Electronic Supplementary Information

Optimisation of the dibromomaleimide (DBM) platform for native antibody conjugation by accelerated post-conjugation hydrolysis

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Synthesis General Remarks

All reagents were purchased from Aldrich, AlfaAesar, Sino Biological Inc or Lumiprobe and were used as received. Alexa Fluor 488® Azide (Ref A10266) was purchased from Molecular Probes™ (Thermo Fisher Scientifics). All reactions were monitored by thin-layer chromatography (TLC) on pre-coated SIL G/UV254 silica gel plates (254 μm) purchased from VWR. Flash column chromatography was carried out with Kieselgel 60M 0.04/0.063 mm (200–400 mesh) silica gel. Detection was by UV (254 nm and 365 nm) or chemical stain (KMnO₄, ninhydrin, iodine). Normal phase silica gel, 40-63 μm, (BDH) and sand (VWR) were used for column chromatography. ¹H and ¹³C NMR spectra were recorded at ambient temperature on a Bruker Avance 300 instrument operating at a frequency of 300 MHz for ¹H and 75 MHz for ¹³C, a Bruker Avance 500 instrument operating at a frequency of 500 MHz for ¹H and 125 MHz for ¹³C, and a Bruker Avance 600 instrument operating at a frequency of 600 MHz for ¹H and 150 MHz for ¹³C in CDCl₃ or CD₃OD (as indicated below). The chemical shifts (δ) for ¹H and ¹³C are quoted relative to residual signals of the solvent on the ppm scale. ¹H NMR peaks are reported as singlet (s), doublet (d), triplet (t), quartet (q), doublet of doublets (dd), triplet of doublets (td), triplet of triplets (tt), m (multiplet) and br (broad). Coupling constants (J values) are reported in Hertz (Hz) and are H-H coupling constants unless otherwise stated. Signal multiplicities in ¹³C NMR were determined using the distortionless enhancement by phase transfer (DEPT) spectral editing technique. Infrared spectra were obtained on a Perkin Elmer Spectrum 100 FTIR Spectrometer operating in ATR mode with frequencies given in reciprocal centimetres (cm⁻¹). Melting points were measured with a Gallenkamp apparatus and are uncorrected. Extinction coefficients were determined from the slopes of curves generated from plotting concentration (M) versus absorbance and linear regression analysis. Absorbance spectra were obtained in 10% DMF in acetate buffer pH 4.5.

Compounds 3,4-dithiophenoyl-maleimide-1-N-(prop-2-yn-1-yl)hexanamide¹ (9: ε₄₂₅ = 3054 M⁻¹ cm⁻¹), 3,4-dithiophenoyl-maleimide-1-N-(prop-2-yn-1-yl) p-benzamide¹ (11: ε₄₂₅ = 6428 M⁻¹ cm⁻¹) and the precursor N-(2-aminoethyl)-5-(dimethylamino)naphthalene-1-sulfonamide 2,2,2-trifluoroacetate² were prepared as previously described.
Synthesis and Characterization of Compounds

Scheme S1 - Synthesis of DBMs 1-6

General procedure to prepare 3,4-Dibromo-maleimide acids
This procedure was adapted from one already reported.3 3,4-dibromomaleic acid (250 mg, 913 μmol) in AcOH (5 mL) was refluxed for 1 h. Then, the amino acid (1.1 eq.) was added and the mixture was stirred under reflux for another 3 h. The mixture was allowed to cool to 20 °C. AcOH was removed by concentrating under vacuum and traces of AcOH were removed by adding toluene (10 mL) and concentrating once more. Purification for each compound is described below.

3,4-Dibromo-maleimide-N-hexanoic acid (1)3

Compound 1 (285 mg; 772 μmol; yield: 85%) was isolated as a white solid after purification by column chromatography (MeOH 0% to 10% in CH₂Cl₂ with 1% AcOH). Characterization data: mp = 123-124 °C. IR (solid) νmax 2936, 2868, 1721, 1695, 1589, 1396, 1046, 946, 842, 733. ¹H NMR (600 MHz, CDCl₃) ²H: 1.34 (quint., J = 7.8 Hz, 2H), 1.68 (m, 4H), 2.36 (t, J = 7.4 Hz, 2H), 3.63 (t, J = 7.2 Hz, 2H); ¹³C NMR (150 MHz, CDCl₃) ²C: 24.1 (CH₂), 26.1 (CH₂), 28.3 (CH₂), 33.4 (CH₂), 39.6 (CH₂), 129.5 (C), 164.1 (CO), 177.4 (CO). HRMS (ESI) calcd. for C₁₀H₁₀NO₄Br₂ [M⁺Br⁺Br-H]⁺ 365.8977, observed: 365.8986.
3,4-Dibromo-maleimide-N-acetic acid (2)

Compound 2 (234 mg; 749 µmol; yield: 82%) was isolated as a white solid after purification by column chromatography (MeOH 0% to 10% in CH₂Cl₂ with 1% AcOH). Characterization data: mp = 205-206 °C. IR (solid) νmax 2984, 2872, 1785, 1715, 1583, 1483, 1046, 1409, 1313, 1109. ¹H NMR (600 MHz, CD₃OD) δH: 4.33 (s, 2H); ¹³C NMR (150 MHz, CD₃OD) δC: 40.7 (CH₂), 130.7 (C), 164.8 (CO), 177.4 (CO). LRMS (ESI) 311 (50, [M⁺⁺Br⁺⁺Br⁺⁺]⁺), 313 (100, [M⁺⁺Br⁺⁺Br⁺⁺]⁺), 315 (50, [M⁺⁺Br⁺⁺Br⁺⁺]⁺⁺); HRMS (ESI) calcd. for C₆H₃NO₄Br₂ [M⁺⁺Br⁺⁺Br⁺⁺]⁺ 310.8428, observed: 310.8439.
3,4-Dibromo-maleimide-N-(p-benzoic acid) (3)

Compound 3 (284 mg; 758 μmol; yield: 89%) was isolated as a yellow solid after washing the dry crude residue with cold MeOH (5 mL) and CH₂Cl₂ (5 mL).

Characterization data: mp > 260 °C. IR (solid) νₘₐₓ 2828, 2544, 1778, 1728, 1689, 1591, 1376, 1286, 1100. ¹H NMR (600 MHz, DMSO-d₆) δ_H: 7.52 (d, J = 8.4 Hz, 2H), 8.06 (d, J = 8.4 Hz, 2H), 13.14 (br s, 1H, COOH); ¹³C NMR (150 MHz, DMSO-d₆) δ_C: 126.6 (CH), 129.8 (C), 130.1 (CH), 130.3 (C), 135.4 (C), 163.1 (CO) 166.6 (CO). HRMS (ESI) calcd. for C₁₁H₅NO₄Br₂ [M⁺Br⁺Br⁻]⁺ 372.8579, observed: 372.8583.
3,4-Dibromo-maleimide-N-(prop-2-yn-1-yl)hexanamide (4)

3,4-Dibromo-maleimide-N-hexanoic acid (1) (150 mg, 407 μmol, 1 eq.) and N-ethoxycarbonyl-2-ethoxy-1,2-dihydroquinoline (EEDQ) (120 mg, 488 μmol, 1.2 eq.) were dissolved in MeCN (5 mL) to give a yellow solution that was stirred for 30 min. at 20 °C, followed by addition of propargylamine (23.4 μL, 366 μmol, 0.9 eq.). After stirring at 20 °C for 1 h, the solvent was evaporated and the pale yellow oil was purified by flash chromatography on silica (MeOH 0% to 10% in CH₂Cl₂) to afford the title compound as an off white solid (85 mg, 208 μmol, 57%).

