Electronic Supplementary Material (ESI) for RSC Advances.

Electronic Supplementary Information

Pyridazinediones deliver potent, stable, targeted and efficacious antibody-drug conjugates (ADCs) with a controlled loading of 4 drugs per antibody

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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. 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 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 then buffer exchanged completely for borate buffer pH 8.0 *via* ultrafiltration (MWCO 10 kDa, Amicon). 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. 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 (H) and light (L) chains.

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 ~35 µM in total 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 ~35 µM in total 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 concentrations for trastuzumab NGM and PD ADCs

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 antibody fragment concentration followed the Beer-Lambert law using ε_{280} = 215380 $M^{-1}\,cm^{-1}$ for trastuzumab. Concentrations of trastuzumab NGM-MMAE and Mal-MMAE conjugates **1** and **2** were calculated using the same extinction coefficient for trastuzumab since the maleamic acid and succinimide groups and MMAE were found to have negligible absorption at 280 nm compared to trastuzumab. Concentrations of trastuzumab PD-MMAE conjugates **3** and **4** were calculated using the same extinction coefficient, applied to a corrected absorption value at 280 nm calculated as follows by subtracting pyridazinedione absorption at 340 nm using 0.28 as a correction factor. A_{280} is measured absorption at 280 nm and A_{340} is pyridazinedione absorption at 340 nm.

$$[NGM\text{–}MMAE\ ADC] = \frac{A_{280}}{l \times \varepsilon_{280}} \qquad [PD\text{–}MMAE\ ADC] = \frac{A_{280} - (0.28 \times A_{340})}{l \times \varepsilon_{280}}$$

Liquid chromatography mass spectrometry (LC-MS)

Trastuzumab and PD-MMAE ADC samples were prepared (50 µL of 7.5 µM solution in BBS) and deglycosylated by addition of PNGase F (Glycerol-free) (1.25 µL, 625 units; New England BioLabs) and incubation at 37 °C for 16 h. The samples were then buffer exchanged and concentrated by ultrafiltration into water (16 µM, 2.4 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 gases 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.

Hydrophobic interaction chromatography (HIC)

Samples of trastuzumab and ADCs (~35 µM) were diluted 1:1 with water and injected (6-12 µL) onto a TSK-Gel Butyl-NPR 4.6 mm x 35 mm, 2.5 µm particle size column from Tosoh Bioscience, connected to an Agilent 1100 HPLC equipped with a diode array for UV-vis detection. Samples were run with a gradient from 100% buffer A (1.5 M ammonium sulfate, 25 mM sodium phosphates, pH 7) to 60% buffer B (25 mM sodium phosphates, 25% isopropanol (v/v), pH 7) over 52 minutes at a flow rate of 0.6 mL/min. The temperature was maintained at 20 °C for the duration of the run. Detection was by UV-vis absorbance at 280 nm. Drug-to-antibody ratio (DAR) species were assigned based on reference to the observed retention times for elution of DAR species 0, 2, 4 and 6 with increasing % of buffer B over time for Mal-MMAE ADC **2**. Average DAR for each ADC was calculated as follows, where DAR_n corresponds to the peak area for a given DAR species with n being the number of MMAE drugs per antibody for that given DAR species.

$$Average\ DAR = \frac{(DAR_1 + 2 \times DAR_2 + 3 \times DAR_3 + 4 \times DAR_4 + 5 \times DAR_5)}{(DAR_0 + DAR_1 + DAR_2 + DAR_3 + DAR_4 + DAR_5)}$$

Size-exclusion chromatography (SEC)

Trastuzumab and ADCs were prepared as 20 µM (2.94 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. Trastuzumab and ADCs were diluted in PBS yielding concentrations covering the range 30-0.12 nM. The dilution series was added, including PBS only and trastuzumab or ADC at the highest concentration 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 trastuzumab or ADC were corrected as follows, where $A_{490corr}$ is corrected absorption, A_{490} is measured absorption, A_{PBS} is absorption for PBS control, A_{mAb30} is absorption for trastuzumab or ADC control at 30 nM and C is the given concentration for the respective data point.

$$A_{490corr} = A_{490} - \left(\frac{A_{PBS} + A_{mAb30} \times C/_{30}}{2}\right)$$

Alexa Fluor 488® conjugate serum stability

Alexa Fluor 488® trastuzumab fluorophore conjugates AFC **5** and AFC **12** were 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 conjugates were diluted with 50% of human blood serum to give a final a concentration of 0.1 mg/mL of **5** or **12** and 1 mM of sodium azide. One aliquot (50 µL) for each conjugate was taken, flash frozen and stored at −80 °C. The remaining solution was incubated at 37 °C under mild shaking (300 rpm) and covered from light. Aliquots (50 µL) were taken at 1, 2, 4 and 7 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 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, with ε_{280} = 215380 M^{-1} cm^{-1} for trastuzumab, ε_{495} = 71000 M^{-1} cm^{-1} for Alexa Fluor 488®, 0.11 x Abs_{495} as a correction factor for the dye absorption at 280 nm and 0.28 x Abs_{340} as a correction factor for PD absorption at 280 nm.

$$FAR = \frac{{}^{(A_{495})}/_{\varepsilon_{495}}}{{}^{(A_{280}-0.11\times A_{495}-0.28\times A_{340})}/_{\varepsilon_{280}}}$$

$$\left[PD-fluorophore\ conjugate\right] = \frac{A_{280}-(0.11\times A_{AF495}+0.28\times A_{340})}{l\times\varepsilon_{280}}$$

Cell Lines

Human breast cancer cell lines HCC-1954 (Her-2-positive) and MCF-7 (Her-2-negative) were purchased from ATCC, and maintained in RPMI-1640, and DMEM medium, respectively, all

supplemented with 10% foetal bovine serum (Life Technologies, UK) at 37 °C, under humidified atmosphere containing 5% CO_2.

In Vitro Cytotoxicity Assessment

In vitro cytotoxicity of the compounds was evaluated in both human breast cancer cell lines (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), trastuzumab (72h) and ADCs **1**, **3** and **4** (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.

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

In Vivo Efficacy Assessment

Female NSG mice (age, 6–8 wk; weight, 20–25 g) were sourced from our breeding colony at the Biological Services Unit (University College London). Animal experiments were performed in accordance with the U.K. Home Office Animals Scientific Procedures Act 1986 and United Kingdom Co-ordinating Committee on Cancer Research Guidelines for the Welfare and Use of Animals in Cancer Research[2] and approved by the University College London Animal Welfare and Ethical Review Body under project license 70-309.

Subcutaneous xenografts were established with the HCC-1954 cell line. Briefly, 2×10^6 cells were injected into the right flank of mice and tumor volumes were caliper-measured and calculated using the formula volume = 4π/3 (1/2 length × 1/2 width × 1/2 height). Therapy was initiated when tumour volumes reached ~ 0.1 cm^3, approximately 14 days post tumour cell inoculation. Animals were then allocated to the following groups (n=8 per group): (1) Untreated; (2) Herceptin; (3) NGM-ADC-**1**; (4) PD-ADC **3**; (5) PD-ADC **4**. Each treatment was administered at a dose of 20 mg/kg 7qX3.

Tumour volumes were measured, and mouse weights monitored throughout the duration of the study. Mice were sacrificed if tumour volume reached 1.5 cm^3 or if a weight loss of <10%

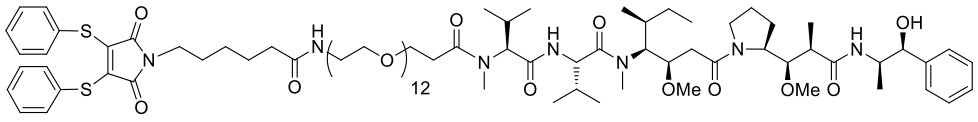
was incurred. In addition to weight loss, disease progression was also evaluated qualitatively by observation of behaviour and muscle wasting, as previously described.[3,4]

Tumour growth curves based on changes in tumour volume over time and survival were used to evaluate treatment efficacy.

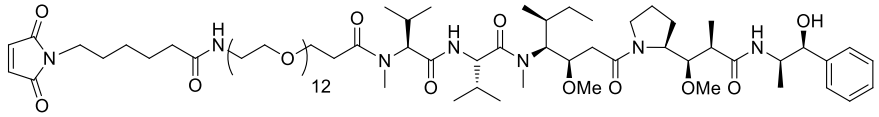
Statistical Analyses

Data were plotted and analyzed using Prism software (version 6.0; GraphPad Software). Differences in tumour growth between the groups were assessed by 2-way ANOVA and multiple *t* tests. Kaplan–Meier survival plots were analyzed using the log-rank test. Results were considered statistically significant at a *P* value of 0.05 or less.

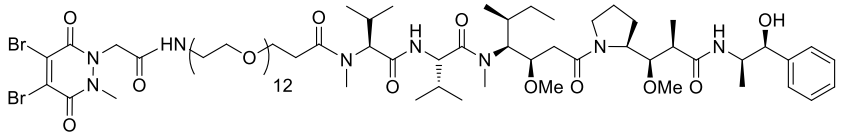
Conjugation protocols



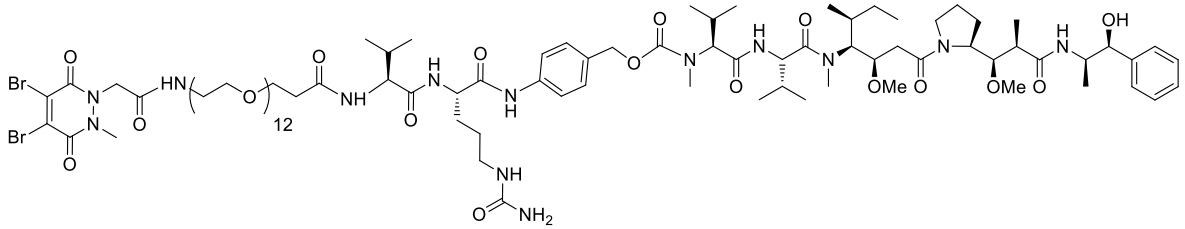
NGM-MMAE 6



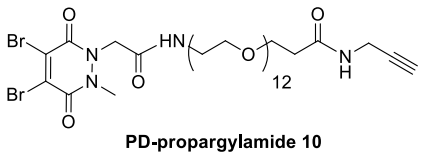
Mal-MMAE 7



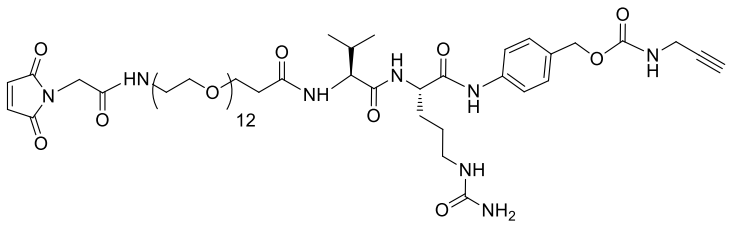
PD-MMAE 8



PD-MMAE 9



PD-propargylamide 10



Mal-propragylamide 11

Figure S1 – Reagents used for conjugation.

Conjugation of trastuzumab with NGM-MMAE 6 (NGM-MMAE ADC 1)

This conjugation was adapted from a previously described protocol.[5] To trastuzumab (40 µM, 5 mL, 0.2 µmol) in BBS pH 8 was added TCEP (10 mM, 120 µL, 6 eq.) and the reaction was incubated at 37 °C for 2 h under mild agitation (400 rpm). Then, the reduced trastuzumab solution was cooled to 4 °C. Next, NGM-MMAE **6** was prepared in dry DMF (10 mM, 120 µL, 6 eq.), diluted with DMF (477 µL) and borate buffer pH 8 (1 mL) and immediately added to the reduced trastuzumab solution, thus giving a solution that was 10% (v/v) in DMF. The reaction was incubated at 4 °C for 30 min. Afterwards, excess reagents were removed by ultrafiltration (10 kDa MWCO) into BBS pH 8.4 and incubated at 20 °C over 72 h to effect hydrolysis of the maleimide unit to maleamic acid. Next, buffer swapped by ultrafiltration (10 kDa MWCO) into PBS to afford the modified trastuzumab NGM-MMAE ADC **1** in PBS. Yield 92%, average DAR by HIC 3.8.

Conjugation of trastuzumab with Mal-MMAE 7 (Mal-MMAE ADC 2)

This conjugation was adapted from a previously described protocol.[5] To trastuzumab (40 µM, 3 mL, 0.12 µmol) in BBS pH 8 was added TCEP (10 mM, 24 µL, 2 eq.) and the reaction was incubated at 37 °C for 3 h under mild agitation (400 rpm). Next, Mal-MMAE **7** was prepared in dry DMF (10 mM, 96 µL, 8 eq.), diluted with DMF (240 µL) and immediately added to the reduced trastuzumab solution, thus giving a solution that was 10% (v/v) in DMF. The reaction was incubated at 37 °C for 45 min under mild agitation. Afterwards, excess reagents were removed by ultrafiltration (10 kDa MWCO) into PBS to afford the modified trastuzumab Mal-MMAE ADC **2** in PBS. Yield 91%, average DAR by HIC 2.9.

Conjugation of trastuzumab with PD-MMAE 8 (PD-MMAE ADC 3)

To trastuzumab (40 µM, 3 mL, 0.12 µmol) in BBS pH 8 was added TCEP (10 mM, 72 µL, 6 eq.) and the reaction was incubated at 37 °C for 2 h under mild agitation (400 rpm). Then, the reduced trastuzumab solution was cooled to 4 °C. Next, PD-MMAE **8** was prepared in dry DMF (10 mM, 96 µL, 8 eq.), diluted with DMF (360 µL) and borate buffer pH 8 (720 µL) and cooled to 4 °C before addition to the reduced trastuzumab solution, thus giving a solution that was 10% (v/v) in DMF. The reaction was incubated at 4 °C for 16 h. Afterwards, excess reagents were removed by ultrafiltration (10 kDa MWCO) into PBS to afford the modified trastuzumab PD-MMAE ADC **3** in PBS. Yield 86%, average DAR by HIC 4.0.

Conjugation of trastuzumab with PD-MMAE 9 (PD-MMAE ADC 4)

To trastuzumab (40 µM, 3 mL, 0.12 µmol) in BBS pH 8 was added TCEP (10 mM, 72 µL, 6 eq.) and the reaction was incubated at 37 °C for 2 h under mild agitation (400 rpm). Then, the reduced trastuzumab solution was cooled to 4 °C. Next, PD-MMAE **9** was prepared in dry DMF (10 mM, 240 µL, 20 eq.), diluted with DMF (60 µL) and cooled to 4 °C before addition to the reduced trastuzumab solution, thus giving a solution that was 10% (v/v) in DMF. The reaction was incubated at 4 °C for 16 h. Afterwards, excess reagents were removed by ultrafiltration (10 kDa MWCO) into PBS to afford the modified trastuzumab PD-MMAE ADC **4** in PBS. Yield 97%, average DAR by HIC 3.9.

Conjugation of trastuzumab with PD-propargylamide 10, followed by copper-catalysed Huisgen 1,3-dipolar cycloaddition (CuAAC) with Alexa Fluor 488® (conjugate AFC 5)

To trastuzumab (40 µM, 100 µL, 0.004 µmol) in BBS pH 8 was added TCEP (10 mM, 2.4 µL, 6 eq.) and the reaction was incubated at 37 °C for 2 h under mild agitation (400 rpm). Then, the reduced trastuzumab solution was cooled to 4 °C. Next, PD-propargylamide **10** was prepared in dry DMF (10 mM, 8 µL, 20 eq.), diluted with DMF (4 µL) and cooled to 4 °C before addition to the reduced trastuzumab solution, thus giving a solution that was 10% (v/v) in DMF. The reaction was incubated at 4 °C for 16 h. Afterwards, excess reagents were removed by ultrafiltration (10 kDa MWCO) into 50 mM phosphate buffer at pH 7.0 to afford the modified trastuzumab PD-propargylamide conjugate. Next, to this conjugate (40 µM, 70 µL, 0.0028 µmol) was added tris(3-hydroxypropyltriazolylmethyl)amine (THPTA, 50 mM, 1.68 µL 30 eq.) and $CuSO_4$ (20 mM, 0.84 µL, 6 eq.). Then, added Alexa Fluor 488® in DMF (5 mM, 5.6 µL, 10 eq.), followed by sodium ascorbate (final concentration 5 mM) and the reaction mixture was incubated at 37 °C for 5 h. Excess reagents were removed by desalting, using a PD MiniTrap G-25 filter (GE Healthcare Life Sciences) eluting with PBS pH 7.4 (10 mM EDTA) followed by ultrafiltration (10 kDa MWCO) into fresh PBS to afford the modified trastuzumab conjugate AFC **5** with 94% yield and a fluorophore to trastuzumab ratio of 3.1.

Conjugation of trastuzumab with Mal-propargylamide 11, followed by copper-catalysed Huisgen 1,3-dipolar cycloaddition (CuAAC) with Alexa Fluor 488® (conjugate AFC 12)

To trastuzumab (40 µM, 300 µL, 0.012 µmol) in BBS pH 8 was added TCEP (10 mM, 2.4 µL, 2 eq.) and the reaction was incubated at 37 °C for 3 h under mild agitation (400 rpm). Then,

Mal-propargylamide **11** was prepared in dry DMF (10 mM, 9.6 µL, 8 eq.) and added to the reduced trastuzumab solution. The reaction was incubated at 20 °C for 45 min. Afterwards, excess reagents were removed by ultrafiltration (5 kDa MWCO) into 50 mM phosphate buffer at pH 7.0 to afford the modified trastuzumab Mal-propargylamide conjugate. Next, added tris(3-hydroxypropyltriazolylmethyl)amine (THPTA, 25 mM, 9.6 µL 40 eq.) and $CuSO_4$ (10 mM, 6.8 µL, 8 eq.). Then, added Alexa Fluor 488® in DMF (5 mM, 24 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 trastuzumab conjugate AFC **12** with 71% yield and a fluorophore to trastuzumab ratio of 2.6.

Conjugation of albumin with Mal-propargylamide 11, 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, Mal-propargylamide **11** 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 73% yield and a fluorophore to albumin ratio of 0.9.

Supplementary figures and tables

Figure S2 – LC-MS data of unmodified trastuzumab

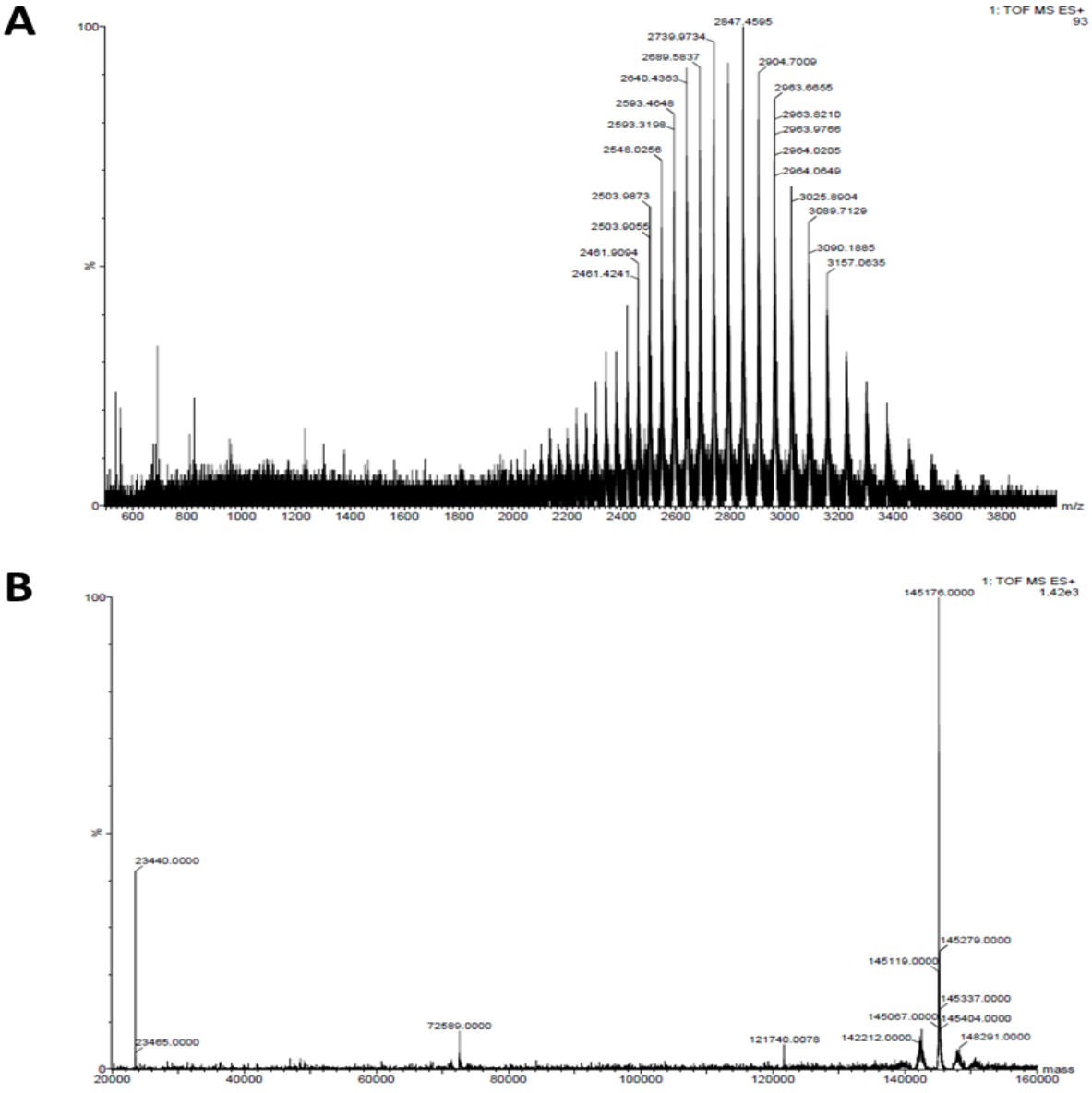


Figure S2 – ESI-MS data for unmodified trastuzumab. A) non-deconvoluted ion series and B) deconvoluted ion series mass spectra. Trastuzumab observed mass of 145176 Da.

