaxial orientation of 74% and 73%, respectively.

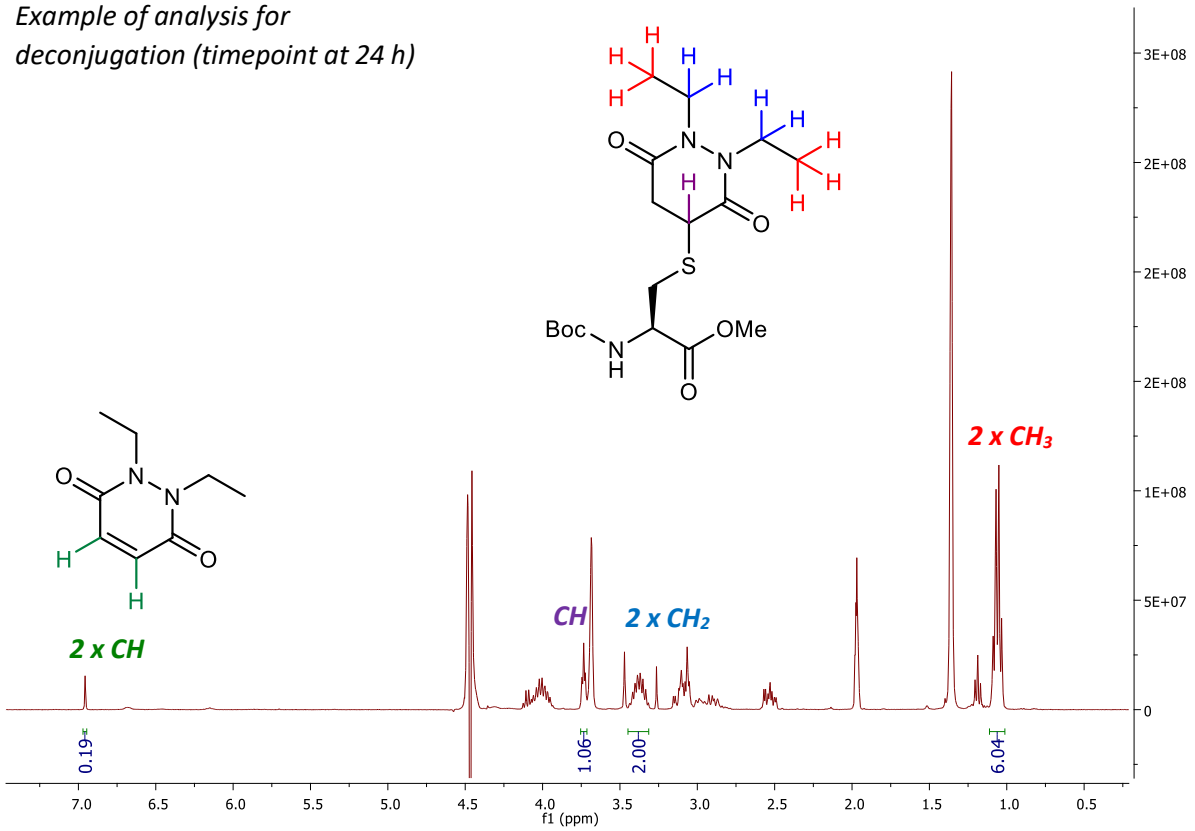
Retro-Michael deconjugation



Scheme S1: Outline of deconjugation of **9**

Retro-Michael mediated deconjugation of methyl *N*-(*tert*-butoxycarbonyl)-(1,2-diethyl-3,6-dioxohexahydropyridazin-4-yl)-L-cysteinate **9** to give *N*-(*tert*-butoxycarbonyl)-L-cysteine methyl ester **10** and 1,2-diethyl-1,2-dihydropyridazine-3,6-dione **11** was performed in a solvent system of 7:3 100 mM PB pH 7.4:CD_3CN containing 1 mM EDTA. Deconjugation was monitored by ^1H NMR, and deconjugation was assessed using integrations of the appearing alkene CH protons of 1,2-diethyl-1,2-dihydropyridazine-3,6-dione **11** (relative to the integrations of protons corresponding to methyl *N*-(*tert*-butoxycarbonyl)-*S*-(1,2-diethyl-3,6-dioxohexahydropyridazin-4-yl)-L-cysteinate **9**).

Example of analysis for deconjugation (timepoint at 24 h)



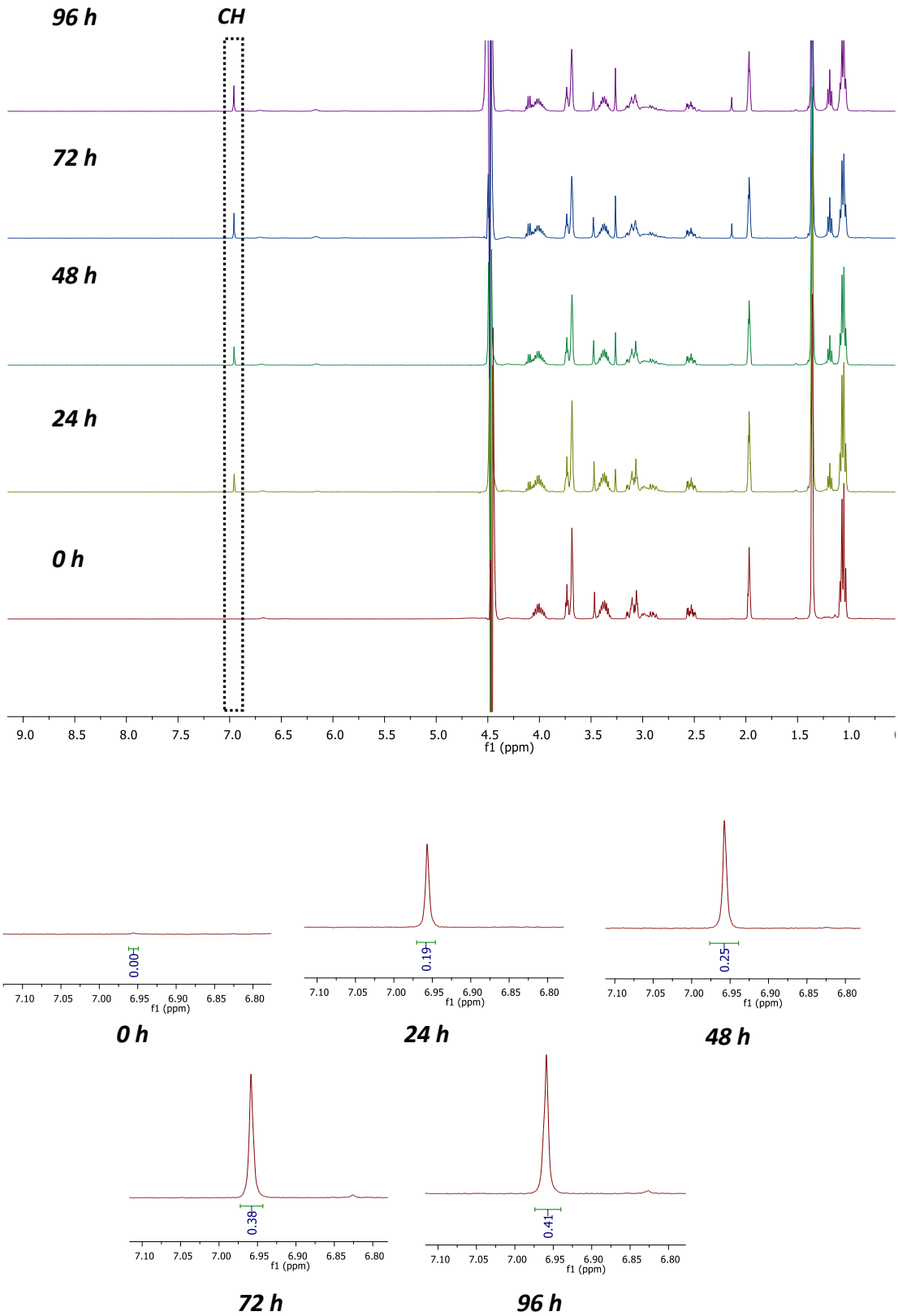
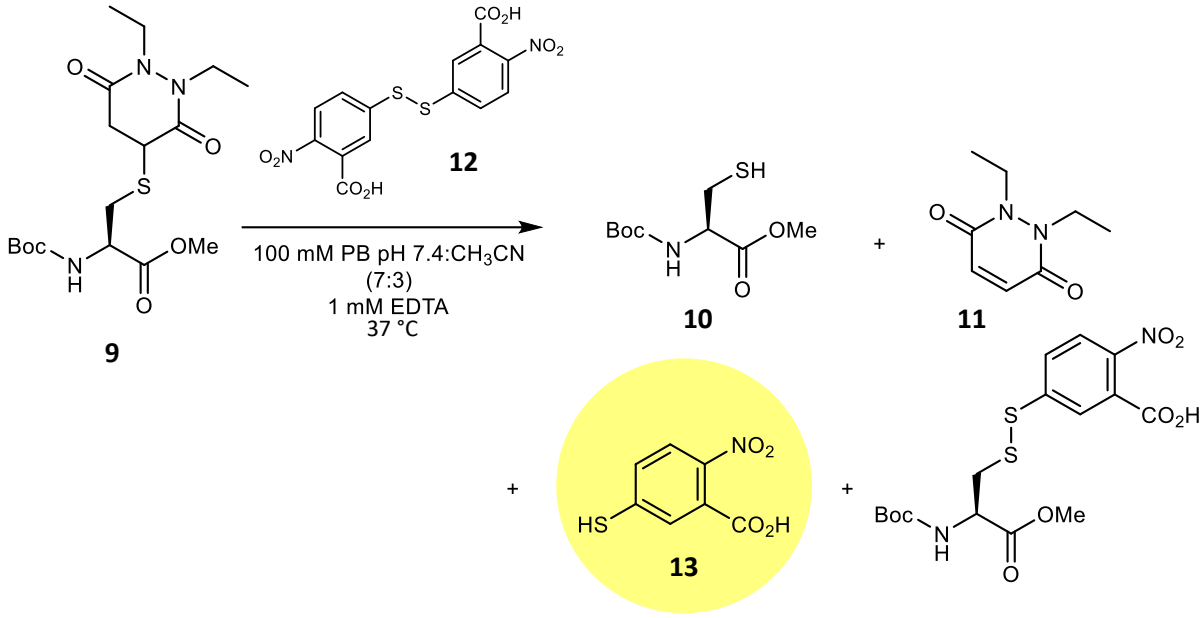


Figure S6: Above: ^{1}H NMR following deconjugation of **9** (outlined in Scheme S1). Peaks corresponding to CH of **11** are highlighted. Below: Integration of peak corresponding to the CH protons of PD **11** during deconjugation.

Small molecule Ellman's test



Scheme S2: Deconjugation of **9** followed by trapping of free thiol using Ellman's reagent **12**, resulting in release of UV active TNB **13**.

Trapping of *N*-(*tert*-butoxycarbonyl)-L-cysteine methyl ester **10** (resulting from the retro-Michael-mediated deconjugation of *methyl N-(tert*-butoxycarbonyl)-(1,2-diethyl-3,6-dioxohexahydropyridazin-4-yl)-L-cysteinate **9**) with Ellman's reagent **12** was performed thus: CH_3CN (476 µL) was added to 100 mM PB pH 7.4, 1 mM EDTA buffer (1112 µL). To this solution was added methyl *N*-(*tert*-butoxycarbonyl)-*S*-(1,2-diethyl-3,6-dioxohexahydropyridazin-4-yl)-L-cysteinate **9** (28 µL, 5 mM in CH_3CN, 1 eq.), followed by addition of Ellman's reagent **12** (57 µL, 3 mM, 1.2 eq.), resulting in a final reaction mixture of 140 µM methyl *N-(tert-butoxycarbonyl)-(1,2-diethyl-3,6-dioxohexahydropyridazin-4-yl)-L-cysteinate* **9** and 170 µM Ellman's reagent **12** in 100 mM PB pH 7.4, 1 mM EDTA buffer: CH_3CN (7:3). The reaction mixture was then allowed to incubate at 37 °C with shaking. An additional control reaction was also performed, wherein 170 µM Ellman's reagent **12** was incubated in 100 mM PB pH 7.4, 1 mM EDTA buffer:CH_3CN (7:3) at 37 °C with shaking. A_{412} of the reaction mixtures were monitored at time points of 24 h, 48 h, 72 h, and 96 h (using 100 mM PB pH 7.4:CH_3CN (7:3), 1 mM EDTA as a blank). Estimated free thiol content (and therefore amount of deconjugation that had occurred) at the given time points was estimated using Equation 1.

$$\frac{[\mathrm{TNB_{Trap}}]-[\mathrm{TNB_{Control}}]}{[\mathrm{CysPD_{Initial}}]} \times 100 = \%\ \text{estimated deconjugation} \qquad (1)$$

Where: TNB_{Trap} = Calculated TNB **13** concentration in trapping reaction, $TNB_{Control}$ = Calculated TNB **13** concentration in control, and $CysPD_{Initial}$ = Initial concentration of **9**

Table S1: Monitoring of A_{412} of 170 µM Ellman's reagent **12** in 100 mM PB pH 7.4, 1 mM EDTA buffer:CH_3CN (7:3), incubating at 37 °C, and the estimated concentration of free TNB **13** in solution

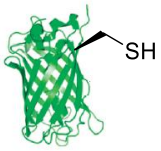
Time point (h)	A_{412}	Estimated conc^n of free TNB 13 (µM)
0	0.026	2
24	0.139	10
48	0.167	12
72	0.153	11
96	0.208	15

Table S2: Monitoring of A_{412} of 140 µM **9** and 170 µM Ellman's reagent **12** in 100 mM PB pH 7.4, 1 mM EDTA buffer:CH_3CN (7:3), incubating at 37 °C, and the estimated concentration of free TNB **13** in solution. Estimated deconjugation of **9** is determined using Equation 1 and Table S1

Time point (h)	A_{412}	Estimated conc^n of free TNB 13 (µM)	Corrected estimated 9 deconjugated (µM)	Corrected estimated 9 deconjugated (%)
0	0.012	1	0	0
24	0.452	33	23	16
48	0.687	50	38	27
72	0.797	58	47	34
96	0.908	66	51	36

Synthesis of proteins:

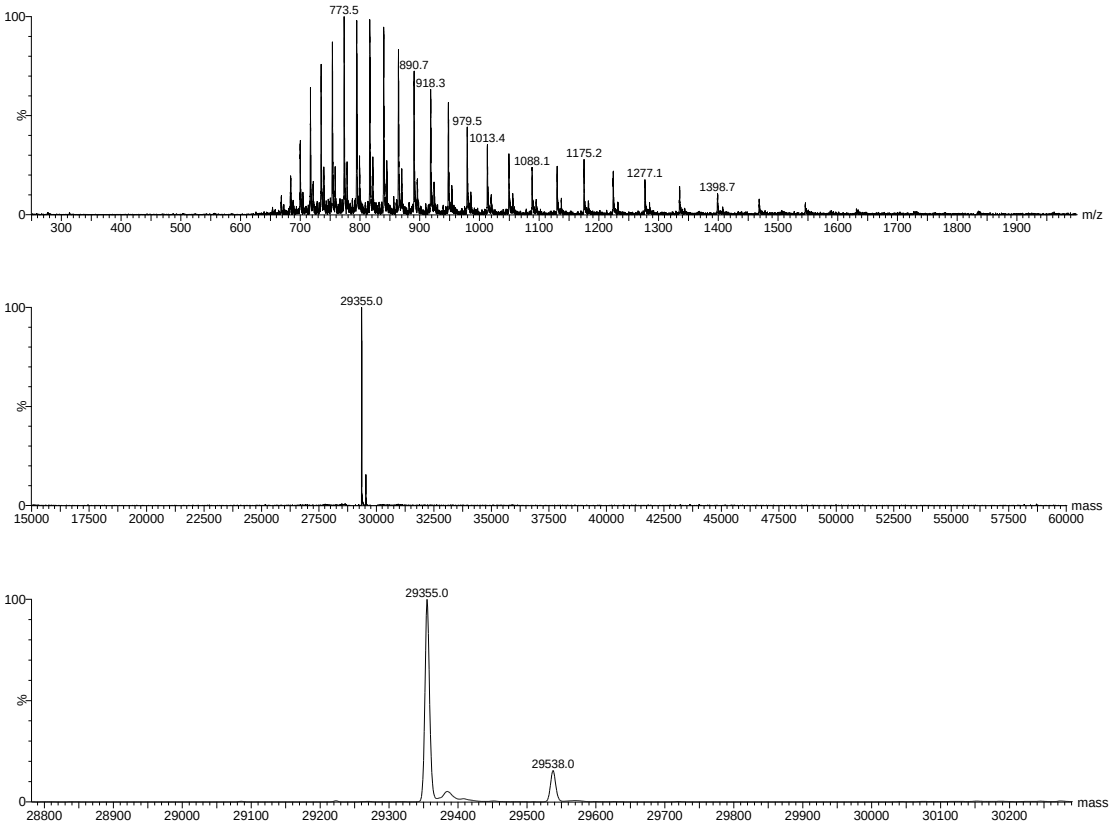
Cysteine Mutant Green Fluorescent Protein (GFPS147C) 14:



14

GFPS147C **14** was expressed in competent E. coli BL21(DE3) cells as described previously.[8] Due to the presence of a small amount of dimer species, GFPS147C **14** was then reduced prior to conjugation and characterised as below:

TCEP (31.3 µL, 20 mM in deionised water, 25 eq.) was added to a solution of GFPS147C **14** (500 µL, 50 µM) in PBS (pH 7.4, 5 mM EDTA) and the solution was incubated at 37 °C for 90 min. Excess reagents were removed by ultrafiltration (6 × 10000 MWCO, VivaSpin®, GE Healthcare) into PBS (pH 7.4, 5 mM EDTA) for further experiments. Samples were desalted (7000 MWCO, ZebaSpin®, Thermo Scientific) prior to LCMS analysis. Concentration was determined photometrically using ε_{280} = 20,500 $M^{-1}\,cm^{-1}$. Observed masses (LCMS Method 1): 29355 Da (GFP(1)-SH **14**), 29538 Da (GFP(2)-SH **14**).



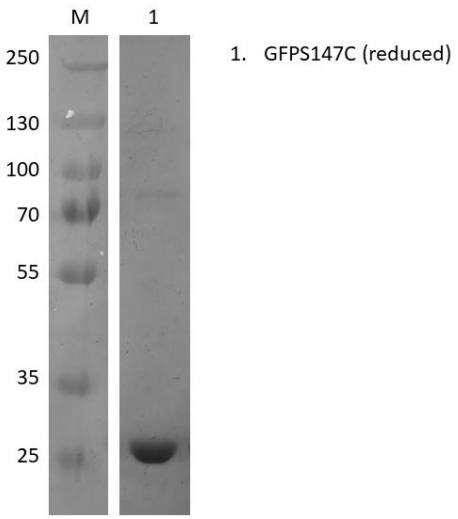
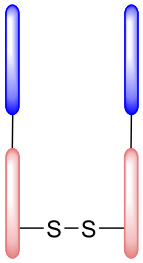


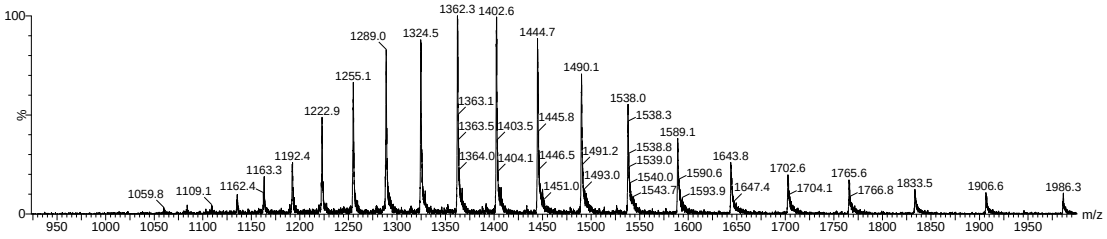
Figure S7: SDS-PAGE gel of reduced GFPS147C **14**

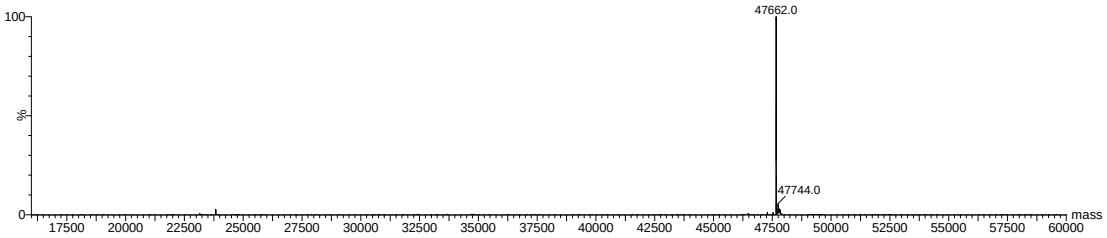
Trastuzumab Fab 15:

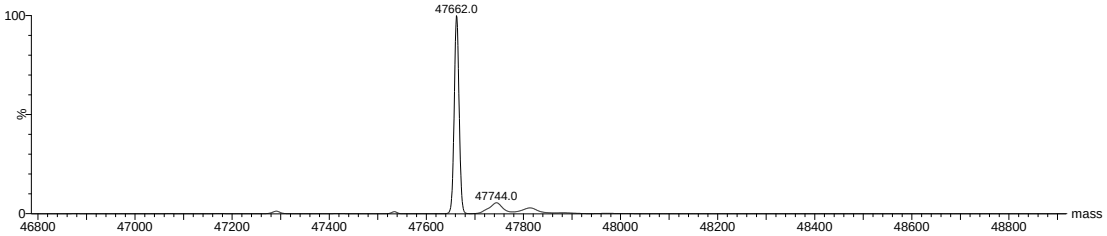


15

Trastuzumab Fab **15** was obtained through pepsin/papain digestion of trastuzumab as described previously.[9] Concentration was determined photometrically using $\varepsilon_{280} = 68{,}590\ M^{-1}\,cm^{-1}$. Observed mass 47662 Da.







