SUPPORTING INFORMATION

Protease-Cleavable Linkers Modulate the Anticancer Activity of Non-Internalizing Antibody-Drug Conjugates.

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List of Abbreviations

AcOEt	Ethyl acetate
ADC	Antibody-Drug conjugate
Ala	Alanine
Arg	Arginine
Cit	Citrulline
CH_2Cl_2	Methylene chloride
δ	Chemical shift
DMF	*N*,*N*-Dimethylformamide
EDC·HCl	*N*-(3-Dimethylaminopropyl)-*N*′-ethylcarbodiimide hydrochloride
ESI	Electrospray ionization
FPLC	Fast protein liquid chromatography
Hex	Hexane
HBSS	Hank's Balanced Salt Solution
iPr_2NEt	*N*,*N*-Diisopropylethylamine
LC	Light chain
Mc	Maleimidocaproyl
MMAE	Monomethyl auristatin E
PABC	*p*-aminobenzyl carbonate/carbamate
PABOH	*p*-aminobenzyl alcohol
PBS	Phosphate buffered saline
ppm	Part per million
SDS	Sodium dodecyl sulfate
SEM	Standard error of the mean
TCEP	tris(2-carboxyethyl)phosphine.
THF	Tetrahydrofuran
Val	Valine

General Remarks and Procedures

Anhydrous solvents were purchased from Acros or Fluka. All other solvents were used as supplied by Fisher Chemicals, Merck or Sigma Aldrich in HPLC or analytical grade. ADC IgG(F16)-Val-Cit-MMAE (**1**),[S1] and linker-drug modules Mc-Val-Arg-MMAE, Mc-Val-Ala-MMAE and Mc-Val-Lys-MMAE[S2] were prepared according to procedure reported previously. Free MMAE and Mc-Val-Cit-MMAE were purchased from Levena Biopharma (No.9 Weidi Road, Qixia District, Nanjing, 210046, China). All other reagents were purchased from Sigma Aldrich, Acros, ABCR or TCI and used as supplied. All reactions using anhydrous conditions were performed using oven-dried glassware under argon atmosphere. Silica for flash column chromatography (high-purity grade, pore size 60 Å) was purchased from Sigma Aldrich. Reactions were monitored by thin layer chromatography using Merck silica gel 60 F254 TLC glass plates. Concentration under reduced pressure was performed by rotary evaporation at 40 °C at the appropriate pressure. Yields refer to chromatographically purified and spectroscopically pure compounds, unless noted otherwise.

Proton (^{1}H) nuclear magnetic resonance (NMR) spectra were recorded on a Bruker AV400 (400 MHz). Carbon (^{13}C) NMR spectra were recorded on a Bruker AV400 (100 MHz) spectrometer. Shifts are given in ppm using residual solvent as the internal standard. Coupling constants (*J*) are reported in Hz with the following abbreviations used to indicate splitting: s = singlet, d = doublet, t = triplet, q = quartet, m = multiplet.

High-Resolution Mass Spectrometry (HRMS) spectra and analytical Reversed-Phase Ultra Performance Liquid Chromatography (UPLC) were recorded on a Waters Xevo G2-XS QTOF coupled to a Waters Acquity UPLC H-Class System with PDA UV detector, using a ACQUITY UPLC BEH C18 Column, 130 Å, 1.7 µm, 2.1 mm × 50 mm at a flow rate of 0.6 ml min^{-1} with linear gradients of solvents A and B (A = Millipore water with 0.1% formic acid [FA], B = MeCN with 0.1% formic acid [FA]). Preparative reversed-phase high-pressure liquid chromatography (RP-HPLC) were per-

formed on a Waters Xterra 5 μm 150 Å 10 ×150 mm C18 column at a flow rate of 2.8 ml min^{-1} with linear gradients of solvents A and B (A = Millipore water with 0.1% trifluoroacetic acid [TFA], B = MeCN with 0.1% trifluoroacetic acid [TFA]).

MS analyses of ADC products were performed on a Waters Xevo G2-XS Qtof instrument (ESI-ToF-MS) coupled to a Waters Acquity UPLC H-Class System using a 2.1 × 50 mm Acquity BEH300 C4 1.7 μm column (Waters). 0.1% formic acid in water (solvent A) and 0.1% formic acid in acetonitrile (solvent B) were used as mobile phase at a flow rate of 0.4 mL/min. Gradient was programmed as follows: after 1.5 min isocratic with 95% solvent A, stepwise change from 95% solvent A to 95% solvent B in 4.5 min (10% increase every 0.5 min), back to 95% solvent A in 0.5 min, linearly to 95% solvent B and back to 95% solvent A in 2.25 min (last step repeated twice). Analytical size exclusion chromatography of ADCs was performed on FPLC (Äkta, GE Healthcare) and protein were separated by a Superdex200 10/300GL column (GE Healthcare).

F16-MMAE Conjugates Preparation

Anti-Tenascin C-A1 IgG(F16) mAb was reduced with 30 equivalents (calculated on the basis of antibody monomers, each containing a single cysteine residue) of tris(2-carboxyethyl)phosphine hydrochloride (TCEP-HCl, ACBR) in PBS (pH = 7.4). The reduced protein was purified by size exclusion chromatography on a HiPrep 26/10 Desalting column (GE Healthcare). PBS was used as a mobile phase at a flow rate of 2 mL/min. The recovered protein was pooled and concentrated using Vivaspin® Turbo 15 (Sartorious) in order to remain in the capacity limit of the FPLC-loop. Ten equivalents of Mc-Linker-MMAE compounds were dissolved in DMSO and added to the reduced protein with a final DMSO content of 10% (*v/v*). The mixture was stirred for 1 hour at room temperature, then cysteine was added at a final concentration of 1 mM and stirred 30 min. Final product was FPLC-purified as described above and characterized by MS, analytical FPLC and SDS-page.

ADCs were then concentrated (Vivaspin®) at suitable concentrations for in vivo assays, snap-frozen in liquid nitrogen and stored at -80 ºC.

Cell Culture

A-431 epidermoid carcinoma cells (ATCC, CRL-1555) and MDA-MB-231 (ATCC, HTB-26) were grown in DMEM (Gibco) supplemented with 10% FBS (Gibco) and Antibiotic-Antimycotic (Gibco) and incubated at 378C in 5% CO_2 atmosphere.

Animal Studies

Ten to twelve weeks old female Balb/c nude mice were obtained from Janvier Laboratories (France). A431 cells (2.8×10^6 in 120 µL HBSS), were implanted subcutaneously in the flank. Animals were sacrificed when tumors volumes reached a maximum of 2,000 mm^3 or weight loss exceeded 15%. Experiments were performed under project licenses issued by the Veterinäramt des Kantons Zürich, Switzerland (Bew. Nr. 42/2012 and Nr. 027/15).

Dose Escalation Study

Healthy Balb/c nude mice (n = 1) were injected with the antibody-drug conjugates **1**-**4**, at increasing doses, from 12.5 to 50 mg/Kg. The body weight of mice was monitored daily.