Characterization data: mp = 111-112 °C. IR (solid) νmax 3282, 2937, 1705, 1633, 1594, 1375, 1039, 933. ¹H NMR (600 MHz, CDCl₃) δH: 1.33 (quint., J = 7.5 Hz, 2H), 1.68 (overlapped quint., J = 7.5 Hz, 4H), 2.19 (t, J = 7.5 Hz, 2H), 2.23 (t, J = 2.5 Hz, 1H), 3.61 (t, J = 7.5 Hz, 2H), 4.05 (dd, J = 5.5, 2.5 Hz, 2H); ¹³C NMR (150 MHz, CDCl₃) δC: 24.8 (CH₂), 26.2 (CH₂), 28.2 (CH₂), 29.2 (CH₂), 36.1 (CH₂), 39.6 (CH₂), 71.7 (CH), 79.6 (C), 129.4 (C), 164.1 (CO), 172.2 (CO). LRMS (ESI) 404 (50, [M²⁹Br²⁹Br]+), 406 (100, [M²⁸¹Br²⁹Br]+), 408 (50, [M²⁸¹Br²⁹Br]+); HRMS (ESI) calcd. for C₁₃H₁₄N₂O₃Br₂ [M²⁹Br²⁹Br-H]+ 403.9477, observed: 403.8948. UV (10% DMF in acetate buffer pH 4.5) ε₃₂⁵ = 662 M⁻¹ cm⁻¹
3,4-Dibromo-maleimide-N-(prop-2-yn-1-yl)acetamide (5)

![Chemical structure of 5](image)

3,4-Dibromo-maleimide-N-acetic acid (2) (70 mg, 225 µmol, 1 eq.) and EEDQ (66 mg, 270 µmol, 1.2 eq.) were dissolved in MeCN (3 mL) to give a yellow solution that was stirred for 30 min. at 20 °C, followed by addition of propargylamine (13 µL, 202 µmol, 0.9 eq.). After stirring at 20 °C for 1 h, the solvent was evaporated and the pale yellow oil was purified by flash chromatography on silica (EtOAc 0% to 50% in n-hexane) to afford the title compound as off white solid (40 mg, 114 µmol, 56%).

Characterization data: mp = 191-192 ºC. IR (solid) νmax 3282, 2984, 2846, 1785, 1716, 1686, 1669, 1549, 1369, 1409, 1316, 1106, 959. ¹H NMR (600 MHz, CD₃OD) δH: 2.61 (t, J = 2.6 Hz, 1H), 3.98 (d, J = 2.6 Hz, 2H), 4.26 (s, 2H); ¹³C NMR (150 MHz, CD₃OD) δC: 29.7 (CH₂), 42.0 (CH₂), 72.5 (CH), 80.0 (C), 130.7 (C), 165.0 (CO), 168.4 (CO). LRMS (ESI) 348 (50, [M⁺+2Br⁻]⁺), 350 (100, [M⁺+3Br⁻]⁺), 352 (50, [M⁺+4Br⁻]⁺); HRMS (ESI) calcd. for C₉H₆N₂O₃Br₂ [M⁺+2Br⁻]⁺ 347.8745, observed: 347.8756. UV (10% DMF in acetate buffer pH 4.5) ε₃₂₅ = 935 M⁻¹ cm⁻¹
3,4-Dibromo-maleimide-N-(prop-2-yn-1-yl) p-benzamide (6)

3,4-Dibromo-maleimide-N-(p-Benzonic acid) (3) (153 mg, 409 µmol, 1 eq.) and EEQD (121 mg, 491 µmol, 1.2 eq.) were dissolved in MeCN (5 mL) to give a cloudy solution that was stirred at 20 °C overnight. Propargylamine (23.5 µL, 368 µmol, 0.9 eq.) was added and the reaction mixture was stirred at 20 °C for 1 h. Then, the solvent was evaporated and the pale yellow oil was purified by flash chromatography on silica (EtOAc 0% to 50% in n-hexane) to afford the title compound as an off white solid (50 mg, 121 µmol, 33%).

Characterization data: mp = 206-207 °C. IR (solid) νmax 3281, 2444, 1740, 1721, 1674, 1586, 1371, 1278, 1159. 1H NMR (600 MHz, DMSO-d6) δH: 3.14 (t, J = 2.5 Hz, 1H), 4.08 (dd, J = 5.5, 2.5 Hz, 2H), 7.49 (d, J = 8.4 Hz, 2H), 7.95 (d, J = 8.4 Hz, 2H), 9.02 (s, 1H); 13C NMR (150 MHz, DMSO-d6) δC: 28.6 (CH2), 73.0 (CH), 81.2 (C), 126.6 (CH), 128.3 (CH), 129.8 (C), 134.0 (C), 163.1 (CO) 165.3 (CO). LRMS (ESI) 410 (50, [M79Br79Br]+), 412 (100, [M81Br79Br]+), 414 (50, [M81Br81Br]+); HRMS (ESI) calcd. for C14H8N2O3Br2 [M79Br79Br-H]+ 409.8979, observed: 409.8983.
3,4-Dibromo-maleimide-\textit{N}-(acetamide-\textit{N}-dimethylamino dansyl) (7)

![Chemical structure]

3,4-Dibromo-maleimide-1-acetic acid (2) (25 mg, 80 µmol, 1.6 eq.) and EEDQ (19 mg, 80 µmol, 1.6 eq.) were dissolved in MeCN (5 mL) and stirred at 20 °C for 30 min. Then, \textit{N}-(2-aminoethyl)-5-(dimethylamino)naphthalene-1-sulfonamide 2,2,2-trifluoroacetate (19 mg, 50 µmol, 1 eq.) previously dissolved in MeCN (0.5 mL) and DIPEA (8.7 µL, 50 µmol, 1 eq.) was added and the mixture was stirred at 20 °C for 1 h. The solvent was evaporated and the pale yellow oil was purified by flash chromatography on silica (EtOAc 20% to 100% in n-hexane) to afford the title compound as pale yellow solid (20 mg, 34 µmol, 67%).

