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Electronic Supplementary Information

Use of a next generation maleimide in combination with THIOMAB™ antibody technology delivers a highly stable, potent and near homogeneous THIOMAB™ antibody-drug conjugate (TDC)

João P. M. Nunes,[a] Vessela Vassileva,[b] Eifion Robinson,[a] Maurício Morais,[a] Mark E. B. Smith,[a] R. Barbara Pedley,[b] Stephen Caddick,[a] James R. Baker*[a] and Vijay Chudasama*[a]

[a]Department of Chemistry, University College London, London, WC1H 0AJ, UK.
Tel.: +44 (0)20 7679 2653 and + 44 (0)20 7679 2077;
E-mail: j.r.baker@ucl.ac.uk and v.chudasama@ucl.ac.uk
[b]UCL Cancer Institute, 72 Huntley Street, London, WC1E 6BT, UK

Sections Included

Conjugation Experiments General Remarks

Conjugation experiments were carried out in standard polypropylene micro test tubes 3810x 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 25 mM sodium borate, 25 mM sodium chloride and 1 mM EDTA at pH 8.0. Phosphate-buffered saline (PBS) was 140 mM sodium chloride and 12 mM sodium phosphates at pH 7.4. Phosphate-buffered saline for SEC-HPLC was 140 mM NaCl, 100 mM sodium phosphates and 0.02% sodium azide at pH 7.0. 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. Solutions of dehydroascorbic acid (dhAA) 10 mM (1.74 mg/mL) were prepared in PBS. 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 5 kDa. Centrifugation was carried out on an eppendorf 5415R fixed angle rotor centrifuge operating at 14000 rcf at 20 °C.

Thio-trastuzumab antibody is a trastuzumab (IgG1) variant with an engineered cysteine on each light chain (LC-V205C), directed against HER2. This THIOMAB™ antibody was obtained as a 3.67 mg/mL solution in 50 mM Tris acetates solution (Genentech). Thio-trastuzumab was buffer exchanged into BBS *via* ultrafiltration (10 kDa MWCO). Concentration was determined by UV-vis absorbance (using ε_{280} = 210896 M^{-1} cm^{-1} or A = 1.422 for 1 mg/mL for thio-trastuzumab), adjusted to 40 µM (5.93 mg/mL) and was stored in aliquots at 4 °C or used for experiments. Thio-trastuzumab drug conjugate (TDC) concentration was determined using the same extinction coefficient as for unmodified thio-trastuzumab (both MMAE and maleamic acid compounds were found to have negligible absorption at 280 nm). The following acronyms are used to describe light chain (LC) and heavy chain (HC).

Analytical methods for antibody-drug conjugates

SDS-PAGE

Non-reducing glycine-SDS-PAGE 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 ~25-30 µM in total thio-trastuzumab) were quenched with maleimide (1 µL of a 10 mM solution in PBS, >110 eq.) and mixed with loading buffer (2 µL, composition for 6× 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 2 minutes. For reducing gel (using β-mercaptoethanol (BME) as reducing agent), samples (3 µL at ~25-30 µM in total thio-trastuzumab) were mixed with loading buffer (2 µL, composition for 4 x SDS: 0.8 mL BME, 0.8 g SDS, 4 mL glycerol, 2.5 mL 0.5 M Tris buffer pH 6.8, 2.5 mL H_2O, 2 mg R-250 dye). 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[1] where 0.12 % of Coomassie G-250 and Coomassie R-250 dyes were added to the staining solution (5:4:1 MeOH:H_2O:AcOH).

Determination of thio-trastuzumab and conjugate concentration

UV-vis spectra were recorded on a Varian Cary 100 Bio UV-visible spectrophotometer, operating at 20 °C using a cell length (l) of 1 cm. Sample buffer was used as blank for baseline correction. Calculation of antibody concentration follows the Beer-Lambert law using ε_{280} = 210896 M^{-1} cm^{-1} for thio-trastuzumab. Thio-trastuzumab drug conjugate (TDC) **3** concentration was calculated as follows using the same extinction coefficient for thio-trastuzumab since the maleamic acid group and MMAE were found to have negligible absorption at 280 nm compared to thio-trastuzumab. Thio-trastuzumab fluorophore conjugate (TFC) **5** concentration was calculated using the same extinction coefficient for thio-trastuzumab, applied to a corrected absorption value at 280 nm calculated as follows by subtracting AlexaFluor® 488 absorption at 280 nm using 0.11 as a correction factor. A_{280} is measured absorption at 280 nm and A_{AF495} is dye absorption at 495 nm.

$$[TDC] = \frac{A_{280}}{l \times \varepsilon_{280}} \qquad [TFC] = \frac{A_{280} - (0.11 \times A_{AF495})}{l \times \varepsilon_{280}}$$

Ellman's assay

Ellman's assay was carried out by mixing a 1 mM solution of 5,5'-dithio-*bis*-(2-nitrobenzoic acid) (Ellman's reagent) in PBS (5 µL) with the thio-trastuzumab or conjugate sample at ~25-30 µM (5 µL) and diluting with PBS (60 µL). The solution was incubated at 20 °C for 2 min and then absorption was measured at 280 nm (protein concentration) and 412 nm (2-nitro-5-thiobenzoic acid). A sample of 1 mM Ellman's reagent in PBS (5 µL) diluted with PBS (65 µL) was used as blank for baseline correction. Each sample in PBS was analysed in the absence and presence of Ellman's reagent, under identical concentration conditions. The sulfhydryl per antibody ratio (SAR) was calculated as follows with ε_{412} = 14150 M^{-1} cm^{-1} for 2-nitro-5-thiobenzoic acid and ε_{280} = 210896 M^{-1} cm^{-1} for thio-trastuzumab, where A_{280} is measured absorption at 280 nm in the absence of Ellman's reagent and A_{412} is absorption at 412 nm in the presence of Ellman's reagent.

$$SAR = \frac{(A_{412})/\varepsilon_{412}}{(A_{280})/\varepsilon_{280}}$$

Liquid chromatography mass spectrometry (LC-MS)

Thio-trastuzumab and TDC **3** samples were prepared in water (6.6 µM, 1 mg/mL). LC-MS analysis was performed on a Hypersil Gold C4 1.9 µm 2.1 × 50 mm column connected to an Agilent 1100 HPLC connected to a Micromass Q-TOF API-US. Detection wavelength was 254 nm. Samples were eluted with 95:5 Water:MeCN (0.1% formic acid) to 5:95 Water: MeCN (0.1% formic acid) gradient over 7 min with a flow rate of 0.4 mL/min. MS Mode: ES+. Scan Range: m/z = 500-4000. Scan time: 1.0 s. Data was obtained in continuum mode. The electrospray source of the MS was operated with a capillary voltage of 3 kV and a cone voltage of 25 V. Nitrogen was used as the nebulizer and desolvation gas at a total flow of 756 L/h. Ion series were generated by integration of the total ion chromatogram (TIC) over the 3.0-4.5 min range. Total mass spectra for protein samples were reconstructed from the ion series using MassLynx V4.0 SP4 software.

Size-exclusion chromatography (SEC)

Thio-trastuzumab and TDC **3** were prepared as 13 µM (2 mg/mL) solutions in PBS. Samples were analysed by SEC-HPLC on a TSK gel G3000SWXL (7.8 mm x 30 cm) column connected to an Agilent 1100 HPLC system equipped with a 1100 series diode array detector. Samples were eluted using PBS 100 mM NaCl, 50 mM sodium phosphates and 0.02% sodium azide at pH 7.0 as mobile phase at a flow rate of 0.5 mL/ min. over 30 min. Detection wavelength was 280 nm.

Enzyme-linked immunosorbent assay (ELISA)

Binding affinity to HER2 receptor was determined by ELISA. PBS 140 mM sodium chloride and 12 mM sodium phosphates at pH 7.4 was used for all solutions. A maxisorp 96-well plate was coated for 2 h at 20 °C with HER2 (100 µL of a 0.25 µg/mL solution in PBS). One row of wells was coated with PBS only as a negative control. Next, each well was washed with PBS and blocked with a 3% BSA solution in PBS (200 µL) overnight at 4 °C. Then, the wells were washed with 0.1% Tween 20 in PBS, followed by PBS. Thio-trastuzumab and TDC **3** were diluted in PBS yielding the following concentrations (nM): 80, 60, 40, 20, 10, 5, 2.5, 1.25, 0.42, and 0.14 nM. The dilution series was added, including PBS only and thio-trastuzumab or TDC **3** at 80 nM in the absence of HER2 as negative controls. The plate was incubated for 2 h at 20 °C. Then, wells were washed and the detection antibody (100 µL of anti-human IgG, Fab-specific-HRP solution, 1:5000 in PBS) was added followed by incubation for 1 h at 20 °C. After another washing step, freshly prepared OPD solution (100 µL of 10 mg/20 mL OPD in phosphate-citrate buffer) was added to each well and the reaction was stopped by addition of 4 M HCl (50 µL). The colorimetric reaction was measured at 490 nm. Absorption values for a given concentration of thio-trastuzumab or TDC **3** were corrected as follows, where $A_{490corr}$ is corrected absorption, $A_{490corr}$ is measured absorption, A_{PBS} is absorption for PBS control, A_{TmAb80} is absorption for thio-trastuzumab or TDC **3** control at 80 nM and C is the given concentration for the respective data point.

$$A_{490corr} = A_{490} - \left(\frac{A_{PBS} + A_{TmAb80 \times C/80}}{2}\right)$$

Alexa Fluor 488® conjugate serum stability

Alexa Fluor 488® thio-trastuzumab fluorophore conjugate TFC **5** was prepared as 0.2 mg/mL solutions in PBS 140 mM sodium chloride 12 mM sodium phosphates and 2 mM sodium azide at pH 7.4. The conjugate was diluted with 50% of human blood serum to give a final a concentration of 0.1 mg/mL of TFC **5** and 1 mM of sodium azide. One aliquot (50 µL) was taken, flash frozen and stored at −80 °C. The remaining solution was incubated at 37 °C under mild shaking (300 rpm) and under the cover of light. Aliquots (50 µL) were taken at 1, 2, 4, 7 and 10 days, flash frozen and stored at −80 °C. Aliquots were thawed, spin-filtered (0.22 µm filter) and diluted 100x with elution buffer. Samples (20 µL) of diluted aliquots were analysed by SEC-HPLC on a TSK gel G3000SWXL (7.8 mm x 30 cm) column connected to an Agilent 1200 HPLC system equipped with a 1200 series diode array detector and a fluorescence detector. Samples were eluted using PBS 140 mM NaCl, 100 mM sodium phosphates and 0.02% sodium azide at pH 7.0 as mobile phase at a flow rate of 0.5 mL/ min. over 30 min. Fluorescence was detected with an excitation wavelength of 495 nm and emission wavelength of 525 nm.

Determination of drug to antibody ratio (DAR)

To TDC **3** (20 µM, 50 µL, 0.001 µmol) in BBS pH 8 was added TCEP (2 mM, 5 µL, 10 eq.) and the reaction was incubated at 37 °C for 3 h under mild agitation (400 rpm). Then, added maleimide (10 mM, 10 µL, 100 eq.) Purified by ultrafiltration (10 kDa MWCO) into water and immediately analysed by LC-MS as described above. Total protein mass spectra were obtained from reconstructed ion series. Light chain (LC) species peaks were integrated to determine number of counts per mass species. The drug to antibody ratio (DAR) was calculated as follows, where LC–$MMAE_{nonHyd}$ is number of counts for light chain species (only proton adduct was observed) modified with one maleimide PEG_{12}-vc-PABC-MMAE payload and one maleimide, LC−$MMAE_{Hyd}$ is number of counts for light chain species (proton and sodium adducts) modified with one maleamic PEG_{12}-vc-PABC-MMAE payload and one maleimide, and LC−Mal is number of counts for light chain species modified with two maleimides alone (only proton adduct was observed).

$$DAR = 2 \times \frac{(LC\text{–}MMAE_{nonHyd} + LC\text{–}MMAE_{Hyd})}{(LC\text{–}MMAE_{nonHyd} + LC\text{–}MMAE_{Hyd}) + (LC\text{–}Mal)}$$

Determination of fluorophore to antibody ratio (FAR)

UV-vis spectra were recorded on a Varian Cary 100 Bio UV-visible spectrophotometer, operating at 20 °C. Sample buffer was used as blank for baseline correction. Calculation of fluorophore to antibody ratio (FAR) follows the formula below, where with ε_{280} = 210896 M^{-1} cm^{-1} for thio-trastuzumab, ε_{495} = 71000 M^{-1} cm^{-1} for Alexa Fluor 488® and 0.11 as a correction factor for the dye absorption at 280 nm.

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

Cell Lines

Three human breast cancer cell lines SKBR-3 (Her-2-positive), HCC-1954 (Her-2-positive) and MCF-7 (Her-2-negative) were purchased from ATCC, and maintained in McCoy's 5A, RPMI-1640, and DMEM medium, respectively, all supplemented with 10% foetal bovine serum (Life Technologies, UK) at 37 °C, 5% CO_2.

***In Vitro* Cytotoxicity Assessment**

In vitro cytotoxicity of the compounds was evaluated in all three human breast cancer cell lines (SKBR-3, HCC-1954 and MCF-7) by the MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide] colourimetric assay. Briefly, 5×10^4 cells were seeded in 96-well plates and incubated overnight. Cells were then exposed to a range of concentrations (0-100 nM) of MMAE (24h), thio-trastuzumab (72h) and TDC **3** (72h). Following each treatment, cells were washed with PBS and incubated with drug-free medium for five days. The MTT reagent (5 mg/ml) was then added to each well and cells were incubated for 2h, followed by the addition of ethanol:DMSO (1:1) solution and optical density (OD) was measured at 540 nm. The percentage of viable cells was calculated as follows:

$$Cell\ viability\ (\%) = \frac{(OD_{treated\ cells})}{(OD_{untreated\ cells})} \times 100$$

Conjugation protocols

Conjugation of thio-trastuzumab with MBM 1 (conjugate TDC 3)

To thio-trastuzumab (40 µM, 200 µL, 0.008 µmol) in BBS pH 8 was added TCEP (10 mM, 8 µL, 10 eq.) and the reaction was incubated at 37 °C for 3 h under mild agitation (400 rpm). The buffer was exchanged to PBS 140 mM sodium chloride and 12 mM sodium phosphates at pH 7.4 by ultrafiltration (5 kDa MWCO) to remove unreacted TCEP and the volume was corrected to 200 µL. Next, added dhAA (10 mM, 16 µL, 20 eq.) and the reaction was incubated at 37 °C for 3 h under mild agitation (400 rpm). The buffer was exchanged to BBS pH 8 by ultrafiltration (5 kDa MWCO) to remove unreacted dhAA and the volume was corrected to 200 µL. Then, MBM **1** was prepared in dry DMF (2 mM, 16 µL, 4 eq.) and added to the thio-trastuzumab solution. The reaction was incubated at 20 °C for 1 h. Afterwards, excess reagents were removed by ultrafiltration (5 kDa MWCO) with PBS to afford the modified thio-trastuzumab conjugate TDC **3** in PBS with 60% yield and drug-to-antibody ratio (DAR) of 1.8.

Conjugation of thio-trastuzumab with MBM 2 (conjugate TAC 4), followed by copper-catalysed Huisgen 1,3-dipolar cycloaddition (CuAAC) with Alexa Fluor 488® (conjugate TFC 5)

To thio-trastuzumab (40 µM, 80 µL, 0.0032 µmol) in BBS pH 8 was added TCEP (10 mM, 3.2 µL, 10 eq.) and the reaction was incubated at 37 °C for 3 h under mild agitation (400 rpm). The buffer was exchanged to PBS 140 mM sodium chloride and 12 mM sodium phosphates at pH 7.4 by ultrafiltration (5 kDa MWCO) to remove unreacted TCEP and the volume was corrected to 80 µL. Next, added dhAA (10 mM, 6.4 µL, 20 eq.) and the reaction was incubated at 37 °C for 3 h under mild agitation (400 rpm). The buffer was exchanged to BBS pH 8 by ultrafiltration (5 kDa MWCO) to remove unreacted dhAA and the volume was corrected to 80 µL. Then, MBM **2** was prepared in dry DMF (10 mM, 1.3 µL, 4 eq.) and added to the thio-trastuzumab solution. The reaction was incubated at 20 °C for 4 h. Afterwards, excess reagents were removed by ultrafiltration (5 kDa MWCO) with 50 mM phosphate buffer at pH 7.0 and the volume corrected to 80 µL to afford the modified thio-trastuzumab conjugate TAC **4** in PBS. Next, added tris(3-hydroxypropyltriazolylmethyl)amine (THPTA, 25 mM, 5.1 µL 40 eq.) and $CuSO_4$ (10 mM, 2.6 µL, 8 eq.). Then, added Alexa Fluor 488® in

DMF (5 mM, 6.4 µL, 10 eq.), followed by sodium ascorbate (final concentration 5 mM) and the reaction mixture was incubated at 20 °C for 1 h. Excess reagents were removed by ultrafiltration (5 kDa MWCO) into fresh PBS to afford the modified thio-trastuzumab conjugate TFC **5** with 40% yield and a fluorophore to thio-trastuzumab ratio of 1.1.

Conjugation of albumin with MBM 2, followed by copper-catalysed Huisgen 1,3-dipolar cycloaddition (CuAAC) with Alexa Fluor 488® (used as control for serum stability study)

To albumin (40 µM, 300 µL, 0.012 µmol) in BBS pH 8 was added dithiothreitol (DTT 10 mM, 6 µL, 5 eq.) and the reaction was incubated at 20 °C for 90 min. under mild agitation (400 rpm). The buffer was exchanged to fresh BBS pH 8 by ultrafiltration (5 kDa MWCO) to remove unreacted DTT and the volume was corrected to 300 µL. In a separate vial, MBM **2** was prepared in dry DMF (10 mM, 6 µL, 5 eq.) and added tris(3-hydroxypopyltriazolylmethyl)amine (THPTA, 50 mM, 2.4 µL, 10 eq.) and $CuSO_4$ (20 mM, 6 µL, 10 eq.). Next, added Alexa Fluor 488® in DMF (5 mM, 18 µL, 7.5 eq.), followed by sodium ascorbate (final concentration 5 mM) in 50 mM phosphate buffer pH 7. This reaction mixture was incubated at 20 °C for 4 h. Then, it was added to the reduced albumin solution and the mixture was incubated at 20 °C for 1 h. Afterwards, excess reagents were removed by ultrafiltration (5 kDa MWCO) with 50 mM phosphate buffer at pH 7.0 and the volume corrected to 300 µL to afford the modified albumin conjugate in PBS with 76% yield and a fluorophore to albumin ratio of 1.0.

Supplementary figures and tables

Table S1 – Sulfhydryl per antibody (SAR) and corresponding calculated DAR from Ellman's analysis of TDC **3**.

Conditions	SAR	DAR (by Ellman's)*	DAR (by LC-MS)
Reduced thio-trastuzumab	10.0	n.a.	n.a.
Reoxidised thio-trastuzumab	2.2	n.a.	n.a.
After conjugation and purification	0.2	1.8	1.8

*Estimated DAR assumes non-free sulfhydryls are NGM-MMAE maleamic disulfide bridges.