Therapy Studies

When tumors reached an average volume of 100–120 mm^3 (typically 7 to 9 days after subcutaneous tumor implantation),mice were randomly grouped (n = 4 or n = 5) and injected intravenously (i.v.) into the lateral tail vein. In a first therapy experiment (**Figure 3B**), mice (n = 4) were injected every 3 days, 4 times in total, with the antibody-drug conjugates, at a dose of 3 mg/Kg, or with vehicle (PBS). In the subsequent therapy study (**Figure 6**), mice (n = 5) were injected every 3 days, 4 times in total, with the antibody–drug conjugates, at a dose of 7 mg/Kg, or with vehicle (PBS). The body weight of mice was monitored daily. Tumor volumes were measured with a digital caliper (*volume*

$= length \times width^2 \times 0.5$). Results were expressed as tumor volume in mm^3 ± SEM. Animals were sacrificed when tumor reached a maximum of 2,000 mm^3 or weight loss exceeded 15%.

Quality Control of ADC Products

Mass Spectrometry

Samples were diluted to about 0.1 mg/mL and LC-MS was performed on a Waters Xevo G2XS Qtof instrument (ESI-ToF-MS) coupled to a Waters Acquity UPLC H-Class System using a 2.1 × 50 mm Acquity BEH300 C4 1.7 µm column (Waters). 0.1% formic acid in water (solvent A) and 0.1% formic acid in acetonitrile (solvent B) were used as mobile phase at a flow rate of 0.4 mL/min. Gradient was programmed as follows: after 1.5 min isocratic with 95% solvent A, stepwise change from 95% solvent A to 95% solvent B in 4.5 min (10% increase every 0.5 min), back to 95% solvent A in 0.5 min, linearly to 95% solvent B and back to 95% solvent A in 2.25 min (last step repeated twice).

Gel Filtration Analysis

100 µL of diluted sample (final concentration 0.3-0.5 mg/mL) were loaded on FPLC (Äkta, GE Healthcare) and protein were separated by a Superdex200 10/300GL column (GE Healthcare) previously equilibrated with 1 CV PBS, using PBS as mobile phase at a flow rate of 0.5 mL/min (column pressure limit set at 1.5 MPa). Proteins were detected by an UV-detector at a wavelength of 280 nm.

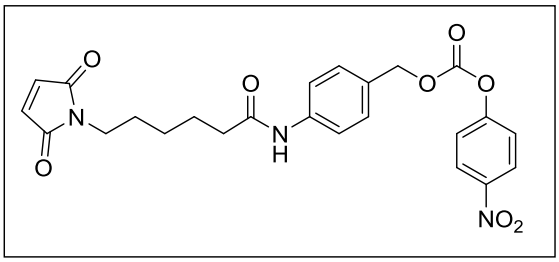
SDS Page

Protein samples were diluted to 0.2-0.3 mg/mL in PBS and mixed with either reducing or non-reducing 5X Loading buffer. Samples were denatured 5’ at 95 °C and loaded on NuPAGE 4-12% Bis-Tris Gel (NP0335, ex. Novex by Life Technologies). 1X MES NuPAGE (NP0002, ex. Novex by Life Technologies) was used as running buffer and elecopphoresis was performed at 180 V, 110 mA for 1 h. The gel was rinsed with deionized water and stained in Coomassie blue for 15-20’ on an orbital shaker. Staining solution was discarded, gel was rinsed 3 times with deionized water and immerged in destaining solution (10% acetic acid /30% MeOH in mQ water) for 3-12 h on and or-

bital shaker. Destaining solution was discarded and recycled, the gel was rinsed with deionized water and scanned.

Synthesis of Mc-NC-MMAE (7)

MC-PABC-PNP (**6**)

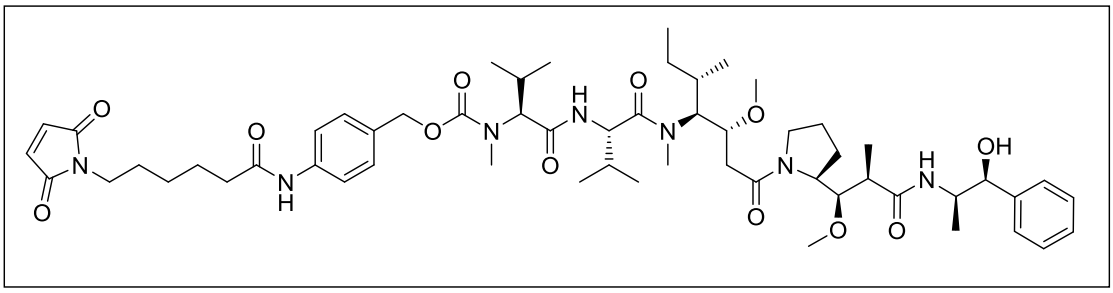


$C_{24}H_{23}N_3O_8$
MW: 481.4610

6-maleimidohexanoic acid (**5**, 200 mg; 0.95 mmol; 1.0 eq) was dissolved in CH_2Cl_2 (9 ml) and the solution was cooled to 0 °C. EDC·HCl (200 mg; 1.04 mmol; 1.1 eq), *i*Pr_2NEt (330 µl; 1.89 mmol; 2 eq) and PABOH (128 mg; 1.04 mmol; 1.1 eq) were added subsequently. The reaction was stirred overnight at room temperature. The mixture was diluted with AcOEt (100 ml), washed with $KHSO_4$ (1M aqueous solution; 3 × 30 ml), $NaHCO_3$ (saturated aqueous solution; 2 × 30 ml) and brine (1 × 20 ml). The organic phase was dried and concentrated, affording 183 mg of amide (61%). The latter was suspended in THF and drops of DMF were added until complete dissolution. 4-nitrophenyl chloroformate (300 mg, 1.5 mmol, 2.6 eq) and pyridine (140 µl; 1.74 mmol; 3 eq) were added and the mixture was stirred at room temperature for 4 hours. The solution was concentrated and the crude was purified by flash column (eluent 1:1 AcOEt/Hex), to afford compound **6** (220 mg; 0.46 mmol; 78% yield).

R_f = 0.39 (AcOEt/Hex 5:3); ^{1}H NMR (400 MHz, $CDCl_3$) δ 8.27 (d, J = 9.3 Hz, 2H), 7.57 (d, J = 8.3 Hz, 2H), 7.40 (d, J = 8.3 Hz, 2H), 7.37 (d, J = 9.3 Hz, 1H), 7.27 (bs, 1H), 6.67 (s, J = 3.1 Hz, 2H), 5.25 (s, 2H), 3.53 (t, J = 7.2 Hz, 2H), 2.36 (t, J = 7.5 Hz, 1H), 1.80-1.73 (m, 2H), 1.68-1.60 (m, 2H), 1.43-1.32 (m, 2H) ppm; ^{13}C NMR (101 MHz, $CDCl_3$) δ 171.0, 155.7, 152.6, 134.2, 129.9, 125.4, 121.9, 120.1, 120.1, 120.0, 70.8, 37.6, 37.6, 28.3, 26.3, 24.9 ppm.

