Electronic Supplementary Material (ESI) for ChemComm.

A Dual-Enzyme Cleavable Linker for Antibody-Drug Conjugates

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Supplementary Information

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1 General Experimental Details

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 (PE) refers to the fraction between 40–60 °C upon distillation. Tetrahydrofuran (THF) 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 visualised by quenching of UV fluorescence (λ_{max} = 254 nm) or by staining with potassium permanganate. Retention factors (Rf) are quoted to 0.01.

Flash column chromatography was carried out using slurry-packed Merck 9385 Kieselgel 60 SiO_2 (230-400 mesh) or Combiflash Rf200 automated chromatography system with Redisep® normalphase silica flash columns (35−70 μm) or 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 Supelcosil™ ABZ+PLUS column (150 mm × 4.6 mm, 3 μm) with a linear gradient system (solvent A: 0.05% (v/v) TFA in H_2O; solvent B: 0.05% (v/v) TFA in MeCN) over 20 min at a flow rate of 1 mL/min, and UV detection (λ_{max} = 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}).

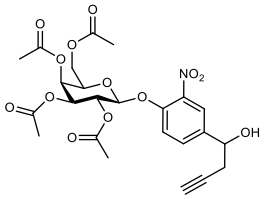
Proton and carbon nuclear magnetic resonance (NMR) were recorded using an internal deuterium lock on Bruker DPX-400 (400 MHz, 101 MHz), Bruker Avance 400 QNP (400 MHz, 101 MHz), Bruker Avance 500 Cryo Ultrashield (500 MHz, 126 MHz) and 600 MHz Bruker Avance 600 BBI (600 MHz) spectrometers. Proton assignments are supported by ^{1}H-^{1}H COSY, ^{1}H-^{1}H NOESY, ^{1}H-^{13}C HSQC or ^{1}H-^{13}C HMBC spectra, or by analogy. Tetramethylsilane was used as an internal standard. 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 ($CDCl_3$: 7.26, DMSO-d_6: 2.50, CD_3OD: 3.31, Acetone-d_6: 2.05, CD_3CN: 1.94). 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; br = broad or as a combination of these, e.g. dd, dt etc.), integration 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 ($CDCl_3$: 77.16, DMSO-d_6, 39.52, CD_3OD: 49.00, Acetone-d_6: 29.84 and 206.26, CD_3CN: 1.32 and 118.26).

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 × 50.5 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 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. Trastuzumab samples were deglycosylated with PNGase F (New England Biolabs) prior to LC-MS analysis.

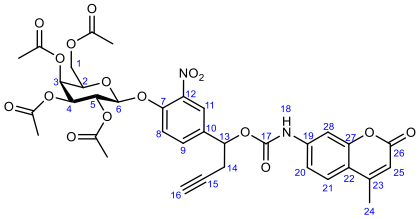
2 Chemical Synthesis

(2*R*,3*S*,4*S*,5*R*,6*S*)-2-(Acetoxymethyl)-6-(4-(1-hydroxybut-3-yn-1-yl)-2-nitrophenoxy)tetrahydro-2*H*-pyran-3,4,5-triyl triacetate (3)



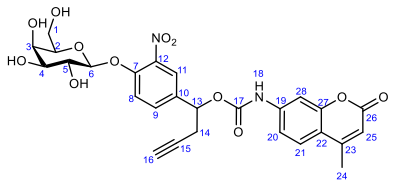
(2*R*,3*S*,4*S*,5*R*,6*S*)-2-(Acetoxymethyl)-6-(4-(1-hydroxybut-3-yn-1-yl)-2-nitrophenoxy)tetrahydro-2*H*-pyran-3,4,5-triyl triacetate (**3**) was synthesised according to the previously reported procedure.[1]

Ac-Gal-AMC (4a)



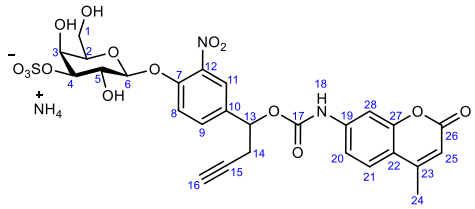
7-Amino-4-methylcoumarin (89.0 mg, 508 µmol) and triphosgene (75.4 mg, 254 µmol) were suspended in toluene (10 mL) and refluxed for 90 min. The reaction mixture was then cooled and concentrated under a stream of nitrogen before a solution of alcohol **3** (273 mg, 508 µmol) in THF (10 mL) was added. Dibutyltin dilaurate (30.1 µL, 50.8 µmol) was added to the resulting suspension and stirred at rt for 48 h, then quenched with H_2O (1 mL) and concentrated *in vacuo*. The crude residue was washed with purified by flash column chromatography (40-50% EtOAc in PE) to yield carbamate **4a** (101 mg, 137 µmol, 27%) as a white solid. **Rf** 0.18 (50% EtOAc in PE); $\boldsymbol{\nu_{max}}$ (neat/cm^{-1}) 3286 (w), 2923 (w), 1738 (br s), 1619 (m), 1578 (w), 1532 (m), 1367 (m), 1211 (s); **^{1}H NMR** (400 MHz, CD_3CN) δ 8.39 (br s, 1H, H^{18}), 7.92 (app t, 1H, *J* = 1.7 Hz, H^{11}), 7.71 (dt, *J* = 8.7, 1.5 Hz, H^{9}), 7.63 (d, 1H, *J* = 8.7 Hz, H^{21}), 7.50 (d, 1H, *J* = 2.1 Hz, H^{28}), 7.43 (dd, 1H, *J* = 8.7, 1.6 Hz, H^{8}), 7.32 (app dt, 1H, *J* = 8.7, 2.1 Hz, H^{20}), 6.15 (d, 1H, *J* = 1.1 Hz, H^{25}), 5.89 (t, 1H, *J* = 6.1 Hz, H^{13}), 5.43 (d, 1H, *J* = 3.4 Hz, H^{3}), 5.36–5.30 (m, 2H, H^{5} and H^{6}), 5.21–5.13 (m, 1H, H^{4}), 4.30–4.25 (m, 1H, H^{2}), 4.24–4.18 (m, 1H, H^{1}), 4.16–4.11 (m, 1H, H^{1}), 2.86 (dd, 2H, *J* = 6.1, 2.6 Hz, H^{14}), 2.39 (d, 3H, *J* = 0.8 Hz, H^{24}), 2.28 (t, 1H, *J* = 2.6 Hz, H^{16}), 2.13–2.09 (m, 6H, C*H*$_3$), 2.04–2.00 (m, 6H, C*H*$_3$); **^{13}C NMR** (101 MHz, CD_3CN) δ 170.2, 169.8, 169.3, 160.5, 154.4, 153.0, 148.7, 132.0, 125.8, 122.9, 118.1, 114.4, 112.6, 105.1, 99.5, 72.7, 71.8, 71.7, 71.5, 70.2, 67.9, 67.1, 61.3, 31.6, 27.2, 25.7, 19.92, 19.91, 19.85, 19.84, 19.79, 19.76, 17.6; **HMRS** (ESI) *m/z* found $[M+Na]^+$ 761.1816, $C_{35}H_{34}N_2O_{16}Na^+$ required 761.1801.

Gal-AMC (6a)



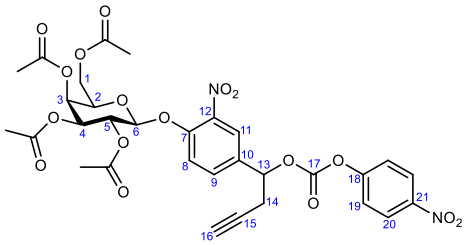
A solution of lithium hydroxide monohydrate (13.4 mg, 320 µmol) in H_2O (1 mL) was added dropwise to a solution of tetra-acetate **4a** (23.6 mg, 32.0 µmol) in MeOH (1 mL) at 0 °C. After 30 min, the reaction was neutralised by stirring with Amberlite® IRC76 (weakly acidic) at 0 °C for 15 min. The reaction mixture was then filtered through a pad of cotton wool, concentrated *in vacuo* and purified by reverse phase flash column chromatography (25-50% MeCN in 0.1 M NH_4OH (aq)) to yield alcohol **6a** (14.0 mg, 24.5 µmol, 77%) as a white solid. $\boldsymbol{\nu_{max}}$ (neat/cm^{-1}) 3297 (w), 1697 (s), 1618 (s), 1536 (m), 1393 (m), 1345 (m); **^{1}H NMR** (500 MHz, CD_3CN) δ 8.40 (br s, 1H, H^{18}), 7.93 (app t, 1H, *J* = 1.8 Hz, H^{11}), 7.69 (dd, 1H, *J* = 8.9, 2.3 Hz, H^9), 7.64 (d, 1H, *J* = 8.8 Hz, H^{21}), 7.50 (app q, 1H, *J* = 1.1 Hz, H^{28}), 7.42 (d, 1H, *J* = 8.7 Hz, H^8), 7.32 (dd, 1H, *J* = 8.6, 2.1 Hz, H^{20}), 6.16 (d, 1H, *J* = 1.3 Hz, H^{25}), 5.89 (t, 1H, *J* = 6.1 Hz, H^{13}), 5.02 (dd, 1H, *J* = 7.7, 3.2 Hz, H^6), 3.87 (t, 1H. *J* = 3.2 Hz, H^3), 3.73–3.64 (m, 4H, H^1, H^2 and H^5), 3.59–3.51 (m, 2H, H^4 and O*H*), 3.38 (d, 1H, *J* = 2.3 Hz, 1H), 3.22 (d, 1H, *J* = 3.4 Hz, C^3-O*H*), 3.02–2.96 (m, 1H, O*H*), 2.86 (dd, 2H, *J* = 6.2, 2.6 Hz, H^{14}), 2.39 (d, 3H, *J* = 1.2 Hz, H^{24}), 2.28 (t, 1H, *J* = 2.6 Hz, H^{16}); **^{13}C NMR** (126 MHz, CD_3CN) δ 161.4, 155.3, 153.9, 153.1, 150.5, 142.9, 141.2, 134.6, 133.0, 126.8, 123.91, 123.90, 118.3, 116.3, 115.3, 113.5, 106.1, 102.43, 102.41, 79.9, 76.6, 74.0, 73.6, 72.6, 71.7, 69.6, 62.1, 26.7, 18.5; **HMRS** (ESI) *m/z* found $[M+Na]^+$ 593.1372, $C_{27}H_{26}N_2O_{12}Na^+$ required 593.1378.