Figure S1 – LC-MS data of unmodified thio-trastuzumab

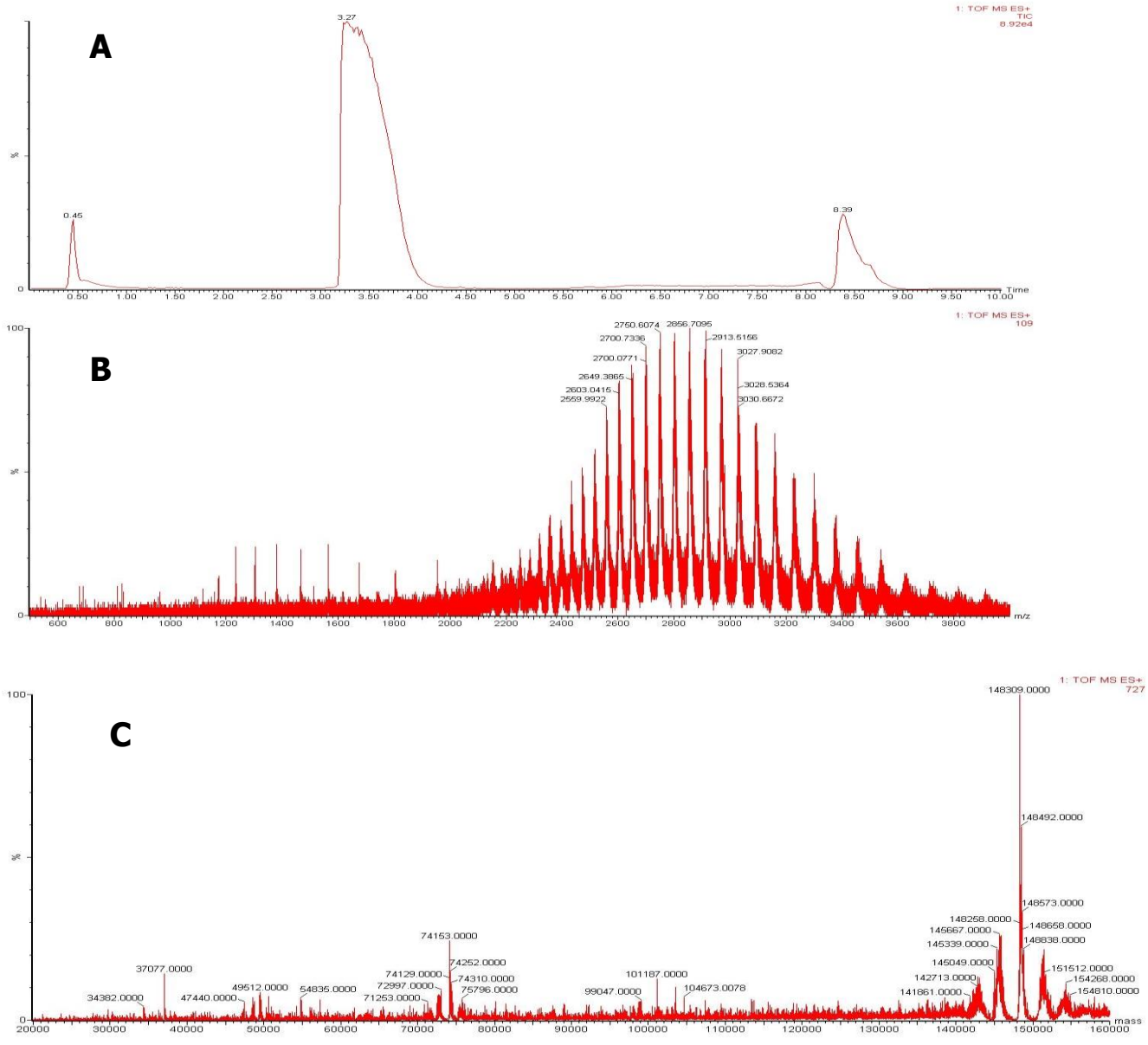


Figure S1 – ESI-MS data for unmodified thio-trastuzumab. A) Total ion count spectra, B) non-deconvoluted ion series and C) deconvoluted ion series mass spectra. Thio-trastuzumab capped with two cysteines observed mass of 148309 Da.

Figure S2 – LC-MS data of reduced thio-trastuzumab

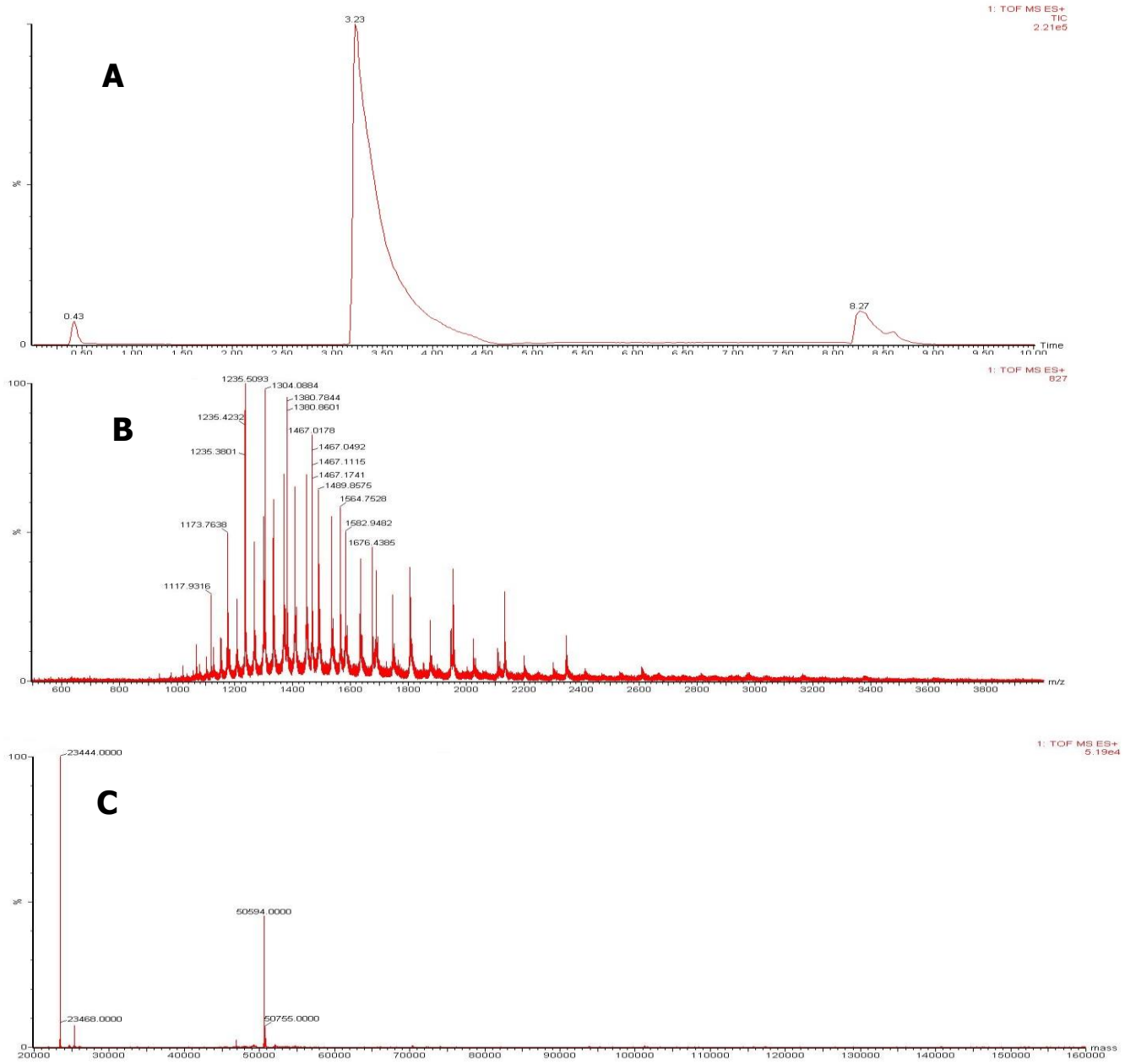


Figure S2 – ESI-MS data for reduced thio-trastuzumab. A) Total ion count spectra, B) non-deconvoluted ion series and C) deconvoluted ion series mass spectra. Light chain (LC) observed mass of 23444 Da and heavy chain (HC) observed mass of 50594 Da.

Figure S3 – LC-MS data of thio-trastuzumab drug conjugate TDC 3

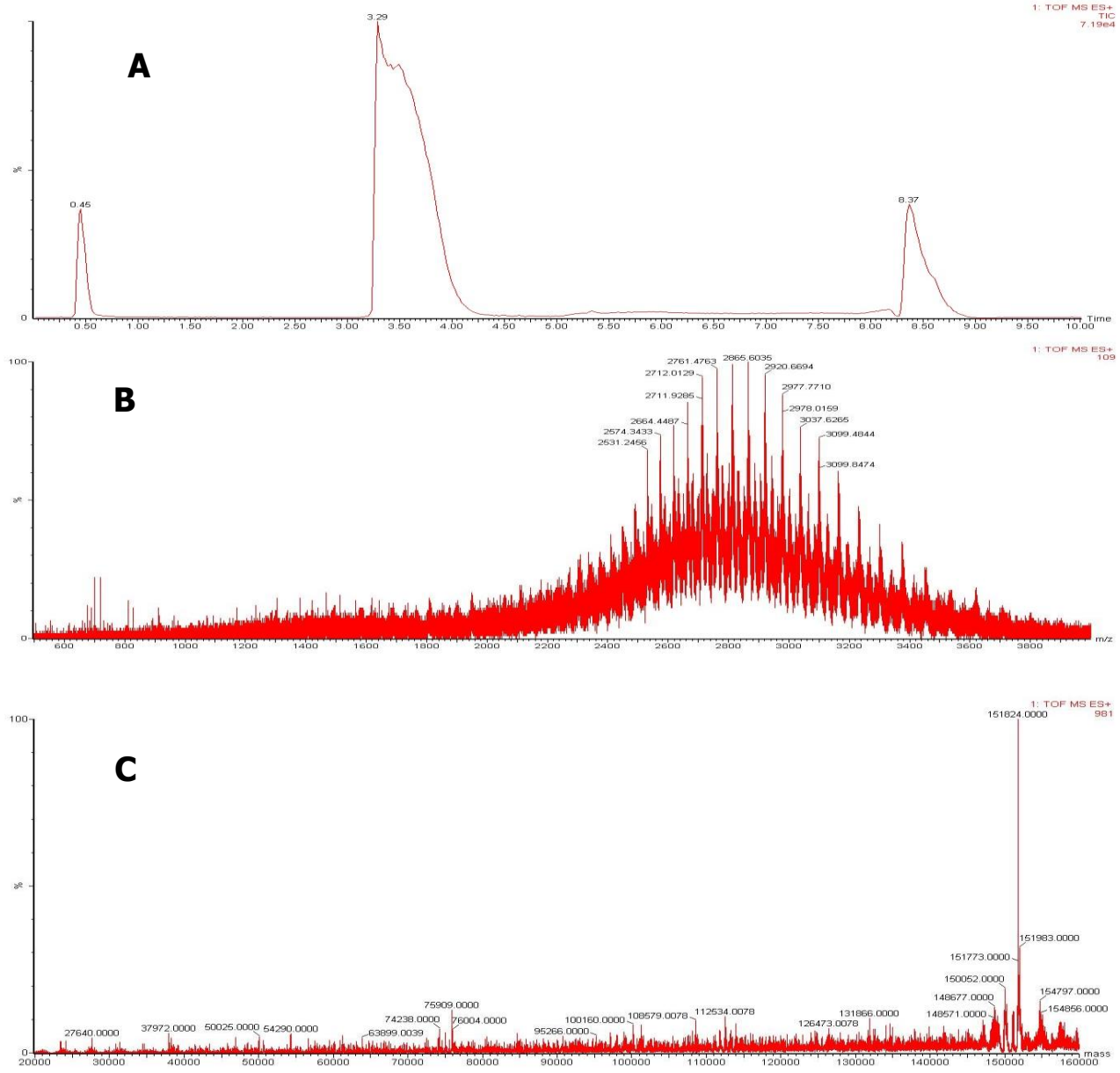
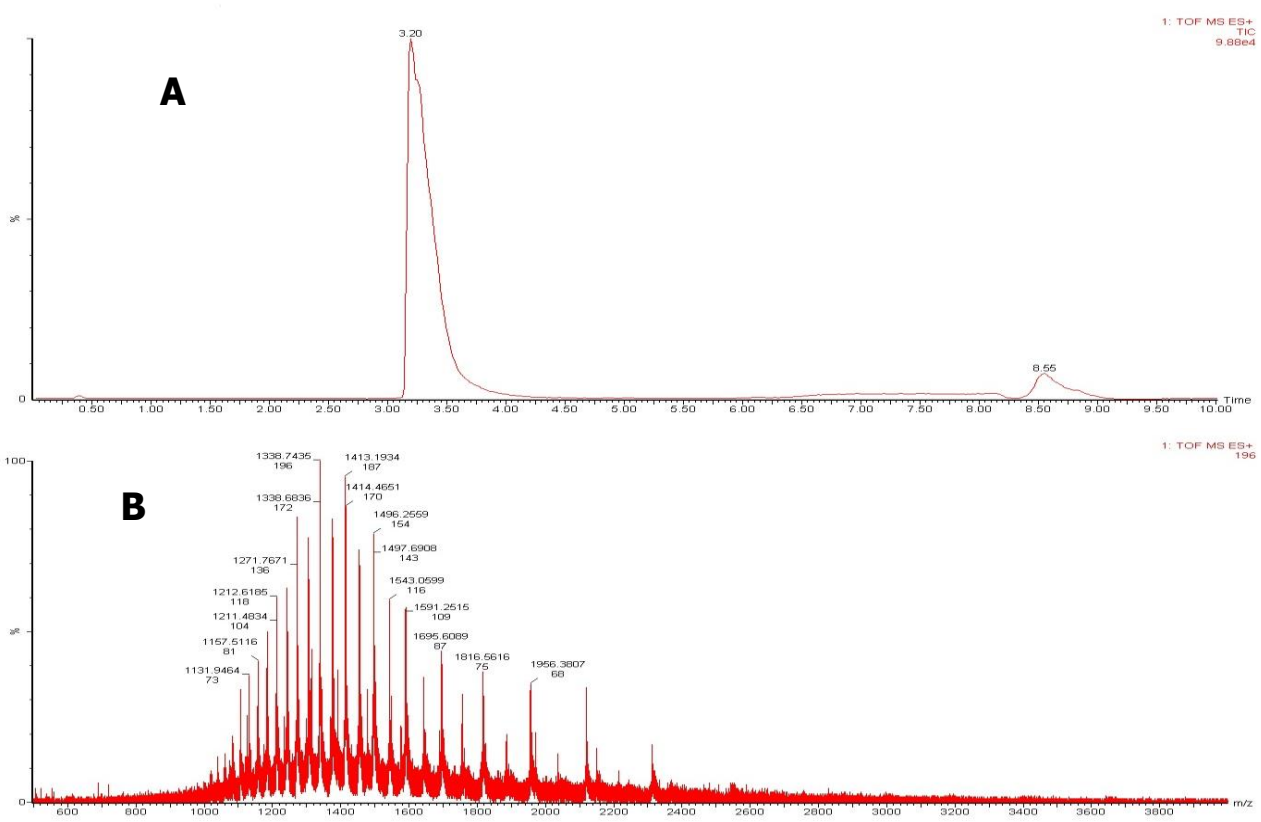


Figure S3 – ESI-MS data for TDC **3**. A) Total ion count spectra, B) non-deconvoluted ion series and C) deconvoluted ion series mass spectra. Thio-trastuzumab drug conjugate TDC **3** observed mass of 151824 Da (calculated 151819 Da).

Figure S4 – LC-MS data of thio-trastuzumab drug conjugate TDC 3 reduced with TCEP and quenched with maleimide



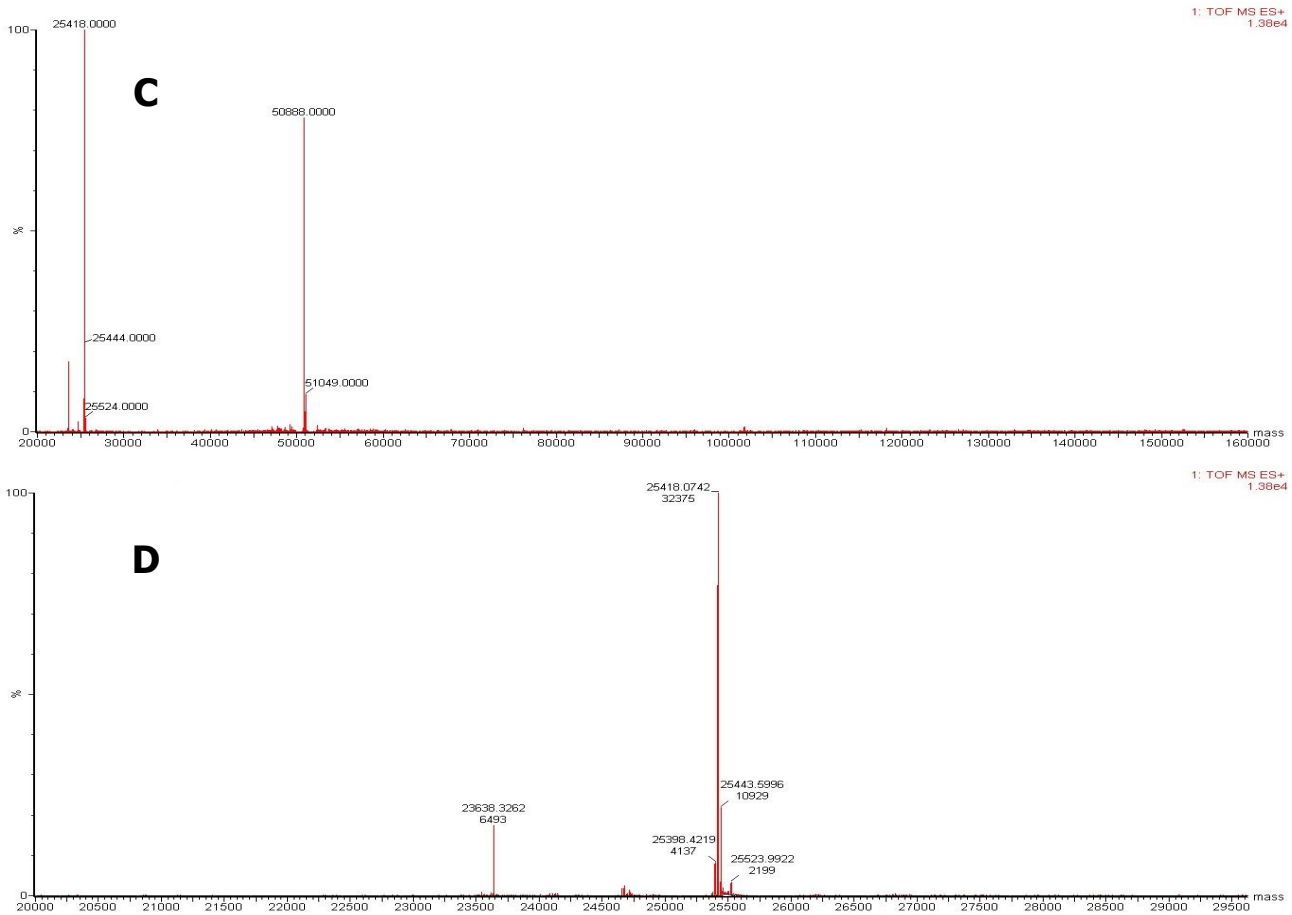


Figure S4 – ESI-MS data for reduced TDC **3** quenched with maleimide. A) Total ion count spectra, B) non-deconvoluted ion series, C) deconvoluted ion series mass spectra and D) detail of light chain species. Light chain conjugates observed masses of 25418 Da (LC-$MMAE_{Hyd}$ modified with one maleamic acid PEG_{12}-vc-PABC-MMAE payload and one maleimide, calculated 25416 Da, proton adduct), 25443 (LC-$MMAE_{Hyd}$ modified with one maleamic acid PEG_{12}-vc-PABC-MMAE payload and one maleimide, calculated 25438 Da, sodium adduct), 25398 Da (LC-$MMAE_{nonHyd}$ modified with one maleimide PEG_{12}-vc-PABC-MMAE payload and one maleimide, calculated 25398 Da, proton adduct), and 23638 Da (LC-Mal modified with two maleimides, calculated 23638 Da).