Characterization data: mp > 250 °C. IR (solid) ν_{max} 3340, 3285, 1722, 1617, 1586, 1542, 1418, 1392, 1309, 1158, 1139, 1103. \textsuperscript{1}H NMR (600 MHz, CD\textsubscript{3}OD) δ\textsubscript{H}: 2.88 (s, 6H), 2.91 (t, J = 6.3 Hz, 2H), 3.21 (t, J = 6.3 Hz, 2H), 4.16 (s, 2H), 7.28 (dd, J = 7.5, 0.7 Hz, 1H), 7.51 – 7.65 (m, 2H), 8.19 (dd, J = 7.3, 1.2 Hz, 1H), 8.31 (d, J = 8.7 Hz, 1H), 8.56 (s, 1H); \textsuperscript{13}C NMR (150 MHz, CD\textsubscript{3}OD) δ\textsubscript{C}: 40.8 (CH\textsubscript{2}), 42.1 (CH\textsubscript{2}), 42.8 (CH\textsubscript{2}), 45.8 (CH\textsubscript{3}), 116.5 (CH), 120.4 (C), 124.3 (CH), 129.2 (CH), 130.2 (CH), 130.7 (CH), 130.9 (CH), 131.2 (CH), 131.3 (C), 136.6 (C), 153.3 (C), 165.0 (CO), 168.9 (CO).

LRMS (ESI) 586 (50, [M\textsuperscript{79}Br\textsuperscript{79}Br]\textsuperscript{+}), 588 (100, [M\textsuperscript{81}Br\textsuperscript{79}Br]\textsuperscript{+}), 590 (50, [M\textsuperscript{81}Br\textsuperscript{81}Br]\textsuperscript{+}); HRMS (ESI) calcd. for C\textsubscript{20}H\textsubscript{20}N\textsubscript{4}O\textsubscript{5}SBr\textsubscript{2} [M\textsuperscript{79}Br\textsuperscript{79}Br]\textsuperscript{+} 586.9600, observed: 586.9598.
3,4-Dibromo-maleimide-N-(acetamide-N-doxorubicin) (8)

3,4-Dibromo-maleimide-1-acetic acid (2) (18 mg, 57 μmol, 1.5 eq.), EEDQ (14 mg, 57 μmol, 1.5 eq.) were dissolved in MeCN and stirred at 20 °C for 30 min.. Then, doxorubicin.HCl (20 mg, 37 μmol, 1 eq.) previously dissolved in DMF (1.0 mL) and DIPEA (6.5 μL, 38 μmol, 1 eq) was added and the mixture was stirred at 20 °C for 1 h. The solvent was evaporated and the crude residue was dissolved in CH₂Cl₂ (5 mL). The organic layer was washed with 5% citric acid solution (3 x 5 mL), water (3 x 5 mL), dried (NaSO₄), filtered and concentrated under vacuum. The obtained red solid was purified by flash chromatography on silica (EtOAc 50% to 100% in CH₂Cl₂) to afford the title compound as red solid (14 mg, 17 μmol, 45%).

Characterization data: mp = decomposes at 184-186 °C. °C. IR (solid) νmax 3385, 2975, 2935, 1725, 1615, 1577, 1397, 1281, 1233, 1205, 1172, 1107, 1086, 1067, 1014. ¹H NMR (600 MHz, CDCl₃) δH: 1.29 (d, J = 6.6 Hz, 3H), 1.77 (td, J = 4.3, 3.9, 4.2 Hz, 1H), 1.88 (dd, J = 5.0, 5.0 Hz, 1H), 2.09 – 2.18 (m, 1H), 2.29 (d, J = 14.6 Hz, 1H), 2.98 (d, J = 18.7 Hz, 1H), 3.24 (dd, J = 1.9, 1.8 Hz, 1H), 3.64 (s, 1H), 4.05 (s, 3H), 4.10-4.16 (m, 2H), 4.23 (s, 2H), 4.73 (s, 2H), 5.24 (d, J = 1.6 Hz, 1H), 5.50 (d, J = 3.9 Hz, 1H), 6.12 (d, J = 8.3 Hz, 1H), 7.37 (d, J = 8.5 Hz, 1H), 7.78 (dd, J = 8.3, 7.8 Hz, 1H), 8.02 (dd, J = 7.7, 0.9 Hz, 1H), 13.20 (s, 1H), 13.97 (s, 1H); ¹³C NMR (150 MHz, CDCl₃) δC: 16.9 (CH₃), 29.9 (CH₂), 34.1 (CH₂), 35.8 (CH₂), 45.9 (CH), 56.8 (CH₃), 65.6 (CH), 67.2 (CH), 69.3 (CH), 100.5 (CH), 111.5 (C), 111.7 (C), 118.5 (C), 120.0 (C), 120.9 (C), 129.9 (C), 133.6 (C), 135.6 (C), 135.9 (C), 155.7 (CO), 156.2 (CO), 161.1 (CO), 163.7 (CO), 164.5 (CO), 186.7 (CO), 187.1 (CO), 213.9 (CO). LRMS (ESI) 836 (50, [M²⁹Br⁷⁹Br⁺]), 838 (100, [M²⁹Br⁷⁹Br⁺]), 840 (50, [M²⁹Br²¹Br⁺]); HRMS (ESI) calcd. for C₃₃H₃₀N₂O₁₄Br₂ [M²⁹Br⁷⁹Br-H]⁺ 836.0134, observed: 835.0063.
3,4-Dithiophenoyl-maleimide-N-(prop-2-yn-1-yl)acetamide (10)

\[
\begin{align*}
\text{S} & \quad \text{S} \\
\text{\sideset{-}{-}{\overset{\text{O}}{\text{N}}} & \quad \text{O} & \quad \text{N} & \quad \text{H} & \quad \text{\sideset{\text{-}}{\text{-}}{\overset{\text{O}}{\text{N}}} \\
\text{\sideset{-}{-}{\overset{\text{O}}{\text{N}}} & \quad \text{O} & \quad \text{N} & \quad \text{H} & \quad \text{\sideset{\text{-}}{\text{-}}{\overset{\text{O}}{\text{N}}}}
\end{align*}
\]

3,4-Dibromo-maleimide-N-(prop-2-yn-1-yl)acetamide (5) (51 mg, 144 µmol, 1 eq.) and sodium acetate (24 mg, 289 µmol, 2 eq.) were dissolved in MeOH (3 mL). A solution of thiophenol (29 µL, 289 µmol, 2 eq.) in MeOH (1 mL) under argon was added dropwise and the reaction mixture was left stirring at 20 °C for 30 min., turning yellow overtime. The mixture was concentrated under vacuum, redissolved in CH₂Cl₂ (2 mL) to give a yellow solid that was purified by flash chromatography on silica (EtOAc 0% to 10% in CH₂Cl₂) to afford the title compound as a yellow solid (41 mg, 101 µmol, 70%).