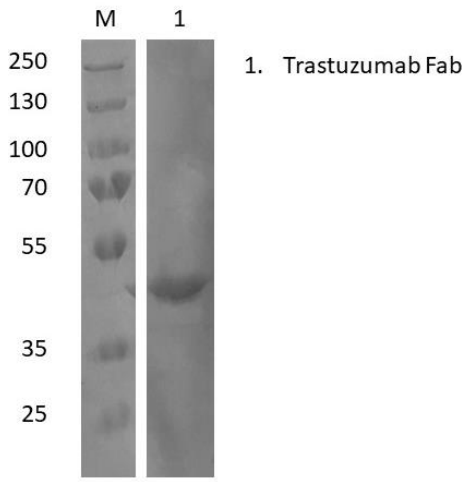


Figure S8: SDS-PAGE gel of Trastuzumab Fab **15**

GFP Experiments

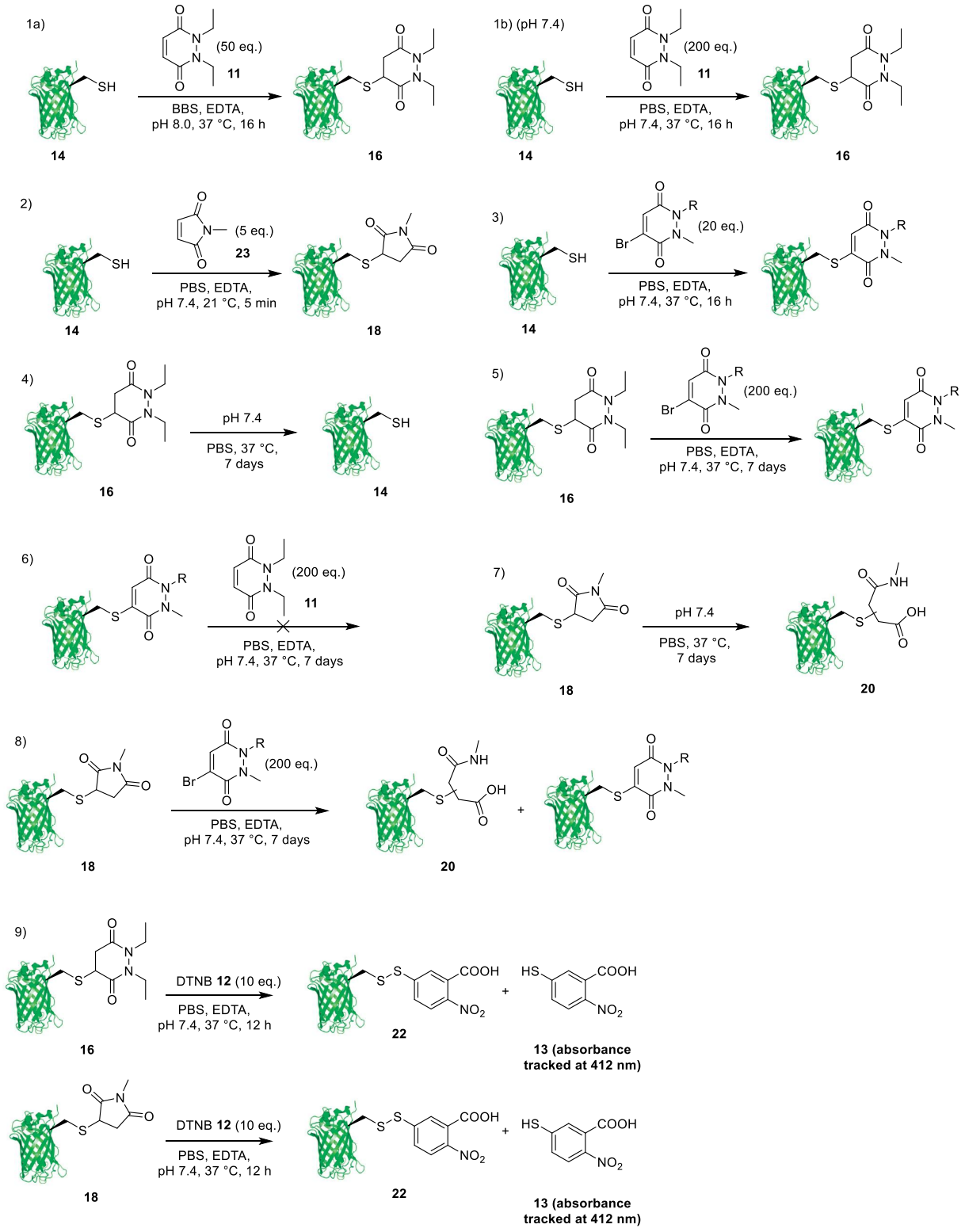
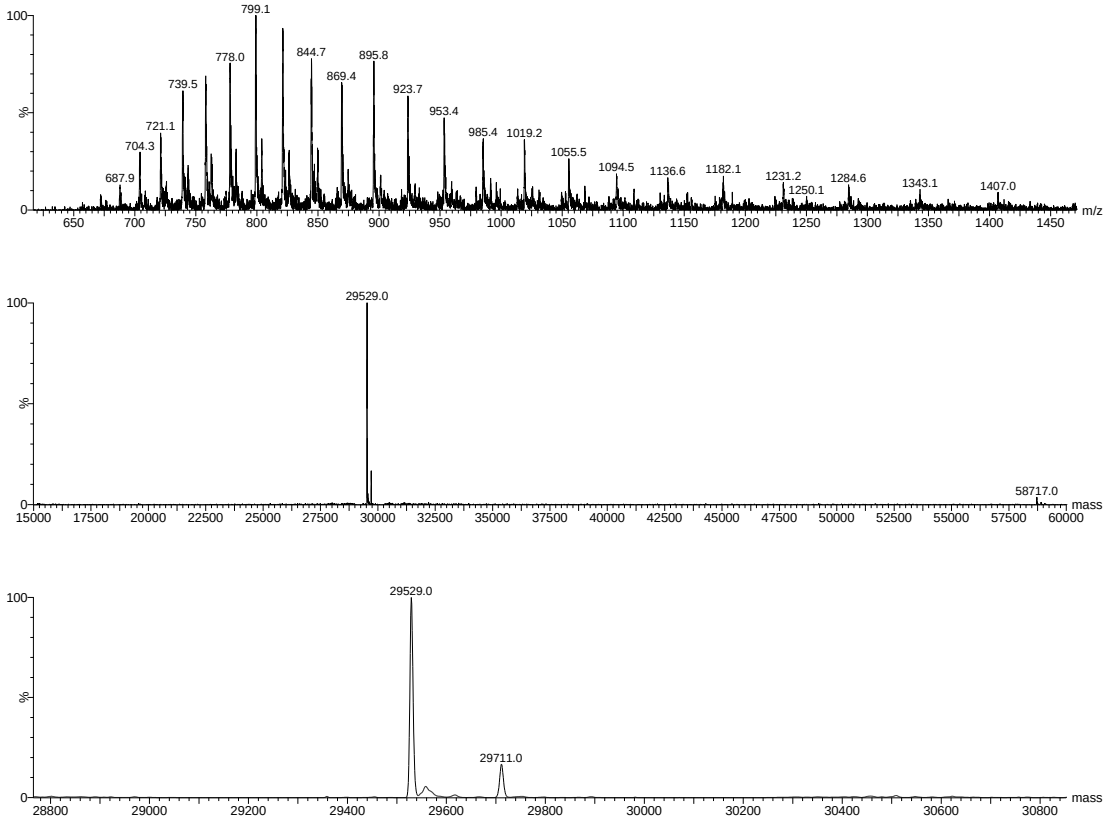


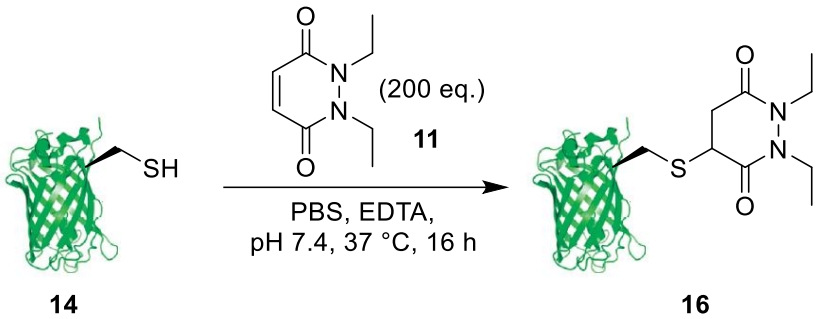
Figure S9: Outline of GFP bioconjugation related experiments.

1a) GFPS147C 14 conjugation with *N,N*-diethyl pyridazinedione 11 (pH 8.0, 50 eq. of PD)

N,N-Diethyl pyridazinedione **11** (25 µL, 50 mM in DMSO, 50 eq.) was added to reduced GFPS147C **14** (500 µL, 50 µM in BBS pH 8.0, 5 mM EDTA) and the solution was incubated at 37 °C for 16 h. Excess reagents were removed using desalting columns (7000 MWCO, ZebaSpin®, Thermo Scientific) prior to LCMS analysis. Expected masses: 29524 Da (GFP(1)-S-PD **16**), 29707 Da (GFP(2)-S-PD **16**). Observed masses (LCMS Method 1): 29529 Da (GFP(1)-S-PD **16**), 29711 Da (GFP(2)-S-PD **16**).



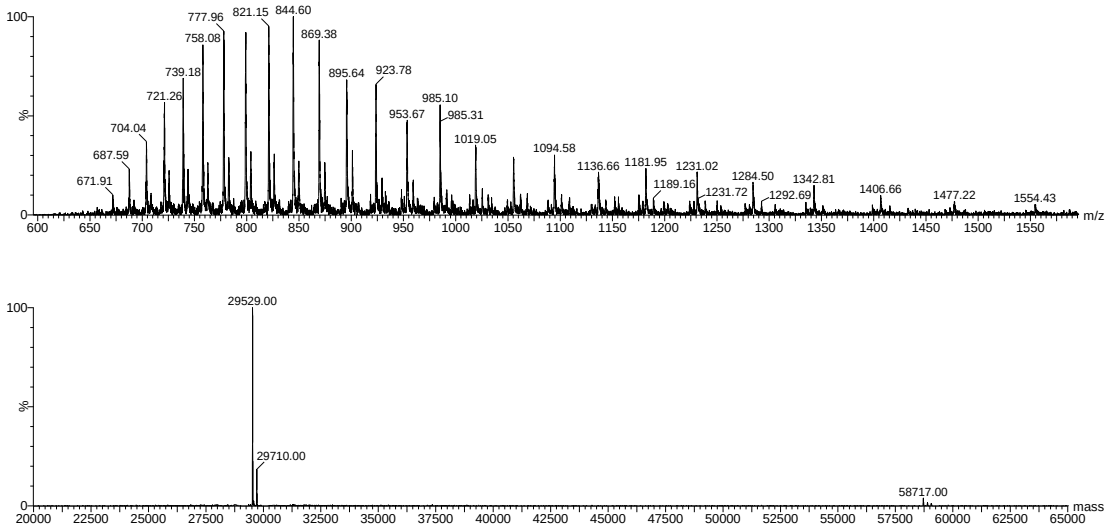
1b) Alternative (lower pH) GFPS147C 14 conjugation with *N,N*-diethyl pyridazinedione 11 (pH 7.4, 200 eq. of PD)



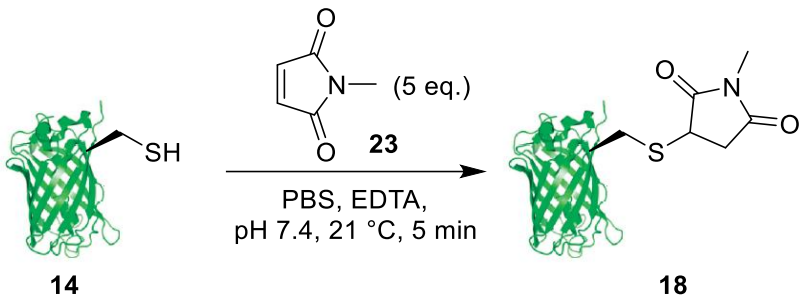
14 **16**

N,N-Diethyl pyridazinedione **11** (25 µL, 200 mM in DMSO, 200 eq.) was added to reduced GFPS147C **14** (500 µL, 50 µM in PBS pH 7.4, 5 mM EDTA) and the solution was incubated at 37 °C for 16 h. Excess reagents were removed using desalting columns (7000 MWCO, ZebaSpin®, Thermo Scientific) prior to

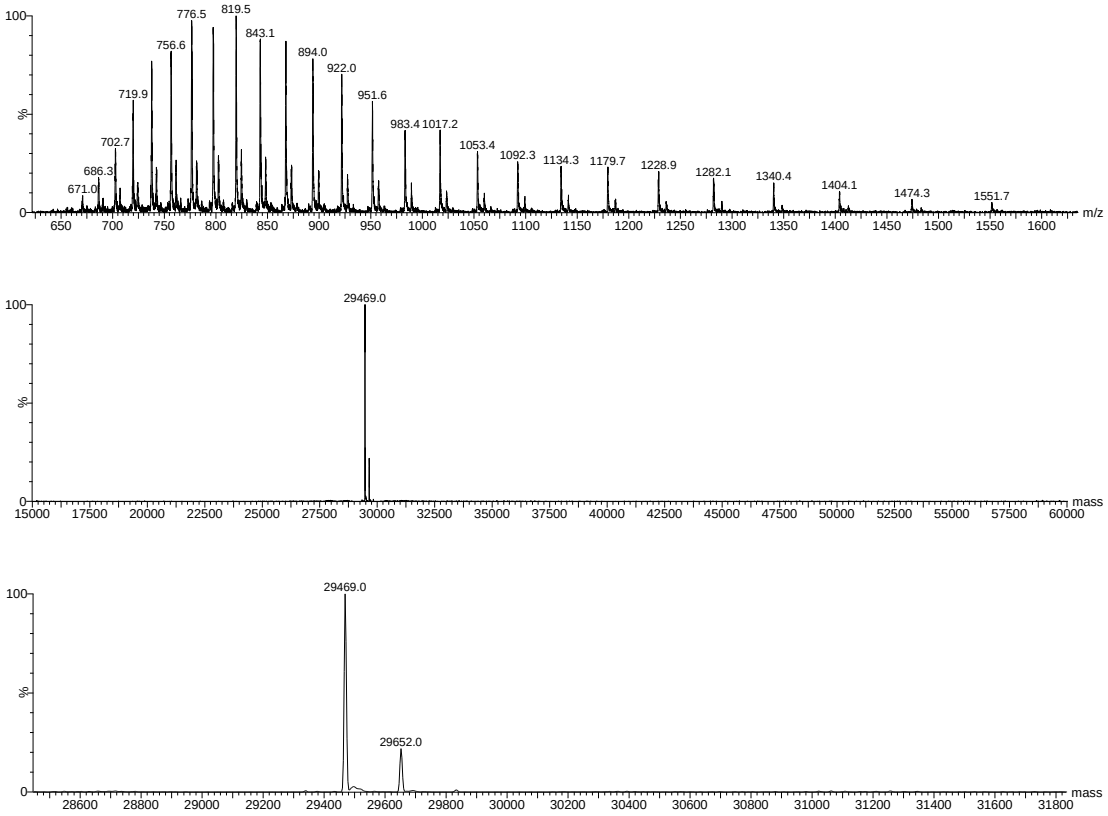
LCMS analysis. Expected masses: 29524 Da (GFP(1)-S-PD **16**), 29707 Da (GFP(2)-S-PD **16**). Observed masses (LCMS Method 1): 29529 Da (GFP(1)-S-PD **16**), 29710 Da (GFP(2)-S-PD **16**).



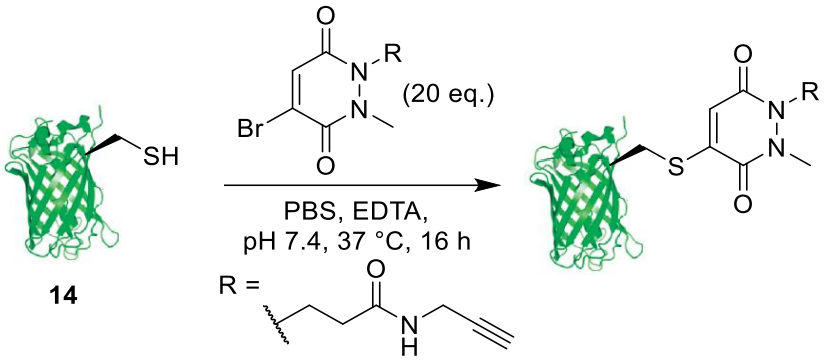
2) GFPS147C 14 conjugation with *N*-methyl maleimide 23



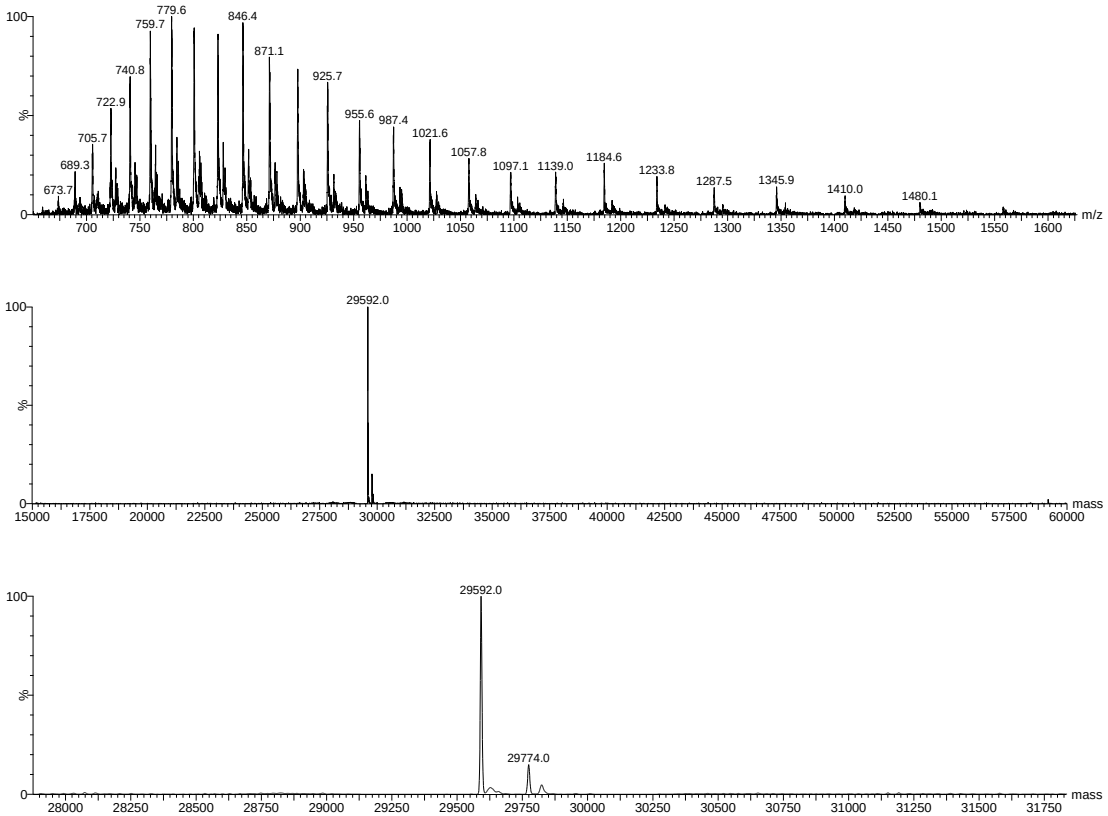
N-Methyl maleimide **23** (15 µL, 5 mM in DMSO, 5 eq.) was added to reduced GFPS147C **14** (300 µL, 50 µM in PBS pH 7.4, 5 mM EDTA) and the solution was incubated at 21 °C for 5 min. Excess reagents were removed using desalting columns (7000 MWCO, ZebaSpin®, Thermo Scientific) prior to LCMS analysis. Expected masses: 29467 Da (GFP(1)-S-Mal **18**), 29650 Da (GFP(2)-S-Mal **18**). Observed masses (LCMS Method 1): 29469 Da (GFP(1)-S-Mal **18**), 29652 Da (GFP(2)-S-Mal **16**).



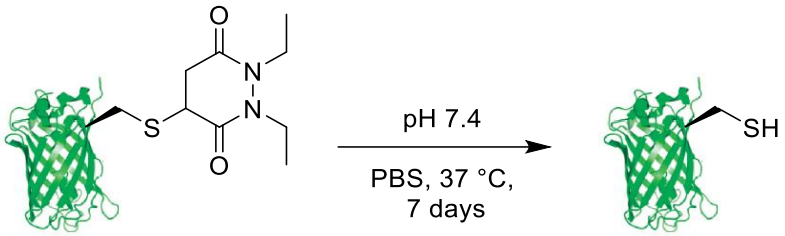
3) GFPS147C 14 conjugation with monoBr *N,N*-methyl propargyl pyridazinedione



MonoBr *N,N*-methyl propargyl pyridazinedione (10 µL, 20 mM in DMSO, 20 eq.) was added to reduced GFPS147C **14** (300 µL, 50 µM in PBS pH 7.4, 5 mM EDTA) and the solution was incubated at 37 °C for 16 h. Excess reagents were removed using desalting columns (7000 MWCO, ZebaSpin®, Thermo Scientific) prior to LCMS analysis. Expected masses: 29589 Da (GFP(1)-S-PD_{unsat}), 29772 Da (GFP(2)-S-PD_{unsat}). Observed masses (LCMS Method 1): 29592 Da (GFP(1)-S-PD_{unsat}), 29774 Da (GFP(2)-S-PD_{unsat}).