Figure S5 – SEC-HPLC chromatograms of thio-trastuzumab and TDC 3

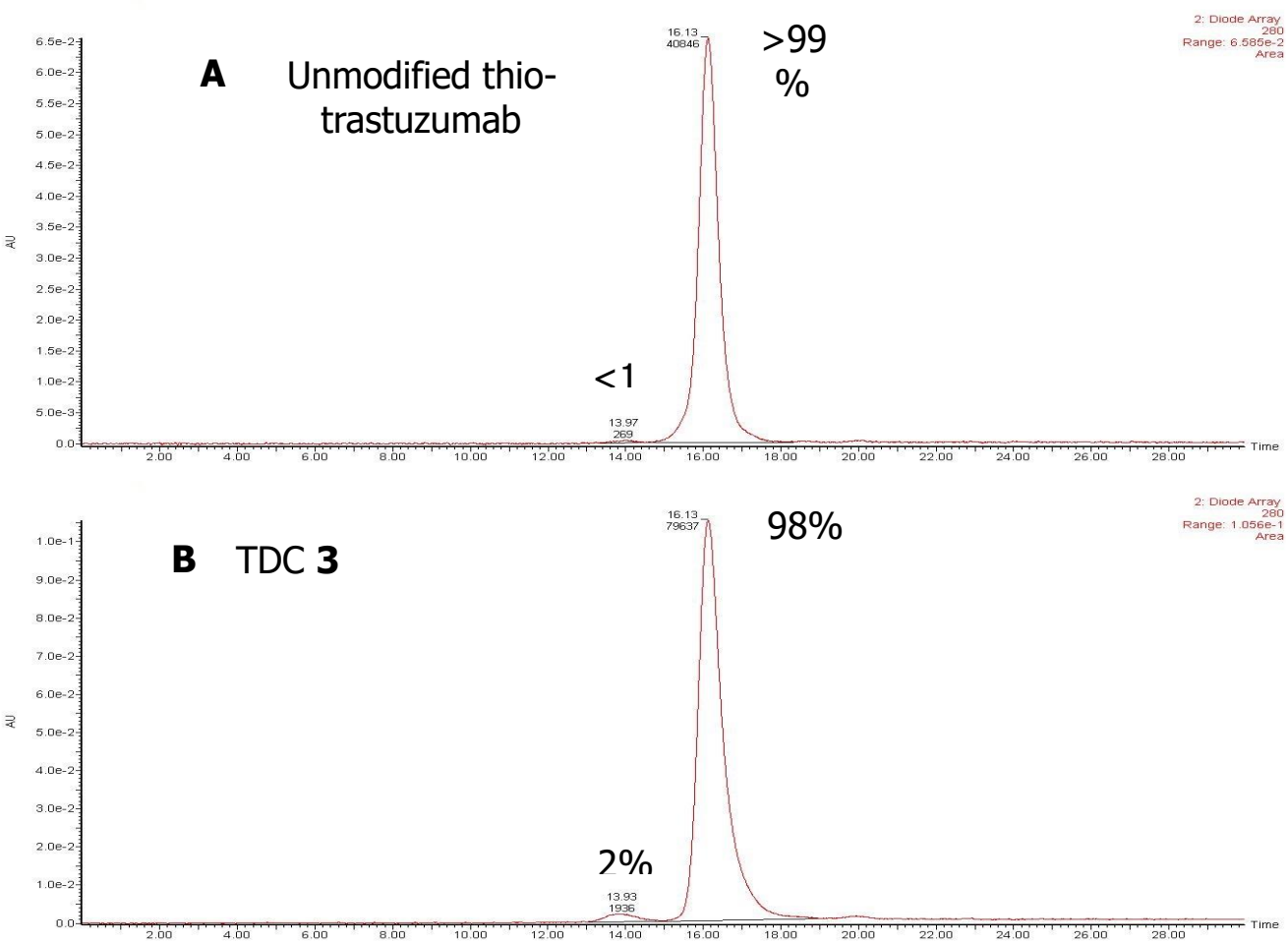


Figure S5 – SEC-HPLC chromatograms with peak percentages of monomeric (rt 16.1 min) and aggregate (rt 13.9 min) species for A) unmodified thio-trastuzumab and B) TDC **3**.

Figure S6 – HER2 ELISA of TDC 3 compared with thio-trastuzumab

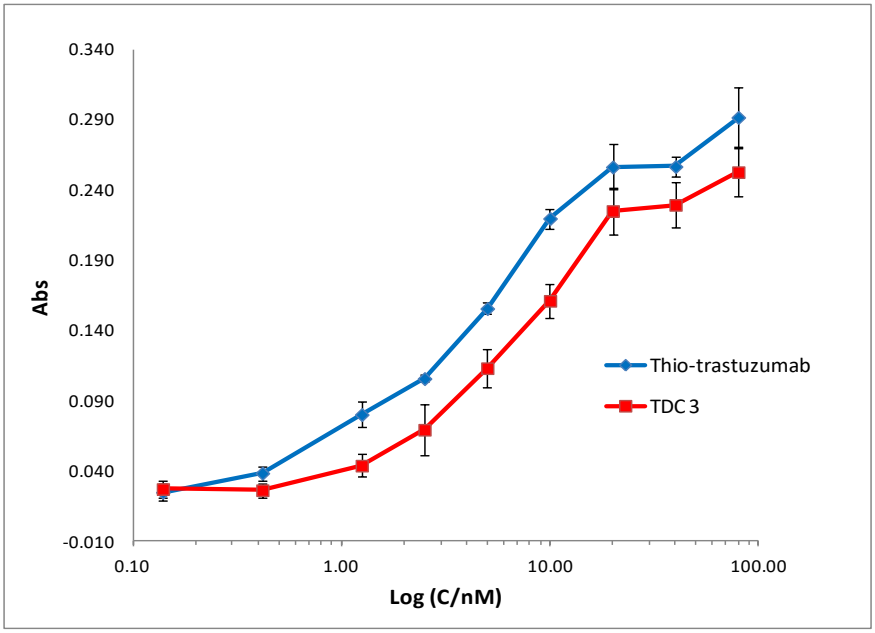


Figure S6 – Binding activity to HER2 of TDC **3** compared with thio-trastuzumab by ELISA assay.

Figure S7 – SEC-HPLC representative chromatograms of TFC 5 and albumin-fluorophore conjugate

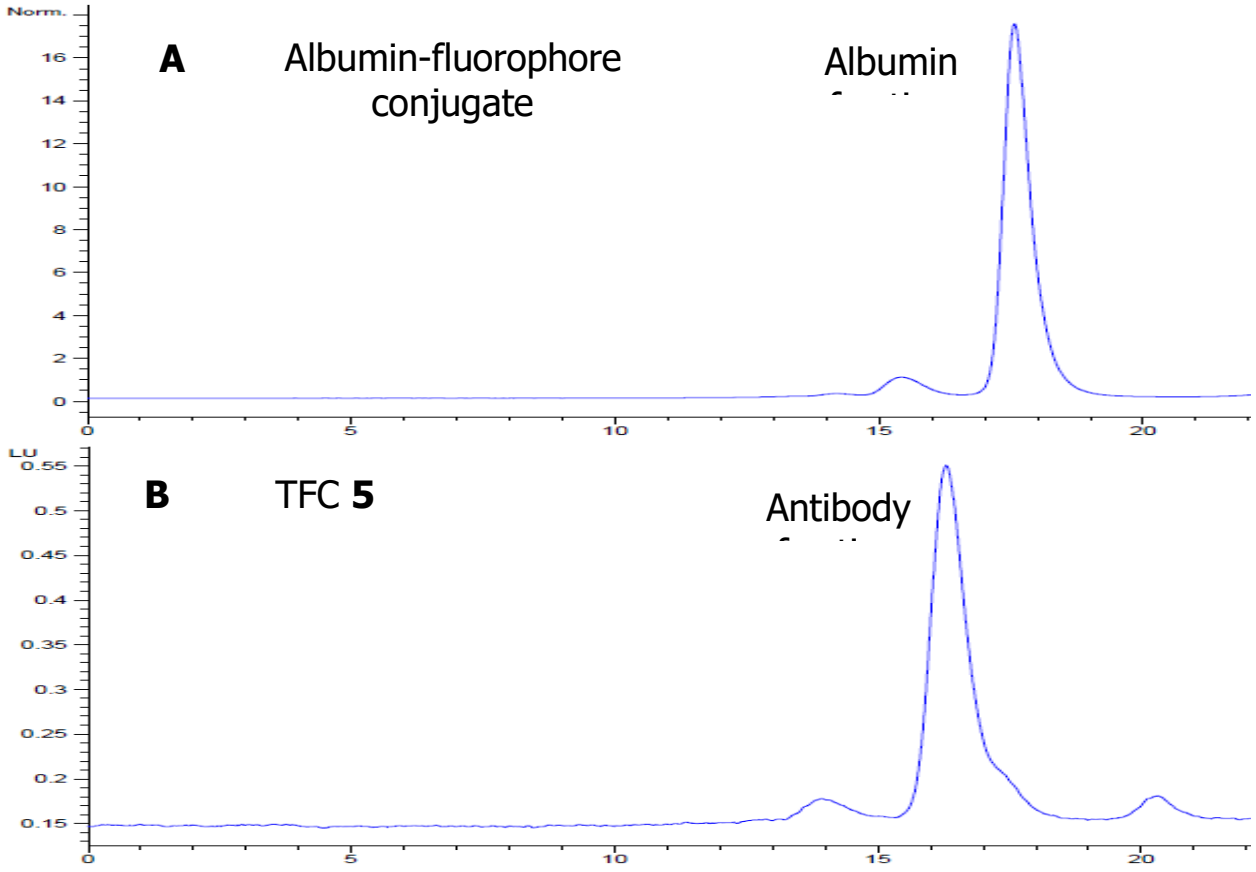
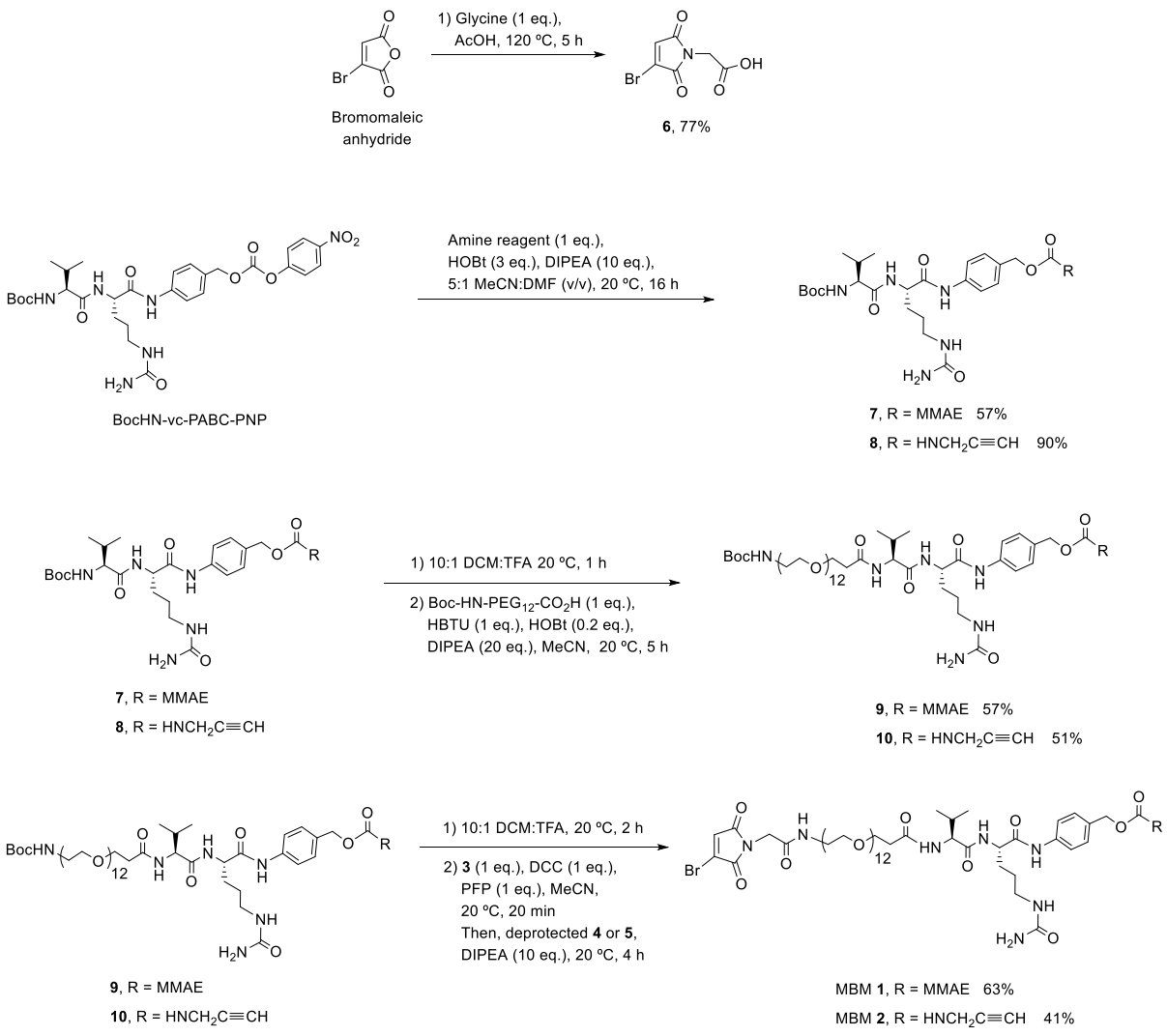


Figure S7 – SEC-HPLC with fluorescence detection (excitation 495 nm, emission 525 nm) chromatograms of A) albumin-fluorophore conjugate and B) TFC **5**.

Synthesis General Remarks

All reactions were carried out at atmospheric pressure with stirring at 20 °C unless otherwise stated. Reagents and solvents were purchased from Sigma Aldrich and Alfa Aesar and used as supplied. H_2N-PEG_{12}-$CH_2CH_2CO_2{}^tBu$ and BocHN-vc-PABC-PNP were purchased from Iris Biotech GmbH was purchased from Iris Biotech GmbH. Reactions were monitored by TLC analysis carried out on silica gel SIL G/UV254 coated onto aluminium plates purchased from VWR. Visualization was carried out under a UV lamp operating at 254 nm wavelength and by staining with a solution of phosphomolybdic acid in ethanol (12 g/250 mL), followed by heating. Flash column chromatography was carried out on silica gel 60 (0.04-0.063 mm, 230-400 mesh) purchased from Merck. Preparative thin-layer chromatography (PLC) was carried out on 20×20 cm glass plates coated with PLC silica gel 60 F_{254} (2 mm) purchased from Merck. Chromatographic and crystallisation purifications used solvents dichloromethane (DCM), methanol (MeOH), ethyl acetate (EtOAc) and petroleum ether 40 °C - 60 °C boiling range, purchased from Sigma Aldrich. Nuclear magnetic resonance spectra were recorded in either $CDCl_3$ or MeOD-d_4 (unless another solvent is stated) on Bruker NMR spectrometers operating at ambient 20 °C probe. ^1H spectra were recorded at 400, 500 or 600 MHz and ^{13}C spectra were recorded at 100, 125 or 150 MHz, using residual solvents as internal reference. Where necessary, DEPT135, COSY, HMQC, HMBC and NOESY spectra have been used to ascertain structure. Data is presented as follows for ^1H: chemical shift in ppm (multiplicity, *J* coupling constant in Hz, nº of H, assignment on structure); and on ^{13}C: chemical shift in ppm (assignment on structure). Multiplicity is reported as follows: s (singlet), d (doublet), t (triplet), q (quartet), quint. (quintet), sext. (sextet), oct. (octet), m (multiplet), br (broad), dd (doublet of doublet). Infrared spectra were recorded on a Perkin Elmer Spectrum 100 FTIR spectrometer operating in ATR mode. Melting points were measured on a Gallenkamp apparatus and are uncorrected. Experimental procedures for all isolated compounds are presented. All yields quoted are isolated yields, unless otherwise stated.

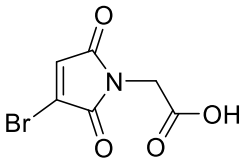
Synthesis of compounds



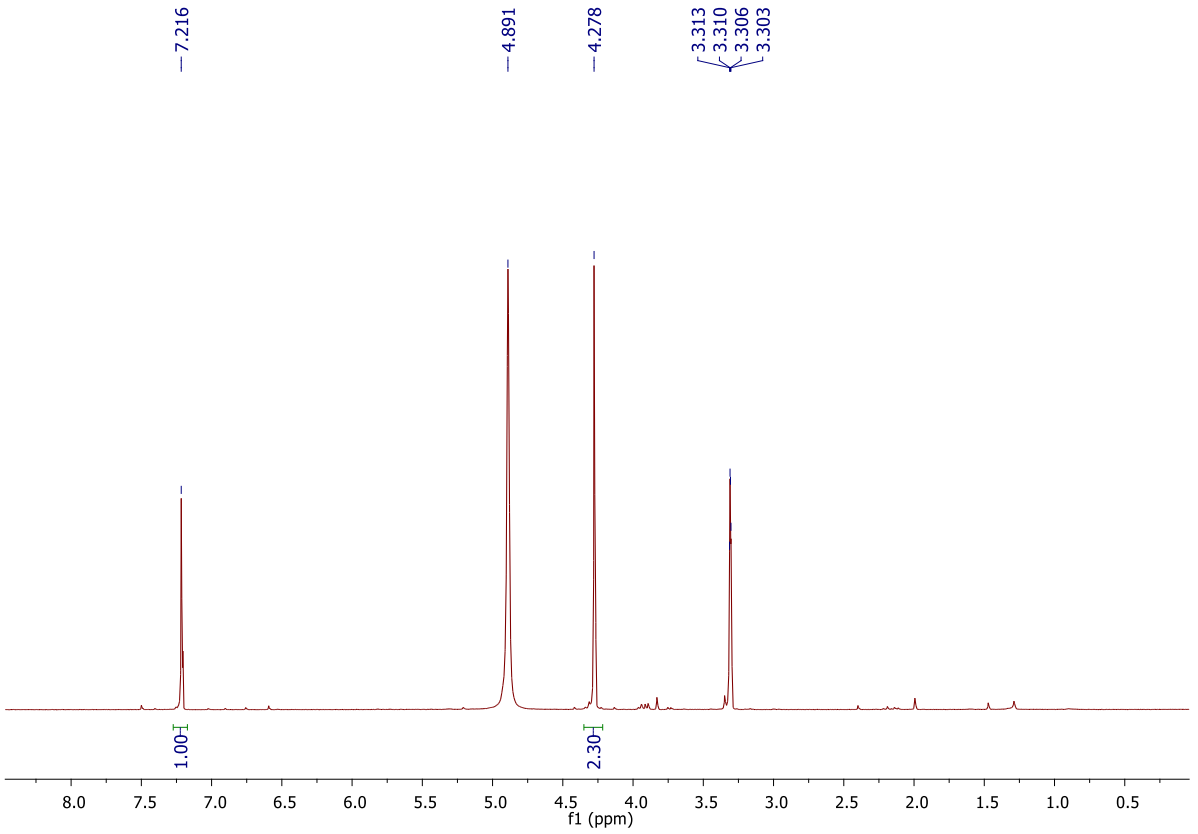
Scheme S1 – Synthesis of MBMs **1** and **2**.