Characterization data: mp = 176-178 °C. IR (solid) v_max 3300, 3262, 2506, 1703, 1649, 1509, 1462, 1392, 1352, 1192. \(^1\)H NMR (600 MHz, CD₃OD) δH: 2.62 (s, 1H), 3.98 (d, J = 2.6 Hz, 2H), 4.19 (s, 1H), 7.17-7.19 (overlapped m, 4H), 7.24-7.29 (overlapped m, 6H); \(^1\)C NMR (150 MHz, CD₃OD) δC: 29.6 (CH₂), 41.4 (CH₂), 72.0 (CH), 80.0 (C), 129.1 (CH), 130.0 (CH), 130.4 (C), 132.3 (CH), 137.1 (CH), 167.9 (CO), 168.7 (CO). LRMS (ESI) 408 (100, [M]+); HRMS (ESI) calcd. for C₂₁H₁₆N₂O₃S₂ [M+H]^+ 408.0654, observed: 408.0616. UV (10% DMF in acetate buffer pH 4.5) ε₄₂₅ = 5569 M⁻¹ cm⁻¹.
3,4-Di-(N-(tert-butoxycarbonyl)-L-cysteine methyl ester)-maleimide-N-(prop-2-yn-1-yl) hexanamide (12)

![Chemical Structure](image)

3,4-Dibromo-maleimide-N-(prop-2-yn-1-yl)hexanamide (4) (51 mg, 126 μmol, 1 eq.) and sodium acetate (21 mg, 253 μmol, 2 eq.) were dissolved in MeOH (5 ml). N-(tert-butoxycarbonyl)-L-cysteine methyl ester (Boc-L-Cys-OMe) (52 μL, 253 μmol, 2 eq.) was added and the solution stirred under argon at 20 °C for 30 min., resulting in a bright yellow solution. All solvent was removed in vacuo, the crude material dissolved in CH₂Cl₂ (10 ml) and washed with 10% aqueous citric acid (20 ml). The organic layer was collected, dried (MgSO₄) and all solvent removed in vacuo. The crude material was purified by flash chromatography (EtOAc 50% to 100% in n-hexane) to yield title compound as a yellow powder (66 mg, 92 μmol, 73%).

Characterization data: mp = 123-124 °C. IR (solid) νmax 3345, 2972, 2936, 1748, 1697, 1678, 1644, 1513, 1434, 1392, 1363, 1320, 1294, 1246, 1206, 1155, 1047, 1013, 977. 

¹H NMR (600 MHz, CDCl₃) δH: 1.32 (d, J = 15.2 Hz, 2H), 1.58 – 1.69 (m, 6H), 1.46 (s, 18H), 2.19 (t, J = 7.4 Hz, 2H), 2.22 (t, J = 2.6 Hz, 1H), 3.49 (t, J = 6.8 Hz, 2H), 3.65 – 3.83 (m, 10H), 4.04 (dd, J = 5.1, 5.1 Hz, 2H), 4.65 (s, 2H), 5.69 (d, J = 7.1 Hz, 1H); 

¹³C NMR (150 MHz, CDCl₃) δC: 24.8 (CH₂), 26.2 (CH₂), 26.9 (C), 28.1 (CH₃), 28.4 (CH₂), 29.2 (CH₂), 33.9 (CH₂), 36.1 (CH₂), 38.5 (CH₂), 52.8 (CH₃), 53.0 (CH₂), 53.8 (CH₂), 71.6 (CH), 79.7 (C), 80.3 (C), 136.1 (C), 155.1 (CO), 166.1 (CO), 170.9 (CO), 172.4 (CO). 

LRMS (ESI) 715 (100, [M]+); LRMS (ESI) calcd. for C₃₁H₄₆N₄O₁₁S₂ [M+H]+ 715.2575. UV (10% DMF in acetate buffer pH 4.5) ε₄₀₆ = 2216 M⁻¹ cm⁻¹
3,4-Di-(\(N\)-(tert-butoxycarbonyl))-L-cysteine methyl ester)-maleimide-\(N\)-(prop-2-yn-1-yl)acetamide (13)

![Chemical structure](image)

3,4-Dibromo-maleimide-\(N\)-(prop-2-yn-1-yl)acetamide (5) (26 mg, 75 \(\mu\)mol, 1 eq.) and sodium acetate (12 mg, 150 \(\mu\)mol, 2 eq.) were dissolved in MeOH (5 ml). Boc-L-Cys-OMe (31 \(\mu\)L, 150 \(\mu\)mol, 2eq.) was added and the solution stirred under argon at 20 \(^\circ\)C for 30 min., resulting in a bright yellow solution. The solvent was removed \textit{in vacuo}, the crude material dissolved in CH\(_2\)Cl\(_2\) (10 ml) and washed with 10% aqueous citric acid (20 ml). The organic layer was collected, dried (MgSO\(_4\)) and all solvent concentrated. The crude material was purified by flash chromatography (EtOAc 50% to 100% in n-hexane) to yield title compound as a yellow powder (35 mg, 53 \(\mu\)mol, 70%).

Characterization data: mp = 155-156 \(^\circ\)C. IR (solid) \(\nu\)\textsubscript{max} 3333, 2977, 1737, 1707, 1679, 1516, 1415, 1390, 1365, 1347, 1318, 1285, 1246, 1213, 1154, 1118, 1055, 1022, 978.

\(^1\)H NMR (600 MHz, CDCl\(_3\)) \(\delta\)\textsubscript{H}: 1.43 (s, 18H), 2.11 – 2.20 (m, 1H), 3.58 (d, \(J = 8.1\) Hz, 1H), 3.68 – 3.90 (m, 8H), 4.66 (s, 2H), 3.96 – 4.25 (m, 5H), 4.66 (s, 2H), 5.52 (d, \(J = 5.4\) Hz, 2H), 6.58 (s, 1H); \(^{13}\)C NMR (150 MHz, CDCl\(_3\)) \(\delta\)\textsubscript{C}: 28.4 (CH\(_3\)), 29.3 (CH\(_2\)), 34.1 (CH\(_2\)), 41.3 (CH\(_2\)), 53.1(CH\(_3\)), 53.5 (CH), 71.7 (CH), 80.7 (C), 136.6 (C), 155.2 (CO), 165.4 (CO), 170.9 (CO). LRMS (ESI) 659 (100, [M]+); HRMS (ESI) calcd. for C\(_{27}\)H\(_{38}\)N\(_4\)O\(_{11}\)S\(_2\) [M+H]+ 659.2057. UV (10% DMF in acetate buffer pH 4.5) \(\varepsilon\)\textsubscript{406} = 1636 M\(^{-1}\) cm\(^{-1}\)
3,4-Di-(N-(tert-butoxycarbonyl)-L-cysteine methyl ester)-maleimide-N-(prop-2-yn-1-yl) p-benzamide (14)

3,4-Dibromo-maleimide-N-(prop-2-yn-1-yl) p-benzamide (6) (10 mg, 25 μmol, 1 eq.) and sodium acetate (4 mg, 49 μmol, 2 eq.) were dissolved in MeOH (5 ml). Boc-L-Cys-OMe (10 μL, 49 μmol, 2 eq.) was added and the solution stirred under argon at 20 °C for 30 min., resulting in a bright yellow solution. The solvent was removed in vacuo, the crude material dissolved in CH₂Cl₂ (10 ml) and washed with 10% aqueous citric acid (20 ml). The organic layer was collected, dried (MgSO₄) and concentrated. The crude material was purified by flash chromatography (EtOAc 50% to 100% in n-hexane) to yield title compound as a yellow powder (12 mg, 17 μmol, 68%).

