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

Efficient and Selective Antibody Modification with Functionalised Divinyltriazines

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Supplementary Information

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General Experimental Information

All solvents and reagents were used as received unless otherwise stated. Ethyl acetate and dichloromethane were distilled from calcium hydride. Petroleum ether (PE) 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 an 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 F_{254} and visualized by quenching of UV fluorescence (λ_{max} = 254 nm) or by staining with potassium permanganate. Retention factors (R_f) are quoted to 0.01. LC-MS was carried out using a Waters ACQUITY H-Class UPLC with an ESCi Multi-Mode Ionisation Waters SQ Detector 2 spectrometer using MassLynx 4.1 software; EI refers to the electrospray ionisation technique; LC system: solvent A: 2 mM NH_4OAc in H_2O/MeCN (95:5); solvent B: MeCN; solvent C: 2% formic acid; column: ACQUITY UPLC® CSH C18 (2.1 mm × 50 mm, 1.7 μm, 130 Å) at 40 °C; gradient: 5 – 95% B with constant 5% C over 1 min at a 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 SiO_2 (230-400 mesh) under a positive pressure of nitrogen.

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 Ultrashield (500 MHz, 126 MHz). In proton NMR, chemical shifts (δ) are reported in parts per million (ppm), to the nearest 0.01 ppm and are referenced to the residual non-deuterated

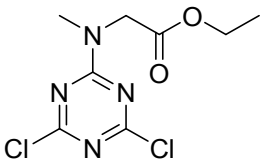
solvent peak ($CDCl_3$: 7.26, DMSO-d_6: 2.50). Coupling constants (J) are reported in Hertz (Hz) to the nearest 0.1 Hz. Data are reported as follows: chemical shift, multiplicity (s = singlet; d = doublet; t = triplet; q = quartet; qn = quintet; sep = septet; m = multiplet; or as a combination of these, e.g. dd, dt *etc.*), integration and coupling constant(s). In carbon NMR, chemical shifts (δ) are quoted in ppm, to the nearest 0.1 ppm, and are referenced to the residual non-deuterated solvent peak ($CDCl_3$: 77.16, DMSO-d_6, 39.52).

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

Chemical Synthetic Procedures

Ethyl *N*-(4,6-dichloro-1,3,5-triazin-2-yl)-*N*-methylglycinate (6)

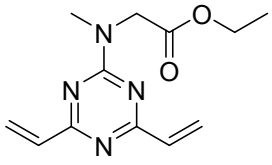


To a solution of cyanuric chloride (500 mg, 2.71 mmol) and sarcosine ethyl ester hydrochloride (416 mg, 2.71 mmol) in acetone (10.0 mL) at 0 °C was added dropwise DIPEA (0.950 mL, 5.45 mmol). The mixture was stirred at 0 °C for 2 h. The mixture was then poured into ice-water and extracted with CH_2Cl_2 (3 × 25 mL). The organic layers were combined and dried over anhydrous $MgSO_4$, filtered, and concentrated *in vacuo*. The residue was purified *via* column chromatography (SiO_2, EtOAc/Petroleum Ether = 1:4) to yield ester **6** as a white solid (358 mg, 1.36 mmol, 50%).

R_f 0.43 (SiO_2, EtOAc/Petroleum Ether = 1:4). **IR** ν_{max} (neat/cm^{-1}) 2983 (w, C-H), 1744 (s, C=O), 1547, 1479, 1234, 1200, 1170. **^{1}H NMR** (400 MHz, $CDCl_3$) δ 4.35 (s, 2H), 4.22 (q, 2H, *J* = 7.1 Hz), 3.25 (s, 3H), 1.28 (t, 3H, *J* = 7.1 Hz). **^{13}C NMR** (101 MHz, $CDCl_3$) δ 170.5, 170.1, 168.1, 165.6, 61.9, 50.9, 36.6, 14.3. **LRMS** (ESI+) *m*/*z* 265.2 $[M+H]^+$.

The spectroscopic data are in agreement with literature.[1]

Ethyl *N*-(4,6-divinyl-1,3,5-triazin-2-yl)-*N*-methylglycinate (7)

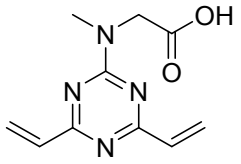


A mixture of ester **6** (500 mg, 1.89 mmol), potassium vinyltrifluoroborate (760 mg, 5.67 mmol) and K_2CO_3 (1.60 g, 11.6 mmol) in 1,4-dioxane (20 mL) and H_2O (2 mL) was degassed with N_2 for 10 min at room temperature. $PdCl_2(dppf)\cdot CH_2Cl_2$ (160 mg, 0.200 mmol) was added and the reaction mixture heated to 90 °C with stirring for 16 h. The reaction mixture was cooled to room temperature, filtered through Celite under vacuum, washing with EtOAc (1 × 30 mL). The filtrate was concentrated *in vacuo* and purified *via* column chromatography (SiO_2, EtOAc/Petroleum Ether = 1:4) to yield DVT **7** as a pale yellow oil (368 mg, 1.48 mmol, 78%).