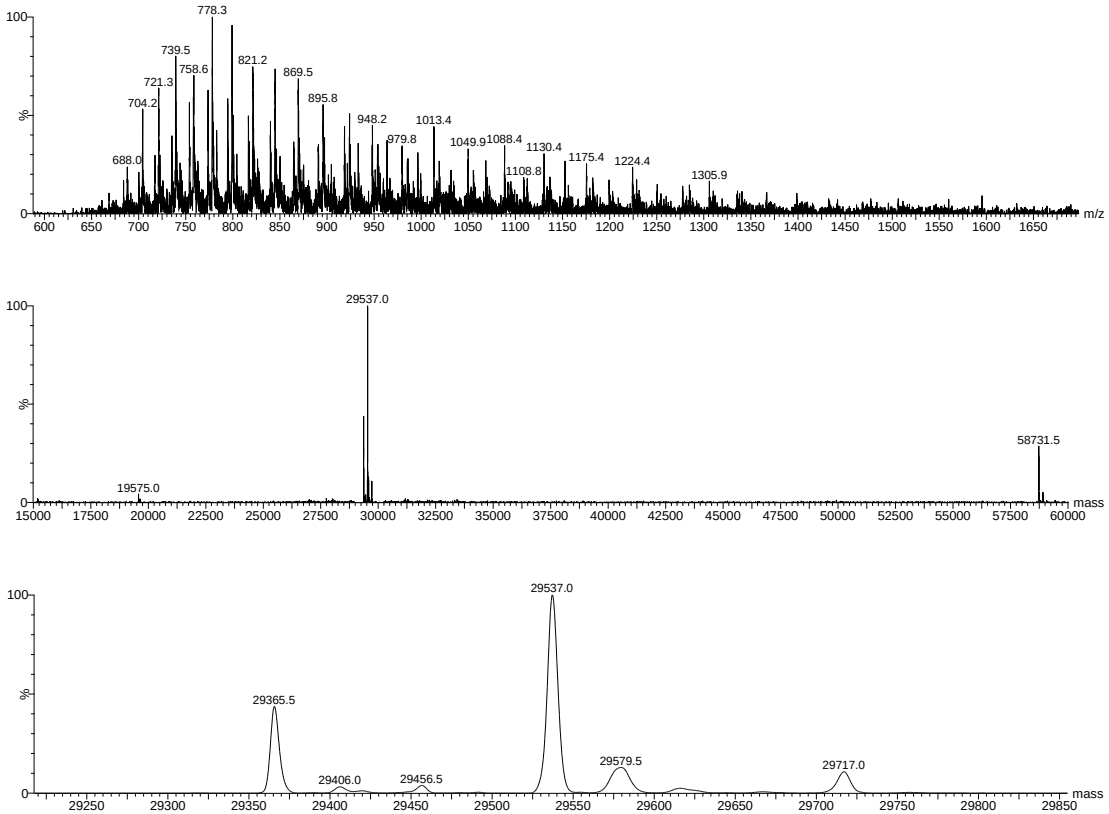


4) Incubation of GFPS147C-PD conjugate 16

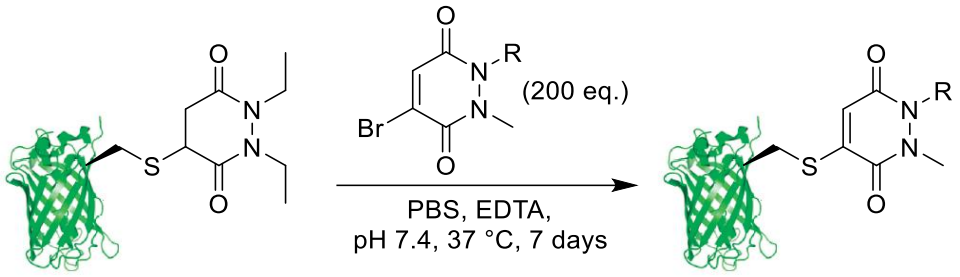


16 **14**

GFPS147C-PD conjugate **16** (200 µL, 50 µM) was buffer exchanged (7000 MWCO, ZebaSpin®, Thermo Scientific) into PBS (pH 7.4). The GFPS147C-PD conjugate **16** was then incubated at 37 °C for a total of 7 days. Samples were desalted (7000 MWCO, ZebaSpin®, Thermo Scientific) before LCMS analysis. Expected masses: 29355 Da (GFP(1)-SH **14**), 29529 Da (GFP(1)-S-PD **16**), 29538 Da (GFP(2)-SH **14**), 29711 Da (GFP(2)-S-PD **16**), Observed masses (LCMS Method 1): 29365 Da (GFP(1)-SH **14**), 29537 Da (GFP(1)-S-PD **14**), 29717 Da (GFP(2)-S-PD **16**).

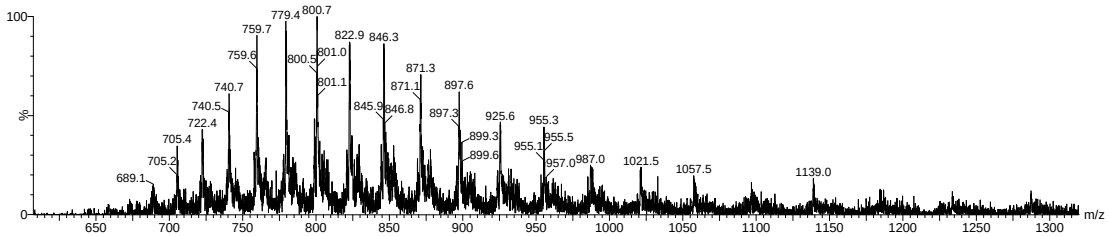


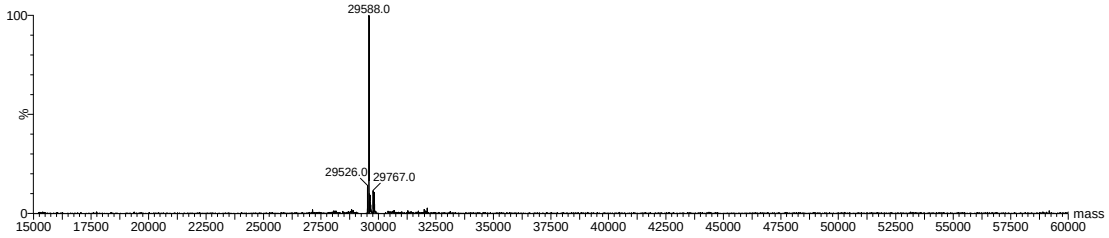
5) Reaction of GFPS147C-PD conjugate 16 with monoBr *N,N*-methyl propargyl pyridazinedione

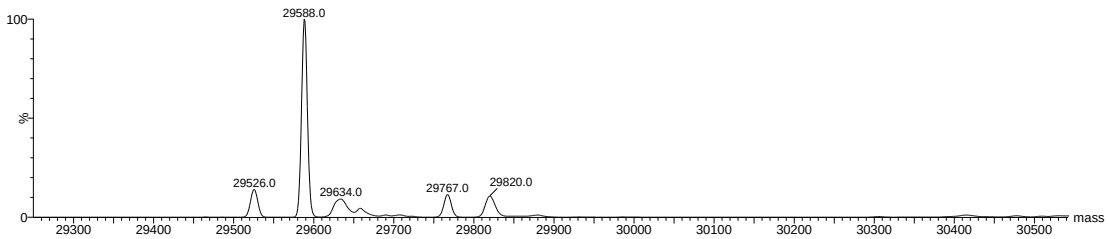


16

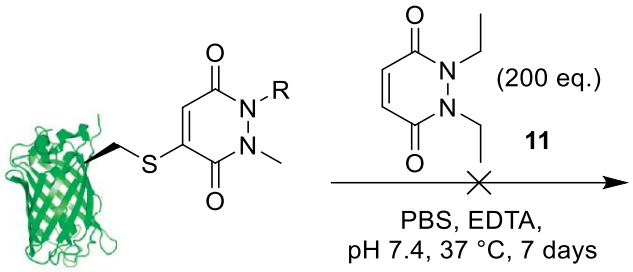
MonoBr *N,N*-methyl propargyl pyridazinedione (5.0 µL, 200 mM in DMSO, 200 eq.) was added to a solution of GFPS147C-PD conjugate **16** (100 µL, 50 µM in PBS pH 7.4, 5 mM EDTA) and the solution was incubated at 37 °C for 7 days. Excess reagents were removed using desalting columns (7000 MWCO, ZebaSpin®, Thermo Scientific) prior to LCMS analysis. Expected masses: 29589 Da (GFP(1)-S-PD$_{unsat}$), 29772 Da (GFP(2)-S-PD$_{unsat}$). Observed masses (LCMS Method 1): 29582 Da (GFP(1)-S-PD$_{unsat}$), 29776 Da (GFP(2)-S-PD$_{unsat}$).



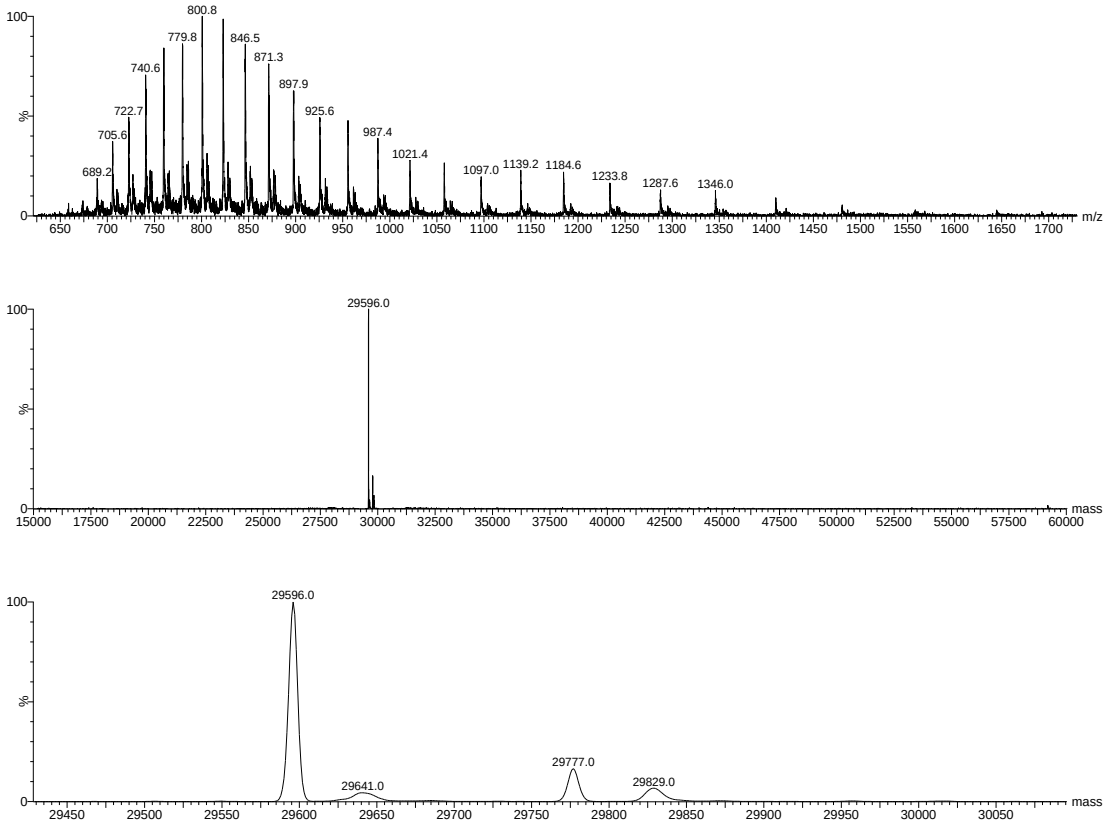




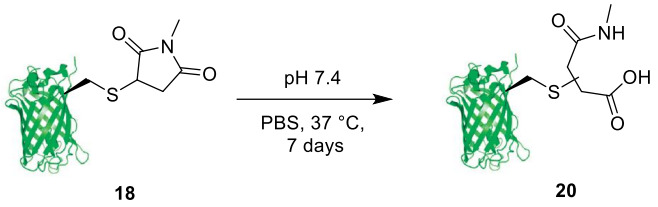
6) Control: Reaction of GFPS147C-PD conjugate with *N,N*-diethyl pyridazinedione



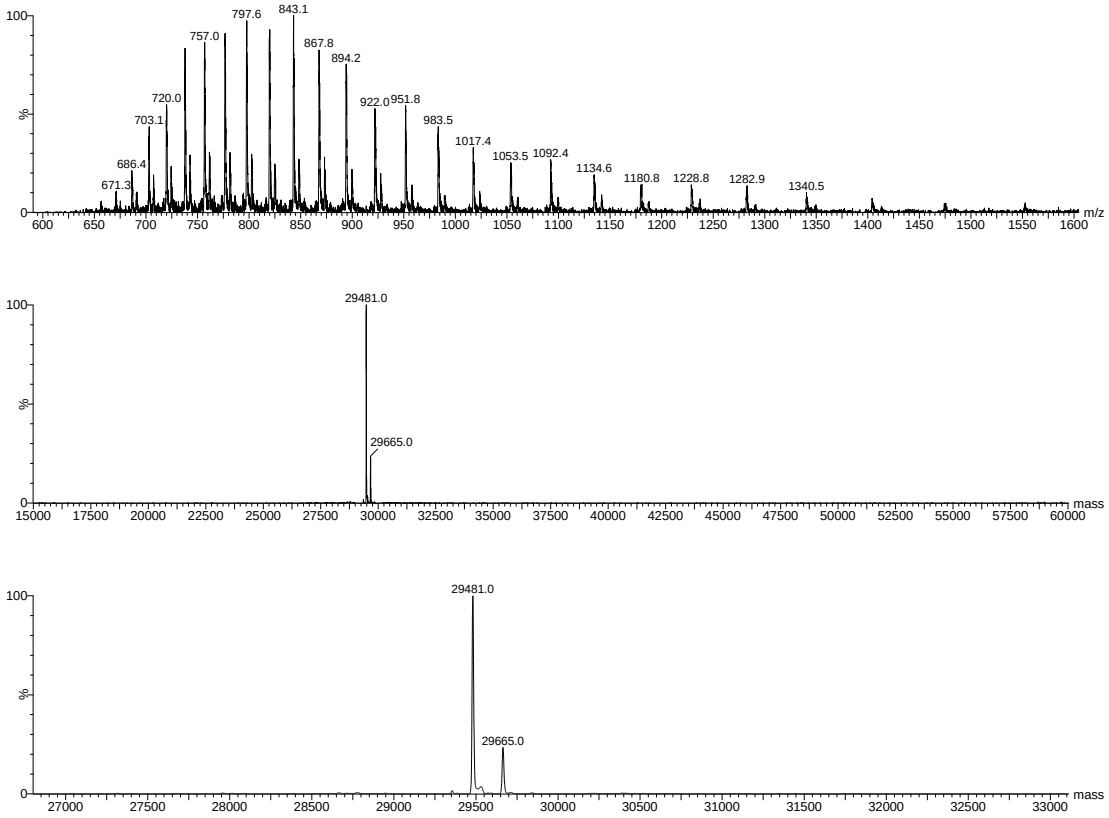
N,N-Diethyl pyridazinedione **11** (5.0 µL, 200 mM in DMSO, 200 eq.) was added to a solution of GFPS147C-PD conjugate (100 µL, 50 µM in PBS = pH 7.4, 5 mM EDTA) and the solution was incubated at 37 °C for 7 days. Excess reagents were removed using desalting columns (7000 MWCO, ZebaSpin®, Thermo Scientific) prior to LCMS analysis. Expected masses: 29589 Da (GFP(1)-S-PD_{unsat}), 29772 Da (GFP(2)-S-PD_{unsat}). Observed masses (LCMS Method 1): 29596 Da (GFP(1)-S-PD_{unsat}), 29777 Da (GFP(2)-S-PD_{unsat}).



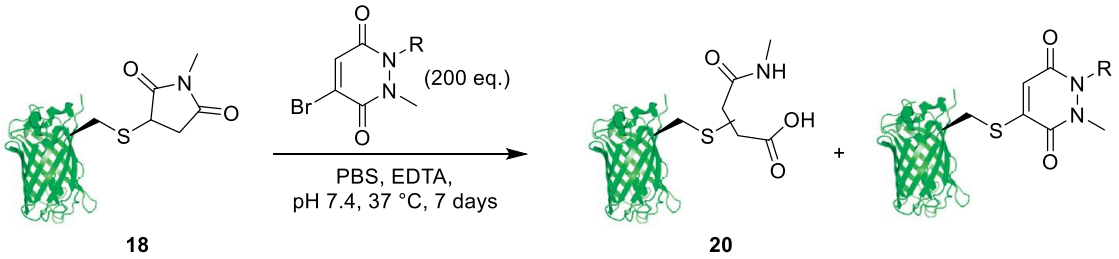
7) Incubation of GFPS147C-maleimide conjugate 18



GFPS147C-maleimide conjugate **18** (200 µL, 50 µM) was buffer exchanged (7000 MWCO, ZebaSpin®, Thermo Scientific) into PBS (pH 7.4). The GFPS147C-maleimide conjugate **18** was then incubated at 37 °C for a total of 7 days. Samples were desalted (7000 MWCO, ZebaSpin®, Thermo Scientific) before LCMS analysis. Expected masses: 29485 Da (GFP(1)-S-Mal$_{hyd}$ **20**), 29668 Da (GFP(2)-S-Mal$_{hyd}$ **20**), Observed masses (LCMS Method 1): 29481 Da (GFP(1)-S-Mal$_{hyd}$ **20**), 29665 Da (GFP(2)-S-Mal$_{hyd}$ **20**).

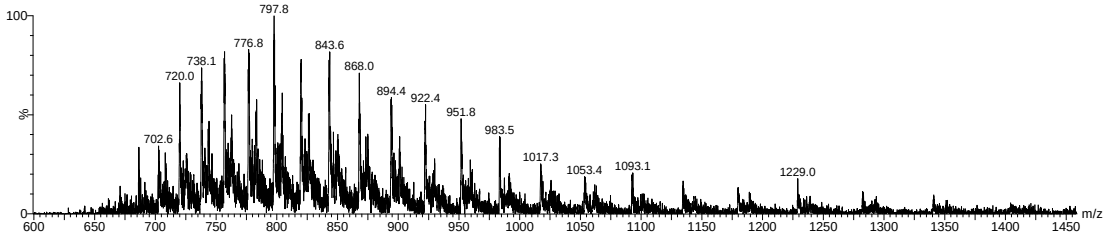


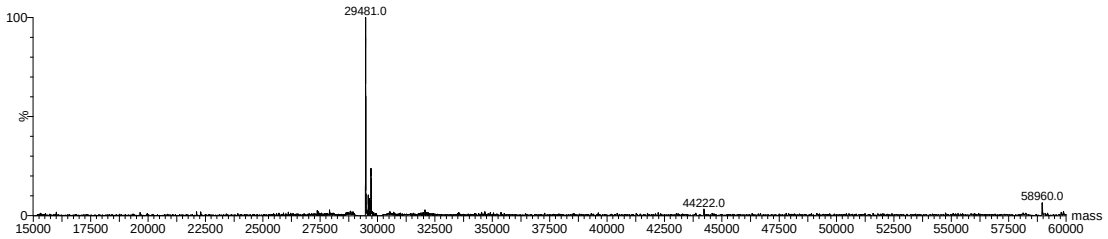
8) Reaction of GFPS147C-PD conjugate 18 with monoBr *N,N*-methyl propargyl pyridazinedione

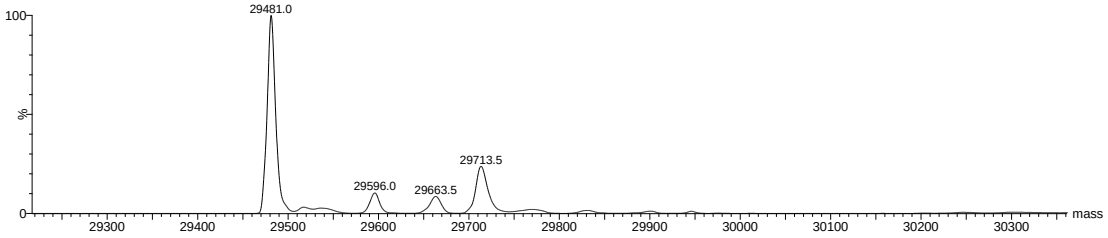


18 **20**

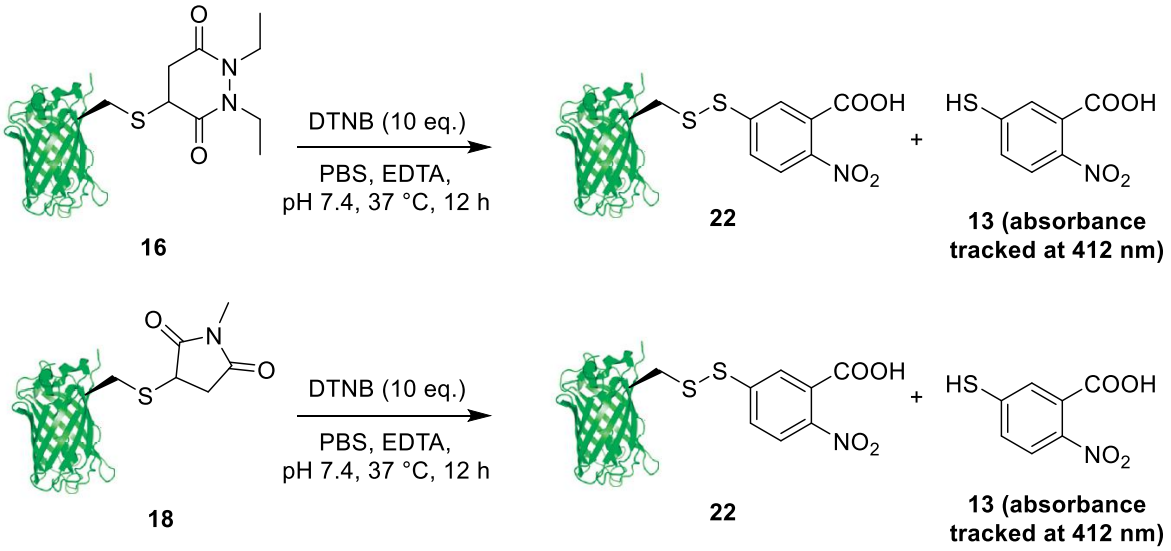
MonoBr *N,N*-methyl propargyl pyridazinedione (5.0 µL, 200 mM in DMSO, 200 eq.) was added to a solution of GFPS147C-maleimide conjugate **18** (100 µL, 50 µM in PBS pH 7.4, 5 mM EDTA) and the solution was incubated at 37 °C for 7 days. Excess reagents were removed using desalting columns (7000 MWCO, ZebaSpin®, Thermo Scientific) prior to LCMS analysis. Expected masses: 29485 Da (GFP(1)-S-Mal$_{hyd}$ **20**), 29589 Da (GFP(1)-S-PD$_{unsat}$), 29668 (GFP(2)-S-Mal$_{hyd}$ **20**), 29772 (GFP(1)-S-PD$_{unsat}$). Observed masses (LCMS Method 1): 29481 Da (GFP(1)-S-Mal$_{hyd}$ **20**), 29596 Da (GFP(1)-S-PD$_{unsat}$), 29663 Da (GFP(2)-S-Mal$_{hyd}$ **20**).







