Electronic Supplementary Material (ESI) for Organic & Biomolecular Chemistry.

Impact of cathepsin B-sensitive triggers and hydrophilic linkers on in vitro efficacy of novel site-specific antibody-drug conjugates.

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NMR data

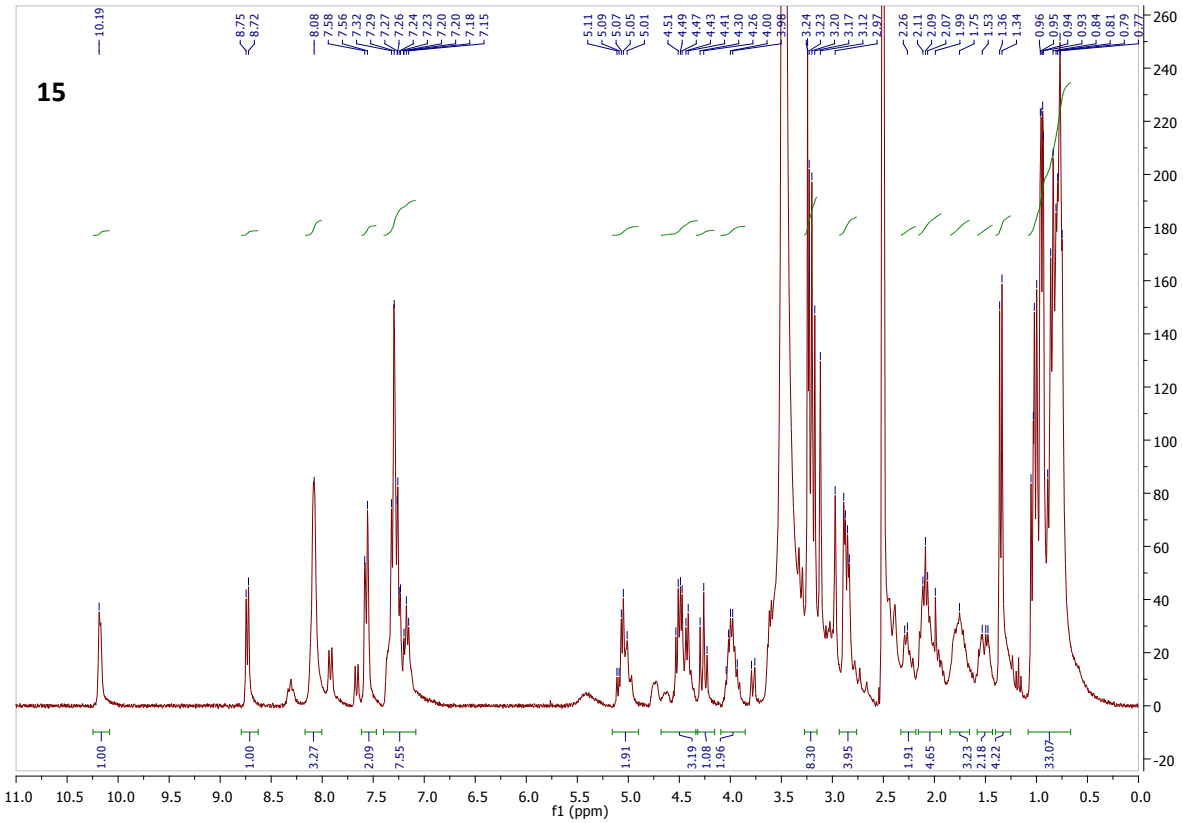


Figure S1: 1H NMR for compound **15**

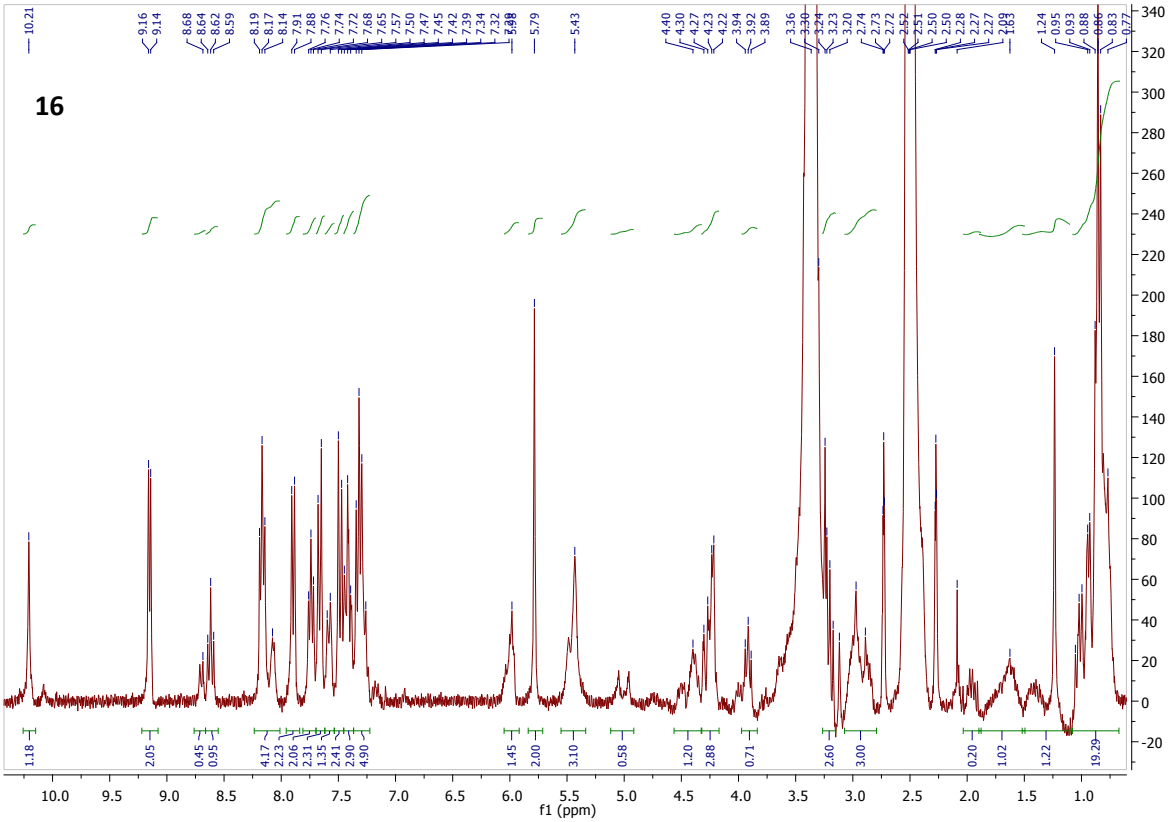


Figure S2: 1H NMR for compound **16**

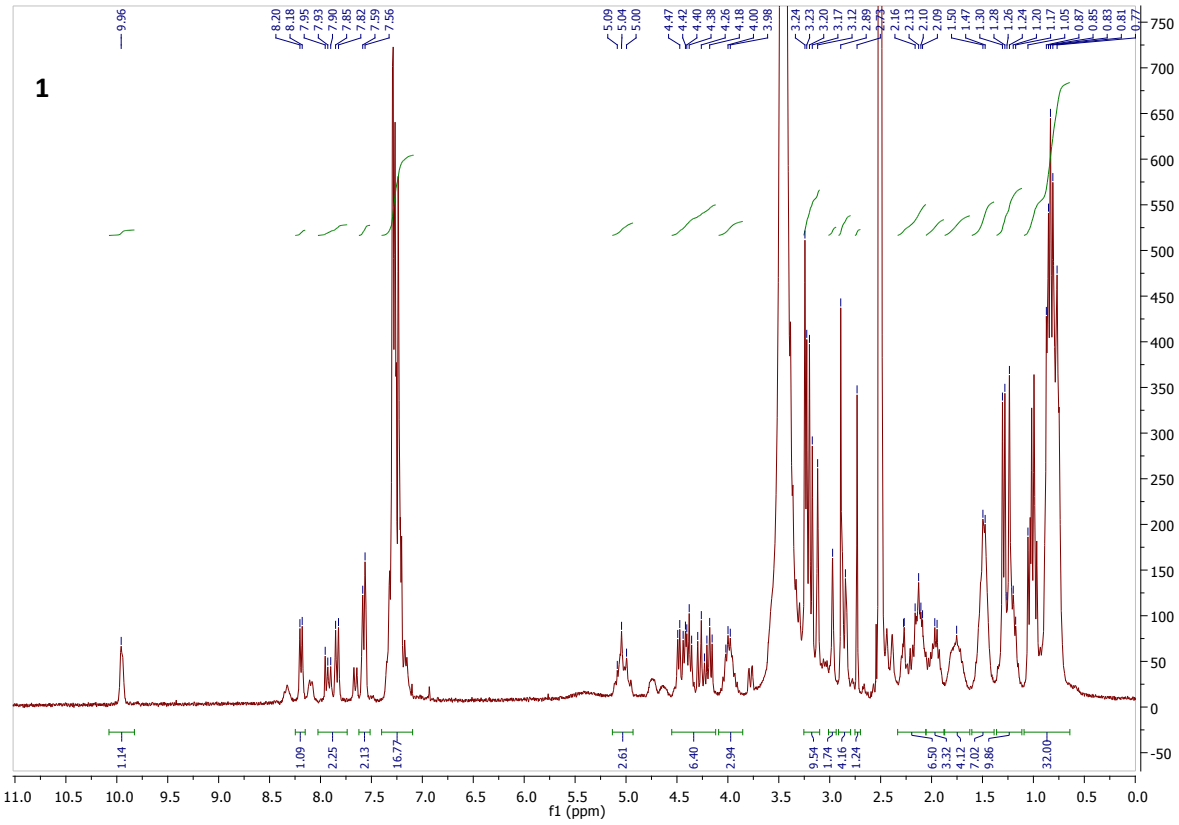


Figure S3: 1H NMR for compound **1**

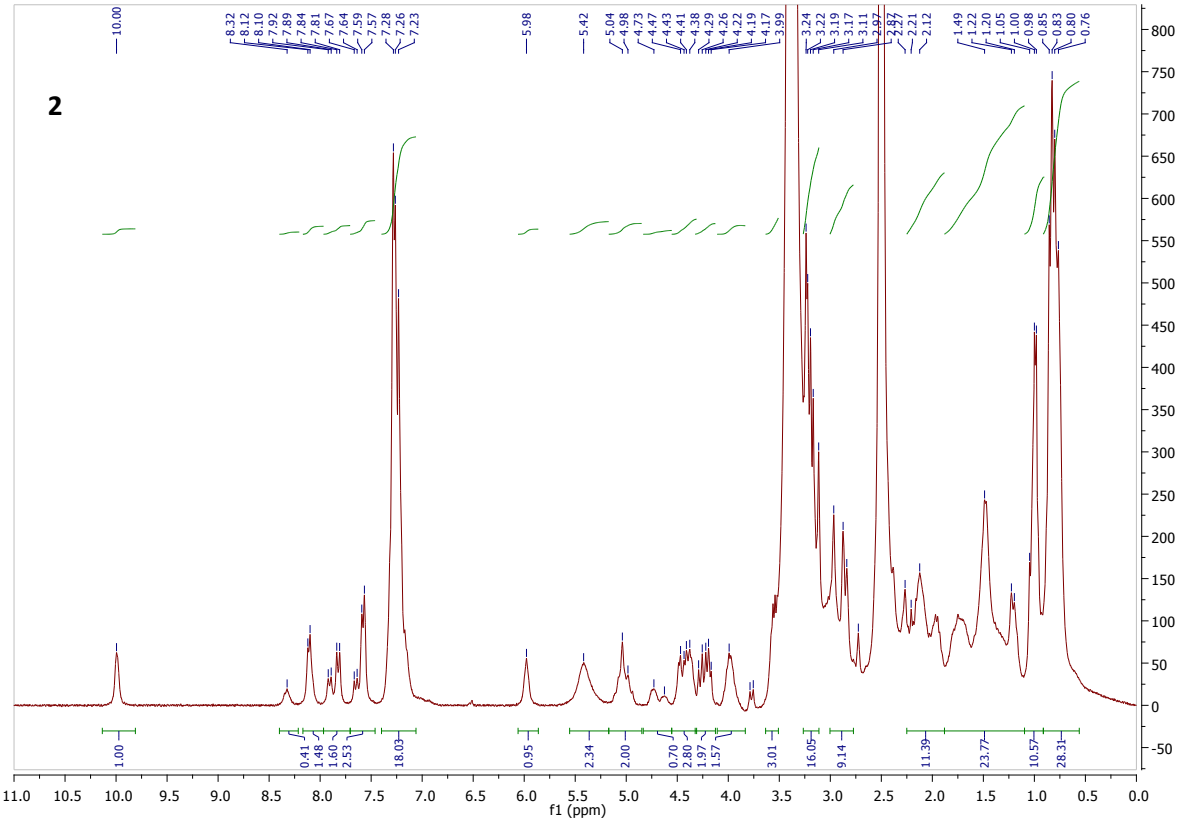


Figure S4: 1H NMR for compound 2

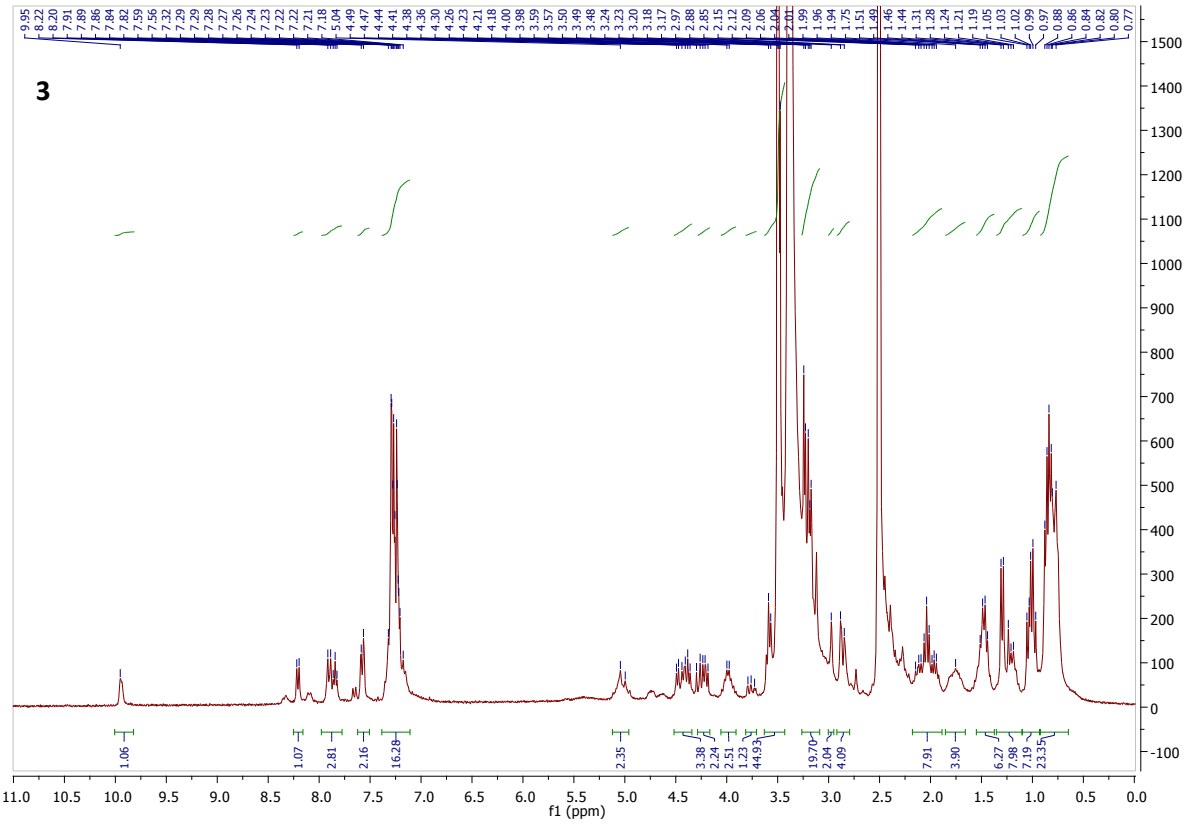


Figure S5: 1H NMR of compound **3**

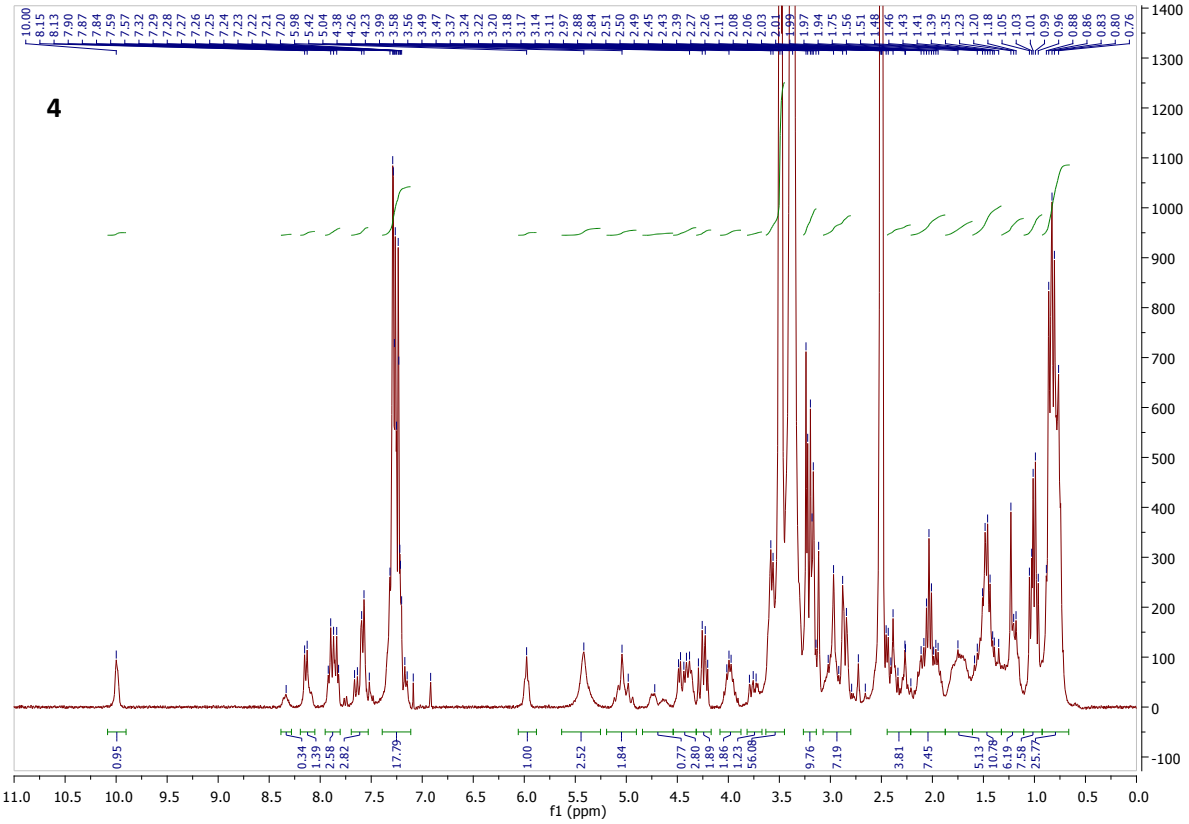


Figure S6: 1H NMR of compound **4**

HIC for native TTZ

Samples of trastuzumab and ADCs were injected (50 µg) onto a TSK-Gel Butyl-NPR 4.6 mm x 10 cm, 2.5 µm particle size column from Tosoh Bioscience, connected to an Waters e2695 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 