R_f 0.51 (SiO_2, EtOAc/Petroleum Ether = 1:4). **IR** ν_{max} (neat/cm^{-1}) 2981 (w, C-H), 1747 (s, C=O), 1639 (w, C=C), 1542, 1513, 1422, 1407, 1196, 846. **^{1}H NMR*** (400 MHz, $CDCl_3$) δ 6.75 – 6.50 (m, 4H), 5.76 – 5.67 (m, 2H), 4.35 (s, 2H), 4.18 (q, 2H, *J* = 7.1 Hz), 3.26 (s, 3H), 1.22 (t, 3H, *J* = 7.1 Hz). **^{13}C NMR*** (101 MHz, $CDCl_3$) δ 170.8, 170.3, 169.7, 165.6, 136.3, 136.2, 126.2, 126.0, 61.1, 50.7, 35.7, 14.3. **LRMS** (ESI+) *m*/*z* 249.3 $[M+H]^+$.

* Extra peaks due to the presence of rotameric forms due to restricted rotation about the C-N bond external to the triazine ring. The spectroscopic data are in agreement with literature.[1]

***N*-(4,6-Divinyl-1,3,5-triazin-2-yl)-*N*-methylglycine (1)**

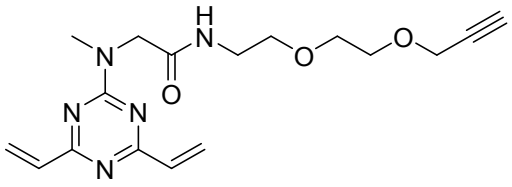


To a solution of ester **5** (147 mg, 0.590 mmol) in THF (4 mL) and H_2O (4 mL) at 0 °C was added $LiOH·H_2O$ (33.0 mg, 0.780 mmol). The mixture was stirred at room temperature for 24 h, and then diluted with H_2O (10 mL) and washed with Et_2O (10 mL). The aqueous layer was carefully neutralized with 1M HCl, and extracted with CH_2Cl_2 (4 × 20 mL). The organic layer was dried over anhydrous Na_2SO_4, filtered, concentrated *in vacuo*, and triturated with petroleum ether to yield DVT **1** as a white solid (110 mg, 0.500 mmol, 85%).

R_f 0.35 (SiO_2, MeOH/$CHCl_3$ = 1:4). **IR** ν_{max} (neat/cm^{-1}) 2917 (w, C-H), 1711 (m, C=O), 1640 (m, C=C), 1541, 1507, 1406, 1224, 1200, 1095, 1029. **^{1}H NMR*** (400 MHz, DMSO-d_6) δ 12.77 (br s, 1H), 6.73 – 6.47 (m, 4H), 5.87 – 5.79 (m, 2H), 4.35 (s, 2H), 3.20 (s, 3H). **^{13}C NMR*** (101 MHz, DMSO-d_6) δ 170.7, 170.0, 169.5, 165.1, 136.0, 135.9, 126.8, 126.7, 50.0, 35.4. **LRMS** (ESI+) *m*/*z* 221.1 $[M+H]^+$.

* Extra peaks due to the presence of rotameric forms due to restricted rotation about the C-N bond external to the triazine ring. The spectroscopic data are in agreement with literature.[1]

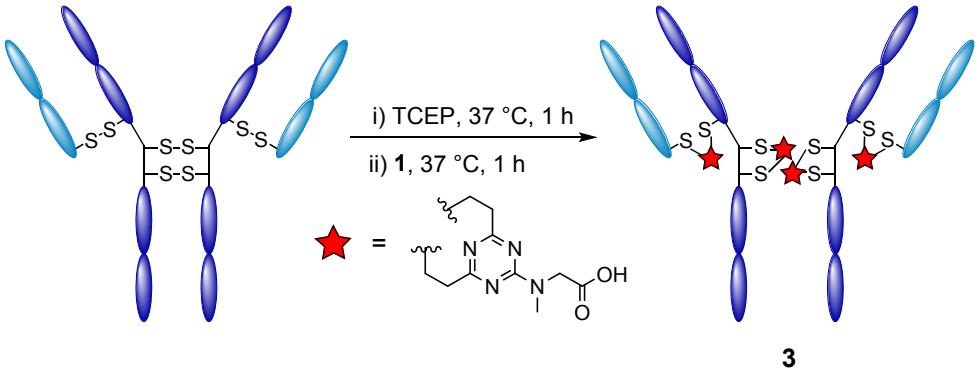
2-((4,6-Divinyl-1,3,5-triazin-2-yl)(methyl)amino)-N-(2-(2-(prop-2-yn-1-yloxy)ethoxy)ethyl)acetamide (2)



DVT **1** (50.0 mg, 0.230 mmol) was dissolved in DMF (3 mL) and cooled to 0 °C. TEA (31.7 μL, 0.230 mmol), EDC·HCl (43.5 mg, 0.230 mmol) and HOBt·H_2O (34.8 mg, 0.230 mmol) were added successively. H_2N-PEG_2-Alkyne (35.4 μL, 0.250 mmol) was then added and the solution allowed to warm to room temperature and left to stir for 17 h. The reaction mixture was diluted with H_2O and extracted with CH_2Cl_2 (4 × 20 mL). The organic phases were combined and washed with brine (1 × 20 mL), dried over anhydrous $MgSO_4$, filtered, and concentrated *in vacuo*. The residue was purified *via* column chromatography (SiO_2, EtOAc) to yield alkynyl DVT **2** as a white powder (34.8 mg, 0.100 mmol, 44%).