Mc-NC-MMAE (**7**)

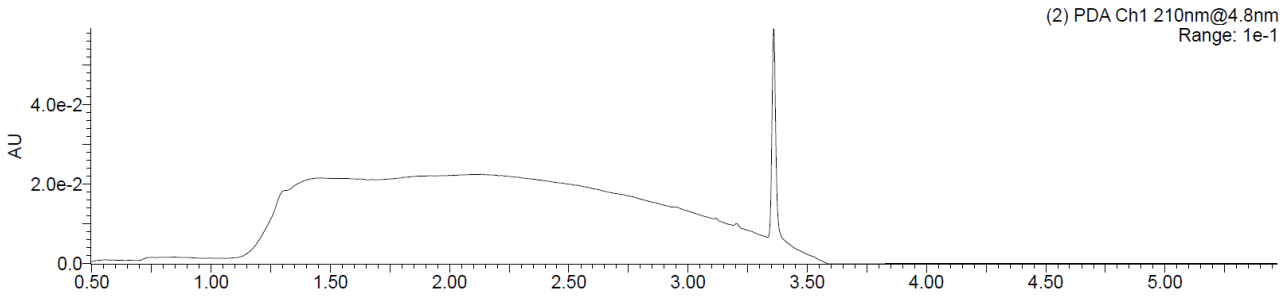


$C_{57}H_{85}N_7O_{12}$
MW: 1060.3440

MMAE·TFA (15 mg; 0.018 mmol; 1.1 eq) was dissolved in dry DMF (300 µl) under nitrogen atmosphere. Compound **6** (8 mg; 0.016 mmol; 1 eq), HOAt (1.1 mg dissolved in 10 µl of DMF; 0.008 mmol; 0.5 eq) and *i*Pr_2NEt (14 µl; 0.083 mmol; 5.0 eq) were added subsequently. The mixture was stirred at room temperature for 48 hours and concentrated under vacuum. The crude was diluted with a H_2O/MeCN 1:1 mixture (1 ml), and purified over RP-HPLC (Waters Xterra 5 µm 150 Å 10 ×150 mm C18 column, 5% MeCN in 0.1% aq. TFA to 80% over 30 min). Product containing fractions were lyophilized overnight to obtain compound **7** as a white solid (6 mg, 5.6 µmol, 35% yield).

MS (ESI) *m/z* calcd. for $[C_{57}H_{86}N_7O_{12}]^+$: 1060.63 $[M+H]^+$, found: 1060.70; *m/z* calcd. for $[C_{57}H_{85}N_7NaO_{12}]^+$: 1082.61 $[M+Na]^+$, found: 1082.70.

HPLC Purity analysis: Parameters: BEH C18 Column, 130 Å, 1.7 µm, 2.1 mm × 50 mm at a flow rate of 0.6 ml min^{-1}, 5% MeCN in 0.1% aq. FA to 80% MeCN in 6 min.



ADC Metabolite Analysis

ADCs were injected in the lateral vain of Balb/c nude mice at a dose of 10 mg/Kg. After 24 or 48 h., mice were sacrificed and blood was punctured from their heart and transferred into Li-Heparin coated tubes (BD). Tubes were centrifuged at 3,000 g for 5 min. Plasma was recovered and purified by affinity chromatography onto an antigen-coated resin, based on TnC A1 coupled to CNBr-activated Sepharose (GE Healthcare), washed with PBS and then eluted with 0.1 M glycine solution (pH = 3). Samples were analyzed by MS spectrometry (**Figure S1**). Deconvolution of raw data was processed in the molar mass ranges of 20-60 and 10-30 Da. Quantitative analysis of ADC metabolites was obtained by calculation of the ratio between the MS peak intensity of individual fragments and the sum of MS signal intensities of all detected peaks (100%).

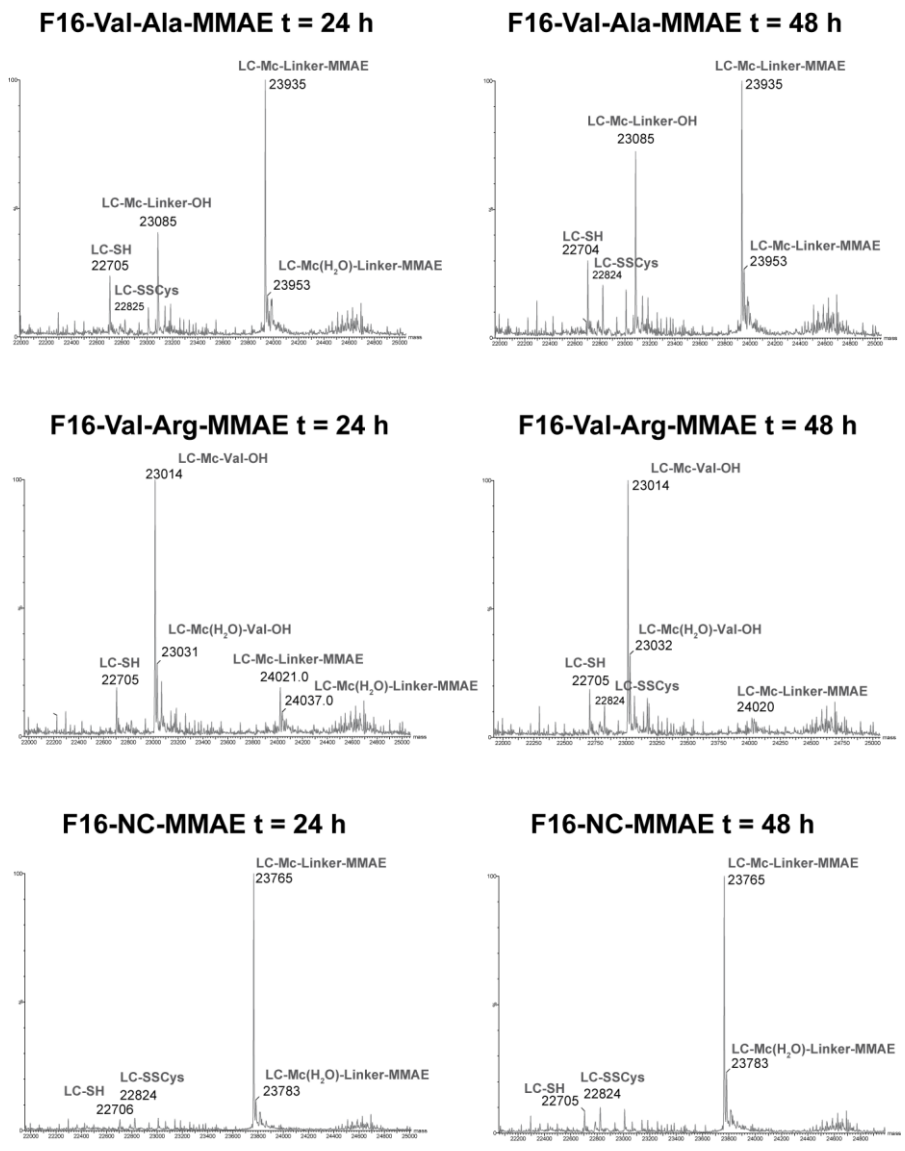


Figure S 1. Representative examples (ADCs: F16-Val-Ala-MMAE; F16-Val-Arg-MMAE and F16-NC-MMAE) of ADC metabolite analysis by MS spectrometry after 24 and 48 h after i. v. injection. The assignment of all the identified peaks is reported in grey. The relative intensity of the observed peaks and the molecular structure of the metabolites is shown in Figure 5.