60 min at a flow rate of 0.6 mL/min. The temperature was maintained at 30 °C for the duration of the run. Detection was by UV-vis absorbance at 280 nm.

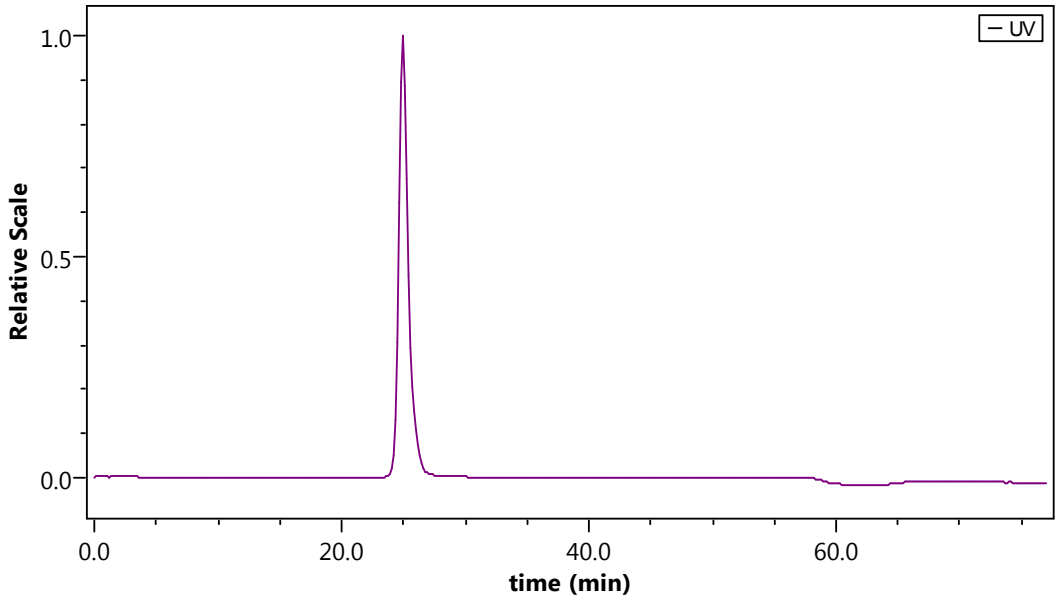


Figure S7: HIC trace of native TTZ

SDS-PAGE of ADCs 1-4

ADCs were analyzed on non-reducing Tris-HCl SDS-PAGE acrylamide gels. A stacking gel at 5% acrylamide and a resolving gel at 7% were used. Samples (2 µg) were mixed with Laemmli buffer (Biorad 4x Laemmli sample buffer), and heated at 96 °C, 10 min. A broad-range MW marker (Invitrogen SeeBlue® Plus2 Prestained Standard) was used to estimate protein weights. The gels were run at 90 V during 15 min, and 120 V during 50 min, in MOPS running buffer (Novex NuPAGE MOPS SDS Running Buffer). After washing in water, the gels were stained with Coomassie dye (Thermo Scientific Imperial TM Protein Stain).

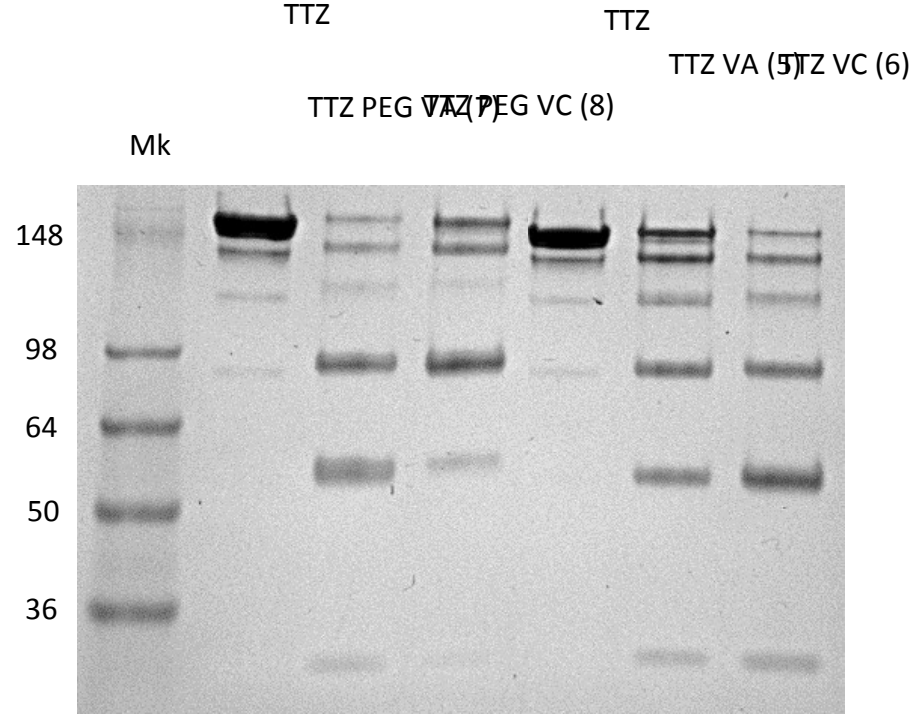


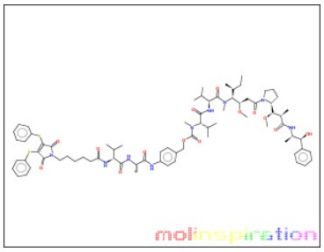
Figure S8: SDS-PAGE of conjugates 5-8 and TTZ.