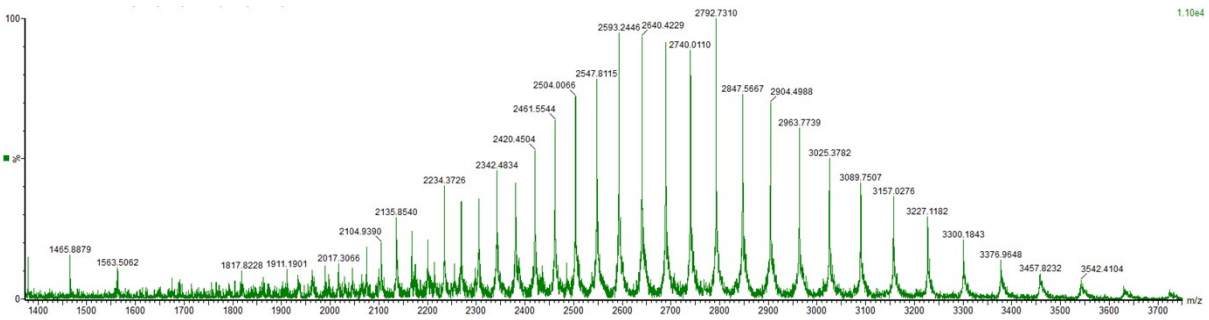
R_f (SiO_2, EtOAc) 0.49. **IR** ν_{max} (neat/cm^{-1}) 3298, 2868, 1664, 1550, 1511, 1423, 1406, 1356, 1283, 1194, 1099, 1027, 991, 956, 845. **^{1}H NMR** ($CDCl_3$, 400 MHz) 6.78—6.51 (m, 5H), 5.78 (m, 2H), 4.31 (s, 2H), 4.12 (d, 2H, J = 2.4 Hz), 3.58—3.44 (m, 8H), 3.30 (s, 3H), 2.41 (t, 1H, J = 2.4 Hz). **^{13}C NMR** ($CDCl_3$, 101 MHz) 169.1, 165.7, 136.1, 126.7, 79.6, 74.1, 70.2, 69.9, 69.0, 58.5, 53.2, 39.2, 35.9. **HRMS** (ESI+) *m/z* found $[M+H]^+$ 346.1881, $C_{17}H_{24}N_5O_3^+$ calc. 346.1874.

Trastuzumab and triazine **1** conjugation

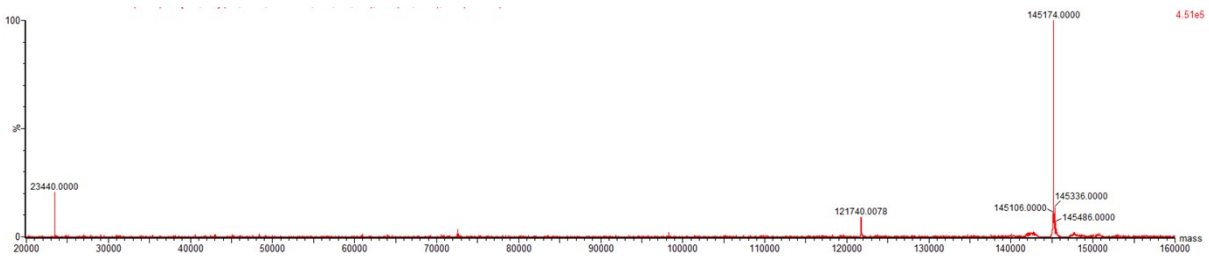


A solution of trastuzumab (49.2 µL, 48.4 µM) in Tris buffer (25 mM Tris HCl, 25 mM NaCl, 0.5 mM EDTA, pH 8) was diluted with Tris buffer (90.8 µL) and TCEP (4.76 µL, 5 mM) added. The mixture was vortexed, incubated at 37 °C for 1 h, and then passed through a Zeba Spin Desalting Column (7000 MWCO, Thermo Scientific). A solution of triazine **1** (0.34 µL, 10 mM in DMSO) was added to the filtrate and incubated at 37 °C for 1 h. The solution was purified by repeated diafiltration into PBS using an Amicron-Ultra centrifugal filter (10000 MWCO, Merck Millipore). LC-MS, SDS-PAGE demonstrated conversation to the bridged conjugate.

a)



b)