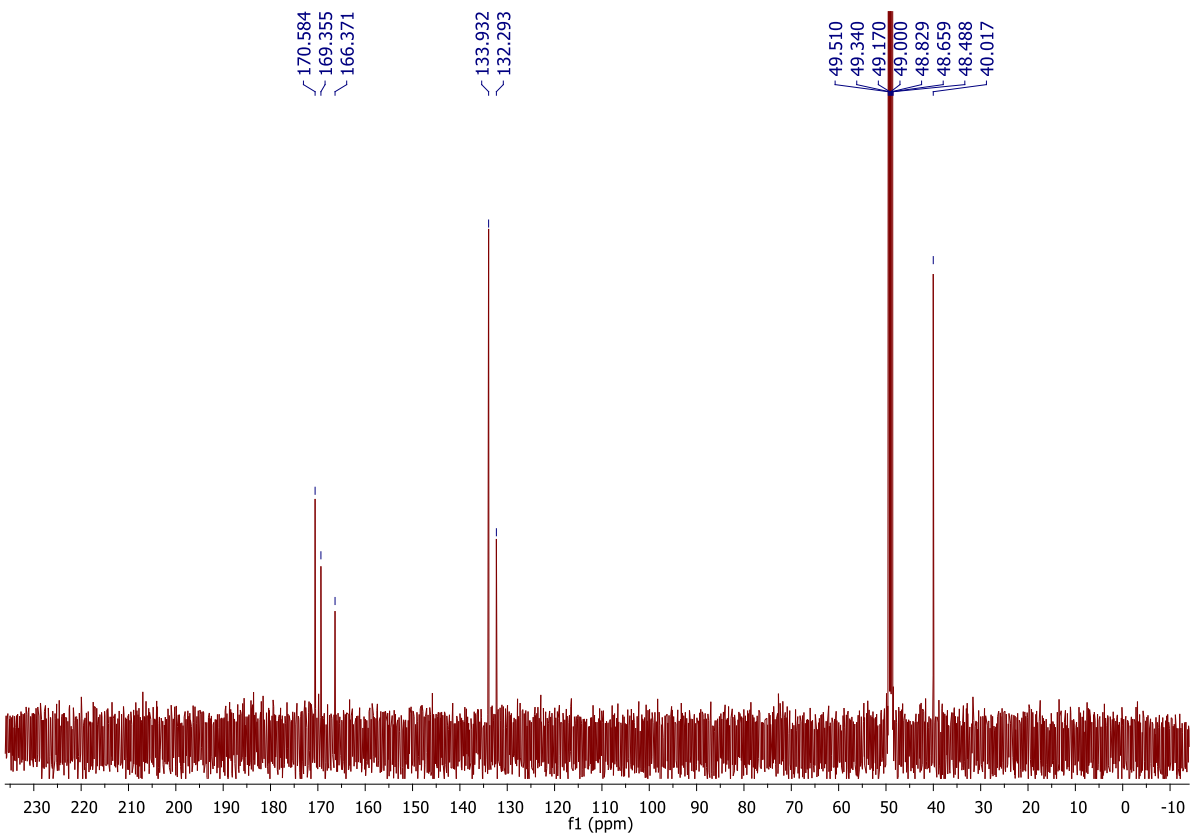
2-(3-bromo-2,5-dioxo-2,5-dihydro-1*H*-pyrrol-1-yl)acetic acid 6

(MBM 6)

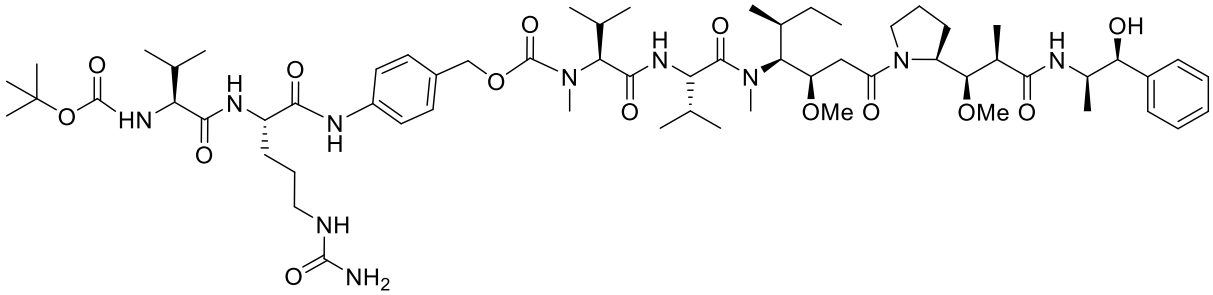


In a 10 mL round-bottom flask, suspended 3-bromomaleic anhydride (186 µL, 354 mg, 2 mmol) and glycine (150 mg, 2 mmol, 1 eq.) in acetic acid (2 mL). The mixture was heated at 120 °C for 5 hours. Then, cooled down to 20 °C and concentrated in vacuum. Added toluene and MeOH and concentrated again. Redissolved in EtOAc:MeOH (15:2 v/v) and ran through a short plug of silica gel 60. Concentrated filtrate under vacuum to afford the title compound as a beige solid (362 mg, 1.55 mmol, 77%). Characterisation data: m.p. 140-142 °C. FTIR ν_{max} (cm^{-1}) 3091, 2935, 1704, 1414, 1381, 1277, 929, 879, 635, 489. ^{1}H NMR (500 MHz, MeOD-d_4) 4.28 (s, 2H, CH_2), 7.22 (s, 1H, CH); ^{13}C NMR (125 MHz, MeOD-d_4) 40.0 (CH_2), 132.3 (CBr), 133.9 (CH), 166.4 (CO), 169.4 (CO), 170.6 (CO). LRMS (NSI) 233.9 (30, $[M^{81}Br-H]^-$), 231.9 (30, $[M^{79}Br-H]^-$); HRMS (NSI) calcd. for $C_6H_3NO_4Br$ $[M^{79}Br-H]^-$ 231.9251, observed: 231.9252.



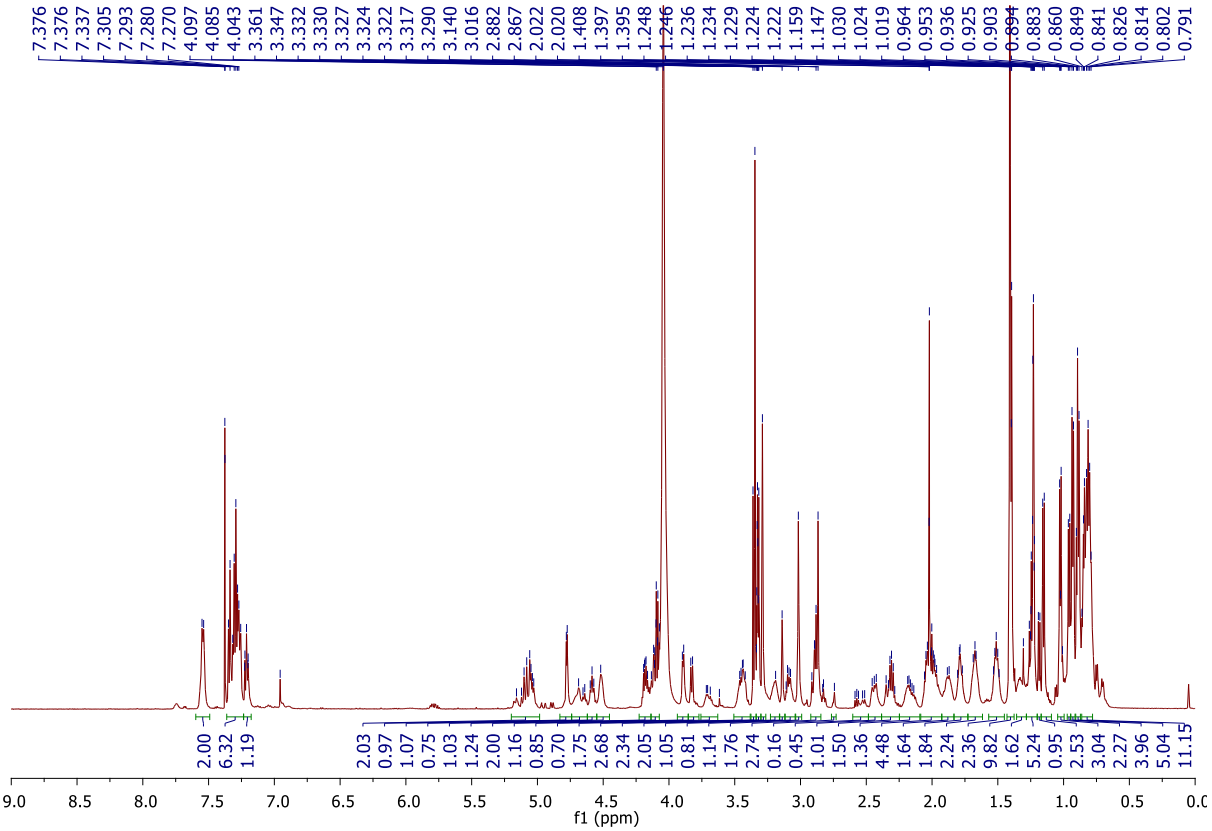


BocHN-vc-PABC-MMAE 7



This protocol is a modification of a literature protocol[2]. In a 10 mL round-bottom flask, suspended BocHN-vc-PABC-PNP (31 mg, 48 µmol, 1.6 eq.), and 1-hydroxybenzotriazole hydrate (HOBt, 12 mg, 90 µmol, 3 eq.) in DMF (0.2 mL). Next, dissolved monomethyl auristatin E (MMAE, 25 mg, 30 µmol, 1 eq.) in MeCN (0.8 mL) and added to the reaction mixture. Then, added DIPEA (52 µL, 300 µmol, 10 eq.). The solution turned yellow over time. The solution was stirred at 20 °C over 16 hours. Then, added DCM (10 mL) and washed with aq. sat. $NaHCO_3$ (3×10 mL), 0.1 M aq. acetates pH 5 (10 mL) and 1:1 water:sat. LiCl (10 mL). The organic layer was dried ($MgSO_4$), filtered and concentrated under vacuum to yield an off-white solid which was purified by preparative liquid chromatography on silica with a

gradient of DCM:EtOAc:MeOH (5:5:2 to 10:3:1 v/v) to afford the title compound as a white solid (21 mg, 17 µmol, 57%). Characterisation data: ^{1}H NMR (600 MHz, $CDCl_3$ and MeOD-d_4 mix) 0.77-0.87 (overlapped d, *J* = 6.6 Hz, 9H, C*H*$_3$ and MMAE), 0.87-0.91 (overlapped d, *J* = 6.6 Hz, 6H, C*H*$_3$ and MMAE), 0.91-0.97 (m, 5H, MMAE), 1.00-1.05 (m, 3H, MMAE), 1.10-1.20 (m, MMAE), 1.21-1.28 (m, MMAE), 1.28-1.36 (m, MMAE), 1.37-1.43 (s, 9H, C*H*$_3$ Boc), 1.46-1.56 (m, *J* = 6.6 Hz, 2H, C*H*$_2$), 1.62-1.72 (m, 2H, C*H*$_2$ and MMAE), 1.75-1.82 (m, 1H, C*H*$_2$), 1.83-2.00 (m, MMAE), 2.00-2.08 (m, *J* = 6.6 Hz, 3H, C*H* and MMAE), 2.10-2.23 (m, MMAE), 2.26-2.60 (m, MMAE), 2.84-2.93 (m, MMAE), 2.97-3.04 (m, MMAE), 3.05-3.11 (m, 1H, C*H*), 3.12-3.15 (br, MMAE), 3.16-3.24 (m, 1H, C*H*), 3.27-3.32 (m, MMAE), 3.34-3.37 (m, MMAE), 3.40-3.50 (m, MMAE), 3.65-3.75 (m, MMAE), 3.80-3.85 (d, *J* = 7.8 Hz, 1H, MMAE), 3.86-3.91 (d, *J* = 6.6 Hz, 1H, C*H*), 3.95-4.22 (overlapped m and H_2O, MMAE), 4.45-4.55 (m, 1H, C*H*), 4.56-4.62 (t, *J* = 8.4 Hz, 1H, MMAE), 4.62-4.74 (overlapped m, MMAE and C*H*$_2$), 4.75-4.82 (d, *J* = 3.6 Hz, 1H, MMAE), 5.00-5.19 (overlapped m, 2H, BocN*H* and C*H*$_2$), 7.16-7.36 (overlapped m, 7H, Ar*H* MMAE and PABC), 7.53-7.56 (d, *J* = 7.2 Hz, 2H, Ar*H* PABC). ^{13}C NMR (150 MHz, $CDCl_3$ and MeOD-d_4 mix) 10.5 (CH_3, MMAE), 13.8 (CH_3, MMAE), 14.0 (CH_3, MMAE), 15.6 (CH_3, MMAE), 17.6 (CH_3, MMAE), 18.3 (CH_3), 18.8 (CH_3), 19.0 (CH_3, MMAE), 24.6 (CH_2, MMAE), 24.7 (CH_2, MMAE), 25.6 (CH_2, MMAE), 26.1 (CH_2), 28.0 (CH_3, Boc), 29.2 (CH, MMAE), 29.4 (CH_2), 29.5 (CH_3, MMAE), 30.1 (CH, MMAE), 30.8 (CH), 31.8 (CH, MMAE), 33.0 (CH, MMAE), 37.3 (CH_2, MMAE), 38.9 (CH_2), 44.7 (CH, MMAE), 47.8 (CH_2, MMAE), 50.7 (CH, MMAE), 53.1 (CH, MMAE), 54.3 (CH), 57.7 (OCH_3, MMAE), 59.7 (CH, MMAE), 60.1 (CH), 60.6, (OCH_3, MMAE), 61.4 (MMAE), 64.8 (CH_2), 67.2 (OCH, MMAE), 75.5 (OCH, MMAE), 78.3 (CH, MMAE), 80.0 (C, Boc), 82.1 (OCH, MMAE), 85.6 (MMAE), 119.9 (ArCH), 126.2 (ArCH, MMAE), 127.2 (ArCH, MMAE), 127.9 (ArCH, MMAE), 128.1 (ArC, MMAE), 128.4 (ArCH), 132.2 (ArC), 137.8 (ArC), 141.3 (ArC, MMAE), 156.4 (CO, Boc), 157.4 (CO), 160.4 (CO), 170.2 (CO, MMAE), 170.3 (CO), 170.4 (CO, MMAE), 172.8 (CO, MMAE), 173.3 (CO), 174.6 (CO, MMAE). LRMS (ESI) 1246 (100, $[M+Na]^+$), 1224 (38, $[M+H]^+$); HRMS (ESI) calcd. for $C_{63}H_{102}N_{10}O_{14}Na$ $[M+Na]^+$ 1245.7469, observed: 1245.7451.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2.00 6.32 1.19 2.03 0.97 1.07 0.75 1.03 1.24 2.00 1.16 0.85 0.70 1.75 2.68 2.34 2.05 1.05 0.81 1.14 1.76 2.74 0.16 0.45 1.01 1.50 1.36 4.48 1.64 1.84 2.24 2.36 9.82 1.62 5.24 0.95 2.53 3.04 2.27 3.96 5.04 11.15
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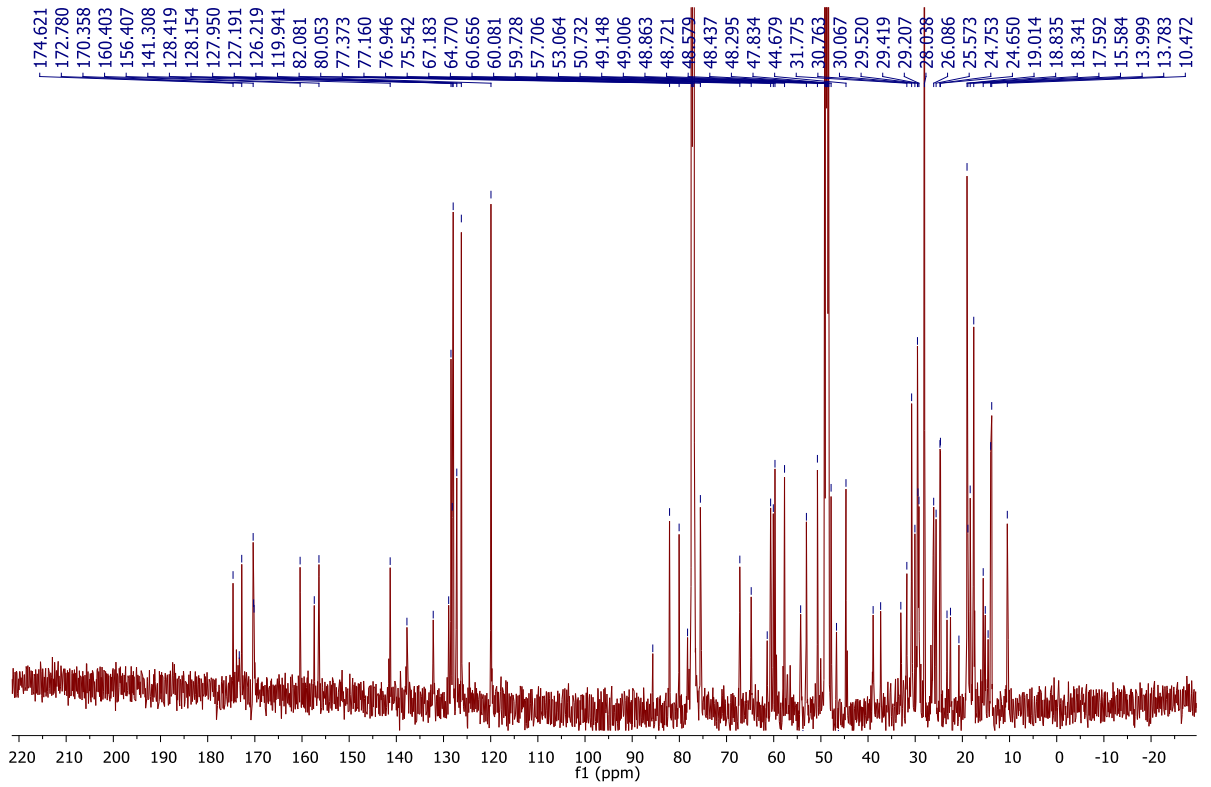
f1 (ppm)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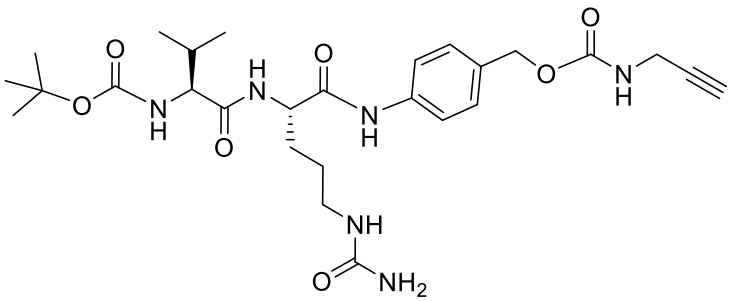
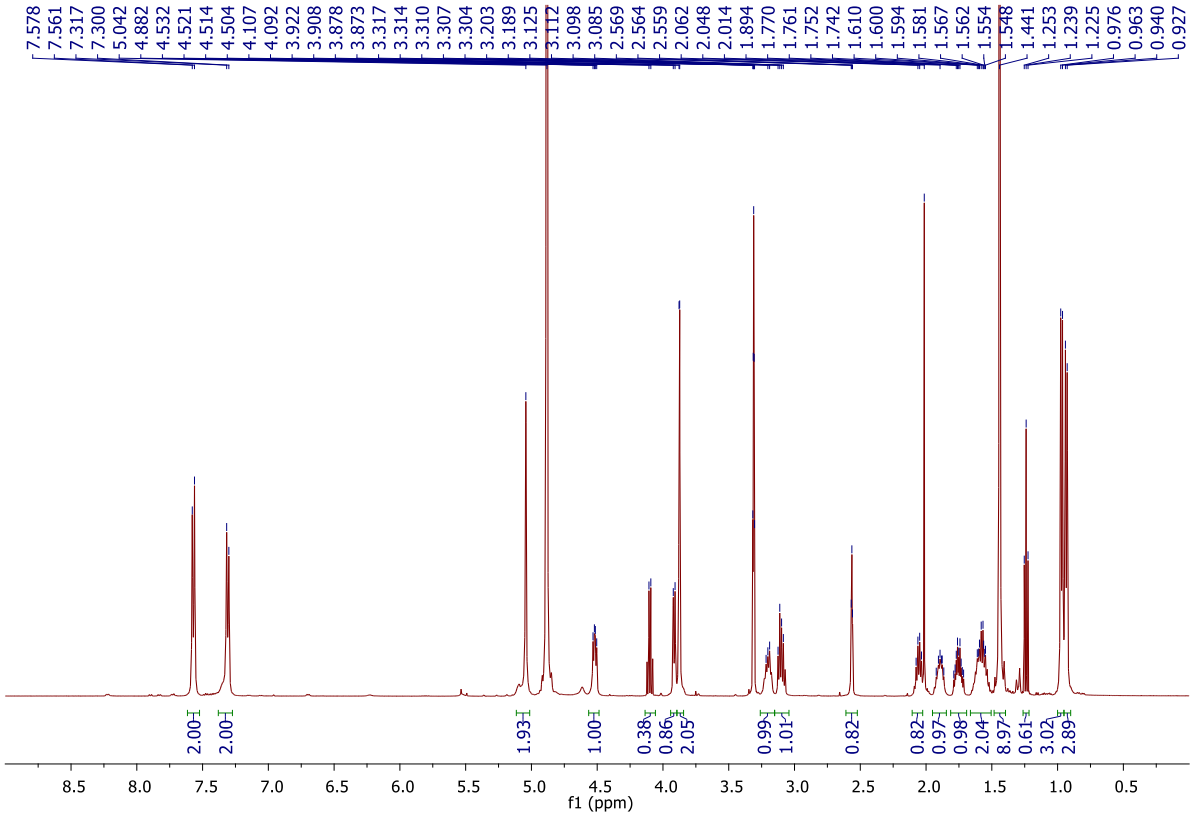
f1 (ppm)