Calculation of cLogP

All cLogP values were calculated using the software provided by MolInspiration at http://www.molinspiration.com/services/logp.html, accessed 29/10/2017.

molinspiration — Calculation of Molecular Properties

originalSMILES O=C(N[C@@H](C(C)C)C(N[C@@H](C)C(NC1=CC=C(COC(N(C)[C@@H](C(C)C)C(N[C@@H](C(C)C)C(N(C)[C@@H]([C@@H](C)CC)[C@H](OC)CC(N2[C@H]([C@H](OC)[C@@H](C)C(N[C@H](C)[C@H](C3=CC=CC=C3)O)=O)CCC2)=O)=O)=O)=O)C=C1)=O)=O)CCCCCN4C(C(SC5=CC=CC=C5)=C(SC6=CC=CC=C6)C4=O)=O
miSMILES: O=C(N[C@@H](C(C)C)C(N[C@@H](C)C(NC1=CC=C(COC(N(C)[C@@H](C(C)C)C(N[C@@H](C(C)C)C(N(C)[C@@H]([C@@H](C)CC)[C@H](OC)CC(N2[C@H]([C@H](OC)[C@@H](C)C(N[C@H](C)[C@H](C3=CC=CC=C3)O)=O)CCC2)=O)=O)=O)=O)C=C1)=O)=O)CCCCCN4C(C(SC5=CC=CC=C5)=C(SC6=CC=CC=C6)C4=O)=O



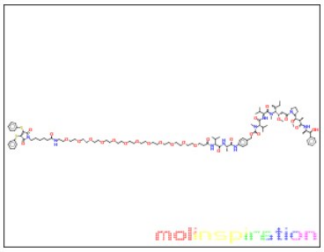
Molinspiration property engine v2016.10

miLogP	8.51
TPSA	293.42
natoms	102
MW	1446.89
nON	23
nOHNH	6
nviolations	4
nrotb	39
volume	1363.86

Get data as text (for copy / paste).