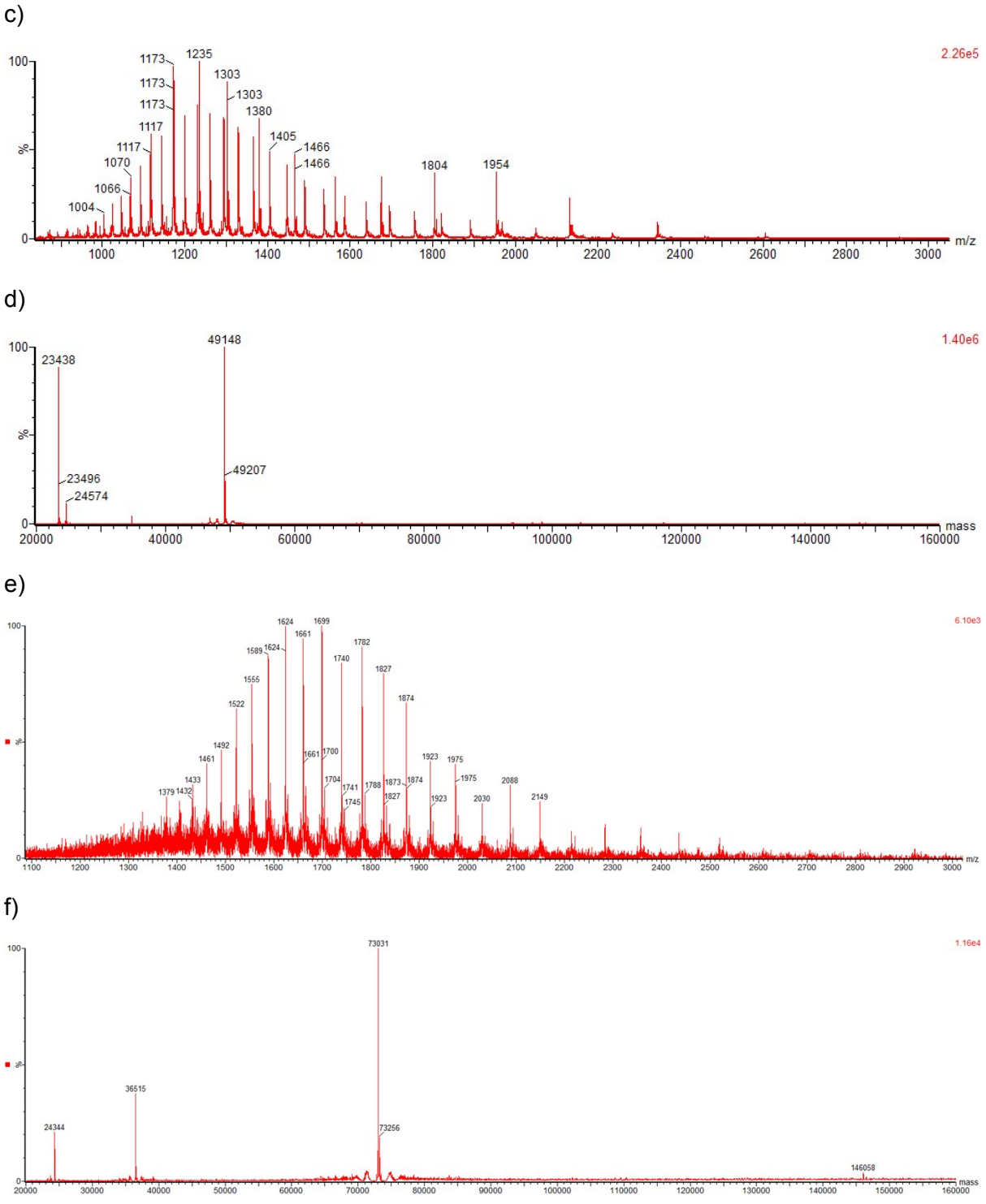


Fig. S1 - LC-MS analysis of reaction between trastuzumab and triazine **1**: a) non-deconvoluted MS of trastuzumab; b) deconvoluted MS of trastuzumab; c) non-deconvoluted MS of trastuzumab following TCEP reduction; d) deconvoluted MS of trastuzumab following TCEP reduction – observed 49148 (HC), 23438 (LC) Da; e) non-deconvoluted MS of trastuzumab conjugate **3**; f) deconvoluted MS of trastuzumab conjugate **3**. Expected 146056, 73028 Da, observed 146058, 73031 Da.

Trastuzumab and triazine **1** conjugation optimisation

Reactions were carried out as described in the previous section under the conditions described in Table 1. At the time points indicated, aliquots were removed, diluted, flash frozen in liquid N_2 and stored at -20 °C until analysis.

Table S1 - Initial screening of conditions for the rebridging conjugation of trastuzumab with triazine **1**. Trastuzumab concentration 2.5 mg/mL, 10% DMSO/Tris (25 mM Tris HCl, 25 mM NaCl, 0.5 mM EDTA, pH 8). Aliquots were removed, diluted, flash frozen and stored at -20 °C until analysis.

Entry	Temp. (°C)	Linker equiv.	Time (h)
1	37	10	1, 2, 4
2	37	20	1, 2, 4
3	37	40	1, 2, 4
4	37	80	1, 2, 4
5	21	10	1, 2, 4
6	21	20	1, 2, 4
7	21	40	1, 2, 4
8	21	80	1, 2, 4
9	4	10	1, 2, 4
10	4	20	1, 2, 4
11	4	40	1, 2, 4
12	4	80	1, 2, 4
13*	37	10	1 ,2, 4, 8, 24
14*	37	20	1 ,2, 4, 8, 24
15*	37	40	1 ,2, 4, 8, 24
16*	37	80	1 ,2, 4, 8, 24
17*	21	10	1 ,2, 4, 8, 24
18*	21	20	1 ,2, 4, 8, 24
19*	21	40	1 ,2, 4, 8, 24
20*	21	80	1 ,2, 4, 8, 24