9) Kinetics: Reaction of GFPS147C conjugates 16 and 18 with DTNB 12



DTNB **12** (5.0 µL, 50 mM in DMSO) was added to GFPS147C-PD **16** (1000 µL, 14.4 µM in PBS pH 7.4, 5 mM EDTA), GFPS147C-maleimide **18** (1000 µL, 12.7 µM in PBS pH 7.4, 5 mM EDTA) and to PBS only (pH 7.4, 5 mM EDTA) as a control. The solutions were incubated in a 1 mL cuvette with stirring at 37 °C for 12 h. UV measurements were taken at 280 nm, 412 nm and 490 nm at 2 min intervals. A_{412} control readings for DTNB **12** only experiments were subtracted from all timepoints for GFP conjugates **16** and **18**. A_{412} for initial readings (t=180 s) were also subtracted from all timepoints for GFP conjugates **16** and **18** to plot ΔA_{412} *vs* time (seconds). ΔA_{412} values were converted into Δ[TNB **13**] values using ε_{280} = 14,150 $M^{-1}\,cm^{-1}$ for TNB **13**. Δ[TNB **13**] values were converted into Δ[GFP-TNB **13**] and subtracted from initial GFP conjugate concentrations (calculated using ε_{490} = 55,000 $M^{-1}\,cm^{-1}$) to form a plot of Δ[GFP-PD **16**] or Δ[GFP-maleimide **18**] vs time. The natural logarithm of Δ[GFP-PD **16**] and Δ[GFP-maleimide **18**] was calculated and plotted against time (seconds). Linear regression was plotted using GraphPad Prism software and pseudo order rate constants were calculated using Equation 2:

$$\ln([\text{GFP conjugate 16 or 18}]) = -kt + \ln([\text{GFP conjugate}]_0) \qquad (2)$$

Table S3: Summary of kinetic data

Data	*GFP-PD conjugate **16***	*GFP-maleimide conjugate **18***
Linear Regression	$y = -2.292\times10^{-5}\,x - 11.07$	$y = -2.342\times10^{-5}x - 11.26$
Rate constant K (s^{-1})	2.29×10^{-5}	2.342×10^{-5}
R^2	0.9906	0.9983

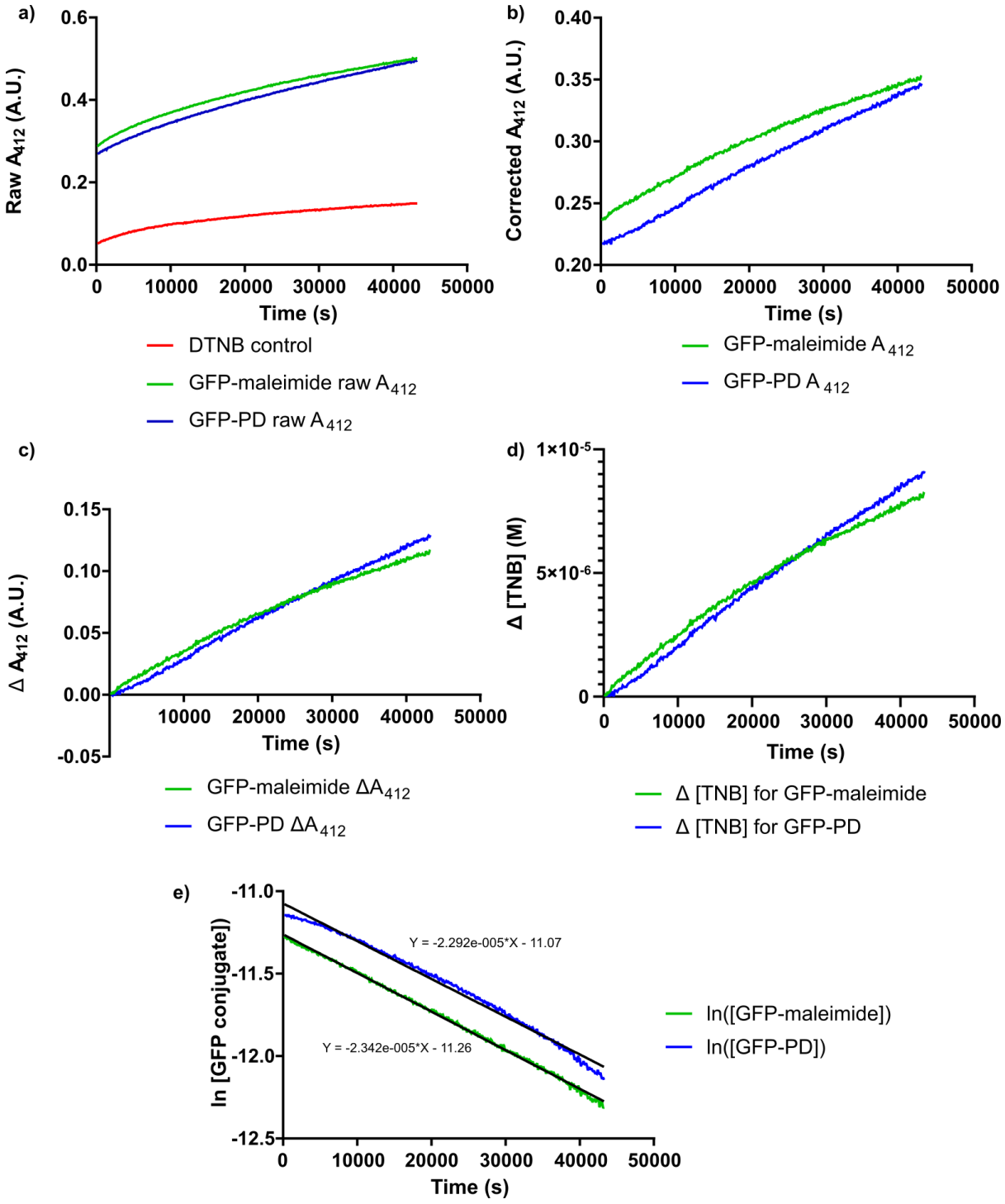


Figure S10: Summary of kinetic data plots a) Raw A_{412} vs time. b) corrected A_{412} vs time. c) change in A_{412} vs time. d) chance in TNB concentration vs time. e) change in logarithm of concentration of GFP conjugates against time.

Trastuzumab Fab Experiments:

Summary:

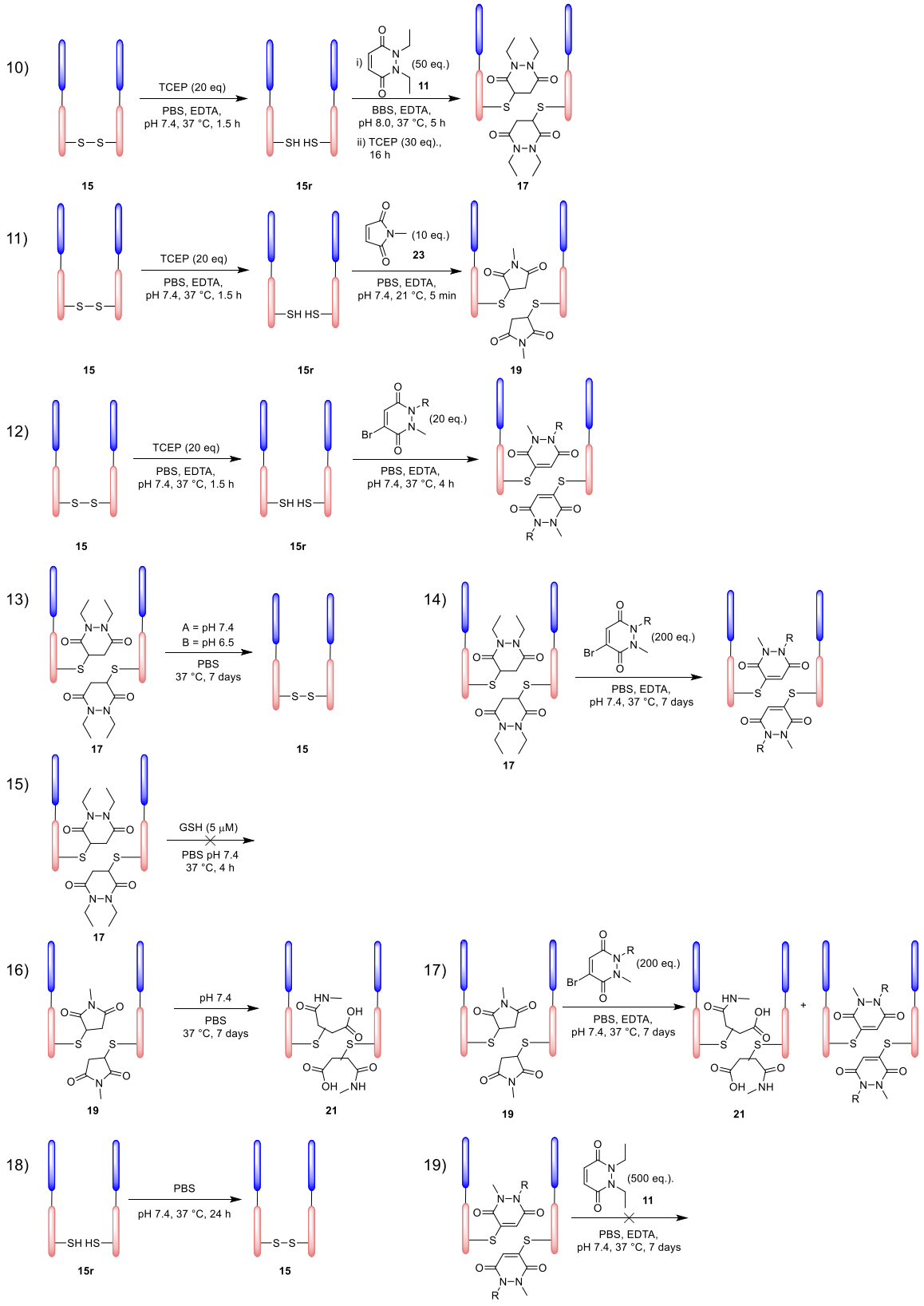
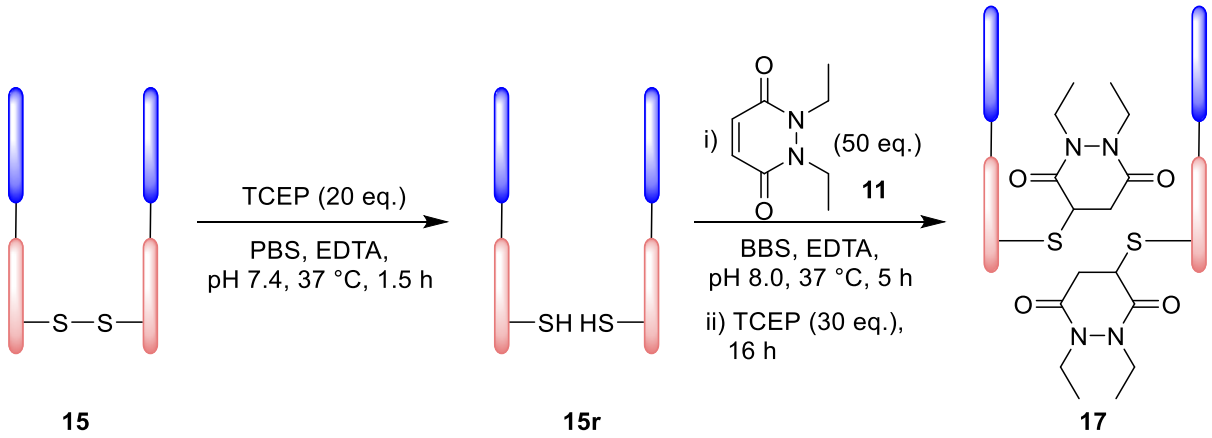


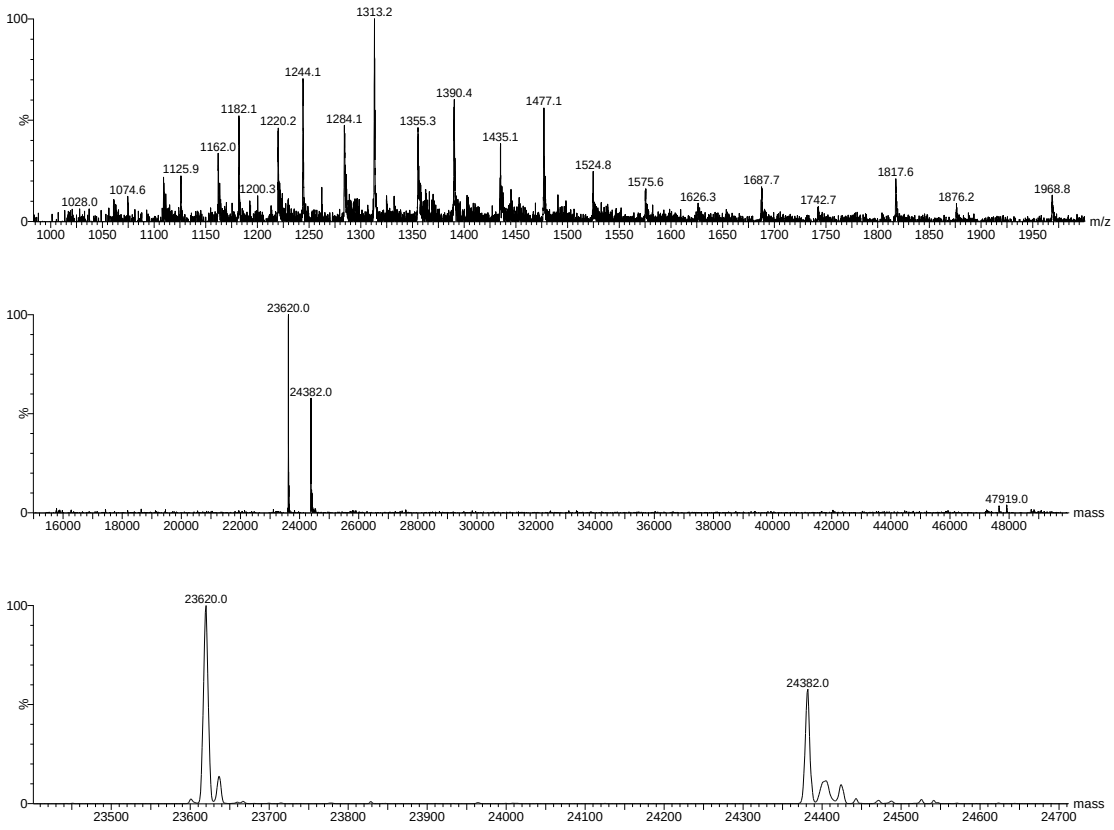
Figure S11: Outline of Trastuzumab Fab bioconjugation related experiments.

10) Conjugation of Trastuzumab Fab 15 with *N,N*-Diethyl Pyridazinedione 11

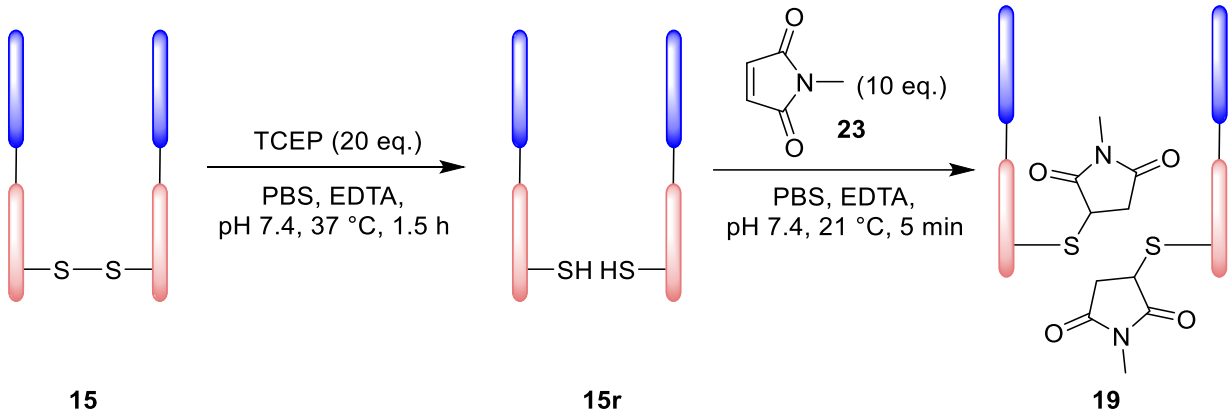


15 **15r** **17**

TCEP (10.0 µL, 20 mM in deionised water, 20 eq.) was added to a solution of Trastuzumab Fab **15** (500 µL, 20 µM) in PBS (pH 7.4) and the solution was incubated at 37 °C for 90 min. Excess TCEP was removed by ultrafiltration (6 × 10000 MWCO, VivaSpin®, GE Healthcare) into PBS (pH 8.0, 5 mM EDTA). PD **11** (10.0 µL, 50 mM in DMSO, 50 eq.) was added to the solution of reduced Trastuzumab Fab **15r** and the solution was incubated at 37 °C for 5 h. After this time, additional TCEP (15.0 µL, 20 mM in deionised water, 30 eq.) was added and the solution was incubated at 37 °C for a further 16 h. Excess reagents were removed by ultrafiltration (6 × 10000 MWCO, VivaSpin®, GE Healthcare) into deionised water prior to LCMS analysis. Expected masses: 23621 Da (Fab(LC)-S-PD **17**), 24382 Da (Fab(HC)-S-PD **17**). Observed masses (LCMS Method 1): 23620 Da (Fab(LC)-S-PD **17**), 24382 Da (Fab(HC)-S-PD **17**).