Statistical Analysis of Therapy Experiments

Differences in tumor volume between therapeutic groups were compared using the two-way ANOVA analysis with Bonferroni post-test of Graphpad Prism 6 (La Jolla, CA, USA).

Tumor Size (mg) - 3 mg/kg experiment (Fig 3B)

*Compound **1** (F16-ValCit-MMAE) vs. Vehicle:*

non-significant differences

*Compound **2** (F16-ValArg-MMAE) vs. Vehicle:*

non-significant differences

*Compound **3** (F16-ValLys-MMAE) vs. Vehicle:*

non-significant differences

*Compound **4** (F16-ValAla-MMAE) vs. Vehicle:*

day 18 $p < 0.05$

from day 19 $p < 0.01$

*Compound **1** (F16-ValCit-MMAE) vs compound **2** (F16-ValArg-MMAE):*

non-significant differences

*Compound **1** (F16-ValCit-MMAE) vs compound **3** (F16-ValLys-MMAE):*

non-significant differences

*Compound **1** (F16-ValCit-MMAE) vs. compound **4** (F16-ValAla-MMAE):*

non-significant differences

*Compound **2** (F16-ValArg-MMAE) vs. compound **3** (F16-ValLys-MMAE):*

non-significant differences

*Compound **2** (F16-ValArg-MMAE) vs. compound **4** (F16-ValAla-MMAE):*

from day 19 $p < 0.05$

*Compound **3** (F16-ValLys-MMAE) vs. compound **4** (F16-ValAla-MMAE):*

non-significant differences

Tumor Size (mg) - 7 mg/kg experiment (Fig 6A)

*Compound **1** (F16-ValCit-MMAE) vs. Vehicle:*

day 14 $p < 0.05$

day 15 $p < 0.01$

day 16 $p < 0.001$

from day 17 $p < 0.0001$

*Compound **4** (F16-ValAla-MMAE) vs. Vehicle:*

day 14 $p < 0.01$

day 15 $p < 0.001$

from day 16 $p < 0.0001$

*Compound **5** (F16-NC-MMAE) vs. Vehicle:*

non-significant differences

*Compound **1** (F16-ValCit-MMAE) vs compound **4** (F16-ValAla-MMAE):*

non-significant differences

*Compound **1** (F16-ValCit-MMAE) vs compound **5** (F16-NC-MMAE):*

day 15 $p < 0.05$

day 16 non-significant differences

day 17 $p < 0.01$

from day 18 $p < 0.0001$

*Compound **4** (F16-ValAla-MMAE) vs. compound **5** (F16-NC-MMAE):*

day 14 $p < 0.05$

day 15 $p < 0.01$

day 16 $p < 0.05$

day 17 $p < 0.001$

from day 18 $p < 0.0001$

Body Weight Change (%) - 7 mg/kg experiment (Fig 6B)