Sulfo-Gal-AMC (1)



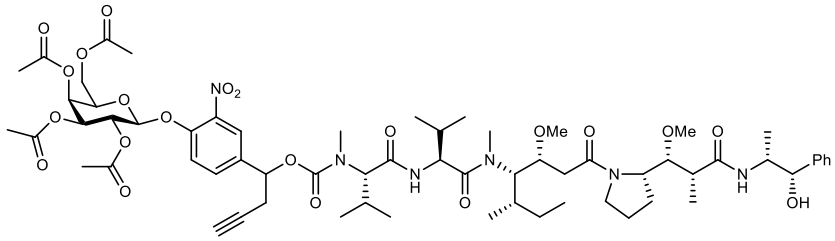
Dibutyltin oxide (5.67 mg, 22.8 µmol) was added to a solution of alcohol **6a** (13.0 mg, 22.8 µmol) in MeOH (0.3 mL) and stirred at 60 °C for 3 h. The reaction mixture was then allowed to cool to rt and concentrated under a stream of nitrogen. The resulting residue was suspended in 1,4-dioxane at 0 °C (0.3 mL) and sulfur trioxide trimethylamine complex (5.67 mg, 22.8 µmol) was added. After 5 min the reaction mixture was warmed to rt and stirred for 90 min, then purified by reverse phase flash column chromatography (15-35% MeCN in 0.1 M NH_4OH (aq)) and lyophilised to yield sulfate **1** (10.2 mg, 15.3 µmol, 67%) as a hygroscopic white solid. $\boldsymbol{\nu_{max}}$ (neat/cm^{-1}) 3385 (w), 3259 (w), 2921 (w), 1687 (s), 1620 (m), 1587 (m), 1533 (s); **^{1}H NMR** (500 MHz, Acetone-*d*^{6}) δ 7.93 (d, 1H, *J* = 2.2 Hz, H^{11}), 7.75 (dd, 1H, *J* = 8.8, 2.0 Hz, H^9), 7.69 (d, 1H, *J* = 8.7 Hz, H^{21}), 7.62 (app t, 1H, *J* = 1.7 Hz, H^{28}), 7.56 (d, 1H, *J* = 8.8 Hz, H^8), 7.49 (dd, 1H, *J* = 8.7, 2.1 Hz, H^{20}), 6.17 (d, 1H, *J* = 1.2 Hz, H^{25}), 5.94 (t, 1H, *J* = 6.3 Hz, H^{13}), 5.21 (dd, 1H, *J* = 7.7, 2.6 Hz, H^6), 4.30 (dd, 1H, *J* = 9.7, 3.3 Hz, H^4), 4.25 (dd, 1H, *J* = 3.3, 1.0 Hz, H^3), 3.99 (dd, 1H, *J* = 9.7, 7.7 Hz, H^5), 3.87 (t, 1H, *J* = 6.0 Hz, H^2), 3.80–3.71 (m, 2H, H^1), 2.96–2.86 (m, 2H, H^{14}), 2.49 (t, 1H, *J* = 2.6 Hz, H^{16}), 2.43 (d, 3H, *J* = 1.2 Hz, H^{24}); **^{13}C NMR** (126 MHz, Acetone-*d*^{6}) δ 161.0, 155.3, 153.7, 153.1, 150.8, 143.3, 141.32, 141.30, 134.11, 134.08, 132.90, 132.86, 126.7, 123.7, 123.6, 118.38, 118.37, 115.9, 115.2, 113.3, 105.9, 102.64, 102.60, 80.4, 79.90, 79.89, 76.5, 73.82, 73.76, 72.92, 72.91, 70.0, 69.3, 68.0, 61.8, 26.5, 18.4; **HMRS** (ESI) *m/z* found $[M]^-$ 649.0975, $C_{27}H_{25}N_2O_{15}S^+$ required 649.0981.

Ac-Gal-PNP (6)



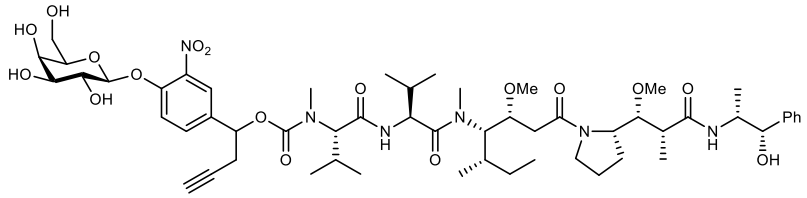
Pyridine (16.5 µL, 205 µmol) was added to a solution of benzylic alcohol **3** (44.0 mg, 81.9 µmol) and 4-nitrophenyl chloroformate (33.1 mg, 164 µmol) in CH_2Cl_2 (1 mL) at 0 °C and the reaction mixture was allowed to warm to rt over 24 h. Sat. $NaHCO_3$ (aq) (10 mL) was then added and the aqueous layer was extracted with CH_2Cl_2 (3 × 30 mL). The combined organic fractions were dried (Na_2SO_4), concentrated *in vacuo* and purified by flash column chromatography (25-50% EtOAc in PE) to yield carbonate **6** (49.2 mg, 70.0 µmol, 86%) as a clear oil. **Rf** 0.43 (50% EtOAc in PE); **^{1}H NMR** (600 MHz, $CDCl_3$) δ 8.30–8.26 (m, 2H, H^{20}), 7.94 (d, 1H, *J* = 2.2 Hz, H^{11}), 7.63 (dd, 1H, *J* = 8.7, 2.3, H^9), 7.43–7.37 (m, 3H, H^8 and H^{19}), 5.85–5.81 (m, 1H, H^{13}), 5.56 (dd, 1H, *J* = 10.5, 7.9 Hz, H^5), 5.49 (dd, 1H, *J* = 3.4, 0.9 Hz, H^3), 5.14–5.10 (m, 2H, H^4 and H^6), 4.30–4.25 (m, 1H, H^1), 4.20–4.15 (m, 1H, H^1), 4.12–4.09 (m, 1H, H^2), 2.98–2.85 (m, 2H, H^{14}), 2.20 (s, 3H, C$\underline{H}_3$), 2.14 (d, 3H, *J* = 0.7 Hz, C$\underline{H}_3$), 2.11 (t, 1H, *J* = 2.6 Hz, H^{16}), 2.08 (s, 3H, C$\underline{H}_3$), 2.03 (s, 3H, C$\underline{H}_3$); **^{13}C NMR** (101 MHz, $CDCl_3$) δ 170.4, 170.24, 170.19, 169.4, 155.3, 151.6, 149.81, 149.79, 145.7, 141.33, 141.28, 133.38, 133.37, 132.2, 132.1, 125.5, 123.7, 123.6, 121.8, 119.8, 119.7, 100.69, 100.66, 77.54, 77.53, 77.4, 72.6, 71.64, 71.6, 70.58, 67.9, 66.8, 61.4, 26.39, 26.36, 20.8, 20.74, 20.66; **LRMS** (ESI) *m/z* found $[M+Na]^+$ 725.3, $C_{31}H_{30}N_2O_{17}Na^+$ required 725.1. Data in accordance with literature.[1]

Ac-Gal-MMAE (4b)



