Rapid and robust cysteine bioconjugation with vinylheteroarenes

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1. General experimental

All solvents and reagents were used as received unless otherwise stated. Ethyl acetate, methanol, dichloromethane, acetonitrile and toluene were distilled from calcium hydride. Diethyl ether was distilled from a mixture of lithium aluminium hydride and calcium hydride. Petroleum ether refers to the fraction between 40–60 °C upon distillation. Tetrahydrofuran was dried using Na wire and distilled from a mixture of lithium aluminium hydride and calcium hydride with triphenylmethane as indicator.

Non-aqueous reactions were conducted under a stream of dry nitrogen using oven dried glassware. Temperatures of 0 °C were maintained using an ice-water bath. Room temperature (rt) refers to ambient temperature.

Yields refer to spectroscopically and chromatographically pure compounds unless otherwise stated. Reactions were monitored by thin layer chromatography (TLC) or liquid chromatography mass spectroscopy (LC-MS). TLC was performed using glass plates pre-coated with Merck silica gel 60 F254 and visualized by quenching of UV fluorescence (λ<sub>max</sub> = 254 nm) or by staining with potassium permanganate or para-anisaldehyde. Retention factors (R<sub>f</sub>) are quoted to 0.01.

LC-MS was carried out using a Waters ACQUITY HClass UPLC with an ESCi Multi-Mode Ionisation Waters SQ Detector 2 spectrometer using MassLynx 4.1 software; EI refers to the electrospray ionisation technique; LC system: solvent A: 2 mM NH₄OAc in H₂O/MeCN (95:5); solvent B: MeCN; solvent C: 2% formic acid; column: ACQUITY UPLC® CSH C18 (2.1 mm × 50 mm, 1.7 μm, 130 Å) at 40 °C; gradient: 5 – 95 % B with constant 5 % C over 1 min at flow rate of 0.6 mL/min; detector: PDA eλ Detector 220 – 800 nm, interval 1.2 nm.

Flash column chromatography was carried out using slurry-packed Merck 9385 Kieselgel 60 SiO₂ (230-400 mesh) under a positive pressure.

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

Analytical high performance liquid chromatography (HPLC) was performed on Agilent 1260 Infinity machine, using a Supelecosi™ ABZ+PLUS column (150 mm × 4.6 mm, 3 μm) with a linear gradient system (solvent A: 0.05% (v/v) TFA in H₂O; solvent B: 0.05% (v/v) TFA in MeCN) over 20 min at a flow rate of 1 mL/min, and UV detection (λ<sub>max</sub> = 220 – 254 nm).
Infrared (IR) spectra were recorded neat on a Perkin-Elmer Spectrum One spectrometer with internal referencing. Selected absorption maxima (ν_max) are reported in wavenumbers (cm⁻¹).

Proton and carbon nuclear magnetic resonance (NMR) were recorded using an internal deuterium lock on Bruker DPX-400 (400 MHz, 101 MHz), Bruker Avance 400 QNP (400 MHz, 101 MHz) and Bruker Avance 500 Cryo Ultrasound (500 MHz, 126 MHz). In proton NMR, chemical shifts (δ_H) are reported in parts per million (ppm), to the nearest 0.01 ppm and are referenced to the residual non-deuterated solvent peak (CHCl₃: 7.26, CHD₂OD: 3.31, HOD: 4.79). Coupling constants (J) are reported in Hertz (Hz) to the nearest 0.1 Hz. Data are reported as follows: chemical shift, integration, multiplicity (s = singlet; d = doublet; t = triplet; q = quartet; qn = quintet; sep = septet; m = multiplet; app = apparent; br = broad; or as a combination of these, e.g. dd, dt etc.), and coupling constant(s). In carbon NMR, chemical shifts (δ_C) are quoted in ppm, to the nearest 0.1 ppm, and are referenced to the residual non-deuterated solvent peak (CDCl₃: 77.16, CD₃OD: 49.00).

High resolution mass spectrometry (HRMS) measurements were recorded with a Micromass Q-TOF mass spectrometer or a Waters LCT Premier Time of Flight mass spectrometer. Mass values are reported within the error limits of ±5 ppm mass units. ESI refers to the electrospray ionisation technique.

Protein LCMS was performed on a Xevo G2-S TOF mass spectrometer coupled to an Acquity UPLC system using an Acquity UPLC BEH300 C4 column (1.7 μm, 2.1 × 50 mm). H₂O with 0.1% formic acid (solvent A) and 95% MeCN and 5% water with 0.1% formic acid (solvent B), were used as the mobile phase at a flow rate of 0.2 mL/min. The gradient was programmed as follows: 95% A for 0.93 min, then a gradient to 100% B over 4.28 min, then 100% B for 1.04 minutes, then a gradient to 95% A over 1.04 min. The electrospray source was operated with a capillary voltage of 2.0 kV and a cone voltage of 40 or 150 V. Nitrogen was used as the desolvation gas at a total flow of 850 L/h. Total mass spectra were reconstructed from the ion series using the MaxEnt algorithm preinstalled on MassLynx software (v4.1 from Waters) according to the manufacturer’s instructions.

Non-reducing Tris-Glycine SDS-PAGE with 12% acrylamide with 4% stacking gel was performed as standard. Broad range molecular weight marker (10-200 kDa, New England BioLabs) was run in all gels. Samples were prepared by mixing with loading dye and heated to 90 °C for 5 minutes. Loading dye containing β-mercaptoethanol was used to prepare samples under reducing conditions. Gels were run at constant voltage (160 V) for 70 min to 90 min in
×1 Laemmli running buffer. All gels were stained with Coomassie brilliant blue dye and imaged on a Syngene gel imaging system.

Monoclonal antibodies were deglycosylated and reduced prior to LCMS analysis. This was typically performed by adding 0.1 μL of peptide:N-glycosidase F (PNGase F; New England BioLabs Catalogue number P0704S) to a solution of antibody (10 μL at 1 μM) and was left to stand at rt for 15 h. To this solution, TCEP-HCl (1 μL, 5 mM in H2O) was added and was left to stand at rt for 10 minutes before analysis.

UV-visible (UV-vis) spectrums were obtained using a NanoDrop™ One spectrophotometer (ThermoFisher). Raw data was plotted using GraphPad Prism software (version 8). The following equation1,2 was used to calculate fluorophore-to-antibody ratio for AlexaFluor488-containing antibodies, where ε280 = 215380 M⁻¹ cm⁻¹ is the molar extinction coefficient trastuzumab at 280 nm; ε495 = 71000 M⁻¹ cm⁻¹ is the molar extinction coefficient for AlexaFluor488 at 495 nm; Abs495 and Abs280 are absorbance at 495 nm and 280 nm, respectively. A correction factor of 0.11 was used to account for AlexaFluor488 absorbance at 280 nm.

\[
\text{Fluorophore to antibody ratio} = \frac{\text{Abs}_{495}/\varepsilon_{495}}{[\text{Abs}_{280} - 0.11\text{Abs}_{495}]/\varepsilon_{280}}
\]

1 Maruani, A.; Savoie, H.; Bryden, F.; Caddick, S.; Boyle, R.; Chudasama, V. Chem. Commun. 2015, 51, 15304.
2 Walsh, S. J.; Omarjee, S.; Galloway, W. R. J. D.; Kwan, T. T.-L.; Sore, H. F.; Parker, J. S.; Hyvönen, M.; Carroll, J. S.; Spring, D. R. Chem. Sci. 2019, 10, 694.
2. Rate determination of conjugate addition

2.1. General procedure

To a solution of linker 1, 2, 3 or 4 [0.7 mL, 20 mM in 3:7 CD₃OD/buffer] in an NMR tube, a solution of Boc-Cys-OMe, Boc-Lys-OMe-HCl or H-Ala-NH₂·HCl [0.7 mL, 20 mM in 3:7 CD₃OD/buffer] was added and mixed by vigorous shaking.

After the first ¹H NMR spectrum (with water suppression) was acquired, subsequent measurements were taken every ~10 minutes for all nucleophile-vinylheteroarene combinations, apart from Table S1, Entries 2–3 and 11–15, where measurements were taken every ~15 seconds. The vinyl peaks at 5.52 ppm, 5.75 ppm, and 5.96 ppm were integrated to determine the concentration of substrates 1, 2 and 3, respectively.

The following equations were used to determine second order rate constants \( k_2 \).

\[
\frac{d[E]}{dt} = -k_2[Nu][E]
\]

When \([Nu] = [E]\),

\[
\frac{d[E]}{dt} = -k_2[E]^2
\]

Integration gives

\[
\frac{1}{[E]_t} = k_2 t + \frac{1}{[E]_{t=0}}
\]

Thus, the second order rate constant \( k_2 \) can be determined by plotting \( y = \frac{1}{[E]_t} \) and \( x = t \); the gradient is equal to \( k_2 \).\(^4\)

\(^3\) For tetrazine 4, only reactivity with Boc-Cys-OMe was examined. For results, refer to Figure S7.

\(^4\) For an example of second order rate constants determined using this method, see Kamber, D. N.; Liang, Y.; Blizzard, R. J.; Liu, F.; Mehl, R. A.; Houk, K. N.; Prescher, J. A. J. Am. Chem. Soc. 2015, 137, 8388.
Table S1. Second order rate constants of vinylheteroarene reaction with Boc-Cys-OMe, Boc-Lys-OMe and H-Ala-NH₂.

| Entry | Linker | Nucleophile | Buffer | Second order rate constant $k_2$ ($\times 10^3$ M⁻¹ s⁻¹) |
|-------|--------|-------------|--------|--------------------------------------------------|
| 1     | 1      | Boc-Cys-OMe | NaPi (pH 8, 50 mM in D₂O) | 4.91 ± 0.01 |
| 2     | 2      |             |        | 375 ± 30                                          |
| 3     | 3      |             |        | 3100 ± 30                                         |
| 4     | 4      |             |        | n/a                                              |
| 5     | 1      | Boc-Lys-OMe HCl | NaPi (pH 8, 50 mM in D₂O) | 0                                      |
| 6     | 2      |             |        | 0                                                  |
| 7     | 3      |             |        | 0.323 ± 0.006                                     |
| 8     | 1      | H-Ala-NH₂ HCl | NaPi (pH 8, 50 mM in D₂O) | 0                                      |
| 9     | 2      |             |        | 0                                                  |
| 10    | 3      |             |        | 0.944 ± 0.090                                     |
| 11    | 2      | Boc-Cys-OMe | NaPi (pH 8, 25 mM in D₂O) | 330 ± 6                                  |
| 12    | 2      |             | NaPi (pH 8, 10 mM in D₂O) | 213 ± 2                                  |
| 13    | 2      | Boc-Cys-OMe | NaPi (pH 7, 50 mM in D₂O) | 510 ± 1                                  |
| 14    | 2      |             | NaPi (pH 6, 50 mM in D₂O) | 636 ± 10                                 |
| 15    | 2      |             | CD₂CO₂Na (pH 5, 50 mM in D₂O) | 851 ± 7 |


2.2. Cysteine reactivity with vinylheteroarenes in CD$_3$OD/NaPi (pH 8, 50 mM)

**Figure S1.** Kinetic data used to calculate second order rate constants for the reaction of Boc-Cys-OMe with vinylpyridine 1 in 3:7 CD$_3$OD/NaPi (pH 8, 50 mM in D$_2$O). Two measurements were taken (plots a and b) and averaged.
Figure S2. Kinetic data used to calculate second order rate constants for the reaction of Boc-Cys-OMe with vinylpyrimidine 2 in 3:7 CD$_3$OD/NaPi (pH 8, 50 mM in D$_2$O). Two measurements were taken (plots a and b) and averaged.
Figure S3. Kinetic data used to calculate second order rate constants for the reaction of Boc-Cys-OMe with vinyltriazine 3 in 3:7 CD$_3$OD/NaPi (pH 8, 50 mM in D$_2$O). Two measurements were taken (plots a and b) and averaged.
Figure S4. Representative $^1$H NMR spectra of the reaction of vinylpyridine 1 with Boc-Cys-OMe in 3:7 CD$_3$OD/NaPi (pH 8, 50 mM in D$_2$O).