*Compound **1** (F16-ValCit-MMAE) vs. Vehicle:*

day 22 $p < 0.05$

day 23 $p < 0.05$

day 24 $p < 0.05$

from day 25 non-significant differences

*Compound **4** (F16-ValAla-MMAE) vs. Vehicle:*

day 22 $p < 0.05$

day 23 $p < 0.01$

day 24 $p < 0.01$

day 25 $p < 0.05$

from day 26 non-significant differences

*Compound **5** (F16-NC-MMAE) vs. Vehicle:*

non-significant differences

*Compound **1** (F16-ValCit-MMAE) vs compound **4** (F16-ValAla-MMAE):*

non-significant differences

*Compound **1** (F16-ValCit-MMAE) vs compound **5** (F16-NC-MMAE):*

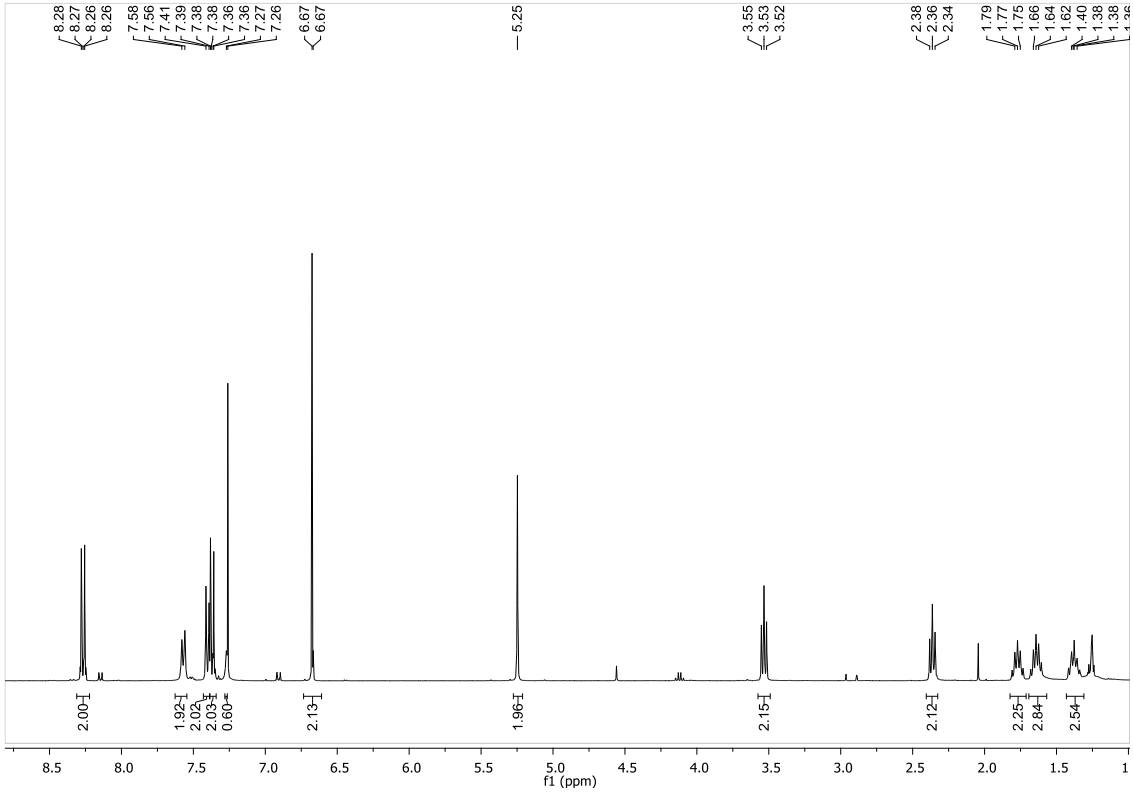
non-significant differences

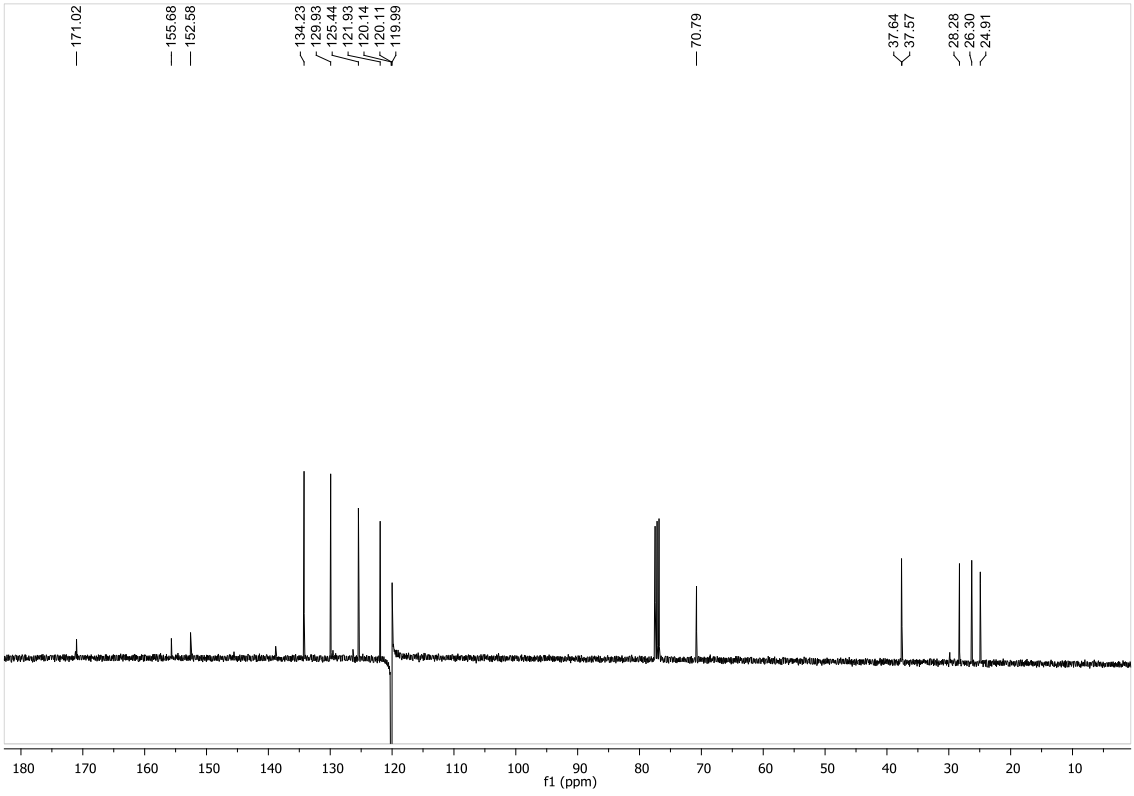
*Compound **4** (F16-ValAla-MMAE) vs. compound **5** (F16-NC-MMAE):*

non-significant differences

Appendix of NMR Data

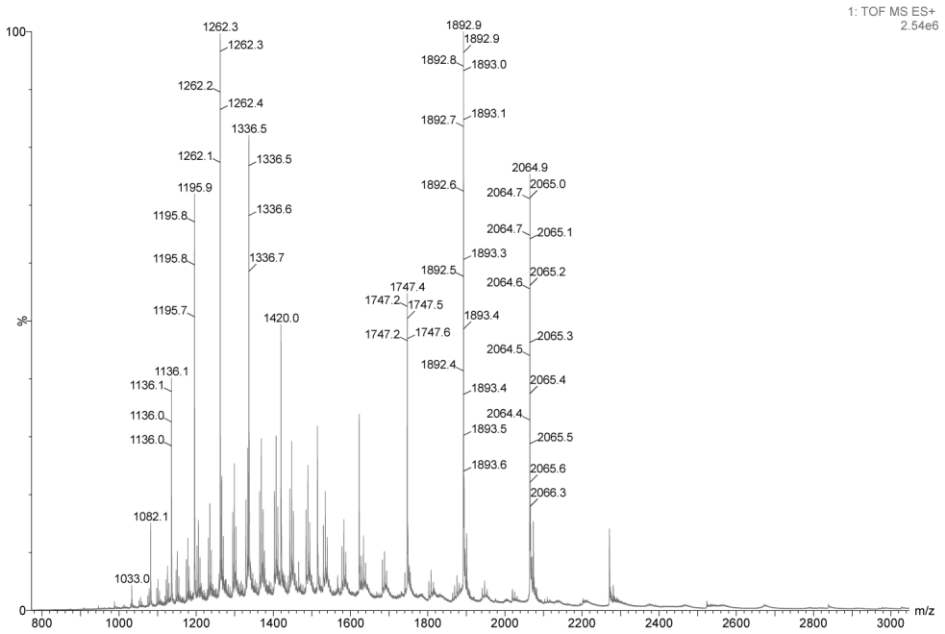
MC-PABC-PNP (**6**)