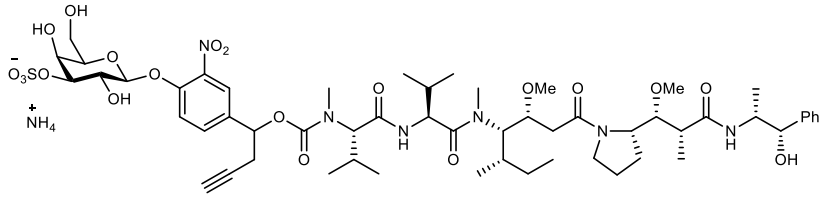
Carbonate **6** (45.1 mg, 64.2 µmol) and MMAE (30.0 mg, 41.8 µmol) were dissolved in 4:1 DMF/pyridine (1 mL) before the addition of HOBt (80%) (5.42 mg, 32.1 µmol) at rt. After 24 h, the reaction mixture was concentrated *in vacuo* and purified by flash column chromatography (1-5% MeOH in CH_2Cl_2) to yield carbamate **4b** (43.0 mg, 33.6 µmol, 80%) as a yellow solid. **Rf** 0.45 (5% MeOH in CH_2Cl_2); **HPLC** (5-95% MeCN/H_2O over 20 min) retention time 13.625 min; **LRMS** (ESI) *m/z* found $[M+H]^+$ 1281.7, $C_{64}H_{93}N_6O_{21}^+$ required 1281.6. Data in accordance with literature.[1]

Gal-MMAE (6b)



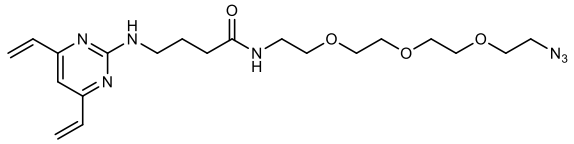
A solution of lithium hydroxide monohydrate (9.64 mg, 0.136 mmol) in H_2O (1 mL) was added dropwise to a solution of tetra-acetate **4b** (20.0 mg, 15.6 µmol) in MeOH (1 mL) at 0 °C. After 30 min, the reaction was neutralised by stirring with Amberlite® IRC76 (weakly acidic) at 0 °C for 30 min. The reaction mixture was then filtered through a pad of cotton wool, concentrated *in vacuo* and purified by flash column chromatography (3-20% MeOH in CH_2Cl_2) to yield alcohol **6b** (15.8 mg, 14.2 µmol, 91%) as a white solid. **Rf** 0.19 (5% MeOH in CH_2Cl_2); **HPLC** (5-95% MeCN/H_2O over 20 min) retention time 10.882, 10.949 min (diastereomers); **LRMS** (ESI) *m/z* found $[M+H]^+$ 1114.7, $C_{56}H_{85}N_6O_{17}^+$ required 1113.6. Data in accordance with literature.[1]

Sulfo-Gal-MMAE (7)



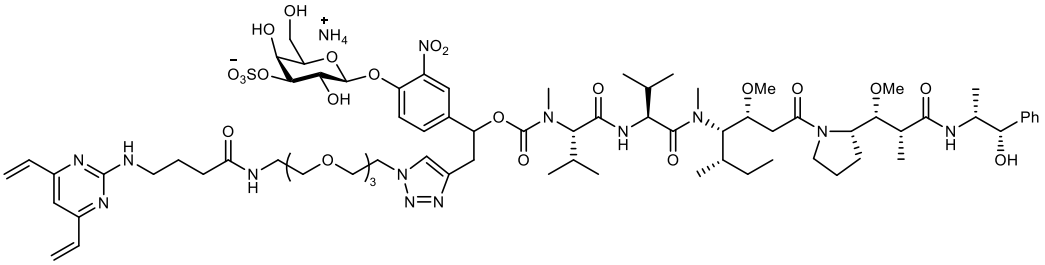
Dibutyltin oxide (2.24 mg, 8.98 µmol) was added to a solution of alcohol **6b** (10.0 mg, 8.98 µmol) in MeOH (0.1 mL) and stirred at 60 °C for 3 h. The reaction mixture was then allowed to cool to rt and concentrated under a stream of nitrogen. The resulting residue was suspended in THF at 0 °C (0.1 mL) and sulfur trioxide trimethylamine complex (2.50 mg, 18.0 µmol) was added. After 5 min the reaction mixture was warmed to rt and stirred for 90 min, then purified by reverse phase flash column chromatography (35-45% MeCN in 0.1 M NH_4OH (aq)) and lyophilised to yield sulfate **7** (6.02 mg, 4.98 µmol, 55%) as a hygroscopic white solid; **HPLC** (5-95% MeCN/H_2O over 20 min) retention time 11.174, 11.264 min (diastereomers); **LRMS** (ESI) *m/z* found $[M]^-$ 1192.5, $C_{56}H_{83}N_6O_{20}S^-$ required 1191.5.

DVP-PEG-N_3



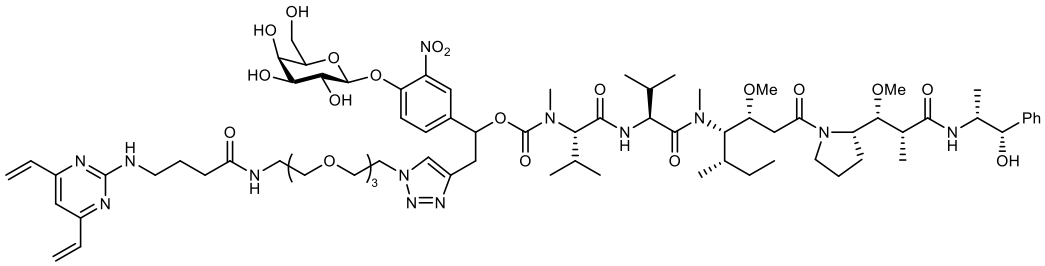
DVP-PEG_3-N_3 was synthesised according to the previously reported procedure.[2]

DVP-PEG$_3$-Sulfo-Gal-MMAE (2)



To a degassed solution of sulfate **7** (6.0 mg, 5.0 µmol) and DVP-PEG$_3$-N$_3$ (59.5 µL of 0.1 M solution in DMSO, 5.95 µmol) in ^{t}BuOH (0.2 mL) was added a degassed solution of $CuSO_4 \cdot 5H_2O$ (0.6 mg, 3 µmol), THPTA (2.2 mg, 5.0 µmol) and sodium ascorbate (2.0 mg, 9.9 µmol) in H_2O/^{t}BuOH (0.2 mL, 1:1) and the reaction mixture was stirred at rt for 60 min. The reaction mixture was then purified by reverse phase flash column chromatography (30-50% MeCN in 0.1 M NH_4OH (aq)) and lyophilised to yield linker-drug **2** (4.5 mg, 2.9 µmol, 58%) as a white solid. **HPLC** (5-95% MeCN/H_2O over 20 min) retention time 9.934, 9.966 min (diastereomers); **LRMS** (ESI) *m/z* found $[M]^-$ 1624.9, $C_{76}H_{114}N_{13}O_{24}S^-$ required 1624.8.

DVP-PEG$_3$-Gal-MMAE (8)

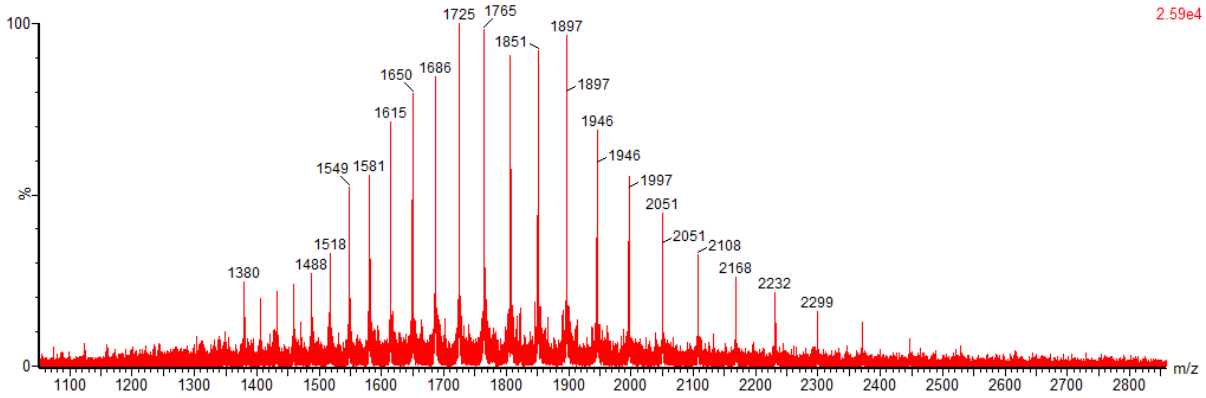


To a degassed solution of alkyne **6b** (3.1 mg, 2.8 µmol) and DVP-PEG$_3$-N$_3$ (33 µL of 0.1 M solution in DMSO, 3.3 µmol) in ^{t}BuOH (0.2 mL) was added a degassed solution of $CuSO_4 \cdot 5H_2O$ (0.4 mg, 1 µmol), THPTA (1.2 mg, 2.8 µmol) and sodium ascorbate (1.1 mg, 5.6 µmol) in H_2O/^{t}BuOH (0.2 mL, 1:1) and the reaction mixture was stirred at rt for 90 min. The reaction mixture was then purified by reverse phase flash column chromatography (30-50% MeCN in 0.1 M NH_4OH (aq)) and lyophilised to yield linker-drug **8** (2.1 mg, 1.4 µmol, 50%) as a white solid. **HPLC** (5-95% MeCN/H_2O over 20 min) retention time 9.637 min (diastereomers); **LRMS** (ESI) *m/z* found $[M+H]^+$ 1546.8, $C_{76}H_{116}N_{13}O_{21}^+$ required 1546.8.