molinspiration — Calculation of Molecular Properties

originalSMILES O=C(N[C@H](C)C(NC1=CC=C(COC(N(C)[C@H](C(C)C)C(N[C@H](C(C)C)C(N(C)[C@H]([C@H](C)CC)[C@@H](OC)CC(N2[C@@H]([C@@H](OC)[C@H](C)C(N[C@@H](C)[C@@H](C3=CC=CC=C3O)=O)CCC2)=O)=O)=O)=O)C=C1)=O)
[C@H](C(C)C)NC(CCOCCOCCOCCOCCOCCOCCOCCOCCOCCOCCOCCOCCOCCNC(CCCCCN4C(C(SC5=CC=CC=C5)=C(SC6=CC=CC=C6)C4=O)=O)=O)=O
miSMILES: O=C(N[C@H](C)C(NC1=CC=C(COC(N(C)[C@H](C(C)C)C(N[C@H](C(C)C)C(N(C)[C@H]([C@H](C)CC)[C@@H](OC)CC(N2[C@@H]([C@@H](OC)[C@H](C)C(N[C@@H](C)[C@@H](C3=CC=CC=C3O)=O)CCC2)=O)=O)=O)=O)C=C1)=O)
[C@H](C(C)C)NC(CCOCCOCCOCCOCCOCCOCCOCCOCCOCCOCCOCCOCCOCCNC(CCCCCN4C(C(SC5=CC=CC=C5)=C(SC6=CC=CC=C6)C4=O)=O)=O)=O



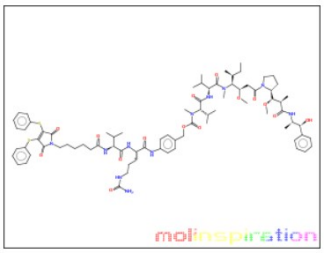
Molinspiration property engine v2016.10

miLogP	5.79
TPSA	433.33
natoms	143
MW	2046.60
nON	37
nOHNH	7
nviolations	4
nrotb	78
volume	1939.91

Get data as text (for copy / paste).

molinspiration Calculation of Molecular Properties

originalSMILES O=C(N[C@@H](C(C)C)C(N[C@@H](CCCNC(N)=O)C(NC1=CC=C(COC(N(C)[C@@H](C(C)C)C(N[C@@H](C(C)C)C(N(C)[C@@H]([C@@H](C)CC)[C@H](OC)CC(N2[C@H]([C@H](OC)[C@@H](C)C(N[C@H](C)[C@H](C3=CC=CC=C3)O)=O)CCC2)=O)=O)=O)=O)C=C1)=O)=O)CCCCCN4C(C(SC5=CC=CC=C5)=C(SC6=CC=CC=C6)C4=O)=O
miSMILES: O=C(N[C@@H](C(C)C)C(N[C@@H](CCCNC(N)=O)C(NC1=CC=C(COC(N(C)[C@@H](C(C)C)C(N[C@@H](C(C)C)C(N(C)[C@@H]([C@@H](C)CC)[C@H](OC)CC(N2[C@H]([C@H](OC)[C@@H](C)C(N[C@H](C)[C@H](C3=CC=CC=C3)O)=O)CCC2)=O)=O)=O)=O)C=C1)=O)=O)CCCCCN4C(C(SC5=CC=CC=C5)=C(SC6=CC=CC=C6)C4=O)=O



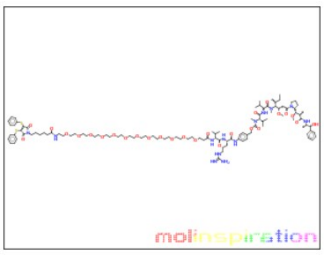
Molinspiration property engine v2016.10

miLogP	7.93
TPSA	348.54
natoms	108
MW	1532.98
nON	26
nOHNH	9
nviolations	4
nrotb	43
volume	1440.38

Get data as text (for copy / paste).

molinspiration Calculation of Molecular Properties

originalSMILES O=C(N[C@H](CCCNC(N)=N)C(NC1=CC=C(COC(N(C)[C@H](C(C)C)C(N[C@H](C(C)C)C(N(C)[C@H]([C@H](C)CC)[C@@H](OC)CC(N2[C@@H]([C@@H](OC)[C@H](C)C(N[C@@H](C)[C@@H](C3=CC=CC=C3)O)=O)CCC2)=O)=O)=O)=O)C=C1)=O)
[C@H](C(C)C)NC(CCOCCOCCOCCOCCOCCOCCOCCOCCOCCOCCOCCOCCOCCOCCNC(CCCCCN4C(C(SC5=CC=CC=C5)=C(SC6=CC=CC=C6)C4=O)=O)=O)=O
miSMILES: O=C(N[C@H](CCCNC(N)=N)C(NC1=CC=C(COC(N(C)[C@H](C(C)C)C(N[C@H](C(C)C)C(N(C)[C@H]([C@H](C)CC)[C@@H](OC)CC(N2[C@@H]([C@@H](OC)[C@H](C)C(N[C@@H](C)[C@@H](C3=CC=CC=C3)O)=O)CCC2)=O)=O)=O)=O)C=C1)=O)
[C@H](C(C)C)NC(CCOCCOCCOCCOCCOCCOCCOCCOCCOCCOCCOCCOCCOCCNC(CCCCCN4C(C(SC5=CC=CC=C5)=C(SC6=CC=CC=C6)C4=O)=O)=O)=O



Molinspiration property engine v2016.10

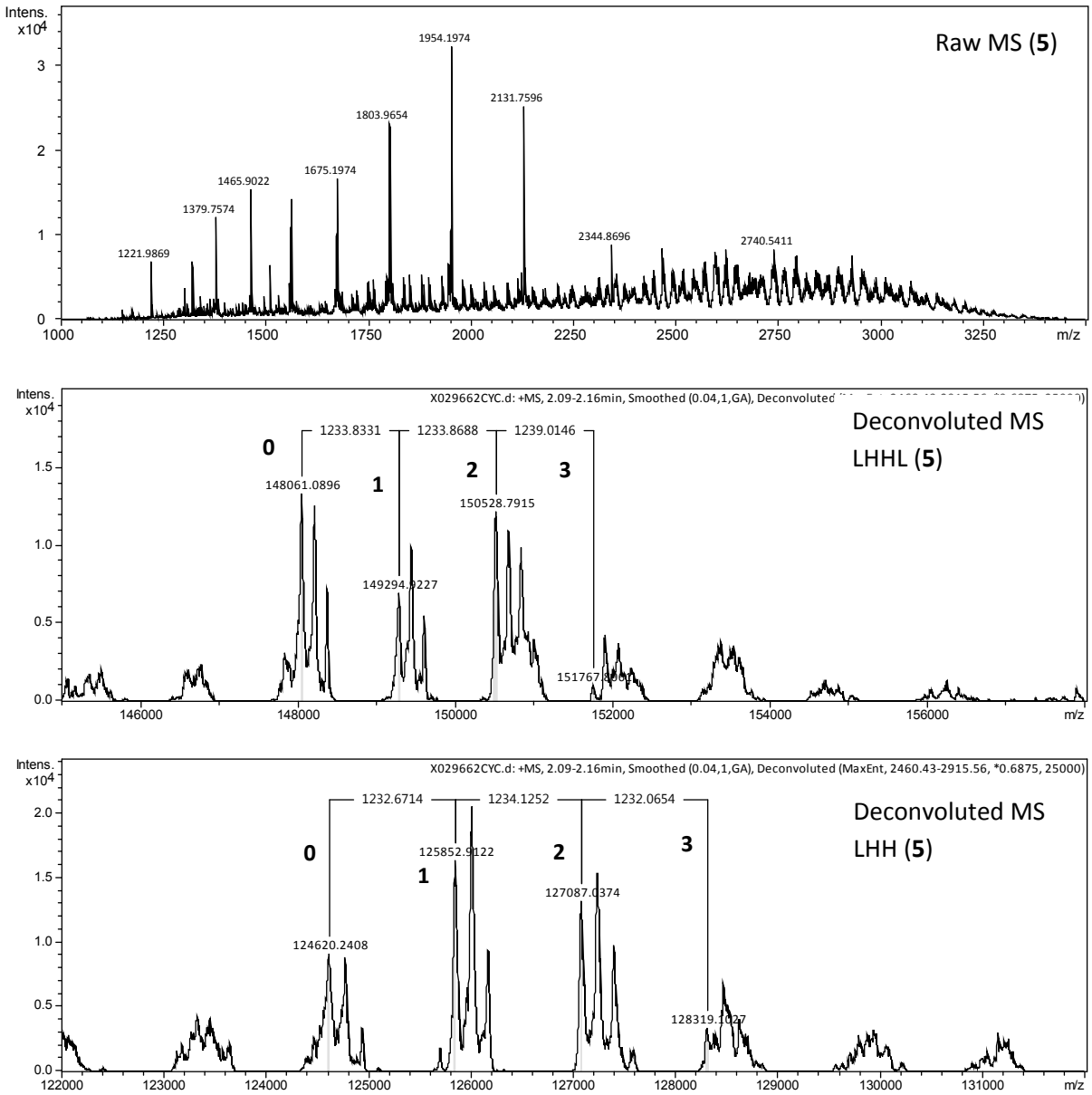
miLogP	4.85
TPSA	495.23
natoms	149
MW	2131.71
nON	40
nOHNH	11
nviolations	3
nrotb	83
volume	2019.75