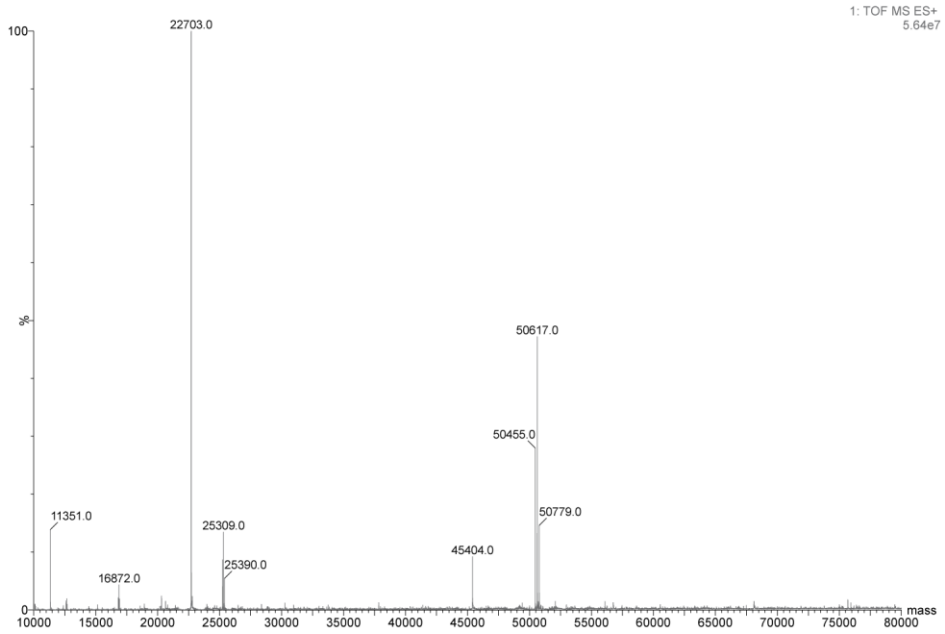




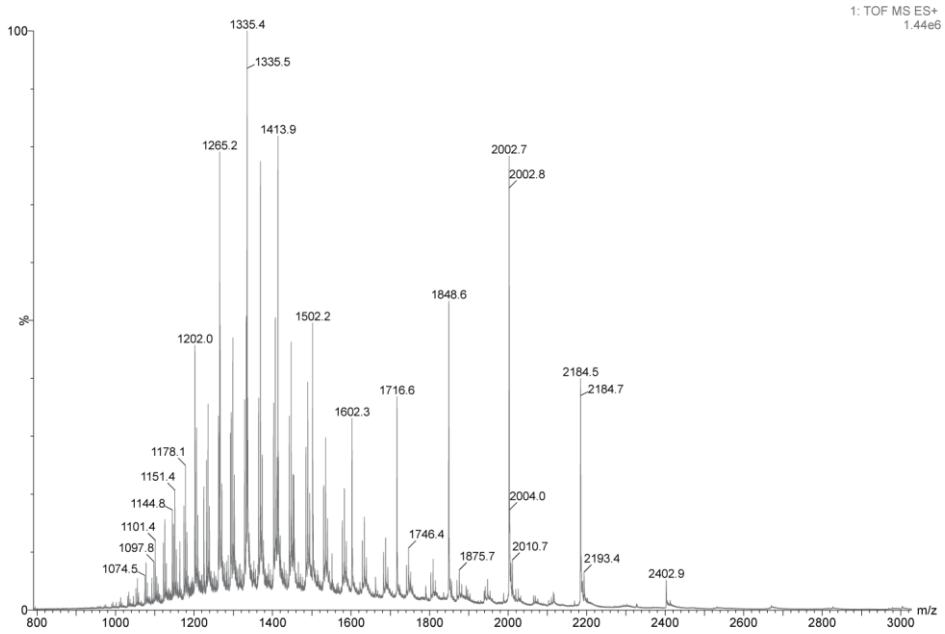
Appendix of Mass Spectrometry Data

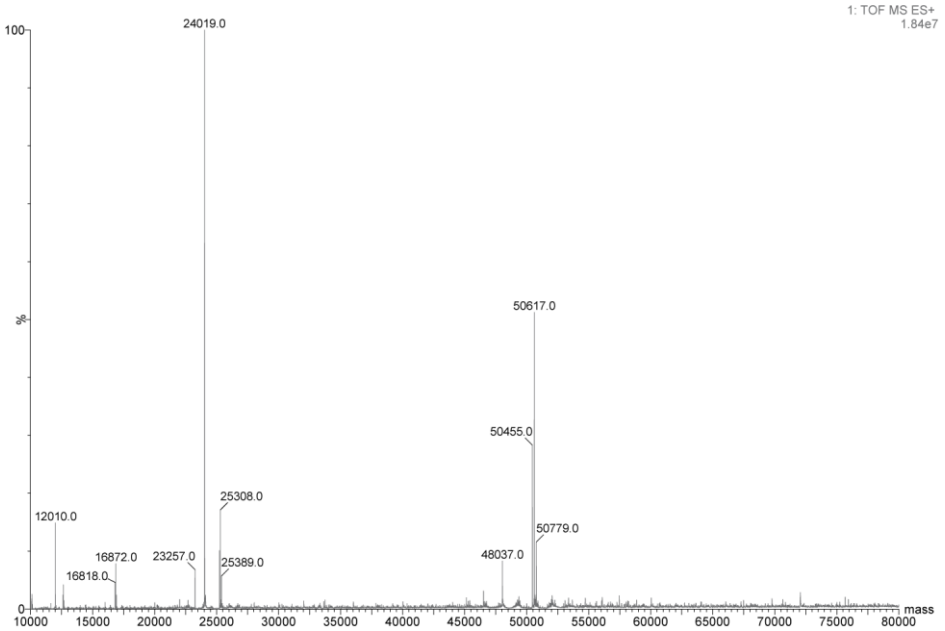
IgG(F16) mAb



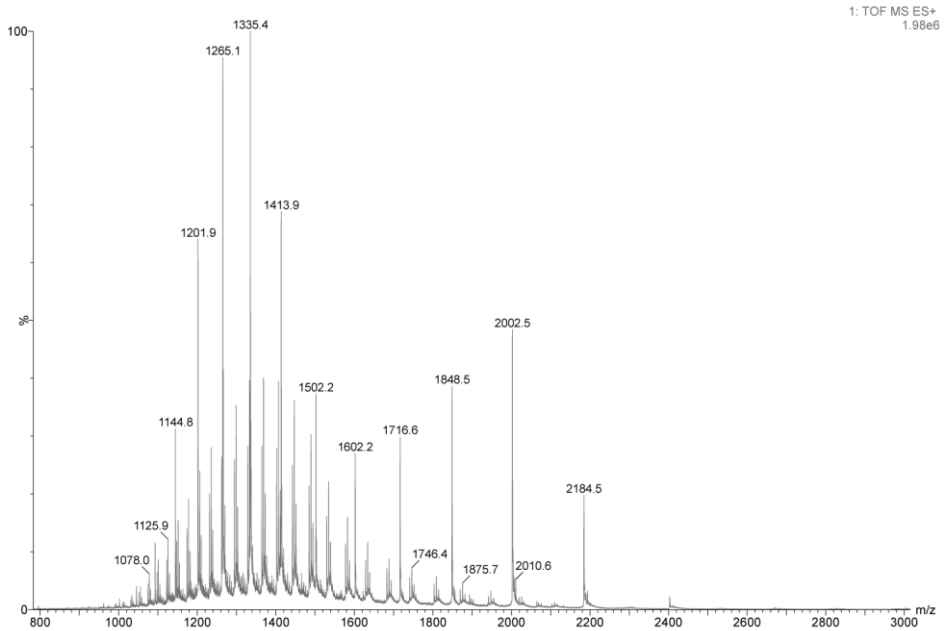