3 Bioconjugation

LCMS Analysis

A



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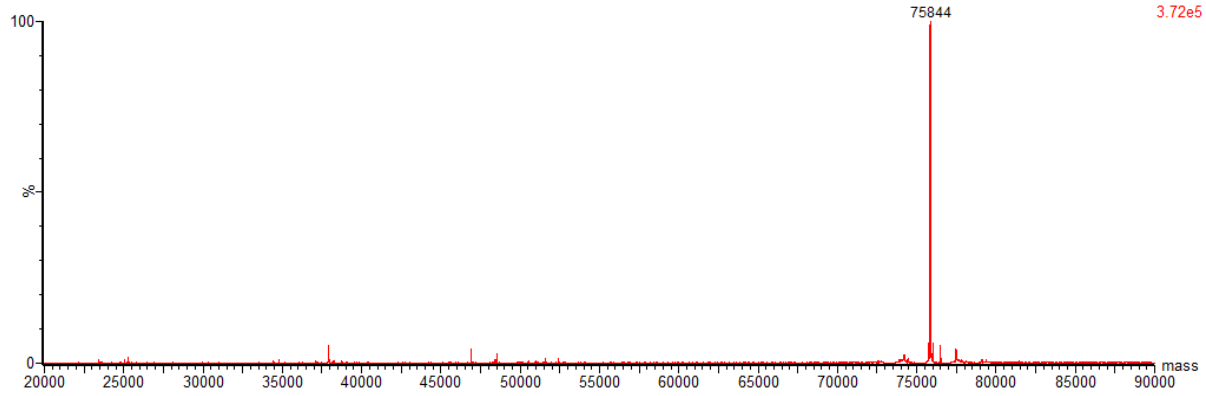

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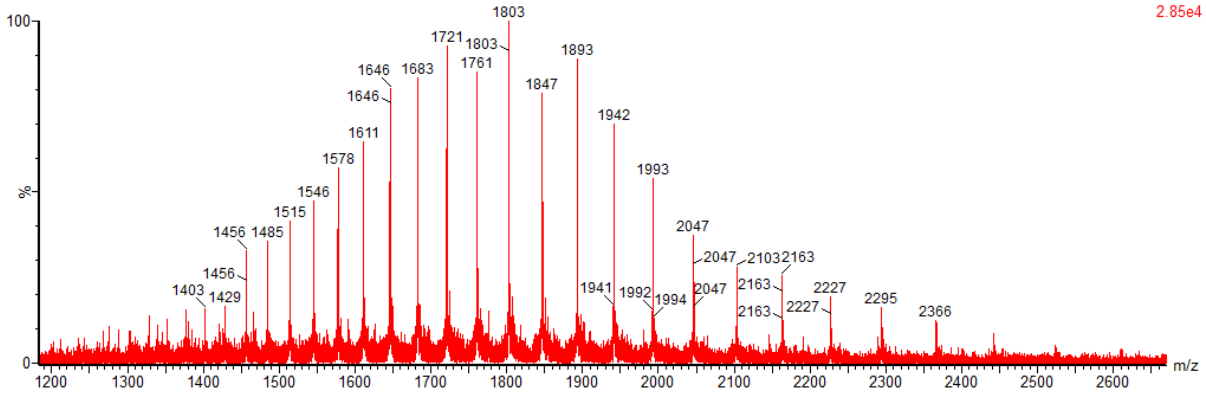


D

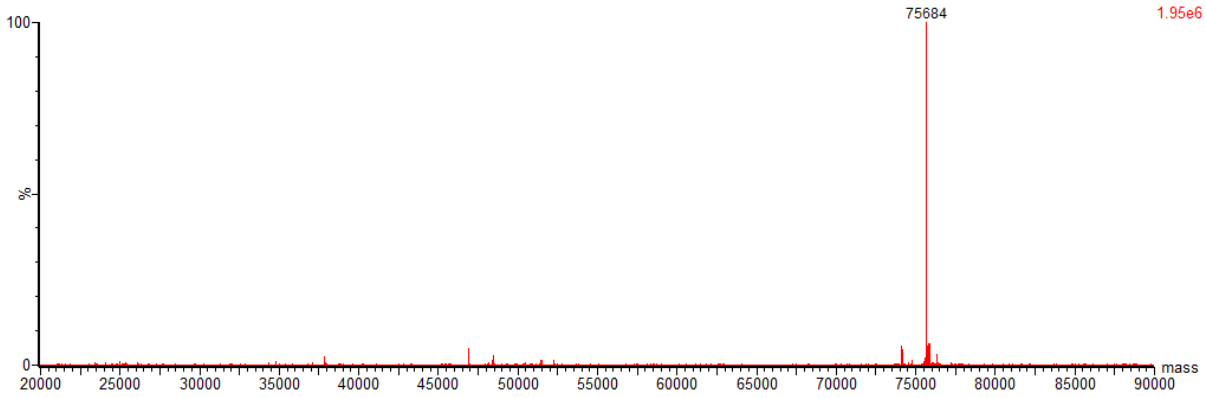


Figure S1: LCMS analysis of **ADC 1** and **ADC 2**. (A) Non-deconvoluted MS of **ADC 1**; (B) deconvoluted MS of **ADC 1**, expected 75,838 Da, observed 75,844; (C) non-deconvoluted MS of **ADC 2**; (D) deconvoluted MS of **ADC 2**, expected 75,678 Da, observed 75,684 Da.

SDS Page Analysis

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 (10 µL of 0.4 mg/mL) were prepared with reducing loading dye (10 µL, containing *β*-mercaptoethanol) and heated to 70 °C for 2 min. Gels were run at constant voltage (180 V) for 60 min in 1 × Laemmli running buffer (LRB). All gels were stained with Coomassie dye and imaged on a Syngene gel imaging system.

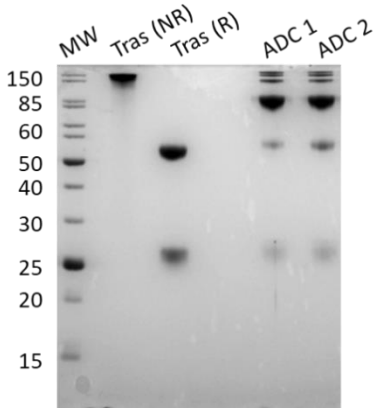


Figure S2: SDS-PAGE analysis of (A) **ADC 1** and **ADC 2** in 12% acrylamide gel. Lane markings: Tras (NR) = trastuzumab, non-reduced (loaded with no β-mercaptoethanol and not heated), Tras (R) = trastuzumab, reduced.

4 Fluorimetry

Human recombinant arylsulfatase A (EC 3.1.6.8) was purchased from R&D Systems. The enzyme molarities reported by the supplier were used to calculate the final sulfatase concentrations.

β-Galactosidase (from Escherichia coli, EC 3.2.1.23) was purchased from Sigma as a lyophilised powder and stored at -20 °C. The supplier's reported units/mg solid were used to calculate the final U/mL.

Human and mouse plasma were purchased from Sigma as lyophilised pellets. Upon reconstitution with distilled H_2O, the plasma was stored at 4 °C and used within one week.

Data were processed using GraphPad Prism Version 7.

Fluorescence was measured with a SpectraMax i3x plate reader (λ_{ex} = 350 nm, λ_{em} = 460 nm).

Cleavage by lysosomal enzymes

ARSA: ARSA (12 µL, 50 µM in 50 mM NaCl (aq)) was added to a vortexed solution of **1** (2.4 µL, 250 µM in DMSO) in NaOAc buffer (48 µL, 0.5 M, pH 5) and H_2O (57.6 µL).

β-Gal: β-Gal (12 µL, 1 U/mL in 50 mM NaCl (aq)) was added to a vortexed solution of **1** (2.4 µL, 250 µM in DMSO) in NaOAc buffer (48 µL, 0.5 M, pH 5) and H_2O (57.6 µL). Enzyme units (U) refer to the enzyme's ability to hydrolyse 2-nitrophenyl β-D-galactoside.

ARSA and β-Gal together: ARSA (6 µL, 100 µM in 50 mM NaCl (aq)) and β-Gal (6 µL, 2 U/mL in 50 mM NaCl (aq)) were added to a vortexed solution of **1** (2.4 µL, 250 µM in DMSO) in NaOAc buffer (48 µL, 0.5 M, pH 5) and H_2O (57.6 µL).