Figure S5. Representative $^1$H NMR spectra of the reaction of vinylpyrimidine 2 with Boc-Cys-OMe in 3:7 CD$_3$OD/NaPi (pH 8, 50 mM in D$_2$O).
**Figure S6.** Representative $^1$H NMR spectra of the reaction of vinyltriazine 3 with Boc-Cys-OMe in 3:7 CD$_3$OD/NaPi (pH 8, 50 mM in D$_2$O).

**Figure S7.** $^1$H NMR spectra of the reaction of vinyltetrazine 4 with Boc-Cys-OMe in 3:7 CD$_3$OD/NaPi (pH 8, 50 mM in D$_2$O). Rather than the depletion of vinyl peaks (which indicate conjugate addition), a new set of vinyl peaks arise over time at 6.04 ppm (dd, $J = 18.0, 11.3$ Hz), 5.70 (overlapping) and 5.48 (dd, $J = 11.3, 1.6$ Hz). This suggests that Boc-Cys-OMe is reacting with the tetrazine ring rather than the vinyl group.
2.3. Lysine reactivity with vinylheteroarenes in CD$_3$OD/NaPi (pH 8, 50 mM)

Figure S8. Kinetic data used to calculate second order rate constants for the reaction of Boc-Lys-OMe·HCl with vinylpyridine 1 in 3:7 CD$_3$OD/NaPi (pH 8, 50 mM in D$_2$O). Two measurements were taken (plots a and b) and averaged.
Figure S9. Kinetic data used to calculate second order rate constants for the reaction of Boc-Lys-OMe·HCl with vinylpyrimidine 2 in 3:7 CD$_3$OD/NaPi (pH 8, 50 mM in D$_2$O). Two measurements were taken (plots a and b) and averaged.
Figure S10. Kinetic data used to calculate second order rate constants for the reaction of Boc-Lys-OMe·HCl with vinyltriazine 3 in 3:7 CD3OD/NaPi (pH 8, 50 mM in D2O). Two measurements were taken (plots a and b) and averaged.
**Figure S11.** Representative $^1$H NMR spectra of the reaction of vinylpyridine 1 with Boc-Lys-OMe·HCl in 3:7 CD$_3$OD/NaPi (pH 8, 50 mM in D$_2$O).

**Figure S12.** Representative $^1$H NMR spectra of the reaction of vinylpyrimidine 2 with Boc-Lys-OMe·HCl in 3:7 CD$_3$OD/NaPi (pH 8, 50 mM in D$_2$O).
Figure S13. Representative $^1$H NMR spectra of the reaction of vinyltriazine 3 with Boc-Lys-OMe·HCl in 3:7 CD$_3$OD/NaPi (pH 8, 50 mM in D$_2$O).
2.4. N-Terminus reactivity with vinylheteroarenes in CD$_3$OD/NaPi (pH 8, 50 mM)

Figure S14. Kinetic data used to calculate second order rate constants for the reaction of H-Ala-NH$_2$HCl with vinylpyridine 1 in 3:7 CD$_3$OD/NaPi (pH 8, 50 mM in D$_2$O). Two measurements were taken (plots a and b) and averaged.
Figure S15. Kinetic data used to calculate second order rate constants for the reaction of H-Ala-NH$_2$HCl with vinylpyrimidine 2 in 3:7 CD$_3$OD/NaPi (pH 8, 50 mM in D$_2$O). Two measurements were taken (plots a and b) and averaged.
Figure S16. Kinetic data used to calculate second order rate constants for the reaction of H-Ala-NH$_2$·HCl with vinyltriazine 3 in 3:7 CD$_3$OD/NaPi (pH 8, 50 mM in D$_2$O). Two measurements were taken (plots a and b) and averaged.
Figure S17. Representative $^1$H NMR spectra of the reaction of vinylpyridine 1 with H-Ala-NH$_2$-HCl in 3:7 CD$_3$OD/NaPi (pH 8, 50 mM in D$_2$O).

Figure S18. Representative $^1$H NMR spectra of the reaction of vinylpyrimidine 2 with H-Ala-NH$_2$-HCl in 3:7 CD$_3$OD/NaPi (pH 8, 50 mM in D$_2$O).
Figure S19. Representative $^1$H NMR spectra of the reaction of vinyltriazine 3 with H-Ala-NH$_2$-HCl in 3:7 CD$_3$OD/NaPi (pH 8, 50 mM in D$_2$O).
2.5. Effect of buffer pH on vinylpyrimidine-cysteine reactivity

**Figure S20.** Kinetic data used to calculate second order rate constants for the reaction of Boc-Cys-OMe with vinylpyrimidine 2 in 3:7 CD$_3$OD/NaPi (pH 7, 50 mM in D$_2$O). Two measurements were taken (plots a and b) and averaged.
Figure S21. Kinetic data used to calculate second order rate constants for the reaction of Boc-Cys-OMe with vinylpyrimidine 2 in 3:7 CD$_3$OD/NaPi (pH 6, 50 mM in D$_2$O). Two measurements were taken (plots a and b) and averaged.
Figure S22. Kinetic data used to calculate second order rate constants for the reaction of Boc-Cys-OMe with vinylpyrimidine 2 in 3:7 CD3OD/CD3CO2Na (pH 5, 50 mM in D2O). Two measurements were taken (plots a and b) and averaged.
Figure S23. Representative $^1$H NMR spectra of the reaction of vinylpyrimidine 2 with Boc-Cys-OMe in 3:7 CD$_3$OD/NaPi (pH 7, 50 mM in D$_2$O).

Figure S24. Representative $^1$H NMR spectra of the reaction of vinylpyrimidine 2 with Boc-Cys-OMe in 3:7 CD$_3$OD/NaPi (pH 6, 50 mM in D$_2$O).
Figure S25. Representative $^1$H NMR spectra of the reaction of vinylpyrimidine 2 with Boc-Cys-OMe in 3:7 CD$_3$OD/CD$_3$CO$_2$Na (pH 5, 50 mM in D$_2$O).

a) 1 min

b) 3 min

c) 6 min

d) 10 min
2.6. Effect of buffer concentration on vinylpyrimidine-cysteine reactivity

Figure S26. Kinetic data used to calculate second order rate constants for the reaction of Boc-Cys-OMe with vinylpyrimidine 2 in 3:7 CD$_3$OD/NaPi (pH 8, 25 mM in D$_2$O). Two measurements were taken (plots a and b) and averaged.
Figure S27. Kinetic data used to calculate second order rate constants for the reaction of Boc-Cys-OMe with vinylpyrimidine 2 in 3:7 CD$_3$OD/NaPi (pH 8, 10 mM in D$_2$O). Two measurements were taken (plots a and b) and averaged.
Figure S28. Representative $^1$H NMR spectra of the reaction of vinylpyrimidine 2 with Boc-Cys-OMe in 3:7 CD$_3$OD/NaPi (pH 8, 25 mM in D$_2$O).

Figure S29. Representative $^1$H NMR spectra of the reaction of vinylpyrimidine 2 with Boc-Cys-OMe in 3:7 CD$_3$OD/NaPi (pH 8, 10 mM in D$_2$O).
3. Thioether stability studies

3.1. General procedure

A solution of linker-difluorobenzyl mercaptan conjugate 5, 6 or 7 (15 mM), 1-thioglycerol (150 mM), and sodium trifluoroacetate (4.5 mM) in 1:1 (v/v) CD3CN/NaPi (pH 7.4, 50 mM in H2O) was prepared. This solution was transferred to an NMR tube, and the atmosphere was purged with argon gas before the tube was sealed. The samples were placed in a water bath at 37 °C for 10 days. 19F NMR spectra was acquired every day, and the integral of 5, 6 or 7 were compared with that of an internal standard (sodium trifluoroacetate) in order to determine the amount of starting material. The CD3CN co-solvent was required to ensure solubilisation of substrates in an aqueous solution.

Figure S30. 19F NMR spectra of incubation mixtures on Day 0 for a) pyrimidine 5; b) triazine 6; and c) succinimide 7. The signal for the internal standard (sodium trifluoroacetate) can be observed at -76.5ppm.
3.2. Stability data for pyrimidine 5

![Image of NMR spectra]

**Figure S31.** Representative $^{19}$F NMR spectra for the stability study of pyrimidine 5. The stacked spectra are offset by an angle of 10°.

**Table S2.** Integration data for the stability of pyrimidine 5.

| Day | Integral of 5 | Integral of CF$_3$CO$_2$Na | Ratio 5 vs CF$_3$CO$_2$Na | Percentage 5 | Integral of 5 | Integral of CF$_3$CO$_2$Na | Ratio 5 vs CF$_3$CO$_2$Na | Percentage 5 |
|-----|--------------|----------------------------|---------------------------|--------------|--------------|----------------------------|---------------------------|--------------|
| 0   | 11406        | 4630                       | 2.46                      | 100          | 9085         | 3697                       | 2.46                      | 100          |
| 1   | 9964         | 4086                       | 2.44                      | 99.0         | 9511         | 3895                       | 2.44                      | 99.4         |
| 2   | 20212        | 8114                       | 2.49                      | 101.1        | 19237        | 7736                       | 2.49                      | 101.2        |
| 3   | 10191        | 4150                       | 2.46                      | 99.7         | 8961         | 3528                       | 2.54                      | 103.4        |
| 4   | 10876        | 4434                       | 2.45                      | 99.6         | 10951        | 4325                       | 2.53                      | 103.0        |
| 5   | 9192         | 3727                       | 2.47                      | 100.1        | 11059        | 4385                       | 2.52                      | 102.6        |
| 6   | 22434        | 9029                       | 2.48                      | 100.8        | 22162        | 8887                       | 2.49                      | 101.5        |
| 7   | 11689        | 4751                       | 2.46                      | 99.9         | 11359        | 4581                       | 2.48                      | 100.9        |
| 8   | 12977        | 5221                       | 2.49                      | 100.9        | 13138        | 5294                       | 2.48                      | 101.0        |
| 9   | 12449        | 5065                       | 2.46                      | 99.8         | 13529        | 5539                       | 2.44                      | 99.4         |
| 10  | 14471        | 5877                       | 2.46                      | 99.9         | 11690        | 4759                       | 2.46                      | 100.0        |
3.3. Stability data for triazine 6

![Figure S32. Representative $^{19}$F NMR spectra for the stability study of triazine 6. The stacked spectra are offset by an angle of 10°.]