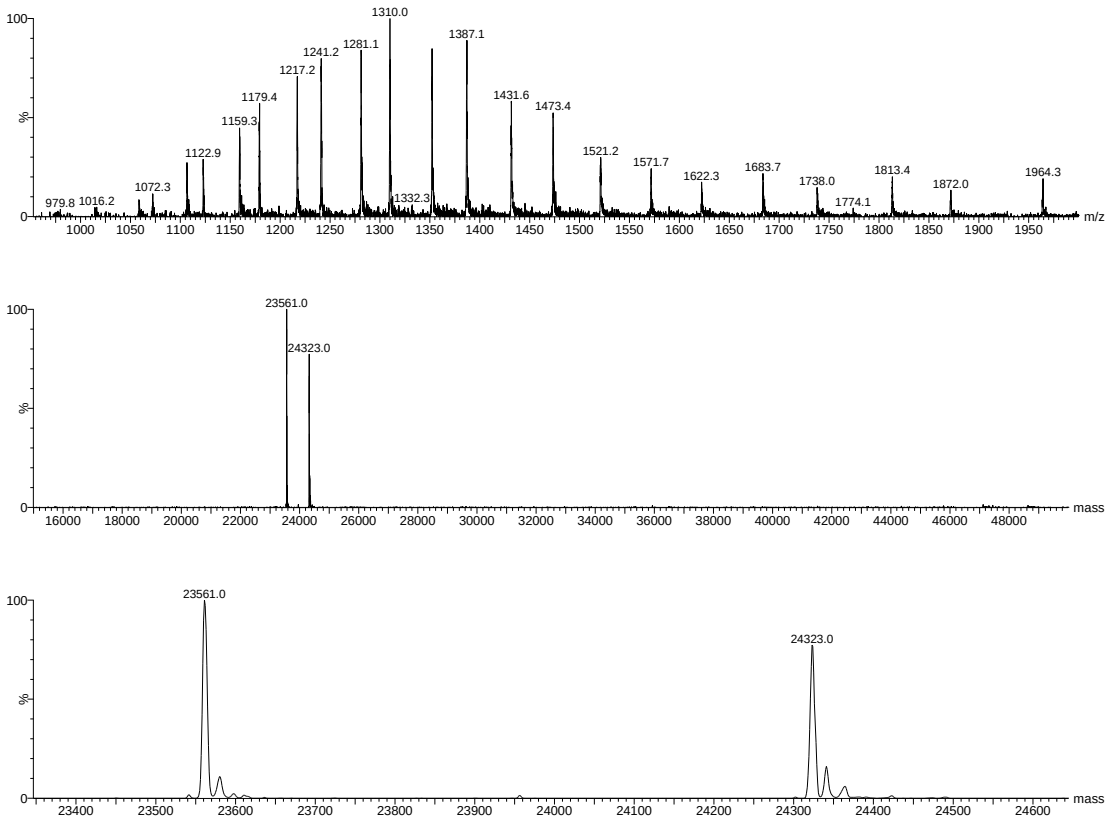


11) Conjugation of Trastuzumab Fab 15 with *N*-methyl maleimide 23

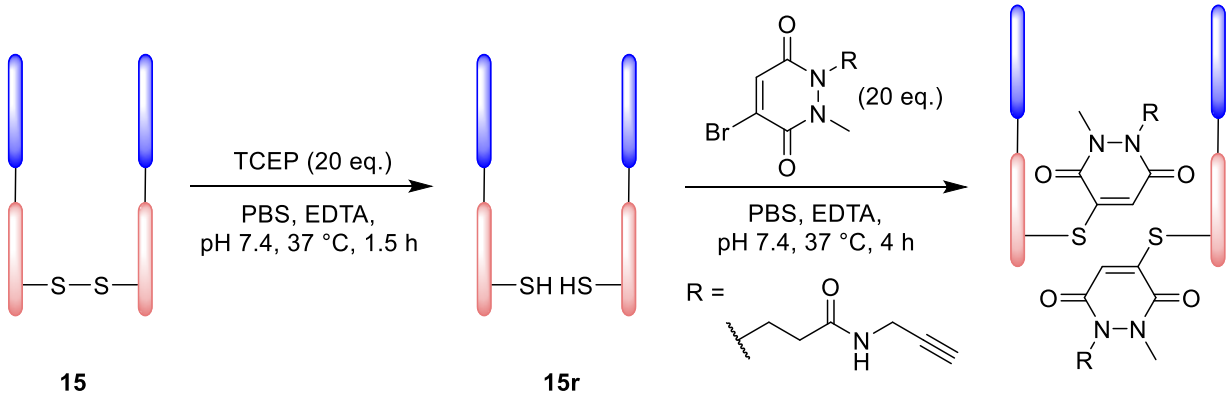


15 **15r** **19**

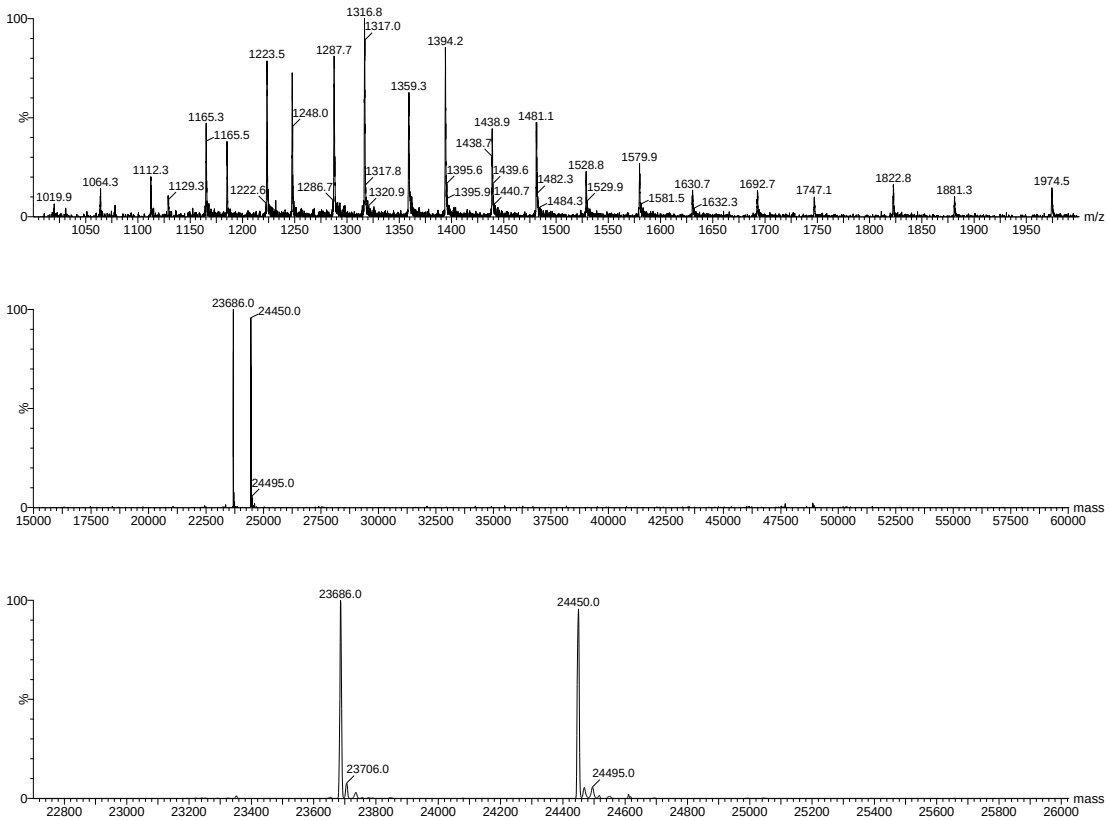
TCEP (10.0 µL, 20 mM in deionised water, 20 eq.) was added to a solution of Trastuzumab Fab **15** (500 µL, 20 µM) in PBS (pH 7.4) and the solution was incubated at 37 °C for 1.5 h. Excess TCEP was removed by ultrafiltration (6 × 10000 MWCO, VivaSpin®, GE Healthcare) into PBS (pH 7.4, 5 mM EDTA). *N*-methyl maleimide **23** (5.0 µL, 20 mM in DMSO, 10 eq.) was added to the solution of reduced Trastuzumab Fab **15r** and the solution was incubated at 21 °C for 5 min. Excess reagents were removed by ultrafiltration (6 × 10000 MWCO, VivaSpin®, GE Healthcare) into deionised water prior to LCMS analysis. Expected masses: 23564 Da (Fab(LC)-S-Mal **19**), 24325 Da (Fab(HC)-S-Mal **19**). Observed masses (LCMS Method 1): 23561 Da (Fab(LC)-S-Mal **19**), 24323 Da (Fab(HC)-S-Mal **19**).



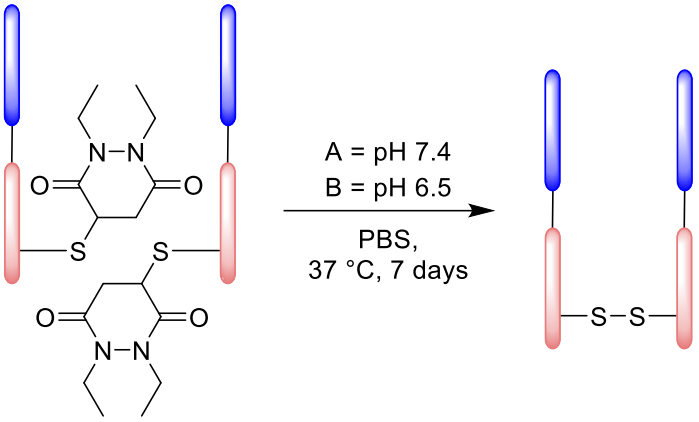
12) Conjugation of Trastuzumab Fab with monoBr *N,N*-methyl propargyl pyridazinedione



TCEP (5.0 µL, 20 mM in deionised water, 20 eq.) was added to a solution of Trastuzumab Fab **15** (250 µL, 20 µM) in PBS (pH 7.4) and the solution was incubated at 37 °C for 1.5 h. Excess TCEP was removed by ultrafiltration (6 × 10000 MWCO, VivaSpin®, GE Healthcare) into PBS (pH 7.4, 5 mM EDTA). MonoBr *N,N*-methyl propargyl pyridazinedione (5.0 µL, 20 mM in DMSO, 20 eq.) was added to the solution of reduced Trastuzumab Fab **15r** and the solution was incubated at 37 °C for 4 h. Excess reagents were removed by ultrafiltration (6 × 10000 MWCO, VivaSpin®, GE Healthcare) into deionised water prior to LCMS analysis. Expected masses: 23686 Da (Fab(LC)-S-PD_{unsat}), 24447 Da (Fab(HC)-S-PD_{unsat}). Observed masses (LCMS Method 1): 23686 Da (Fab(LC)-S-PD_{unsat}), 24450 Da (Fab(HC)-S-PD_{unsat}).



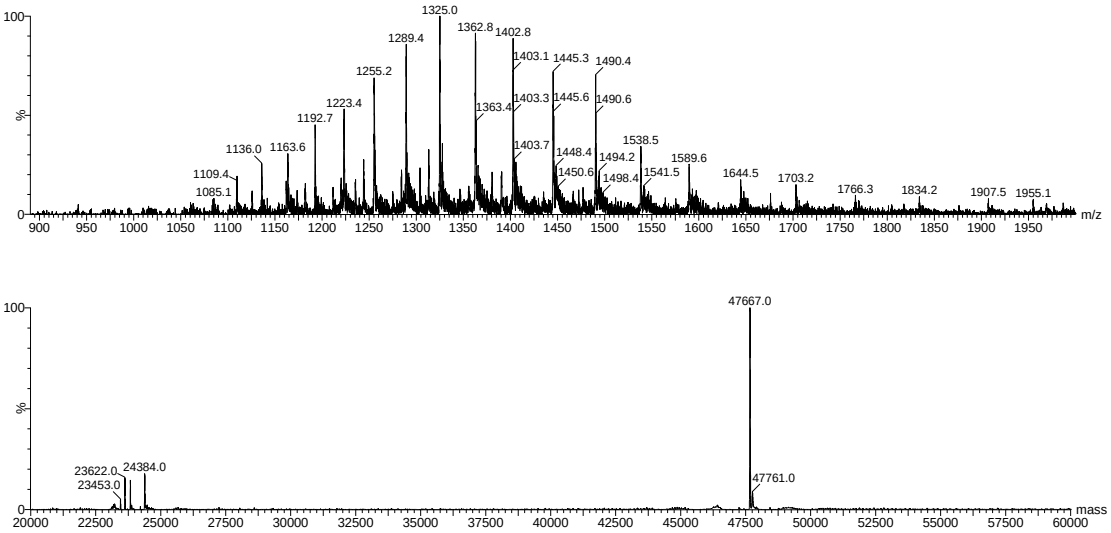
13) Release of *N,N*-Diethyl PD 11 from Trastuzumab Fab-PD conjugate 17



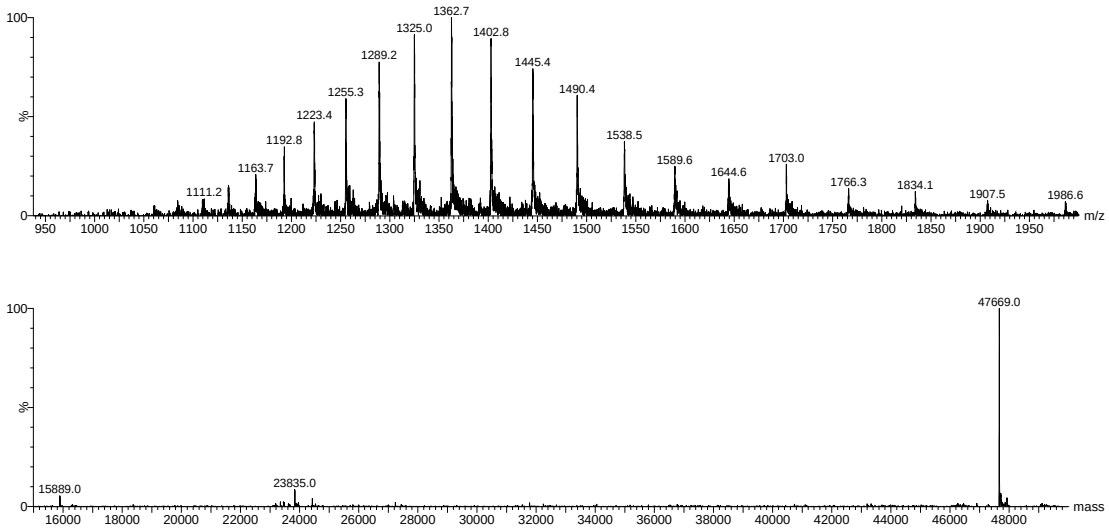
17 **15**

Trastuzumab conjugate **17** (200 µL, 20 µM) was buffer exchanged (7000 MWCO, ZebaSpin®, Thermo Scientific) into PBS (pH 7.4 and pH 6.5). The Trastuzumab conjugates were then incubated at 37 °C for a total of 7 days. Samples were desalted (7000 MWCO, ZebaSpin®, Thermo Scientific) before LCMS analysis. Expected masses: 23452 Da (Fab(LC)-SH **15r**), 23621 Da (Fab(LC)-S-PD **17**), 24213 Da (Fab(HC)-SH **15r**), 24384 Da (Fab(HC)-S-PD **17**), 47663 Da (Fab **15**). Observed masses (LCMS Method 1): 23453 Da (Fab(LC)-SH **15r**), 23622 Da (Fab(LC)-S-PD **17**), 24217 Da (Fab(HC)-SH **15r**), 24385 Da (Fab(HC)-S-PD **17**), 47669 Da (Fab **15**).

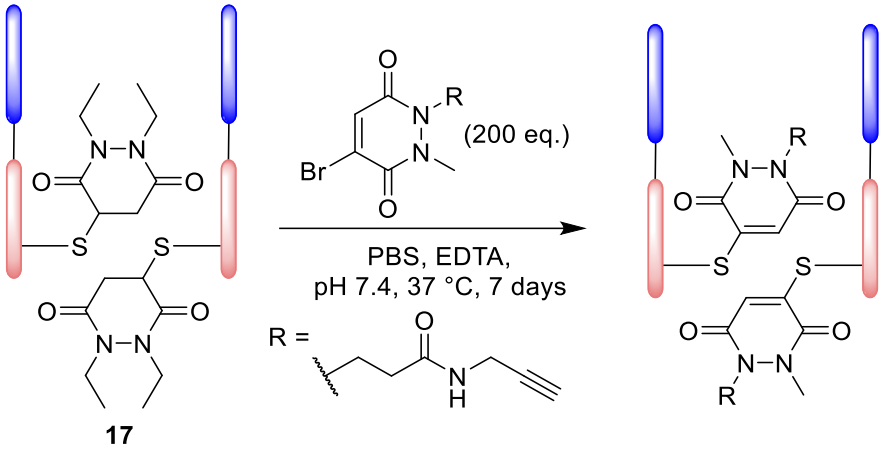
pH 6.5



pH 7.4

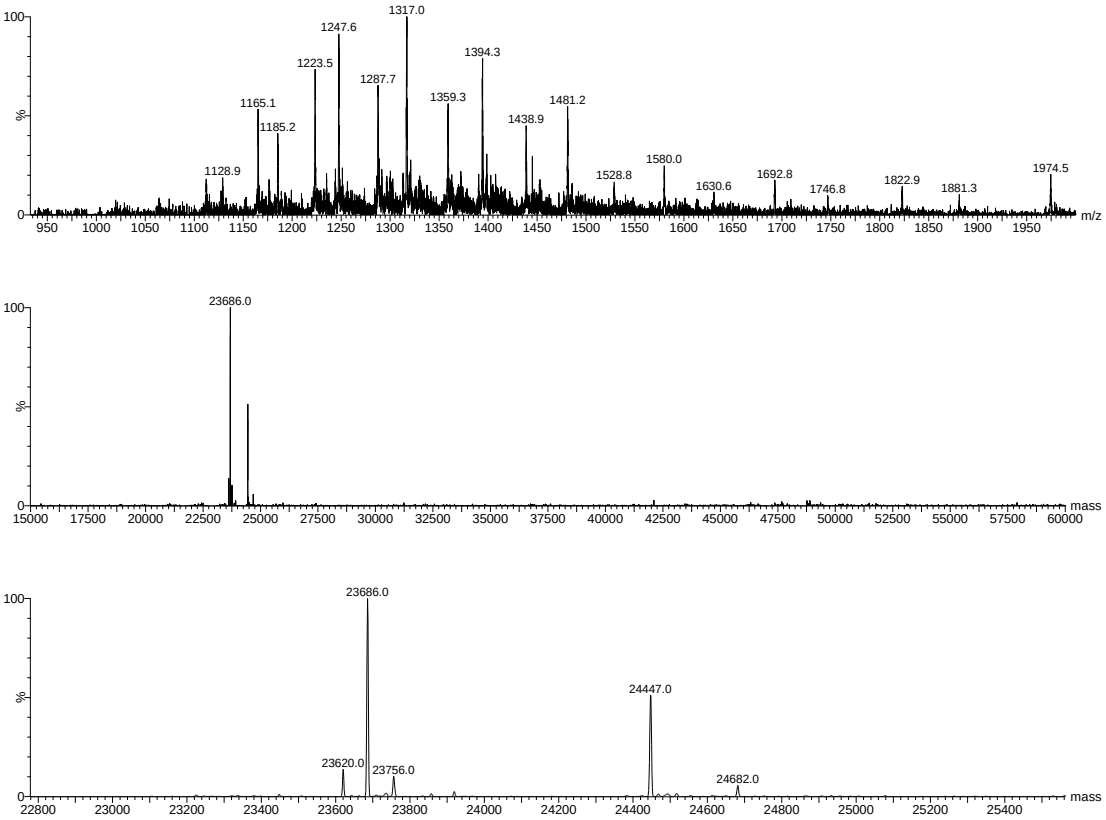

100
%
0
1362.7
1325.0
1402.8
1289.2
1445.4
1255.3
1490.4
1223.4
1538.5
1192.8
1589.6
1163.7
1644.6
1703.0
1111.2
1766.3
1834.1
1907.5
1986.6
950 1000 1050 1100 1150 1200 1250 1300 1350 1400 1450 1500 1550 1600 1650 1700 1750 1800 1850 1900 1950
m/z
100
%
0
47669.0
15889.0
23835.0
16000 18000 20000 22000 24000 26000 28000 30000 32000 34000 36000 38000 40000 42000 44000 46000 48000
mass

14) Reaction of Trastuzumab-PD conjugate 17 with monoBr *N,N*-methyl propargyl pyridazinedione

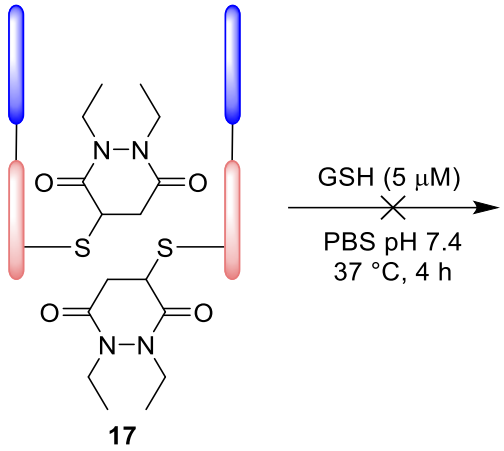


17

MonoBr *N,N*-methyl propargyl pyridazinedione (2.0 µL, 200 mM in DMSO, 200 eq.) was added to the solution of Trastuzumab conjugate **17** (100 µL, 20 µM in PBS pH 7.4, 5 mM EDTA) and the solution was incubated at 37 °C for 7 days. Excess reagents were removed using desalting columns (7000 MWCO, ZebaSpin®, Thermo Scientific) prior to LCMS analysis. Expected masses: 23686 Da (Fab(LC)-S-PD_{unsat}), 24447 Da (Fab(HC)-S-PD_{unsat}). Observed masses (LCMS Method 1): 23620 Da (Fab(LC)-S-PD **17**), 23686 Da (Fab(LC)-S-PD_{unsat}), 24447 Da (Fab(HC)-S-PD_{unsat}).

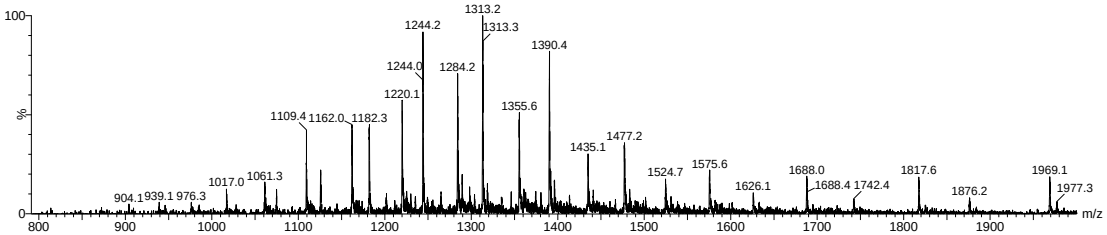


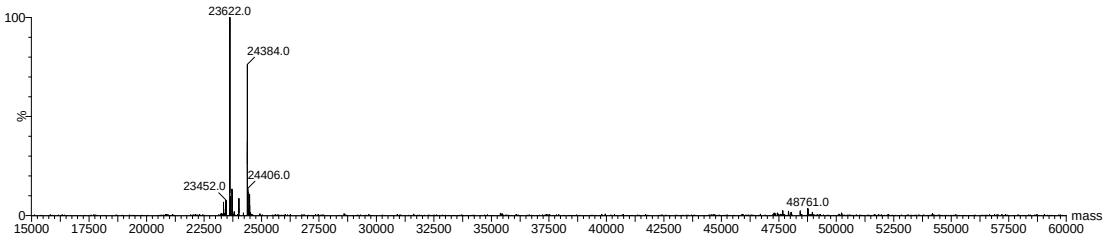
15) Stability of Trastuzumab-PD conjugate 17 to glutathione

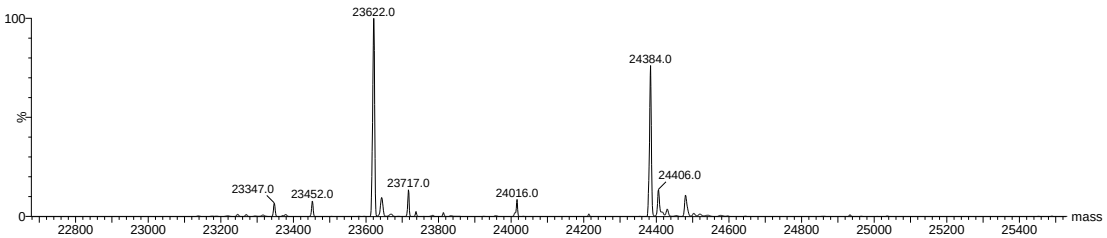


17

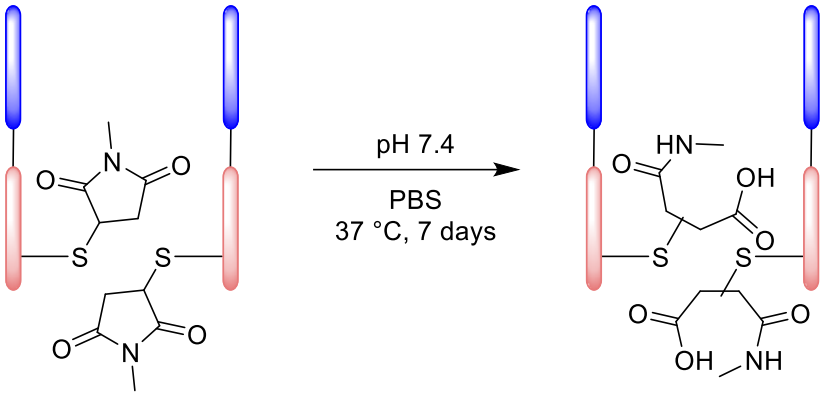
Glutathione (1.0 µL, 500 µM in PBS adjusted to pH 7.4) was added to the solution of Trastuzumab conjugate **17** (99 µL, 20 µM in PBS = pH 7.4) and the solution was incubated at 37 °C for 4 h. Excess reagents were removed using desalting columns (7000 MWCO, ZebaSpin®, Thermo Scientific) prior to LCMS analysis. Expected masses: 23620 Da (Fab(LC)-S-PD **17**), 24385 Da (Fab(HC)-S-PD **17**). Observed masses (LCMS Method 1): 23622 Da (Fab(LC)-S-PD **17**), 24384 Da (Fab(HC)-S-PD **17**).