Figure S3 – LC-MS data of PD-MMAE ADC 3

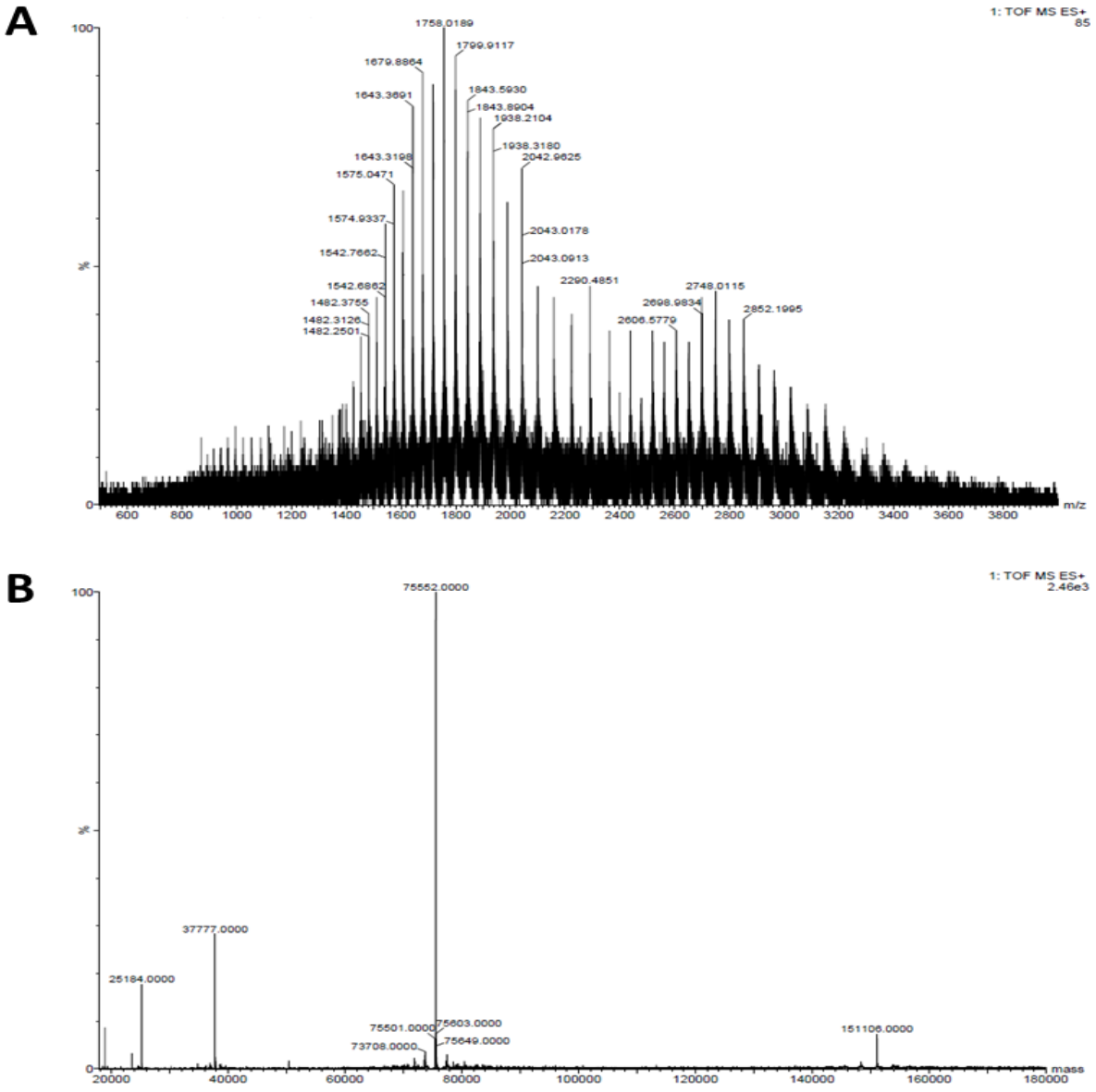


Figure S3 – ESI-MS data for PD-MMAE ADC **3**. A) non-deconvoluted ion series and B) deconvoluted ion series mass spectra. observed mass of 151106 Da (calculated 151099 Da).

Figure S4 – LC-MS data of PD-MMAE ADC 4

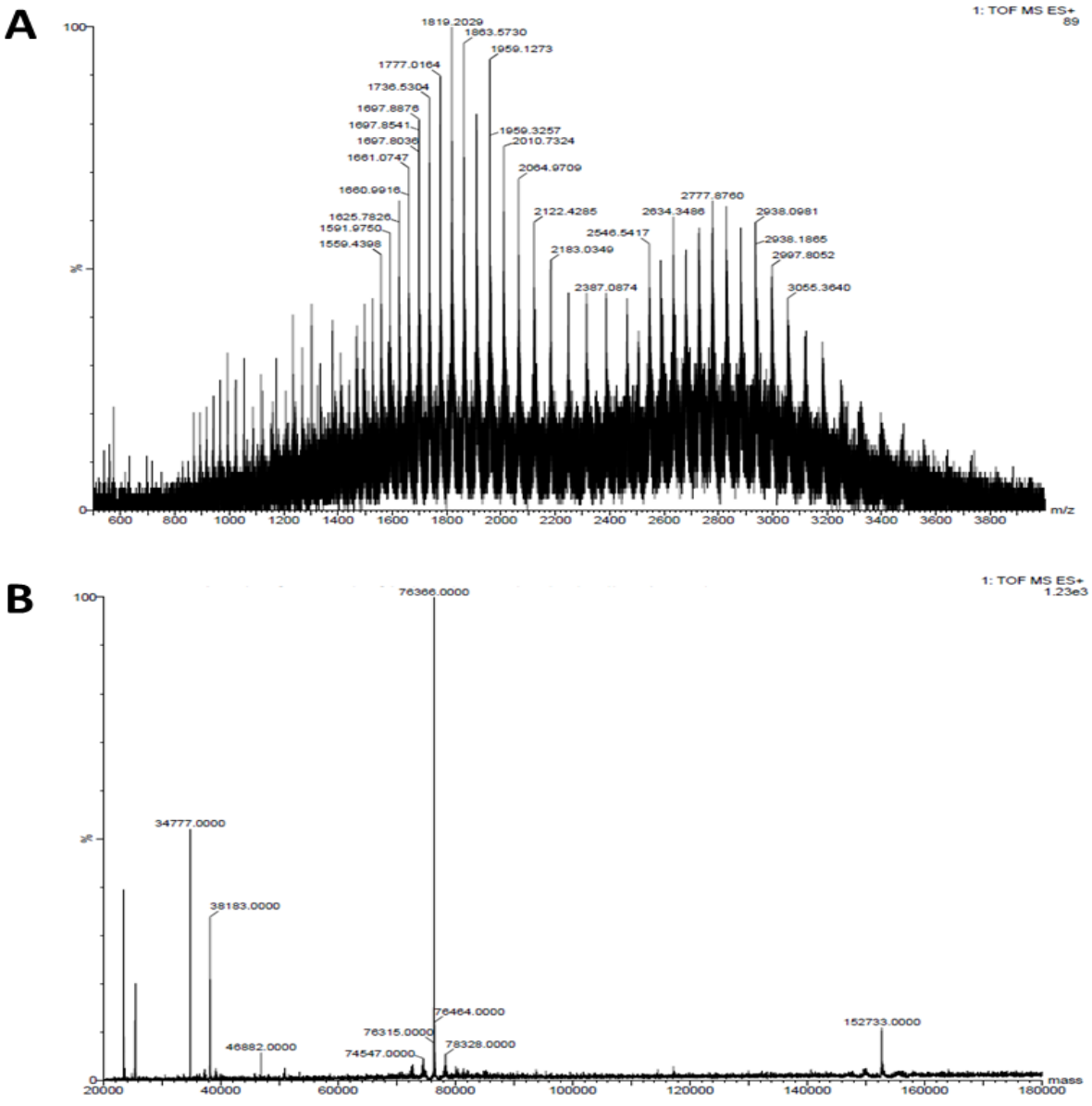
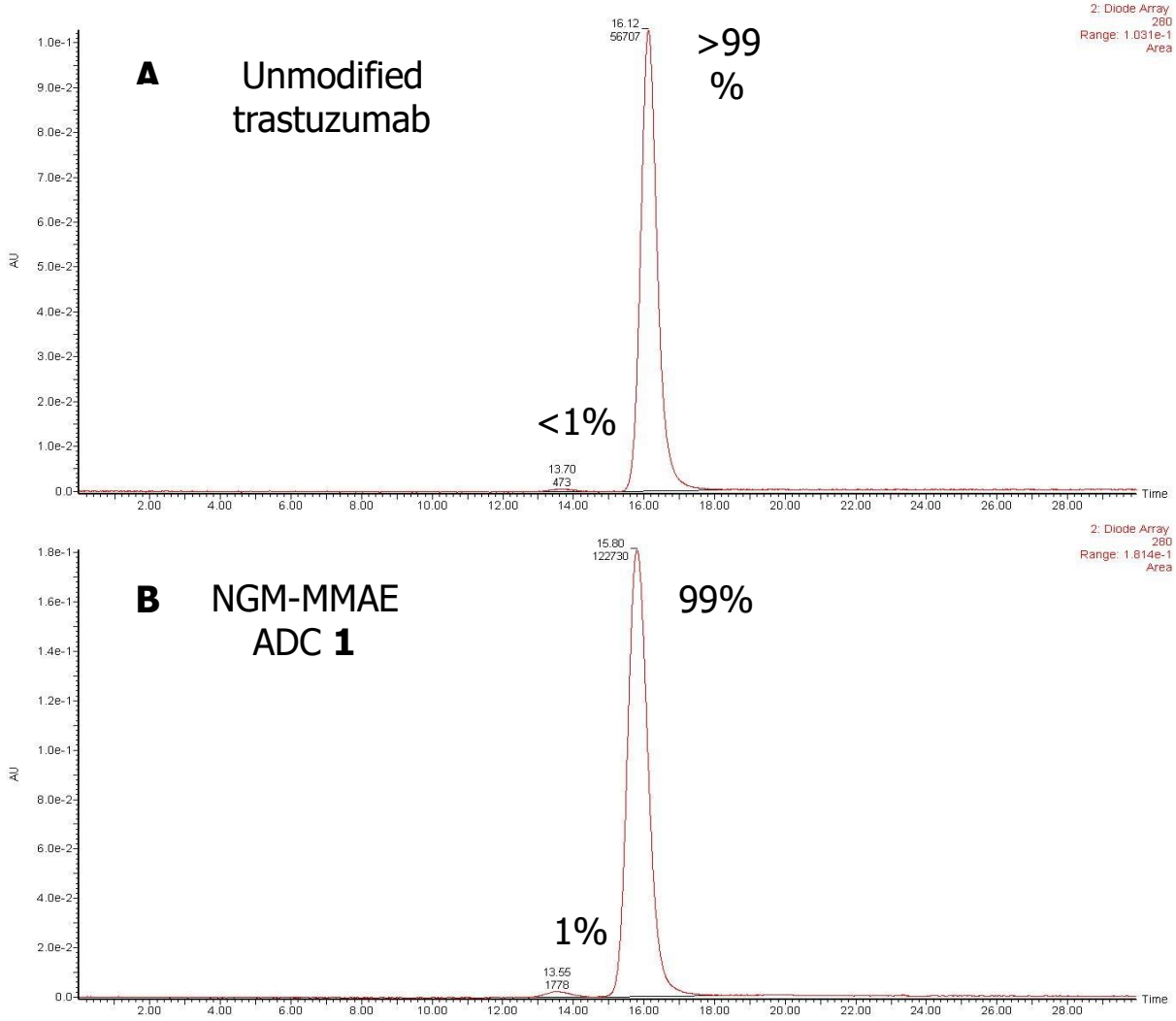
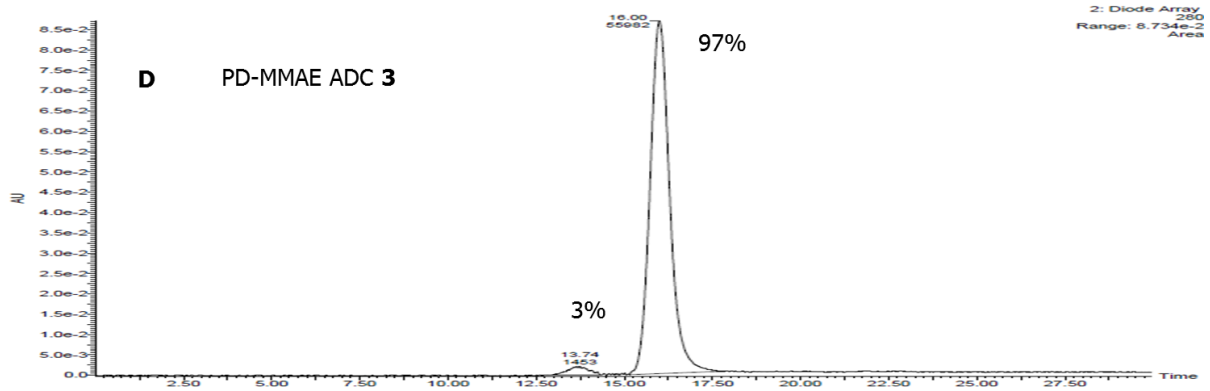
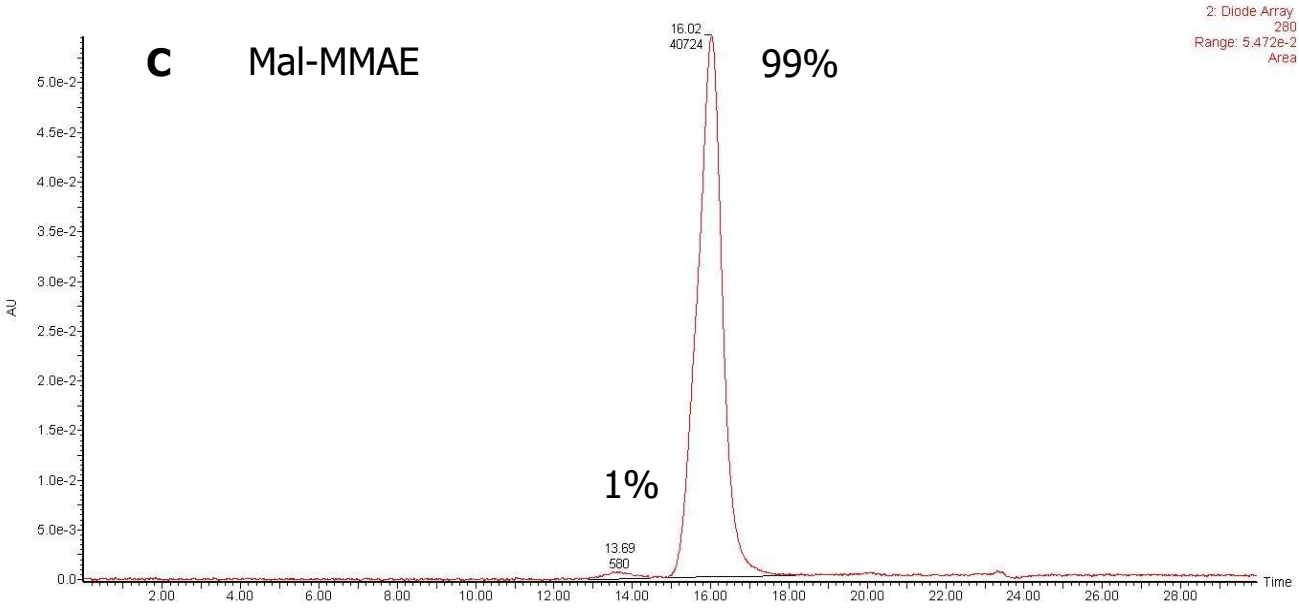


Figure S4 – ESI-MS data for PD-MMAE ADC **4**. A) non-deconvoluted ion series and B) deconvoluted ion series mass spectra. PD-MMAE ADC **4** observed mass of 152733 Da (calculated 152717 Da).

Figure S5 – SEC-HPLC chromatograms of trastuzumab, NGM-MMAE ADC XX and Mal-MMAE ADC XX.




D
PD-MMAE ADC 3
97%
3%
16.00
555682
13.74
1453
2: Diode Array
280
Range: 8.734e-2
Area
AU
Time


C
Mal-MMAE
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1%
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13.69
580
2: Diode Array
280
Range: 5.472e-2
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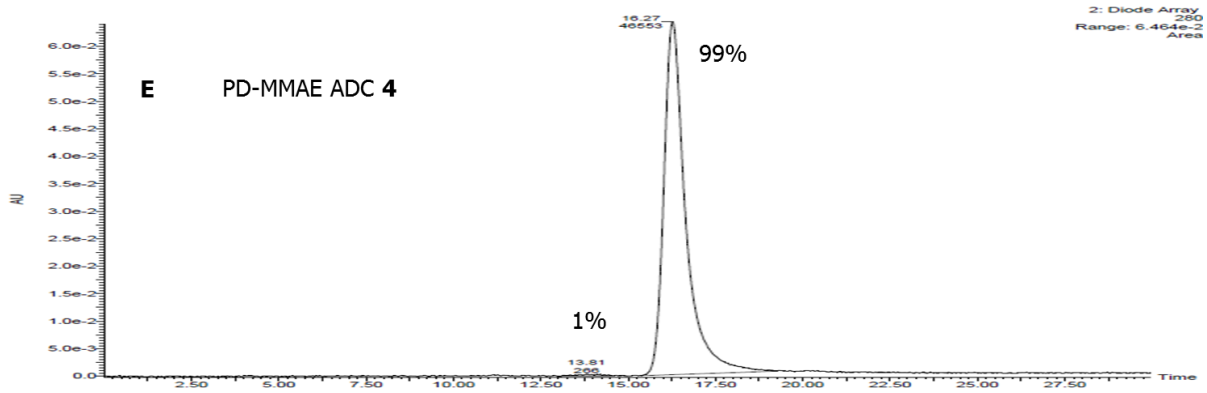


Figure S5 – SEC-HPLC chromatograms with peak percentages of monomeric (rt 15.8-16.2 min) and aggregate (rt 13.6-13.7 min) species for A) unmodified trastuzumab, B) NGM-MMAE ADC **1**, C) Mal-MMAE ADC **2**, C) PD-MMAE ADC **3**, and E) PD-MMAE ADC **4**.

Figure S6 – HER2 ELISA of PD-MMAE ADC 3 & **4 compared with trastuzumab**

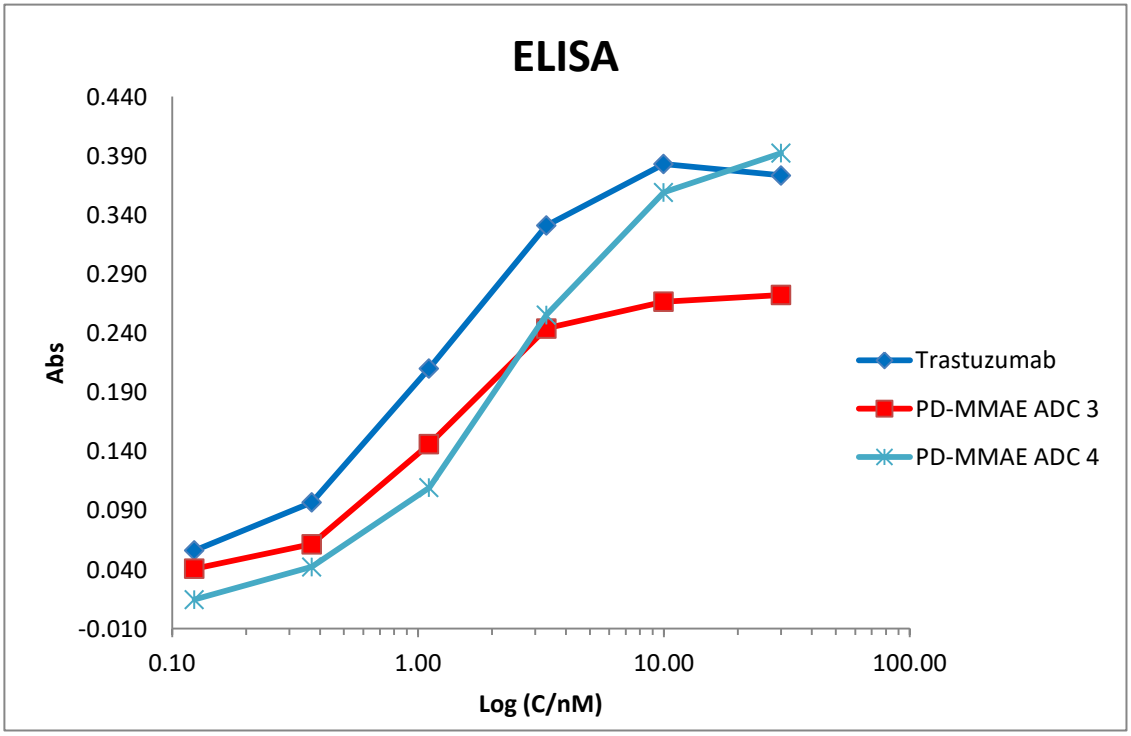


Figure S6 – Binding activity to HER2 of PD-MMAE ADC **3** & **4** compared with trastuzumab by ELISA assay.