Table S3. Integration data for the stability of triazine 6.

| Day | Integral of 6 | Integral of CF$_3$CO$_2$Na | Ratio 6 vs CF$_3$CO$_2$Na | Percentage 6 | Integral of 6 | Integral of CF$_3$CO$_2$Na | Ratio 6 vs CF$_3$CO$_2$Na | Percentage 6 |
|-----|---------------|-----------------------------|---------------------------|--------------|---------------|-----------------------------|---------------------------|--------------|
| 0   | 8720          | 3728                        | 2.34                      | 100          | 6350          | 2484                        | 2.56                      | 100          |
| 1   | 8754          | 3726                        | 2.35                      | 100.4        | 8928          | 3588                        | 2.49                      | 97.3         |
| 2   | 7325          | 3065                        | 2.39                      | 102.2        | 6385          | 2457                        | 2.60                      | 101.6        |
| 3   | 8589          | 3638                        | 2.36                      | 101.0        | 8476          | 3381                        | 2.51                      | 98.1         |
| 4   | 7388          | 3082                        | 2.40                      | 102.5        | 7685          | 3053                        | 2.52                      | 98.5         |
| 5   | 9504          | 4052                        | 2.35                      | 100.3        | 7573          | 2931                        | 2.58                      | 101.1        |
| 6   | 8766          | 3720                        | 2.36                      | 100.8        | 8921          | 3615                        | 2.47                      | 96.5         |
| 7   | 8525          | 3608                        | 2.36                      | 101.0        | 8992          | 3636                        | 2.47                      | 96.7         |
| 8   | 7174          | 3017                        | 2.38                      | 101.7        | 9028          | 3609                        | 2.50                      | 97.8         |
| 9   | 14606         | 6310                        | 2.31                      | 99.0         | 9687          | 3947                        | 2.45                      | 96.0         |
| 10  | 14365         | 6267                        | 2.29                      | 98.0         | 9635          | 3968                        | 2.43                      | 95.0         |
3.4. Stability data for succinimide 7

Figure S33. Representative $^{19}$F NMR spectra for the stability study of succinimide 7. The stacked spectra are offset by an angle of 10°. A number of new fluorinated peaks emerged over the ten-day incubation period.

Table S4. Integration data for the stability of succinimide 7.

| Day | Replicate 1 | Replicate 2 |
|-----|-------------|-------------|
|     | Integral of  | Integral of  | Percentage  | Integral of  | Integral of  | Percentage  |
|     | 7           | CF$_3$CO$_2$Na | 7     | 7           | CF$_3$CO$_2$Na | 7     |
| 0   | 7705        | 3185         | 2.42  | 100        | 6714        | 3701         | 2.35  | 100        |
| 1   | 8487        | 3694         | 2.30  | 95.0       | 8344        | 3707         | 2.25  | 95.6       |
| 2   | 7809        | 3593         | 2.17  | 89.9       | 7743        | 3659         | 2.12  | 89.9       |
| 3   | 7215        | 3520         | 2.05  | 84.7       | 6170        | 3060         | 2.02  | 85.7       |
| 4   | 7059        | 3599         | 1.96  | 81.1       | 5905        | 3073         | 1.92  | 81.6       |
| 5   | 5741        | 3085         | 1.86  | 76.9       | 6101        | 3628         | 1.68  | 71.4       |
| 6   | 6576        | 3687         | 1.78  | 73.7       | 6582        | 3813         | 1.73  | 73.3       |
| 7   | 5255        | 3031         | 1.73  | 71.7       | 6332        | 3823         | 1.66  | 70.3       |
| 8   | 6133        | 3702         | 1.66  | 68.5       | 6184        | 3876         | 1.60  | 67.8       |
| 9   | 6320        | 4038         | 1.57  | 64.7       | 6300        | 4198         | 1.50  | 63.8       |
| 10  | 5962        | 4012         | 1.49  | 61.4       | 4041        | 2737         | 1.48  | 62.7       |
4. Modification of human serum albumin

4.1. Reduction of human serum albumin

The purity of commercially available recombinant human serum albumin (HSA; purchased from Sigma Aldrich; product code A9731) was determined by protein LCMS. This revealed a mixture of the free Cys-34 form (66445 Da) and the cysteinylated form (i.e. disulfide bonded with cysteine; 66566 Da).

![Deconvoluted mass spectrum of commercially available HSA.](image)

**Figure S34.** Deconvoluted mass spectrum of commercially available HSA. The mass difference between the two signals corresponds to cysteinylation of the Cys-34 residue. Expected mass difference from cysteinylation: 119 Da; observed mass difference: 121 Da.

A sample of the free Cys-34 HSA was required for bioconjugation. This was achieved via reduction with dithiothreitol (DTT).

![Reduction of dithiothreitol](image)

Dithiothreitol (DTT, 1.81 μL, 5 mM in PBS (×1)) was added to a solution of HSA (17.9 μL, 253 μM) in PBS (100 μL, ×1)). The resulting solution was vortexed, and incubated at 37 °C for 2 h. Removal of excess reagents and buffer exchange to the required solvent was achieved by repeated ultracentrifugation into PBS (×1) or Tris-HCl (pH 8, 50 mM) using an Amicon-Ultra centrifugal filter (10k MWCO, Merck Millipore).
Figure S35. Non-deconvoluted (top) and deconvoluted (bottom) mass spectrum of the free Cys-34 form of HSA.
4.2. Optimisation for the reaction between HSA and vinylpyrimidine 2

Table S5. Screening of conjugation reactions between HSA (free Cys-34 form) and vinylpyrimidine 2. Reactions were performed at 37 °C in buffer solutions containing 5% DMSO. Aliquots of the reaction mixture were taken and quenched using 1-thioglycerol at the required timepoints prior to LCMS analysis. Percentage conversion reported to the nearest 5%. Refer to following sections for a representative experimental.

| Entry | [HSA] | Linker eq. | Percentage conversion |
|-------|-------|------------|-----------------------|
|       |       |            | PBS (×1) | Tris-HCl (pH 8, 50 mM) |
|       |       |            | 1 h | 2 h | 3 h | 4 h | 1 h | 2 h | 3 h | 4 h |
| 1     | 35 μM | 5          | 30  | 40  | 50  | 60  | 40  | 60  | 70  | 75  |
| 2     | 10    | 45         | 45  | 65  | 70  | 75  | 50  | 70  | 75  | >95 |
| 3     | 20    | 65         | 65  | 75  | 75  | 80  | 75  | >95 | -   | -   |
| 4     | 10 μM | 20         | 35  | 45  | 55  | 65  | 50  | 70  | 75  | >95 |
| 5     | 50    | 55         | 55  | 70  | 70  | 75  | 75  | >95 | -   | -   |
4.3. Synthesis of HSA-2 using optimised conditions

Linker 2 (0.77 μL, 20 mM in DMSO), Tris·HCl (18.8 μL, pH 8, 50 mM) and DMSO (0.33 μL) were added to a solution of HSA (Cys-34 free form, 2.0 μL, 384 μM in Tris·HCl (pH 8, 50mM)). After incubation at 37 °C for 2 h, the solution was purified via Zeba Spin desalting column (7k MWCO, ThermoFisher, pre-equilibrated with Tris·HCl (pH 8, 50 mM)) to give HSA-2.

**Figure S36.** Non-deconvoluted (top) and deconvoluted (bottom) mass spectrum of HSA-2. Expected 66565 Da; observed 66565 Da.
4.4. Synthesis of HSA-8

Linker 8 (16.8 µL, 20 mM in DMSO), Tris·HCl (413 µL, pH 8, 50 mM) and DMSO (7.15 µL) were added to a solution of HSA (Cys-34 free form, 43.9 µL, 384 μM in Tris·HCl (pH 8, 50mM)). After incubation at 37 °C for 2 h, the solution was purified via Zeba Spin desalting column (7k MWCO, ThermoFisher, pre-equilibrated with Tris·HCl (pH 8, 50 mM)) to give HSA-8.

Figure S37. Non-deconvoluted (top) and deconvoluted (bottom) mass spectrum of HSA-8. Expected 66692 Da; observed 66694 Da.
4.5. Synthesis of HSA-9

Linker 9 (0.58 µL, 20 mM in DMSO), Tris-HCl (14.1 µL, pH 8, 50 mM) and DMSO (0.25 µL) were added to a solution of HSA (Cys-34 free form, 1.5 µL, 384 µM in Tris-HCl (pH 8, 50 mM)). After incubation at 37 °C for 2 h in the dark, the solution was purified via Zeba Spin desalting column (7k MWCO, ThermoFisher, pre-equilibrated with Tris-HCl (pH 8, 50 mM)) to give HSA-9.

Figure S38. Non-deconvoluted (top) and deconvoluted (bottom) mass spectrum of HSA-9. Expected 67066 Da; observed 67070 Da.
4.6. Synthesis of HSA-10

Linker 10 (0.58 µL, 20 mM in DMSO), Tris∙HCl (14.1 µL, pH 8, 50 mM) and DMSO (0.25 µL) were added to a solution of HSA (Cys-34 free form, 1.5 µL, 384 µM in Tris∙HCl (pH 8, 50mM)). After incubation at 37 °C for 2 h, the solution was purified via Zeba Spin desalting column (7k MWCO, ThermoFisher, pre-equilibrated with Tris∙HCl (pH 8, 50 mM)) to give HSA-10.

Figure S39. Non-deconvoluted (top) and deconvoluted (bottom) mass spectrum of HSA-10. Expected 67137 Da; observed 67135 Da.
4.7. Optimisation for the reaction between HSA and vinyltriazine 3

![Chemical structures of HSA and vinyltriazine 3](image)

Table S6. Screening of conjugation reactions between HSA (free Cys-34 form) and vinyltriazine 3. Reactions were performed at 37 °C in buffer solutions containing 5% DMSO. Aliquots of the reaction mixture were taken and quenched using 1-thioglycerol at the required timepoints prior to LCMS analysis. Percentage conversion reported to the nearest 5%. Refer to following sections for a representative experimental.

| Entry | [HSA] | Linker eq. | PBS (×1) | Tris-HCl (pH 8) |
|-------|-------|------------|----------|-----------------|
| 1     | 35 μM | 1          | <5       | 25              | <5 | <5 | - | - |
| 2     | 10 μM | 2          | <5       | 40              | <5 | 25 | - | - |
| 3     | 20 μM | 5          | <5       | 45              | 35 | 45 | - | - |
| 4     | 10 μM | 10         | 35       | 70              | 55 | 70 | - | - |
| 5     | 50 μM | 20         | 50       | >95             | 75 | >95 | - | - |
| 6     | 10 μM | 1          | <5       | <5              | <5 | <5 | - | - |
| 7     | 20 μM | 2          | <5       | <5              | <5 | <5 | - | - |
| 8     | 10 μM | 10         | <5       | 25              | 30 | 40 | - | - |
| 9     | 50 μM | 20         | 25       | 35              | 40 | 55 | 70 | >95 |
| 10    | 10 μM | 50         | -        | -               | - | >95 | - | - |
4.8. Synthesis of HSA-3 under optimised conditions

Linker 3 (0.35 µL, 20 mM in DMSO), PBS (8.01 µL, ×1) and DMSO (0.15 µL) were added to a solution of HSA (Cys-34 free form, 1.56 µL, 226 µM in PBS (×1)). After incubation at 37 °C for 1 h, the solution was purified via Zeba Spin desalting column (7k MWCO, ThermoFisher, pre-equilibrated with PBS (×1)) to give HSA-3.