BocHN-vc-PABC-propargylamide 8



In a 10 mL round-bottom flask, BocHN-vc-PABC-PNP (129 mg, 0.2 mmol, 1 eq.) was dissolved in DMF (2 mL). Next, added HOBt hydrate (54 mg, 0.4 mmol, 2 eq.), followed by addition of propargylamine (13 µL, 0.2 mmol, 1 eq.) and DIPEA (104 µL, 0.6 mmol, 3 eq.). The solution was stirred at 20 °C for 8 h. Then, concentrated under vacuum, redissolved in MeOH and concentrated under vacuum again. Purified by flash chromatography on silica with a gradient of DCM:EtOAc:MeOH (10:3:1 to 10:3:2 v/v) to afford the title compound as an off-white solid (101 mg, 0.18 mmol, 90%). Characterisation data: no melting point starts to decompose at 196-198 °C. FTIR ν_{max} (cm^{-1}): 3297, 2957, 2927, 2870, 2422, 1694, 1669, 1634, 1607, 1405, 1165. ^{1}H NMR (500 MHz, MeOD-d4) 0.93-0.98 (overlapped d, *J* = 6.5 Hz, 6H, C*H*$_3$), 1.42-1.46 (s, 9H, C*H*$_3$), 1.50-1.65 (m, *J* = 7.0 Hz, 2H, C*H*$_2$), 1.70-1.80 (m, *J* = 9.5 Hz, 1H, C*H*$_2$), 1.85-1.95 (m, 1H, C*H*$_2$), 2.01-2.10 (oct., *J* = 7.0 Hz, 1H, C*H*), 2.56-2.57 (t, *J* = 2.5 Hz, 1H, C*H*), 3.06-3.14 (m, *J* = 7.0 Hz, 1H, C*H*$_2$), 3.16-3.25 (m, *J* = 7.0 Hz, 1H, C*H*$_2$), 3.86-3.88 (d, *J* = 2.5 Hz, 2H, C*H*$_2$), 3.90-3.93 (d, *J* = 7.0 Hz, 1H, C*H*), 4.50-4.53 (dd, *J* = 8.5 and 5.0 Hz, 1H, C*H*), 5.01-5.08 (br, 2H, C*H*$_2$), 7.30-7.32 (d, *J* = 8.5 Hz, 2H, Ar*H*), 7.56-7.58 (d, *J* = 8.5 Hz, 2H, Ar*H*); ^{13}C NMR (125 MHz, MeOD-d4) 18.6 (CH_3), 19.8 (CH_3), 27.8 (CH_2), 28.7 (CH_3), 30.5 (CH_2), 31.0 (CH_2), 31.9 (CH), 40.2 (CH_2), 54.9 (CH), 61.7 (CH), 67.3 (CH_2), 72.0 (CH), 80.7 (C), 81.1 (C), 121.2 (ArCH), 129.6 (ArCH), 133.9 (ArC), 139.4 (ArC), 158.2 (CO), 158.5 (CO), 162.3 (CO), 172.3, (CO), 174.7 (CO). LRMS (NSI) 561 (100, $[M+H]^+$), 583 (35, $[M+Na]^+$); HRMS (NSI) calcd. for $C_{27}H_{41}N_6O_7$ $[M+H]^+$ 561.3031, observed: 561.3024.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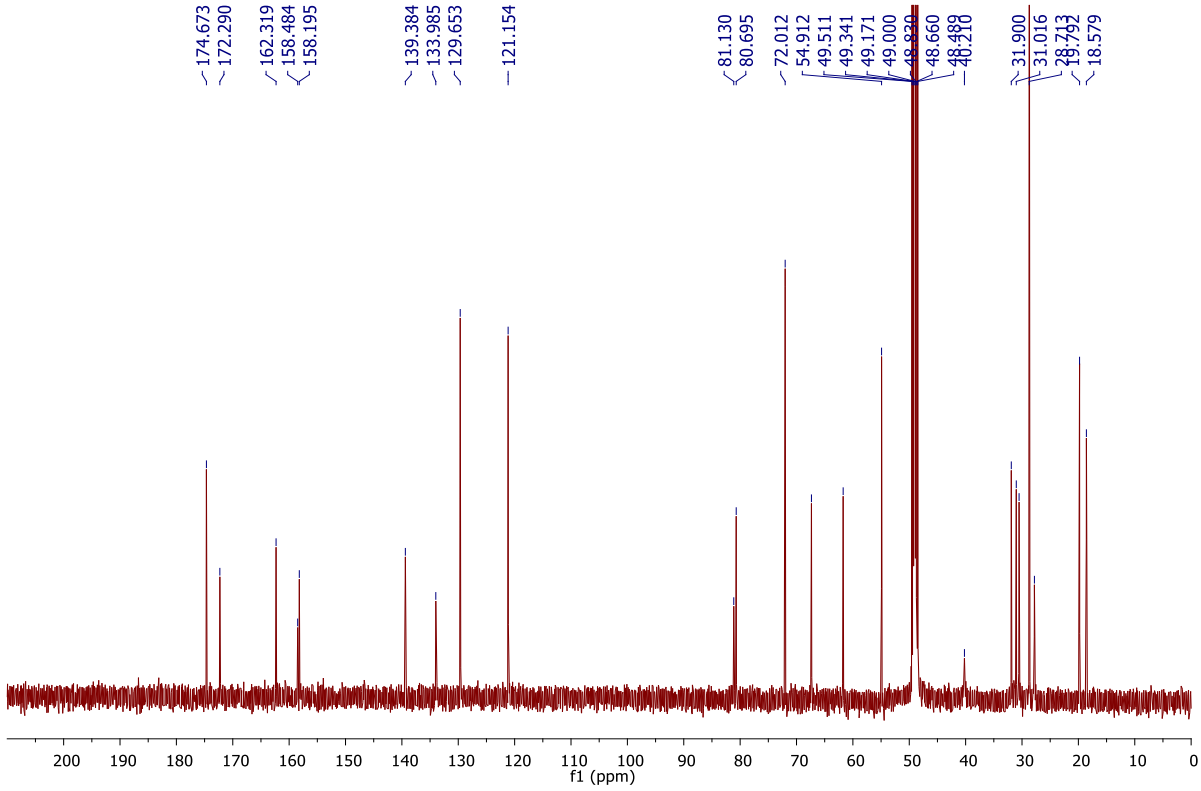
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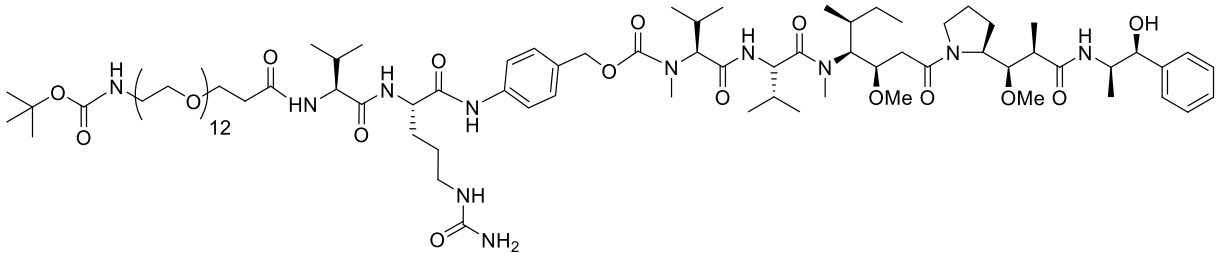
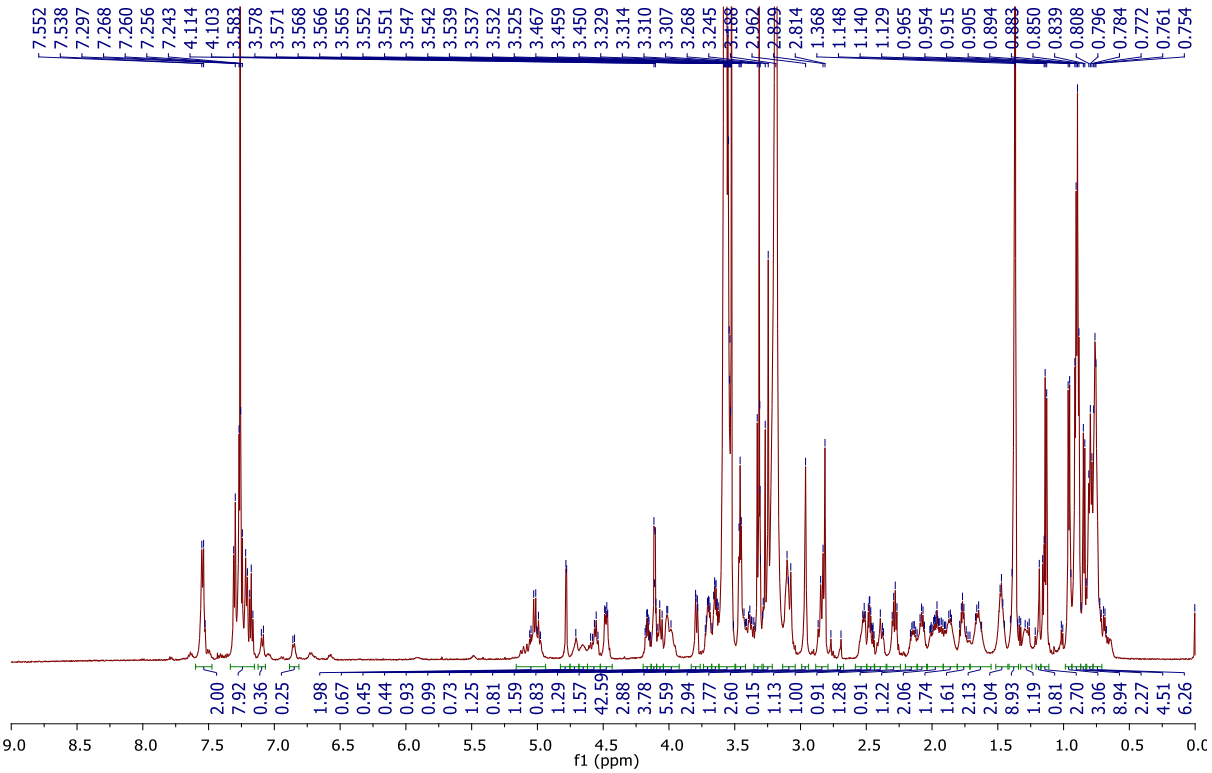
BocHN-PEG$_{12}$-vc-PABC-MMAE 9



In a 10 mL round-bottom flask, dissolved BocHN-vc-PABC-MMAE **7** (34 mg, 28 µmol, 1 eq.) in dry DCM (0.9 mL) and added trifluoroacetic acid (TFA, 0.1 mL). Stirred at 20 °C over 2 hours. Next, concentrated under vacuum to dryness, redissolved in dry DCM, concentrated again, redissolved in dry MeCN and concentrated once more under vacuum. Dissolved the H_2N-vc-PABC-MMAE TFA salt in dry MeCN (2 mL) and added DIPEA (74 µL, 425 µmol, 15 eq.). In a separate flask, dissolved BocHN-PEG$_{12}$-CH_2CH_2-CO_2H (20 mg, 28 µmol, 1 eq.) in dry MeCN (1 mL). Next, added (2-(1*H*-benzotriazol-1-yl)-1,1,3,3-tetramethyluronium hexafluorophosphate) (HBTU, 10.7 mg, 28 µmol, 1 eq.), HOBt hydrate (0.8 mg, 2.8 µmol, 0.1 eq.) and DIPEA (25 µL, 144 µmol, 5 eq.). The solution was stirred at 20 °C for 20 min. Then, added the H_2N-vc-PABC-MMAE solution with DIPEA previously prepared and stirred at 20 °C over 5 hours. Next, added DCM (20 mL) and washed with 15% aq. citric acid (10 mL), sat. aq. $NaHCO_3$ (10 mL) and water (10 mL). The organic layer was dried ($MgSO_4$), filtered and concentrated under vacuum to yield a colourless oil which was purified by preparative thin layer chromatography on silica with a gradient of DCM:EtOAc:MeOH (10:3:2 v/v) to DCM:MeOH (10:2 v/v) to afford the title compound as a colourless oil (29 mg, 16 µmol, 57%). Characterisation data: ^{1}H NMR (600 MHz, $CDCl_3$ and MeOD-d_4 mix) 0.71-0.83 (overlapped m, 10H, C*H*$_3$ and MMAE), 0.83-0.86 (d, *J* = 6.6 Hz, 2H, MMAE), 0.87-0.93 (overlapped m, 9H, MMAE and C*H*$_3$), 0.94-0.98 (d, *J* = 6.6 Hz, 3H, MMAE), 1.10-1.19 (overlapped d, *J* = 6.6 Hz, 3H, MMAE), 1.19-1.21 (m, MMAE), 1.25-1.32 (m, MMAE), 1.34-1.41 (s, 9H, C*H*$_3$ Boc), 1.43-1.52 (m, *J* = 6.6 Hz, 2H, C*H*$_2$), 1.58-1.72 (m, 2H, C*H*$_2$ and MMAE), 1.73-1.81 (m, 1H, C*H*$_2$), 1.81-2.04 (m, MMAE), 2.04-2.11 (m, *J* = 6.6 Hz, 1H, C*H* and MMAE), 2.11-2.20 (m, MMAE), 2.25-2.33 (t, *J* = 7.2 Hz, 1H, MMAE), 2.35-2.58 (m, MMAE), 2.80-2.88 (m, MMAE), 2.94-2.99 (m, MMAE), 3.05-3.14 (overlapped m, 3H, C*H* and MMAE), 3.21-3.28 (m, PEG C*H*$_2$ and MMAE), 3.30-3.34 (m, MMAE), 3.44-3.48 (t, *J* = 5.4 Hz, 2H, PEG C*H*$_2$), 3.51-3.62 (overlapped m, PEG C*H*$_2$), 3.62-3.67 (m, 2H, PEG C*H*$_2$), 3.67-3.74 (m, MMAE), 3.77-3.82