Figure S7 – SEC-HPLC chromatograms of serum stability study with AFC 5 and AFC 12

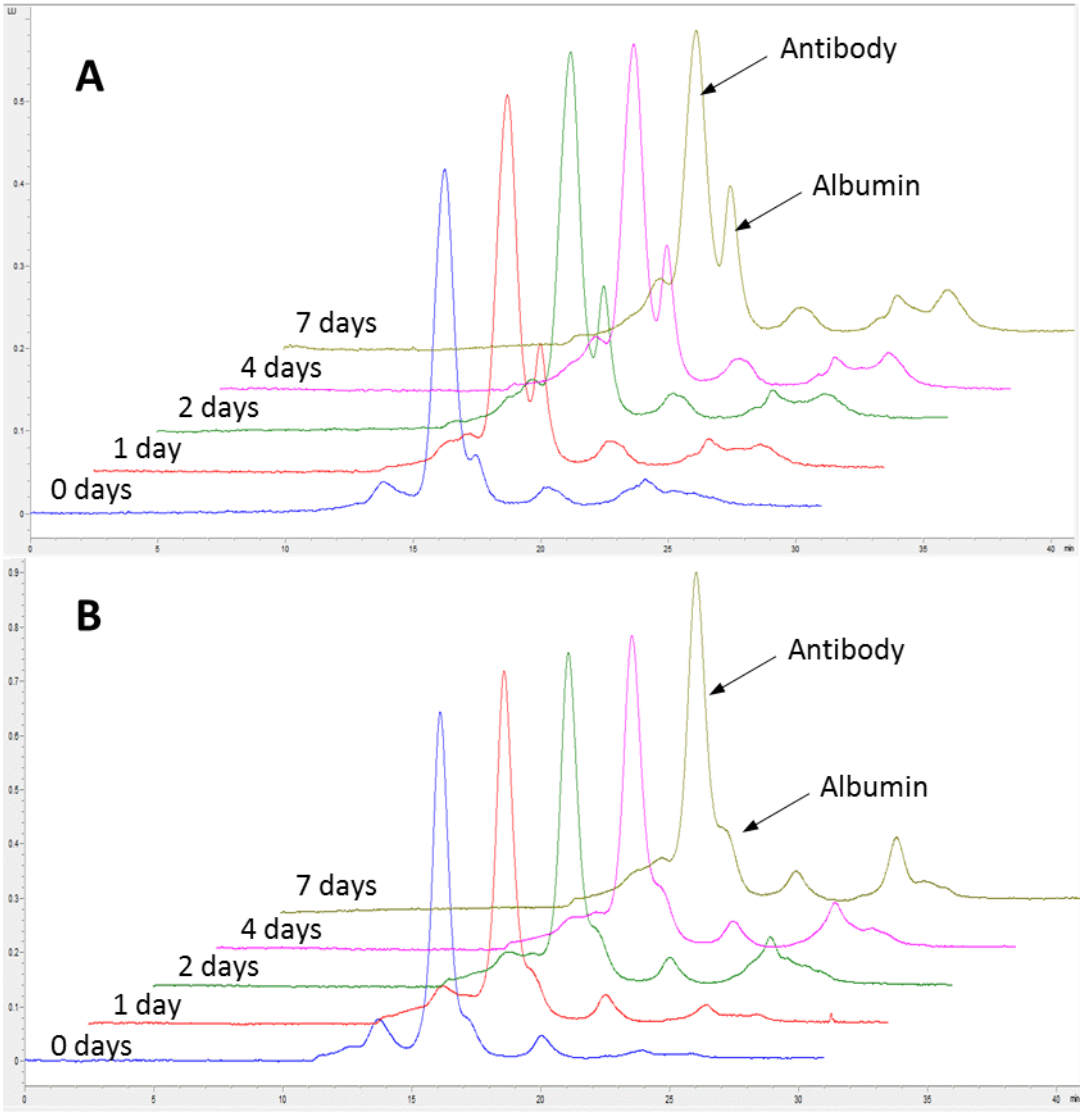


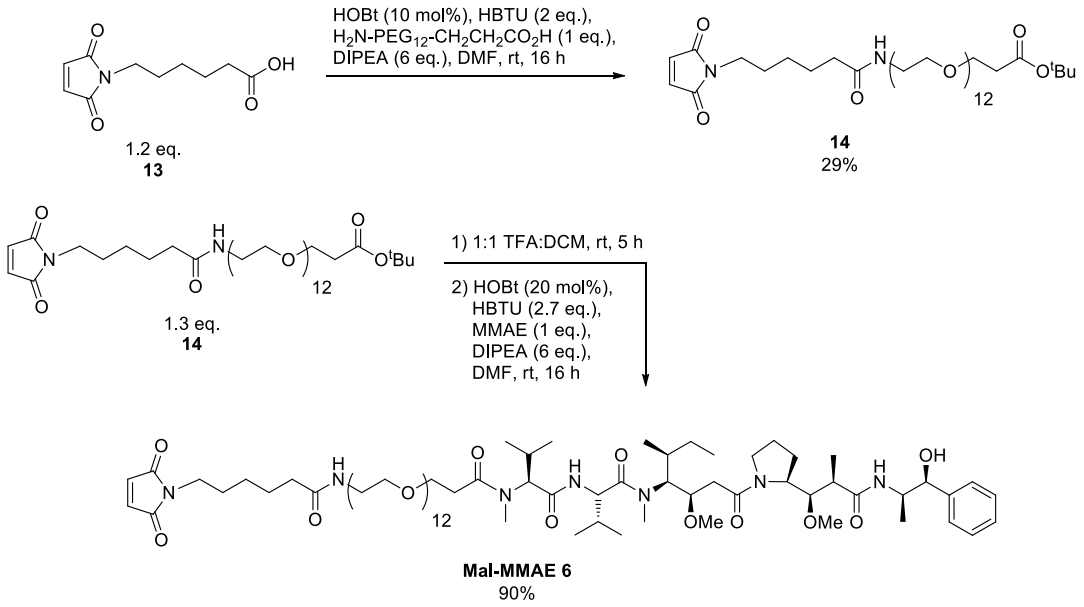
Figure S7 – SEC-HPLC with fluorescence detection (excitation 495 nm, emission 525 nm) chromatograms of antibody-fluorophore conjugates A) AFC **12**, and B) AFC **5,** incubated in blood serum for 7 days and analysed on days 0, 1, 2, 4 and 7. Transfer of fluorophore can be seen over 7 days for the classical maleimide conjugate (AFC **12**) whereas a significant reduction in transfer is seen for the PD conjugate (AFC **5**).

Synthesis General Remarks

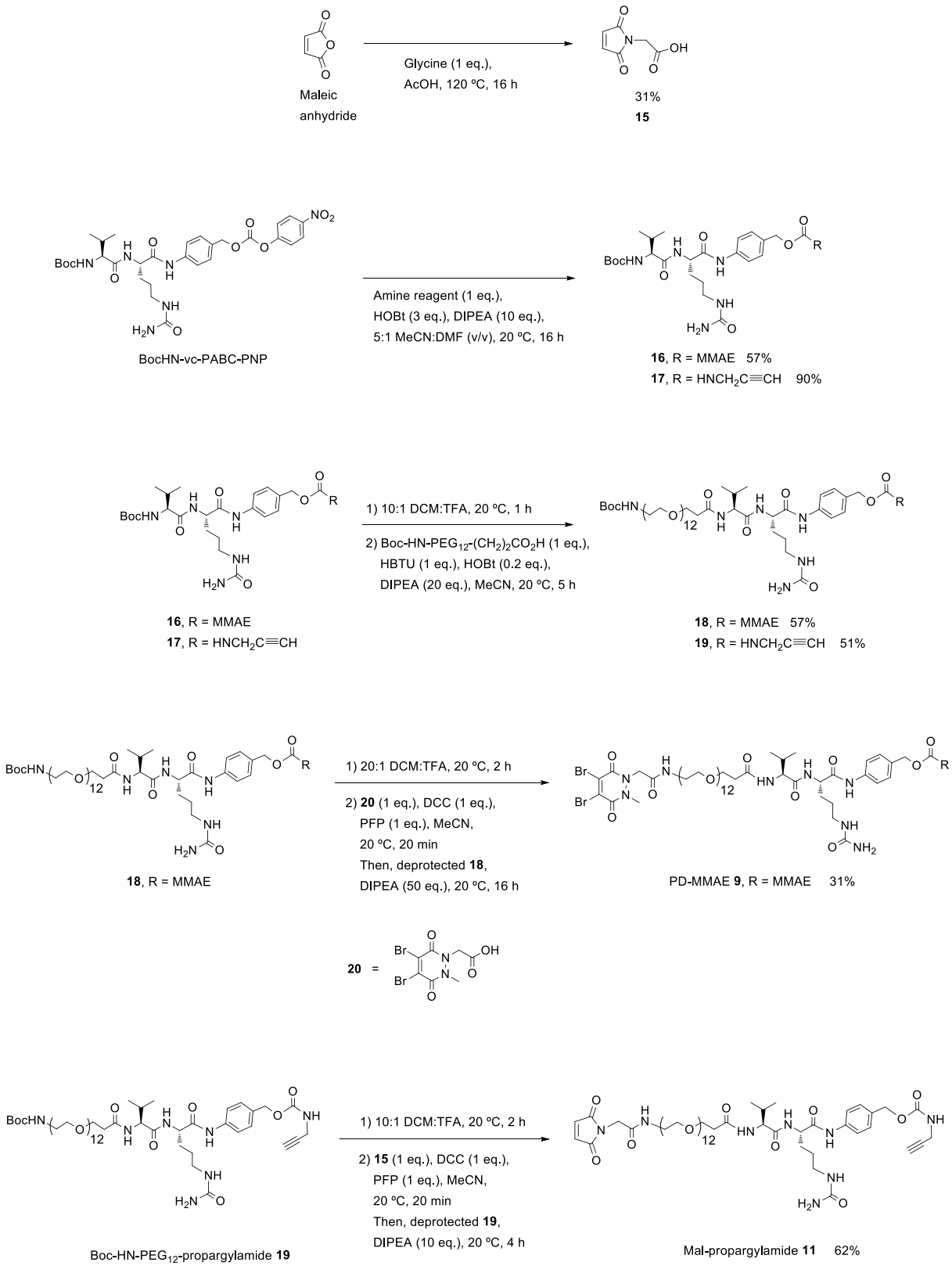
All reactions were carried out at atmospheric pressure with stirring at 20 °C unless otherwise stated. Reagents and solvents were purchased from Sigma Aldrich and Alfa Aesar and used as supplied. H_2N-PEG_{12}-$CH_2CH_2CO_2{}^tBu$ and BocHN-vc-PABC-PNP were purchased from Iris Biotech GmbH. Reactions 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 (PTLC) 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

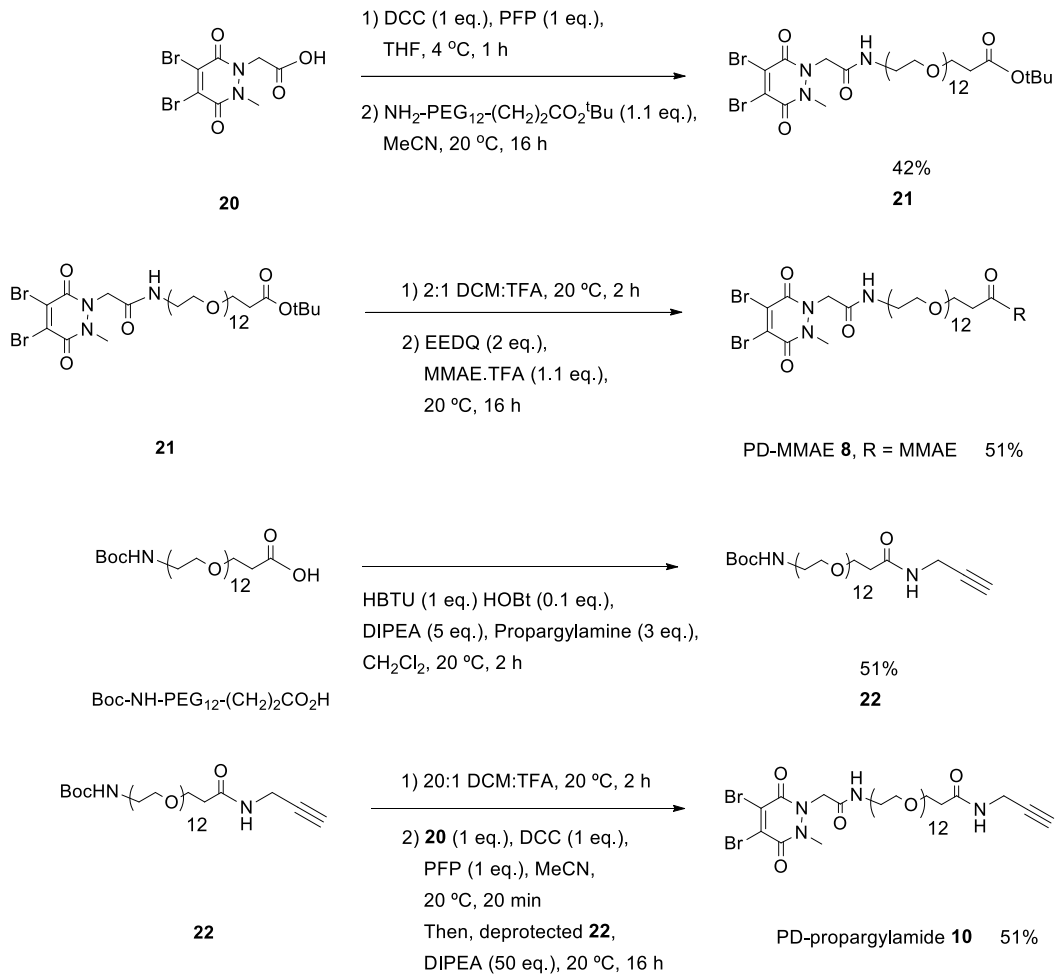
Synthesis of NGM-MMAE **6** has been previously described.[2]



Scheme S1 – Synthesis of Mal-MMAE **6**.



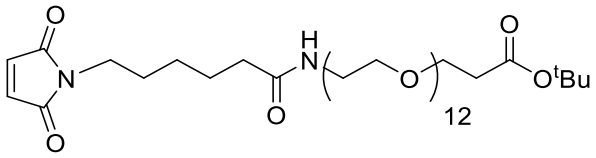
Scheme S2 – Synthesis of PD-MMAE **9** and Mal-propargylamide **11**.



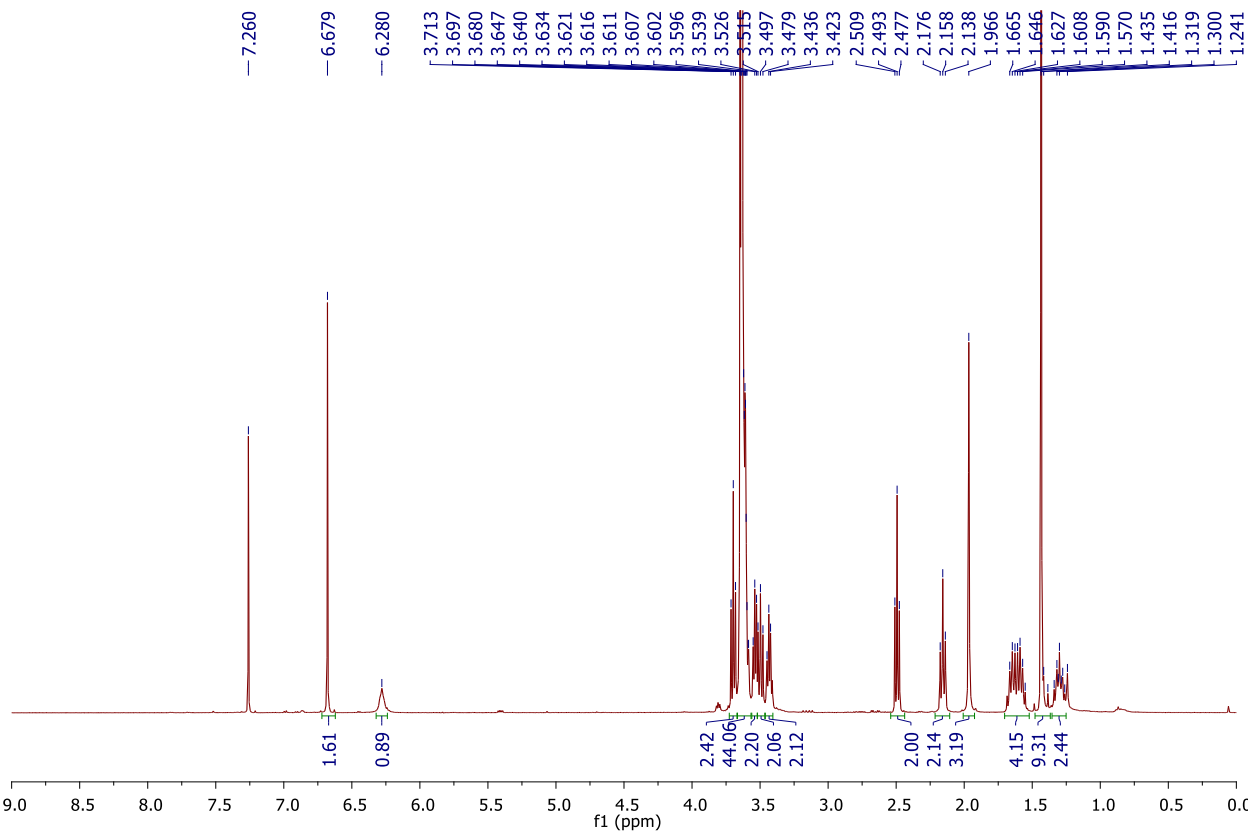
Scheme S3 – Synthesis of PD-MMAE **8** and PD-propargylamide **10**.

***tert*-butyl-46-(2,5-dioxo-2,5-dihydro-1*H*-pyrrol-1-yl)-41-oxo-4,7,10,13,16,19,22,25,28,31,34,37-dodecaoxa-40-azahexatetracontanoate 14**

Maleimide-C6-PEG$_{12}$-*tert*-butyl ester 14



In a 10 mL round-bottom flask, oven dried, maleimide-*N*-hexanoic acid (11 mg, 53 µmol, 1.2 eq.), prepared as reported,[6] HOBt hydrate (1.2 mg, 9 µmol, 0.2 eq) and HBTU (34 mg, 90 µmol, 2 eq.) were dissolved in DMF (0.5 mL). Then, added DIPEA (23 µL, 183 µmol, 3 eq.). The solution was stirred at 20 °C for 10 min. Next, added H_2N-PEG$_{12}$-$CH_2CH_2CO_2{}^tBu$ (30 mg, 44 µmol, 1 eq.) in DMF (0.5 mL), followed by addition of DIPEA (23 µL, 183 µmol, 3 eq.). The solution was stirred at 20 °C for 16 h. Then, concentrated under vacuum, added DCM (10 mL) and washed with 15% aq. citric acid (10 mL), aq. sat. $NaHCO_3$ (10 mL) and 40% aq. LiCl (2×10 mL). The organic layer was dried ($MgSO_4$), filtered and concentrated under vacuum to yield a pale yellow oil which was purified by flash chromatography on silica with DCM:EtOAc:MeOH (15:5:1 to 15:5:2 v/v) to afford the title compound as a colourless oil (11 mg, 13 µmol, 29%). Characterisation data: FTIR ν_{max} (cm^{-1}) 2866, 1704, 1668, 1365, 1248, 1095, 947, 832, 695. ^{1}H NMR (400 MHz, $CDCl_3$) 1.23-1.35 (quint., *J* = 7.6 Hz, 2H, CH_2), 1.44 (s, 9H, CH_3), 1.54-1.62 (overlapped quint., *J* = 7.6 Hz, 4H, CH_2), 2.14-2.17 (t, *J* = 7.6 Hz, 2H, CH_2), 2.48-2.51 (t, *J* = 6.4 Hz, 2H, CH_2), 3.40-3.46 (t, *J* = 5.2 Hz, 2H, CH_2), 3.47-3.52 (t, *J* = 7.6 Hz, 2H, CH_2), 3.52-3.56 (t, *J* = 5.2 Hz, 2H, OCH_2), 3.57-3.67 (overlapped multiplets, 44H, OCH_2), 3.68-3.72 (t, *J* = 6.4 Hz, 2H, OCH_2), 6.25-6.32 (br, 1H, N*H*), 6.70 (s, 2H, C*H*); ^{13}C NMR (100 MHz, $CDCl_3$) 25.2 (*C*H_2), 26.5 (*C*H_2), 28.2 (*C*H_3), 28.4 (*C*H_2), 36.4 (*C*H_2), 36.5 (*C*H_2), 37.8 (*C*H_2), 39.2 (*C*H_2), 67.0 (*C*H_2, PEG), 70.1 (*C*H_2, PEG), 70.3 (*C*H_2, PEG), 70.5 (*C*H_2, PEG), 70.6-70.8 (overlapped *C*H_2, PEG), 80.6 (*C*), 134.1 (*C*), 170.9 (*C*O), 171.0 (*C*O), 172.8 (*C*O). LRMS (ESI+) 889 (100, $[M+Na]^+$), 867 (50, $[M+H]^+$); HRMS (ESI+) calcd. for $C_{41}H_{75}N_2O_{17}$ $[M+H]^+$ 867.5066, observed: 867.5069.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3.640
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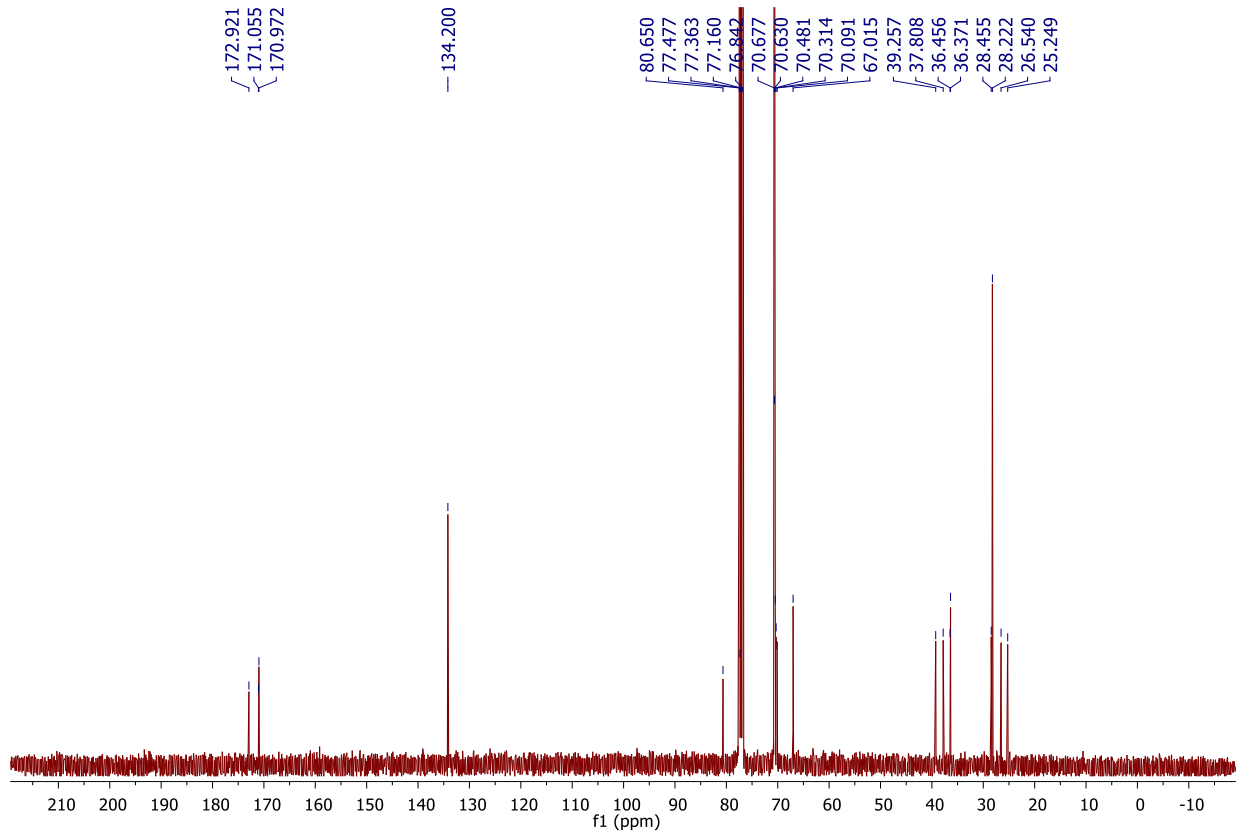
3.607
3.602
3.596
3.539
3.526
3.515
3.497
3.479
3.436
3.423
2.509
2.493
2.477
2.176
2.158
2.138
1.966
1.665
1.646
1.627
1.608
1.590
1.570
1.435
1.416
1.319
1.300
1.241
1.61
0.89
2.42
44.06
2.20
2.06
2.12
2.00
2.14
3.19
4.15
9.31
2.44
f1 (ppm)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171.055
170.972
134.200
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77.160
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70.091
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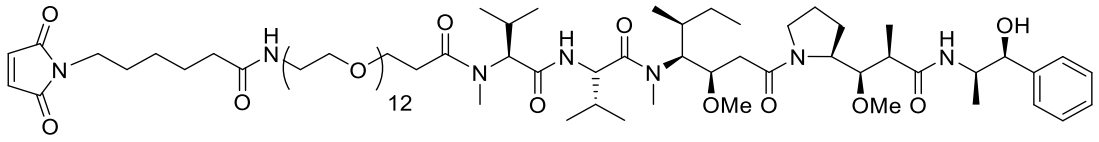
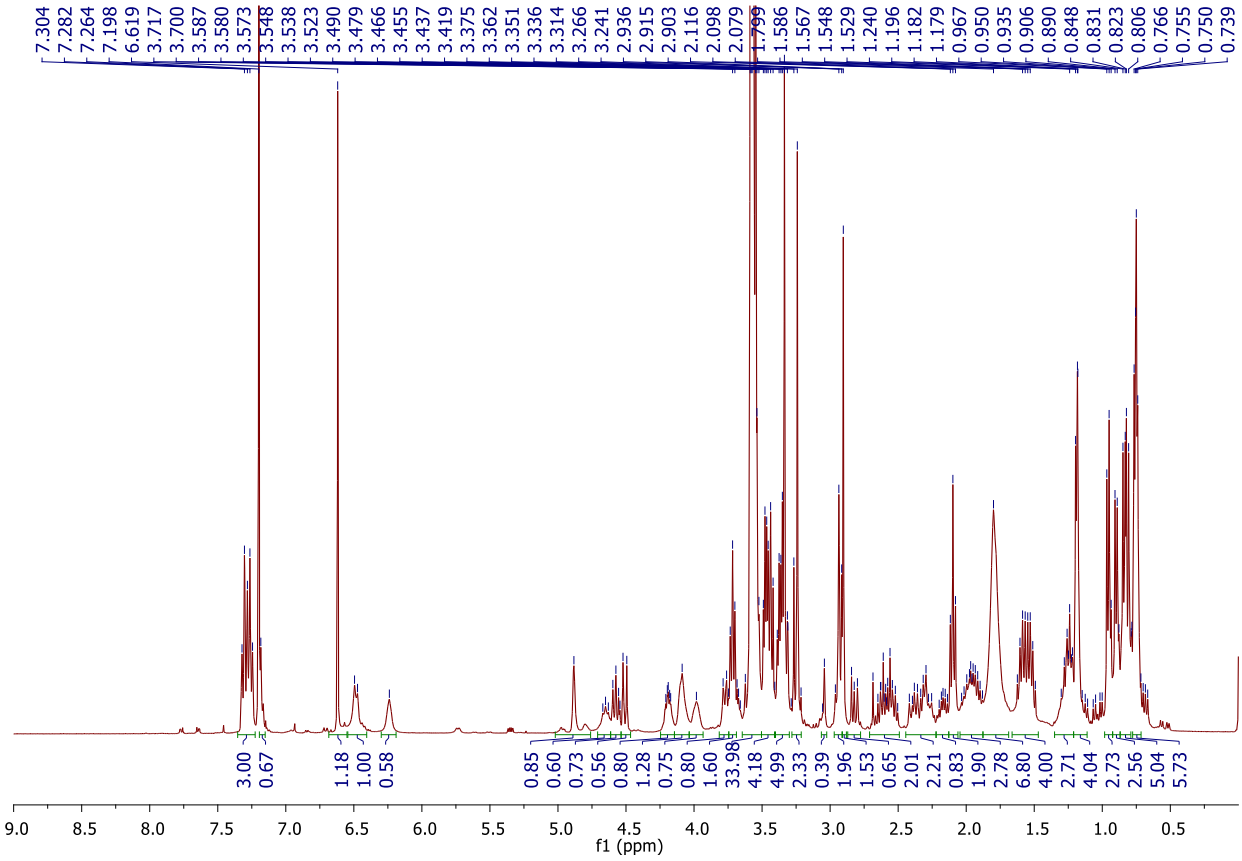
36.456
36.371
28.455
28.222
26.540
25.249
f1 (ppm)