![Synthesis reaction diagram](image)

**Figure S40.** Non-deconvoluted (top) and deconvoluted (bottom) mass spectrum of HSA-3. Expected 66567 Da; observed 66566 Da.
4.9. SDS-PAGE analysis of HSA conjugates

**Figure S41.** SDS-PAGE analysis with 12% acrylamide gel under non-reducing conditions. SDS-PAGE was analysed by in-gel fluorescence and coomassie brilliant blue staining. MW=molecular weight marker.
5. Modification of cysteine-engineered antibody

5.1. Reduction of cysteine-engineered antibody mAb₁

Based on a literature protocol,⁵ the engineered cysteines of mAb₁ were uncapped; all subsequent cysteine conjugation reactions were performed on the reduced mAb₁ prepared via the following method.

TCEP·HCl (4.5 µL, 50 mM in H₂O) and Tris·HCl (40 µL, pH 8, 50 mM containing 1 mM EDTA) were added to a solution of mAb₁ (40 µL, 70.7 µM in PBS (×1)), and the resulting solution was incubated at 37 °C for 4 h. Removal of excess reagents and buffer exchange to the required solvent was achieved by repeated ultracentrifugation into Tris·HCl (pH 8, 50 mM containing 1 mM EDTA) using an Amicon-Ultra centrifugal filter (10k MWCO, Merck Millipore). To this solution, dehydroascorbic acid (2.26 µL, 25 μM in H₂O) was added, and was left to stand at rt for 2 h, before the solution was purified via Zeba Spin desalting column (7k MWCO, ThermoFisher, pre-equilibrated with Tris·HCl (pH 8, 50 mM containing 1 mM EDTA)). Prior to LCMS analysis, samples were deglycosylated and reduced with TCEP·HCl.

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⁵ Dimasi, N.; Fleming, R.; Zhong, H.; Bezabeh, B.; Kinneer, K.; Christie, R. J.; Fazenbaker, C.; Wu, H.; Gao, C. Mol. Pharm. 2017, 14, 1501.
Figure S42. Non-deconvoluted (top) and deconvoluted (bottom) mass spectrum of mAb1 after deglycosylation with PNGase F and reduction with TCEP·HCl. Observed 23440 Da (LC) and 49284 Da (HC).
5.2. Synthesis of mAb1-2

Linker 2 (0.29 μL, 20 mM in DMSO) and DMSO (0.22 μL) were added to a solution of mAb1 (cysteine uncapped form, 9.5 μL, 31.6 μM in Tris-HCl (pH 8, 50 mM containing 1 mM EDTA)), and the resulting solution was incubated at 37 °C for 4 h. The solution was purified via Zeba Spin desalting column (7k MWCO, ThermoFisher, pre-equilibrated with Tris-HCl (pH 8, 50 mM containing 1 mM EDTA)) to give mAb1-2. Prior to LCMS analysis, samples were deglycosylated with PNGase F and reduced with TCEP-HCl.

Figure S43. Non-deconvoluted (top) and deconvoluted (bottom) mass spectrum of mAb1-2. Expected 23440 Da (LC) and 49405 Da (HC); observed 23440 Da (LC) and 49403 Da (HC). Prior to LCMS analysis, samples were deglycosylated with PNGase F and reduced with TCEP-HCl.
5.3. Synthesis of mAb1-8

Linker 8 (0.50 μL, 30 mM in DMSO) was added to a solution of mAb1 (cysteine uncapped form, 9.5 μL, 31.6 μM in Tris·HCl (pH 8, 50 mM containing 1 mM EDTA)), and the resulting solution was incubated at 37 °C for 4 h. The solution was purified via Zeba Spin desalting column (7k MWCO, ThermoFisher, pre-equilibrated with Tris·HCl (pH 8, 50 mM)) to give mAb1-8. Prior to LCMS analysis, samples were deglycosylated with PNGase F and reduced with TCEP·HCl.

Figure S44. Non-deconvoluted (top) and deconvoluted (bottom) mass spectrum of mAb1-8. Expected 23440 Da (LC) and 49531 Da (HC); observed 23439 Da (LC) and 49530 Da (HC). Prior to LCMS analysis, samples were deglycosylated with PNGase F and reduced with TCEP·HCl. The signal at 34774 Da corresponds to PNGase F.
5.4. Synthesis of mAb1-8-12

Aqueous THPTA (0.29 μL, 20 mM), aqueous CuSO₄·5H₂O (0.23 μL, 5 mM), aqueous sodium ascorbate (0.34 μL, 50 mM), DMSO (1 μL) and azido-biotin 12 (0.57 μL, 20 mM in DMSO) were added sequentially to a solution of mAb1-8 (10 μL, 11.4 μM in PBS (×1)). The resulting solution was incubated at 37 °C for 15 h before purification via Zeba Spin desalting column (7k MWCO, ThermoFisher, pre-equilibrated with PBS (×1)) to give mAb1-8-12 Prior to LCMS analysis, samples were deglycosylated with PNGase F and reduced with TCEP-HCl.

Figure S45. Non-deconvoluted (top) and deconvoluted (bottom) mass spectrum of mAb1-8-12. Expected 23440 Da (LC) and 49976 Da (HC); observed 23440 Da (LC) and 49976 Da (HC). Prior to LCMS analysis, samples were deglycosylated with PNGase F and reduced with TCEP-HCl. The signals at 34774 Da and 46880 Da corresponds to PNGase F and light chain dimer, respectively.
5.5. Synthesis of mAb1-8-13

Aqueous THPTA (0.29 µL, 80 mM), aqueous CuSO₄·5H₂O (0.23 µL, 20 mM), aqueous sodium ascorbate (0.34 µL, 200 mM), DMSO (1 µL) and Alexa Fluor 488 azide 13 (0.57 µL, 20 mM in DMSO, purchased from Invitrogen) were added sequentially to a solution of mAb1-8 (10 µL, 11.4 µM in PBS (×1)). The resulting solution was incubated at 37 °C for 6 h before purification via Zeba Spin desalting column (7k MWCO, ThermoFisher, pre-equilibrated with PBS (×1)). The resulting conjugate was analysed by UV-Vis spectroscopy, which revealed conjugate mAb1-8-13 to have a fluorophore-to-antibody ratio of 1.9.

5.6. Synthesis of mAb1-8-14

Aqueous THPTA (2.5 µL, 80 mM), aqueous CuSO₄·5H₂O (3.5 µL, 20 mM), aqueous sodium ascorbate (1.7 µL, 200 mM) and azido-MMAE 14 (1.7 µL, 20 mM in DMSO) were added sequentially to a solution of mAb1-8 (25 µL, 27.3 µM in PBS (×1)). The resulting solution was incubated at 37 °C for 6 h before purification via Zeba Spin desalting column (two rounds of purification, 7k MWCO, ThermoFisher, pre-equilibrated with PBS (×1)) and further ultracentrifugation to PBS (×1) using Amicon-Ultra centrifugal filter (10k MWCO, Merck Millipore) to give mAb1-8-14. Prior to LCMS analysis, samples were deglycosylated with PNGase F and reduced with TCEP·HCl.

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6 Refer to General Experimental for procedure.
Figure S46. Non-deconvoluted (top) and deconvoluted (bottom) mass spectrum of mAb-8-14. Expected 23440 Da (LC; no modification) and 50806 Da (HC); observed 23440 Da (LC) and 50707 Da (HC). Prior to LCMS analysis, samples were deglycosylated with PNGase F and reduced with TCEP·HCl. The signal at 46878 Da corresponds to light chain dimer.
5.7. SDS-PAGE analysis of mAb1 conjugates

SDS-PAGE analysis of mAb1 and mAb1 conjugates under non-reducing (NR) and reducing (R) conditions. Analysis of mAb1-8, mAb1-12 and mAb1-13 reveals that conjugates are found predominantly as the “full antibody” forms (~146 kDa), with all native interchain disulfide bonds present. In-gel fluorescence of Alexa Fluor 488 conjugate mAb1-8-13 under reducing conditions revealed the linker modification to be on the heavy chain (~51 kDa), consistent with mass spectrometry analysis; fluorescence was not observed for the light chain (~23 kDa).

![SDS-PAGE analysis with 12% acrylamide gel under non-reducing (NR) and reducing (R) conditions. SDS-PAGE was analysed by in-gel fluorescence and coomassie brilliant blue staining. MW=molecular weight marker.](image)

**Figure S47.** SDS-PAGE analysis with 12% acrylamide gel under non-reducing (NR) and reducing (R) conditions. SDS-PAGE was analysed by in-gel fluorescence and coomassie brilliant blue staining. MW=molecular weight marker.
6. Preparation of fluorophore-modified trastuzumab and plasma stability

6.1. Mass spectra of native trastuzumab

Figure S48. Non-deconvoluted (top) and deconvoluted (bottom) mass spectrum of native trastuzumab mAb2. Prior to LCMS analysis, samples were deglycosylated with PNGase F and reduced with TCEP-HCl.
6.2. Synthesis of mAb2-8

Tris-buffered saline (25 mM pH 8 aqueous Tris-HCl, 25 mM NaCl, 0.5 mM EDTA; 9.0 μL) and aqueous TCEP-HCl (0.4 μL, 5 mM) were added to a solution of native trastuzumab mAb2 (3.0 μL, 45 μM), and the resulting solution was incubated at 37 °C for 1 h. To this solution, DMSO (0.27 μL) and 8 (1.07 μL, 12.5 mM in DMSO) were added, and the resultant solution was incubated at 37 °C for 6 h, before purification via Zeba Spin desalting column (7k MWCO, ThermoFisher, pre-equilibrated with PBS (×1)). Finally, buffer exchange to PBS (×1) was achieved by repeated ultracentrifugation using an Amicon-Ultra centrifugal filter (10k MWCO, Merck Millipore) to give mAb2-8. Prior to LCMS analysis, samples were deglycosylated with PNGase F and treated with TCEP-HCl.
Figure S49. Non-deconvoluted (top) and deconvoluted (bottom) mass spectrum of native trastuzumab mAb modified with vinylpyrimidine linker 8. The light chain and heavy chain are modified with one and three linkers, respectively, to give a linker-to-antibody ratio of 8. Expected 23686 Da (LC) and 49890 Da (HC). Found 23686 Da (LC) and 49892 Da (HC). Prior to LCMS analysis, samples were deglycosylated with PNGase F and treated with TCEP-HCl.
6.3. Synthesis of mAb2-8-13

Aqueous THPTA (1.62 μL, 80 mM), aqueous CuSO₄·5H₂O (1.08 μL, 20 mM), aqueous sodium ascorbate (1.08 μL, 200 mM) and Alexa Fluor 488 azide 13 (2.16 μL, 20 mM in DMSO, purchased from Invitrogen) were added sequentially to a solution of mAb2-8 (30 μL, 12.6 μM in PBS (×1)). The resulting solution was incubated at 37 °C for 15 h before purification via Zeba Spin desalting column (7k MWCO, ThermoFisher, pre-equilibrated with PBS (×1)). The resulting conjugate was analysed by UV-Vis spectroscopy, which revealed conjugate mAb2-8-13 to have a fluorophore-to-antibody ratio of 6.0.