Characterization data: mp = 112-113 °C. IR (solid) νmax 3348, 3279, 2969, 1700, 1674, 1645, 1502, 1434, 1387, 1365, 1327, 1284, 1240, 1215, 1157, 1122, 1054, 1011. ¹H NMR (600 MHz, CDCl₃) δH: 1.41 (s, 18H), 2.30 (t, J = 2.6 Hz, 1H), 3.76 (s, 10H), 4.26 (dd, J = 5.2, 2.6 Hz, 2H), 4.72 (s, 2H), 5.76 (d, J = 6.9 Hz, 2H), 6.36 (s, 1H), 7.51 (d, J = 8.2 Hz, 2H), 7.87 (d, J = 8.2 Hz, 2H); ¹³C NMR (150 MHz, CDCl₃) δC: 28.3 (CH₃), 29.9 (CH₂), 34.0 (CH₂), 52.9 (CH₃), 53.9 (CH), 72.1 (CH), 79.3 (C), 80.4 (C), 125.6 (CH), 127.8 (CH), 136.8 (C), 155.1 (CO), 164.4 (CO), 166.1 (CO), 170.9 (CO). LRMS (ESI) 719 (100, [M+H]+); HRMS (ESI) calcd. for C₃₂H₄₀N₄O₁₁S₂: 721.2135 (100, [M+H]+) observed: 721.2135. UV (10% DMF in acetate buffer pH 4.5) ε₄06 = 4224 M⁻¹ cm⁻¹
Hydrolysis of 5 and 10

3,4-Dibromo (25 mg, 71 μmol) or 3,4-Dithio-maleimide-N-(prop-2-yn-1-yl)acetamide (25 mg, 61 μmol) were dissolved in a mixture of MeCN: borate buffered saline (BBS) (2:8, 50 mM, pH 8.5) and stirred at 20 °C for 3 h. The pH was adjusted to 7.0 and the solvent were removed under reduced pressure. Desired products were isolated as oil and off white solid after purification by SEPpack (MeOH 0% to 50% in H2O) (7 mg, 19 μmol, 27%; and 20 mg, 47 μmol, 77%, respectively).

2,3-Dibromo-4-oxo-4-((2-oxo-2-(prop-2-yn-1-ylamino)ethyl)amino)but-2-enoic acid (h5)
Characterization data: IR (solid) νmax 3273, 2966, 1706, 1658, 1599, 15005, 1486, 1386, 1277. 1H NMR (600 MHz, CD3OD) δH: 2.50 (broad s, 1H), 3.88 (s, 5H), 4.00 (d, J = 2.5 Hz, 5H); 13C NMR (150 MHz, CD3OD) δC: 29.3 (CH2), 44.0 (CH2), 79.5 (CH), 124.6 (C), 132.3 (C), 166.1 (CO), 168.0 (CO), 171.6 (CO). LRMS (ESI) 366 (50, [M+Br]+), 368 (100, [M+Br]+), 370 (50, [M+Br]+); HRMS (ESI) calcd. for C9H8NO4Br2 [M+Br]+ 367.8835, observed 367.8836.
4-oxo-4-((2-oxo-2-(prop-2-yn-1-ylamino)ethyl)amino)-2,3-bis(phenylthio)but-2-enoic acid (h10)

Characterization data: IR (solid) \( \nu_{\text{max}} \) 3268, 2973, 1709, 1657, 1602, 1511, 1474, 1439, 1415, 1396, 1352, 1252. \(^1\)H NMR (600 MHz, CD\(_3\)OD) \( \delta_{\text{H}} \): 2.47 (t, \( J = 2.5 \) Hz, 1H), 3.53 (s, 2H), 3.90 (d, \( J = 2.5 \) Hz, 2H), 7.24 – 7.36 (m, 6H), 7.44 – 7.53 (m, 4H); \(^{13}\)C NMR (150 MHz, CD\(_3\)OD) \( \delta_{\text{C}} \): 29.3 (CH\(_2\)), 43.8 (CH\(_2\)), 71.6 (CH), 80.6 (C), 128.7 (CH), 128.8 (CH), 129.8 (CH), 130.0 (CH), 132.6 (CH), 133.3 (CH), 134.1 (CH), 134.3 (CH), 166.1, 167.2 (CO), 169.5 (CO), 171.8 (CO). LRMS (ESI) 427 (100, [M]); HRMS (ESI) calcd. for C\(_{21}\)H\(_{18}\)N\(_2\)O\(_4\)S\(_2\) [M+H]\(^+\) 427.0678, observed 427.0679.
Figure S1 – Absorbance spectrum of $h_5$ (left) and $h_{10}$ (right) in a 250 $\mu$M solution of 10% DMF in water.
Analytical methods for antibody conjugates

Experiments on trastuzumab

Conjugation and reoxidation experiments were carried out in standard polypropylene micro test tubes 3810x (1.5 mL) at atmospheric pressure with mixing at 20 °C unless otherwise stated. Reagents and solvents were purchased from commercial sources and used as supplied. All buffer solutions were prepared with double-deionised water and filter-sterilised. Borate buffered saline (BBS) was 50 mM Sodium Borate, 50 mM NaCl and 5 mM ethylenediaminetetraacetic acid (EDTA) at pH 8.0 or 8.5. Phosphate-buffered saline (PBS) was 50 mM sodium chloride and 50 mM sodium phosphates at pH 7.4. Ultrapure DMF was purchased from Sigma-Aldrich and kept under dry conditions. Solutions of tris(2-carboxyethyl)phosphine hydrochloride (TCEP) 10 mM (2.87 mg/mL) were prepared in BBS. Filtration of particulates was carried out through Spin-X 0.22 µm cellulose acetate filters. Ultrafiltration was carried out in vivaspin 500 polyethersulfone (PES) membrane concentrators with a molecular weight cut-off (MWCO) of 10 kDa or in Amicon Ultra-15 low binding cellulose filters with 10 kDa MWCO. Centrifugation was carried out on an eppendorf 5415R fixed angle rotor centrifuge operating at 14000 rcf at 20 °C or in an eppendorf 5810 swing-bucket rotor centrifuge operating at 3220 rcf at 20 °C. Trastuzumab is a chimeric IgG1 full length antibody directed against HER2. The antibody was obtained in its clinical formulation (Roche, lyophilised), dissolved in 10 ml sterile water and the buffer exchanged completely for BBS pH 8.0 via ultrafiltration with 10 kDa amicon molecular weight cut-off (MWCO). Concentration was determined by UV/Vis absorbance (using ε_{280} = 215380 M⁻¹ cm⁻¹ for trastuzumab mAb), adjusted to 40 µM (5.88 mg/mL) and was stored in flash frozen aliquots at −20 °C. For experiments, aliquots were thawed and used immediately. ADC concentration was determined using the same extinction coefficient as for native trastuzumab (maleamic acids were found to have negligible absorbance at 280 nm). The following acronyms are used to describe antibody fragments based on their constituent heavy and light chains: heavy-heavy-light (HHL), heavy-heavy (HH), heavy-light (HL, a.k.a. half antibody), heavy chain (HC) and light chain (LC). Expected mass was calculated according to MS data observed for IgG1 sub units and full antibody (LC: 23440 kDa; HC: 49150 kDa; HL: 72584 kDa; HHL: 145167 kDa).
Reoxidation study of trastuzumab

To trastuzumab (22.9 μM, 100 μL, 2.3 nmol, 1 eq.) in BBS (pH 8.0) was added TCEP (10 mM, 1.4 μL, 13.7 nmol, 8 eq.) and the reaction was incubated at 37 °C for 2 h under mild agitation. Then 5,5-dithio-bis-(2-nitrobenzoic acid) (DTNB) prepared in water (10 mM, 3.8 μL, 10 eq.) was added to the reduced trastuzumab. After incubation at 20 °C for 5 min., the excess DTNB was washed away using a PD10 column and then concentrated up to 10 μM via ultrafiltration (10 kDa MWCO). Cysteamine prepared in water (10 mM, 3.8 μL, 10 eq.) was added and the reaction was incubated at 20 °C for 24 h. The species at each stage were characterized by liquid chromatography–mass spectrometry (LC-MS).