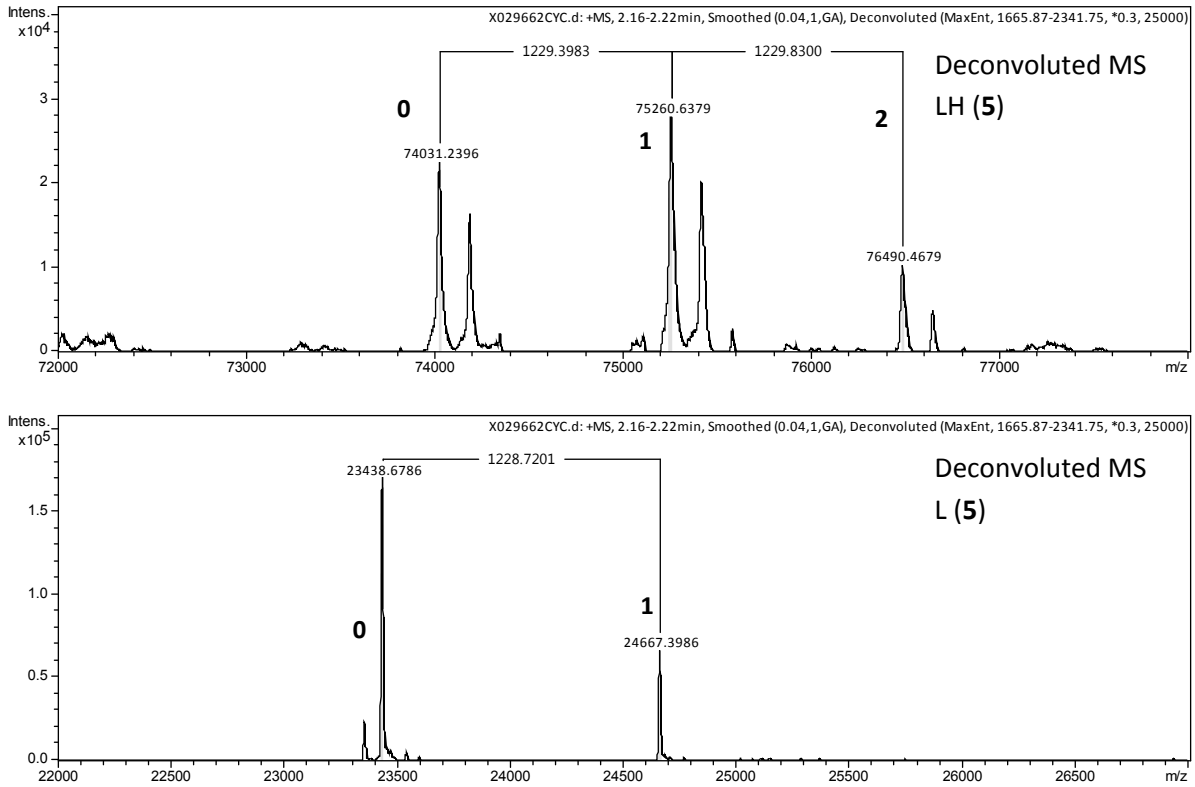
Get data as text (for copy / paste).

Raw and deconvoluted mass spectrometry of TTZ ADCs 5-8

Mass spectrometric analyses of ADCs were performed on a Bruker maXis mass spectrometer coupled to a Dionex Ultimate 3000 RSLC system. Prior to MS analysis, samples (ca. 5 µg) were desalted on a MassPREP desalting cartridge (2.1 x 10 mm, Waters) heated at 80 °C using 0.1% formic acid as solvent A and 0.1% formic acid in acetonitrile as solvent B at 500 µL/min. After 1 min, a linear gradient from 5 to 90% B in 1.5 min was applied; the first 1.5 min were diverted to waste. MS data were acquired in positive mode with ESI source over the m/z range from 900 up to 5000 at 1 Hz. Data were processed using DataAnalysis 4.4 software (Bruker) and MaxEnt algorithm for spectral deconvolution. Deconvolution was carried out in the range 20-180 kDa, with results recorded for full antibody as well as fragments comprising L, LH, LHH as a result of antibody dissociation within the MS. Not all species were observed for all ADCs. DAR was approximated for each species as an average of the percentage abundance of each DAR species present, with the quantities calculated by peak integration of the first glycosylation peak.