110 µL of each resulting solution was added to a 384 well plate (Greiner, black, clear bottomed) and the fluorescence intensity was measured over 20 h at 37 °C, with readings taken at 2 minute intervals. An adhesive film (Bio-Rad) was used to prevent solvent evaporation. The reactions were performed in triplicate and the plotted values are the mean values.

Plasma Stability

Plasma (66 µL) was added to a vortexed solution of **1** (2.4 µL, 250 µM in DMSO) in PBS (51.6 µL). 110 µL of the resulting solution was added to a 384 well plate (Greiner, black, clear bottomed). The fluorescence intensity was measured over 20 h at 37 °C, with readings taken at 2 minute intervals. An adhesive film (Bio-Rad) was used to prevent solvent evaporation. The reactions were performed in triplicate and the plotted values are the mean values.

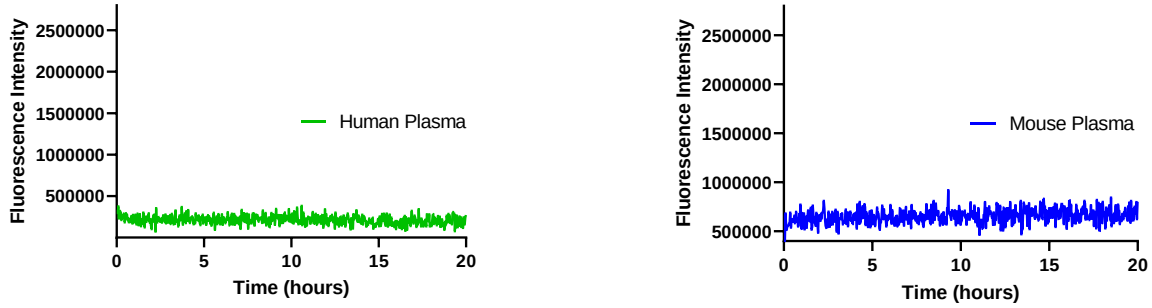


Figure S3: Fluorometric measurement of AMC release from probe **1** by incubation with human (left) and mouse (right) plasma.

5 Cell Lines

HER2-positive SKBR3 cells were obtained from the American Type Culture Collection (ATCC) and HER2-negative MCF7 cells were obtained from the European Collection of Authenticated Cell Cultures (ECACC) and ATCC, respectively. 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% heat-inactivated fetal-bovine serum (FBS), 2 mM Lglutamine, 50 U/mL penicillin and 50 µg/mL streptomycin. All cell lines were incubated at 37 °C with 5% CO_2.

6 Cell Viability

Cells were seeded in 96-well plates for 24 h at 37 °C with 5% CO_2. SKBR3 cells were seeded at 15,000 cells/well and MCF7 cells were seeded at 7,500 cells/well. Serial dilutions of **ADC 1** and **ADC 2** were added to the cells in complete growth medium and incubated at 37 °C with 5% CO_2 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.

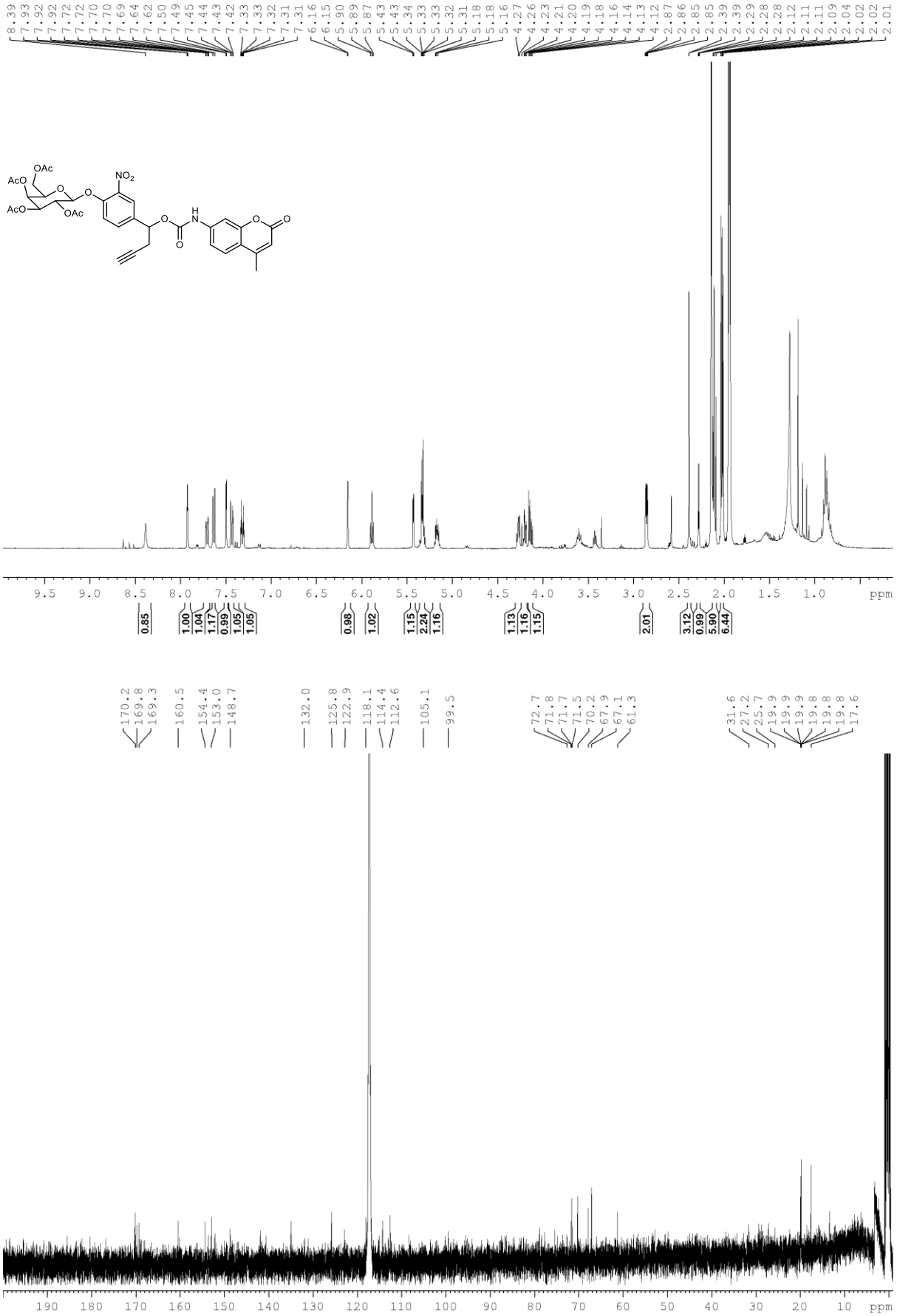
7 References

1 T. Legigan, J. Clarhaut, I. Tranoy-Opalinski, A. Monvoisin, B. Renoux, M. Thomas, A. Le-Pape, S. Lerondel and S. Papot, *Angew. Chemie - Int. Ed.*, 2012, **51**, 11606–11610.

2 J. D. Bargh, S. J. Walsh, A. Isidro-Llobet, S. Omarjee, J. S. Carroll and D. R. Spring, *Chem. Sci.*, 2020, **11**, 2375–2380.