(dd, *J* = 8.4 and 1.8 Hz, 1H, MMAE), 3.94-4.03 (m, MMAE), 4.03-4.09 (m, 1H, MMAE), 4.09-4.13 (overlapped d and m, *J* = 6.6 Hz, 1H, C*H* and MMAE), 4.13-4.20 (m, 1H, MMAE), 4.44-4.52 (dd, *J* = 9.6 and 4.8 Hz, 1H, C*H*), 4.52-4.62 (m, MMAE), 4.62-4.74 (overlapped m, MMAE and CH_2), 4.76-4.83 (d, *J* = 3.6 Hz, 1H, CH_2), 4.95-5.15 (overlapped m, 2H, BocN*H* and MMAE), 7.14-7.36 (overlapped m, 7H, Ar*H* MMAE and PABC), 7.53-7.56 (d, *J* = 8.4 Hz, 2H, Ar*H* PABC). ^{13}C NMR (150 MHz, $CDCl_3$ and MeOD-d_4 mix) 10.7 (CH_3, MMAE), 13.9 (CH_3, MMAE), 15.8 (CH_3, MMAE), 18.1 (CH_3, MMAE), 18.5 (CH_3), 19.0 (CH_3), 19.2 (CH_3, MMAE), 24.8 (CH_2, MMAE), 24.9 (CH_2, MMAE), 25.7 (CH_2, MMAE), 26.3 (CH_2), 28.4 (CH_3, Boc), 29.2 (CH_2), 29.6 (CH_3, MMAE), 30.3 (CH, MMAE), (CH_2), 30.8 (CH_2), 31.9 (CH, MMAE), 33.2 (CH, MMAE), 36.4 (CH_2, PEG), 37.6 (CH_2, MMAE), 38.9 (CH_2), 40.2 (PEG CH_2), 43.7 (CH, MMAE), 47.9 (CH_2, MMAE), 50.9 (CH, MMAE), 53.2 (CH, MMAE), 53.3 (CH), 57.9 (OCH_3, MMAE), 59.5 (CH), 59.9 (OCH_3, MMAE), 60.9 (CH, MMAE), 64.9 (CH_2), 67.2 (OCH, MMAE), 67.3 (CH_2, PEG), 70.1 (CH_2, PEG), 70.1-70.5 (overlapped CH_2, PEG), 75.6 (OCH, MMAE), 78.5 (CH, MMAE), 79.4 (C, Boc), 82.2 (OCH, MMAE), 120.0 (ArCH), 126.3 (ArCH, MMAE), 127.3 (ArCH, MMAE), 128.1 (ArCH, MMAE), 128.3 (ArC, MMAE), 128.5 (ArCH), 132.2 (ArC), 138.0 (ArC), 141.3 (ArC, MMAE), 156.4 (CO, Boc), 157.5 (CO), 160.4 (CO), 170.2 (CO, MMAE), 170.4 (CO), 170.6 (CO, MMAE), 172.2 (CO), 172.9 (CO, MMAE), 173.4 (CO), 174.8 (CO, MMAE). LRMS (NSI) 589 (100, $[M+NH_4+H]^{2+}$), 1182 (10, $[M+Na]^+$); HRMS (NSI) calcd. for $C_{54}H_{93}N_7O_{20}Na$ $[M+Na]^+$ 1182.6368, observed: 1182.6369.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2.00 7.92 0.36 0.25 1.98 0.67 0.45 0.44 0.93 0.99 0.73 1.25 0.81 1.59 0.83 1.29 1.57 42.59 2.88 3.78 5.59 2.94 1.77 2.60 0.15 1.13 1.00 0.91 1.28 0.91 1.22 2.06 1.74 1.61 2.13 2.04 8.93 1.19 0.81 2.70 3.06 8.94 2.27 4.51 6.26
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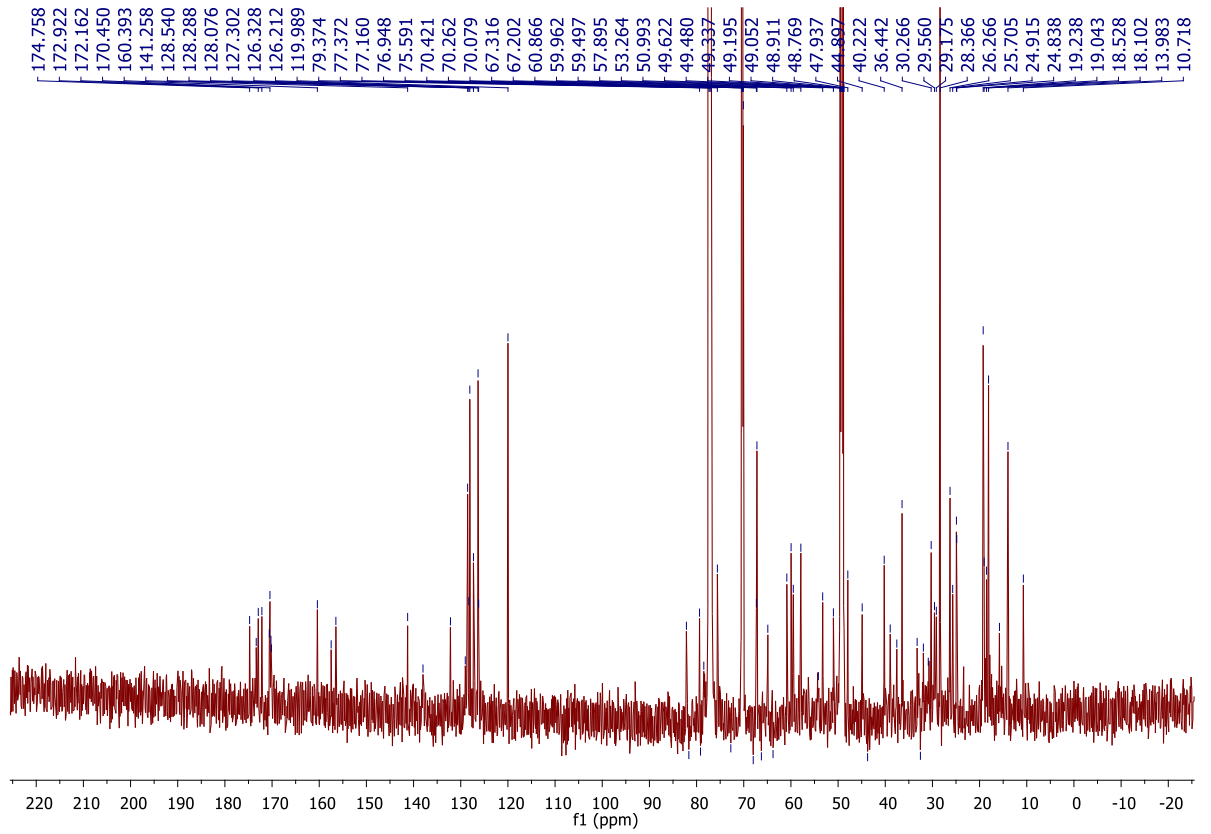
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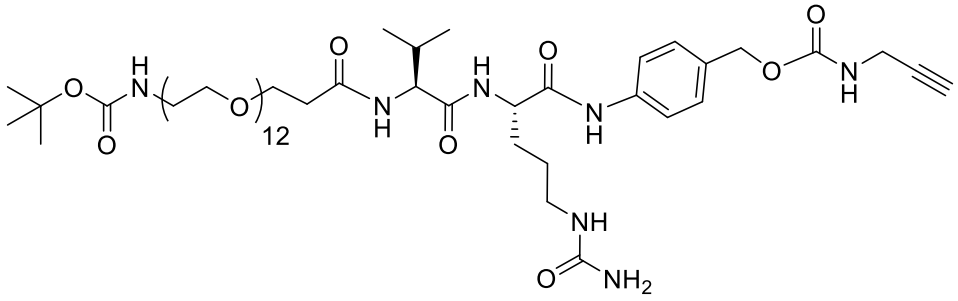
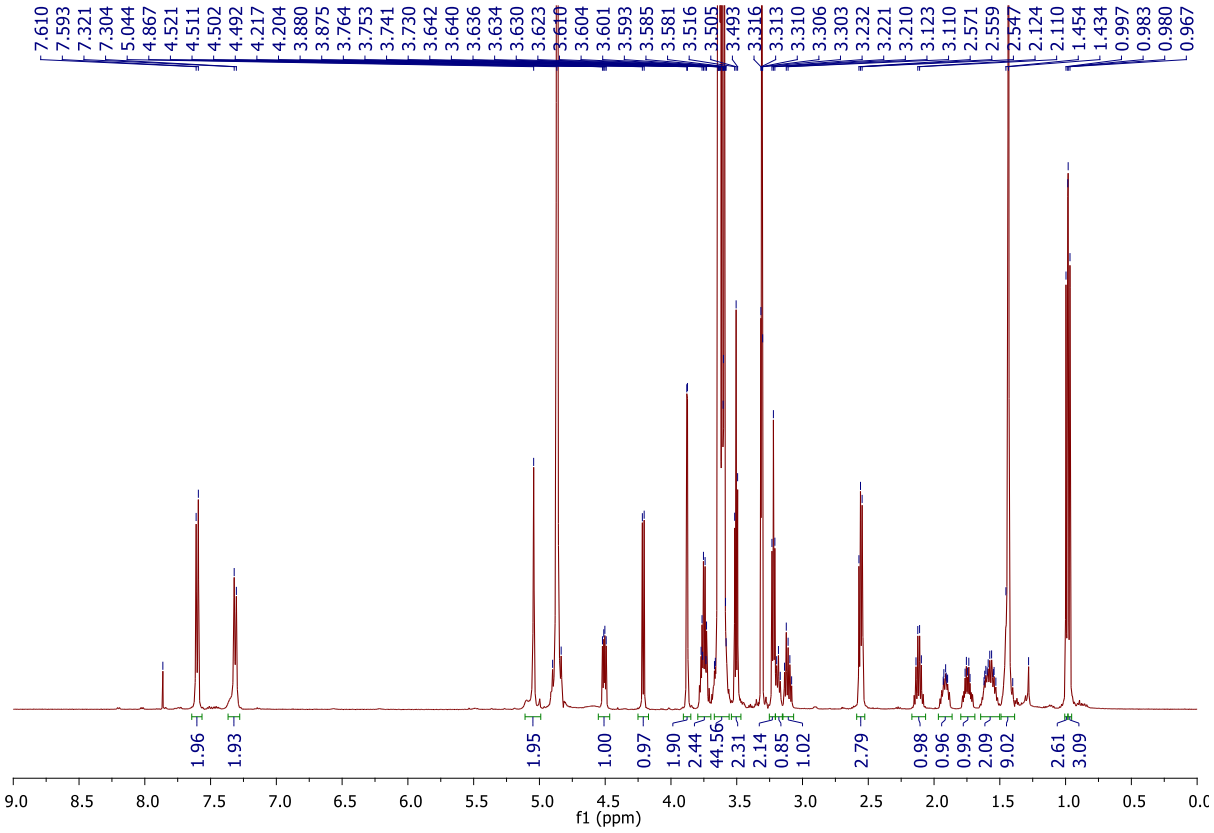
f1 (ppm)

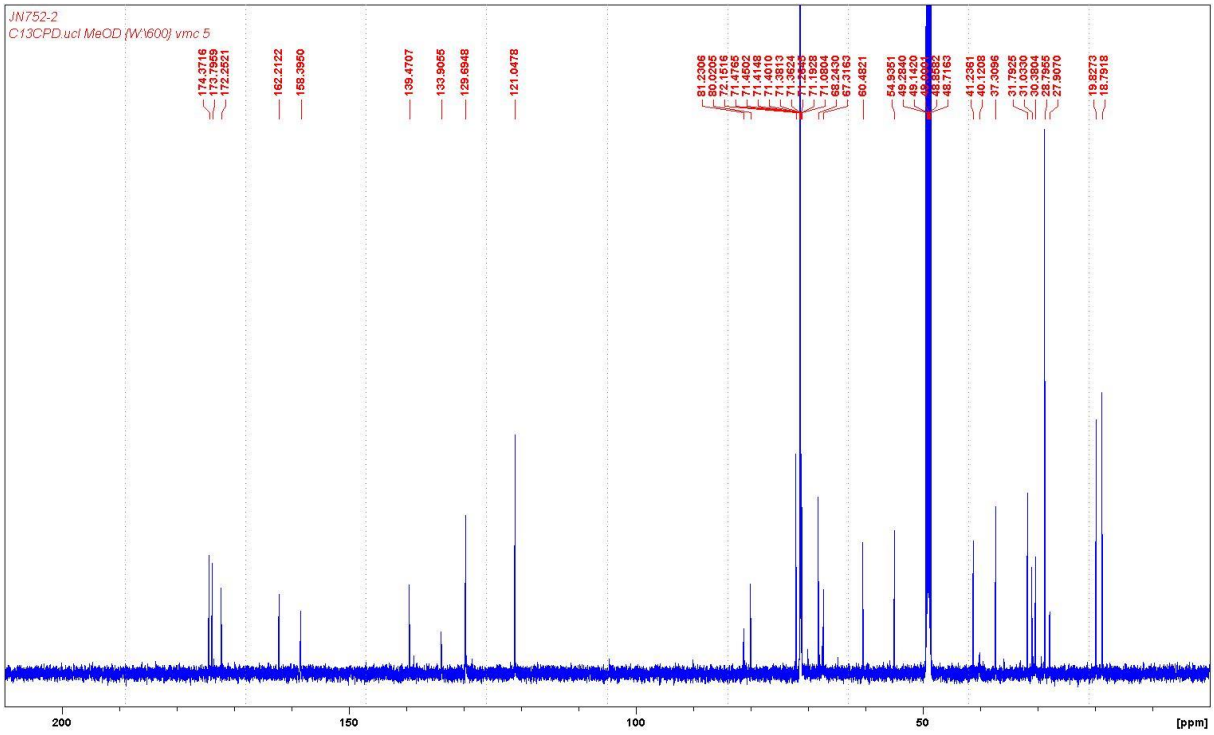
BocHN-PEG$_{12}$-vc-PABC-propargylamide 10



In a 10 mL round-bottom flask, BocHN-vc-PABC-propargylamide **8** (210 mg, 0.37 mmol, 1 eq.) in dry DCM (1.8 mL) and added TFA (0.2 mL). Stirred at 20 °C over 2 hours. Next, concentrated under vacuum to dryness, redissolved in dry DCM, concentrated again, redissolved in dry MeCN and concentrated once more under vacuum. Dissolved H_2N-vc-PABC-propargylamide TFA salt in dry MeCN (4 mL) and added DIPEA (260 µL, 1.5 mmol, 4 eq.). In a separate flask, dissolved BocHN-PEG$_{12}$-CH_2CH_2-CO_2H (268 mg, 0.37 µmol, 1 eq.) in dry MeCN (4 mL). Next, added HBTU (142 mg, 0.37 mmol, 1 eq.), HOBt hydrate (5.0 mg, 37 µmol, 0.1 eq.) and DIPEA (130 µL, 0.75 mmol, 2 eq.). Stirred at 20 °C for 20 min. Then, added the H_2N-vc-PABC-propargylamide solution with DIPEA previously prepared and stirred at 20 °C over 16 hours. Next, added DCM (320 mL) and washed with 15% aq. citric acid (20 mL), sat. aq. $NaHCO_3$ (20 mL). Each aqueous layer was further extracted with DCM (2×40 mL). The combined organic layer was dried ($MgSO_4$), filtered and concentrated under vacuum to yield an oil which was purified by flash chromatography on silica with a gradient of DCM:EtOAc:MeOH (10:3:2 v/v) to DCM:MeOH (10:2 v/v) to afford the title compound as a light-brown oil (223 mg, 0.19 mmol, 51%). Characterisation data: FTIR ν_{max} (cm^{-1}): 3271, 2868, 1697, 1650, 1631, 1532, 1249, 1097. ^{1}H NMR (500 MHz, MeOD-d4 and $CHCl_3$ mix) 0.96-0.99 (overlapped d, *J* = 7.0 Hz, 6H, C*H*$_3$), 1.41-1.47 (s, 9H, C*H*$_3$), 1.51-1.64 (m, *J* = 7.0 Hz, 2H, C*H*$_2$), 1.70-1.79 (m, *J* = 9.0 Hz, 1H, C*H*$_2$), 1.87-1.96 (m, 1H, C*H*$_2$), 2.06-2.17 (oct., *J* = 7.0 Hz, 1H, C*H*), 2.52-2.58 (t overlapped with m, *J* = 6.0 Hz, 3H, PEG C*H*$_2$ and C*H*), 3.08-3.14 (m, *J* = 7.0 Hz, 1H, C*H*$_2$), 3.16-3.20 (m, *J* = 7.0 Hz, 1H, C*H*$_2$), 3.20-3.24 (t, *J* = 5.5 Hz, 2H, PEG C*H*$_2$), 3.49-3.52 (t, *J* = 6.0 Hz, 2H, PEG C*H*$_2$), 3.58-3.65 (overlapped m, 44H, PEG C*H*$_2$), 3.72-3.78 (t, *J* = 6.0 Hz, 2H, PEG C*H*$_2$), 3.87-3.88 (d, *J* = 2.5 Hz, 2H, C*H*$_2$), 4.20-4.22 (d, *J* = 7.0 Hz, 1H, C*H*), 4.49-4.52 (dd, *J* = 9.5 and 5.0 Hz, 1H, C*H*), 5.01-5.08 (br, 2H, C*H*$_2$), 7.29-7.32 (d, *J* = 8.5 Hz, 2H, Ar*H*), 7.58-7.60 (d, *J* = 8.5 Hz, 2H, Ar*H*); ^{13}C NMR (150 MHz, MeOD-d4) 18.8 (CH_3), 19.8 (CH_3), 27.9 (CH_2), 28.8 (CH_3), 30.4(CH_2), 31.0 (CH_2), 31.8 (CH), 37.3 (CH_2, PEG), 40.1 (CH_2), 41.2 (CH_2,

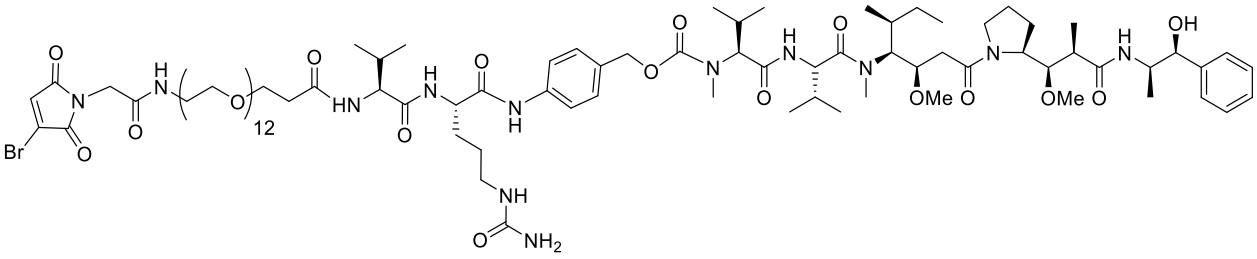
PEG), 54.9 (CH), 60.5 (CH), 67.3 (CH_2), 68.2 (CH_2, PEG), 71.1-71.5 (overlapped CH_2, PEG), 72.1 (CH), 80.0 (C), 81.2 (C), 121.0 (ArCH), 129.7 (ArCH), 133.9 (ArC), 139.5 (ArC), 158.4 (CO), 162.2 (CO), 172.2, (CO), 173.8 (CO), 174.4 (CO). LRMS (NSI) 589 (100, $[M+NH_4+H]^{2+}$), 1183 (5, $[M+Na]^+$); HRMS (NSI) calcd. for $C_{54}H_{93}N_7O_{20}Na$ $[M+Na]^+$ 1182.6368, observed: 1182.6369.





2-(3-bromo-2,5-dioxo-2,5-dihydro-1*H*-pyrrol-1-yl)-*N*-(-PEG$_{12}$-vc-PABC-MMAE)acetamide 1

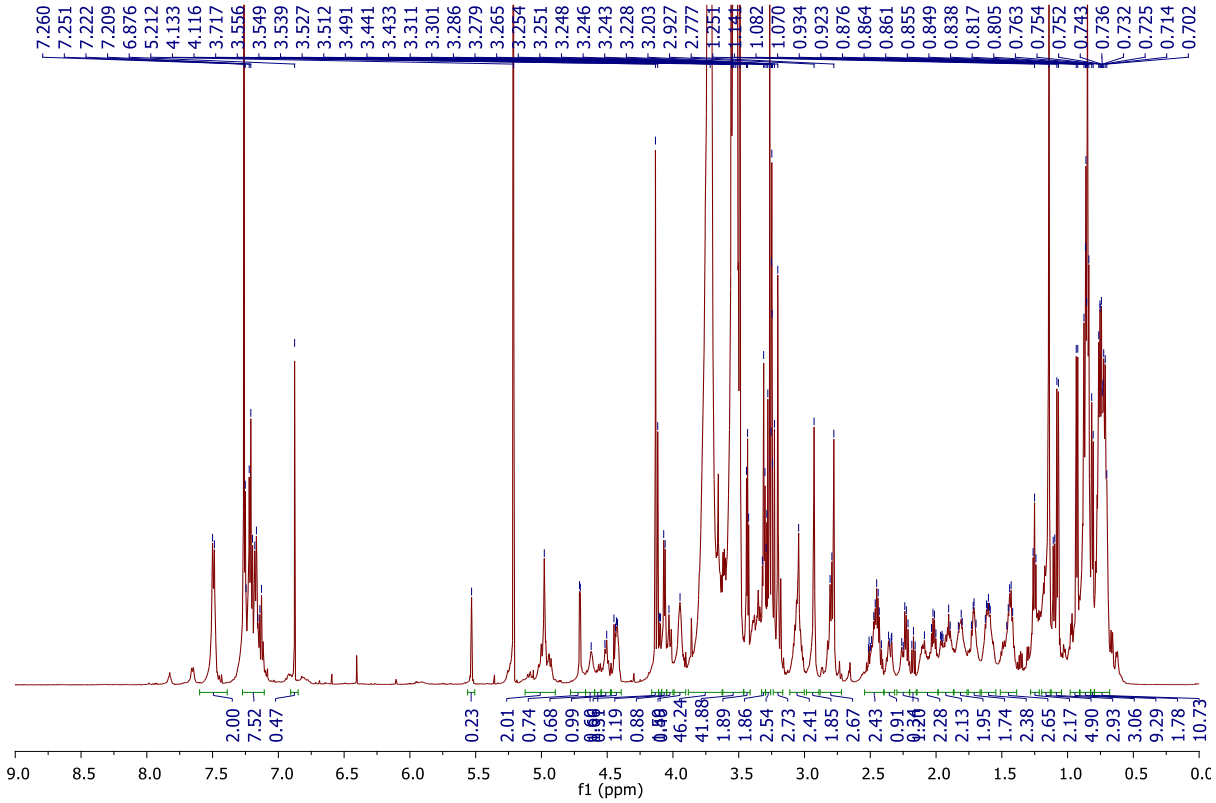
MBM 1

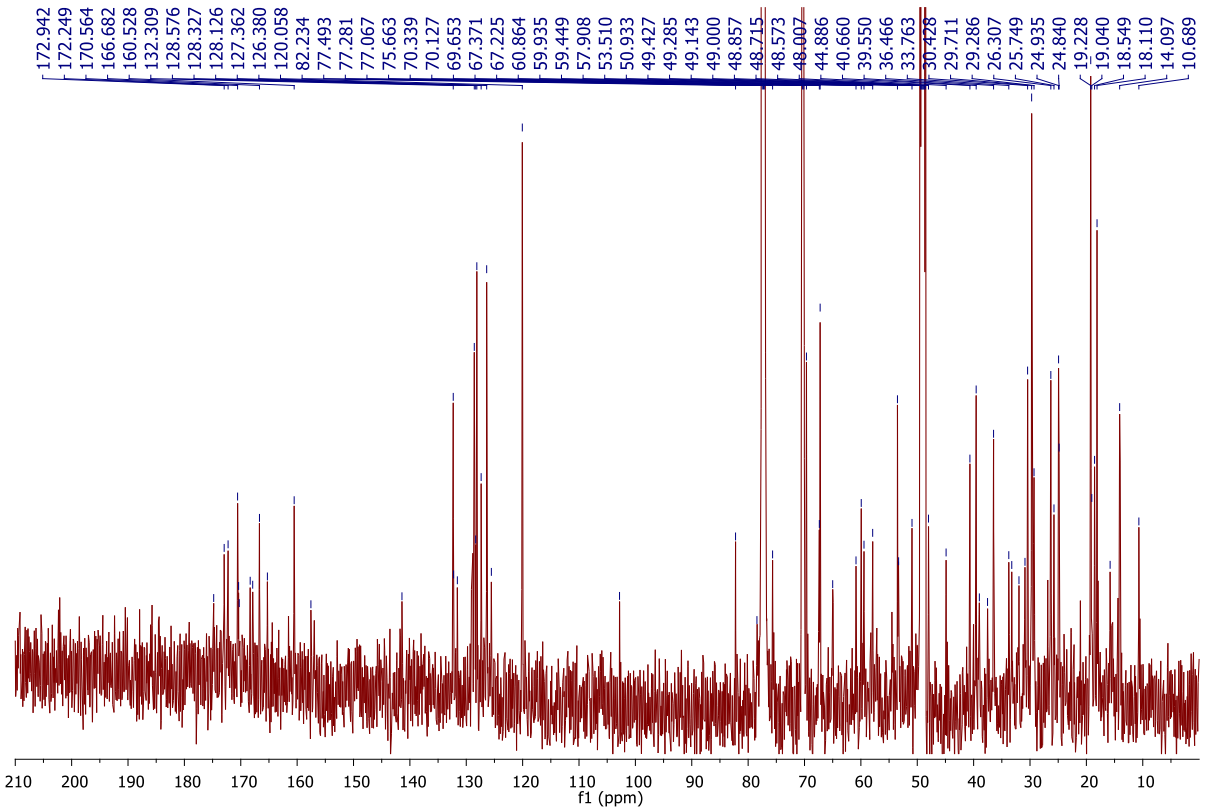


In a 10 mL round-bottom flask, dissolved BocHN-PEG$_{12}$-vc-PABC-MMAE **9** (29 mg, 16 µmol, 1 eq.) in dry DCM (0.9 mL) and added TFA (0.1 mL). Stirred at 20 °C over 2 hours. Next, concentrated under vacuum to dryness, redissolved in dry DCM, concentrated again, redissolved in dry MeCN and concentrated once more under vacuum. Dissolved H$_2$N-PEG$_{12}$-vc-PABC-MMAE TFA salt in dry MeCN (2 mL) and added DIPEA (14 µL, 80 µmol, 5 eq.). In a separate flask, dissolved 2-(3-bromo-2,5-dioxo-2,5-dihydro-1*H*-pyrrol-1-yl)acetic acid (MBM **6**, 3.8 mg, 16 µmol, 1 eq.) in dry MeCN (1 mL). Next, added dicyclohexylcarbodiimide (DCC, 3.3 mg, 16 µmol, 1 eq.) and stirred at 20 °C for 20 min. Then, added pentafluorophenol (1.7 µL, 16 µmol, 1 eq.) and stirred at 20 °C for 90 min. Filtered this mixture through a short