* indicates TCEP was removed *via* filtration prior to linker addition

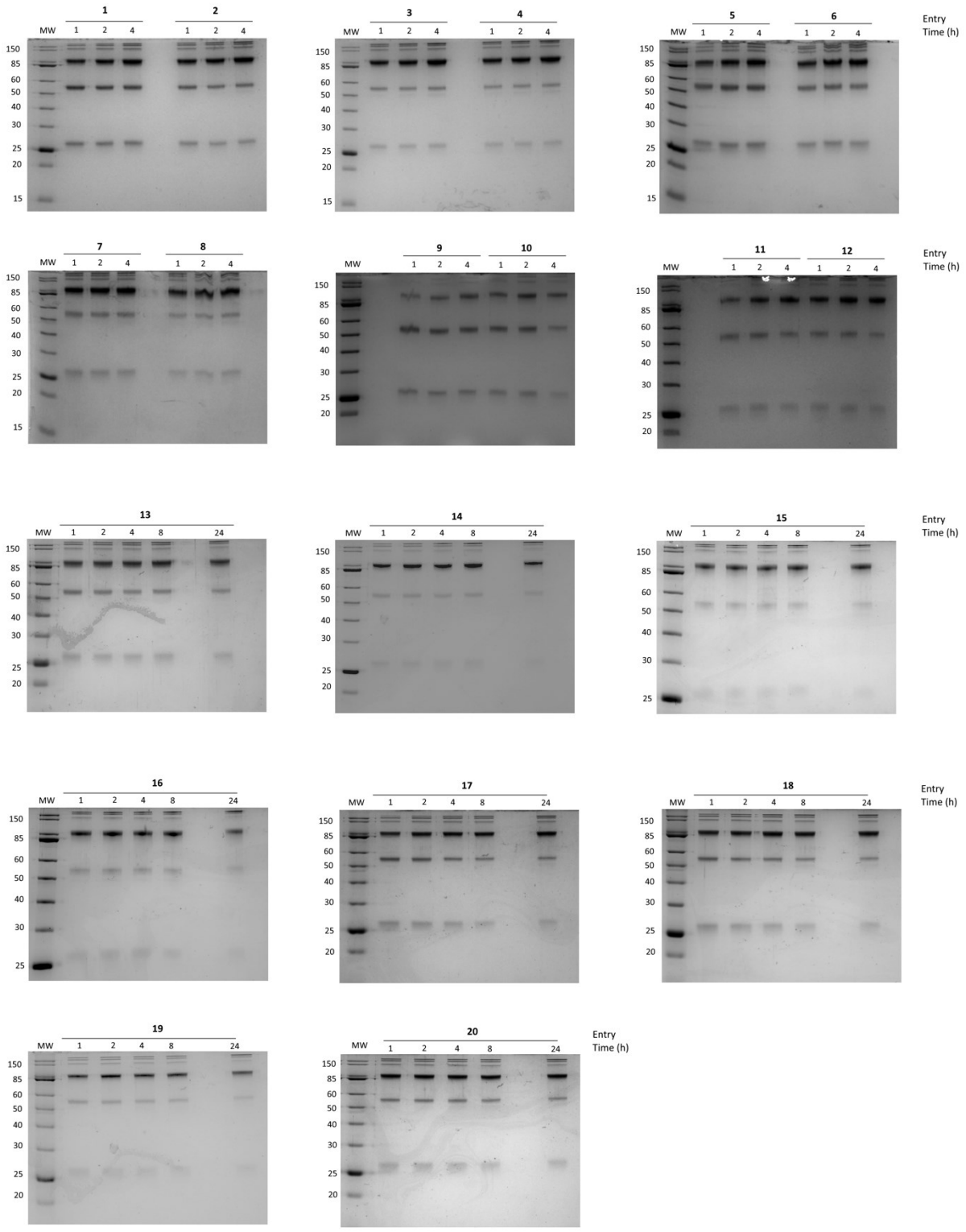


Fig. S2 - SDS-PAGE analysis of the initial screen of conditions in Table S1. All samples were reduced with loading dye containing β-mercaptoethanol. The lanes are labelled according to the entry in Table 1 and the time the aliquot was removed from the reaction and frozen until analysis.