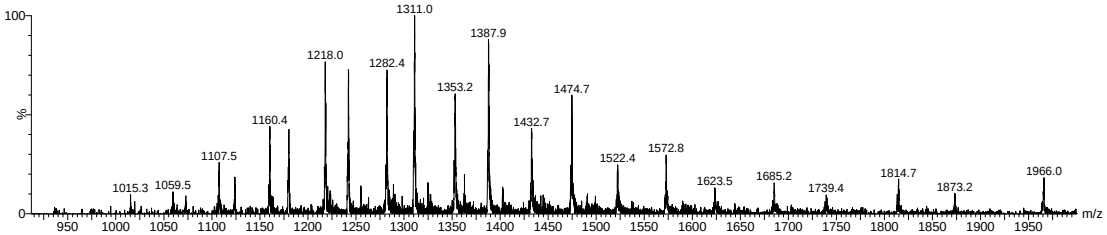


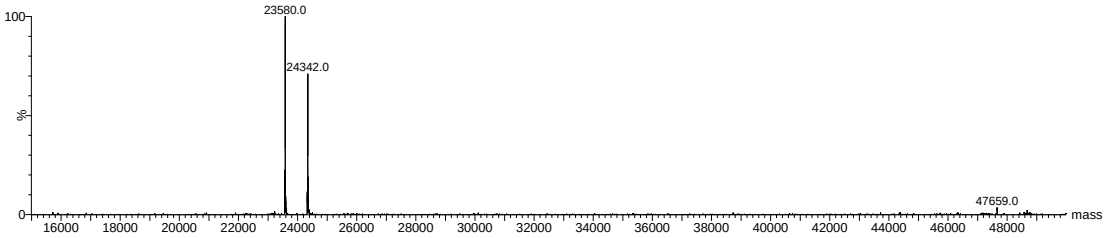


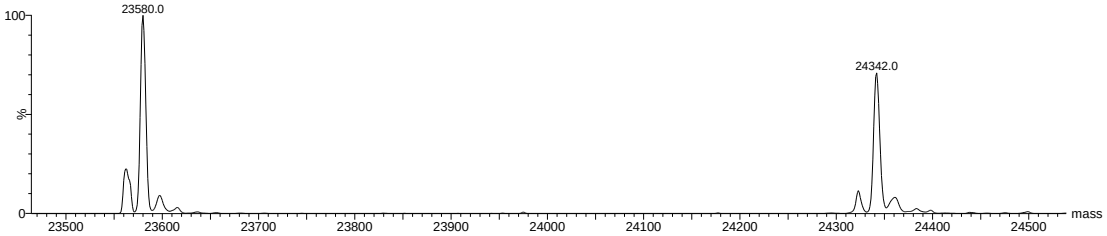
16) Incubation of Trastuzumab Fab-Maleimide conjugate 19



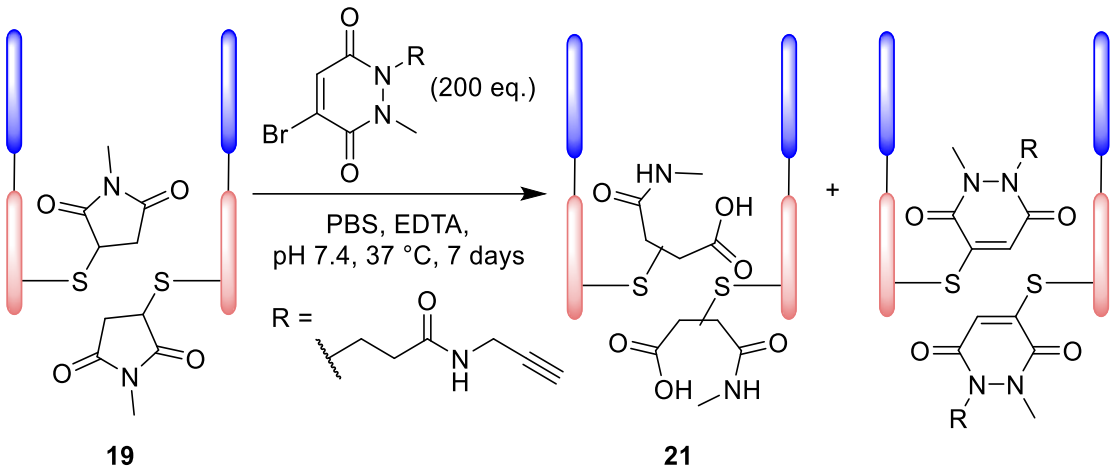
Trastuzumab conjugate **19** (200 µL, 20 µM) was buffer exchanged (7000 MWCO, ZebaSpin®, Thermo Scientific) into PBS (pH 7.4). The Trastuzumab conjugate was then incubated at 37 °C for a total of 7 days. Samples were desalted (7000 MWCO, ZebaSpin®, Thermo Scientific) before LCMS analysis. Expected masses: 23582 Da (Fab(LC)-S-Mal$_{hyd}$ **21**), 24343 Da (Fab(HC)-S-Mal$_{hyd}$ **21**). Observed masses (LCMS Method 1): 23580 Da (Fab(LC)-S-Mal$_{hyd}$ **21**), 24342 Da (Fab(HC)-S-Mal$_{hyd}$ **21**).



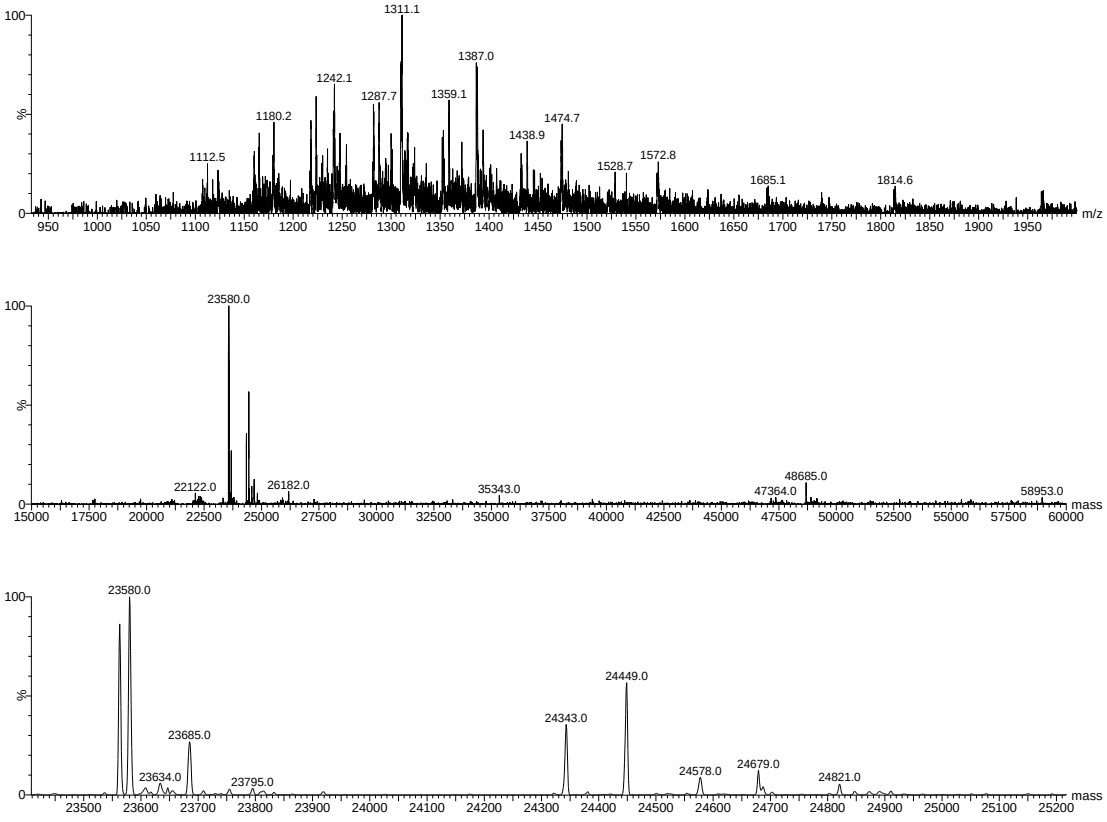




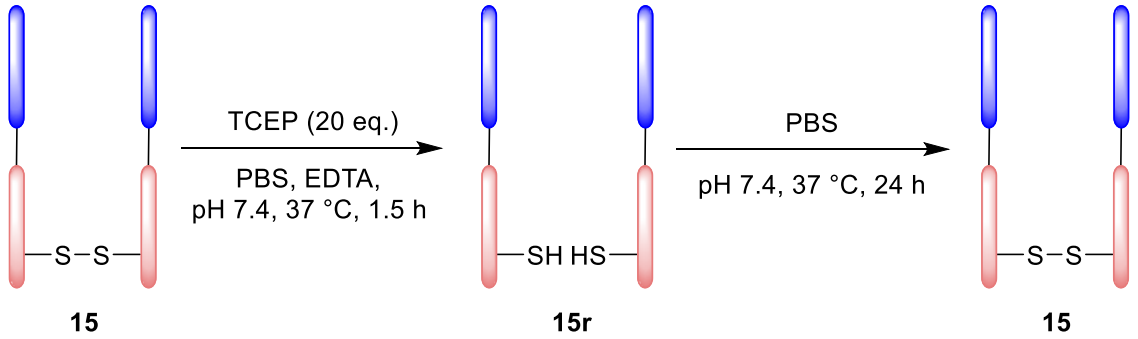
17) Reaction of Trastuzumab-maleimide conjugate 19 with monoBr *N,N*-methyl propargyl pyridazinedione



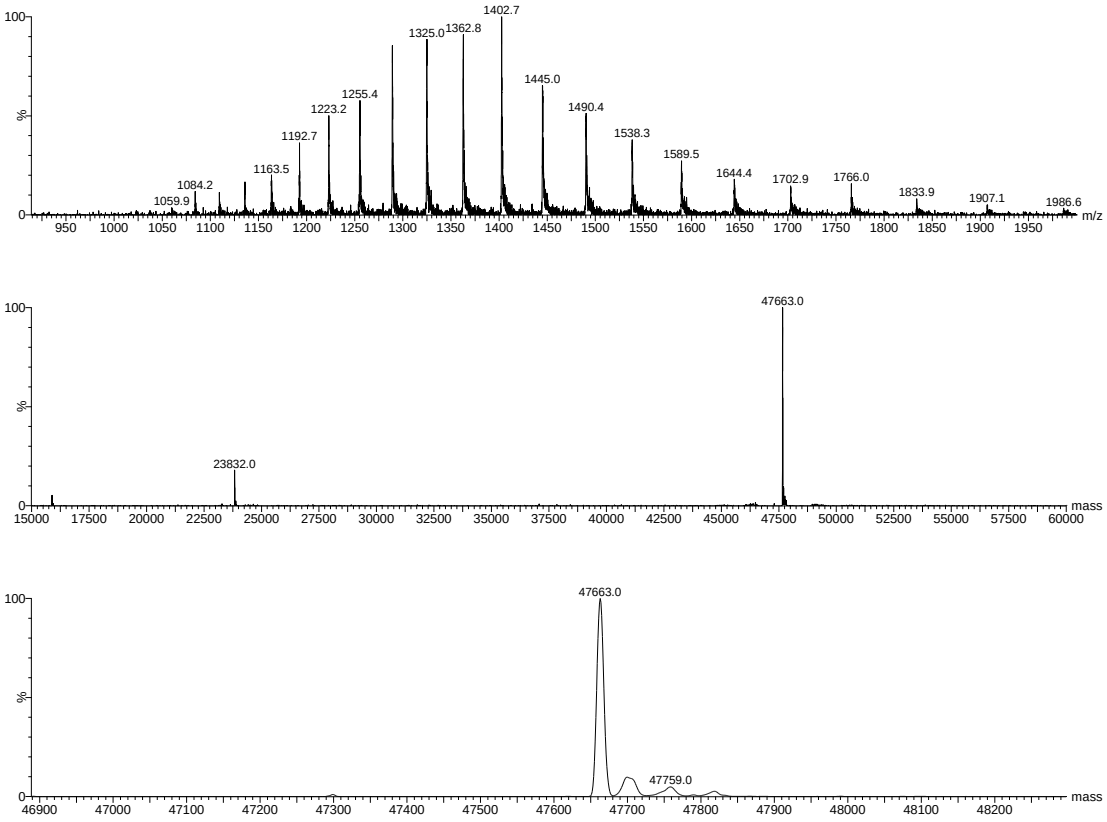
MonoBr *N,N*-methyl propargyl pyridazinedione (2.0 µL, 200 mM in DMSO, 200 eq.) was added to the solution of Trastuzumab conjugate **19** (100 µL, 20 µM in PBS pH = 7.4, 5 mM EDTA) and the solution was incubated at 37 °C for 7 days. Excess reagents were removed using desalting columns (7000 MWCO, ZebaSpin®, Thermo Scientific) prior to LCMS analysis. Expected masses: 23582 Da (Fab(LC)-S-Mal$_{hyd}$ **21**), 23686 Da (Fab(LC)-S-PD$_{unsat}$), 24343 Da (Fab(HC)-S-Mal$_{hyd}$ **21**), 24447 Da (Fab(HC)-S-PD$_{unsat}$). Observed masses (LCMS Method 1): 23562 Da (Fab(LC)-S-Mal **19**), 23580 Da (Fab(LC)-S-Mal$_{hyd}$ **21**), 23685 Da (Fab(LC)-S-PD$_{unsat}$), 24343 Da (Fab(HC)-S-Mal$_{hyd}$ **17**), 24449 Da (Fab(HC)-S-PD$_{unsat}$).



18) Control: Reoxidation of reduced Trastuzumab Fab 15

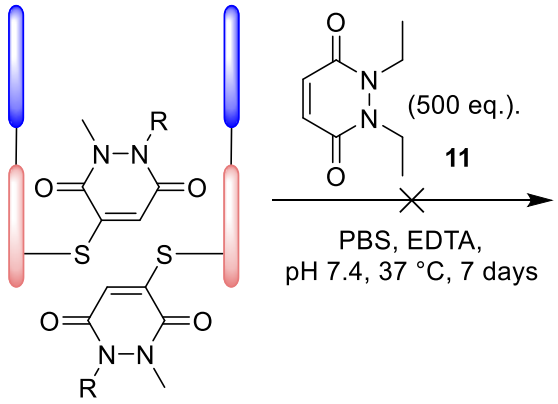


TCEP (2.0 µL, 20 mM in deionised water, 20 eq.) was added to a solution of Trastuzumab Fab **15** (100 µL, 20 µM) in PBS = pH 7.4, 5 mM EDTA) and the solution was incubated at 37 °C for 1.5 h. Excess TCEP was removed by ultrafiltration (6 × 10000 MWCO, VivaSpin®,GE Healthcare) into PBS (pH = 7.4). Reduced Trastuzumab Fab **15r** was then incubated at 37 °C for 24 h. Samples were desalted (7000 MWCO, ZebaSpin®, Thermo Scientific) before LCMS analysis. Expected masses: 47663 Da (Fab **15**). Observed masses (LCMS Method 1): 23832 Da* (Fab/2 **15**), 47663 Da (Fab **15**).

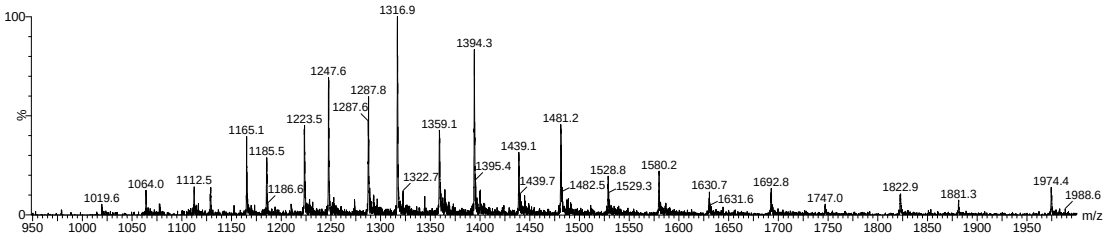


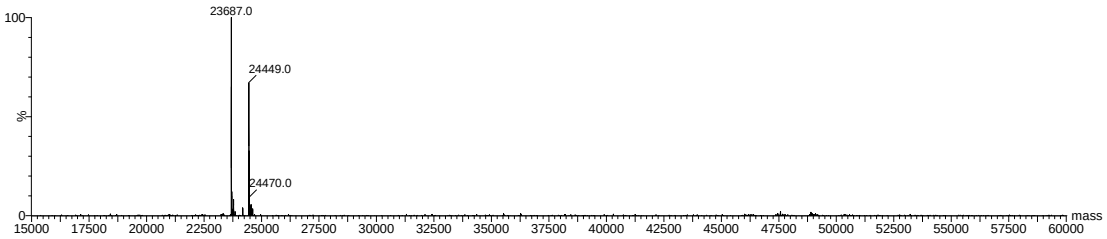
* indicates deconvolution artefact

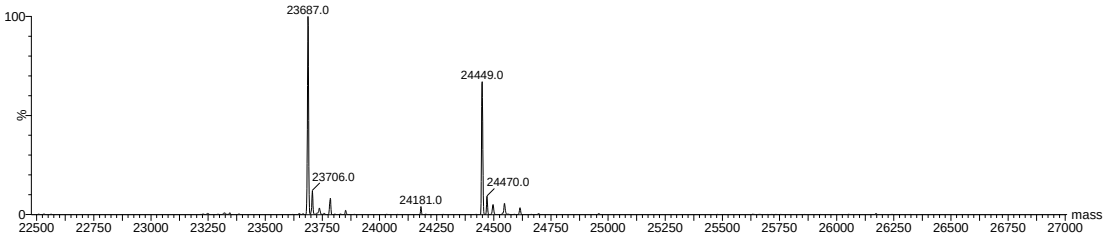
19) Control: Reaction of Trastuzumab-PD conjugate with *N,N*-diethyl pyridazinedione 11



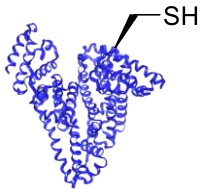
N,N-Diethyl pyridazinedione (5.0 µL, 200 mM in DMSO, 500 eq.) was added to the solution of Trastuzumab conjugate (100 µL, 20 µM in PBS = pH 7.4, 5 mM EDTA) and the solution was incubated at 37 °C for 7 days. Excess reagents were removed using desalting columns (7000 MWCO, ZebaSpin®, Thermo Scientific) prior to LCMS analysis. Expected masses: 23686 Da (Fab(LC)-S-PD_{unsat}), 24447 Da (Fab(HC)-S-PD_{unsat}). Observed masses (LCMS Method 1): 23686 Da (Fab(LC)-S-PD_{unsat}), 24447 Da (Fab(HC)-S-PD_{unsat}).