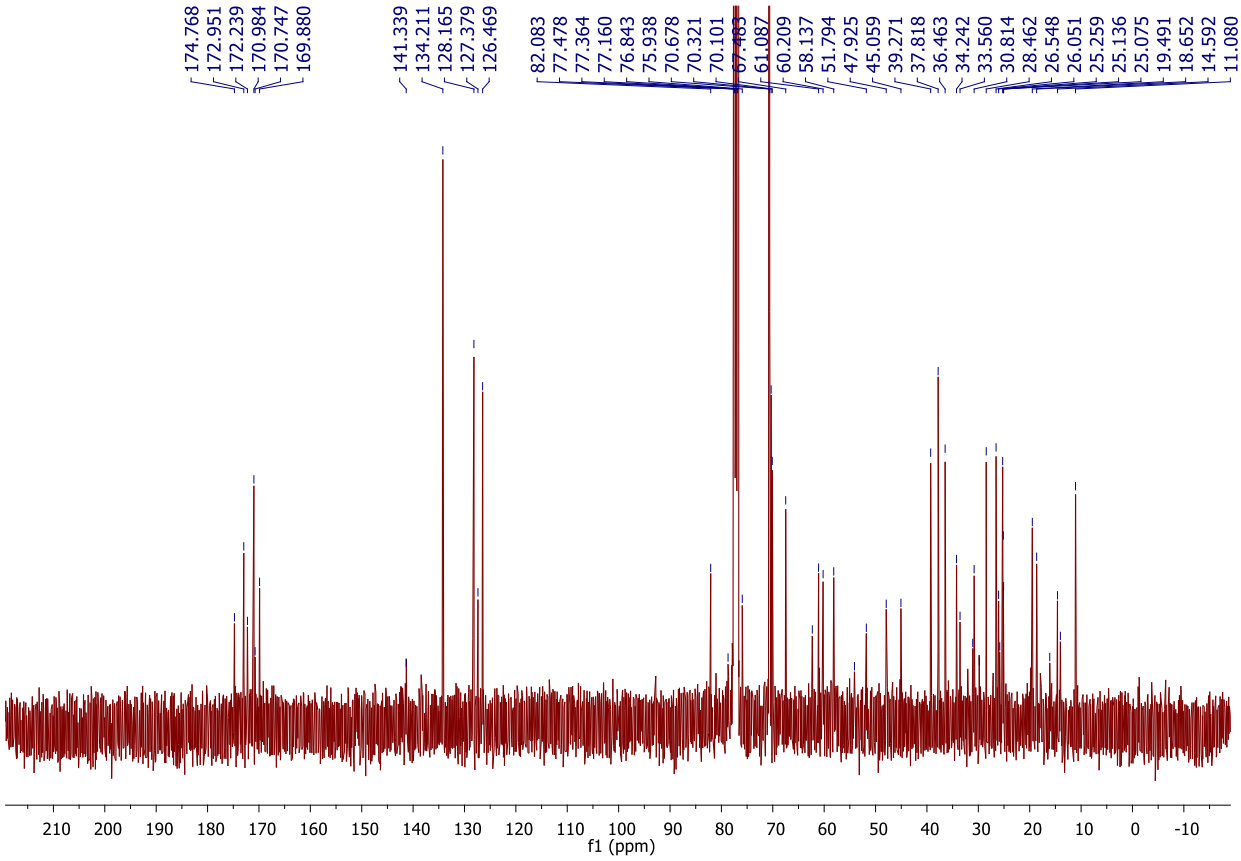
Mal-MMAE 7



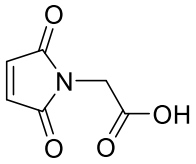
In a 10 mL round-bottom flask, maleimide-C6-PEG_{12}-*tert*-butyl ester **14** (10 mg, 12 µmol, 1.3 eq.) was dissolved in DCM (0.5 mL). Then, added trifluoroacetic acid (0.5 mL, 6.4 mmol). The solution was stirred at 20 °C for 5 h. Then, concentrated under vacuum, redissolved in DCM (10 mL) and washed with 10 mM aq. HCl (10 mL). The organic layer was dried ($MgSO_4$), filtered and concentrated under vacuum to afford a colourless oil. Dissolved the Maleimide-C6-PEG_{12}-CO_2H thus formed in DMF (0.5 mL) and added HOBt hydrate (0.2 mg, 1.5 µmol, 0.2 eq.) and HBTU (9.1 mg, 24 µmol, 2.7 eq.). Then, added DIPEA (4.7 µL, 27 µmol, 3 eq.). The solution was stirred at 20 °C for 10 min. Next, added MMAE (6.4 mg, 8.9 µmol, 1 eq.) in DMF (0.5 mL), followed by DIPEA (4.7 µL, 27 µmol, 3 eq.). The solution was stirred at 20 °C for 16 h. Then, concentrated under vacuum, added DCM (10 mL) and washed with 15% aq. citric acid (10 mL), aq. sat. $NaHCO_3$ (10 mL) and 40% aq. LiCl (2×10 mL). The organic layer was dried ($MgSO_4$), filtered and concentrated under vacuum to yield a pale yellow oil which was purified by flash chromatography on silica with a gradient from DCM:EtOAc:MeOH (5:5:1 v/v) to DCM:MeOH (10:1 v/v) to afford the title compound as a colourless oil (12.1 mg, 8 µmol, 90%). Characterisation data: ^{1}H NMR (400 MHz, $CDCl_3$) 0.72-0.78 (dd, *J* = 6.4, 6H, MMAE), 0.78-0.87 (m, 6H, MMAE), 0.87-0.92 (m, 3H, MMAE), 0.93-0.99 (m, 3H, MMAE), 1.15-1.34 (m, MMAE overlapped with CH_2), 1.47-1.63 (overlapped quint., *J* = 7.6 Hz, 4H, CH_2), 1.71-1.88 (m, MMAE), 1.88-2.05 (m, MMAE), 2.06-2.11 (t, *J* = 7.6 Hz, 2H, CH_2), 2.12-2.23 (m, MMAE), 2.23-2.44 (m, MMAE), 2.48-2.71 (m, MMAE), 2.76-2.86 (m, MMAE), 2.88-2.97 (m, 4H, MMAE overlapped with CH_2), 3.02-3.11 (m, MMAE), 3.21-3.27 (m, MMAE), 3.30-3.39 (m, MMAE overlapped with PEG CH_2), 3.41-3.46 (t, *J* = 7.2 Hz, 2H, CH_2), 3.46-3.49 (t, *J* = 4.8 Hz, 2H, PEG OCH_2), 3.50-3.62 (m, PEG OCH_2), 3.68-3.73 (t, *J* = 6.8 Hz, 2H, PEG OCH_2), 3.73-3.80 (m, MMAE), 3.93-4.02 (m, MMAE), 4.02-4.13 (m, MMAE), 4.13-4.24 (m, MMAE), 4.49-4.52 (d, *J* = 11.2 Hz, 1H, MMAE), 4.54-4.60 (t, *J* = 8.4 Hz, 1H, MMAE), 4.61-4.70 (m, MMAE), 4.75-5.00 (m, MMAE), 6.20-6.28 (br, 1H, N*H*), 6.40-6.53 (m, MMAE), 6.62 (s, 2H, C*H*), 7.16-7.34 (m, 5H, Ar*H* MMAE). ^{13}C NMR (100 MHz, $CDCl_3$) 11.1 (*C*H_3, MMAE), 14.0 (*C*H_3, MMAE), 14.8 (*C*H_3, MMAE), 16.0 (*C*H_3, MMAE), 18.6 (*C*H_3, MMAE), 19.5 (*C*H_3, MMAE), 25.1

(CH_2, MMAE), 25.1 (CH_2, MMAE), 25.2 (CH_2), 25.8 (CH_2, MMAE), 26.1 (CH_2, MMAE), 26.1 (CH_2), 28.5 (CH_2), 29.8 (MMAE), 30.8 (CH, MMAE), 31.1 (CH, MMAE), 33.6 (CH, MMAE), 34.2 (CH_2), 36.5 (CH_2, PEG), 37.8 (CH_2), 39.3 (CH_2, PEG), 45.1 (CH, MMAE), 47.9 (CH_2, MMAE), 51.8 (CH, MMAE), 54.1 (CH, MMAE), 58.1 (OCH_3, MMAE), 60.2 (CH, MMAE), 61.1 (OCH_3, MMAE), 62.3 (MMAE), 67.5 (overlapped CH_2 and OCH, PEG and MMAE), 70.1 (CH_2, PEG), 70.3 (CH_2, PEG), 70.5-70.8 (overlapped CH_2, PEG), 75.9 (CH, MMAE), 78.7 (CH, MMAE), 82.1 (CH, MMAE), 126.5 (ArCH, MMAE), 127.4 (ArCH, MMAE), 128.2 (ArCH, MMAE), 134.2 (C), 141.3 (MMAE), 169.9 (CO), 170.7 (CO), 171.0 (CO), 172.2 (CO), 172.9 (CO), 174.8 (CO). LRMS (ESI+) 1532 (100, $[M+Na]^+$), 1510 (15, $[M+H]^+$); HRMS (ESI+) calcd. for $C_{76}H_{132}N_7O_{23}$ $[M+H]^+$ 1510.9375, observed: 1510.9379.



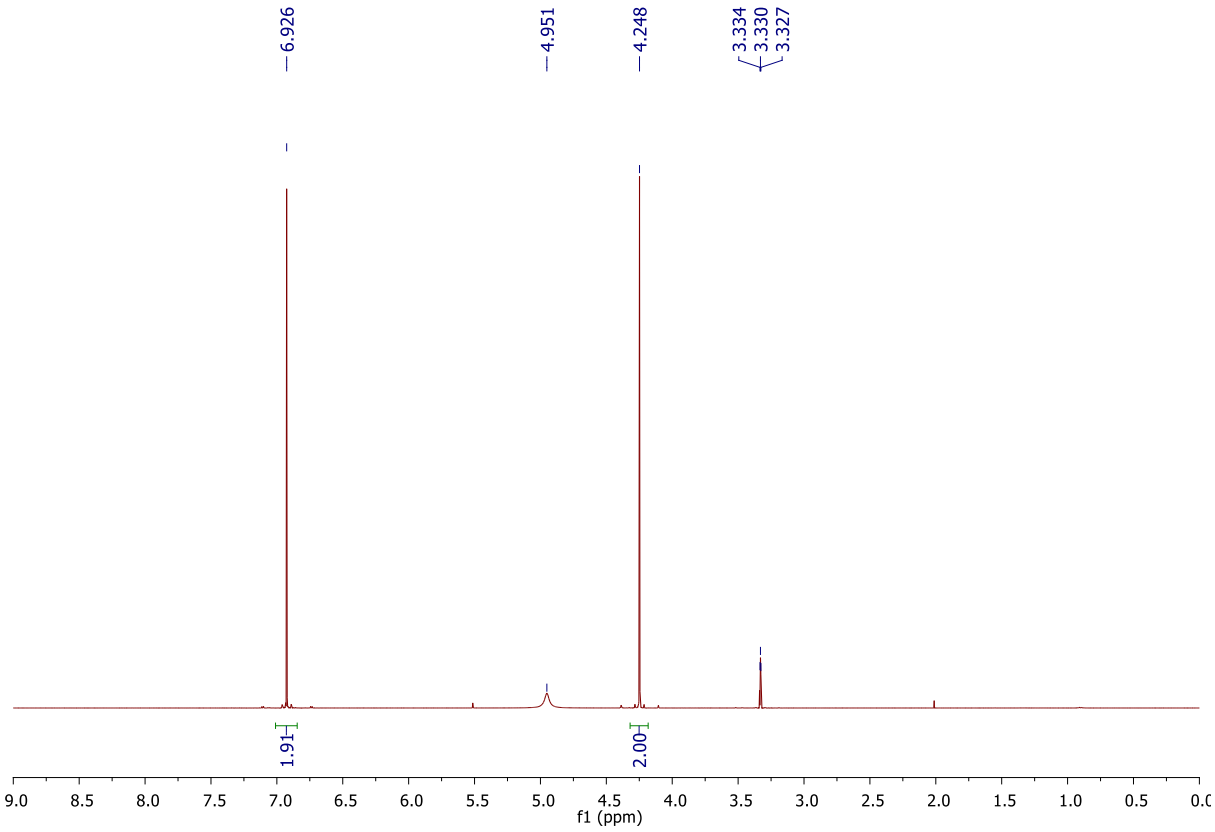


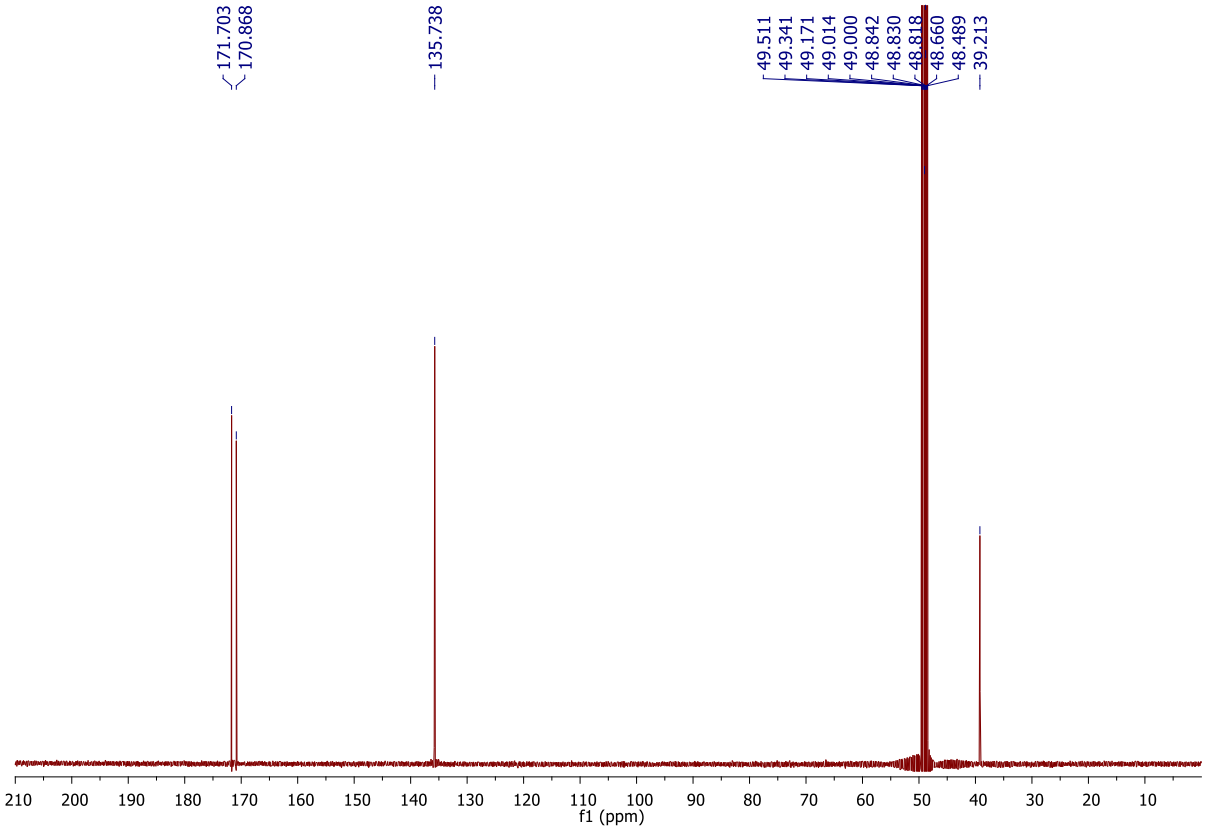
2-(2,5-dioxo-2,5-dihydro-1*H*-pyrrol-1-yl)acetic acid 15



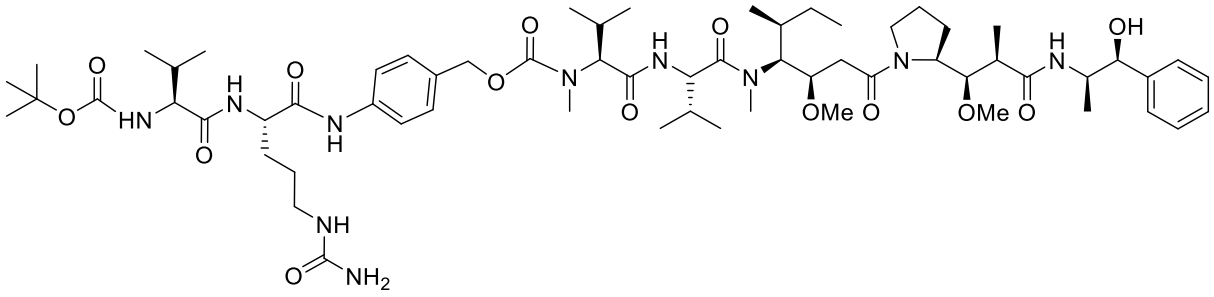
In a 10 mL round-bottom flask, maleic anhydride (196 mg, 2 mmol) and glycine (150 mg, 2 mmol, 1 eq.) were suspended in acetic acid (4 mL). The mixture was heated at 140 °C for 16 hours. Then, cooled down to 20 °C and concentrated in vacuum. Purified by column chromatography in silica with DCM:AcOH (95:5 v/v). Next, recrystallised by dissolving in refluxing DCM:hexanes (1:1 v/v), cooling down and keeping at 4 °C over 48 h to crystallise. Filtered and dried under vacuum to afford the title compound as a white solid (95 mg, 0.61 mmol, 31%). Characterisation data: m.p. 110-111 °C. FTIR ν_{max} (cm^{-1}) 3100-2600 (broad), 2940, 1704, 1431, 1258, 832, 689. ^{1}H NMR (500 MHz, MeOD-d_4) 4.24-4.25 (s, 2H, CH_2), 6.92-6.93 (s, 2H, C*H*); ^{13}C NMR (125 MHz, MeOD-d_4) 39.2 (*C*H_2), 135.7 (*C*H), 170.9 (*C*O), 171.7 (*C*O).

LRMS (NSI) 154 (100, $[M-H]^-$); HRMS (ESI) calcd. for $C_6H_4NO_4$ $[M-H]^-$ 154.0146, observed: 154.0147.