Figure S9: raw and deconvoluted MS for TTZ Val-Ala **(5)**





L loading: 0.28

LHH loading: 1.78

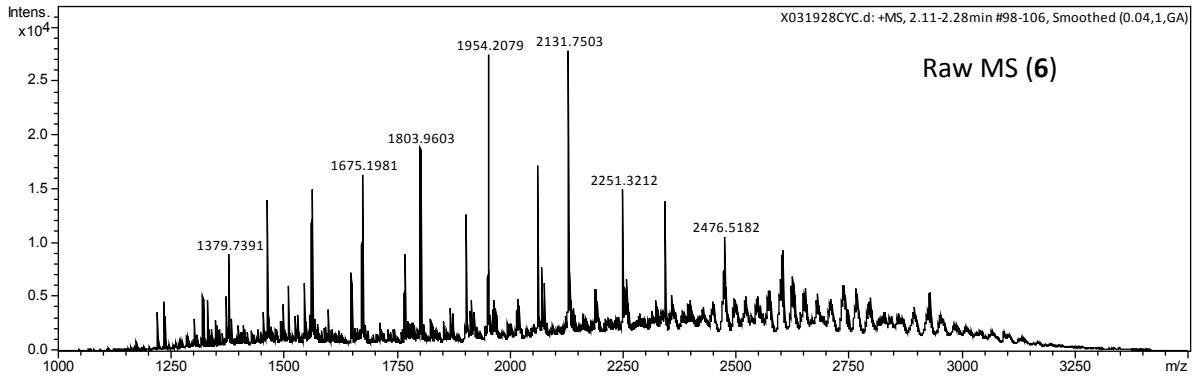
DAR (L+LHH) : 2.06

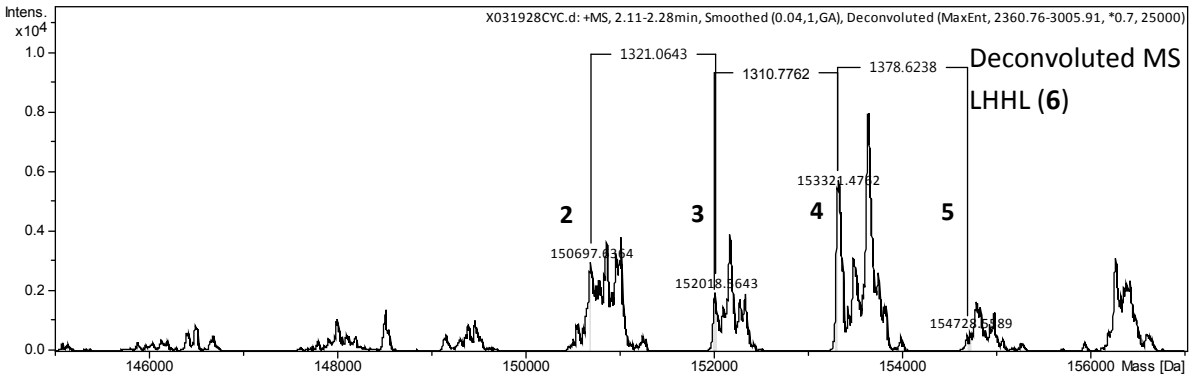
LH loading : 0.79

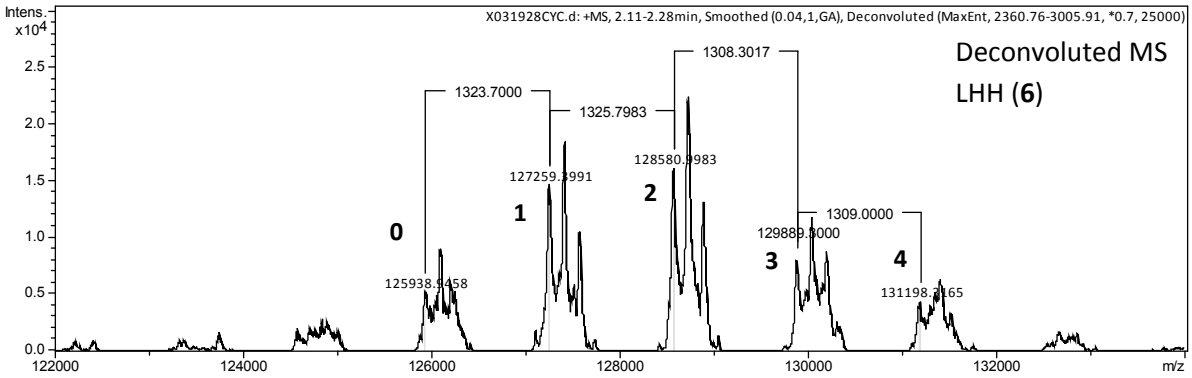
DAR (LH+LH) : 1.59

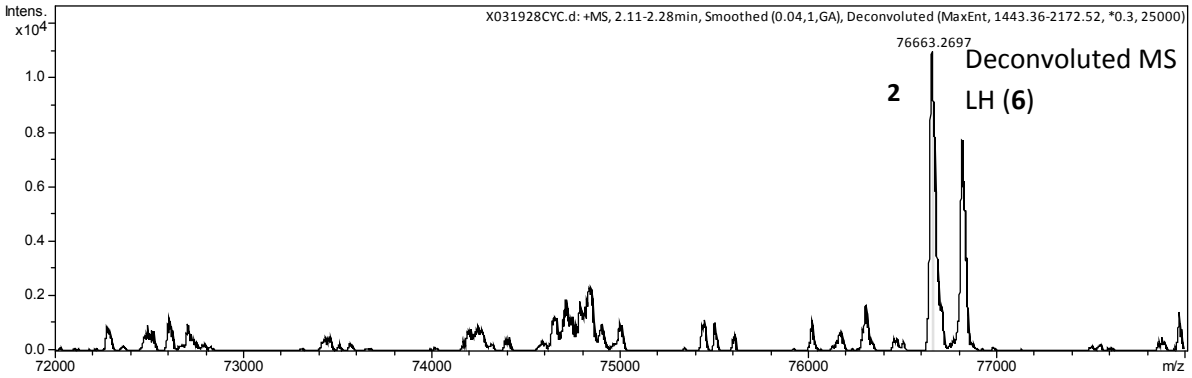
DAR LHHL : 1.17

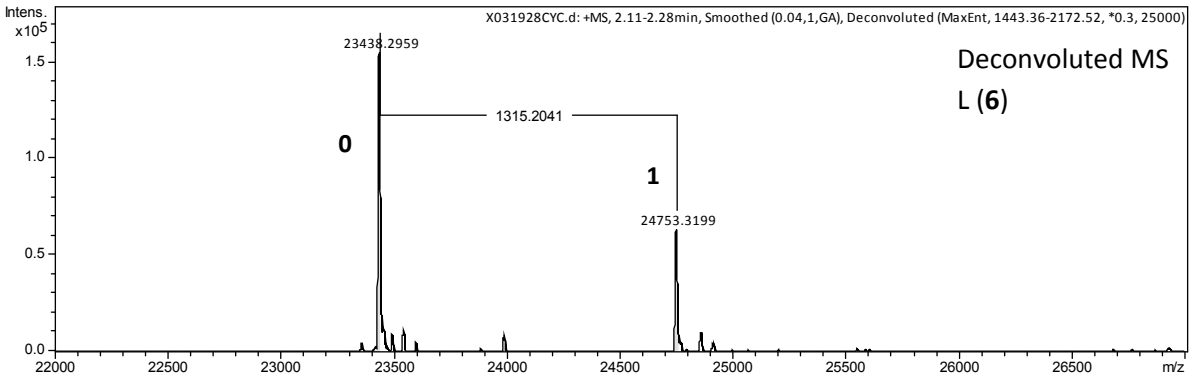
Figure S10: raw and deconvoluted MS for TTZ Val-Cit **(6)**