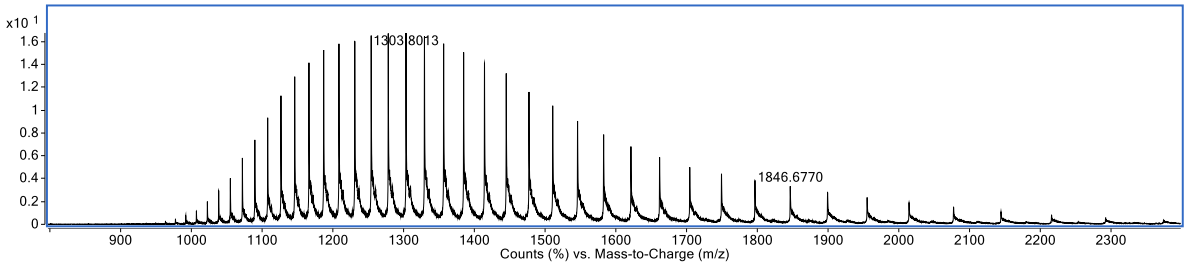


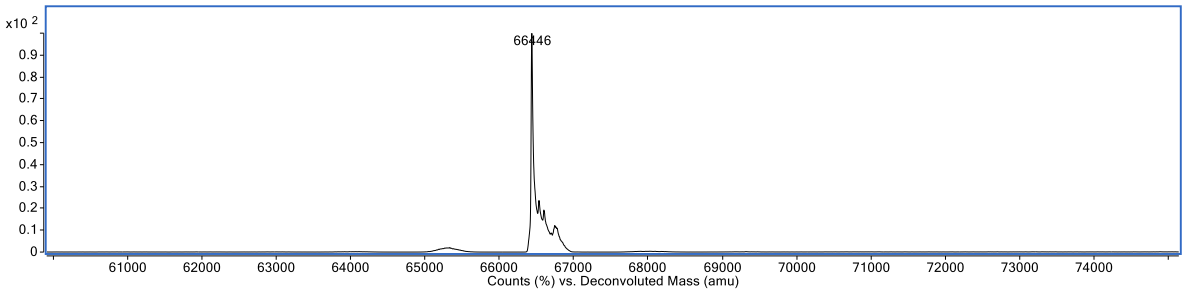


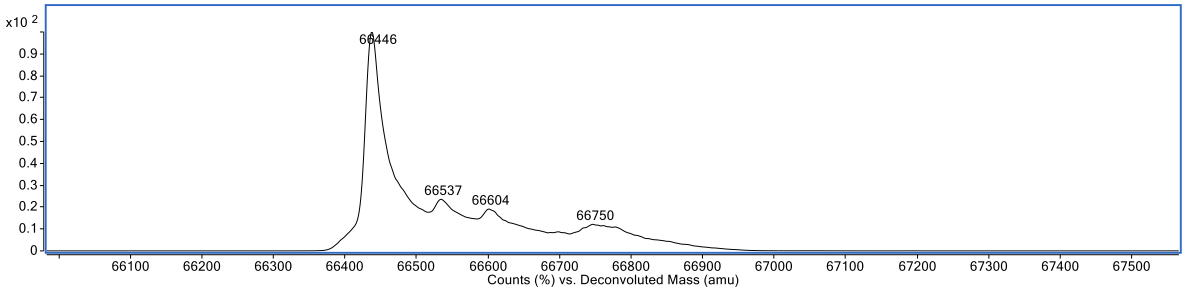
Human Serum Albumin 5



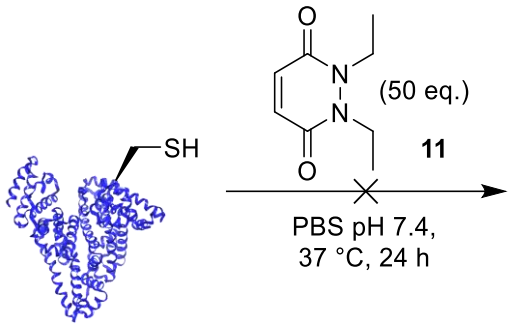
5





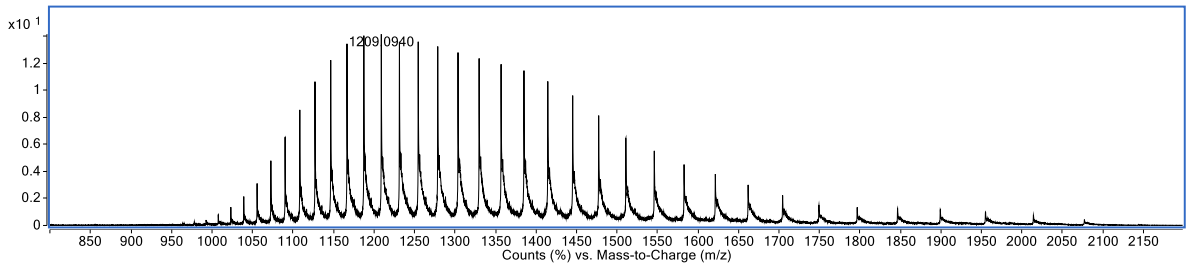


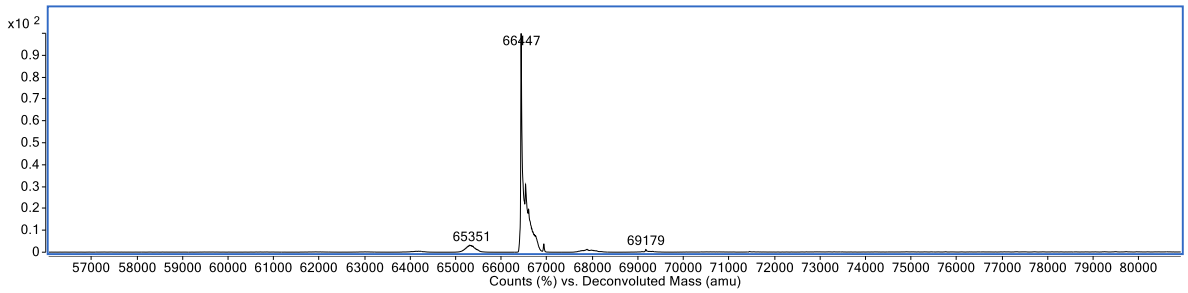
Reaction of human serum albumin 5 and *N,N*-diethyl pyridazinedione 11

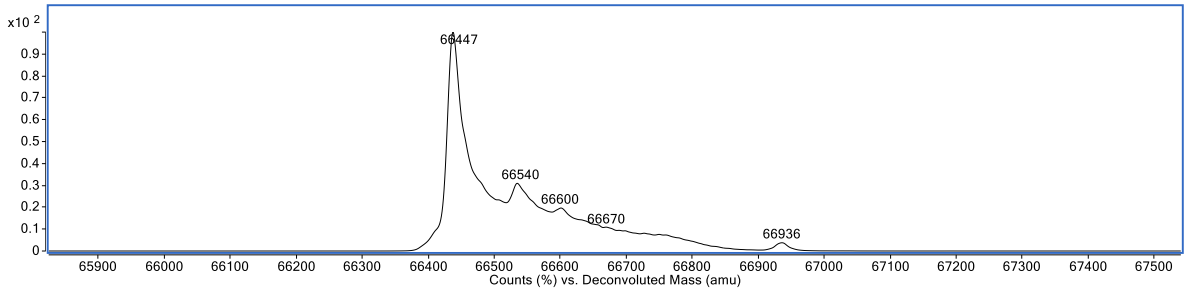


5

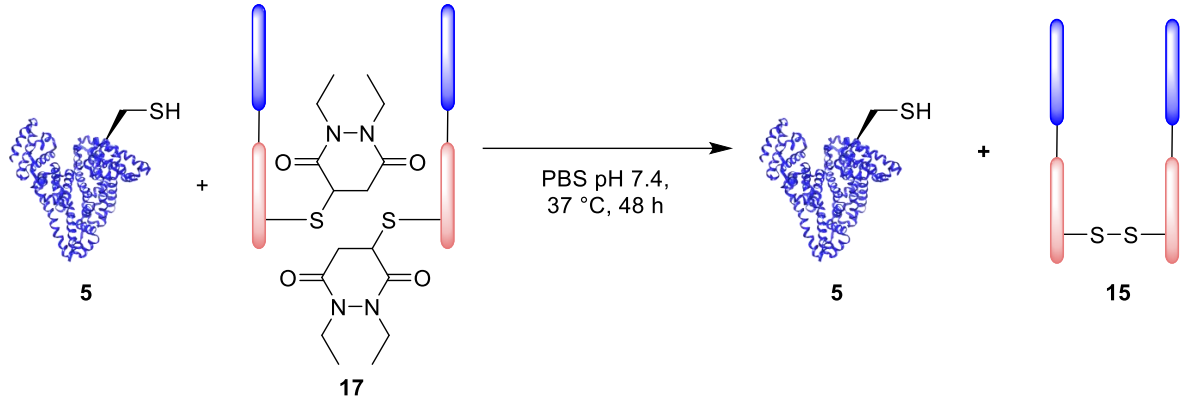
N,N-Diethyl pyridazinedione **11** (1.25 µL, 200 mM in DMSO, 50 eq.) was added to a solution of human serum albumin **5** (100 µL, 50 µM in PBS pH 7.4) and the solution was incubated at 37 °C for 24 h. Excess reagents were removed using desalting columns (7000 MWCO, ZebaSpin®, Thermo Scientific) prior to LCMS analysis. Expected mass: 66446 Da (HSA-SH **5**). Observed mass (LCMS Method 2): 66447 Da (HSA-SH **5**).



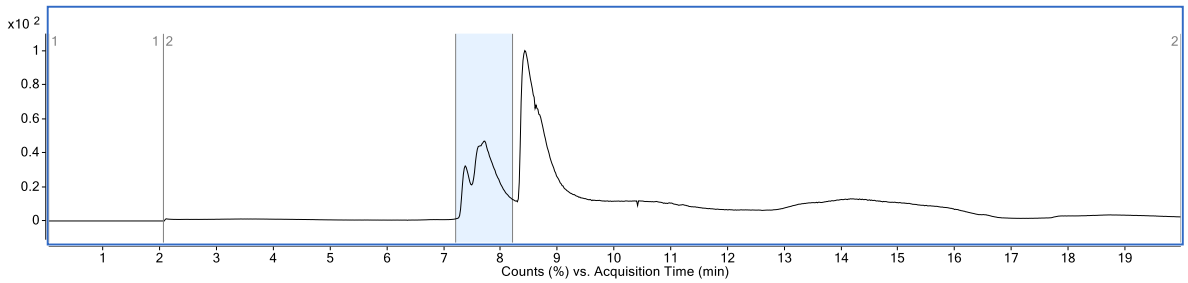


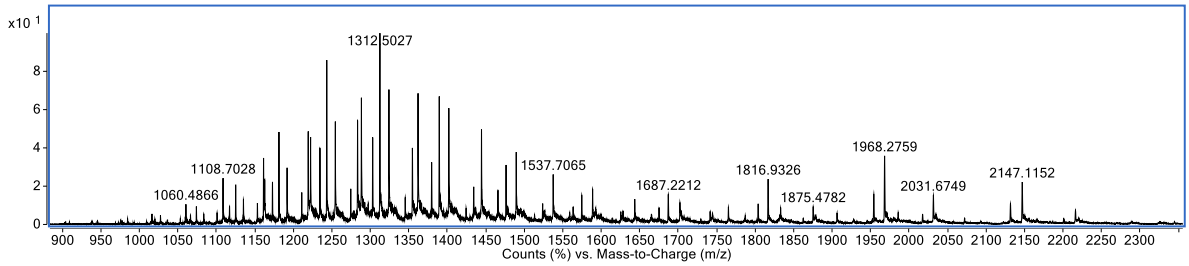


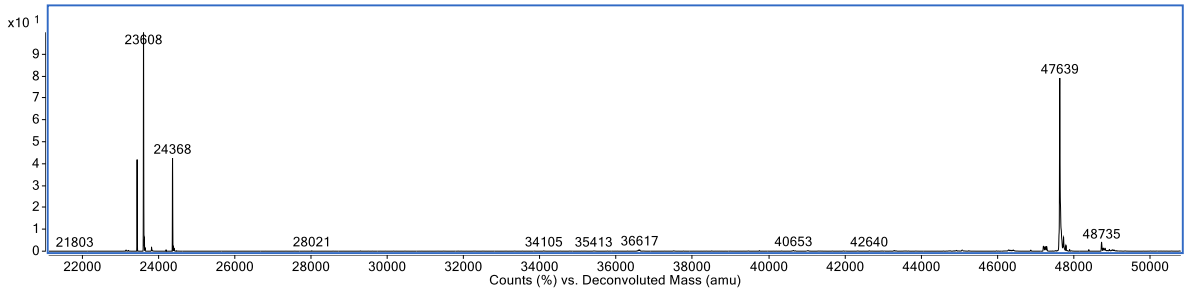
Reaction of human serum albumin 5 and Trastuzumab Fab-PD conjugate 17

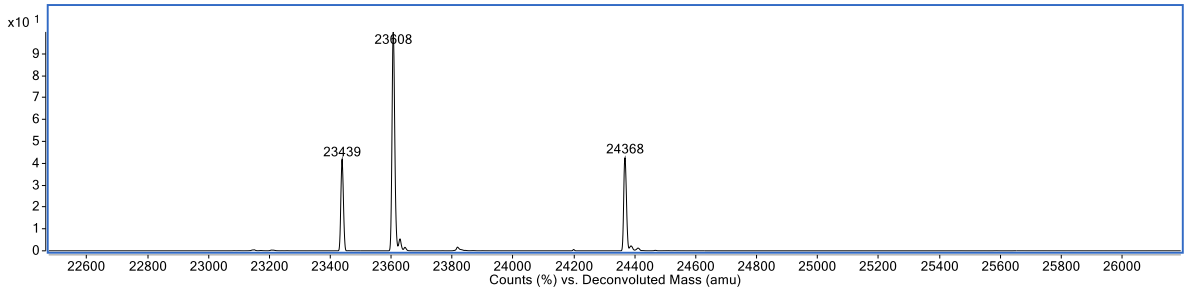


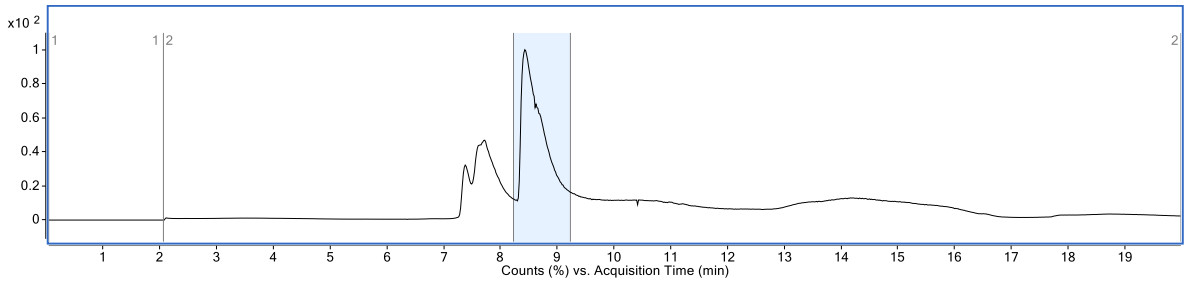
Human serum albumin **5** (25 µL, 100 µM in PBS pH 7.4) was added to a solution of Trastuzumab Fab-PD conjugate **17** (100 µL, 25 µM in PBS) to form a 1:1 solution (125 µL, 20 µM in PBS pH 7.4). The solution was incubated at 37 °C for 48 h. Samples were desalted using desalting columns (7000 MWCO, ZebaSpin®, Thermo Scientific) prior to LCMS analysis. Expected masses: 66449 Da (HSA-SH **5**). Observed mass (LCMS Method 2): 66450 Da (HSA-SH **5**).

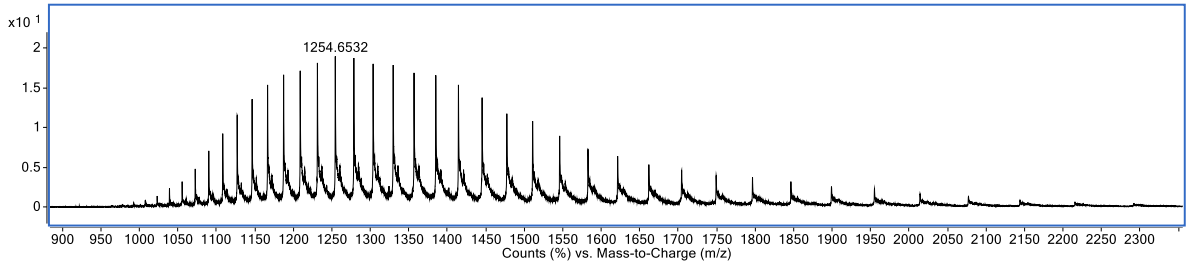


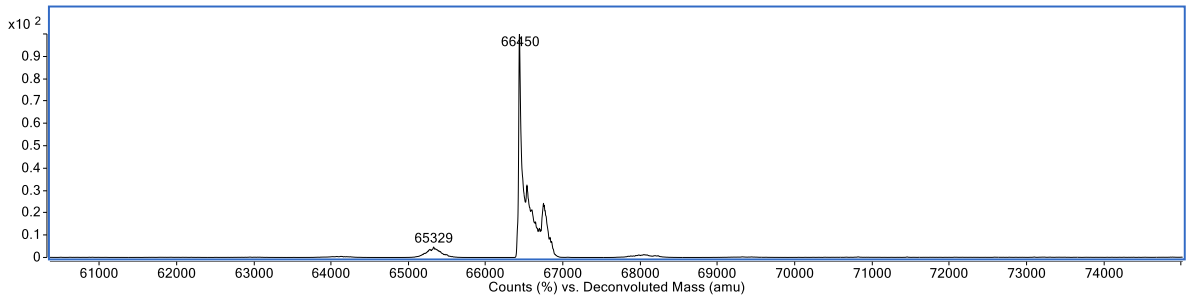


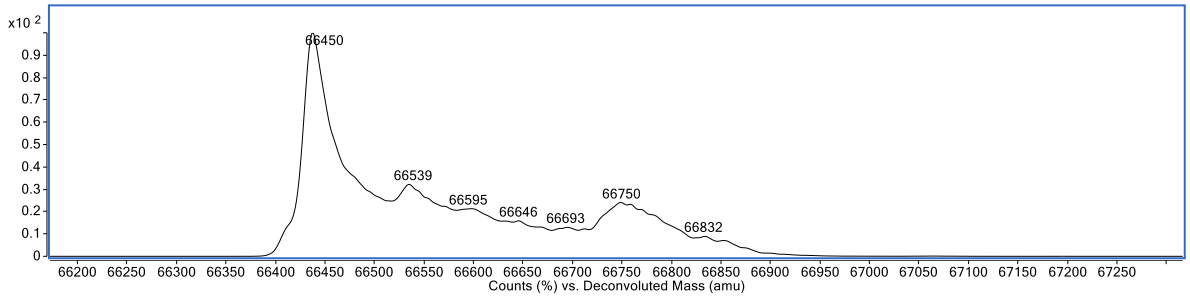



x10 1
23608
23439
24368
Counts (%) vs. Deconvoluted Mass (amu)

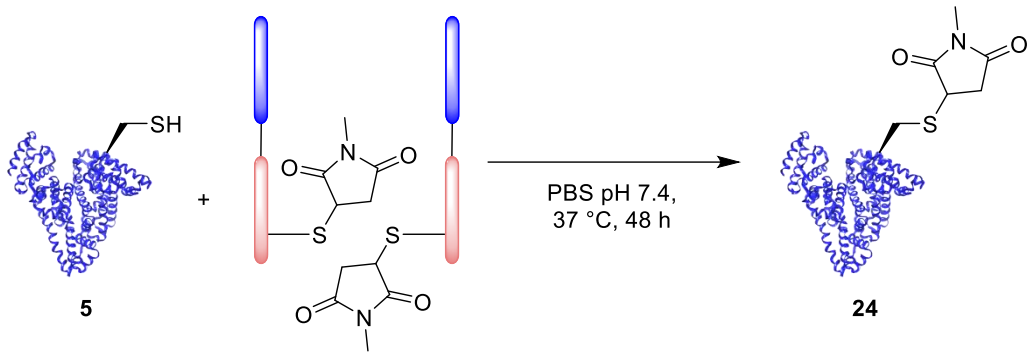

x10 2
Counts (%) vs. Acquisition Time (min)


x10 1
1254.6532
Counts (%) vs. Mass-to-Charge (m/z)


x10 2
66450
65329
Counts (%) vs. Deconvoluted Mass (amu)

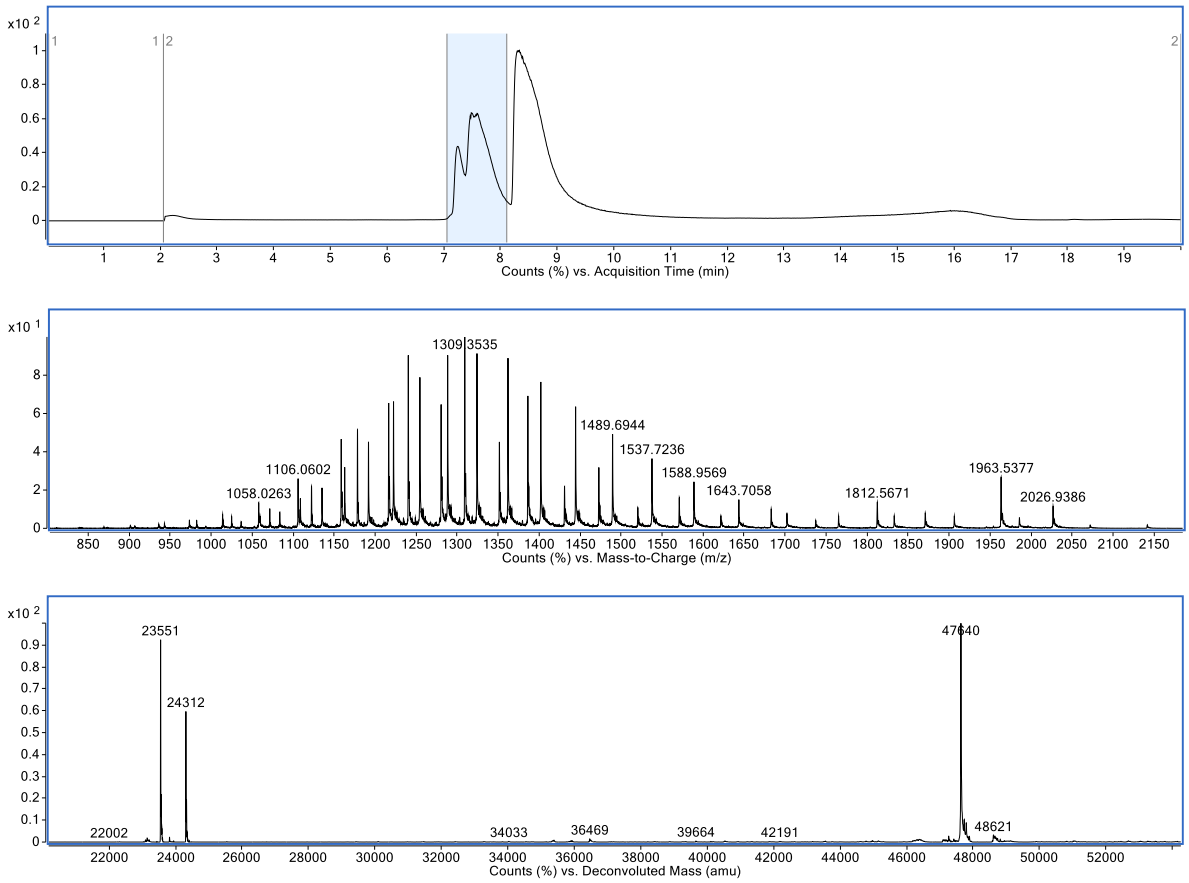

x10 2
66450
66539
66595
66646
66693
66750
66832
Counts (%) vs. Deconvoluted Mass (amu)

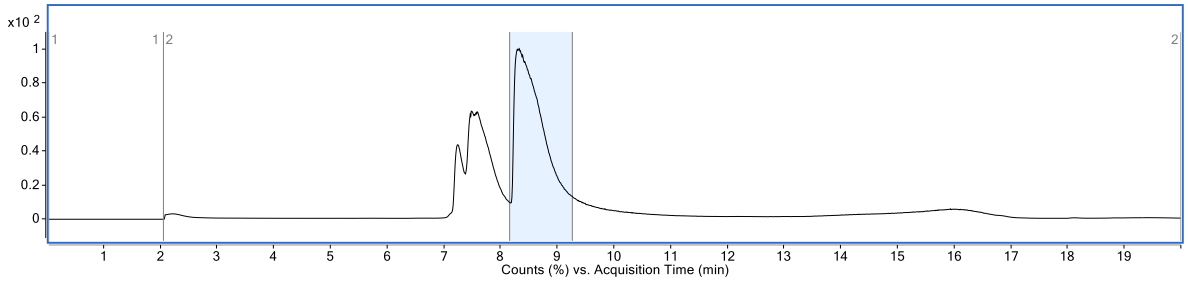
Reaction of human serum albumin 5 and Trastuzumab Fab-maleimide conjugate 19



5 **19** **24**

Human serum albumin **5** (25 µL, 100 µM in PBS pH 7.4) was added to a solution of Trastuzumab Fab-maleimide conjugate **19** (100 µL, 25 µM in PBS) to form a 1:1 solution (125 µL, 20 µM in PBS pH 7.4). The solution was incubated at 37 °C for 48 h. Samples were desalted using desalting columns (7000 MWCO, ZebaSpin®, Thermo Scientific) prior to LCMS analysis. Expected mass: 66560 Da (HSA-S-Mal **24**). Observed mass (LCMS Method 2): 66559 Da (HSA-S-Mal **24**).