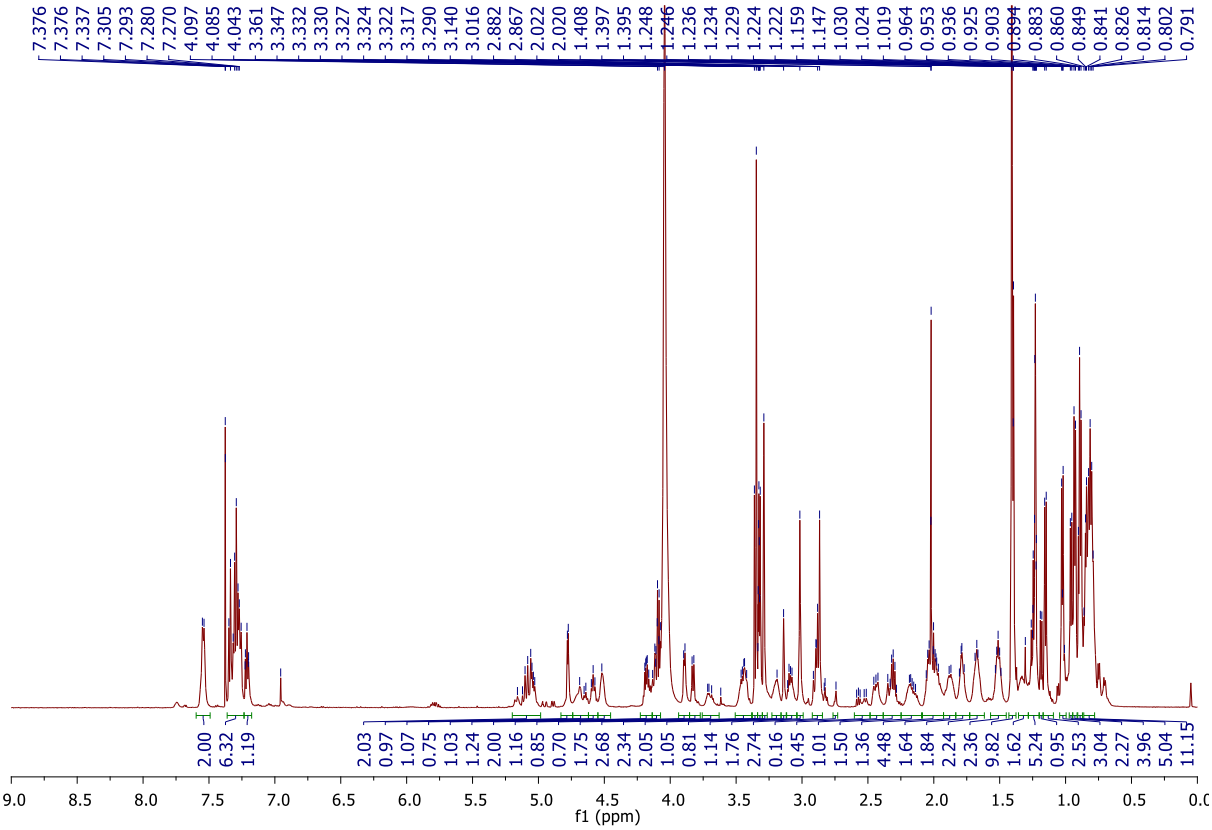


BocHN-vc-PABC-MMAE 16



This protocol is a modification of a literature protocol[4]. 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 (*C*H_3, MMAE), 13.8 (*C*H_3, MMAE), 14.0 (*C*H_3, MMAE), 15.6 (*C*H_3, MMAE), 17.6 (*C*H_3, MMAE), 18.3 (*C*H_3), 18.8 (*C*H_3), 19.0 (*C*H_3, MMAE), 24.6 (*C*H_2, MMAE), 24.7 (*C*H_2, MMAE), 25.6 (*C*H_2, MMAE), 26.1 (*C*H_2), 28.0 (*C*H_3, Boc), 29.2 (*C*H, MMAE), 29.4 (*C*H_2), 29.5 (*C*H_3, MMAE), 30.1 (*C*H, MMAE), 30.8 (*C*H), 31.8 (*C*H, MMAE), 33.0 (*C*H, MMAE), 37.3 (*C*H_2, MMAE), 38.9 (*C*H_2), 44.7 (*C*H, MMAE), 47.8 (*C*H_2, MMAE), 50.7 (*C*H, MMAE), 53.1 (*C*H, MMAE), 54.3 (*C*H), 57.7 (O*C*H_3, MMAE), 59.7 (*C*H, MMAE), 60.1 (*C*H), 60.6, (O*C*H_3, MMAE), 61.4 (MMAE), 64.8 (*C*H_2), 67.2 (O*C*H, MMAE), 75.5 (O*C*H, MMAE), 78.3 (*C*H, MMAE), 80.0 (*C*, Boc), 82.1 (O*C*H, MMAE), 85.6 (MMAE), 119.9 (Ar*C*H), 126.2 (Ar*C*H, MMAE), 127.2 (Ar*C*H, MMAE), 127.9 (Ar*C*H, MMAE), 128.1 (Ar*C*, MMAE), 128.4 (Ar*C*H), 132.2 (Ar*C*), 137.8 (Ar*C*), 141.3 (Ar*C*, MMAE), 156.4 (*C*O, Boc), 157.4 (*C*O), 160.4 (*C*O), 170.2 (*C*O, MMAE), 170.3 (*C*O), 170.4 (*C*O, MMAE), 172.8 (*C*O, MMAE), 173.3 (*C*O), 174.6 (*C*O, 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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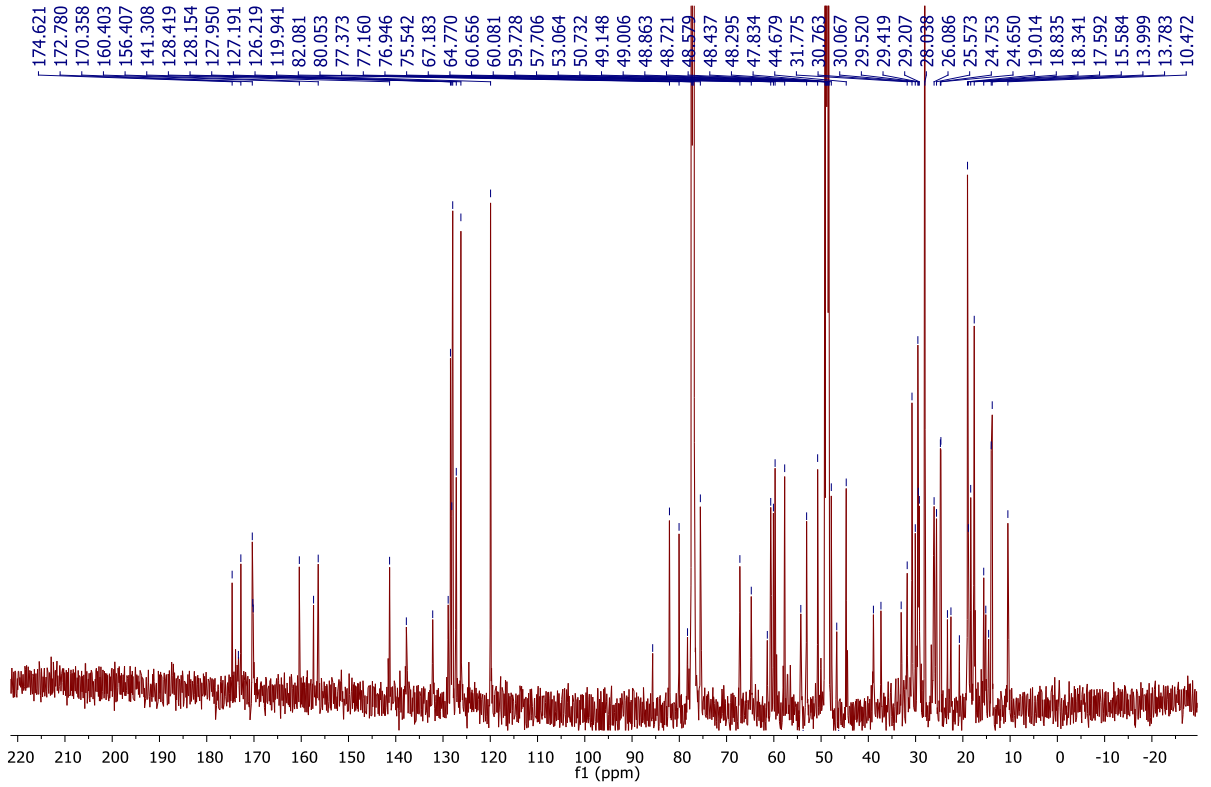
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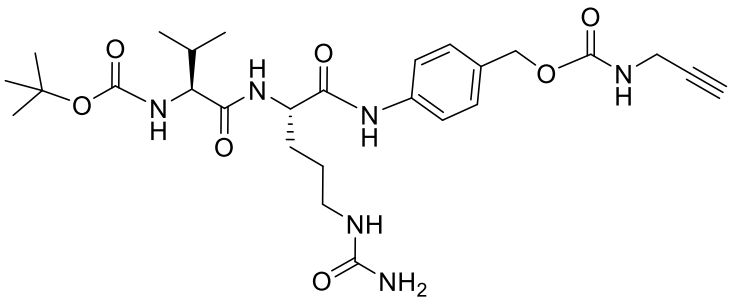
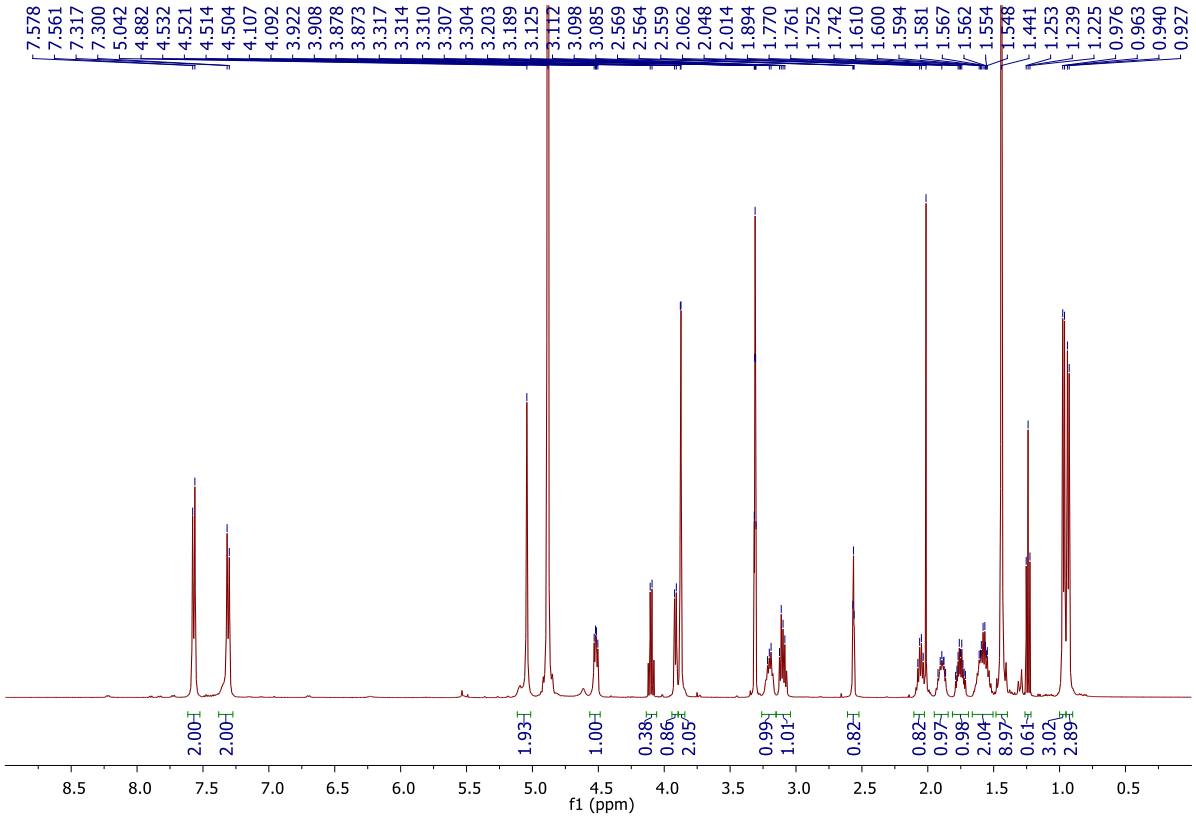
BocHN-vc-PABC-propargylamide 17



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, CH_3), 1.42-1.46 (s, 9H, CH_3), 1.50-1.65 (m, *J* = 7.0 Hz, 2H, CH_2), 1.70-1.80 (m, *J* = 9.5 Hz, 1H, CH_2), 1.85-1.95 (m, 1H, CH_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, CH_2), 3.16-3.25 (m, *J* = 7.0 Hz, 1H, CH_2), 3.86-3.88 (d, *J* = 2.5 Hz, 2H, CH_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, CH_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 (*C*H_3), 19.8 (*C*H_3), 27.8 (*C*H_2), 28.7 (*C*H_3), 30.5 (*C*H_2), 31.0 (*C*H_2), 31.9 (*C*H), 40.2 (*C*H_2), 54.9 (*C*H), 61.7 (*C*H), 67.3 (*C*H_2), 72.0 (*C*H), 80.7 (*C*), 81.1 (*C*), 121.2 (Ar*C*H), 129.6 (Ar*C*H), 133.9 (Ar*C*), 139.4 (Ar*C*), 158.2 (*C*O), 158.5 (*C*O), 162.3 (*C*O), 172.3, (*C*O), 174.7 (*C*O). 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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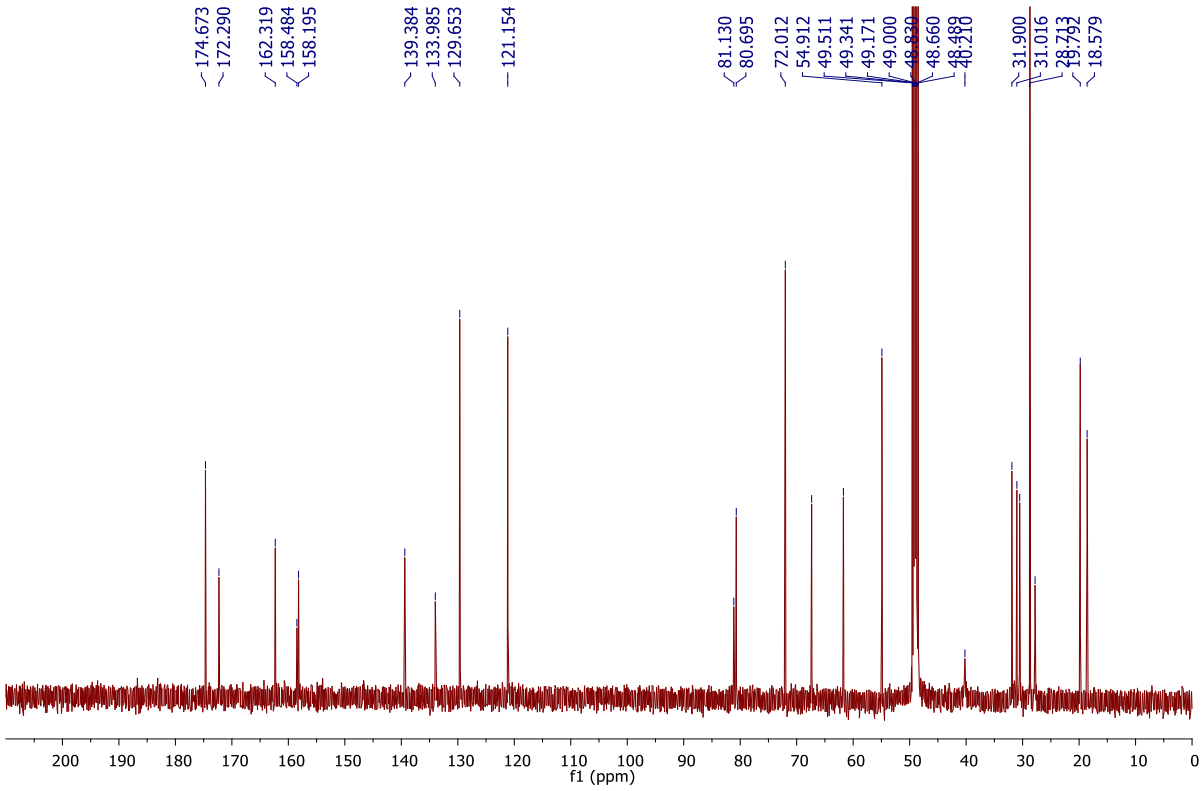
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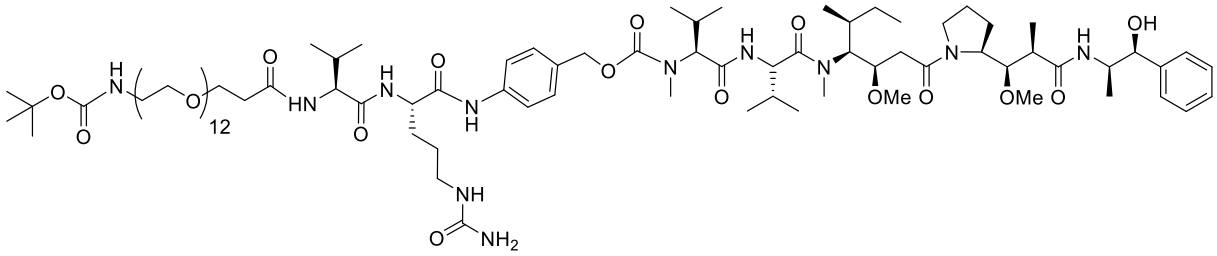
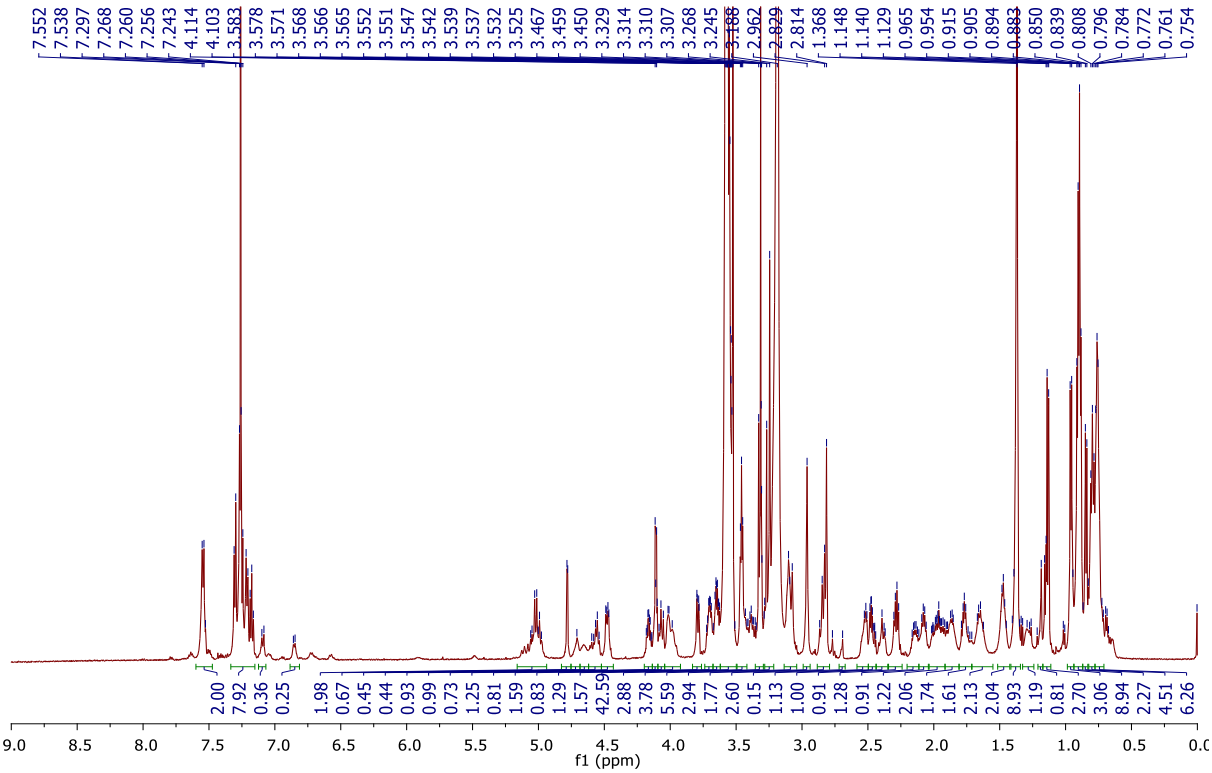
BocHN-PEG$_{12}$-vc-PABC-MMAE 18