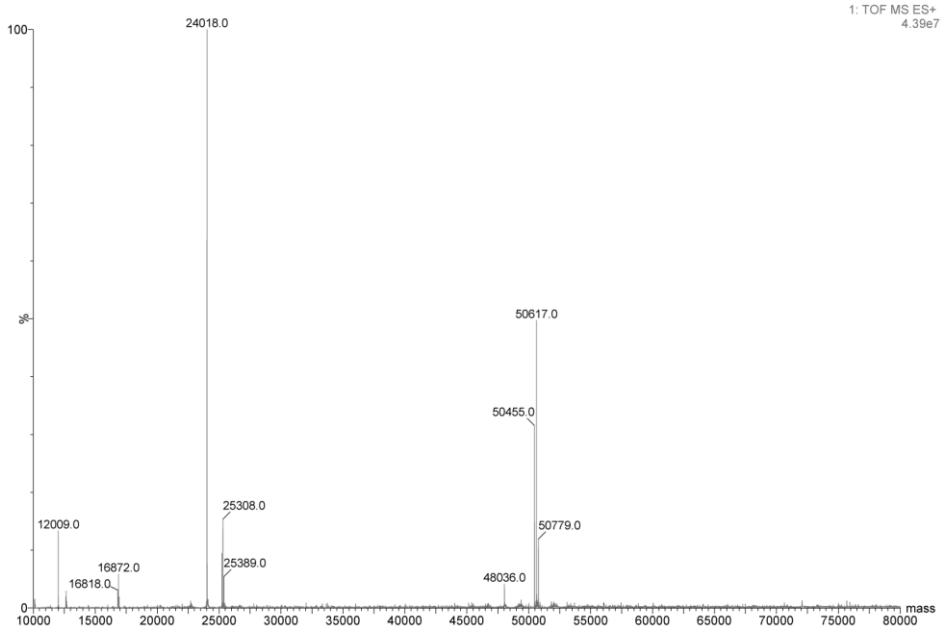
F16-Val-Cit-MMAE (**1**)



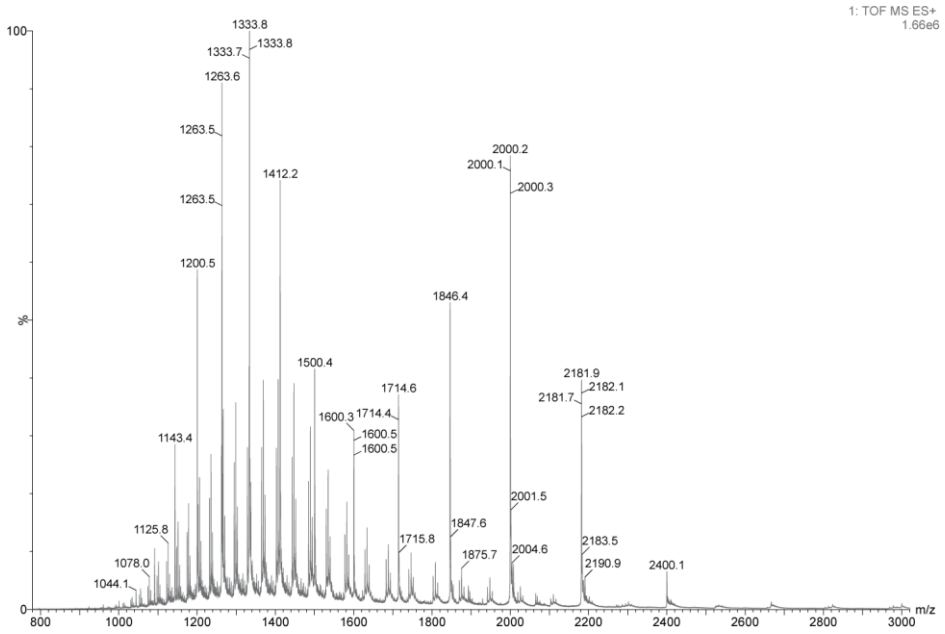


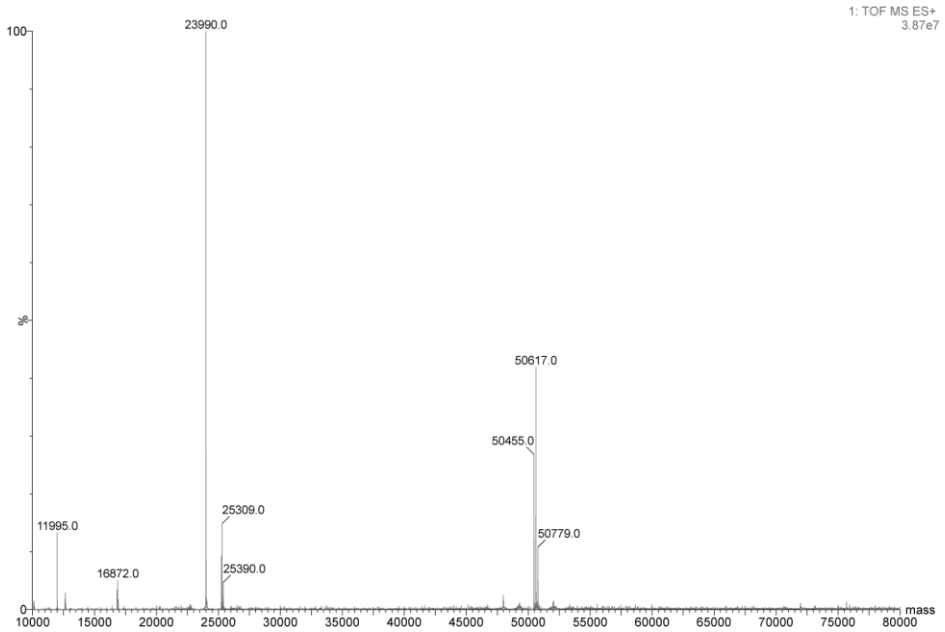
F16-Val-Arg-MMAE (**2**)