6.4. Synthesis of mAb2-15

This bioconjugate was prepared based on a literature procedure.⁷

Aqueous TCEP·HCl (1.2 μL, 5 mM) was added to a solution of native trastuzumab mAb2 (20 μL, 29.7 μM in PBS (×1)) and the solution was incubated at 37 °C for 1 h. To this solution, DMSO (1.7 μL) and maleimide 15 (0.6 μL, 20 mM in DMSO) were added. The resulting solution was left to stand at rt for 1.5 h, before purification via Zeba Spin desalting column (7k

⁷ Li, W.; Veale, K. H.; Qiu, Q.; Sinkevicius, K. W.; Maloney, E. K.; Costoplus, J. A.; Lau, J.; Evans, H. L.; Setiady, Y.; Ab, O.; et al. ACS Med. Chem. Lett. 2019, 10, 1386.
MWCO, ThermoFisher, pre-equilibrated with PBS (×1)) to give mAb2-15. Prior to LCMS analysis, samples were deglycosylated with PNGase F and treated with TCEP-HCl.

Figure S50. Non-deconvoluted (top) and deconvoluted (bottom) mass spectrum of native trastuzumab mAb2 with maleimide linker 15. The reaction gave a mixture of reacted and unreacted cysteine residues. Light chain and heavy chain are modified with one and three linkers, respectively, to give a linker-to-antibody ratio of 8. The observed masses correspond to the following species: 23439 Da (unmodified LC; expected 23439 Da); 23662 Da (LC modified with one linker; expected 23662 Da); 49375 Da (HC modified with one linker; expected 49372 Da); 49599 Da (HC modified with two linkers; expected 49595 Da); 49822 Da (HC modified with three linkers; expected 49818 Da). Prior to LCMS analysis, samples were deglycosylated with PNGase F and treated with TCEP-HCl.
6.5. Synthesis of mAb2-15-13

Aqueous THPTA (1.62 μL, 80 mM), aqueous CuSO₄·5H₂O (1.08 μL, 20 mM), aqueous sodium ascorbate (1.08 μL, 200 mM) and Alexa Fluor 488 azide 13 (2.16 μL, 20 mM in DMSO, purchased from Invitrogen) were added sequentially to a solution of mAb2-15 (30 μL, 12.6 μM in PBS (×1)). The resulting solution was incubated at 37 °C for 15 h before purification via Zeba Spin desalting column (7k MWCO, ThermoFisher, pre-equilibrated with PBS (×1)). The resulting conjugate was analysed by UV-Vis spectroscopy, which revealed conjugate mAb2-15-13 to have a fluorophore-to-antibody ratio of 5.8.
6.6. Plasma stability studies

Solutions containing 0.5 μM of bioconjugate mAb₂-8-13 (fluorophore-to-antibody ratio = 6.0) or mAb₂-15-13 (fluorophore-to-antibody ratio = 5.8) were prepared in PBS (×1) containing 10% human plasma. These solutions were incubated at 37 °C. On day 0, 2, 4, 6 and 8, aliquots were taken and frozen at -80 °C until analysis. These samples were analysed by sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS-PAGE),⁸ where 1.25 pmol of antibody was loaded in each lane (Figure S51).

An analogous stability study was also conducted using human serum instead of human plasma (Figure S52).

![Figure S51](image)

**Figure S51.** Plasma stability analysis for a) mAb₂-8-13 and b) mAb₂-15-13 In-gel fluorescence analysis displays no transfer of fluorescence for mAb₂-8-13, whereas fluorescence transfer to serum proteins was observed for mAb₂-15-13. All lanes were prepared under reducing conditions.

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⁸ For an example of stability determination using SDS-PAGE, see Kolodych, S.; Koniev, O.; Baatarkhuu, Z.; Bonnefoy, J. Y.; Debaene, F.; Cianférani, S.; Van Dorsselaer, A.; Wagner, A. *Bioconjug. Chem.* **2015**, *26*, 197.
Figure S52. Serum stability analysis for a) mAb2-8-13 and b) mAb2-15-13. In-gel fluorescence analysis displays no transfer of fluorescence for mAb2-8-13, whereas fluorescence transfer to serum proteins was observed for mAb2-15-13. All lanes were prepared under reducing conditions.
7. *In vitro* cytotoxicity studies

SKBR3 cells were obtained from the American Type Culture Collection (ATCC) and MCF7 cells were obtained from the European Collection of Authenticated Cell Cultures (ECACC). SKBR3 cells were maintained in high glucose McCoy’s 5A medium, supplemented with 10% heat-inactivated foetal-bovine serum (FBS), 50 U/mL penicillin and 50 μg/mL streptomycin. MCF7 cells were maintained in Dulbecco’s Modified Eagle Medium (DMEM) supplemented with 10% FBS, 2 mM L-glutamine, 50 U/mL penicillin and 50 μg/mL streptomycin. All cell lines were incubated at 37 °C with 5% CO2.

Cells were seeded in 96-well plates for 24 h at 37 °C with 5% CO2. SKBR3 cells were seeded at 20,000 cells/well and MCF7 cells seeded at 7,500 cells/well. Serial dilutions of mAb-8-14 was added to the cells in complete growth medium and incubated at 37 °C with 5% CO2 for 96 h. Cell viability was measured using CellTiter-Glo viability assay (Promega) according to the manufacturer’s instructions. Cell viability was plotted as a percentage of untreated cells. Each measurement was taken in triplicate and three independent repeats were performed.
8. Cell lysate labelling

8.1. Preparation of cell lysate

MCF7 cells (2 × T175 flasks) were trypsinised and washed with PBS (3 × 10 mL). Cell pellets were reconstituted in RIPA buffer (Thermo, 89900) supplemented with protease inhibitors (Roche). Cells were then lysed by sonication (Diagenode, 2 × 30 s cycles) and centrifuged at 21,000 ×g at 4 °C for 15 min. The protein-containing supernatant was removed, and protein concentration (~5 mg/mL) measured using a Direct Detect Spectrometer (Millipore). Samples were diluted to 1 mg/mL with PBS, aliquoted, flash frozen and stored at -20 °C until use.

8.2. Labelling of cell lysate

A ×20 stock solution of vinylpyrimidine-alkyne 8 (2.5 µL; 20 µL, 200 µL, 1 mM, 2 mM, 4 mM, or 8 mM in DMSO) or neat DMSO (2.5 µL; control reaction) was added to MCF7 cell lysate (50 µL, 1 mg/mL), to give final linker concentrations of 1 µM, 10 µM, 50 µM, 100 µM, 200 µM, 400 µM, and 0 µM, respectively. The resulting solutions were left to stand at rt for 2 h, before purification via Zeba Spin desalting column (7k MWCO, ThermoFisher, pre-equilibrated with PBS (×1)). To each solution, THPTA (500 mM in H2O, 1 µL, 500 pmol), CuSO4·5H2O (50 mM in H2O, 1 µL, 50 pmol), sodium ascorbate (1 M in H2O, 1 µL, 1 μmol) and Alexa Fluor 488 azide 13 (5 mM in DMSO, 2 µL, 10 pmol) were added sequentially, and the resulting mixture was left to stand at rt for 1.5 h, before purification via Zeba Spin desalting column (7k MWCO, ThermoFisher, pre-equilibrated with PBS (×1)). Material corresponding to a 7.5 µL aliquot of this resulting solution was used for SDS-PAGE analysis. Samples were prepared under reducing conditions and run using methods described in the general experimental.

For cysteine blockade studies, MCF7 cell lysate (50 µL, 1 mg/mL) was pre-incubated with iodoacetamide (20 mM) at rt for 1 h, prior to the addition of vinylpyrimidine-alkyne 8.
Figure S53. SDS-PAGE analysis of MCF7 cell lysate labelling studies. Cell lysates were first labelled with varying concentrations of probe 8, and further modified with Alexa Fluor 488 azide 13 via a CuAAC reaction. For cysteine blockade studies, cell lysates were first pre-incubated with iodoacetamide. a) In-gel fluorescence of cell lysates. b) Coomassie staining of cell lysates. All lanes were prepared under reducing conditions. Molecular weight ladder in kDa.
9. Chemical synthesis

4-Vinylpyridin-2-amine 1

\[
\begin{align*}
\text{A stirred mixture of 4-bromopyridin-2-amine (250 mg, 1.45 mmol), potassium} \\
\text{vinyltrifluoroborate (223 mg, 1.66 mmol), Pd(dppf)Cl}_2\cdot\text{CH}_2\text{Cl}_2 (59 mg, 0.072 mmol) and} \\
\text{K}_2\text{CO}_3 (240 mg, 1.74 mmol) in THF/H}_2\text{O (10:1, 5.5 mL) was heated at 70 °C for 14 h. The} \\
\text{reaction mixture was filtered through Celite® (eluent EtOAc) and the resultant filtrate was} \\
\text{concentrated in vacuo. The resulting residue was purified by flash column chromatography} \\
\text{(MeOH/CH}_2\text{Cl}_2, 1:19 with 0.5% Et}_3\text{N) to give vinylpyridine 1 (115 mg, 0.957 mmol, 66%) as} \\
\text{a dark brown solid.}
\end{align*}
\]

\[
\text{R}_f \text{ (SiO}_2; \text{MeOH/CH}_2\text{Cl}_2 1:10 with 1% Et}_3\text{N) 0.60; } \nu_{\text{max}} \text{ (neat/cm}^{-1}) 3434, 3291, 3160, 1598, 1541; \text{ }^1\text{H NMR (CD}_3\text{OD, 400 MHz) } \delta \text{ 7.82 (1H, d, } J = 5.6 \text{ Hz), 6.70 (1H, dd, } J = 5.6, \text{ 1.6 Hz), 6.65 – 6.54 (2H, m), 5.93 (1H, dd, } J = 17.6, 0.8 \text{ Hz), 5.42 (1H, d, } J = 10.8 \text{ Hz); } ^{13}\text{C NMR (CD}_3\text{OD, 101 MHz) } \delta \text{ 161.3, 148.5, 147.9, 136.5, 118.5, 111.1, 107.7; HRMS (ESI) } m/z \text{ found [M+H}^+ \text{] 121.0763, } C_7\text{H}_9\text{N}_2^+ \text{ required 121.0760.}
\]

4-Vinylpyrimidin-2-amine 2

\[
\begin{align*}
\text{A stirred mixture of 4-chloropyrimidin-2-amine (257 mg, 1.98 mmol), potassium} \\
\text{vinyltrifluoroborate (798 mg, 5.96 mmol), Pd(dppf)Cl}_2\cdot\text{CH}_2\text{Cl}_2 (162 mg, 0.198 mmol) and} \\
\text{K}_2\text{CO}_3 (1.65 g, 11.9 mmol) in THF/H}_2\text{O (10:1, 6.6 mL) was heated to 70 °C for 16 h. The} \\
\text{reaction mixture was filtered through Celite® (eluent EtOAc) and the filtrate was concentrated in vacuo. The} \\
\text{resulting residue was purified by flash column chromatography (EtOAc/petroleum ether, 2:1) to give vinylpyrimidine 2 (175 mg, 1.44 mmol, 73%) as a light brown solid.}
\end{align*}
\]

\[
\text{R}_f \text{ (SiO}_2; \text{EtOAc/petroleum ether, 2:1) 0.28; } \nu_{\text{max}} \text{ (neat/cm}^{-1}) 3335, 3173, 1655, 1565; \text{ }^1\text{H NMR (CD}_3\text{OD, 400 MHz) } \delta \text{ 8.20 (1H, d, } J = 5.2 \text{ Hz), 6.72 (1H, d, } J = 5.2 \text{ Hz), 6.60 (1H, dd, } J = 17.5, \text{ 10.7 Hz), 6.35 (1H, dd, } J = 17.4, 1.3 \text{ Hz), 5.62 (1H, dd, } J = 10.7, 1.3 \text{ Hz); } ^{13}\text{C NMR (CD}_3\text{OD,}
\]

65
101 MHz) δ 165.4, 164.7, 159.6, 136.7, 123.0, 108.7; **HRMS** (ESI) m/z found [M+H]^+ 122.0715, C_6H_8N_3^+ required 122.0713.