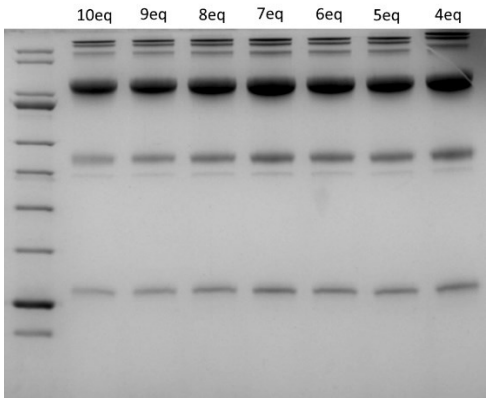
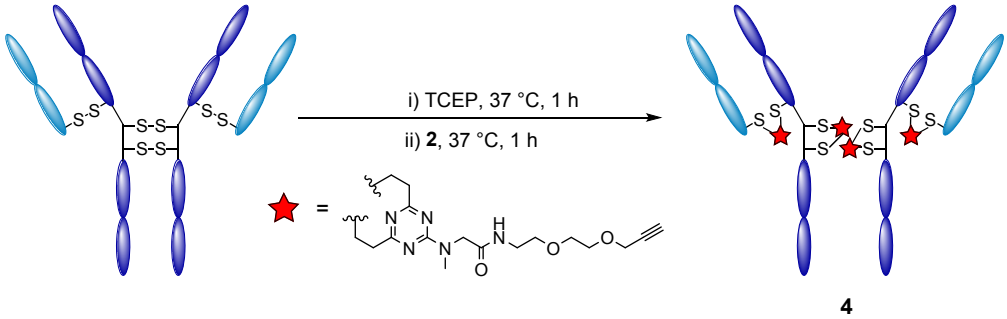


Fig. S3 – SDS-PAGE analysis following conjugation of reduced trastuzumab (following purification with a desalting column) and triazine **1** (equivalents specified above lane).

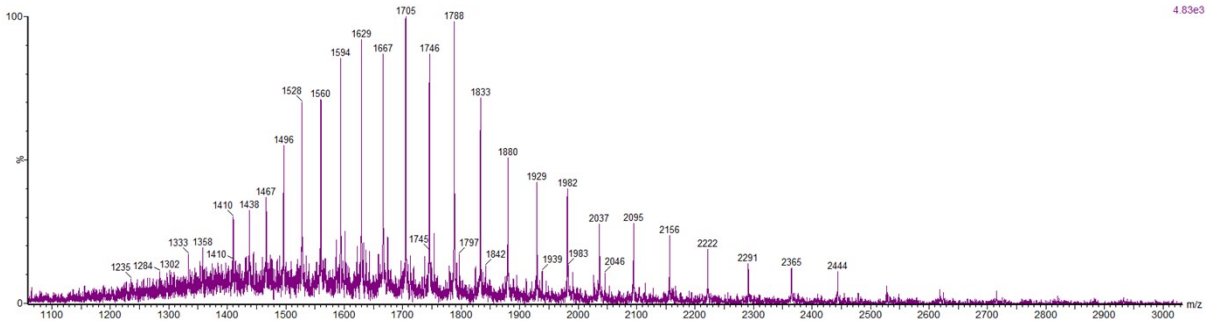
Trastuzumab and triazine **2** conjugation



4

A solution of trastuzumab (133 µL, 75 µM) in Tris buffer (25 mM Tris HCl, 25 mM NaCl, 0.5 mM EDTA, pH 8) was diluted with Tris buffer (260 µL) and TCEP (20 µL, 5 mM) added. The mixture was vortexed, incubated at 37 °C for 1 h, and then passed through a Zeba Spin Desalting Column (7000 MWCO, Thermo Scientific). A solution of triazine **2** (6.59 µL, 10 mM in DMSO) was added to the diluted filtrate (390 uL, 50.7 uM) and incubated at 37 °C for 1 h. The solution was purified by repeated diafiltration into PBS using an Amicron-Ultra centrifugal filter (10000 MWCO, Merck Millipore). LC-MS, SDS-PAGE demonstrated conversation to the bridged conjugate.

a)



b)

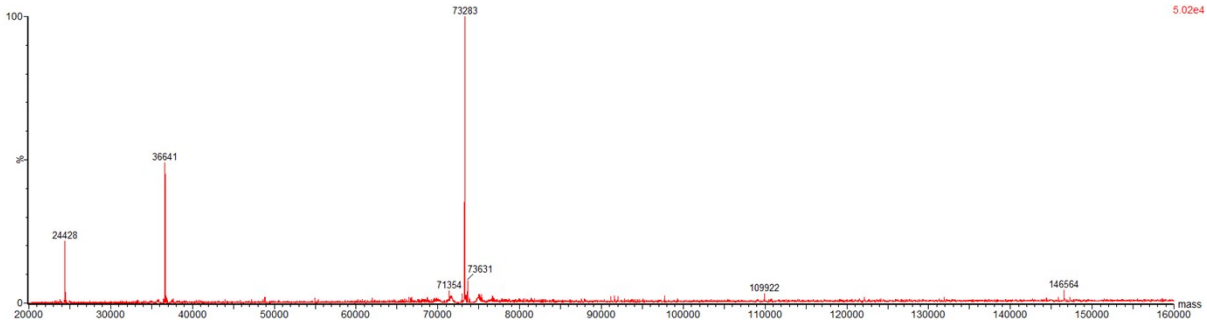
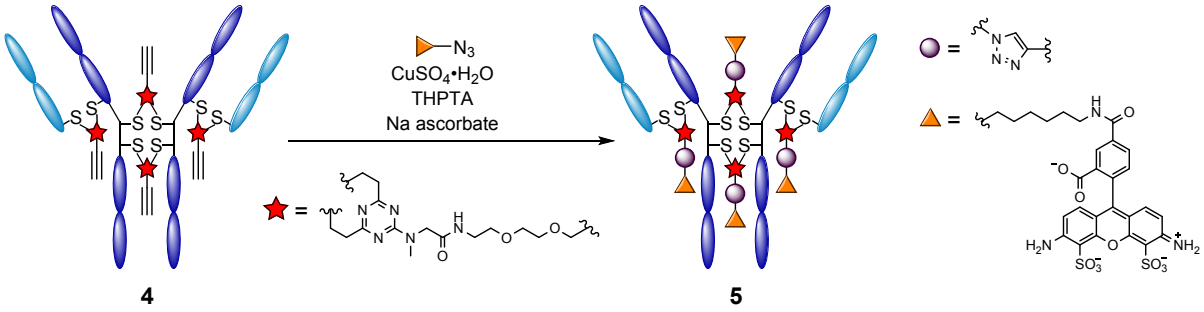