plug of cotton wool and added the filtrate to the H_2N-PEG_{12}-vc-PABC-MMAE solution with DIPEA previously prepared. This solution was stirred at 20 °C over 5 hours. Next, concentrated under vacuum, redissolved in DCM (10 mL) and washed with 15% aq. citric acid (10 mL), sat. aq. $NaHCO_3$ (10 mL) and water (10 mL). The organic layer was dried ($MgSO_4$), filtered and concentrated under vacuum to yield a pale yellow oil which was purified by preparative thin layer chromatography on silica with DCM:MeOH (15:2 v/v) to afford the title compound as a pale yellow oil (20 mg, 10 µmol, 63%). Characterisation data: ^{1}H NMR (600 MHz, $CDCl_3$ and MeOD-d_4 mix) 0.68-0.80 (overlapped m, 10H, CH_3 and MMAE), 0.80-0.83 (d, *J* = 6.6 Hz, 2H, MMAE), 0.83-0.91 (overlapped m, 9H, MMAE and CH_3), 0.91-0.95 (d, *J* = 6.6 Hz, 3H, MMAE), 1.05-1.12 (overlapped d, *J* = 6.6 Hz, 3H, MMAE), 1.13-1.20 (m, MMAE), 1.23-1.27 (t, *J* = 6.6 Hz, 2H, MMAE), 1.38-1.52 (m, *J* = 6.6 Hz, 2H, CH_2), 1.54-1.67 (m, 2H, CH_2 and MMAE), 1.67-1.75 (m, 1H, CH_2), 1.76-1.98 (m, MMAE), 1.98-2.05 (m, *J* = 6.6 Hz, 1H, C*H*), 2.05-2.14 (m, MMAE), 2.15-2.30 (m, 1H, MMAE), 2.30-2.56 (m, MMAE), 2.75-2.85 (m, MMAE), 2.90-2.94 (m, MMAE), 3.00-3.12 (overlapped m, 3H, C*H* and MMAE), 3.16-3.23 (m, MMAE), 3.27-3.29 (m, MMAE), 3.30-3.32 (t, *J* = 5.4 Hz, 2H, PEG CH_2), 3.42-3.45 (t, *J* = 5.4 Hz, 2H, PEG CH_2), 3.47-3.59 (overlapped m, PEG CH_2), 3.91-3.99 (m, MMAE), 3.99-4.05 (m, MMAE), 4.05-4.08 (overlapped d and m, *J* = 6.6 Hz, 1H, C*H* and MMAE), 4.08-4.12 (m, MMAE), 4.12-4.14 (s, 2H, CH_2), 4.38-4.47 (m, 1H, C*H*), 4.47-4.57 (m, MMAE), 4.57-4.66 (overlapped m, MMAE and CH_2), 4.66-4.74 (d, *J* = 4.2 Hz, 1H, CH_2), 4.89-5.04 (m, MMAE), 6.87-6.88 (s, 1H, C*H*), 7.09-7.30 (overlapped m, 7H, Ar*H* MMAE and PABC), 7.45-7.53 (d, *J* = 8.4 Hz, 2H, Ar*H* PABC); ^{13}C NMR (150 MHz, $CDCl_3$ and MeOD-d_4 mix) 10.7 (CH_3, MMAE), 14.0 (CH_3, MMAE), 15.8 (CH_3, MMAE), 18.1 (CH_3, MMAE), 18.5 (CH_3), 19.0 (CH_3), 19.2 (CH_3, MMAE), 24.8 (CH_2, MMAE), 24.9 (CH_2, MMAE), 25.7 (CH_2, MMAE), 26.3 (CH_2), 29.3 (CH_2), 29.7 (CH_3, MMAE), 30.4 (CH, MMAE), (CH_2), 30.9 (CH_2), 31.9 (CH, MMAE), 33.2 (CH, MMAE), 36.4 (CH_2, PEG), 37.5 (CH_2, MMAE), 38.9 (CH_2), 39.5 (CH_2), 40.7 (CH_2, PEG), 44.9 (CH, MMAE), 48.0 (CH_2, MMAE), 50.9 (CH, MMAE), 53.3 (CH, MMAE), 53.5 (CH), 57.9 (OCH_3, MMAE), 59.4 (CH), 59.9 (OCH_3, MMAE), 60.9 (CH, MMAE), 64.9 (CH_2), 67.2 (OCH, MMAE), 67.4 (CH_2, PEG), 69.6 (CH_2, PEG), 70.0-70.5 (overlapped CH_2, PEG), 75.7 (OCH, MMAE), 78.4 (CH, MMAE), 82.2 (OCH, MMAE), 102.8 (CH), 120.0 (ArCH), 126.4 (ArCH, MMAE), 127.4 (ArCH, MMAE), 128.1 (ArCH, MMAE), 128.3 (ArC, MMAE), 128.6 (ArCH), 131.6 (C), 132.3 (ArC), 141.4 (ArC, MMAE), 157.6 (CO), 160.5 (CO), 165.3 (CO), 166.7 (CO), 168.3 (CO), 170.2 (CO, MMAE), 170.4 (CO), 170.6 (CO, MMAE), 172.2 (CO), 172.9 (CO, MMAE), 174.8 (CO, MMAE). LRMS (NSI) 649 (28,

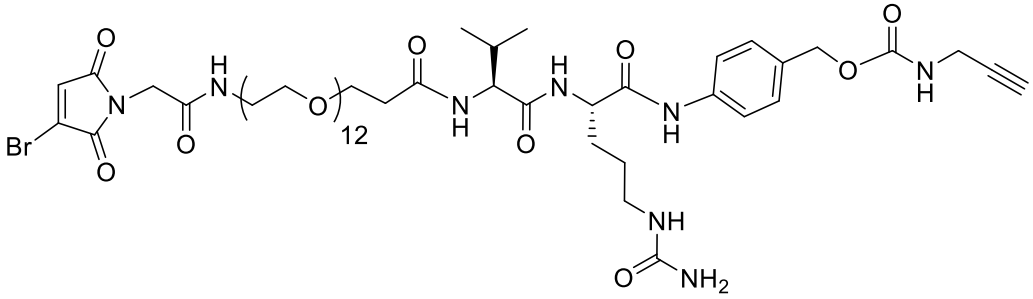
$[M^{81}Br+3H]^{3+}$), 647 (62, $[M^{79}Br+3H]^{3+}$), 971 (7, $[M^{81}Br+2H]^{2+}$), 969 (15, $[M^{79}Br+2H]^{2+}$); HRMS (NSI) calcd. for $C_{91}H_{151}N_{12}O_{28}Br$ $[M^{79}Br+2H]^{2+}$ 969.4967, observed: 969.4961.





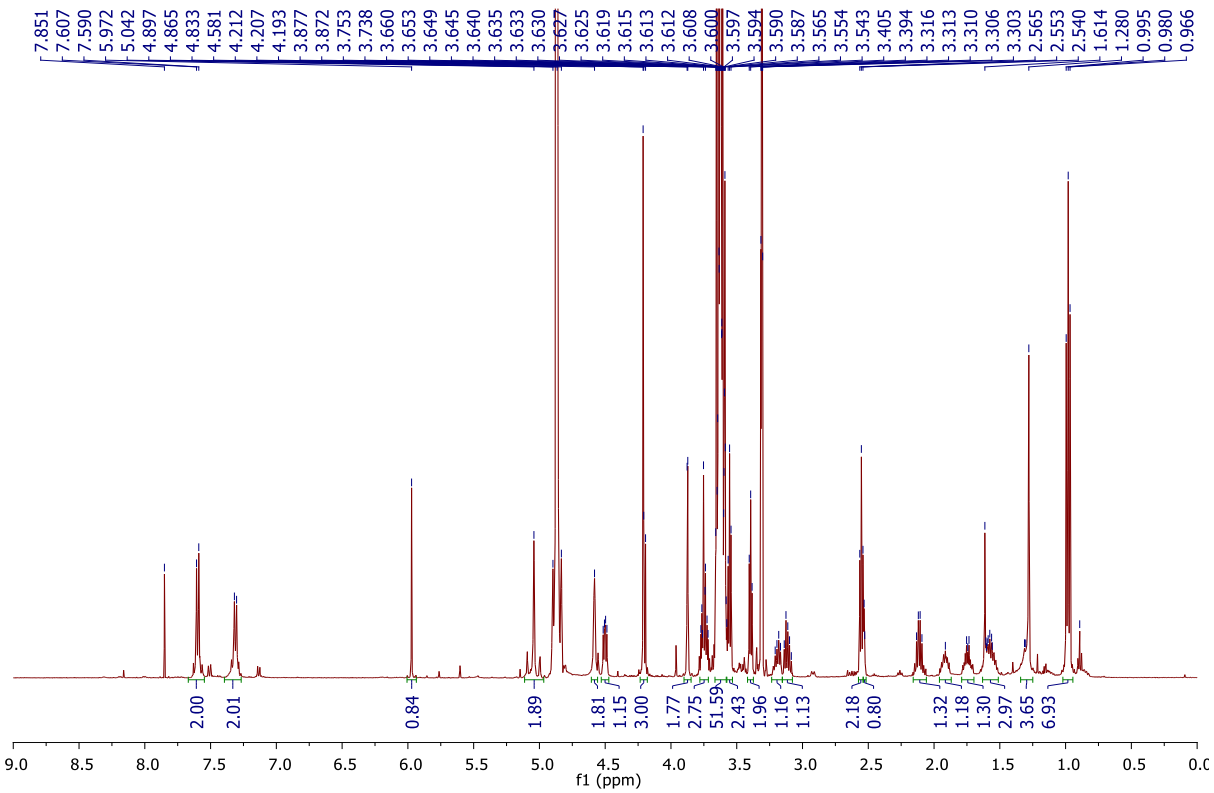
2-(3-bromo-2,5-dioxo-2,5-dihydro-1*H*-pyrrol-1-yl)-*N*-(-PEG$_{12}$-vc-PABC-propargylamide)acetamide 2

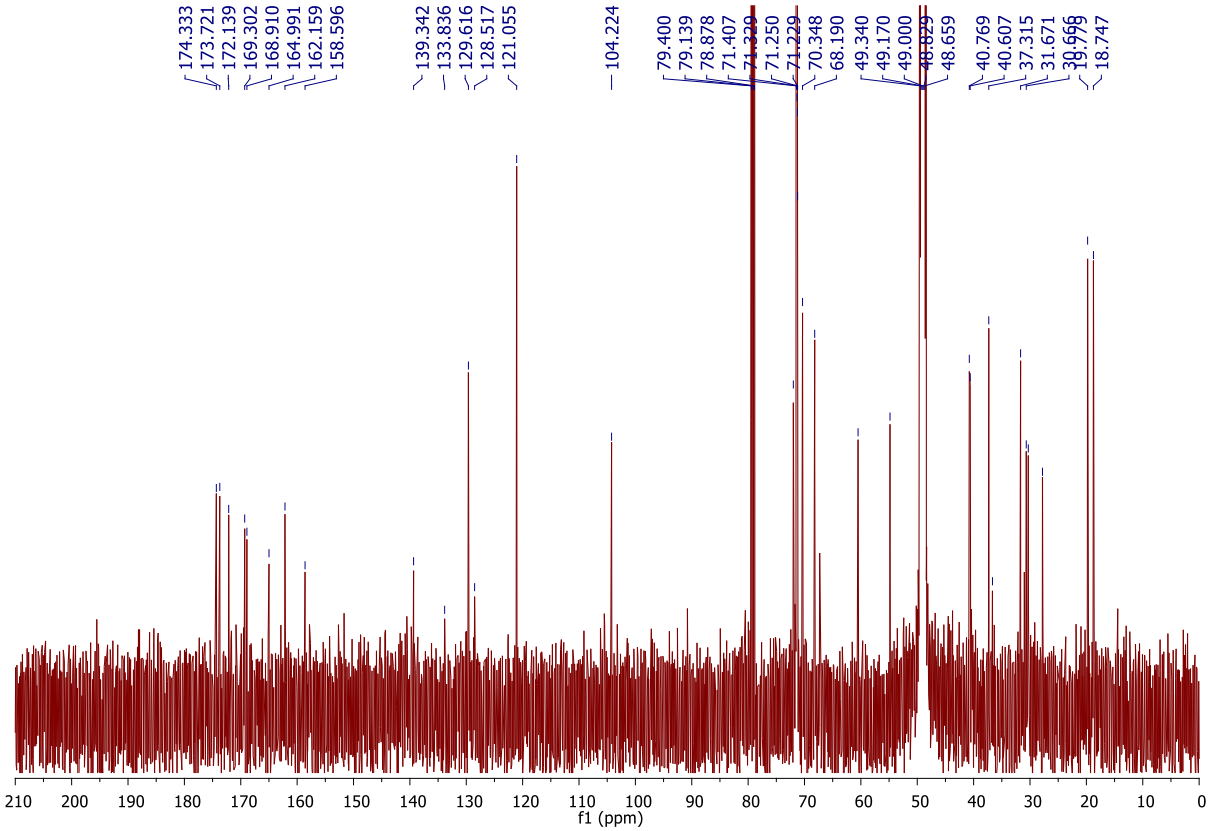
MBM 2



In a 10 mL round-bottom flask, dissolved BocHN-PEG$_{12}$-vc-PABC-propargylamide **10** (34 mg, 29 µmol, 1 eq.) in dry DCM (0.9 mL) and added TFA (0.1 mL). Stirred at 20 °C over 2 hours. Next, concentrated under vacuum to dryness, redissolved in dry DCM, concentrated again, redissolved in dry MeCN and concentrated once more under vacuum. Dissolved H_2N-PEG$_{12}$-valine-citruline-PABC-Alk TFA salt in dry MeCN (1 mL) and added DIPEA (26 µL, 147 µmol, 5 eq.). In a separate flask, dissolved 2-(3-bromo-2,5-dioxo-2,5-dihydro-1*H*-pyrrol-1-yl)acetic acid (MBM **6**, 6.9 mg, 29 µmol, 1 eq.) in dry MeCN (1 mL). Next, added dicyclohexylcarbodiimide (DCC, 6.0 mg, 29 µmol, 1 eq.) and stirred at 20 °C for 10 min. Then, added pentafluorophenol (3.1 µL, 29 µmol, 1 eq.) and stirred at 20 °C for 15 min. Filtered this mixture through a short plug of cotton wool and added the filtrate to the H_2N-PEG$_{12}$-vc-PABC-propargylamide solution with DIPEA previously prepared. This solution was stirred at 20 °C over 4 hours. Next, concentrated under vacuum, redissolved in EtOAc (30 mL) and washed with 15% aq. citric acid (2×5 mL) and brine (5 mL). The organic layer was dried ($MgSO_4$), filtered and concentrated under vacuum to yield a pale yellow oil which was purified by preparative thin layer chromatography on silica with DCM:MeOH (10:1 v/v) to afford the title compound as a yellow solid (15.4 mg, 12.1 µmol, 41%). Characterisation data: m.p. 96-98 °C. FTIR ν_{max} (cm^{-1}): 3303, 3074, 2914, 2867, 2407, 1722, 1683, 1652, 1624, 1517, 1441, 1089. ^{1}H NMR (500 MHz, MeOD-d_4 and $CHCl_3$ mix) 0.95-1.00 (overlapped d, *J* = 6.5 Hz, 6H, C*H*$_3$), 1.51-1.64 (m, *J* = 6.5 Hz, 2H, C*H*$_2$), 1.69-1.79 (m, *J* = 9.0 Hz, 1H, C*H*$_2$), 1.87-1.96 (m, 1H, C*H*$_2$), 2.06-2.16 (oct., *J* = 6.5 Hz, 1H, C*H*), 2.52-2.54 (t, *J* = 2.5 Hz, 1H, C*H*), 2.54-2.57 (t, *J* = 6.0 Hz, 2H, PEG C*H*$_2$), 3.08-3.14 (m, *J* = 7.0 Hz, 1H, C*H*$_2$), 3.16-3.22 (m, *J* = 7.0 Hz, 1H, C*H*$_2$), 3.38-3.41 (t, *J* = 5.5 Hz, 2H, PEG C*H*$_2$), 3.54-3.56 (t. *J* = 5.5 Hz, 2H, PEG C*H*$_2$), 3.58-3.67 (overlapped m, PEG C*H*$_2$), 3.70-3.78 (t, *J* = 6.0 Hz, 2H, PEG C*H*$_2$), 3.86-3.88 (d, *J* = 2.5 Hz, 2H,

CH_2), 4.19-4.21 (d, *J* = 7.0 Hz, 1H, C*H*), 4.21-4.22 (s, 2H, CH_2), 4.48-4.51 (dd, *J* = 9.0 and 5.0 Hz, 1H, C*H*), 4.99-5.10 (br, 2H, CH_2), 5.96-5.98 (s, 1H, C*H*), 7.28-7.33 (d, *J* = 8.5 Hz, 2H, Ar*H*), 7.57-7.61 (d, *J* = 8.5 Hz, 2H, Ar*H*); ^{13}C NMR (125 MHz, MeOD-d4 and $CHCl_3$ mix) 18.7 (CH_3), 19.8 (CH_3), 27.8 (CH_2), 30.3 (CH_2), 30.7 (CH_2), 31.7 (CH), 36.7 (CH_2), 37.3 (CH_2, PEG), 40.6 (CH_2), 40.8 (CH_2, PEG), 54.8 (CH), 60.5 (CH), 67.3 (CH_2), 68.2 (CH_2, PEG), 70.3 (CH_2, PEG), 71.2-71.5 (overlapped CH_2, PEG), 71.9 (CH), 78.0 (C overlapped with $CDCl_3$), 104.2 (CH), 121.0 (ArCH), 128.5 (C), 129.6 (ArCH), 133.8 (ArC), 139.3 (ArC), 158.6 (CO), 162.1 (CO), 165.0 (CO), 168.9 (CO), 169.3 (CO), 172.1, (CO), 173.7 (CO), 174.3 (CO). LRMS (ESI) 1299 (5, [M^{81}Br+Na]$^+$), 1297 (5, [M^{79}Br+Na]$^+$); HRMS (ESI) calcd. for $C_{55}H_{87}N_8O_{21}BrNa$ [M^{79}Br+Na]$^+$ 1297.5067, observed: 1297.5058.