Conjugation to trastuzumab

Conjugation of DBM reagents 4 – 6 to trastuzumab at pH 8.0

This conjugation protocol was performed in BBS (pH 8.0) following a previous method\(^3\) described in the literature with some modifications. Briefly, to trastuzumab (22.9 μM, 100 μL, 2.3 nmol, 1 eq.) was added TCEP (10 mM, 1.8 μL, 18.4 nmol, 8 eq.) and the reaction was incubated at 37 °C for 2 h under mild agitation. Next, the DBM reagent in dry DMF (10 mM, 2.3 μL, 23 nmol, 10 eq.) was added to the reduced trastuzumab. The concentration of DMF was corrected to 10% (v/v) and the reaction was incubated at 20 °C for 30 min.. Afterwards, excess reagents were removed by ultrafiltration (10 kDa MWCO) with BBS to afford the modified trastuzumab. Hydrolysis was achieved by incubating the conjugates for 48 h in conjugation buffer, at 37 °C. The final trastuzumab conjugates were deglycosylated and characterized by LC-MS. All conjugation reactions were carried out in triplicate.

Optimized conjugation of DBM reagents 4 – 8 to trastuzumab at pH 8.5

The conjugation protocol was performed in BBS (pH 8.5) as follows: to trastuzumab (22.9 μM, 100 μL, 2.3 nmol, 1 eq.) was added TCEP (10 mM, 1.4 μL, 13.8 nmol, 6 eq.) and the reaction was incubated at 37 °C for 2 h under mild agitation. Next, the DBM reagent in dry DMF (10 mM, 1.8 μL, 18.4 nmol, 8 eq.)* was added to the reduced trastuzumab and the reaction was left at 20 °C for 5 min.. Afterwards, excess reagents were removed by ultrafiltration (10 kDa MWCO) with BBS pH 8.5. To promote complete hydrolysis, the conjugates containing NGM-C\(_2\)/Ph and NGM-C\(_6\) linker were
incubated in the conjugation buffer for 1 h (at 25 °C) and 48 h (at 37 °C), respectively. The final trastuzumab conjugates were deglycosylated and characterized by LC-MS.

* Reagent 8 added at 6.5 eq., to minimize reagent excess

**One-pot conjugation and hydrolysis protocol**

Conjugation protocol was performed as described above in BBS (pH 8.5) using 5 and 6 with one modification; excess reagent was only removed by ultrafiltration after hydrolysis step. LC-MS data matched that obtained above.
SDS-PAGE

Non-reducing glycin-SDS-PAGE at 12% acrylamide gels were performed following standard lab procedures. A 4% stacking gel was used and a broad-range MW marker (10-250 kDa, BioLabs) was co-run to estimate protein weights. Samples (3 μL at ~35 μM in total mAb) were mixed with loading buffer (2 μL, composition for 6 x SDS: 1 g SDS, 3 mL glycerol, 6 mL 0.5 M Tris buffer pH 6.8, 2 mg R-250 dye) and heated at 65 °C for 5 min. The gel was run at constant current (30-35 mA) for 40 min in 1× SDS running buffer. All gels were stained following a modified literature protocol where 0.12% of the Coomassie G-250 and the Coomassie R-250 dyes were added to the staining solution (5:4:1 MeOH:H₂O:AcOH). Dithiotreitol (DTT) was used as reducing agent in reducing gels. Samples (3 μL at ~35 μM in total mAb) were mixed with loading buffer (2 μL, composition for 4 x SDS, 0.8 g SDS, 4 mL glycerol, 2.5 mL 0.5 M Tris buffer pH 6.8, 2.5 mL H₂O, 2 mg R 250 dye) and DTT (1 μL: 10 mM, 100 eq.). The gel was run at constant current (30-35 mA) for 40 min in 1× SDS running buffer. All gels were stained following a modified literature protocol where 0.12% of the Coomassie G-250 and the Coomassie R-250 dyes were added to the staining solution (5:4:1 MeOH:H₂O:AcOH). Notably, NGM conjugates (non-hydrolysed) are cleaved under reducing gel conditions, whereas NGM conjugates (hydrolysed) are not cleaved under reducing gel conditions.

Copper-catalysed Huisgen 1,3-dipolar cycloaddition to trastuzumab conjugates

To a solution of trastuzumab-alkyne conjugate (50 μM, 50 μL, 0.0025 μmol) in phosphate-buffered saline (PBS, pH 7.4) was added tris(3-hydroxypropyltriazolylmethyl)amine (THPTA, 40 eq.) and CuSO₄ (8 eq.). Next, added Alexa Fluor 488® azide in DMF (10 mM, 2.5 μL, 10 eq.), followed by sodium ascorbate (final concentration 5 mM) and the reaction mixture was incubated at 25 °C for 1 h. Excess reagents were removed by PD-10 desalting column and by ultrafiltration (GE Healthcare, 10 kDa MWCO) with fresh buffer (PBS with 5 mM EDTA).
Determination of fluorophore to antibody ratio (FAR) and drug to antibody ratio (DAR)

UV/Vis spectra were recorded on a Varian Cary 100 Bio UV/Vis spectrophotometer, operating at 20 °C. Sample buffer was used as blank for baseline correction. Calculation of fluorophore-antibody ratio (FAR) and drug-antibody ratio (DAR) follows the formula below using $\epsilon_{280} = 215380 \text{ M}^{-1} \text{ cm}^{-1}$ for trastuzumab mAb, $\epsilon_{495} = 71000 \text{ M}^{-1} \text{ cm}^{-1}$ for Alexa Fluor 488® (0.11 as a correction factor (Cf) of the dye Excitation at 280 nm) and $\epsilon_{495} = 8030 \text{ M}^{-1} \text{ cm}^{-1}$ for doxorubicin (0.724 as a correction factor of the drug absorbance at 280 nm), respectively.

$$\text{FAR} = \frac{\text{Abs}_{495}}{\epsilon_{495} \left( \text{Abs}_{280} - \text{Cf} \times \text{Abs}_{495} \right)}$$