L loading: 0.29

LHH loading: 1.81

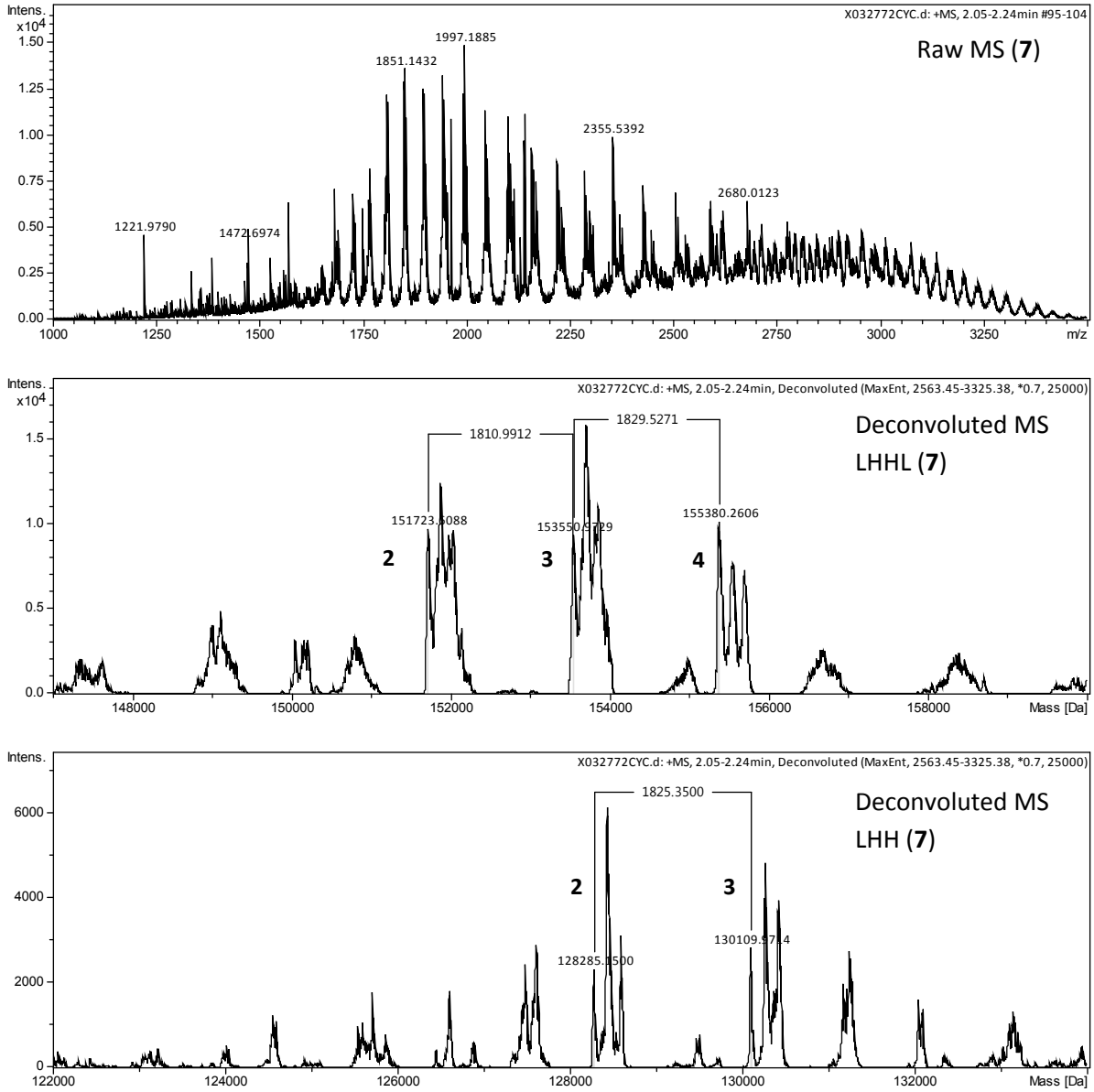
DAR (L+LHH) : 2.10

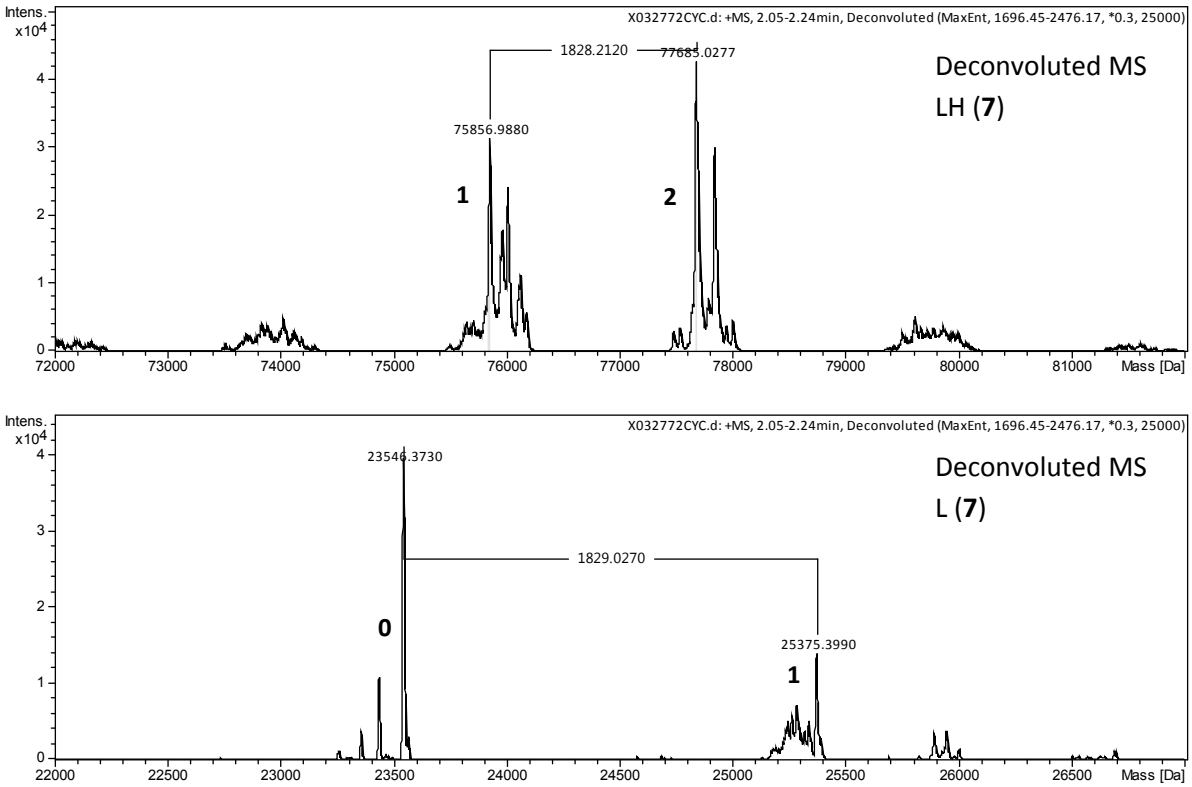
LH loading : 2.00

DAR (LH+LH) : 4.00

DAR LHHL : 3.34

Figure S11: raw and deconvoluted MS for TTZ PEG Val-Ala **(7)**





L loading: 0.27

LHH loading: 2.55

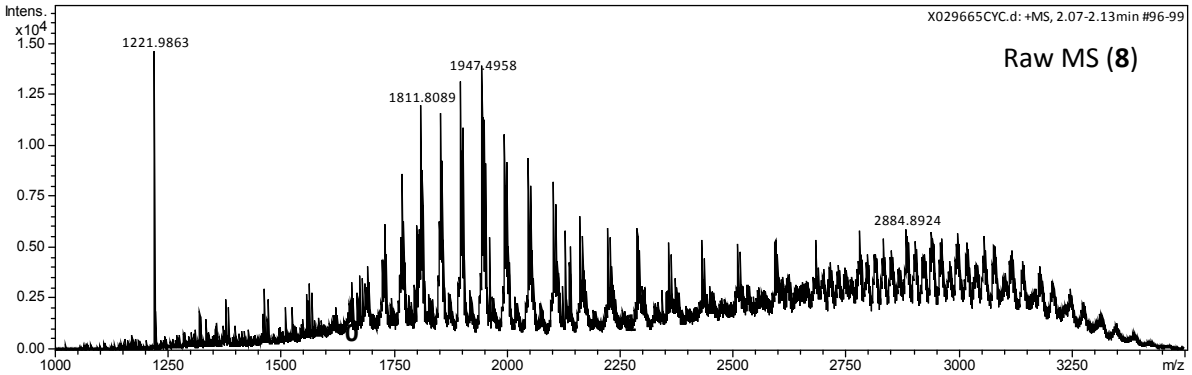
DAR (L+LHH) : 2.82

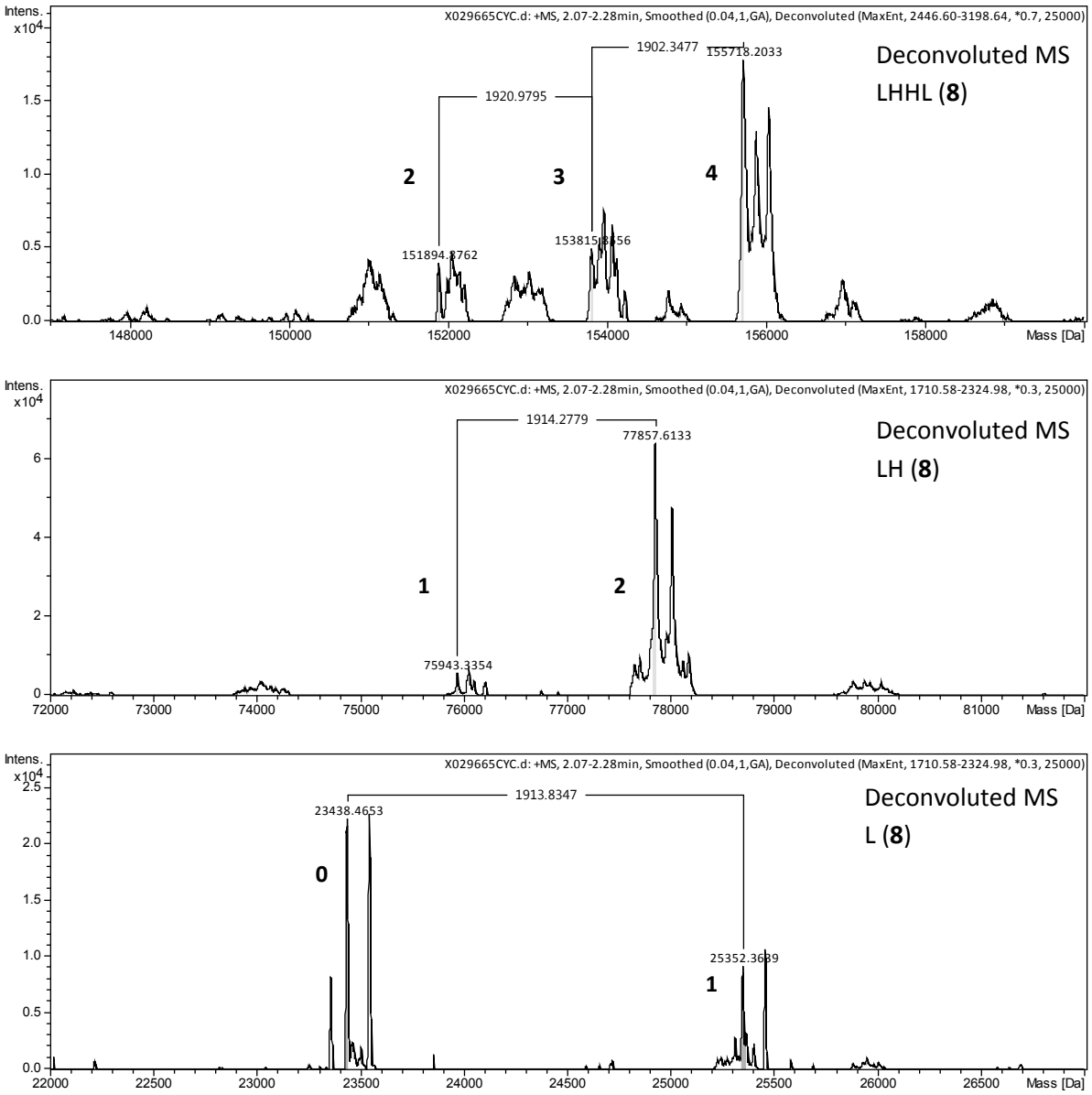
LH loading : 1.57

DAR (LH+LH) : 3.04

DAR LHHL : 3.01

Figure S12: raw and deconvoluted MS for TTZ PEG Val-Cit **(8)**





L loading: 0.29