8 NMR Spectra and HPLC Traces

Ac-Gal-AMC (4a)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OAc
AcO
AcO
OAc
NO2
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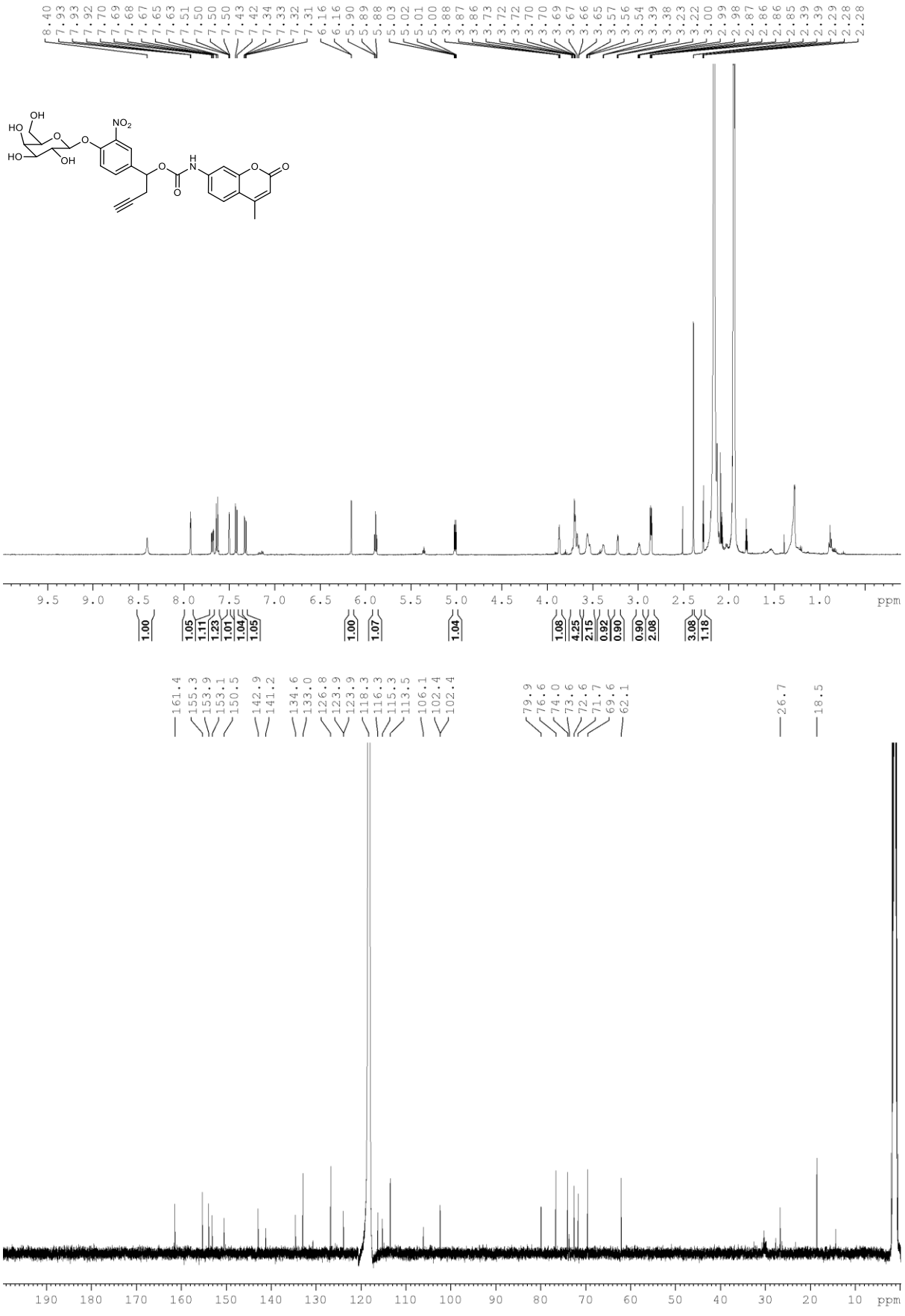
N
O
O
O
O
O
9.5 9.0 8.5 8.0 7.5 7.0 6.5 6.0 5.5 5.0 4.5 4.0 3.5 3.0 2.5 2.0 1.5 1.0 ppm
0.85 1.00 1.04 1.17 0.99 1.05 1.05 0.98 1.02 1.15 2.24 1.16 1.13 1.16 1.15 2.01 3.12 0.99 5.90 6.44
170.2 169.8 169.3 160.5 154.4 153.0 148.7 132.0 125.8 122.9 118.1 114.4 112.6 105.1 99.5 72.7 71.8 71.7 71.5 70.2 67.9 67.1 61.3 31.6 27.2 25.7 19.9 19.9 19.9 19.8 19.8 19.8 17.6
190 180 170 160 150 140 130 120 110 100 90 80 70 60 50 40 30 20 10 ppm

Gal-AMC (6a)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NO2
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N
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9.5 9.0 8.5 8.0 7.5 7.0 6.5 6.0 5.5 5.0 4.5 4.0 3.5 3.0 2.5 2.0 1.5 1.0 ppm
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161.4 155.3 153.9 153.1 150.5 142.9 141.2 134.6 133.0 126.8 123.9 123.9 118.3 116.3 115.3 113.5 106.1 102.4 102.4 79.9 76.6 74.0 73.6 72.6 71.7 69.6 62.1 26.7 18.5
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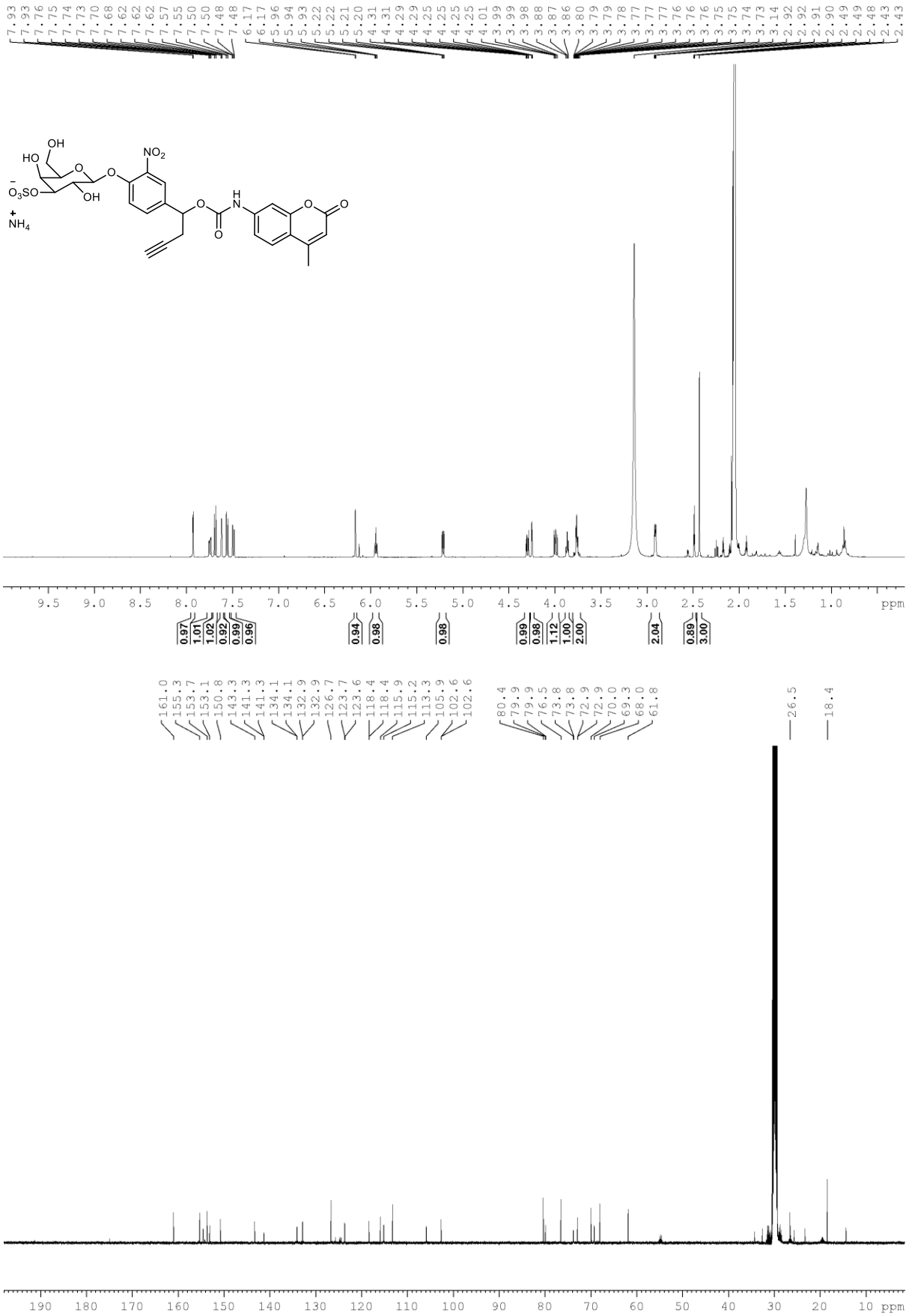