Data in accordance with literature.⁹

**4-Chloro-1,3,5-triazin-2-amine S1**

![4-Chloro-1,3,5-triazin-2-amine S1](image)

2,4-Dichloro-1,3,5-triazine (500 mg, 3.33 mmol) was added to a stirred solution of 35% aqueous NH₃ (22.2 mL, 402 mmol) at −20 °C, and the resulting solution was stirred for 20 min. The reaction mixture was then extracted with EtOAc/PrOH (10:1, ×5). The combined organic extracts were washed with brine (×1), dried with Na₂SO₄, and concentrated *in vacuo*. The residue was purified by flash column chromatography (EtOAc/petroleum ether, 2:3) to give aminotriazine S1 (162 mg, 1.24 mmol, 37%) as a white solid.

**Rf** (SiO₂; EtOAc/petroleum ether, 1:1) 0.46; **vₘₐₓ** (neat/cm⁻¹) 3239; 2480, 2396, 2322, 1632, 1500; **¹H NMR** (CD₃OD, 400 MHz) δ 8.31 (1H, s); **¹³C NMR** (CD₃OD, 101 MHz) δ 171.2, 168.7, 168.2; **HRMS** (ESI) m/z found [M+H]^+ 131.0121, C₃H₄ClN₄^+ required 131.0119.

**4-Vinyl-1,3,5-triazin-2-amine 3**

![4-Vinyl-1,3,5-triazin-2-amine 3](image)

A mixture of 4-chloro-1,3,5-triazin-2-amine S1 (148 mg, 1.20 mmol), potassium vinyltrifluoroborate (243 mg, 1.82 mmol), Pd(dippf)Cl₂·CH₂Cl₂ (50 mmol, 0.061 mmol) and K₂CO₃ (200 mg, 1.45 mmol) in 1,4-dioxane/H₂O (10:1, 4.5 mL) was stirred at 90 °C for 15 h. The resulting reaction mixture was filtered through Celite® (eluent EtOAc) and the filtrate was concentrated *in vacuo*. The resulting residue was purified by flash column chromatography (EtOAc/petroleum ether, 2:1) to give vinyltriazine 3 (107 mg, 0.876 mmol, 72%) as a white solid.

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⁹ Walsh, S. J.; Omarjee, S.; Galloway, W. R. J. D.; Kwan, T. T.-L.; Sore, H. F.; Parker, J. S.; Hyvönen, M.; Carroll, J. S.; Spring, D. R. *Chem. Sci.* **2019**, *10*, 694.
Rf (SiO₂; EtOAc/petroleum ether, 4:1) 0.47; v max (neat/cm⁻¹) 3291, 3142, 1689, 1537, 1504, 1433; ¹H NMR (CD₃OD, 400 MHz) δ 8.43 (1H, s), 6.67 (1H, dd, J = 17.3, 1.9 Hz), 6.53 (1H, dd, J = 17.3, 10.4 Hz), 5.81 (1H, dd, J = 10.4, 1.9 Hz); ¹³C NMR (CD₃OD, 101 MHz) δ 171.8, 168.1, 167.2, 136.3, 127.6; HRMS (ESI) m/z found [M+H]⁺ 123.0668, C₅H₇N₄⁺ required 123.0665.

6-Vinyl-1,2,4,5-tetrazin-3-amine 4

![Chemical structure of 6-vinyl-1,2,4,5-tetrazin-3-amine 4](image)

This compound was synthesised based on a literature procedure.¹⁰,¹¹

To a stirred solution of 3,6-dichloro-1,2,4,5-tetrazine (100 mg, 0.662 mmol) in tBuOMe (3 mL) at rt, ammonia gas was continuously bubbled from a cannister for 10 min. The reaction mixture was then filtered through Celite®, and the resulting solution was concentrated in vacuo. The residue was dissolved in 1,4-dioxane/H₂O (5:1, 4 mL), and potassium vinyltrifluoroborate (124 mg, 0.927 mmol), Brettphos Pd G3 (30 mg, 0.033 mmol) and Cs₂CO₃ (647 mg, 2.00 mmol) was added. The resulting mixture was stirred at 70 °C over 15 h, filtered through Celite®, and the filtrate was concentrated in vacuo. The resulting residue was purified by flash column chromatography (petroleum ether/EtOAc, 3:1) to give vinyltetrazine 4 (26 mg, 0.21 mmol, 31% over two steps) as a bright red solid.

Rf (SiO₂; EtOAc/petroleum ether, 1:1) 0.52; v max (neat/cm⁻¹) 3291, 3152, 1618, 1553, 1505; ¹H NMR (CD₃OD, 400 MHz) δ 6.94 (1H, dd, J = 17.6, 11.1 Hz), 6.51 (1H, dd, J = 17.6, 1.3 Hz), 5.68 (1H, dd, J = 11.0, 1.3 Hz); ¹³C NMR (CD₃OD, 101 MHz) δ 164.3, 161.1, 131.8, 121.2; LRMS (ESI) m/z found [M+H]⁺ 124, C₄H₆N₅⁺ required 124.

¹⁰ Novák, Z.; Bostai, B.; Csékei, M.; Lőrincz, K.; Kotschy, A. Heterocycles 2003, 60, 2653.
¹¹ Bender, A. M.; Chopko, T. C.; Bridges, T. M.; Lindsley, C. W. Org. Lett. 2017, 19, 5693.
4-(2-((2,6-Difluorobenzyl)thio)ethyl)pyrimidin-2-amine 5

A solution of vinylpyrimidine 2 (112 mg, 0.938 mmol) and (2,6-difluorophenyl)methanethiol (300 mg, 1.88 mmol) in MeOH (5 mL) and aqueous NaPi (pH 8, 500 mM, 0.5 mL) was stirred at rt for 15 h. The reaction mixture was concentrated in vacuo, and the resulting residue was diluted with H2O. The aqueous phase was extracted with CH2Cl2 (×3), and the combined organic extracts were dried with Na2SO4, and concentrated in vacuo. The resulting residue was purified via flash column chromatography (EtOAc/petroleum ether, 2:1) to give thioether 5 (248 mg, 0.883 mmol, 94%) as a white solid.

Rf (SiO2; EtOAc/petroleum ether, 2:1) 0.36; \nu_{max} (neat/cm^{-1}) 3333, 3158, 1662, 1646, 1563; \textbf{1H NMR} (CDCl3, 400 MHz) δ 8.18 (1H, d, J = 5.0 Hz), 7.21 (1H, tt, J = 8.4, 6.4 Hz), 6.95 – 6.83 (2H, m), 6.48 (1H, d, J = 5.1 Hz), 5.07 (2H, s), 3.79 (2H, t, J = 1.2 Hz), 2.92 – 2.80 (4H, m); \textbf{13C NMR} (CDCl3, 101 MHz) δ 169.7, 163.1, 161.3 (dd, J = 248.6, 7.9 Hz), 158.3, 128.8 (t, J = 10.3 Hz), 115.4 (t, J = 19.3 Hz), 111.5 (dd, J = 19.0, 6.6 Hz), 111.0, 37.6, 30.5, 22.9 (t, J = 2.9 Hz); \textbf{19F NMR} (CDCl3, 376 MHz) δ -114.96 (t, J = 6.8 Hz); \textbf{HRMS} (ESI) [M+H]+ required for C13H14F2N3S+ 282.0876, found 282.0886.

4-(2-((2,6-Difluorobenzyl)thio)ethyl)-1,3,5-triazin-2-amine 6

A solution of vinyltriazine 3 (114 mg, 0.938 mmol) and (2,6-difluorophenyl)methanethiol (300 mg, 1.88 mmol) in MeOH (5 mL) and aqueous NaPi (pH 8, 500 mM, 0.5 mL) was stirred at rt for 15 h. The reaction mixture was concentrated in vacuo, and the resulting residue was diluted with H2O. The aqueous phase was extracted with CH2Cl2 (×3), and the combined organic extracts were dried with Na2SO4, and concentrated in vacuo. The resulting residue was purified via flash column chromatography (EtOAc/petroleum ether, 2:1) to give thioether 6 (132 mg, 0.468 mmol, 50%) as a white solid.

Rf (SiO2; EtOAc/petroleum ether, 2:1) 0.29; \nu_{max} (neat/cm^{-1}) 3302, 3171, 3171, 1673, 1626, 1578; \textbf{1H NMR} (CDCl3, 400 MHz) δ 8.52 (1H, s), 7.20 (1H, tt, J = 8.4, 6.5 Hz), 6.94 – 6.83
(2H, m), 5.42 (2H, s), 3.81 (2H, t, $J = 1.2$ Hz), 3.04 – 2.92 (4H, m); $^{13}$C NMR (CDCl$_3$, 101 MHz) $\delta$ 177.8, 166.6, 166.3, 161.3 (dd, $J = 248.7, 7.9$ Hz), 128.7 (t, $J = 10.3$ Hz), 115.4 (t, $J = 19.3$ Hz), 111.5 (dd, $J = 19.0, 6.6$ Hz), 38.4, 29.0, 22.84 (t, $J = 2.9$ Hz); $^{19}$F NMR (CDCl$_3$, 376 MHz) $\delta$ -114.93 (t, $J = 6.7$ Hz); HRMS (ESI) [M+H]$^+$ required for C$_{12}$H$_{13}$F$_2$N$_4$S$^+$ 283.0829, found 283.0818.

3-((2,6-Difluorobenzyl)thio)-1-methylpyrrolidine-2,5-dione 7

A solution of 1-methyl-1H-pyrrole-2,5-dione (100 mg, 0.901 mmol) and (2,6-difluorophenyl)methanethiol (216 mg, 1.35 mmol) in MeOH (5 mL) and aqueous NaPi (pH 8, 500 mM, 0.5 mL) was stirred at rt for 15 h. The reaction mixture was concentrated in vacuo, and the resulting residue was purified via flash column chromatography (EtOAc/petroleum ether, 1:5) to give thioether 7 (241 mg, 0.888 mmol, 99%) as a light yellow oil.

R$_f$ (SiO$_2$; EtOAc/petroleum ether, 1:5) 0.14; $\nu_{\text{max}}$ (neat/cm$^{-1}$) 1694; $^1$H NMR (CDCl$_3$, 400 MHz) $\delta$ 7.31 – 7.19 (1H, m), 6.97 – 6.86 (2H, m), 4.25 (1H, dt, $J = 13.6, 1.1$ Hz), 3.99 (1H, dt, $J = 13.6, 1.3$ Hz), 3.80 (1H, dd, $J = 9.1, 4.1$ Hz), 3.11 (1H, dd, $J = 18.7, 9.2$ Hz), 3.02 (3H, s), 2.50 (1H, dd, $J = 18.7, 4.1$ Hz); $^{13}$C NMR (CDCl$_3$, 101 MHz) $\delta$ 176.5, 174.7, 161.3 (dd, $J = 249.3, 7.7$ Hz), 129.5 (t, $J = 10.3$ Hz), 114.2 (t, $J = 19.3$ Hz), 111.7 (dd, $J = 19.0, 6.4$ Hz), 39.7, 36.2, 25.3, 23.1 (t, $J = 3.1$ Hz); $^{19}$F NMR (CDCl$_3$, 376 MHz) $\delta$ -114.46 (t, $J = 6.8$ Hz); HRMS (ESI) [M+H]$^+$ required for C$_{12}$H$_{13}$F$_2$NO$_2$S$^+$ 272.0557, found 272.0546.