In a 10 mL round-bottom flask, dissolved BocHN-vc-PABC-MMAE **16** (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 (*C*H_3, MMAE), 13.9 (*C*H_3, MMAE), 15.8 (*C*H_3, MMAE), 18.1 (*C*H_3, MMAE), 18.5 (*C*H_3), 19.0 (*C*H_3), 19.2 (*C*H_3, MMAE), 24.8 (*C*H_2, MMAE), 24.9 (*C*H_2, MMAE), 25.7 (*C*H_2, MMAE), 26.3 (*C*H_2), 28.4 (*C*H_3, Boc), 29.2 (*C*H_2), 29.6 (*C*H_3, MMAE), 30.3 (*C*H, MMAE), (*C*H_2), 30.8 (*C*H_2), 31.9 (*C*H, MMAE), 33.2 (*C*H, MMAE), 36.4 (*C*H_2, PEG), 37.6 (*C*H_2, MMAE), 38.9 (*C*H_2), 40.2 (*C*H_2, PEG), 43.7 (*C*H, MMAE), 47.9 (*C*H_2, MMAE), 50.9 (*C*H, MMAE), 53.2 (*C*H, MMAE), 53.3 (*C*H), 57.9 (O*C*H_3, MMAE), 59.5 (*C*H), 59.9 (O*C*H_3, MMAE), 60.9 (*C*H, MMAE), 64.9 (*C*H_2), 67.2 (O*C*H, MMAE), 67.3 (*C*H_2, PEG), 70.1 (*C*H_2, PEG), 70.1-70.5 (overlapped *C*H_2, PEG), 75.6 (O*C*H, MMAE), 78.5 (*C*H, MMAE), 79.4 (*C*, Boc), 82.2 (O*C*H, MMAE), 120.0 (Ar*C*H), 126.3 (Ar*C*H, MMAE), 127.3 (Ar*C*H, MMAE), 128.1 (Ar*C*H, MMAE), 128.3 (Ar*C*, MMAE), 128.5 (Ar*C*H), 132.2 (Ar*C*), 138.0 (Ar*C*), 141.3 (Ar*C*, MMAE), 156.4 (*C*O, Boc), 157.5 (*C*O), 160.4 (*C*O), 170.2 (*C*O, MMAE), 170.4 (*C*O), 170.6 (*C*O, MMAE), 172.2 (*C*O), 172.9 (*C*O, MMAE), 173.4 (*C*O), 174.8 (*C*O, 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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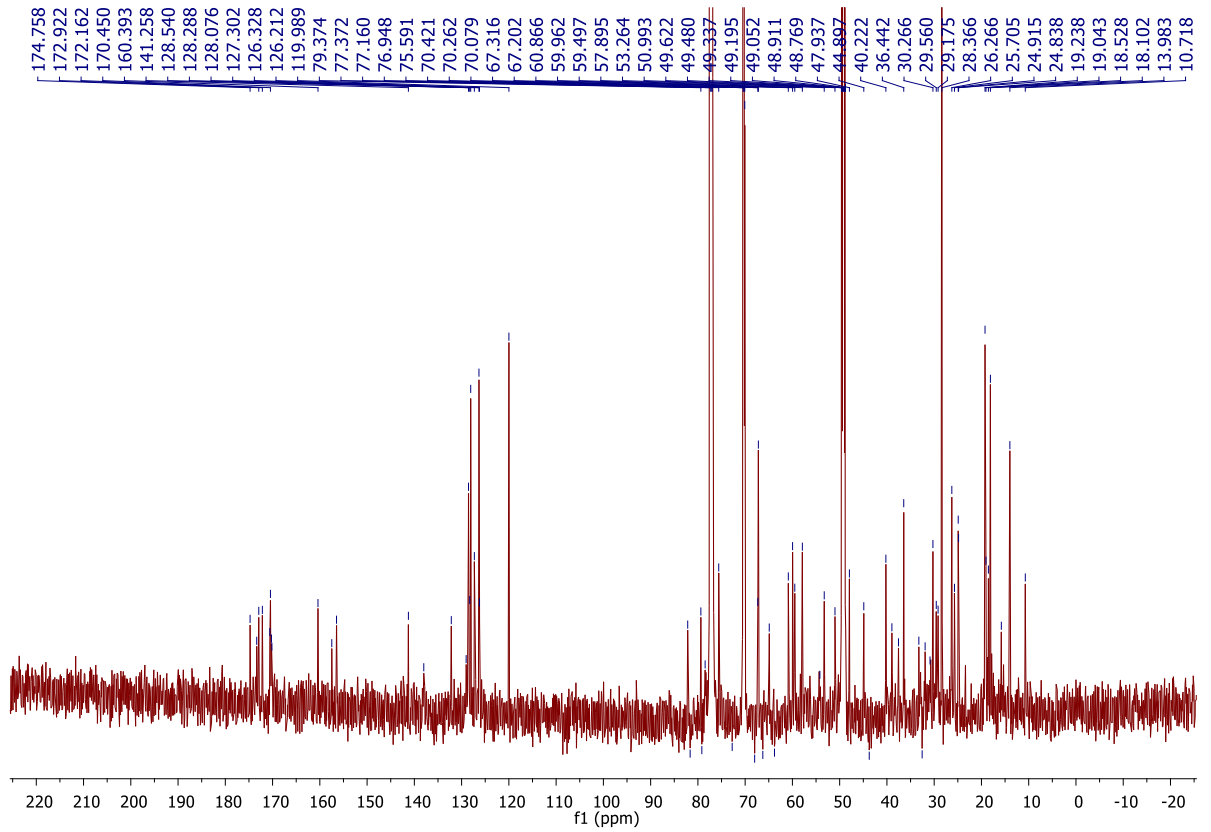
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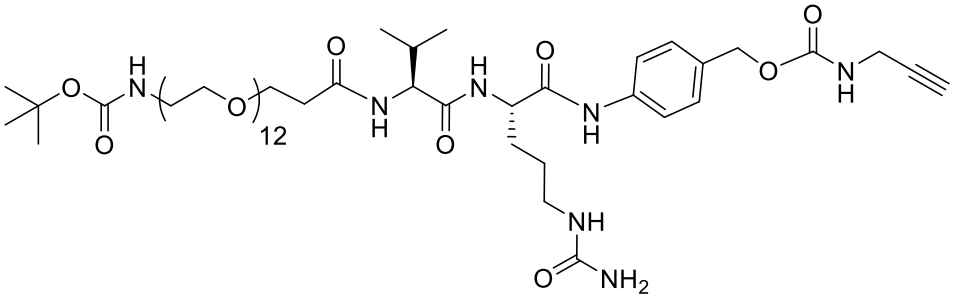
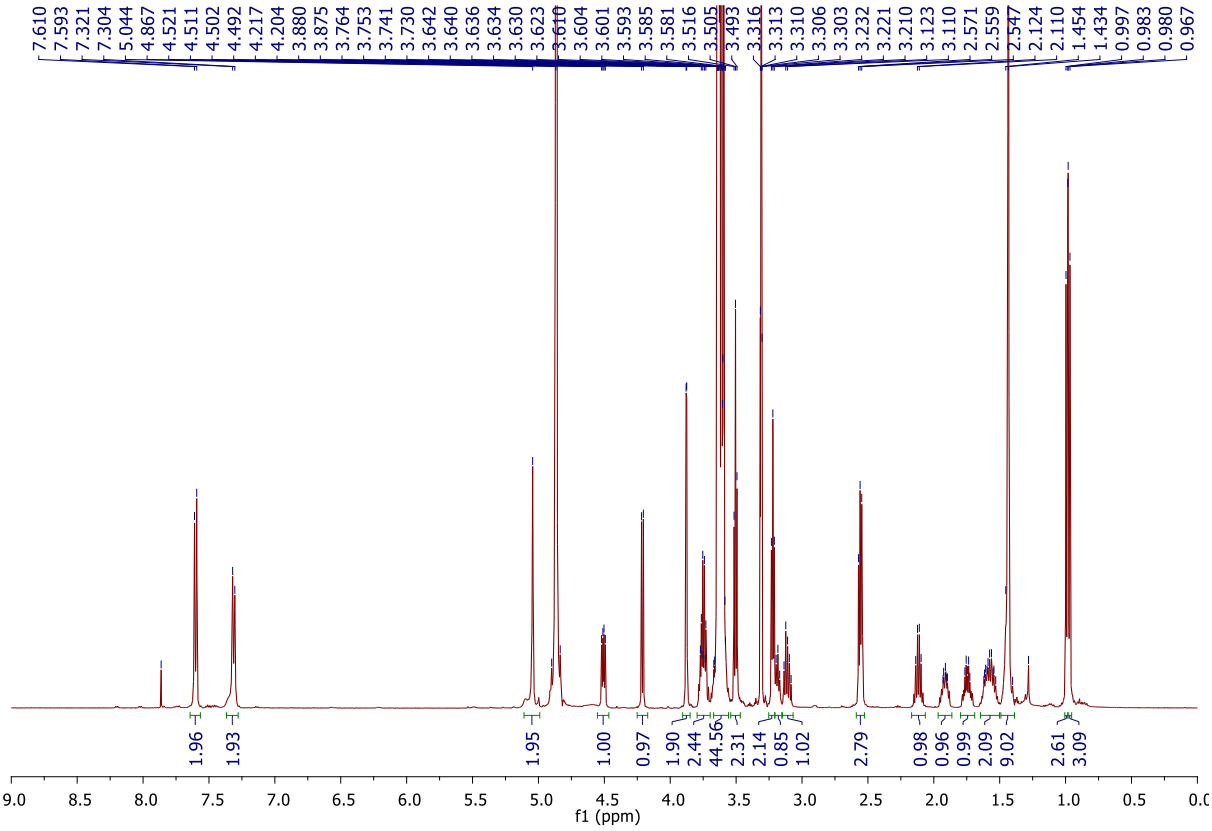
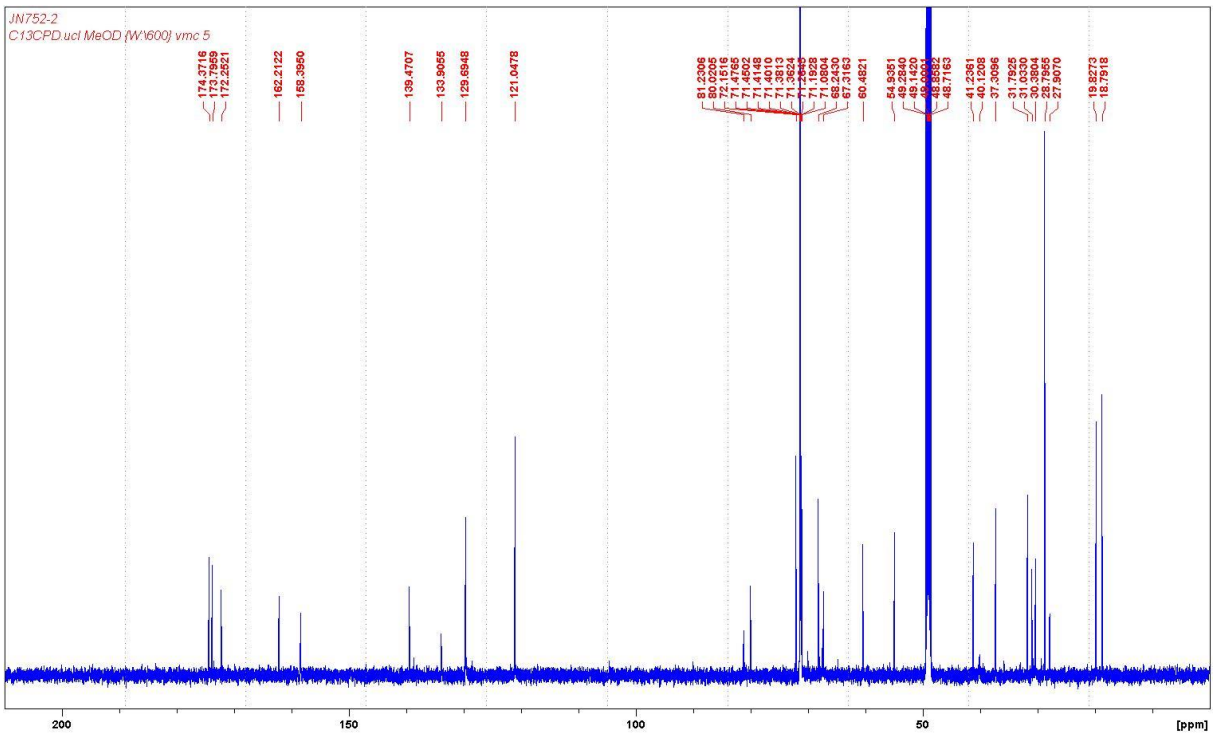
BocHN-PEG$_{12}$-vc-PABC-propargylamide 19



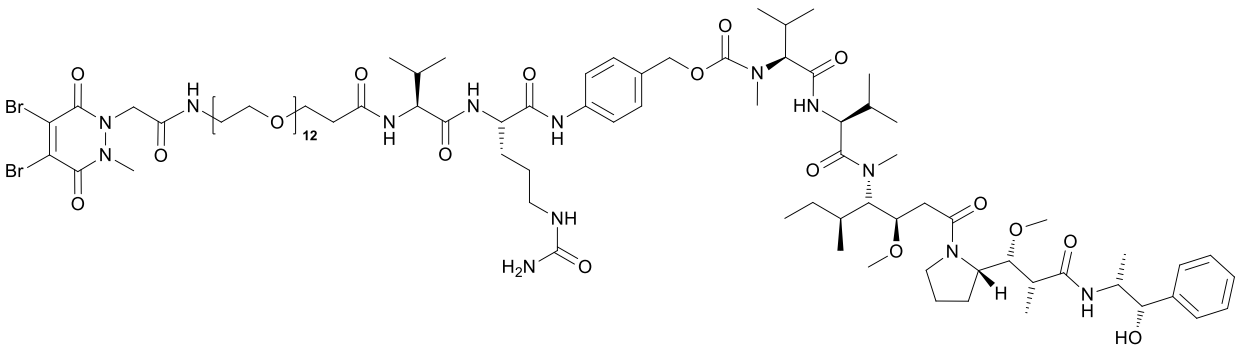
In a 10 mL round-bottom flask, BocHN-vc-PABC-propargylamide **17** (210 mg, 0.37 mmol, 1 eq.) in dry DCM (1.8 mL) and added TFA (0.2 mL). Stirred at 20 °C over 2 hours. Next, concentrated under vacuum to dryness, redissolved in dry DCM, concentrated again, redissolved in dry MeCN and concentrated once 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 $CDCl_3$ 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 (*C*H_3), 19.8 (*C*H_3), 27.9 (*C*H_2), 28.8 (*C*H_3), 30.4(*C*H_2), 31.0 (*C*H_2), 31.8 (*C*H), 37.3 (*C*H_2, PEG), 40.1 (*C*H_2), 41.2 (*C*H_2,

PEG), 54.9 (*C*H), 60.5 (*C*H), 67.3 (*C*H_2), 68.2 (*C*H_2, PEG), 71.1-71.5 (overlapped *C*H_2, PEG), 72.1 (*C*H), 80.0 (*C*), 81.2 (*C*), 121.0 (Ar*C*H), 129.7 (Ar*C*H), 133.9 (Ar*C*), 139.5 (Ar*C*), 158.4 (*C*O), 162.2 (*C*O), 172.2, (*C*O), 173.8 (*C*O), 174.4 (*C*O). 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.





PD-MMAE 9

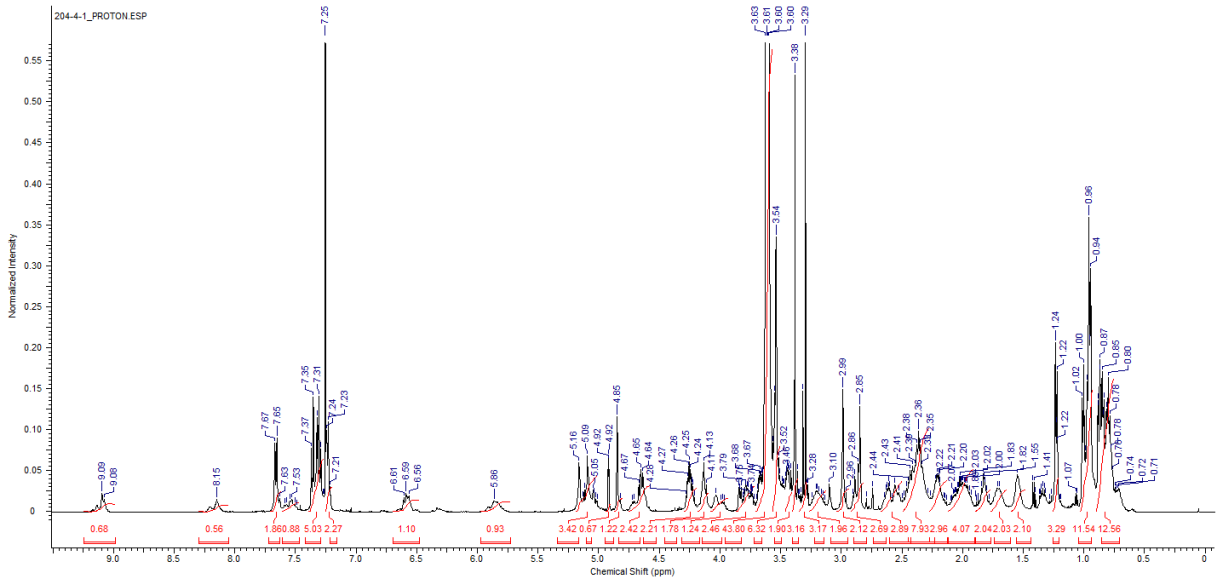


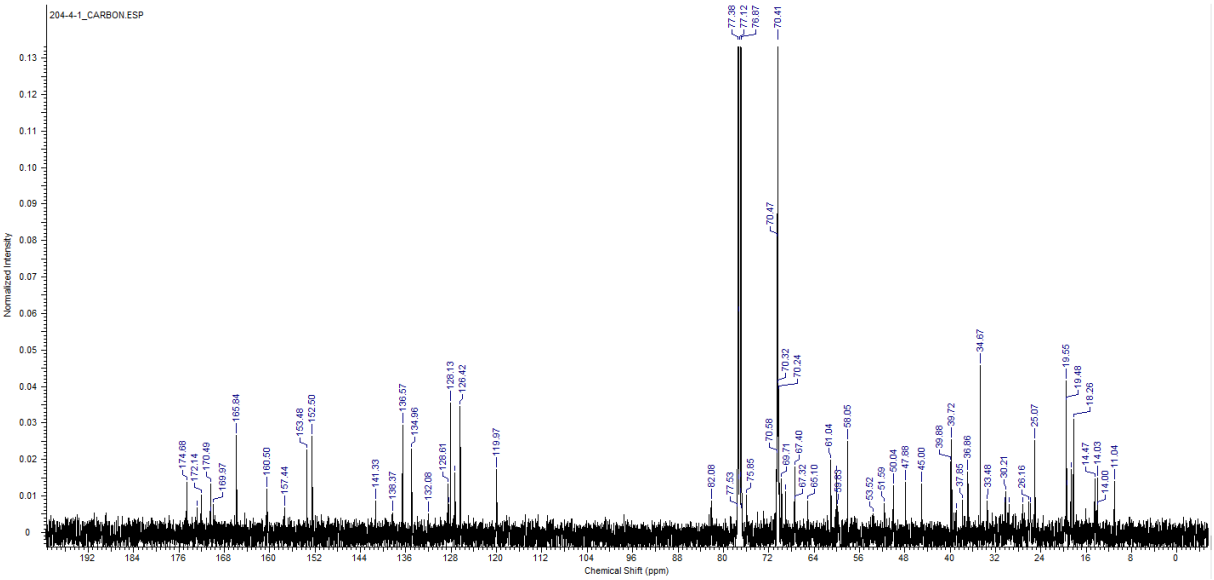
To an oven-dried RBF was added a solution of diBrPD-Me/-Acid **20** (4.1 mg, 12 µmol) in dry THF (200 µL). The stirred solution was cooled to 4 °C with an ice/water bath before addition of DCC (2.47 mg, 12 µmol) and stirring continued at 4 °C for 10 min. Pentafluorophenol (1.26 µL, 12 µmol) was then added and the reaction mixture allowed to reach ambient temperature (≈20 °C) and stirred for 2 h. The reaction mixture was then filtered through a pipette with celite plug and solids washed with cold THF. The filtrate was concentrated *in*

vacuo then taken up in a minimum volume of cold THF and filtered again to ensure no precipitates remained. The PFP-activated ester was then dissolved in dry MeCN (200 µL).

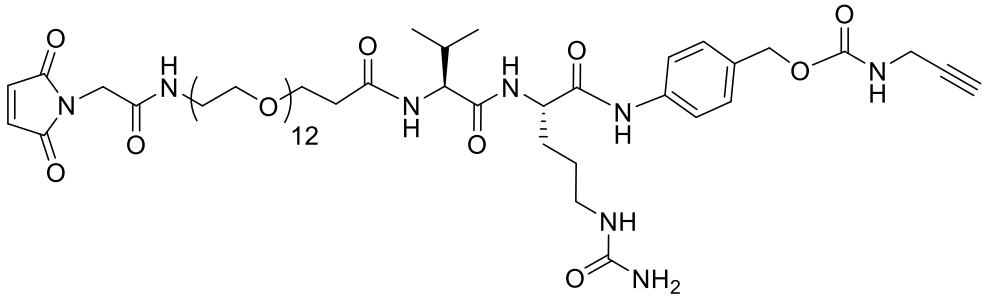
To a solution of BocHN-vc-PABC-MMAE **16** (20 mg, 11 µmol) in CH_2Cl_2 (950 µL) was added TFA (50 µL, 654 µmol) and the reaction mixture stirred at 20 °C for 90 min. The mixture was concentrated *in vacuo* and re-suspended in CH_2Cl_2 (2 mL) repeatedly (5x) to evaporate any residual TFA. The remaining oil was dissolved in MeCN (500 µL), to which was added DIPEA (95 µL, 550 µmol (50 eq.)), and the mixture was added to the solution of PFP-activated ester. The reaction mixture was stirred at ambient temperature for 4 h before concentrating *in vacuo*. Purification of the oily residue by preparative TLC (CH_2Cl_2/EtOAc/MeOH 8:2:2) gave title compound **9** as a light brown oil (7 mg, 31%). Characterisation data: ^{1}H NMR (500 MHz, $CDCl_3$ and MeOD-d_4 mix) 0.75-0.90 (overlapped m, 12H, MMAE and Val CH_3), 0.94-1.02 (overlapped m, 12H, MMAE and Val CH_3), 1.22-1.24 (d, *J* = 7.45 Hz, 3H, MMAE CH_3), 1.50-1.58 (m, 2H, Cit CH_2), 1.65-1.76 (m, 2H, Cit CH_2), 1.78-1.84 (m, 2H, MMAE), 1.90-2.10 (m, 4H, MMAE), 2.15-2.25 (m, 3H, MMAE), 2.28-2.48 (m, 8H, MMAE), 2.50-2.64 (m, 3H, Cit CH_2 and C*H*), 2.85-2.90 (m, 3H, MMAE), 2.99 (m, 2H, MMAE), 3.14-3.24 (m, 2H), 3.27-3.33 (s overlapped with m, 3H, PEG CH_2 and MMAE), 3.38 (overlapped s, 3H, MMAE CH_3), 3.42-3.46 (m, 2H, MMAE), 3.51-3.56 (overlapped m, PEG CH_2), 3.60-3.70 (m, 47H, PEG CH_2 and PD NCH_3), 3.72-3.84 (m, MMAE), 3.94-4.06 (m, 2H, MMAE), 4.11-4.15 (m, 2H, MMAE), 4.21-4.28 (m, 2H, MMAE), 4.60-4.74 (overlapped m, MMAE and CH_2), 4.85 (s, 1H, PD NCH_2), 4.92 (d, *J* = 2.9 Hz, 1H, C*H*, MMAE), 5.01-5.16 (overlapped m, 3H), 5.86 (m, 1H, N*H*), 6.50-6.61 (m, 1H, NH), 7.21-7.37 (overlapped m, 7H, Ar*H* MMAE and PABC), 7.49-7.59 (m, 1H, N*H*), 7.63-7.67 (d, *J* = 8.59 Hz, 2H, Ar*H* PABC), 8.15 (m, 1H, N*H*), 9.09 (m, 1H, N*H*). ^{13}C NMR (125 MHz, $CDCl_3$ and MeOD-d_4 mix) 11.0 (*C*H_3, MMAE), 14.0 (*C*H_3, MMAE), 14.5 (*C*H_3, MMAE), 18.3 (*C*H_3, MMAE), 18.7 (*C*H_3), 19.4 (*C*H_3), 19.5 (*C*H_3), 19.6 (*C*H_3, MMAE), 25.0 (*C*H_2, MMAE), 25.1 (*C*H_2, MMAE), 25.8 (*C*H_2, MMAE), 26.2 (*C*H_2), 27.2 (*C*H_2), 29.7 (*C*H_2), 30.2 (*C*H_3, MMAE), 30.3 (*C*H, MMAE), 33.5 (*C*H_2), 34.7 (N*C*H_3, PD), 36.9 (*C*H_2, PEG), 37.9 (*C*H_2, MMAE), 38.9 (*C*H, MMAE), 39.7 (*C*H_2NH, PEG), 39.9 (*C*H_2), 45.0 (*C*H, MMAE), 47.9 (*C*H_2, MMAE), 50.0 (N*C*H_2, PD), 51.6 (*C*H, MMAE), 53.5 (*C*H), 58.1 (O*C*H_3, MMAE), 59.8 (*C*H), 59.83 (O*C*H_3, MMAE), 60.1 (*C*H, MMAE), 61.0 (*C*H_2), 65.1 (*C*H, MMAE), 67.3 (O*C*H, MMAE), 67.4 (*C*H_2, PEG), 69.0 (*C*H_2), 69.7 (*C*H_2, PEG), 70.2-70.6 (overlapped *C*H_2, PEG), 75.9 (O*C*H, MMAE), 77.5 (*C*H, MMAE), 82.1 (O*C*H, MMAE), 120.0 (Ar*C*H), 126.4 (Ar*C*H, MMAE), 127.4 (Ar*C*H, MMAE), 128.1 (Ar*C*H, MMAE), 128.4 (Ar*C*, MMAE), 128.6 (Ar*C*H), 132.1 (Ar*C*), 135.0 (*C*Br), 136.6 (*C*Br), 138.4 (Ar*C*),

141.3 (Ar*C*, MMAE), 152.5 (Ar*C*O), 153.5 (Ar*C*O), 157.4 (*C*O), 160.5 (*C*O), 165.8, 170.0 (*C*O, MMAE), 170.4 (*C*O), 170.5 (*C*O, MMAE), 172.1 (*C*O), 172.9 (*C*O, MMAE), 174.7 (*C*O, MMAE). LRMS (NSI) 304 (100), 1032 (10, $[M+H+Na]^{2+}$); HRMS (NSI) calcd. for $C_{92}H_{153}Br_2N_{13}O_{28}$ $[M+2H]^{2+}$ 1022.9652, observed: 1022.9646.