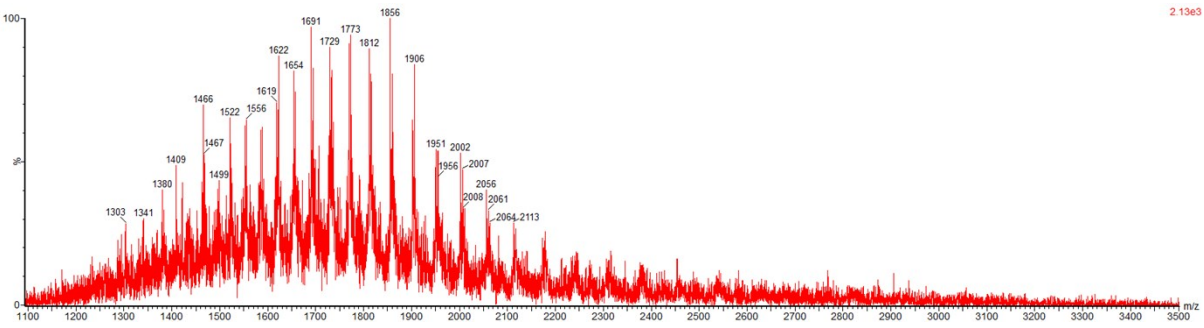
Fig. S4 - LCMS analysis of reaction between trastuzumab and triazine **2**: a) non-deconvoluted MS of trastuzumab conjugate **4**; b) deconvoluted MS of trastuzumab conjugate **4**. Expected 146557, 73278 Da, observed 146564, 73283.

Trastuzumab conjugate **4** - AlexFluor488™ azide CuAAC



To a solution of trastuzumab conjugate **4** (120 µL, 37.2 µM) in PBS was added AlexaFluor™-488 Azide (ThermoFisher) (5 mM in DMSO, final concentration of 290 µM), THPTA (final concentration of 2.42 mM) and sodium ascorbate (final concentration of 3.59 mM). The mixture was vortexed and incubated at 37 °C for 4 h. The excess reagents were removed by repeated diafiltration into PBS using an Amicron-Ultra centrifugal filter (10000 MWCO, Merck Millipore). LC-MS and UV-*vis* evidenced conversion to an antibody-fluorophore conjugate (AFC) with average fluorophore-antibody ratio (FAR) of 4.02.

a)



b)

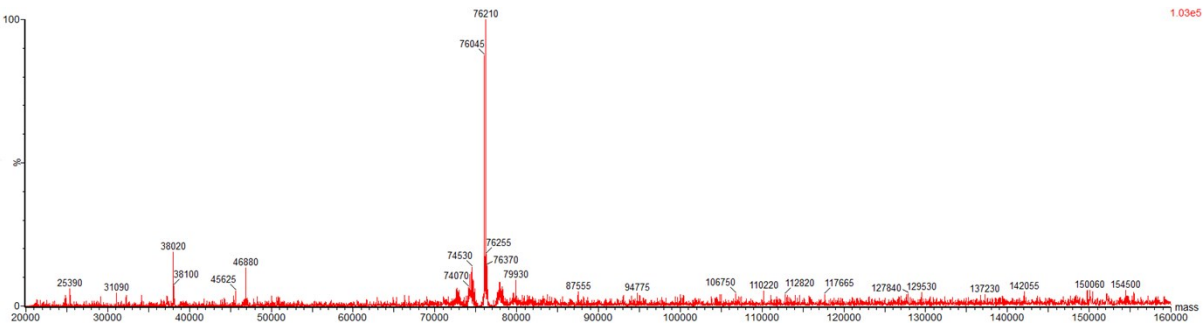


Fig. S5 - LCMS analysis of trastuzumab fluorophore conjugate **5**: a) non-deconvoluted MS; b) deconvoluted MS. Expected 76198/76040 Da, observed 76210/76045 (conjugate was not deglycosylated prior to analysis).

UV-vis FAR Calculation

Sample buffer was used as a baseline for analysis. FAR was calculated using the following formula:

$$FAR = \frac{Abs_{495}/\varepsilon_{495}}{(Abs_{280} - 0.11 \times Abs_{495})/\varepsilon_{280}}$$

where;

Abs_{495} = 1.17

Abs_{280} = 1.01

ε_{495} = 71000 M^{-1} cm^{-1} for AlexaFluor488-Azide

ε_{280} = 215380 M^{-1} cm^{-1} for trastuzumab

and a correction factor of 0.11 for AlexaFluor488-Azide absorption at 280 nm.