174.333
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164.991
162.159
158.596
139.342
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104.224
79.400
79.139
78.878
71.407
71.329
71.250
71.229
70.348
68.190
49.340
49.170
49.000
48.829
48.659
40.769
40.607
37.315
31.671
30.666
19.779
18.747
210 200 190 180 170 160 150 140 130 120 110 100 90 80 70 60 50 40 30 20 10 0
f1 (ppm)

SEC-HPLC chromatograms of TFC 5 incubated in blood serum

Figure S8 – SEC-HPLC chromatogram of TFC 5 incubated in blood serum at 0 days

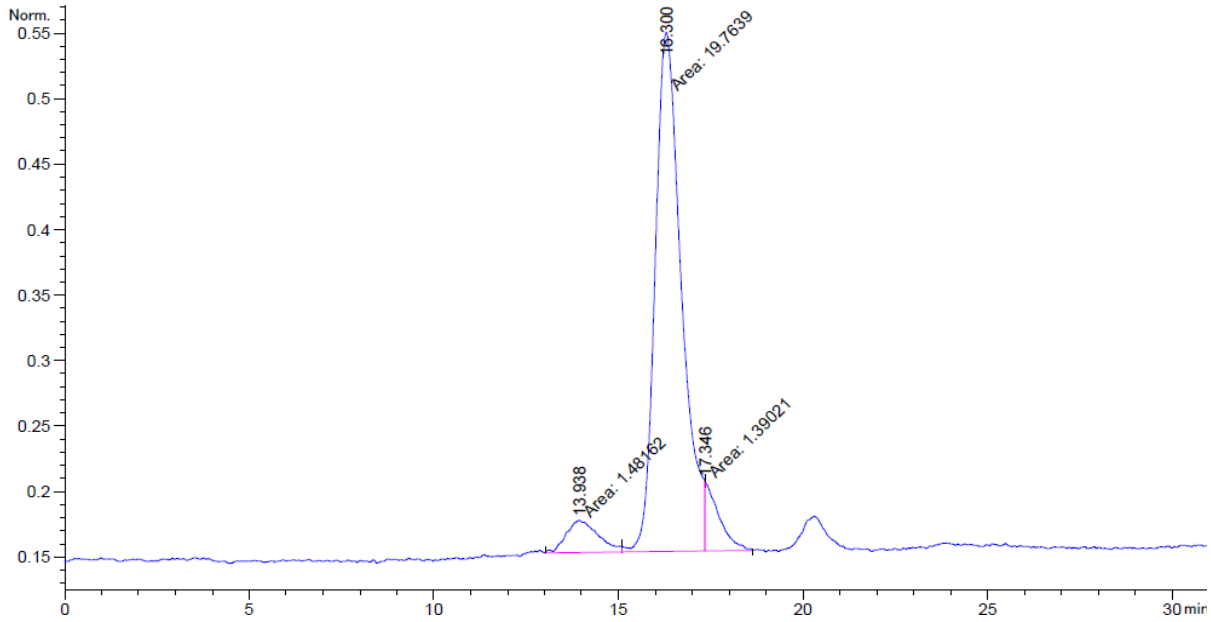

Figure S8 – SEC-HPLC with fluorescence detection (excitation 495 nm, emission 525 nm) chromatogram of TFC **5** incubated in blood serum at 0 days.

Figure S9 – SEC-HPLC chromatogram of TFC 5 incubated in blood serum for 1 day

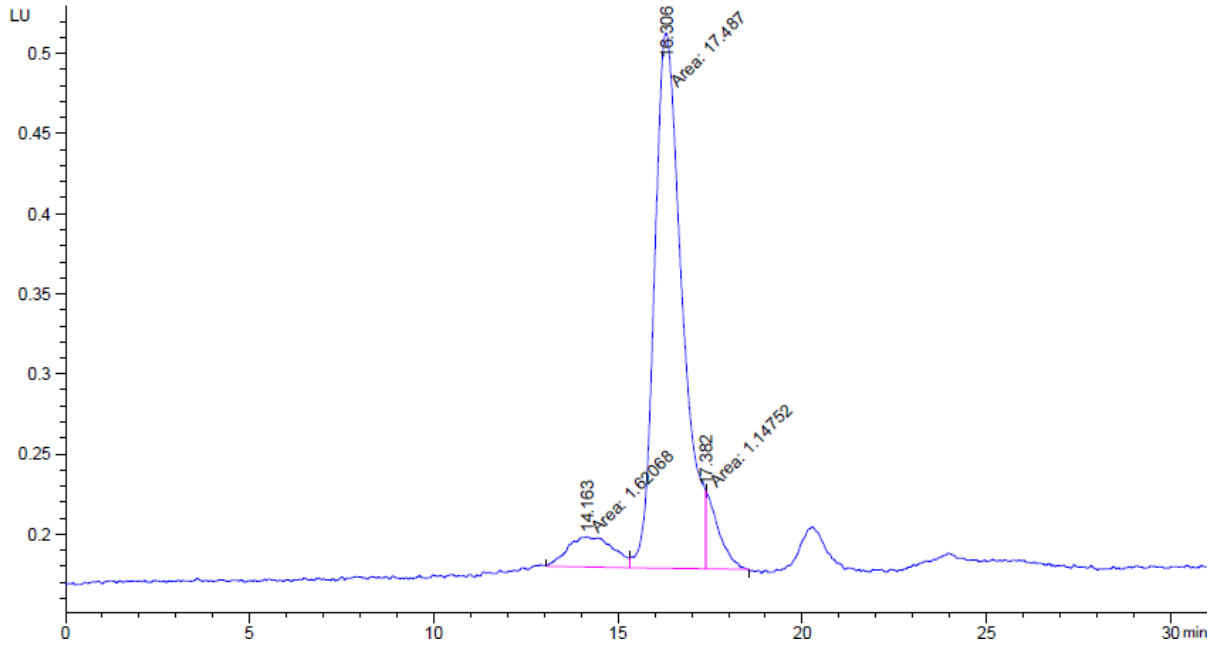

Figure S9 – SEC-HPLC with fluorescence detection (excitation 495 nm, emission 525 nm) chromatogram of TFC **5** incubated in blood serum for 1 day.

Figure S10 – SEC-HPLC chromatogram of TFC 5 incubated in blood serum for 2 days

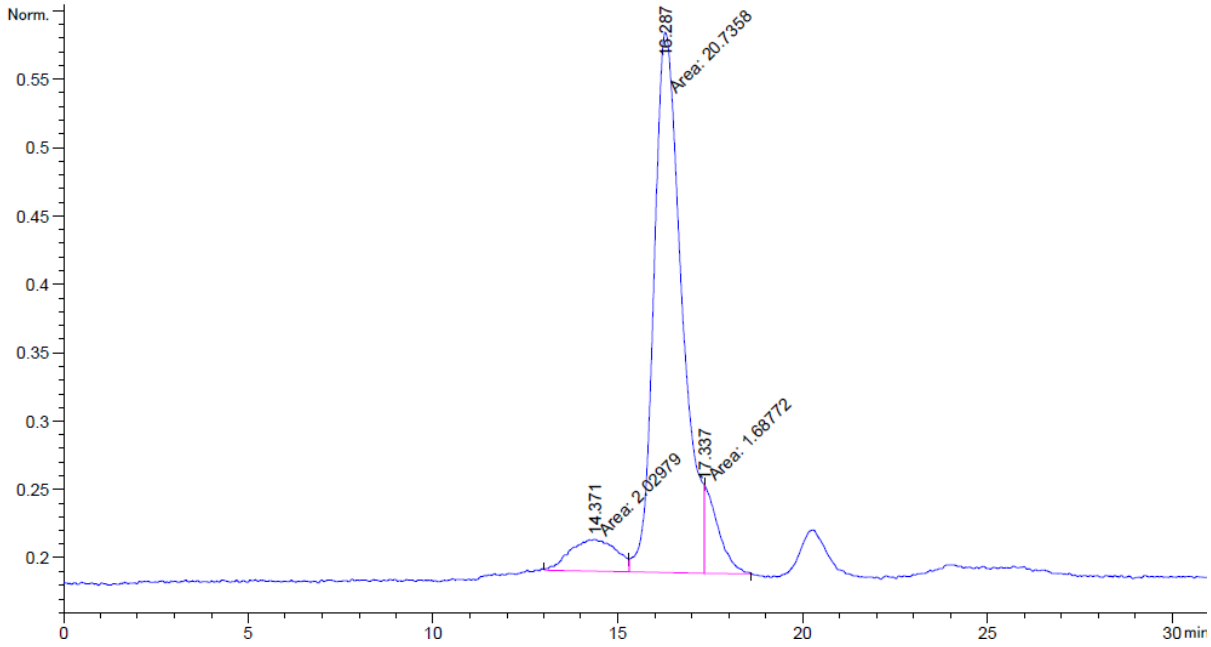

Figure S10 – SEC-HPLC with fluorescence detection (excitation 495 nm, emission 525 nm) chromatogram of TFC **5** incubated in blood serum for 2 days.

Figure S11 – SEC-HPLC chromatogram of TFC 5 incubated in blood serum for 4 days

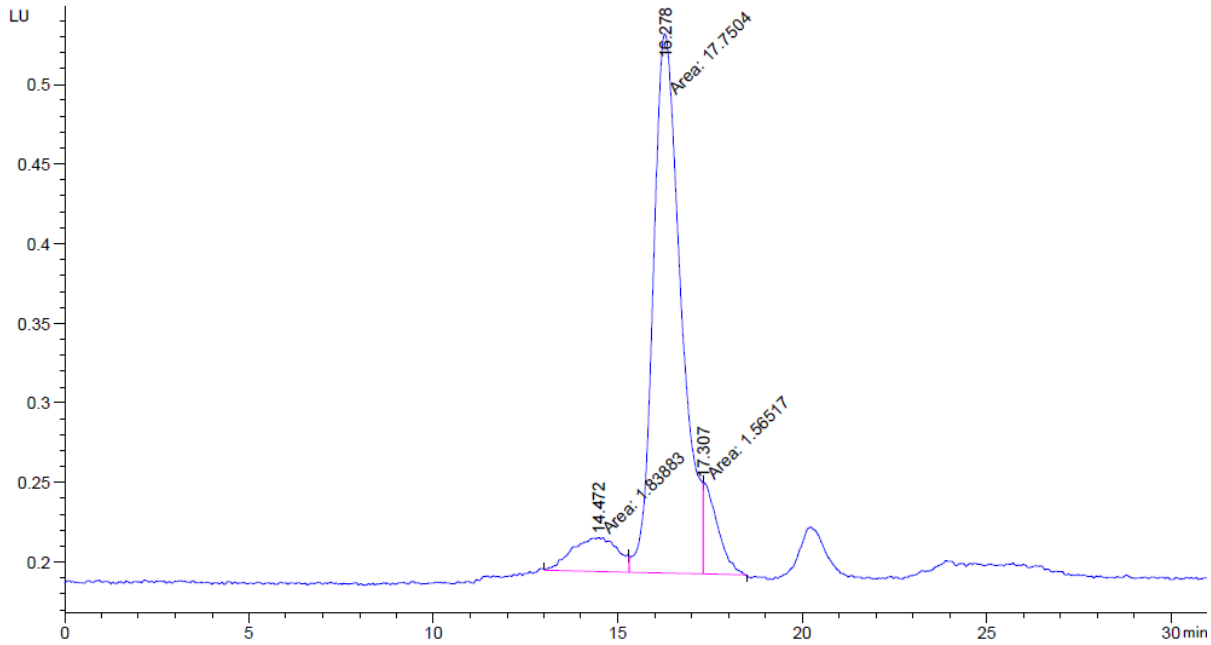

Figure S11 – SEC-HPLC with fluorescence detection (excitation 495 nm, emission 525 nm) chromatogram of TFC **5** incubated in blood serum for 4 days.

Figure S12 – SEC-HPLC chromatogram of TFC 5 incubated in blood serum for 7 days

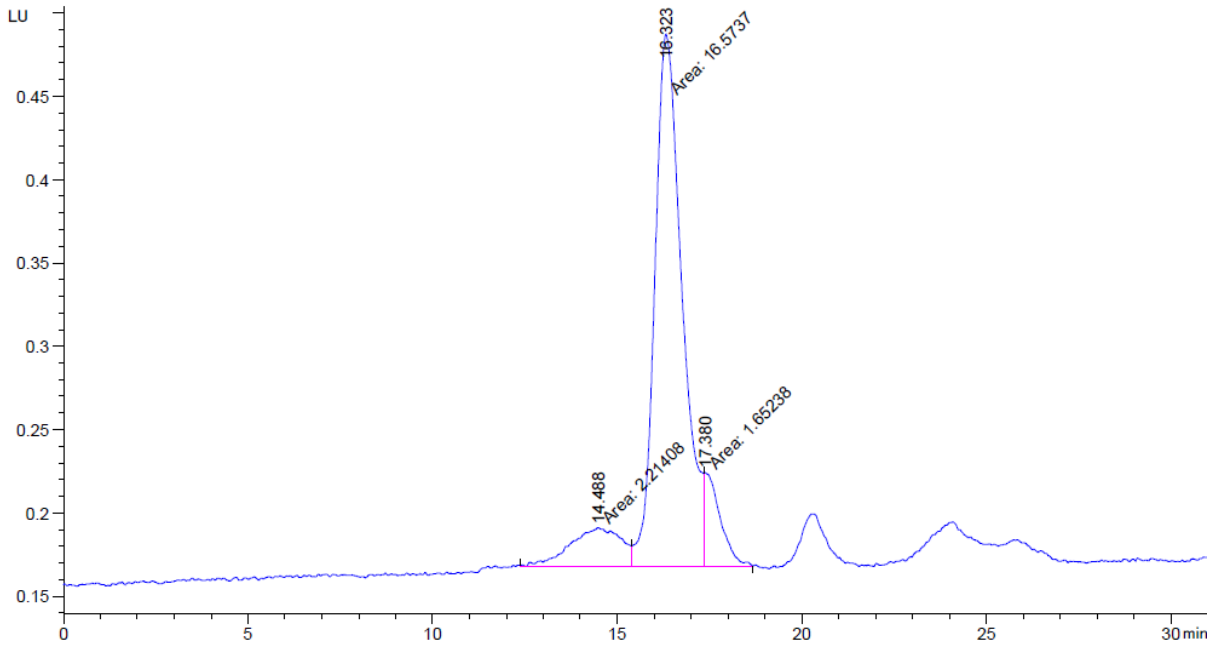

Figure S12 – SEC-HPLC with fluorescence detection (excitation 495 nm, emission 525 nm) chromatogram of TFC **5** incubated in blood serum for 7 days.

Figure S13 – SEC-HPLC chromatogram of TFC 5 incubated in blood serum for 10 days

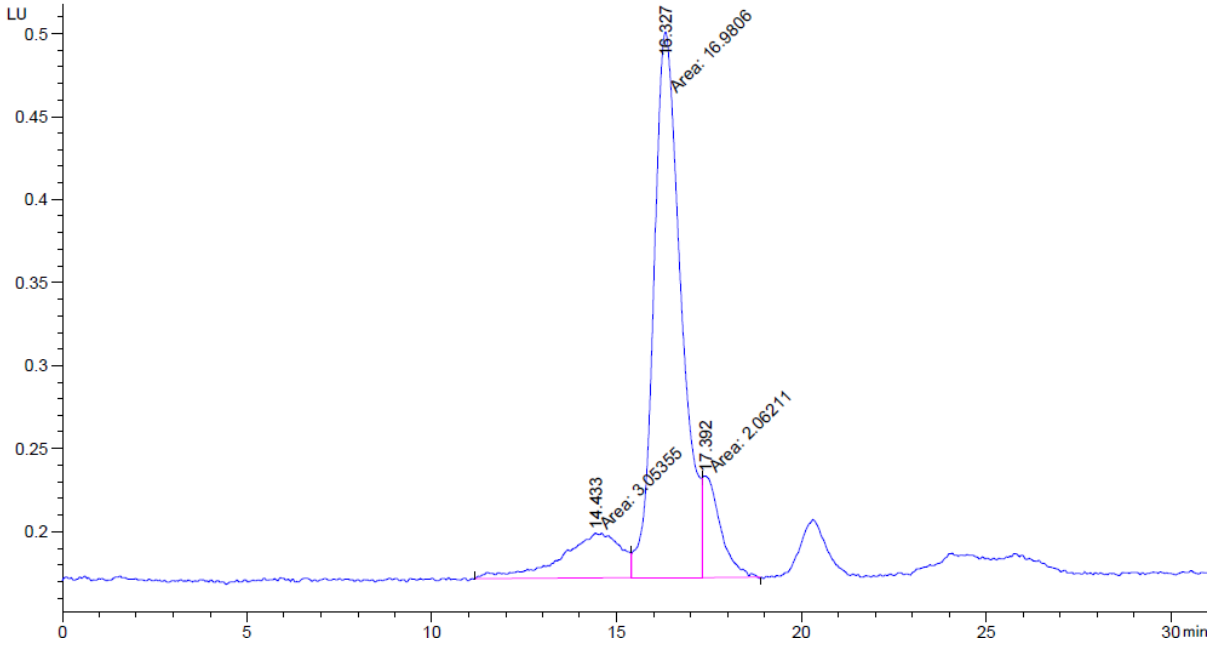

Figure S13 – SEC-HPLC with fluorescence detection (excitation 495 nm, emission 525 nm) chromatogram of TFC **5** incubated in blood serum for 10 days.

Figure S14 – Overlay of SEC-HPLC chromatograms of TFC 5 incubated in blood serum

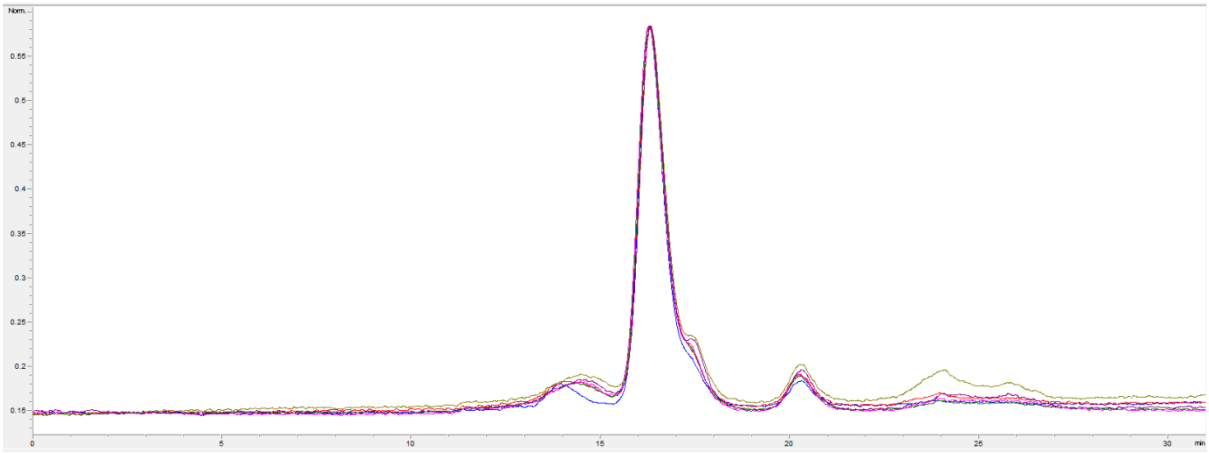

Figure S14 – SEC-HPLC with fluorescence detection (excitation 495 nm, emission 525 nm) chromatogram of TFC **5** incubated in blood serum for 0 days (blue), 1 day (red), 2 days (green), 4 days (pink), 7 days (beige) and 10 days (purple). All chromatograms are normalised to the antibody fraction.

References

1. G. Candiano, M. Bruschi, L. Musante, L. Santucci, G. M. Ghiggeri, B. Carnemolla, P. Orecchia, L. Zardi and P. G. Righetti, *Electrophoresis*, 2004, **25**, 1327-1333.
2. S. O. Doronina, B. E. Toki, M. Y. Torgov, B. A. Mendelsohn, C. G. Cerveny, D. F. Chace, R. L. DeBlanc, P. R. Gearing, T. D. Bovee, C. B. Siegall, J. A. Francisco, A. F. Wahl, D. L. Meyer and P. D. Senter, *Nat. Biotech.*, 2003, **21**, 778-784.