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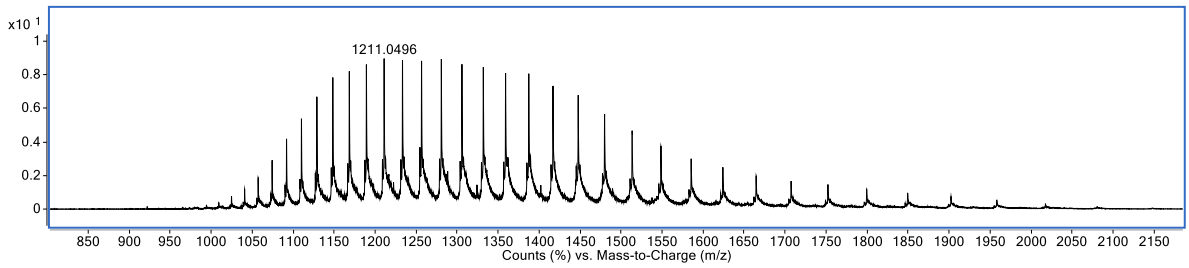
0.8
0.6
0.4
0.2
0
1 2
2
1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18 19
Counts (%) vs. Acquisition Time (min)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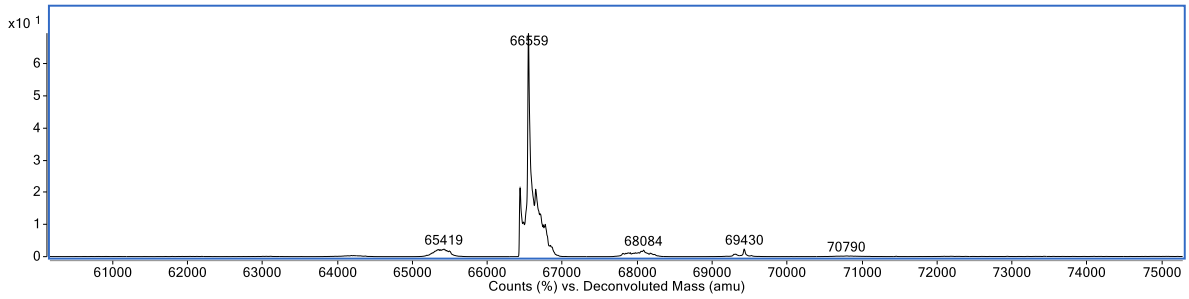
Counts (%) vs. Mass-to-Charge (m/z)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4
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2
1
0
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65419
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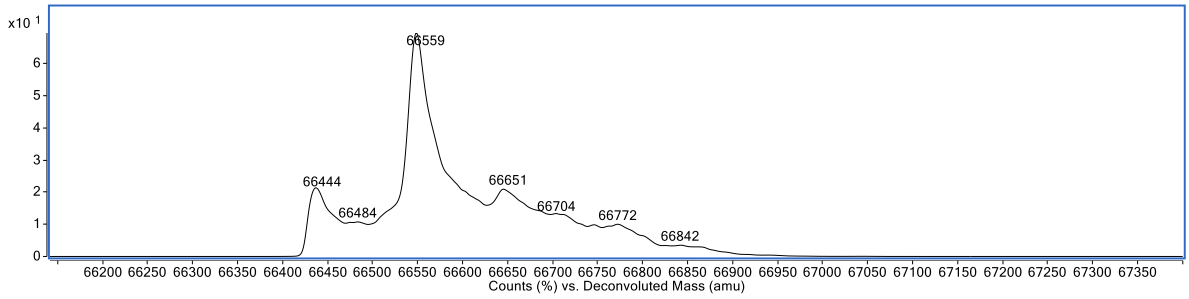
61000 62000 63000 64000 65000 66000 67000 68000 69000 70000 71000 72000 73000 74000 75000
Counts (%) vs. Deconvoluted Mass (amu)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4
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2
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0
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66444
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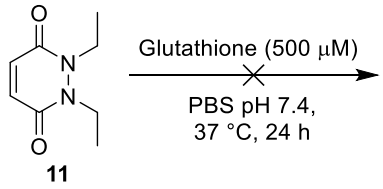
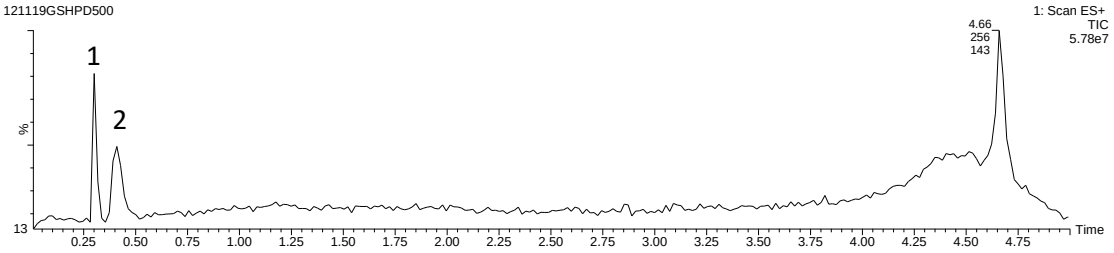
Counts (%) vs. Deconvoluted Mass (amu)

Reaction of glutathione and *N,N*-diethyl pyridazinedione 11

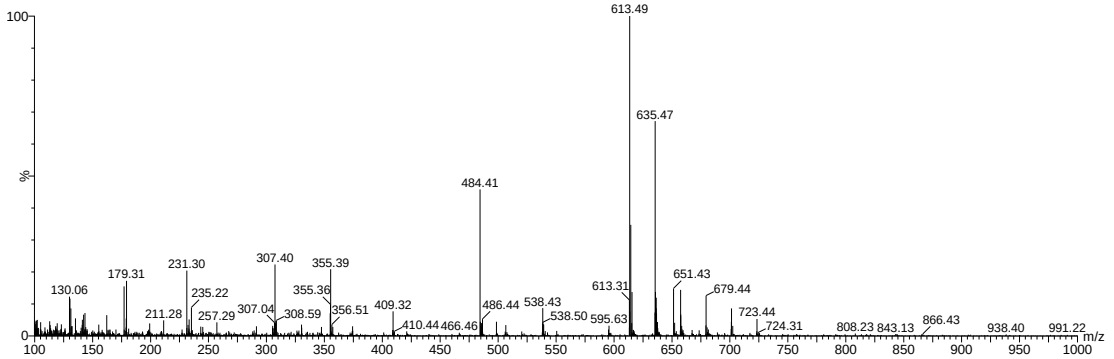


11

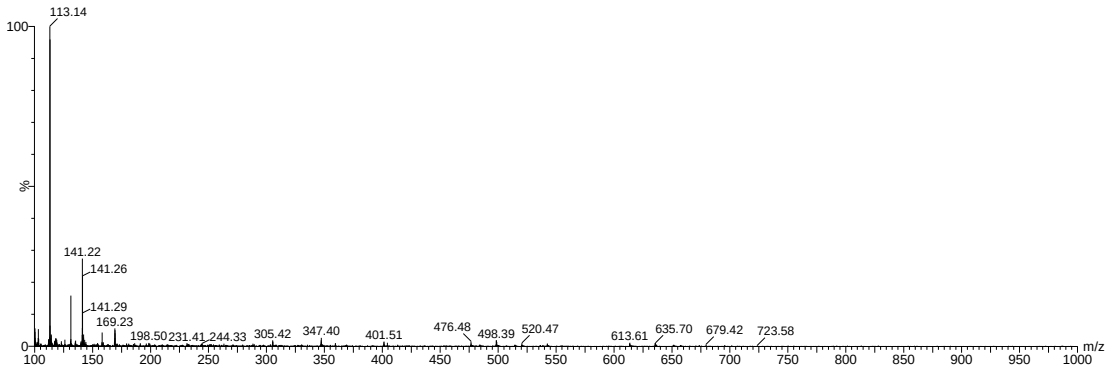
N,N-Diethyl pyridazinedione **11** (5 μL, 20 mM in MeCN, 1 eq.) was added to a solution of glutathione (200 μL, 500 μM in PBS pH 7.4) and the solution was incubated at 37 °C for 24 h. Results were obtained directly from the reaction mixture through LCMS analysis. Expected mass: 166 Da (PD **11**), 307 Da (GSH), 477 Da (GSH-PD), 612 Da (GSH-GSH). Observed mass (LCMS Method 1): 169 Da (PD **11**), 307 Da (GSH), 612 Da (GSH-GSH).



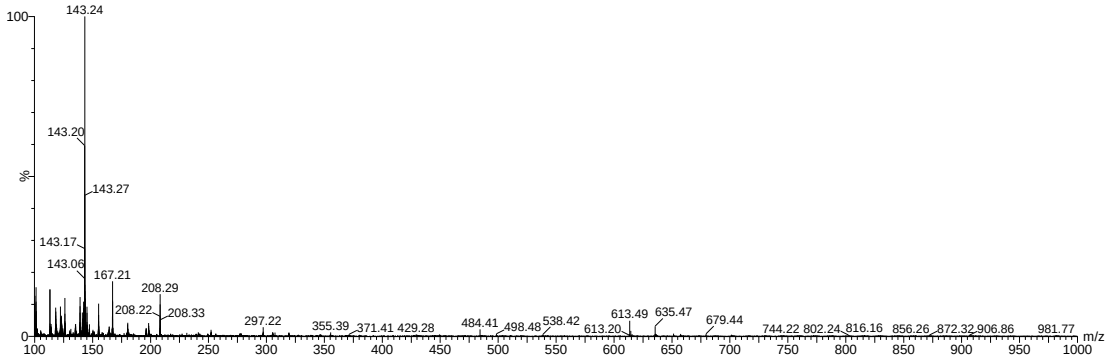
Peak 1



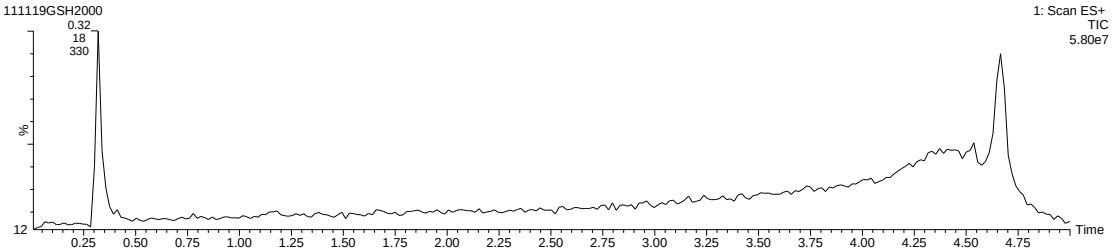
Peak 2

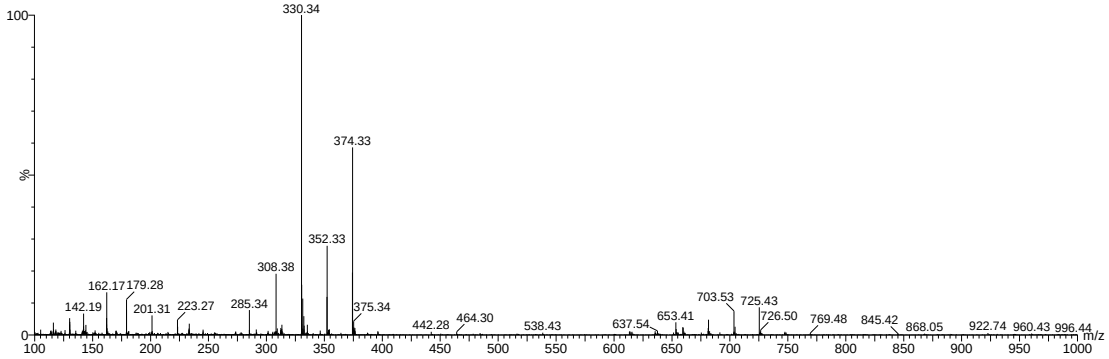


Entire Spectrum

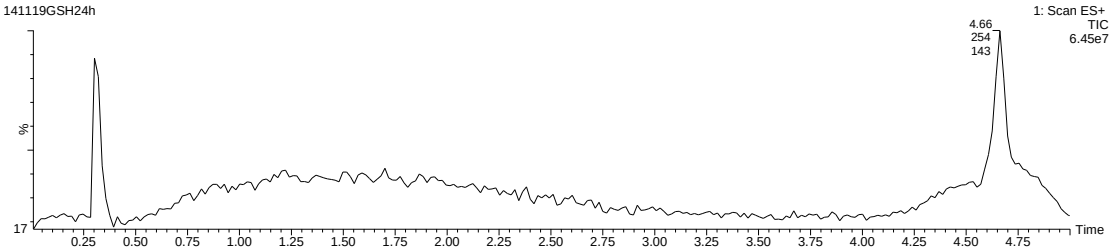


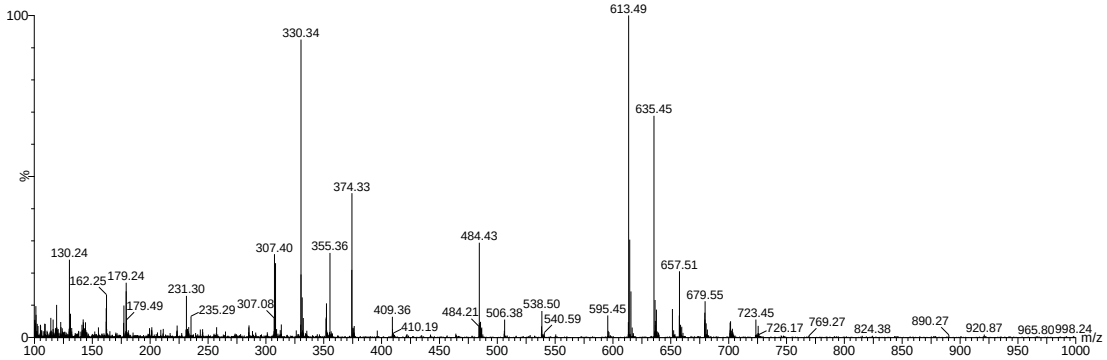
Glutathione reference:



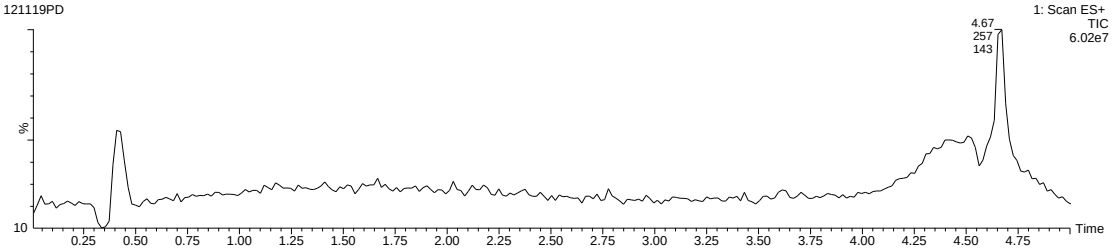


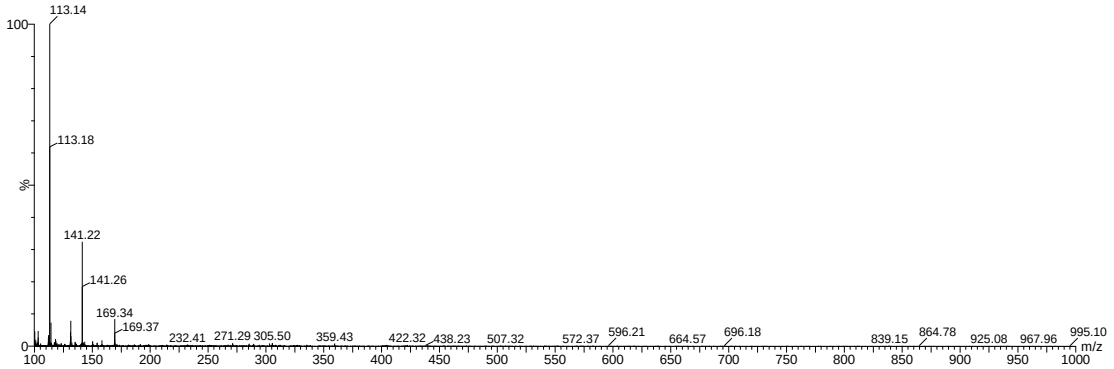
Glutathione control (24 h, 37 °C)





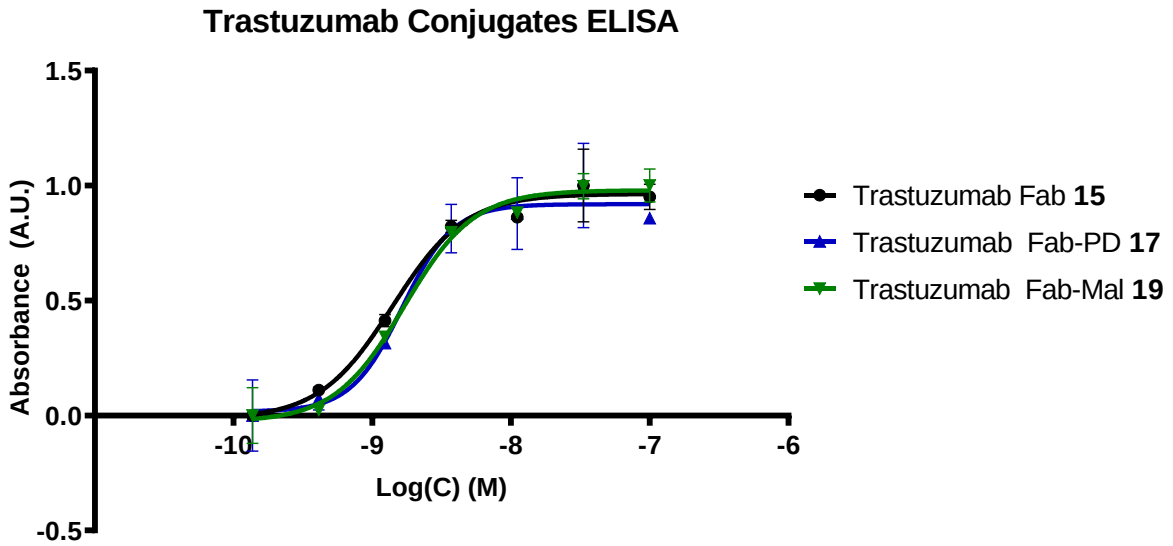
N,N-diethyl pyridazinedione **11** reference





ELISA for Trastuzumab Fab conjugates 17 and 19

Binding affinity to HER2 receptor was determined by ELISA. A 96-well plate was coated overnight at 4 °C with HER2 (Human HER2/ErbB2 Protein (His Tag) from Sino Biological) (100 µL of a 0.25 $\mu g \cdot mL^{-1}$ solution in PBS), including coating one row of wells with PBS only for negative controls. Next, the coating solutions were removed and each well washed with PBS twice. Then, the wells were coated with a 2% BSA solution in PBS (200 µL) for 1 h at 21 °C. Next, the wells were washed with PBST (0.1% Tween 20 in PBS) twice and with PBS three times. Solutions of Trastuzumab Fab and Trastuzumab Fab conjugates (in 0.2% BSA, PBST) were prepared with the following dilution series: 100 nm, 33 nM, 11 nM, 3.7 nM, 1.24 nM, 0.41 nM and 0.14 nM. Wells were coated with the dilution series solutions in triplicate, including a PBS only at 100 nM in the absence of HER2 as negative controls, and incubated for 2 h at 21 °C. Then, the solutions were removed and the wells washed with PBST twice and with PBS three times. Detection antibody (100 µL of anti-human IgG, Fab-specific-HRP solution, prepared by taking 4 µL of a 1:5000 diluted solution and further diluting with 20mL of 0.2% BSA in PBST) was then added and incubated for 1 h at 21 °C. Then, the solutions were removed and the wells washed with PBST twice and with PBS three times. OPD solution (100 µL of 10 mg/20 mL OPD in phosphate-citrate buffer with sodium perborate, prepared by dissolving 1 capsule in 100 mL water) was added to each well. After 30 s the reaction was stopped through addition of 4 M HCl (50 µL). The absorption was measured at 450 nm and corrected by subtracting the average of negative controls. The results obtained were analysed with GraphPad Prism using a regression with variable slope (four parameters).



Conjugate	IC_{50} (M)
Trastuzumab Fab **15**	**1.397×10^{-9}**
Trastuzumab Fab-PD **17**	**1.830×10^{-9}**
Trastuzumab Fab-Mal **19**	**1.703×10^{-9}**

Figure S12: ELISA data of trastuzumab conjugates.

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