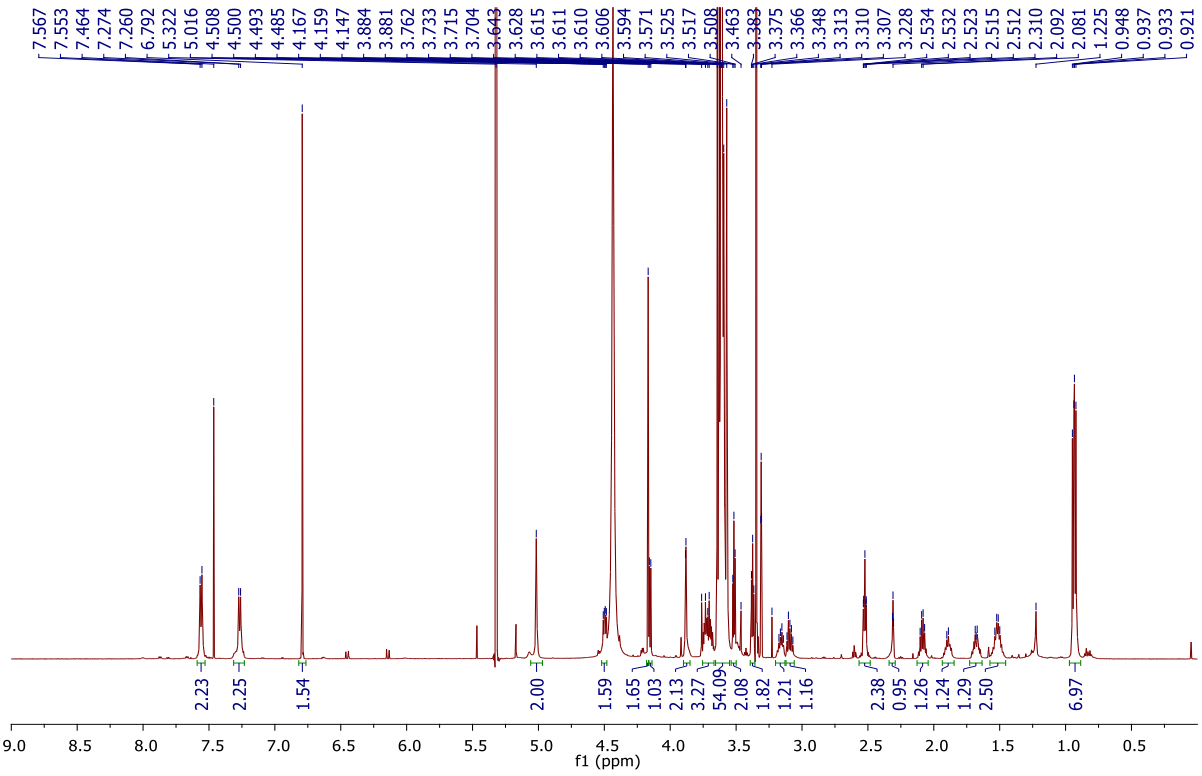


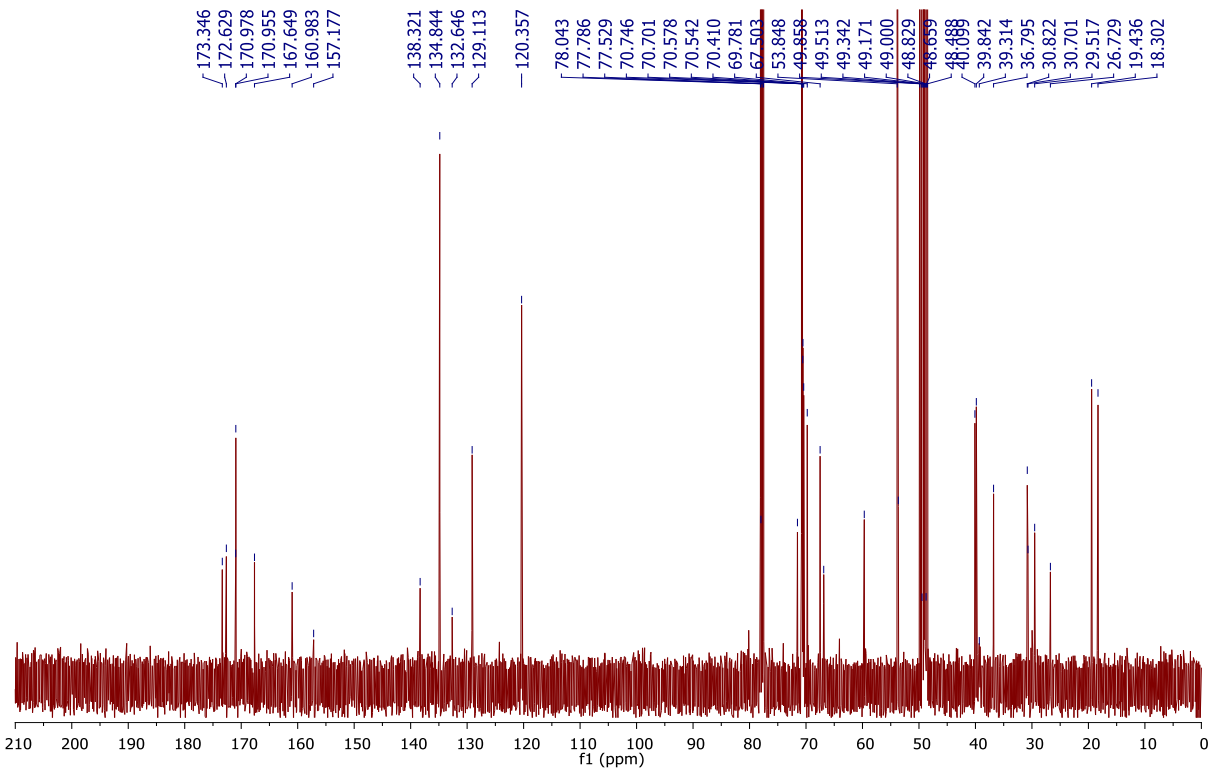
Mal-propargylamide 11



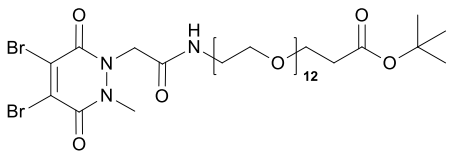
In a 10 mL round-bottom flask, dissolved Boc-HN-PEG_{12}-vc-PABC-propargylamide **19** (46 mg, 40 µmol, 1 eq.) in dry DCM (0.9 mL) and added TFA (0.1 mL). Stirred at 20 °C over 2 hours. Next, concentrated under vacuum to dryness, redissolved in dry DCM, concentrated again, redissolved in dry MeCN and concentrated once more under vacuum. Dissolved H_2N-PEG_{12}-valine-citruline-PABC-Alk TFA salt in dry MeCN (1 mL) and added DIPEA (35 µL, 200 µmol, 5 eq.). In a separate flask, dissolved 2-(2,5-dioxo-2,5-dihydro-1*H*-pyrrol-1-yl)acetic acid (M-C2, 6.2 mg, 40 µmol, 1 eq.) in dry MeCN (1 mL). Next, added dicyclohexylcarbodiimide (DCC, 8.3 mg, 40 µmol, 1 eq.) and stirred at 20 °C for 10 min. Then, added pentafluorophenol (4.2 µL, 40 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}-valine-citruline-PABC-Alk solution with DIPEA previously prepared. This solution was stirred at 20 °C over 4 hours. Next, concentrated under vacuum, redissolved in DCM (30 mL) and washed with 0.1 M sodium acetate buffer pH 5 (5 mL) and brine (5 mL). The organic layer was dried ($MgSO_4$), filtered and concentrated under vacuum, followed by purification by preparative thin layer chromatography on silica with DCM:MeOH (10:1 v/v) to afford the title compound as a beige solid (30 mg, 25 µmol, 62%). Characterisation data: no melting point, starts to decompose at 120-122 °C. FTIR ν_{max} (cm^{-1}): 3293, 2869, 2412, 1713, 1684, 1652, 1626, 1444, 1091. ^{1}H NMR (600 MHz, MeOD-d4 and $CDCl_3$ mix) 0.92-0.95 (overlapped d, *J* = 6.6 Hz, 6H, C*H*$_3$), 1.46-1.56 (m, *J* = 7.0 Hz, 2H, C*H*$_2$), 1.64-1.72 (m, *J* = 9.0 Hz, 1H, C*H*$_2$), 1.86-1.93 (m, *J* = 7.2 Hz, 1H, C*H*$_2$), 2.04-2.13 (oct., *J* = 6.5 Hz, 1H, C*H*), 2.30-2.32 (t, *J* = 2.4 Hz, 1H, CH), 2.51-2.53 (t, *J* = 6.6 Hz, 2H, PEG C*H*$_2$), 3.06-3.12 (m, *J* = 6.6 Hz, 1H, C*H*$_2$), 3.13-3.20 (m, *J* = 6.6 Hz, 1H, C*H*$_2$), 3.36-3.38 (t, *J* = 5.4 Hz, 2H, PEG C*H*$_2$), 3.51-3.53 (t. *J* = 5.4 Hz, 2H, PEG C*H*$_2$), 3.55-3.65 (overlapped m, PEG C*H*$_2$), 3.67-3.76 (m, *J* = 6.0 Hz, 2H, PEG C*H*$_2$), 3.87-3.89 (d, *J* = 2.4 Hz, 2H, C*H*$_2$), 4.15-4.16 (d, *J* = 7.2 Hz, 1H, C*H*), 4.16-4.17 (s, 2H, C*H*$_2$), 4.48-4.51 (dd, *J* = 9.0 and 4.8 Hz, 1H, C*H*), 5.00-5.03 (br, 2H, C*H*$_2$), 6.79-6.80 (s, 2H, C*H*), 7.25-7.28 (d, *J* = 8.4 Hz, 2H, Ar*H*), 7.54-7.57 (d, *J* =

8.4 Hz, 2H, Ar*H*); ^{13}C NMR (150 MHz, MeOD-d4 and $CDCl_3$ mix) 18.3 (*C*H_3), 19.4 (*C*H_3), 26.7 (*C*H_2), 29.5 (*C*H_2), 30.7 (*C*H_2), 30.8 (*C*H), 36.8 (*C*H_2, PEG), 39.3 (*C*H_2), 39.8 (*C*H_2), 40.1 (*C*H_2, PEG), 53.7 (*C*H), 59.7 (*C*H), 66.9 (*C*H_2), 67.5 (*C*H_2, PEG), 69.8 (*C*H_2, PEG), 70.4 (*C*H_2, PEG), 70.5 (*C*H_2, PEG), 70.6 (*C*H_2, PEG), 70.7-70.8 (overlapped *C*H_2, PEG), 71.5 (*C*H), 77.9 (*C* overlapped with $CDCl_3$) 120.4 (Ar*C*H), 129.1 (Ar*C*H), 132.6 (Ar*C*), 134.8 (*C*H), 138.3 (Ar*C*), 157.2 (*C*O), 161.0 (*C*O), 167.6 (*C*O), 170.9 (*C*O), 171.0 (*C*O), 172.6, (*C*O), 173.4 (*C*O). LRMS (NSI) 608 (100, $[M+NH_4+H]^+$), 1219 (6, $[M+Na]^+$); HRMS (NSI) calcd. for $C_{55}H_{88}N_8O_{21}Na$ $[M+Na]^+$ 1219.5962, observed: 1219.5960.



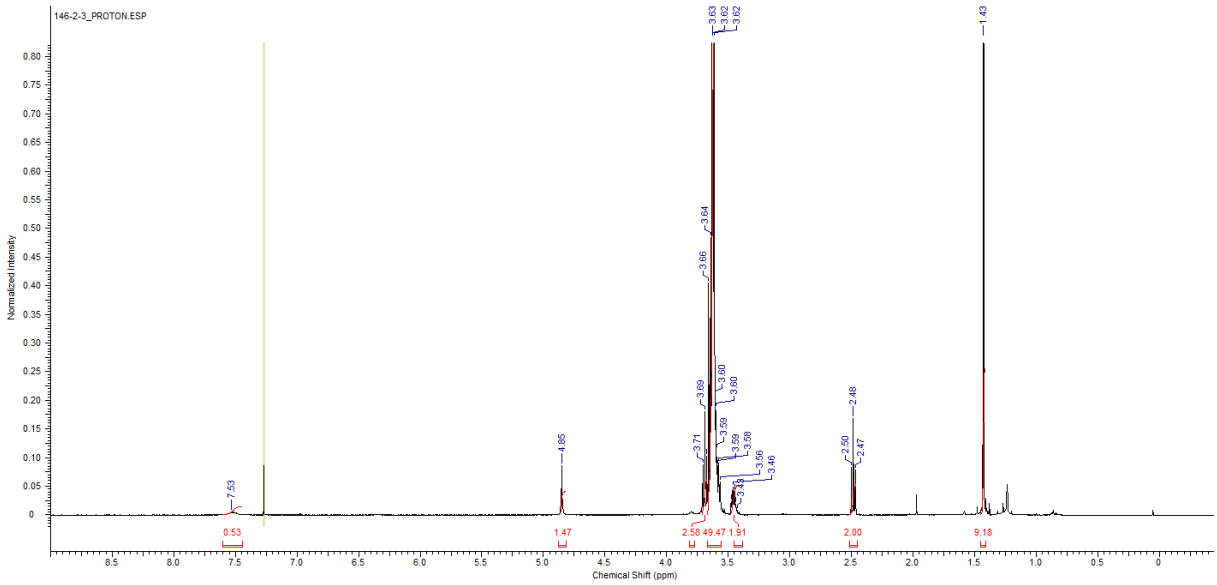


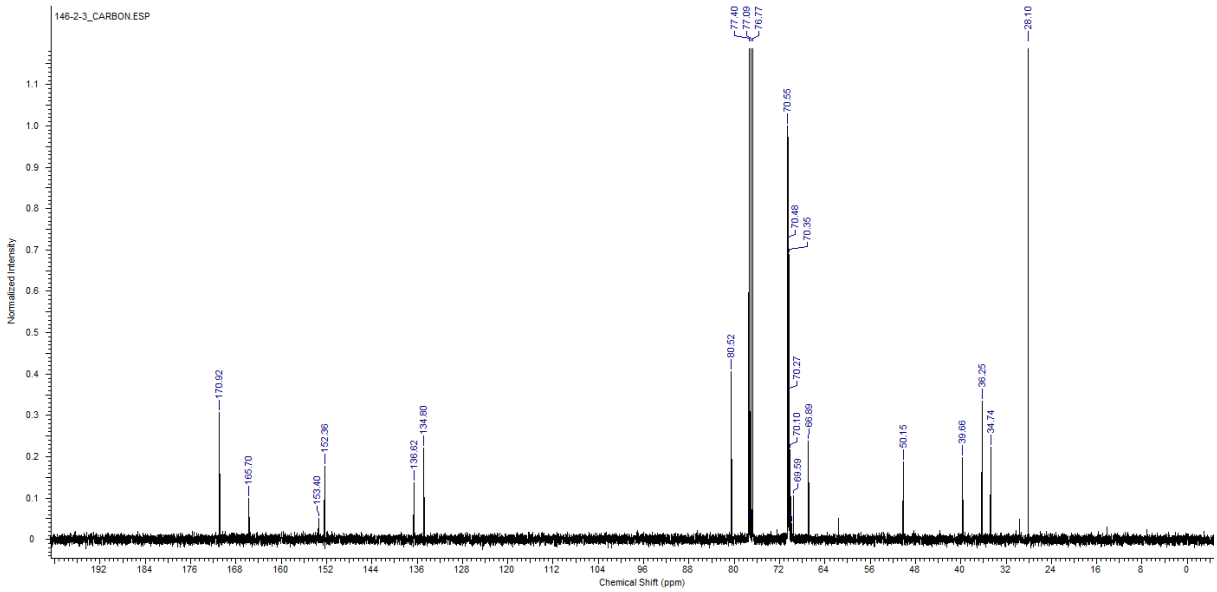
PD-PEG$_{12}$-CO$_2$^{t}Bu 21



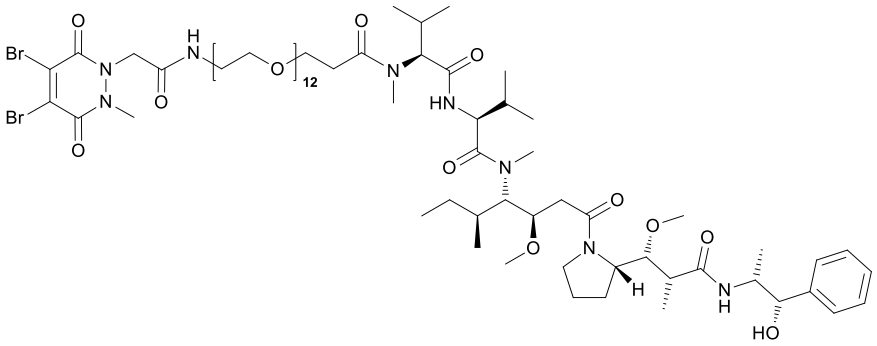
To an oven-dried RBF was added a solution of diBrPD-Me/-Acid **20** (50 mg, 146 µmol) dissolved in dry THF (2 mL) and the stirred solution cooled to 4 °C with an ice/water bath. DCC (28.8 mg, 140 µmol) was added and stirring continued at 4 °C for 10 min. Pentafluorophenol (14.6 µL, 140 µmol) was then added and the reaction mixture allowed to reach ambient temperature (≈20 °C) and stirred for 2 h. The reaction mixture was then filtered by vacuum filtration through a sinter funnel and any solids washed with cold THF. The filtrate was concentrated *in vacuo* then taken up in cold THF and filtered again. The filtration-resuspension process was repeated until concentrating the filtrate resulted in oil residue only. The PFP-activated ester was dissolved in dry MeCN (1 mL) and to the solution was added NH$_2$-PEG$_{12}$-CO$_2$^{t}Bu (109 mg, 161 µmol) The reaction mixture was stirred at ambient temperature for 16 h before concentrating *in* vacuo to leave an oily residue, which

was purified by flash column chromatography (CH_2Cl_2/EtOAc/MeOH 8:4:0 to 8:4:0.6 to 8:2:1) to afford the title compound **21** as a light brown oil (61 mg, 61 µmol, 42%). Characterisation data: FTIR ν_{max} (cm^{-1}): 2869, 1727, 1688, 1640, 1106. ^{1}H NMR (500 MHz, $CDCl_3$): 1.43 (s, 9H, ^{t}Bu CH_3), 2.48 (t, J = 6.6 Hz, 2H, PEG CH_2CO$_2$), 3.43-3.48 (m, 2H, PEG CH_2NH), 3.56-3.66 (overlapped m, 49H, PEG CH_2 and PD NCH_3), 3.69 (t, J = 6.6 Hz, 2H, PEG CH_2CH$_2$CO$_2$), 4.85 (s, 2H, PD NCH_2), 7.53 (br, 1H, NH); ^{13}C NMR (125 MHz, $CDCl_3$) 28.1 (C(*C*H$_3$)$_3$), 34.7 (NCH$_3$), 36.3 (*C*H$_2$CO$_2$), 39.7 (*C*H$_2$NH), 50.2 (*C*H$_2$N), 66.9 (*C*H$_2$CH$_2$CO$_2$), 69.6 (*C*H$_2$, PEG), 69.8 (*C*H$_2$, PEG), 70.1 (*C*H$_2$, PEG), 70.2 (*C*H$_2$, PEG), 70.3-70.6 (overlapped *C*H$_2$, PEG), 80.5 (*C*(CH)$_3$), 134.8 (Ar*C*Br), 136.6 (Ar*C*Br), 152.4 (Ar*C*O), 153.4 (Ar*C*O), 165.7 (NH*C*O), 170.9 (*C*O$_2$).



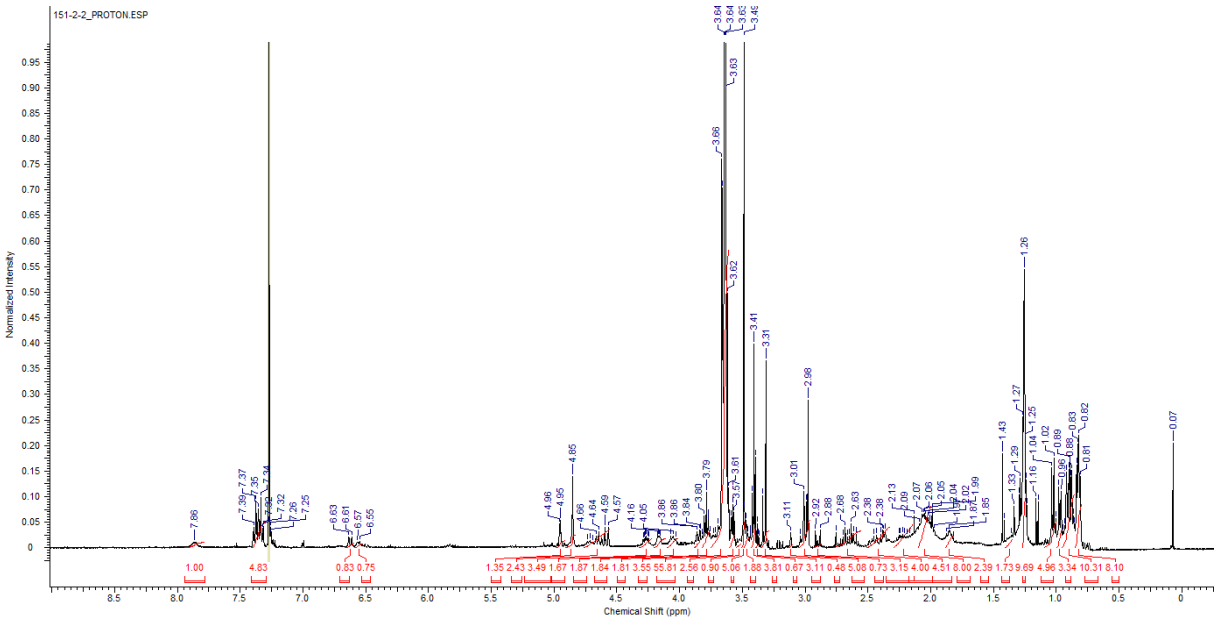


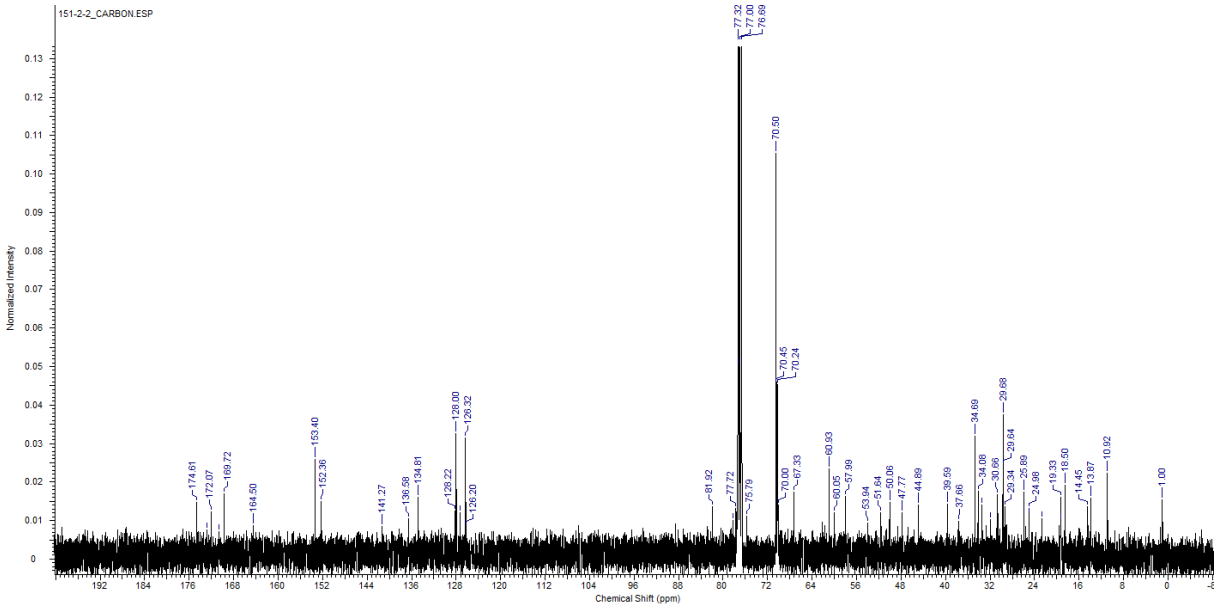
PD-MMAE 8



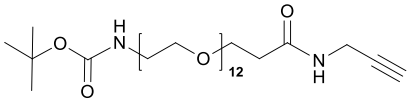
To a solution of ester **21** (40 mg, 0.04 mmol) in CH_2Cl_2 (1 mL) was added TFA (614 µL, 8.02 mmol) and the reaction mixture stirred at room temperature for 2 h. The mixture was concentrated *in vacuo* and azeotroped with toluene (3x 5 mL) to ensure removal of excess TFA. A portion of the resulting acid (10.3 mg, 10.93 µmol) was dissolved in MeCN (200 µL), to which was added EEDQ (5.4 mg, 21.86 µmol) and the reaction mixture was stirred at 20 °C for 5 min before addition of MMAE.TFA (10 mg, 12.02 µmol). Stirring continued at 20 °C for 16 h. Reaction mixture was concentrated *in vacuo* and the residue purified by flash column chromatography (CH_2Cl_2/EtOAc/MeOH 0:9:1 until MMAE eluted, then 6:3:1 to 8:2:1) to give title compound **8** as a light brown viscous oil (9 mg, 51%). Characterisation data: ^{1}H NMR (500 MHz, $CDCl_3$ and MeOD-d_4 mix) 0.81-0.85 (m MMAE), 0.85-0.95 (m, MMAE), 0.96-0.98 (m, 3H, MMAE), 1.00-1.08 (m, 5H, MMAE), 1.23-1.29 (m, MMAE), 1.33-1.41 (m, MMAE),