4-Chloro-N-(2-(2-(prop-2-yn-1-yloxy)ethoxy)ethyl)pyrimidin-2-amine S2

A mixture of 2,4-dichloropyrimidine (458 mg, 3.07 mmol), 2-(2-(prop-2-yn-1-yloxy)ethoxy)ethan-1-amine (400 mg, 2.79 mmol), and Cs$_2$CO$_3$ (2.73 g, 8.37 mmol) in dioxane (8 mL) was heated under reflux for 15 h. The resulting suspension was filtered through cotton wool, and the filtrate was concentrated in vacuo. The resulting residue was purified via flash
column chromatography (EtOAc/petroleum ether, 2:3) to give aminopyrimidine S2 (181 mg, 0.708 mmol, 25%) as a colourless oil.

**Rr** (SiO₂; EtOAc/petroleum ether, 1:1) 0.45; $\nu_{\text{max}}$ (neat/cm$^{-1}$) 3286, 1573, 1522; $^1$H NMR (CDCl$_3$, 400 MHz) $\delta$ 8.14 (1H, d, $J = 5.2$ Hz), 6.55 (1H, d, $J = 5.1$ Hz), 5.80 (1H, br s), 4.21 (2H, d, $J = 2.4$ Hz), 3.72 – 3.59 (8H, m), 2.46 (1H, t, $J = 2.4$ Hz); $^{13}$C NMR (CDCl$_3$, 101 MHz) $\delta$ 162.4, 161.4, 159.2, 110.1, 79.7, 74.8, 70.3, 69.8, 69.2, 58.6, 41.3; HRMS (ESI) [M+H]$^+$ required for C$_{11}$H$_{15}$ClN$_3$O$_2$ + 256.1505, found 255.9449.

**N-(2-(2-(Prop-2-yn-1-yloxy)ethoxy)ethyl)-4-vinylpyrimidin-2-amine 8**

A mixture of S2 (592 mg, 2.31 mmol), potassium vinyltrifluoroborate (930 mg, 6.94 mmol), K$_2$CO$_3$ (1.92 g, 13.9 mmol) and Pd(dppf)Cl$_2$·CH$_2$Cl$_2$ (189 mg, 0.232 mmol) in THF/H$_2$O (10:1, 8 mL) was stirred at 70 °C for 15 h. The resulting suspension was cooled to rt, and EDTA (300 mg, 0.721 mmol) was added. The solution was stirred at rt for 1 h, filtered through Celite®, and the filtrate was concentrated in vacuo. The resulting residue was purified via flash column chromatography (EtOAc/petroleum ether, 1:2) to give vinylpyrimidine 8 (97 mg, 0.39 mmol, 17%) as a colourless oil.

**Rr** (SiO₂; EtOAc/petroleum ether, 1:1) 0.40; $\nu_{\text{max}}$ (neat/cm$^{-1}$) 3290, 1571; $^1$H NMR (CDCl$_3$, 500 MHz) $\delta$ 8.24 (1H, d, $J = 5.0$ Hz), 6.62 – 6.51 (2H, m), 6.34 (1H, dd, $J = 17.4, 1.5$ Hz), 5.57 (1H, dd, $J = 10.6, 1.5$ Hz), 5.50 (1H, s), 4.22 (2H, d, $J = 2.4$ Hz), 3.74 – 3.62 (8H, m), 2.45 (1H, t, $J = 2.4$ Hz); $^{13}$C NMR (CDCl$_3$, 126 MHz) $\delta$ 163.2, 162.5, 158.6, 136.0, 121.8, 108.0; 79.7, 74.8, 70.3, 70.2, 69.2, 58.6, 41.2; HRMS (ESI) [M+H]$^+$ required for C$_{15}$H$_{18}$N$_3$O$_2$ + 248.2051, found 248.1396.
N-(2-(2-(2-Azidoethoxy)ethoxy)ethoxy)ethyl)-7-nitrobenzo[c][1,2,5]oxadiazol-4-amine S3

A solution of 4-chloro-7-nitrobenzo[c][1,2,5]oxadiazole (200 mg, 1.00 mmol), 2-(2-(2-azidoethoxy)ethoxy)ethanol-1-amine (238 µL, 1.20 mmol) and Et3N (139 µL, 1.00 mmol) in DMF (6 mL) was stirred at rt for 4 h. The resulting solution was diluted with 3 M LiCl, and extracted with EtOAc (×3). The combined organic extracts were washed sequentially with 1 M aqueous HCl (×3), 3 M aqueous LiCl (×3), saturated aqueous Na2CO3 (×3), and brine (×2). The organic extracts were dried with Na2SO4, and concentrated in vacuo to provide NBD-azide S3 (280 mL, 0.734 mmol, 73%) as an intensely coloured brown oil.

$\nu_{\text{max}}$ (neat/cm$^{-1}$) 2097, 1579; $^1$H NMR (CD$_3$OD, 400 MHz) $\delta$ 8.38 (1H, d, $J = 8.9$ Hz), 6.32 (1H, d, $J = 8.9$ Hz), 3.79 (2H, t, $J = 5.1$ Hz), 3.70 – 3.53 (12H, m), 3.37 – 3.27 (1H, app m, overlapping with solvent peak); $^{13}$C NMR (CD$_3$OD, 101 MHz) $\delta$ 146.7, 145.7, 145.4, 138.3, 123.1, 100.1, 71.65, 71.59, 71.54, 71.47, 71.1, 69.8, 51.7, 44.8; HRMS (ESI) [M+H]$^+$ required for C$_{14}$H$_{20}$N$_7$O$_6$+ 382.1470, found 382.1429.

7-Nitro-N-(2-(2-(2-(4-(2-(4-vinylpyrimidin-2-yl)amino)ethoxy)ethoxy)methyl)-1H-1,2,3-triazol-1-yl)ethoxy)ethoxy)ethoxy)ethyl)benzo[c][1,2,5]oxadiazol-4-amine 9

A solution of 8 (24 mg, 0.097 mmol), S3 (56 mg, 0.147 mmol), CuSO$_4$·5H$_2$O (30 mg, 0.12 mmol), sodium ascorbate (100 mg, 0.500 mmol) and THPTA (130 mg, 0.300 mmol) in tBuOH/H$_2$O/CH$_2$Cl$_2$ (1:1:1, 6 mL) was stirred at rt for 15 h. To this solution, EDTA (100 mg, 0.240 mmol) was added, and the solution was stirred at rt for 1 h. The resulting solution was concentrated under a stream of N$_2$, and the resulting residue was purified by reverse phase flash column chromatography (10 – 90% solvent B in solvent A). Solvent A: 0.1 M aqueous ammonium hydroxide. Solvent B: MeCN) and lyophilised to give vinylpyrimidine-NBD 9 (25 mg, 0.040 mmol, 41%) as a brown oil.
**1H NMR** (CD$_3$OD, 700 MHz) δ 8.48 (1H, d, J = 8.7 Hz), 8.16 (1H, d, J = 5.1 Hz), 8.01 (1H, s), 6.61 (1H, d, J = 5.1 Hz), 6.55 (1H, dd, J = 17.4, 10.7 Hz), 6.38 (1H, d, J = 8.8 Hz), 6.33 (1H, d, J = 17.4 Hz), 5.57 (1H, dd, J = 10.6, 1.4 Hz), 4.60 (2H, s), 4.53 (2H, t, J = 5.1 Hz), 3.84 (2H, t, J = 5.0 Hz), 3.78 (2H, t, J = 5.2 Hz), 3.71 – 3.62 (10H, m), 3.58 – 3.52 (8H, m); 13C NMR (CD$_3$OD, 176 MHz) δ 164.8, 163.6, 159.4, 146.7, 145.9, 145.7, 145.5, 138.5, 136.9, 125.9, 123.2, 122.6, 108.5, 100.2, 71.6, 71.44, 71.39, 71.3, 70.84, 70.78, 70.3, 69.8, 65.0, 51.4, 44.8, 42.0; HRMS (ESI) [M+H]$^+$ required for C$_{27}$H$_{37}$N$_{10}$O$_8$ 629.2769, found 629.2819.

N-(2-(2-(2-azidoethoxy)ethoxy)ethoxy)ethyl)-5-((3aR,4R,6aS)-2-oxohexahydro-1H-thieno[3,4-d]imidazol-4-yl)pentanamide 12

A solution of biotin (460 mmol, 1.89 mmol), EDC·HCl (725 mg, 3.78 mmol), DMAP (460 mg, 3.76 mmol) and 1Pr$_2$EtN (983 µL, 5.65 mmol) in DMF (15 mL) was stirred at rt for 20 min. To this solution, 2-(2-(2-(azidoethoxy)ethoxy)ethoxy)ethan-1-amine (557 µL, 2.81 mmol) was added before stirring at rt for 15 h. The solution was concentrated in vacuo and the resulting residue was purified via flash column chromatography (neat CH$_2$Cl$_2$ with 1% AcOH → 1:10 MeOH/CH$_2$Cl$_2$ with 1% AcOH) to give biotin-azide 12 (686 mg, 1.54 mmol, 84%) as a white solid.

Rf (SiO$_2$; CH$_2$Cl$_2$/MeOH, 10:1) 0.30; υ$_{max}$ (neat/cm$^{-1}$) 2103, 1682, 1639; 1H NMR (CD$_3$OD, 400 MHz) δ 4.50 (1H, ddd, J = 7.9, 5.0, 1.0 Hz), 4.31 (1H, dd, J = 7.9, 4.4 Hz), 3.71 – 3.60 (10H, m), 3.55 (2H, t, J = 5.5 Hz), 3.40 – 3.34 (4H, m), 3.21 (1H, ddd, J = 8.8, 5.9, 4.5 Hz), 2.93 (1H, dd, J = 12.8, 5.0 Hz), 2.71 (1H, d, J = 12.7 Hz), 2.22 (2H, t, J = 7.4 Hz), 1.80 – 1.53 (4H, m), 1.44 (2H, qu, J = 7.5 Hz); 13C NMR (CD$_3$OD, 101 MHz) δ 174.7, 164.7, 70.24, 70.21, 70.1, 69.9, 69.7, 69.2, 62.0, 60.2, 55.6, 50.4, 39.7, 39.0, 35.3, 28.4, 28.1, 25.5; HRMS (ESI) [M+Na]$^+$ required for C$_{18}$H$_{32}$N$_6$O$_5$SNa$^+$ 467.2047, found 467.1966.
A solution of 8 (30 mg, 0.12 mmol), 12 (107 mg, 0.243 mmol) CuSO₄·5H₂O (37 mg, 0.15 mmol), THPTA (158 mg, 0.364 mmol) and sodium ascorbate (121 mg, 0.607 mmol) in tBuOH/CH₂Cl₂/H₂O (1:1:1, 6 mL) was stirred at rt for 15 h. To this solution, EDTA (100 mg, 0.240 mmol) was added, and the solution was stirred at rt for 1 h. The resulting solution was concentrated under a stream of N₂, and the resulting residue was purified by reverse phase flash column chromatography (10 – 90% solvent B in solvent A. Solvent A: 0.1 M aqueous ammonium hydroxide. Solvent B: MeCN) and lyophilised to give vinylpyrimidine-biotin 10 (19 mg, 0.028 mmol, 23%) as a white solid.