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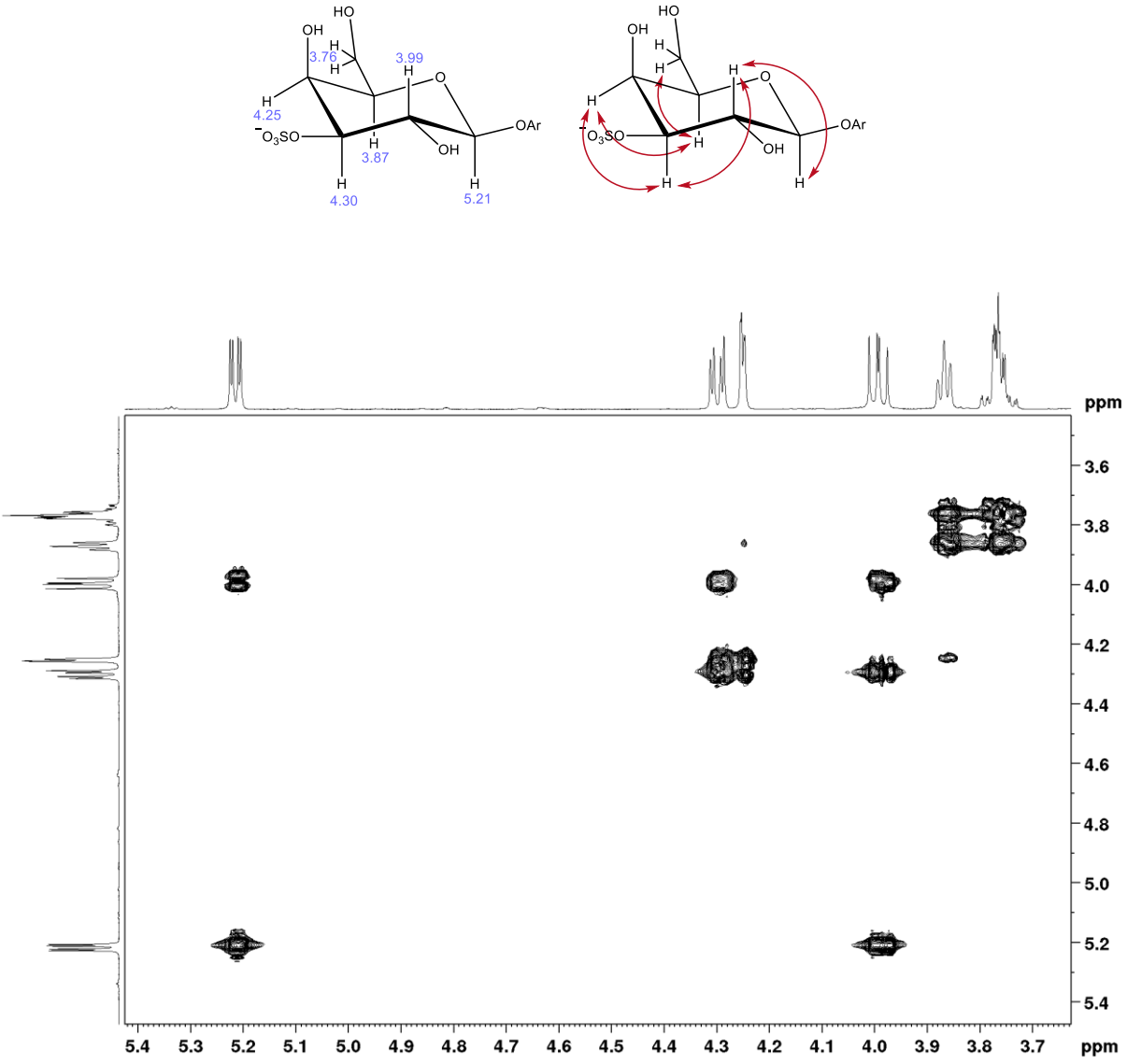
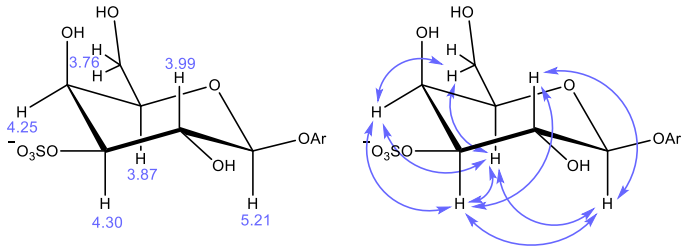
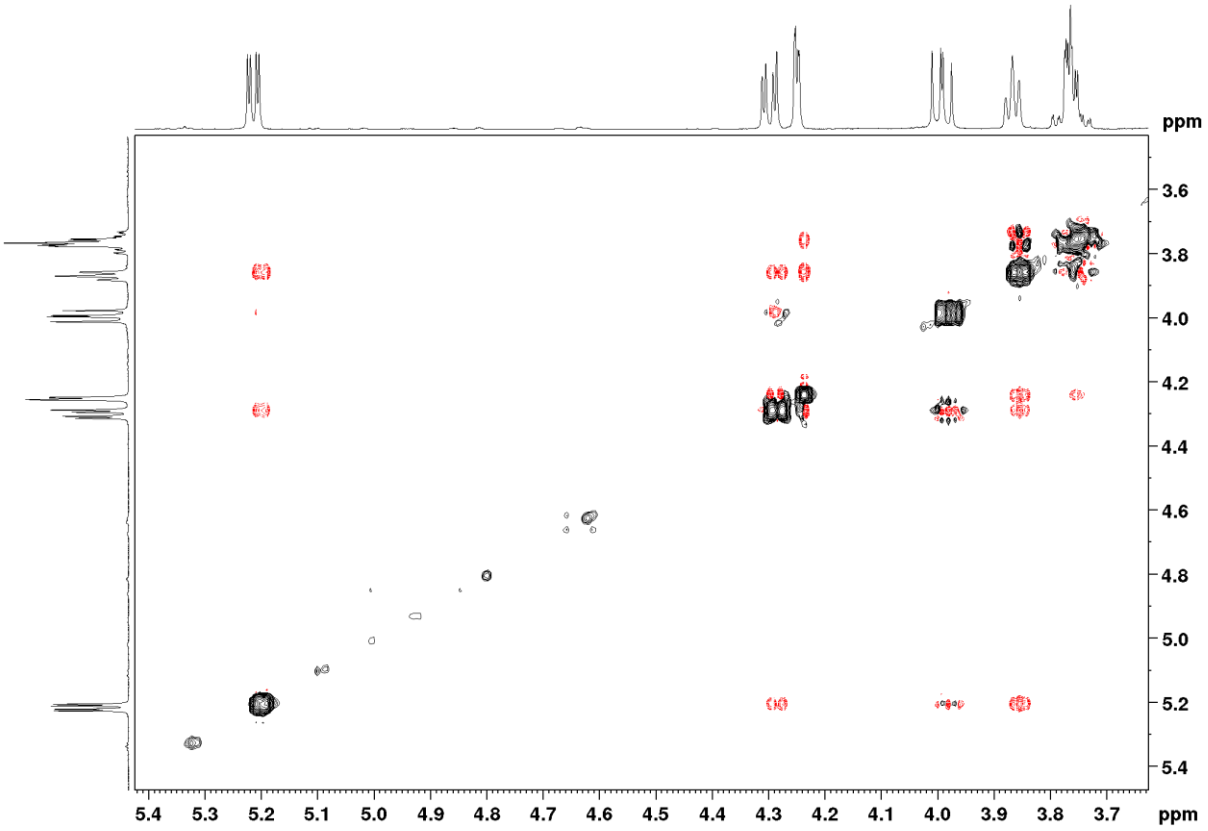


