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 chromatography was carried out with pre-loaded GraceResolv™ Silica Flash cartridges (Grace™) or FlashPure EcoFlex cartridges (Büchi) on a Biotage® Isolera Spektra One flash chromatography system (Biotage®). 1H NMR spectra were obtained at 300 MHz, 400 MHz, 500 MHz, 600 MHz or 700 MHz. 13C 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 1H NMR and 13C 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-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

1H NMR spectra for the deconjugation and transfer experiments were obtained using a Bruker Avance III 400 instrument with 32 scans using CD3CN 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 spectrophotometer (Jenway) or a NanoDrop One 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 GFP147C 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. 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 (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**

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<th>Time (min)</th>
<th>Solvent A (%)</th>
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<tr>
<td>25</td>
<td>85</td>
<td>15</td>
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</table>

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

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 \textit{in vacuo} with toluene co-evaporation (3 x 20 mL, as an azeotrope). Residual toluene was subsequently removed \textit{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\(_3\)) \(\delta\) 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\(_3\)) \(\delta\) 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
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$_{29}$Na$_3$O$_5$S [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(2H)-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(2H)yl) propanoate\(^1\) (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\(_2\)CO\(_3\) (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(2H)-yl)-N-(prop-2-yn-1-yl)propanamide and 3-(5-bromo-2-methyl-3,6-dioxo-3,6-dihydropyridazin-1(2H)-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) \(\delta\) 7.39 (s, 1H), 7.35 (s, 1H), 6.25–6.03 (m, 2H), 4.44 (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); \(^1\)\(^3\)C NMR (150 MHz, CDCl\(_3\), regioisomers) \(\delta\) 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\(_3\)), 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\(_2\)N\(_3\)O\(_3\) [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. 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 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 \( p_{Sax} \) and S-pseudoequatorial \( p_{Seq} = 100 - p_{Sax} \) orientations are calculated using \( \bar{J} = J^{ax} p_{Seq} + J^{ee} p_{Sax} \), where \( \bar{J} \) is the measured averaged coupling constant, whereas \( J^{ax} \) 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 \( J^{ax} \) 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 \( J^{ax} = 12.18 \) 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\(_3\)CN + H\(_2\)O, 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 \( \pm 0.5 \) Hz, the corresponding uncertainty in the predicted populations is estimated to be within \( \pm 3\% \). 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\(_3\)CN containing 1 mM EDTA. Deconjugation was monitored by \(^1\)H 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 \( N\)-\( \text{tert}\)-butoxycarbonyl-\( \lambda \)-cysteine methyl ester 10 (resulting from the retro-Michael-mediated deconjugation of methyl \( N\)-\( \text{tert}\)-butoxycarbonyl-\( \text{(1,2-diethyl-3,6-dioxohexahydropyridazin-4-yl)}\)-\( \lambda \)-cysteinate 9) with Ellman’s reagent 12 was performed thus: CH\(_3\)CN (476 µL) was added to 100 mM PB pH 7.4, 1 mM EDTA buffer (1112 µL). To this solution was added methyl \( N\)-\( \text{tert}\)-butoxycarbonyl-S-\( \text{(1,2-diethyl-3,6-dioxohexahydropyridazin-4-yl)}\)-\( \lambda \)-cysteinate 9 (28 µL, 5 mM in CH\(_3\)CN, 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 \( N\)-\( \text{tert}\)-butoxycarbonyl-S-\( \text{(1,2-diethyl-3,6-dioxohexahydropyridazin-4-yl)}\)-\( \lambda \)-cysteinate 9 and 170 µM Ellman’s reagent 12 in 100 mM PB pH 7.4, 1 mM EDTA buffer: CH\(_3\)CN (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\(_3\)CN (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\(_3\)CN (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.

\[
\frac{[\text{TNB}_{\text{Trap}}] - [\text{TNB}_{\text{Control}}]}{[\text{CysPD}_{\text{Initial}}]} \times 100 = \% \text{ estimated deconjugation} \quad (1)
\]

Where: \( \text{TNB}_{\text{Trap}} \) = Calculated TNB 13 concentration in trapping reaction, \( \text{TNB}_{\text{Control}} \) = Calculated TNB 13 concentration in control, and \( \text{CysPD}_{\text{Initial}} \) = 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$_3$CN (7:3), incubating at 37 °C, and the estimated concentration of free TNB 13 in solution.

<table>
<thead>
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<th>Time point (h)</th>
<th>A$_{412}$</th>
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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$_3$CN (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.