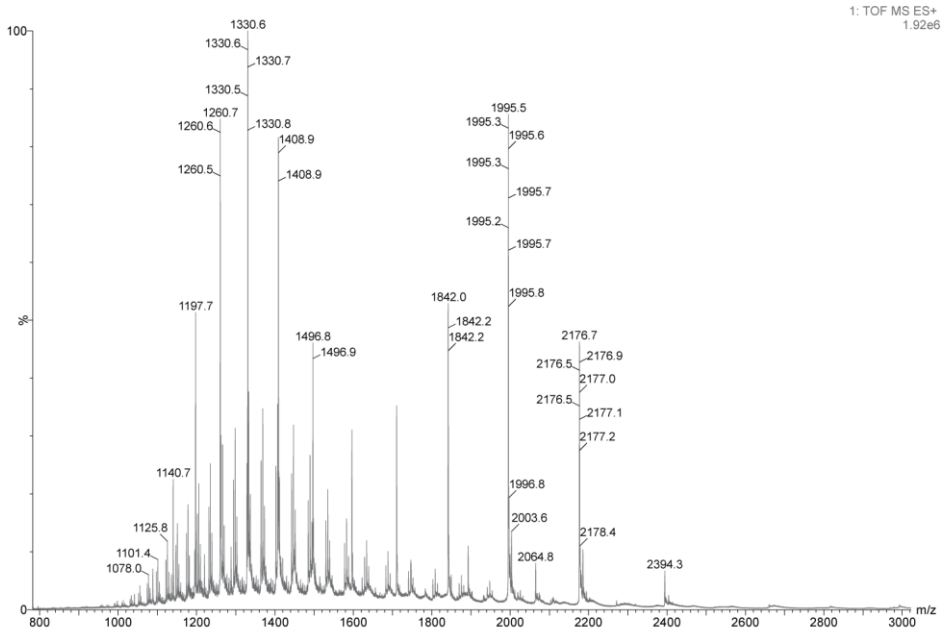


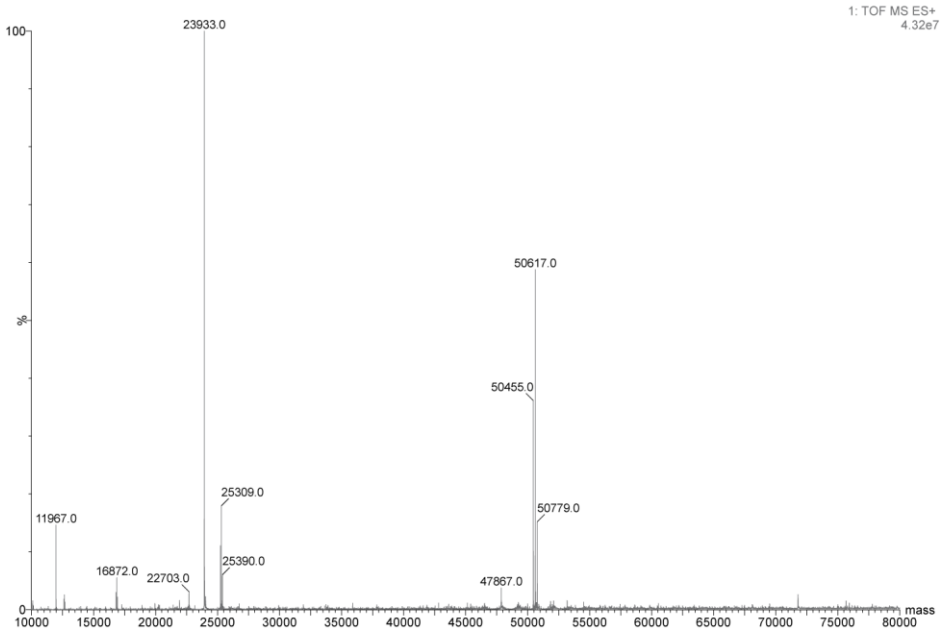
F16-Val-Lys-MMAE (**3**)



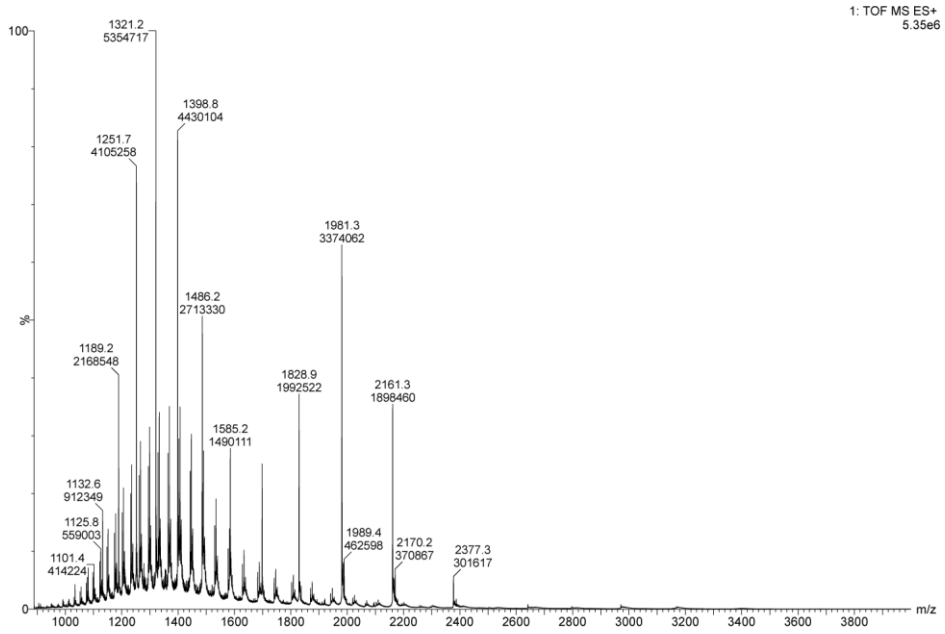


F16-Val-Ala-MMAE (**4**)





F16-NC-MMAE (**8**)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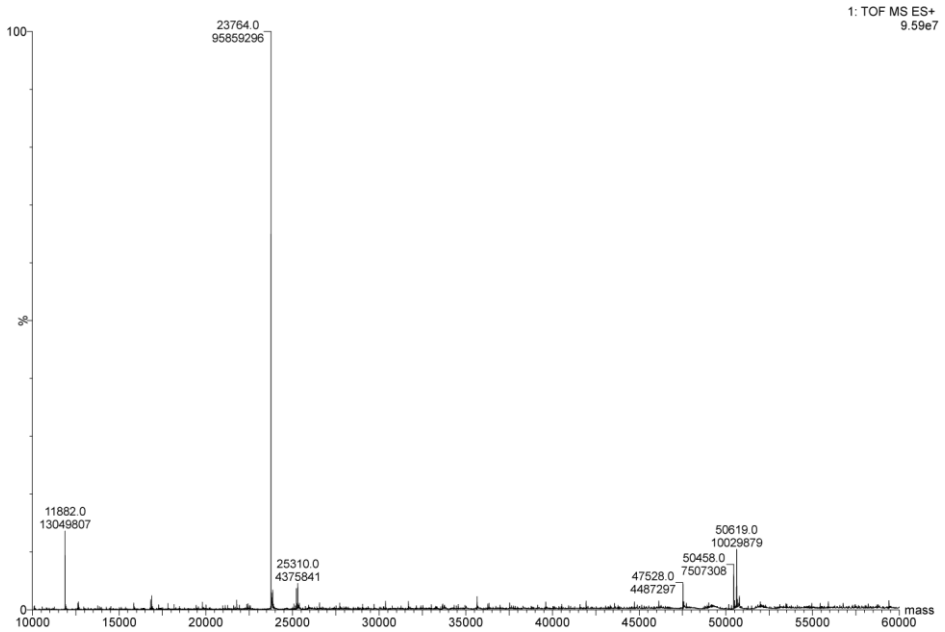
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S1) Gébleux, R., Stringhini, M., Casanova, R., Soltermann, A., and Neri, D. (2017) Non-internalizing antibody-drug conjugates display potent anti-cancer activity upon proteolytic release of monomethyl auristatin E in the sub-endothelial extracellular matrix. *Int. J. Cancer 140*, 1670-1679.

S2) Cazzamalli, S., Dal Corso, A., and Neri, D. (2016) Linker stability influences the anti-tumor activity of acetazolamide-drug conjugates for the therapy of renal cell carcinoma. *J. Control. Release 246*, 39-45.