NMR Spectra of Novel Compounds

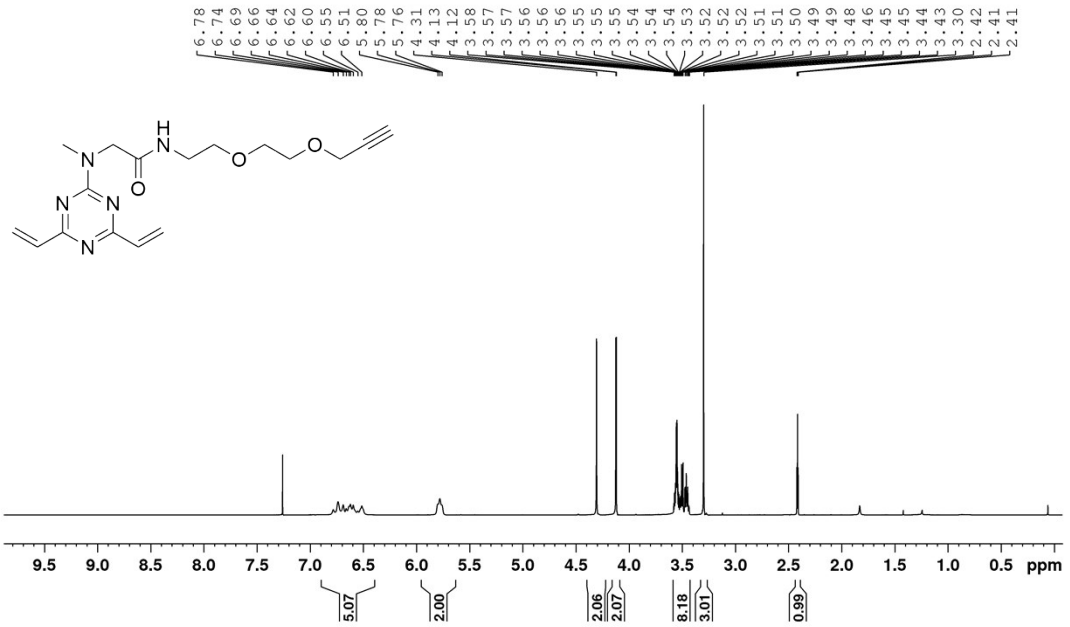


Fig. S6 - ^{1}H spectrum of triazine **2** (400 MHz, $CDCl_3$)

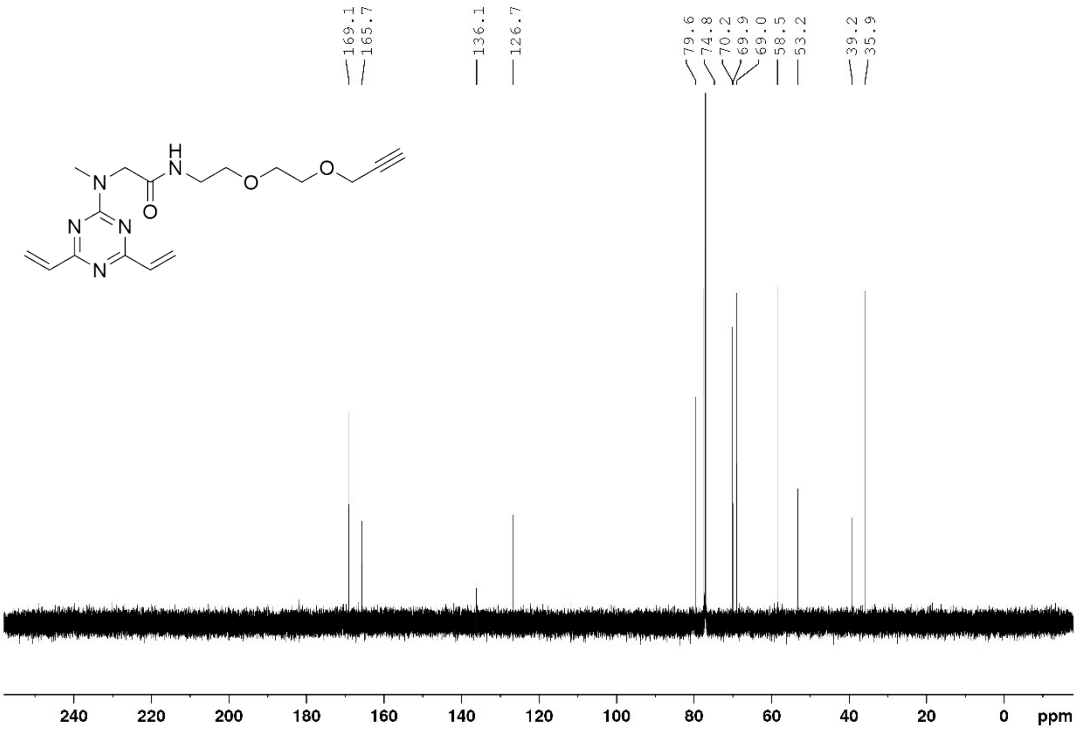


Fig. S7 - ^{13}C spectrum of triazine **2** (100 MHz, $CDCl_3$)

References

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