<table>
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<tr>
<th>Time point (h)</th>
<th>A$_{412}$</th>
<th>Estimated conc$^a$ of free TNB 13 (µM)</th>
<th>Corrected estimated 9 deconjugated (µM)</th>
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Synthesis of proteins:

**Cysteine Mutant Green Fluorescent Protein (GFPS147C) 14:**

GFPS147C 14 was expressed in competent E. coli BL21(DE3) cells as described previously. 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 $\varepsilon_{280} = 20,500$ 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 GFP5147C 14
Trastuzumab Fab 15:

Trastuzumab Fab 15 was obtained through pepsin/papain digestion of trastuzumab as described previously. Concentration was determined photometrically using $\epsilon_{280} = 68,590 \text{ M}^{-1} \text{cm}^{-1}$. 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)

\(N,N\)-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 conjugation with \( N \)-methyl maleimide 23

\( N \)-Methyl maleimide 23 (15 \( \mu \)L, 5 mM in DMSO, 5 eq.) was added to reduced GFPS147C 14 (300 \( \mu \)L, 50 \( \mu \)M 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, ZeaSpin®, 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

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

MonoBr \(N,N\)-methyl propargyl pyridazinedione (5.0 \(\mu\)L, 200 mM in DMSO, 200 eq.) was added to a solution of GFPS147C-PD conjugate 16 (100 \(\mu\)L, 50 \(\mu\)M 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\(\text{unsat}\)), 29772 Da (GFP(2)-S-PD\(\text{unsat}\)). Observed masses (LCMS Method 1): 29582 Da (GFP(1)-S-PD\(\text{unsat}\)), 29776 Da (GFP(2)-S-PD\(\text{unsat}\)).
6) Control: Reaction of GFPS147C-PD conjugate with \(N,N\)-diethyl pyridazinedione

\(N,N\)-Diethyl pyridazinedione 11 (5.0 \(\mu\)L, 200 mM in DMSO, 200 eq.) was added to a solution of GFPS147C-PD conjugate (100 \(\mu\)L, 50 \(\mu\)M 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\(^\text{®}\), Thermo Scientific) prior to LCMS analysis. Expected masses: 29589 Da (GFP(1)-S-PD\(_{\text{unsat}}\)), 29772 Da (GFP(2)-S-PD\(_{\text{unsat}}\)). Observed masses (LCMS Method 1): 29596 Da (GFP(1)-S-PD\(_{\text{unsat}}\)), 29777 Da (GFP(2)-S-PD\(_{\text{unsat}}\)).
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-Malhyd 20), 29668 Da (GFP(2)-S-Malhyd 20), Observed masses (LCMS Method 1): 29481 Da (GFP(1)-S-Malhyd 20), 29665 Da (GFP(2)-S-Malhyd 20).
8) Reaction of GFPS147C-PD conjugate 18 with monoBr N,N-methyl propargyl pyrazinedione

MonoBr N,N-methyl propargyl pyrazinedione (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-Malhyd 20), 29589 Da (GFP(1)-S-PDunsat), 29668 (GFP(2)-S-Malhyd 20), 29772 (GFP(1)-S-PDunsat). Observed masses (LCMS Method 1): 29481 Da (GFP(1)-S-Malhyd 20), 29596 Da (GFP(1)-S-PDunsat), 29663 Da (GFP(2)-S-Malhyd 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. A412 control readings for DTNB 12 only experiments were subtracted from all timepoints for GFP conjugates 16 and 18. A412 for initial readings (t=180 s) were also subtracted from all timepoints for GFP conjugates 16 and 18 to plot ΔA412 vs time (seconds). ΔA412 values were converted into Δ[TNB 13] values using ε280 = 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 ε490 = 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:

\[
\ln\left(\frac{[\text{GFP conjugate}]_t}{[\text{GFP conjugate}]_0}\right) = -kt + \ln([\text{GFP conjugate}]_0)
\]  \hspace{1cm} (2)

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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) change 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

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

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\textsubscript{unsat}), 24447 Da (Fab(HC)-S-PD\textsubscript{unsat}). Observed masses (LCMS Method 1): 23686 Da (Fab(LC)-S-PD\textsubscript{unsat}), 24450 Da (Fab(HC)-S-PD\textsubscript{unsat}).
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 7.4
14) Reaction of Trastuzumab-PD conjugate 17 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 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\textsubscript{unsat}), 24447 Da (Fab(HC)-S-PD\textsubscript{unsat}). Observed masses (LCMS Method 1): 23620 Da (Fab(LC)-S-PD\textsubscript{17}), 23686 Da (Fab(LC)-S-PD\textsubscript{unsat}), 24447 Da (Fab(HC)-S-PD\textsubscript{unsat}).
15) Stability of Trastuzumab-PD conjugate 17 to glutathione

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-Malhyd 21), 24343 Da (Fab(HC)-S-Malhyd 21). Observed masses (LCMS Method 1): 23580 Da (Fab(LC)-S-Malhyd 21), 24342 Da (Fab(HC)-S-Malhyd 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-PDunsat), 24343 Da (Fab(HC)-S-Mal hyd 21), 24447 Da (Fab(HC)-S-PDunsat). 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-PDunsat), 24343 Da (Fab(HC)-S-Mal hyd 17), 24449 Da (Fab(HC)-S-PDunsat).
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 \(\mu\)L, 200 mM in DMSO, 500 eq.) was added to the solution of Trastuzumab conjugate (100 \(\mu\)L, 20 \(\mu\)M 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

SH

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

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

\[ \text{Glutathione (500} \ \mu\text{M)} \quad \text{PBS pH 7.4,} \quad 37 \ \degree\text{C, 24 h} \]

\[ 11 \]

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

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 μg·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).

![Trastuzumab Conjugates ELISA](image)

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<th>Conjugate</th>
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Figure S12: ELISA data of trastuzumab conjugates.
References