1.82-1.87 (m, MMAE), 1.97-2.09 (m, H_2O and MMAE), 2.17-2.25 (m, MMAE), 2.36-2.50 (m, MMAE), 2.57-2.76 (m, MMAE), 2.84-2.93 (m, MMAE), 2.98-3.04 (m, MMAE), 3.11 (br, MMAE), 3.27-3.34 (m, MMAE), 3.37-3.39 (m, MMAE), 3.40-3.42 (m, MMAE), 3.44-3.48 (m, MMAE), 3.47-3.50 (m, MMAE), 3.50-3.55 (m, 1H, MMAE), 3.57-3.59 (t, *J* = 5.3 Hz, 2H, PEG CH_2), 3.60-3.75 (overlapped m, PEG CH_2, MMAE and PD NCH_3), 3.75-3.82 (overlapped m, 3H, MMAE C*H* and PEG CH_2), 3.82-3.86 (m, 2H, PEG CH_2), 4.03-4.09 (m, 2H, MMAE), 4.15-4.18 (m, 2H, MMAE), 4.25-4.29 (m, 2H, MMAE), 4.55-4.75 (m, 3H, MMAE), 4.85 (s, 2H, PD NCH_2), 4.95-4.96 (d, *J* = 3.0 Hz, 1H, MMAE), 6.56 (d, *J* = 6.6 Hz, 1H, N*H*), 6.62 (d, *J* = 9.4 Hz, 1H, N*H*), 7.32-7.39 (m, 5H, Ar*H* MMAE), 7.86 (br, 1H, N*H*). ^{13}C NMR (125 MHz, $CDCl_3$ and MeOD-d_4 mix) 10.9 (*C*H_3, MMAE), 13.9 (*C*H_3, MMAE), 14.5 (*C*H_3, MMAE), 18.5 (*C*H_3, MMAE), 19.3 (*C*H_3, MMAE), 22.7 (*C*H_3, MMAE), 24.9 (*C*H_2, MMAE), 25.0 (*C*H_2, MMAE), 25.9 (*C*H_2, MMAE), 29.3 (*C*H, MMAE), 29.7 (*C*H_3, MMAE), 30.7 (*C*H, MMAE), 31.9 (*C*H, MMAE), 33.4 (*C*H, MMAE), 34.1 (*C*H_2CON, PEG), 34.7 (N*C*H_3, PD), 37.7 (*C*H_2, MMAE), 39.6 (*C*H_2NH, PEG), 44.9 (*C*H, MMAE), 47.8 (*C*H_2, MMAE), 50.1 (N*C*H_2, PD), 51.6 (*C*H, MMAE), 53.9 (*C*H, MMAE), 58.0 (O*C*H_3, MMAE), 60.1 (*C*H, MMAE), 60.9, (O*C*H_3, MMAE), 67.3 (O*C*H, MMAE), 70.0 (*C*H_2, PEG), 70.2-70.5 (overlapped *C*H_2, PEG), 75.8 (O*C*H, MMAE), 78.2 (*C*H, MMAE), 81.9 (O*C*H, MMAE), 126.3 (Ar*C*H, MMAE), 127.2 (Ar*C*, MMAE), 128.0 (Ar*C*H, MMAE), 134.8 (Ar*C*Br), 136.6 (Ar*C*Br), 141.3 (Ar*C*, MMAE), 152.4 (Ar*C*O), 153.4 (Ar*C*O), 164.5 (PD NCH$_2$*C*O), 169.7 (CO) 170.6 (*C*O, MMAE), 172.1 (*C*O, MMAE), 172.8 (*C*O, MMAE), 174.6 (*C*O, MMAE). LRMS (NSI) 304 (100), 838 (40, $[M+2NH_4]^{2+}$), 829 (38, $[M+H+NH_4]^{2+}$); HRMS (NSI) calcd. for $C_{73}H_{129}Br_2N_9O_{23}$ $[M+H+NH_4]^{2+}$ 828.8779, observed: 828.8780.



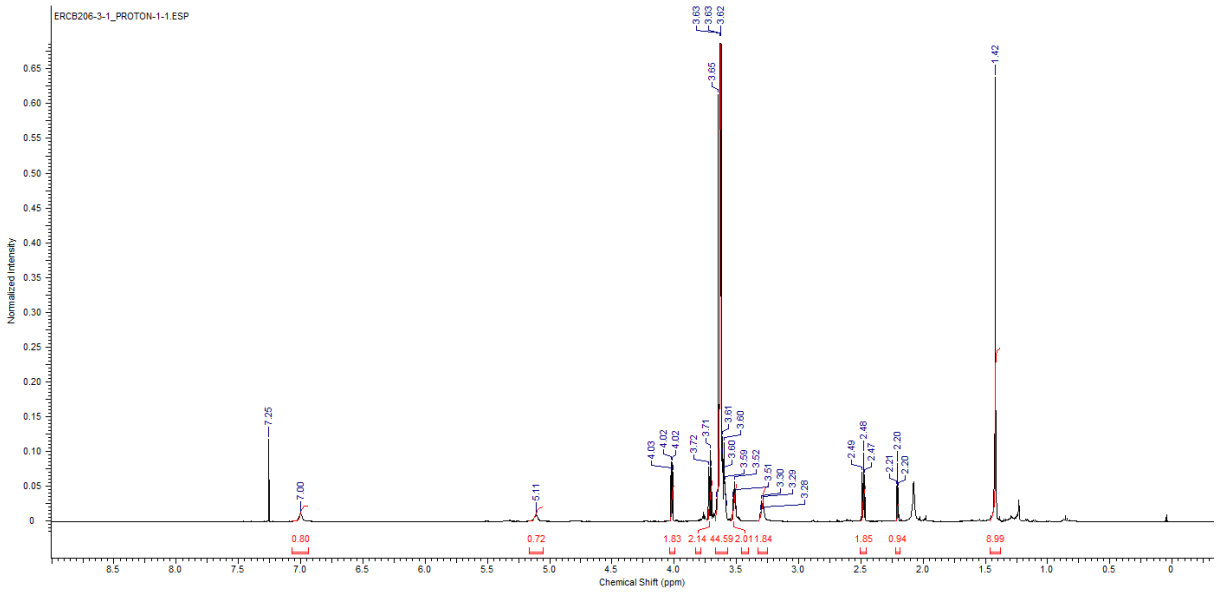


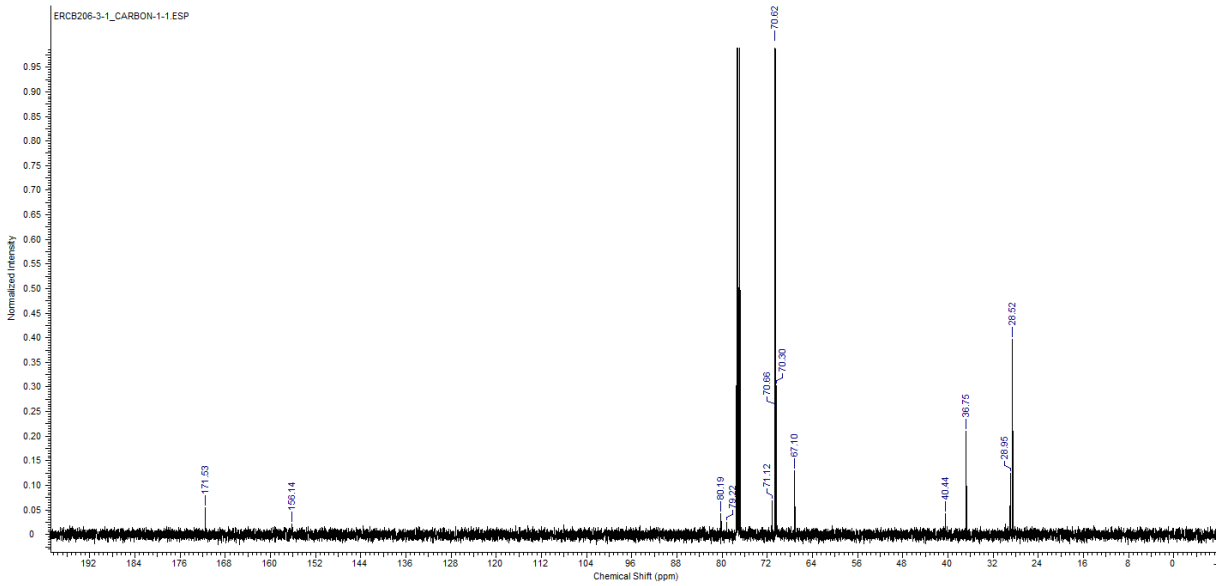
BocNH-PEG$_{12}$-Propargylamide 22



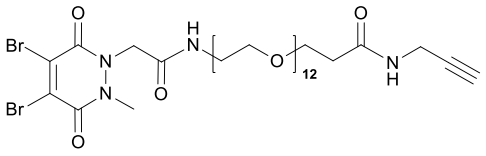
To a solution of BocNH-PEG$_{12}$-CO$_2$H (30 mg, 42.0 µmol) in dry CH_2Cl_2 (1000 µL) was added HBTU (15.9 mg, 42.0 µmol) and HOBt (0.1 mg, 4.2 µmol) followed by DIPEA (36.4 µL, 210 µmol). The reaction mixture was stirred at 20 °C for 5 min before addition of a solution of propargylamine (8.0 µL, 125.0 µmol) in dry CH_2Cl_2 (1000 µL) and stirring continued for 5 h. Reaction mixture was concentrated *in vacuo* and then taken up in CH_2Cl_2 (2 mL) and concentrated again (2x) to eliminate excess propargylamine. Residue taken up in CH_2Cl_2 (20 mL) and washed with 15% citric acid (10 mL), sat. $NaHCO_3$ aq. solution (10 mL), and H_2O (10 mL). Organic layer was dried ($MgSO_4$) and concentrated *in vacuo*. Crude material was purified by flash column chromatography (CH_2Cl_2/MeOH 100:0 to 95:5) to give title compound **22** as a colourless oil (21 mg, 66%). Characterisation data: FTIR ν_{max} (cm^{-1}): 3305, 2869, 1707, 1667, 1249, 1100, 844. ^{1}H NMR (500 MHz, $CDCl_3$): 1.42 (s, 9H, ^{t}Bu C*H*$_3$), 2.20 (t, *J* = 2.3 Hz, 1H, CC*H*), 2.48 (t, *J* = 5.7 Hz, 2H, PEG C*H*$_2$CONH), 3.28-3.31 (m, 2H, PEG C*H*$_2$), 3.51-3.53 (m, 2H, PEG C*H*$_2$), 3.59-3.66 (overlapped m, 45H, PEG C*H*$_2$ and PD NC*H*$_3$), 3.70-3.72 (t, *J* =

5.7 Hz, 2H, PEG CH_2), 4.02 (dd, J = 5.2, 2.3 Hz, 2H, CH_2CC), 5.11 (br, 1H, NH), 7.00 (br, 1H, NH); ^{13}C NMR (125 MHz, $CDCl_3$): 28.5 (^{t}Bu CH_3), 29.0 (CH_2CC), 36.8 (CH_2CH_2CONH), 40.4 (CH_2NHCO$_2$), 67.1 (CH_2CH_2CONH), 70.3 (PEG CH_2), 70.6-70.7 (overlapped PEG CH_2), 71.1 (CCH), 79.2 ($C(CH_3)_3$), 80.2 (CCH), 156.1 (NHCO$_2$), 171.5 (NHCOCH$_2$). LRMS (ESI+) 772 (100, $[M+NH_4]^+$), 755 (95, $[M+H]^+$); HRMS (ESI^+) calcd. for $C_{35}H_{67}N_2O_{15}$ $[M+H]^+$ 755.4542, observed: 755.4543.





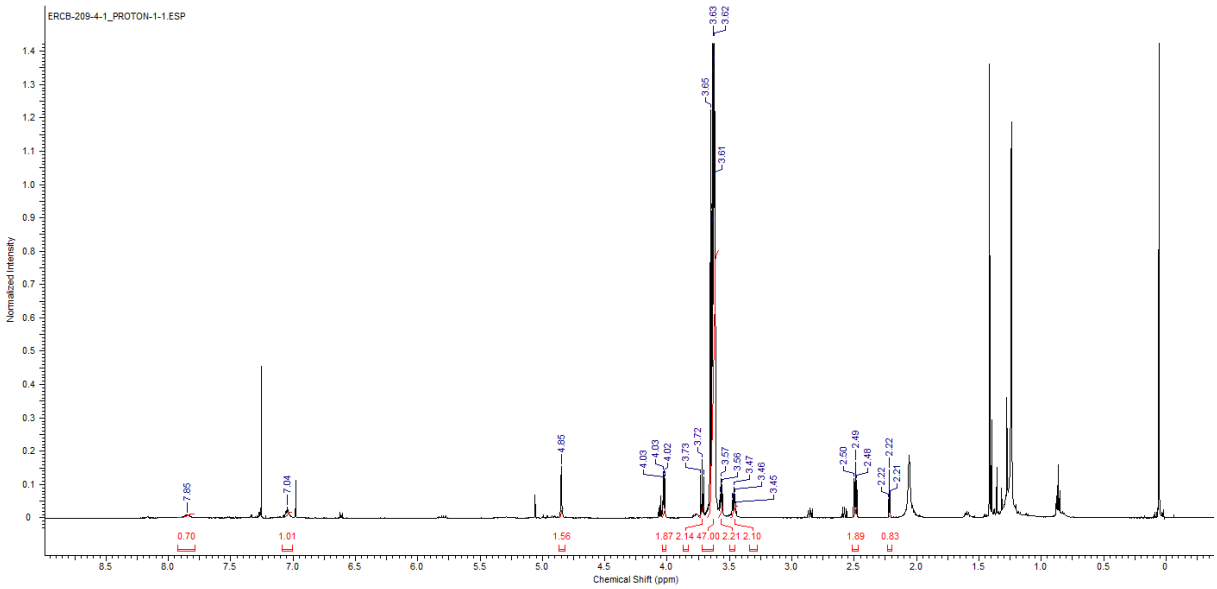
PD-propargylamide 10

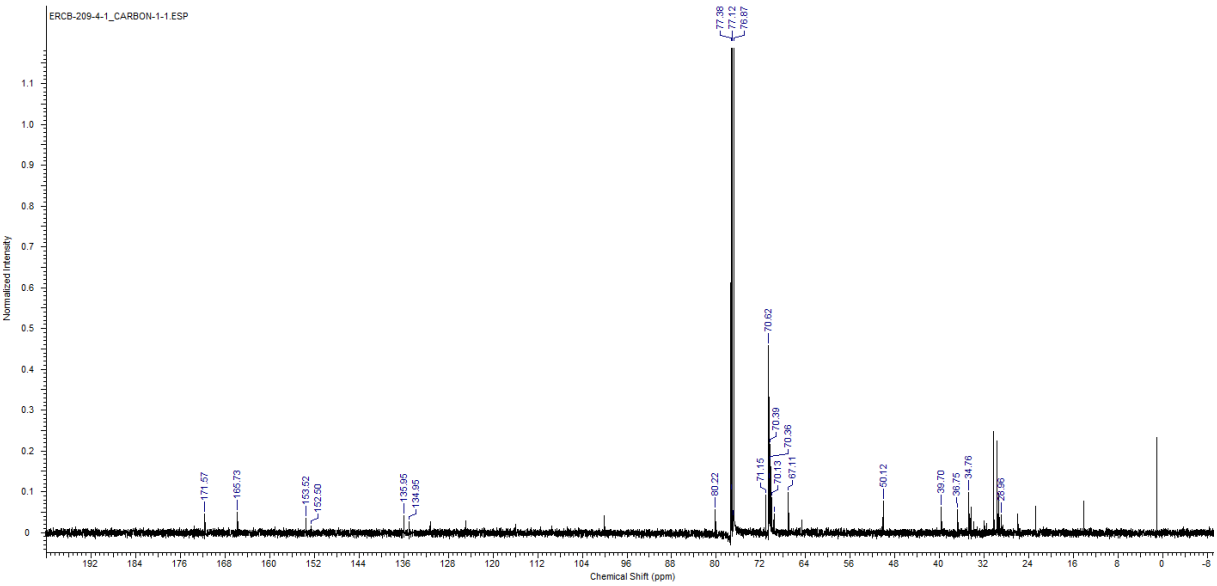


To an oven-dried RBF was added a solution of diBrPD-Me/-Acid **20** (9.5 mg, 28 µmol) in dry THF (200 µL). The stirred solution was cooled to 4 °C with an ice/water bath before addition of DCC (5.73 mg, 28 µmol) and stirring continued at 4 °C for 10 min. Pentafluorophenol (2.91 µL, 28 µmol) was then added and the reaction mixture allowed to reach ambient temperature (≈20 °C) and stirred for 2 h. The reaction mixture was then filtered through a pipette with celite plug and solids washed with cold THF. The filtrate was concentrated *in vacuo* then taken up in a minimum volume of cold THF and filtered again to ensure no precipitates remained. The PFP-activated ester was then dissolved in dry MeCN (200 µL).

To a solution of BocNH-PEG_{12}-propargylamide **22** (21 mg, 28 µmol) in CH_2Cl_2 (950 µL) was added TFA (50 µL, 654 µmol) and the reaction mixture stirred at 20 °C for 90 min. The mixture was concentrated *in vacuo* and re-suspended in CH_2Cl_2 (2 mL) repeatedly (5x) to evaporate any residual TFA. The remaining oil was dissolved in MeCN (500 µL), to which was added DIPEA (244 µL, 1400 µmol (50 eq.)), and the mixture was added to the solution of PFP-activated ester. The reaction mixture was stirred at ambient temperature for 16 h before concentrating *in vacuo*. Purification of the oily residue by flash column chromatography (CH_2Cl_2/EtOAc/MeOH 1:1:0 to 9:9:2 to 9:9:3) resulted in co-elution of two major fractions, which were separated by preparative TLC (CH_2Cl_2/EtOAc/MeOH 8:2:1) to give title compound **10** as a light brown viscous oil (14 mg, 51%) - NMR showed product to contain hydrocarbon impurities. Characterisation data: FTIR ν_{max} (cm^{-1}): 2149, 1647, 1390, 1099, 662. ^{1}H NMR (500 MHz, $CDCl_3$): 2.22 (t, *J* = 2.3 Hz, 1H, CC*H*), 2.49 (t, *J* = 5.7 Hz, 2H, PEG CH_2CONH), 3.45-3.48 (m, 2H, PEG CH_2), 3.56-3.58 (m, 2H, PEG CH_2), 3.61-3.65 (overlapped m, 47H, PEG CH_2 and PD NCH_3), 3.72 (t, *J* = 5.7 Hz, 2H, PEG CH_2), 4.02 (dd, *J* = 5.2, 2.3 Hz, 2H, CH_2CC), 4.85 (s, 2H, NCH_2CONH), 7.04 (br, 1H, N*H*), 7.85 (br, 1H, N*H*); ^{13}C NMR (125 MHz, $CDCl_3$): 29.0 (*C*H_2CC), 34.8 (N*C*H_3), 36.8 (CH_2*C*H_2CONH), 39.7 (CH_2*C*H_2NHCO), 50.1 (N*C*H_2CONH), 67.1 (*C*H_2CH$_2$CONH), 69.7 (PEG *C*H_2), 70.1 (PEG *C*H_2), 70.3-70.7 (overlapped PEG *C*H_2), 71.2 (C*C*H), 80.2 (*C*CH), 135.0 (Ar*C*Br), 136.0 (Ar*C*Br), 152.5 (Ar*C*O), 153.5 (Ar*C*O),

165.7 (NCH_2CONH), 171.6 ($NHCOCH_2CH_2$). LRMS (NSI) 217 (100), 304 (91), 996 (3, $[M+NH_4]^+$); HRMS (NSI) calcd. for $C_{37}H_{66}Br_2N_5O_{16}$ $[M+NH_4]^+$: 994.2866, observed: 994.2855.





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. Workman P, Aboagye EO, Balkwill F, et al*. Guidelines for the welfare and use of animals in cancer research. Br J Cancer. 2010;102:1555–1577*
3. Vassileva V, Allen CJ, Piquette-Miller M*. Effects of sustained and intermittent paclitaxel therapy on tumor repopulation in ovarian cancer. Mol Cancer Ther. 2008;7:630–637.*
4. Ullman-Culleré MH, Foltz CJ*. Body condition scoring: a rapid and accurate method for assessing health status in mice. Lab Anim Sci. 1999;49:319–323.*
5. J. P. M. Nunes, M. Morais, V. Vassileva, E. Robinson, V. S. Rajkumar, M. E. B. Smith, R. B. Pedley, S. Caddick, J. R. Baker and V. Chudasama, *Chem. Commun.*, 2015, **51**, 10624-10627.
6. F. F. Schumacher, J. P. M. Nunes, A. Maruani, V. Chudasama, M. E. B. Smith, K. A. Chester, J. R. Baker and S. Caddick, *Org. Biomol. Chem*, 2014, **12**, 7261-7269.
7. 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.