Analysis of antibodies under denaturing LC-MS conditions

An aliquot (20 μL) of trastuzumab or trastuzumab conjugates in ammonium acetate buffer (50 mM, pH 6.9) is diluted to concentration of 0.7 μM (0.1 mg·ml$^{-1}$) and deglycosylated as follows: PNGase F (1 μL, 15,000 units·mL$^{-1}$, in 20 mM Tris-HCl, 50mM NaCl, 5 mM EDTA, pH 7.5) (purchased from New England BioLabs Inc.) was added to trastuzumab or conjugates and the resultant solution was incubated at 37 °C overnight. After this time the solution was analysed on an Agilent 6510 QTOF LC-MS system (Agilent, UK). Ten μL of each sample was injected onto a PLRP-S, 1000 Å, 8 μm, 150 mm x 2.1 mm column, which was maintained at 60 °C. Chromatographic separation was optimised for the separation of a light and heavy chains and PNGase F using mobile phase A (5% MeCN in aqueous 0.1% formic acid) and B (95% MeCN, 5% water, 0.1% formic acid) using a gradient elution. The flow rate was 300 μL/min and the gradient as follows: 15% B for the first 2 min followed by increase to 32% B over 1 min, remained at 32% B for 1 min. After 4 min, mobile phase B was increased to 50% over 10 min, with further increase of B to 95% over 4 min and maintained at 95% B for 2 min.. At 22 min., the mobile phase B was changed to 15% B for the next 3 min to initial conditions.
The column effluent was continuously electrosprayed into capillary ESI source of the Agilent 6510 QTOF mass spectrometer and ESI mass spectra were acquired in positive electrospray ionization (ESI) mode using the m/z range 800–5,000 in profile mode. The acquisition rate was 1 spectra/sec and acquisition time 1000 msec/spectrum corresponding to 9,652 transients/spectrum. The raw data was converted to zero charge mass spectra using maximum entropy deconvolution algorithm over the region between the vertical lines in the total ion chromatogram (TIC) with MassHunter software (version B.07.00).

**Alexa Fluor 488® conjugate linker pH stability**

To provide an estimate of the relative stability of the linkers as an estimate of the hydrolytic stability of the conjugates in different biological environments, dilute aqueous solution (0.1 mg/ mL) of the Alexa Fluor conjugates were incubated in pH 5.5 and 7.4 buffers (50 mM citrate-phosphate) at 37 °C for different time points (3 h and 24 h) and the appearance of free dye was monitored by size exclusion high performance liquid chromatography (SEC-HPLC). Aliquots were removed and analysed straightforward on a TSK gel G3000SWXL (7.8 mm x 30 cm) column connected to a Varian ProStar pump 500 coupled to a Varian ProStar 350 LC UV/Vis and fluorescent detector. Each analysis was carried out for 30 min. at a flow rate of 0.5 mL/ min and using PBS pH 7.4 (0.1 M) containing 0.02% NaN₃ as mobile phase. Fluorescence was detected with an excitation wavelength of 495 nm and emission wavelength of 525 nm.
Characterization of antibody conjugates by SDS-PAGE

**Figure S2** – SDS-PAGE of trastuzumab and conjugates prepared in BBS (pH 8.0)

**Figure S3** – SDS-PAGE of trastuzumab-NGM-linker-alkyne conjugates prepared following optimized conjugation protocol in BBS (pH 8.5).

**Figure S4** – SDS-PAGE of modular trastuzumab-NGM-conjugates prepared following optimized conjugation protocol.
Characterization of trastuzumab and conjugates by LC-MS

A)

Figure S5 – LC-MS data of trastuzumab. A) TIC, B) non-deconvoluted ion series and C) deconvoluted ion series mass spectra. Full antibody observed mass of 145167 Da (expected 145167).
Figure S6 – LC-MS data of reduced trastuzumab. A) TIC, B) non-deconvoluted ion series and C) deconvoluted ion series mass spectra. HC observed mass of 49149 Da (expected 49150) and LC observed mass of 23440 Da (expected 23440).
Figure S7 – LC-MS data of trastuzumab in Ellman’s reoxidation. A) TIC, B) non-deconvoluted ion series and C) deconvoluted ion series mass spectra. Full antibody observed mass of 145167 Da (expected 145167) and half antibody observed mass 72584 Da (expected 72584).
Figure S8 – LC-MS data of reoxidized trastuzumab with DTNB and cysteamine. A) TIC, B) non-deconvoluted ion series and C) deconvoluted ion series mass spectra. Full antibody observed mass of 145167 Da (expected 145167).
Figure S9 – LC-MS data of trast-NGM-C6-alkyne conjugate prepared in BBS pH 8.0. A) TIC, B) non-deconvoluted ion series C) deconvoluted ion series mass spectra and D) Expansion of the deconvoluted ion series mass spectra (70000-76500 amu). Double addition of reagent to HL observed mass 73118 Da (expected 73117) and double addition of reagent plus incomplete deglycosylation (one N-acetylglucosamine unit) to HL observed mass 73322 Da (expected 73320). Double addition of reagent to HC observed mass 49638 Da (expected 49683). Decarboxylation of LC observed mass 23361 Da (expected 23440 for intact LC).
Figure S10 – LC-MS data of **trast-NGM-C6-alkyne** conjugate prepared in BBS pH 8.5. A) TIC, B) non-deconvoluted ion series and C) deconvoluted ion series mass spectra. Double addition of reagent to HL observed mass 73123 Da (expected 73117) and double addition of reagent to HC observed mass 49680 Da (expected 49683).
Figure S11 – LC-MS data of trast-NGM-C2-alkyne conjugate prepared in BBS pH 8.0. A) TIC, B) non-deconvoluted ion series and C) deconvoluted ion series mass spectra. Fully modified conjugate observed mass of 145994 Da (expected 146008) and half antibody fully modified 73001 Da (expected 73005). LC (2x) observed mass 46878 Da (expected: 46880) and single addition to LC observed mass 23648 Da (expected 23650).
Figure S12 – LC-MS data of trast-NGM-C2-alkyne conjugate prepared in BBS pH 8.5. A) TIC, B) non-deconvoluted ion series and C) deconvoluted ion series mass spectra. Fully modified conjugate observed mass of 145991 Da (expected 146008) and HL fully modified observed mass 73001 Da (expected 73005).
Figure S13 – LC-MS data of trast-NGM-Ph-alkyne conjugate prepared in BBS pH 8.0. A) TIC, B) non-deconvoluted ion series and C) deconvoluted ion series mass spectra. Fully modified conjugate observed mass of 146247 Da (expected 146249) and HL fully modified observed mass 73125 Da (expected 73125). LC (2x) observed mass 46878 Da (expected: 46880) and LC observed mass 23439 Da (expected 23440).
Figure S14 – LC-MS data of trast-NGM-Ph-alkyne conjugate prepared in BBS pH 8.5. A) TIC, B) non-deconvoluted ion series and C) deconvoluted ion series mass spectra. Fully modified conjugate observed mass of 146247 Da (expected 146249) and HL fully modified observed mass 73125 Da (expected 73125).
Figure S15 – LC-MS data of PNGase F (peak between 14-16 min) in MS of trast-NGM-Ph-alkyne conjugate prepared in BBS pH 8.5. A) TIC, B) non-deconvoluted ion series and C) deconvoluted ion series mass spectra. PNGase F observed mass of 34775 Da (expected 34775).
Figure S16 – LC-MS data of **trast-NGM-C2-dansyl** conjugate prepared in BBS pH 8.5. A) TIC, B) non-deconvoluted ion series and C) deconvoluted ion series mass spectra. Fully modified conjugate observed mass of 146942 Da (expected 146959) and HL fully modified observed mass 73468 Da (expected 73480). MS data acquired after 1 h deglycosylation at 37 °C to prevent payload loss under acidic conditions of MS analysis.
Figure S17 – LC-MS data of trast-NGM-C₂-doxorubicin conjugate prepared in BBS pH 8.5. A) TIC, B) non-deconvoluted ion series and C) deconvoluted ion series mass spectra. Fully modified conjugate observed mass of 147936 Da (expected 147959) and HL fully modified observed mass 73976 Da (expected 73980). Conjugation performed using 6.5 eq. of 8.
Figure S18 – Expansion of the deconvoluted ion series mass spectra (72000-76400 amu) of trast-NGM-C₂-doxorubicin. HL fully modified observed mass 73976 Da (expected 73980) and HL containing one drug and one maleic anhydride observed mass 73388 Da (expected 73380).