¹H NMR (CD₃OD, 400 MHz) δ 8.20 (1H, d, J = 5.1 Hz), 8.04 (1H, s), 6.66 (1H, d, J = 5.2 Hz), 6.60 (1H, dd, J = 17.4, 10.6 Hz), 6.37 (1H, dd, J = 17.5, 1.5 Hz), 5.60 (1H, dd, J = 10.7, 1.5 Hz), 4.64 (2H, s), 4.58 (2H, dd, J = 5.6, 4.6 Hz), 4.48 (1H, ddd, J = 7.9, 5.0, 1.0 Hz), 4.29 (1H, dd, J = 7.9, 4.4 Hz), 3.89 (2H, dd, J = 5.6, 4.6 Hz), 3.70 – 3.57 (16H, m), 3.52 (2H, t, J = 5.5 Hz), 3.34 (2H, t, J = 5.5 Hz), 3.19 (1H, ddd, J = 8.9, 5.9, 4.4 Hz), 2.91 (1H, dd, J = 12.7, 5.0 Hz), 2.69 (1H, d, J = 12.7 Hz), 2.19 (2H, t, J = 7.5 Hz), 1.79 – 1.52 (4H, m), 1.49 – 1.34 (2H, m); ¹³C NMR (CD₃OD, 101 MHz) δ 176.1, 166.1, 164.9, 163.7, 159.5, 145.8, 137.0, 125.9, 122.6, 108.5, 71.55, 71.46, 71.4, 71.3, 71.2, 70.84, 70.82, 70.6, 70.4, 65.1, 63.4, 61.6, 57.0, 51.4, 42.0, 41.0, 40.3, 36.7, 29.8, 29.5, 26.8; HRMS (ESI) [M+H]+ required for C₃₁H₅₀N₁₀O₇S⁺ 692.3549, found 692.3698.

4-Chloro-N-(2-(2-(prop-2-yn-1-yloxy)ethoxy)ethyl)-1,3,5-triazin-2-amine S4

2-(2-(Prop-2-yn-1-yloxy)ethoxy)ethan-1-amine (99 µL, 0.698 mmol) was added to a solution of 2,4-dichloro-1,3,5-triazine (104 mg, 0.698 mmol) and 'Pr₂EtN (365 µL, 0.838 mmol) in THF (5 mL) at 0 °C. The resulting solution was stirred at 0 °C for 2 h. The solution was concentrated in vacuo, and the resulting residue was purified via flash column chromatography
(EtOAc/petroleum ether, 1:2) to give aminotriazine **S4** (90 mg, 0.362 mmol, 52%) as a colourless oil.

**Rr** (SiO$_2$; EtOAc/petroleum ether, 1:1) 0.22; $\nu_{\text{max}}$ (neat/cm$^{-1}$) 3279, 2118, 1587, 1561; $^1$H NMR (CDCl$_3$, 400 MHz, mixture of rotamers) $\delta$ 8.37 (1H, app d, $J = 29.6$ Hz), 6.40 (1H, app d, $J = 73.1$ Hz), 4.22 (2H, app dd, $J = 2.4$, 1.3 Hz), 3.76 – 3.62 (8H, m), 2.46 (1H, app td, $J = 2.4$, 1.0 Hz); $^{13}$C NMR (CDCl$_3$, 101 MHz, mixture of rotamers) $\delta$ 170.9, 170.1, 167.4, 166.7, 165.7, 165.6, 79.54, 79.53, 74.9, 70.5, 70.4, 69.31, 69.29, 69.17, 69.16, 58.58, 58.58, 41.2, 41.0; HRMS (ESI) [M+H]$^+$ required for C$_{10}$H$_{14}$ClN$_4$O$_2$+ 257.0800, found 257.0795.

**N-(2-(2-(Prop-2-yn-1-yloxy)ethoxy)ethyl)-4-vinyl-1,3,5-triazin-2-amine 11**

A mixture of **S4** (369 mg, 1.44 mmol), potassium vinyltrifluoroborate (630 mg, 4.71 mmol), Cs$_2$CO$_3$ (3.07 g, 9.42 mmol) and Pd(dppf)Cl$_2$-CH$_2$Cl$_2$ (128 mg, 0.157 mmol) in dioxane/H$_2$O (10:1, 8 mL) was heated to 90 °C for 15 h. The resulting mixture was cooled to rt, and EDTA (300 mg, 0.721 mmol) was added. The suspension was stirred at rt for 1 h, filtered through Celite®, and the filtrate was concentrated *in vacuo*. The resulting residue was purified *via* flash column chromatography (EtOAc/petroleum ether, 1:1) to give vinyltriazine **11** (200 mg, 0.805 mmol, 56%) as a colourless oil.

**Rr** (SiO$_2$; EtOAc) 0.58; $\nu_{\text{max}}$ (neat/cm$^{-1}$) 1591; $^1$H NMR (CDCl$_3$, 400 MHz, mixture of rotamers) $\delta$ 8.53 (1H, app d, $J = 31.0$ Hz), 6.73 – 6.47 (2H, m), 6.11 (1H, app d, $J = 44.5$ Hz, NH), 5.79 (1H, dd, $J = 10.3$, 2.2 Hz), 4.22 (2H, d, $J = 2.4$ Hz), 3.69 (8H, ddd, $J = 8.6$, 6.2, 3.4 Hz), 2.46 (1H, t, $J = 2.4$ Hz); $^{13}$C NMR (CDCl$_3$, 101 MHz, mixture of rotamers) $\delta$ 170.8, 170.5, 166.4, 166.1, 165.4, 165.3, 136.0, 135.6, 126.9, 79.6, 74.9, 70.4, 69.7, 69.2, 58.6, 40.8, 40.6.
Azide-PEG₃-arylsulfate-MMAE 14

A degassed solution of CuSO₄·5H₂O (0.24 mg, 0.95 μmol), THPTA (0.83 mg, 1.9 μmol) and sodium ascorbate (0.76 mg, 3.8 μmol) in H₂O/tBuOH (0.2 mL, 1:1) was added to a degassed solution of alkynyl-arylsulfate-MMAE¹² (2.00 mg, 1.91 μmol) and N₃-PEG₃-N₃ (1.99 μL, 9.54 μmol) in H₂O/tBuOH (0.1 mL, 1:1). The reaction mixture was stirred at rt for 15 min before being purified by reverse-phase flash column chromatography (25-100% solvent B in solvent A. Solvent A: 0.1 M NH₄OH (aq). Solvent B: MeCN) and lyophilised to yield azide 14 (1.56 mg, 1.21 μmol, 63%) as a white solid.

LRMS (ESI) m/z found [M-H⁺] 1274.0 C₅₈H₈₉N₁₂O₁₈³²S⁻ required 1273.6; HPLC (5-95% MeCN/H₂O over 20 min) retention time 12.180 min.

1-(2-(2-(Prop-2-yn-1-yloxy)ethoxy)ethyl)-1H-pyrrole-2,5-dione 15

A solution of 2-(2-(prop-2-yn-1-yloxy)ethoxy)ethan-1-amine (122 mg, 0.852 mmol) and maleic anhydride (84 mg, 0.85 mmol) was heated under reflux for 15 h. The resulting solution was concentrated in vacuo and purified via flash column chromatography (EtOAc/petroleum ether, 1:2) to give maleimide 15 (94 mg, 0.42 mmol, 49%) as a light yellow oil.

Rᵣ (SiO₂; EtOAc/petroleum ether, 1:1) 0.47; νmax (neat/cm⁻¹) 1699; ¹H NMR (CDCl₃, 400 MHz) δ 6.72 (2H, s), 4.17 (2H, d, J = 2.4 Hz), 3.74 (2H, app td, J = 5.6, 0.9 Hz), 3.67 – 3.62 (6H, m), 2.44 (1H, t, J = 2.4 Hz); ¹³C NMR (CDCl₃, 101 MHz) δ 170.7, 134.2, 79.7, 74.6, 69.9, 69.1, 67.9, 58.4, 37.1; HRMS (ESI) [M+H]^+ required for C₁₁H₁₄NO₄⁺ 224.0918, found 224.1000.

¹² This compound was synthesised following a literature procedure.

Bargh, J. D.; Walsh, S. J.; Isidro-Llobet, A.; Omarjee, S.; Carroll, J. S.; Spring, D. R. Chem. Sci. 2020, 11, 2375.
10. Spectra of small molecules

4-Vinylpyridin-2-amine 1
4-Vinylpyrimidin-2-amine 2
4-Chloro-1,3,5-triazin-2-amine S1
4-Vinyl-1,3,5-triazin-2-amine 3
6-Vinyl-1,2,4,5-tetrazin-3-amine 4
4-(2-((2,6-Difluorobenzyl)thio)ethyl)pyrimidin-2-amine 5
4-(2-((2,6-Difluorobenzyl)thio)ethyl)-1,3,5-triazin-2-amine 6
3-((2,6-Difluorobenzyl)thio)-1-methylpyrrolidine-2,5-dione 7
4-Chloro-N-(2-(2-(prop-2-yn-1-yl)oxy)ethoxy)ethyl)pyrimidin-2-amine S2
$N$-(2-(2-(Prop-2-yn-1-ylxy)ethoxy)ethyl)-4-vinylpyrimidin-2-amine 8
$N$-(2-(2-(2-Azidoethoxy)ethoxy)ethoxy)ethyl)-7-nitrobenzo[\textit{c}][1,2,5]oxadiazol-4-amine

S3
7-Nitro-N-(2-(2-(2-((4-vinylpyrimidin-2-yl)amino)ethoxy)ethoxy)methyl)-1H-1,2,3-triazol-1-yl(ethoxy)ethoxy)ethoxy)ethyl)benzo[c][1,2,5]oxadiazol-4-amine 9
$N$-(2-(2-(2-azidoethoxy)ethoxy)ethoxy)ethyl)-5-((3aR,4R,6aS)-2-oxohexahydro-1H-thieno[3,4-d]imidazol-4-yl)pentanamide 12
5-((3aS,4S,6aR)-2-Oxohexahydro-1H-thieno[3,4-d]imidazol-4-yl)-N-(2-(2-(4-((2-((4-vinylpyrimidin-2-yl)amino)ethoxy)ethoxy)methyl)-1H-1,2,3-triazol-1-yl)ethoxy)ethoxy)ethyl)pentanamide 10
4-Chloro-N-(2-(2-(prop-2-yn-1-yl)oxy)ethoxy)ethyl)-1,3,5-triazin-2-amine S4
$N$-(2-(2-(Prop-2-yn-1-yloxy)ethoxy)ethyl)-4-vinyl-1,3,5-triazin-2-amine 11
1-(2-(2-(Prop-2-yn-1-yloxy)ethoxy)ethyl)-1H-pyrrole-2,5-dione 15
11. HPLC Traces

Azide-PEG₃-arylsulfate-MMAE 14

Absorbance at 254 nm. Solvent system: 5-95% MeCN/H₂O over 20 min.