Sulfo-Gal-AMC (1) – ^{1}H–^{1}H NOESY





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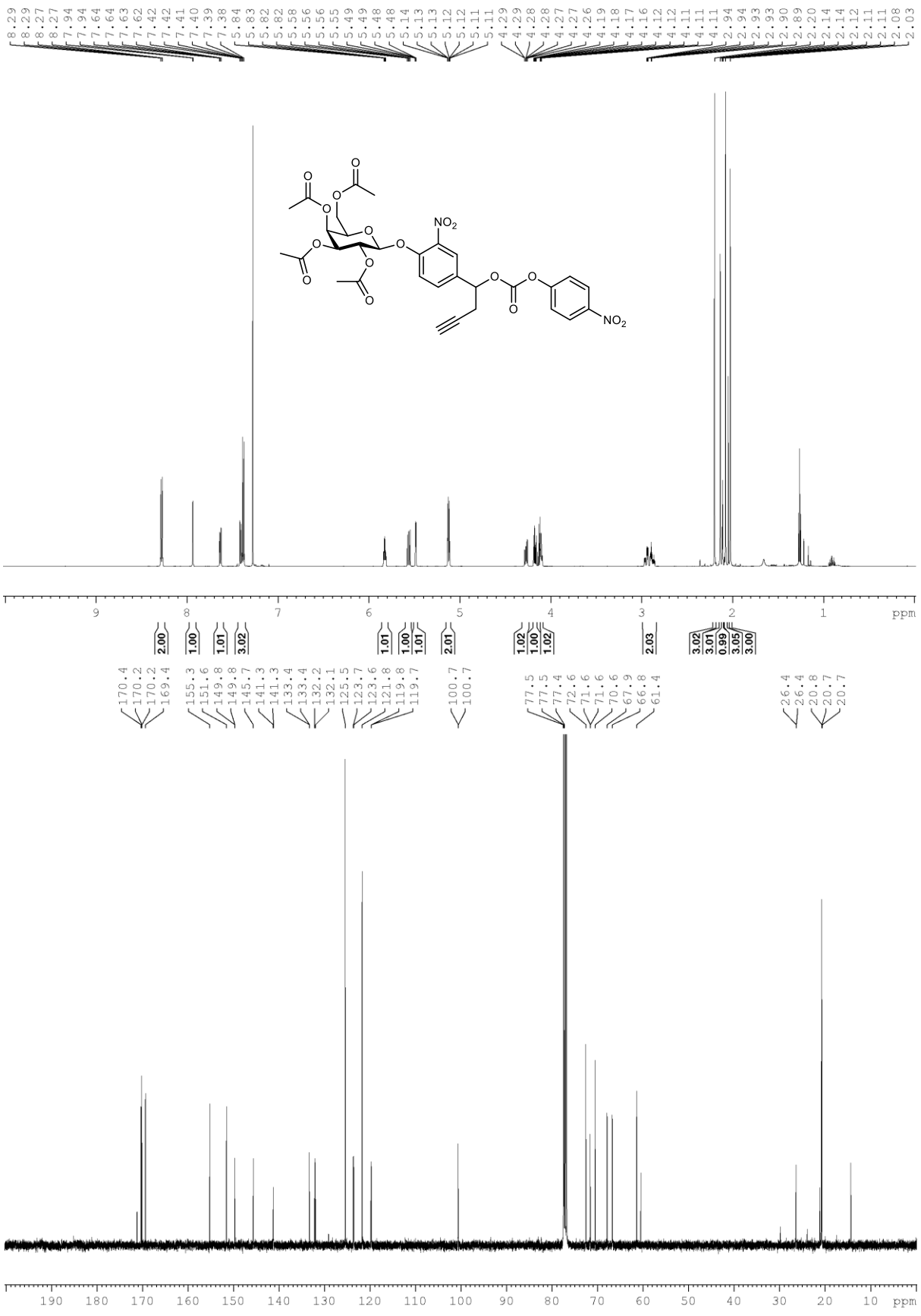
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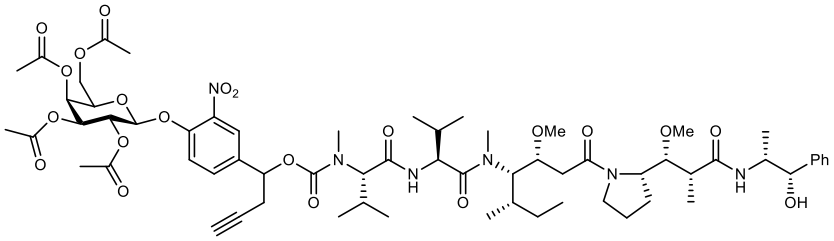
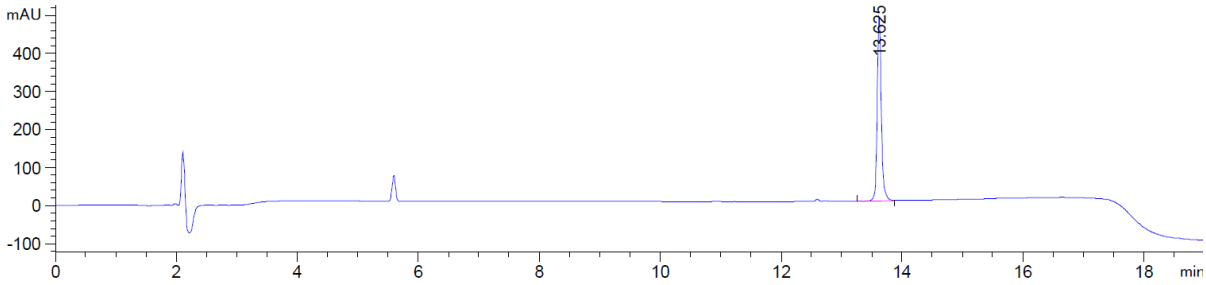
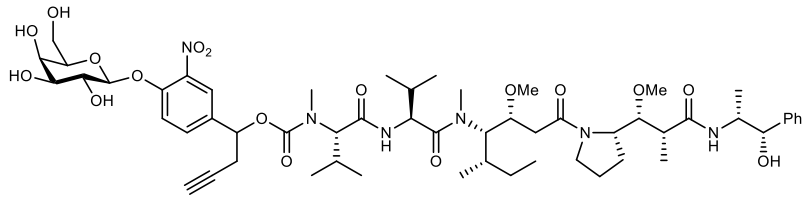
Ac-Gal-MMAE (4b)





Gal-MMAE (6b)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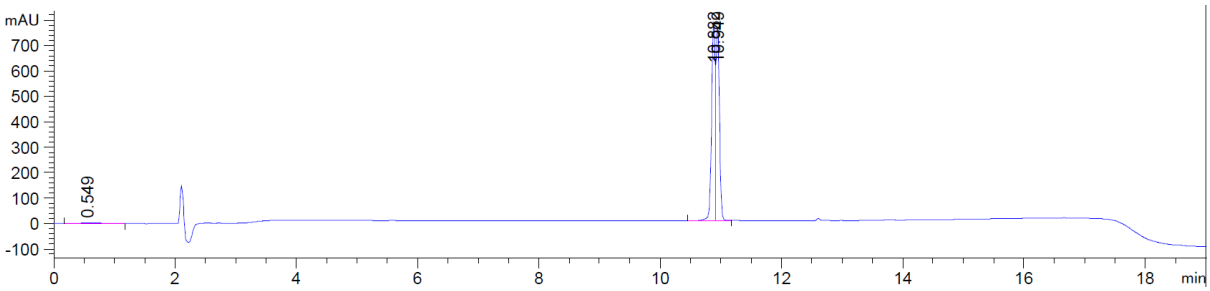
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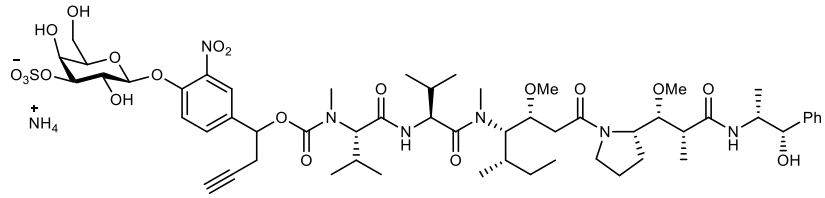
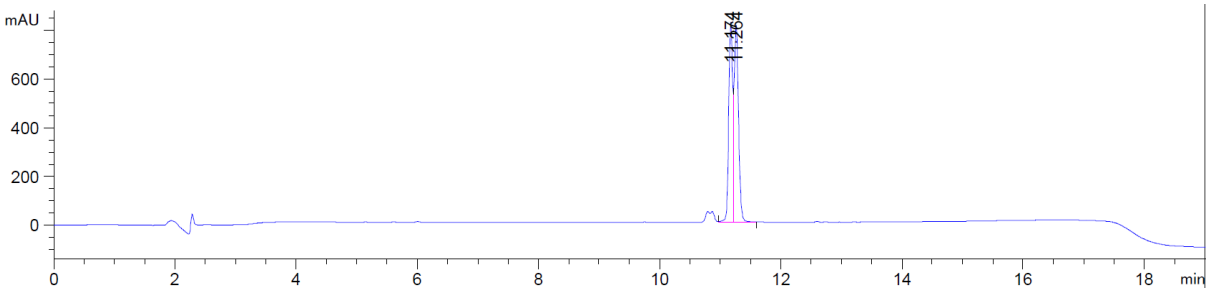
Sulfo-Gal-MMAE (7)





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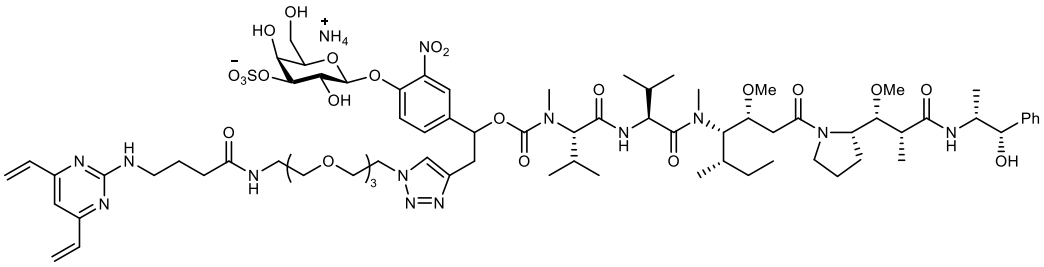

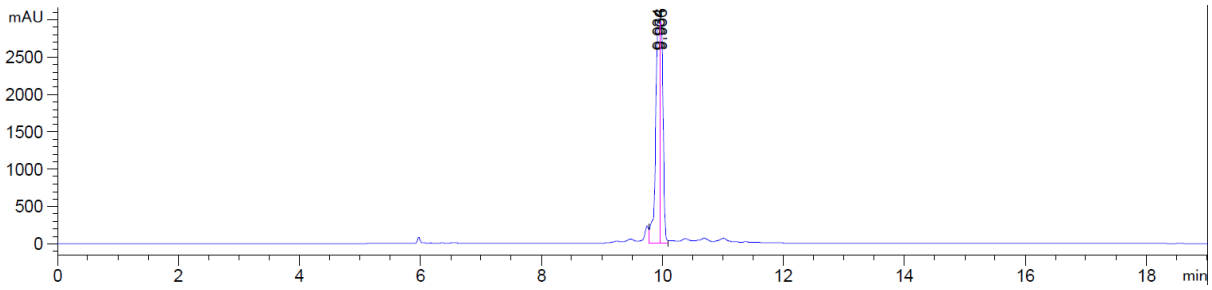

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