Figure S19 – Expansion of the deconvoluted ion series mass spectra (143500-153000 amu) of trast-NGM-C₂-doxorubicin. Fully modified antibody observed mass 147939 Da (expected 147959) and full antibody containing three drugs and one maleic anhydride observed mass 147357 Da (expected 147359). Payload loss during LC-MS analysis in acidic conditions is clearly observed in this example.

Figure S20 – UV/Vis spectrum of trast-NGM-C₂-doxorubicin (DAR = 4.1)
**Hydrolysis Study**

Hydrolysis kinetics study pre – and post – conjugation of NGMs was performed at an initial concentration of approximately 230 μM. Hydrolysis data were further analysed in units of molar concentration to determine kinetic constants. Pseudo first-order rate constants were determined from the slopes of curves generated from plotting ln [NGM] versus time and linear regression analysis. Hydrolysis reaction half-lives were calculated from the pseudo first order rate constants using equation below:

\[ t_{1/2} = \frac{0.693}{k_{1, \text{obs}}} \]

**Pre-conjugation**

Hydrolysis kinetics of DBMs (4 – C₆ linker and 5 – C₂ linker) and DTPMs (9 – C₆ linker, 10 – C₂ linker and 11 – Ph linker) alkynes was performed in BBS pH 8.0 with an initial concentration of 230 μM. Hydrolysis kinetics of DBMs and DTPMs reagents was monitored by spectrophotometry, using absorbance at 325 nm and 425 nm, respectively.

Absorbance spectrum of 4 and 5

![Absorbance spectrum of 4 and 5](image)

**Figure S21** – Absorbance spectrum of 4 (left) and 5 (right) in BBS pH 8.0, 22 °C.
**Figure S22** – Hydrolysis kinetics of 4 (left) and 5 (right) in BBS pH 8.0, 22 °C.

**Figure S23** – Determination of pseudo first order reaction rate constants for 4 (left) and 5 (right) in BBS pH 8.0, 22 °C. Pseudo first order rate constants were determined from the –slope of the best fit line.
**Figure S24** – Absorbance spectrum of 9 (left), 10 (center) and 11 (right) in BBS pH 8.0, 22 °C.

**Figure S25** – Hydrolysis kinetics of 9 (left), 10 (center) and 11 (right) in BBS pH 8.0, 22 °C.

**Figure S26** – Determination of pseudo first order reaction rate constants of 9 (left), 10 (center) and 11 (right) in BBS pH 8.0, 22 °C. Pseudo first order rate constants were determined from the –slope of the best fit line.
Table S1 – Rate constants and half-life of NGM maleimide hydrolysis pre-conjugation in BBS pH 8.0, 22 °C

| Compound | $k_{1,obs}$ (s$^{-1}$) | $t_{1/2}$ (min) |
|----------|------------------------|-----------------|
| 4        | $1.6 \times 10^{-3}$    | 7.2             |
| 5        | $17.7 \times 10^{-3}$   | 0.6             |
| 9        | $2 \times 10^{-4}$      | 57.8            |
| 10       | $3 \times 10^{-4}$      | 38.5            |
| 11       | $3 \times 10^{-4}$      | 38.5            |
Post-conjugation

Hydrolysis kinetics of maleimide in trastuzumab-NGM conjugates at pH 8.5 was monitored by spectrophotometry, using absorbance at 406 nm, 402 nm and 406 nm for trast-NGM-C₆-Alk, trast-NGM-C₂-Alk and trast-NGM-Ph-Alk, respectively. Samples (200 μL) were prepared as described above (conjugation section). Extinction coefficient of dicysteine maleimide-alkyne (12, 13 and 14) prepared above was used to estimate the concentration of conjugated reagent.

Figure S27 – Absorbance spectrum of trast-NGM-C₆-alkyne (left), trast-NGM-C₂-alkyne (center) and trast-NGM-Ph-alkyne (right) in BBS pH 8.5, 22 °C.

Figure S28 – Hydrolysis kinetics of trast-NGM-C₆-alkyne (left), trast-NGM-C₂-alkyne (center) and trast-NGM-Ph-alkyne (right) in BBS pH 8.5, 22 °C.
Figure S29 – Determination of pseudo first order reaction rate constants of trast-NGM-C₆-alkyne (left), trast-NGM-C₂-alkyne (center) and trast-NGM-Ph-alkyne (right) in BBS pH 8.5, 22 °C. Pseudo first order rate constants were determined from the slope of the best fit line.

Figure S30 – Absorbance spectrum of trast-NGM-C₂-alkyne conjugate containing hydrolyzed bridging agent, in BBS pH 8.5.

Table S2 – Rate constants and half-life of NGM maleimide hydrolysis post-conjugation in BBS pH 8.5, 22 °C.

| Conjugate             | k₁,obs (s⁻¹) | t₁/₂ (min) |
|-----------------------|--------------|------------|
| trast-NGM-C₆-alkyne   | 4 x 10⁻⁴     | 2887.5     |
| trast-NGM-C₂-alkyne   | 6 x 10⁻⁴     | 19.3       |
| trast-NGM-Ph-alkyne   | 7 x 10⁻⁴     | 16.5       |
pH stability study of NGM linker in antibody conjugate

Copper-catalysed Huisgen 1,3-dipolar cycloaddition to trastuzumab conjugates

FAR values found for \textit{trast-NGM-C\textsubscript{2}-alkyne} and \textit{trast-NGM-Ph-alkyne} was 3.00.

\begin{figure}
\centering
\includegraphics[width=\textwidth]{figs/15621 FIG3.png}
\caption{Absorbance spectrum of conjugates \textit{trast-NGM-C\textsubscript{2}-Alexa} and \textit{trast-NGM-Ph-Alexa}.}
\end{figure}

\begin{figure}
\centering
\includegraphics[width=\textwidth]{figs/15621 FIG4.png}
\caption{SEC-HPLC chromatograms of conjugates \textit{trast-NGM-C\textsubscript{2}-Alexa} and \textit{trast-NGM-Ph-Alexa} in citrate-phosphate buffer (pH 7.4) at different time points. No payload release detected for at least 10 days.}
\end{figure}
Figure S33 – SEC-HPLC chromatograms of conjugates trast-NGM-C₂-Alexa and trast-NGM-Ph-Alexa in citrate-phosphate buffer (pH 5.5) at different time points. Fluorophore release after 24 h was 4% for the aryl linker; not detected for C2 linker.
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