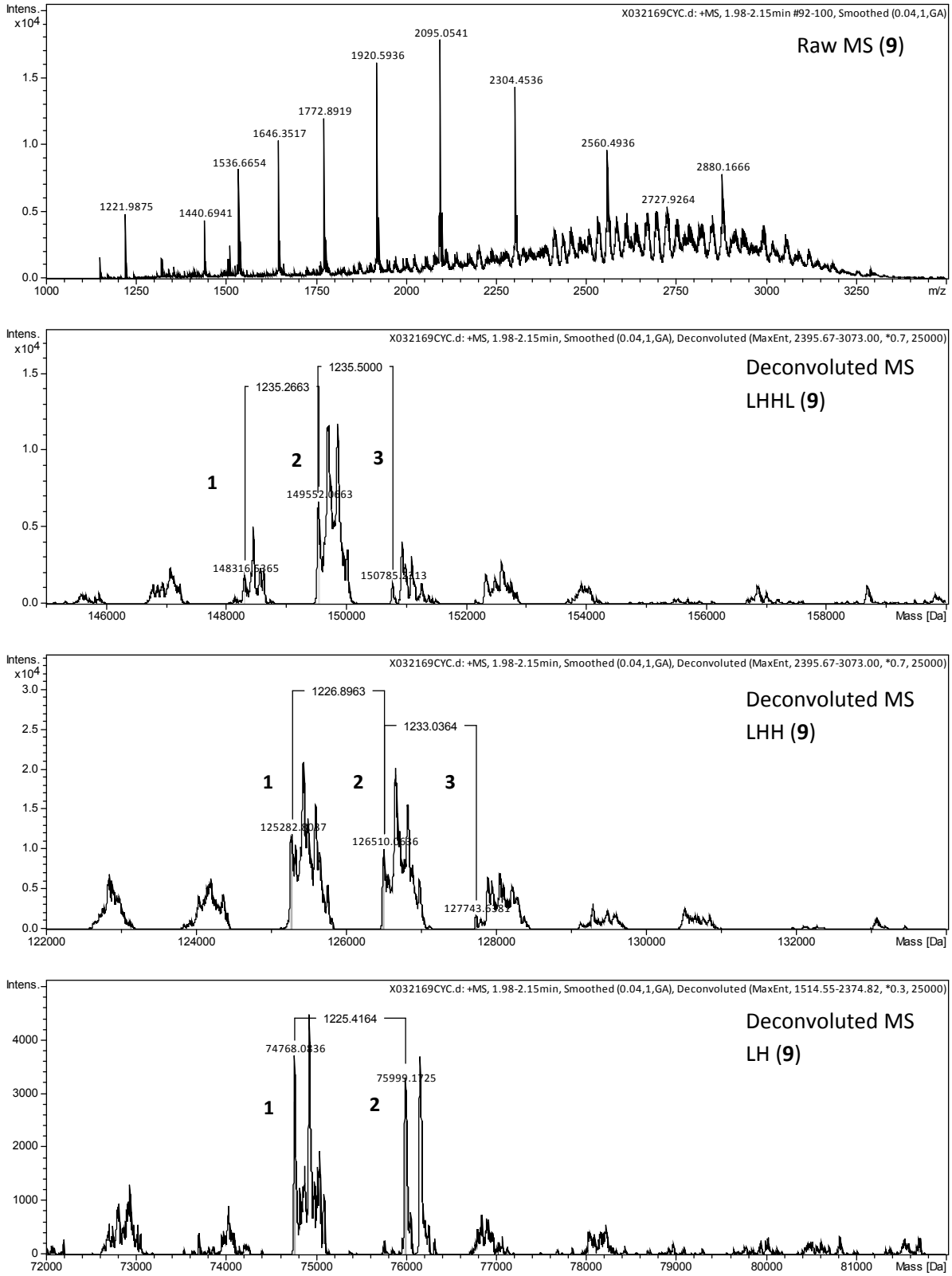
LH loading : 1.92

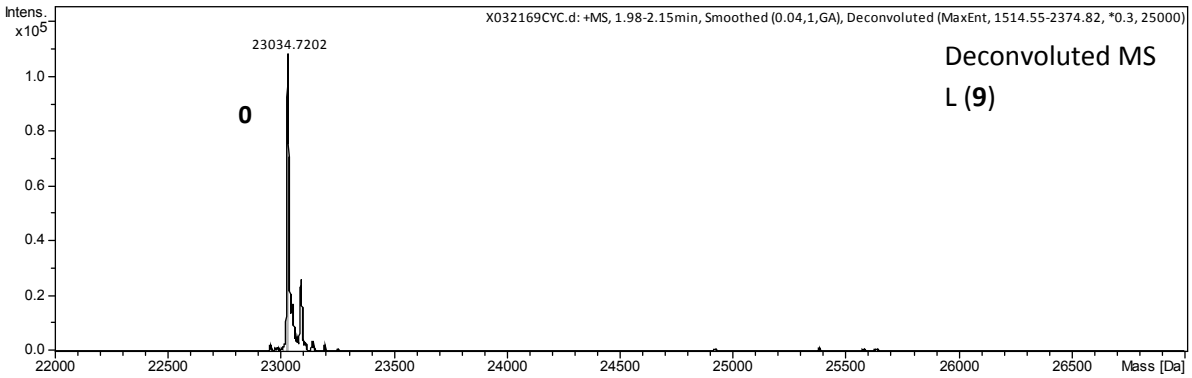
DAR (LH+LH) : 3.84

DAR LHHL : 3.51

Convoluted and deconvoluted mass spectrometry of RTX control ADCs 9-12

Figure S13: raw and deconvoluted MS for RTX Val-Ala **(9)**





L loading: 0

LHH loading: 1.57

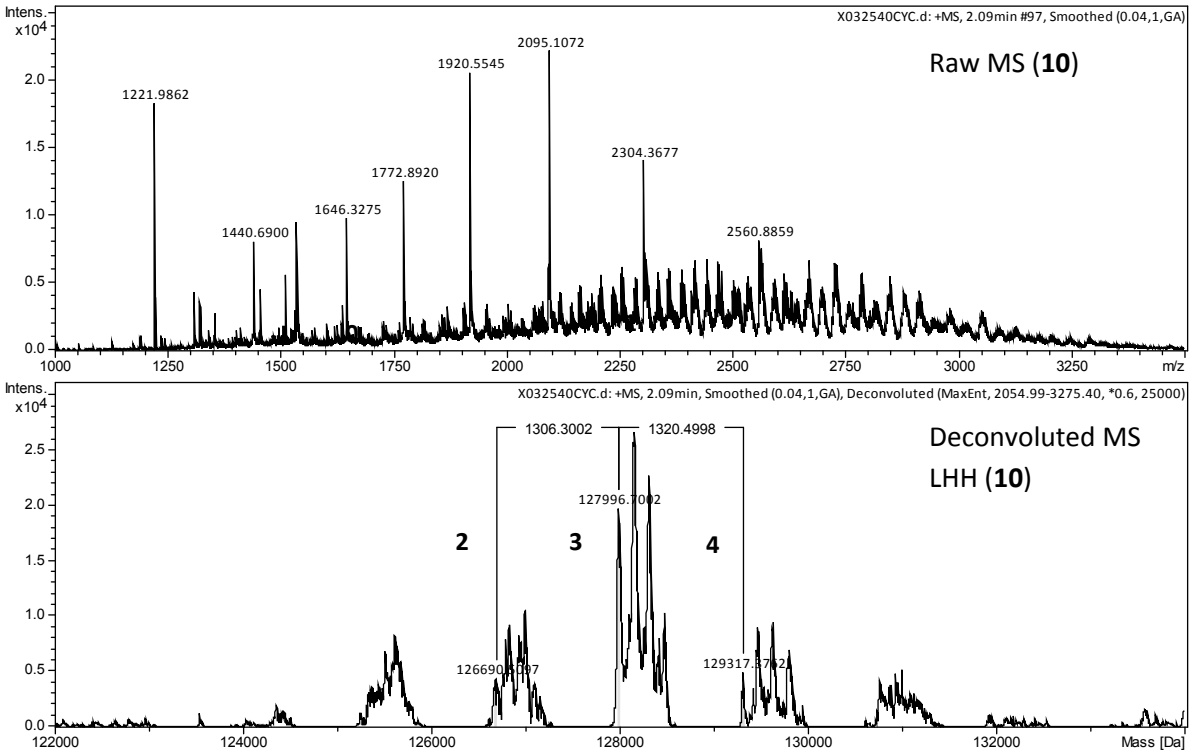
DAR (L+LHH) : 1.57

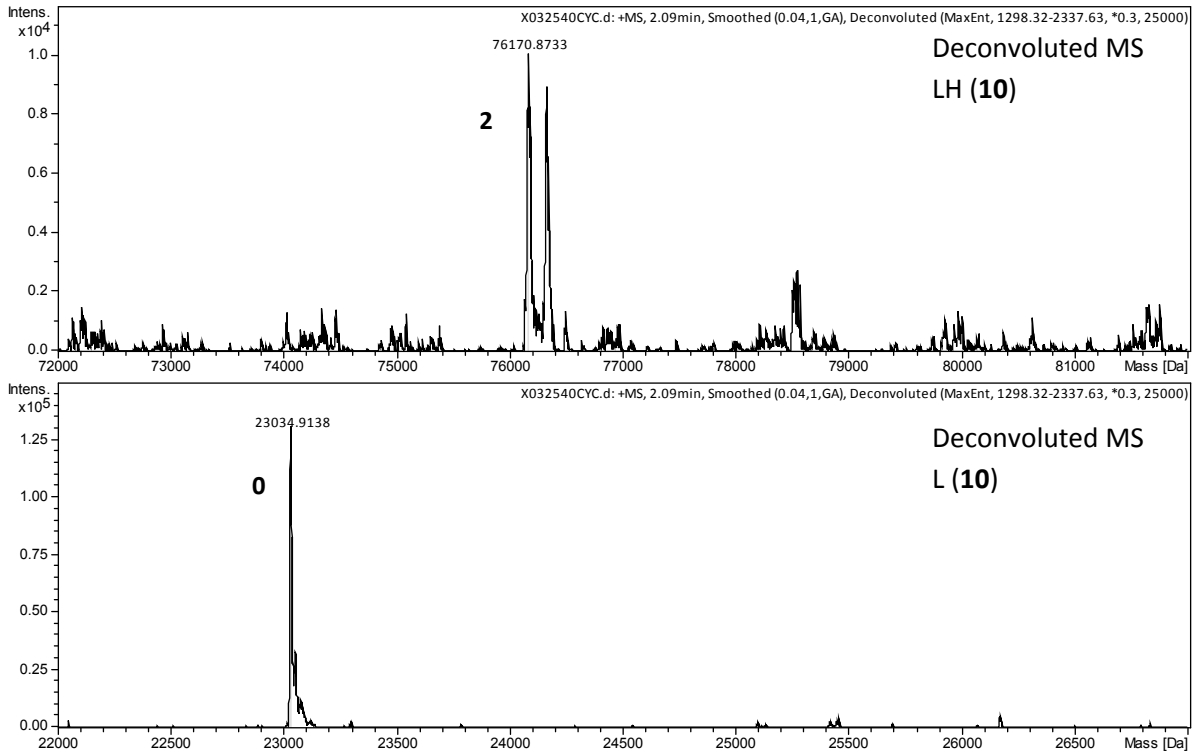
LH loading : 1.46

DAR (LH+LH) : 2.92

DAR LHHL : 1.95

Figure S14: raw and deconvoluted MS for RTX Val-Cit **(10)**





L loading: 0

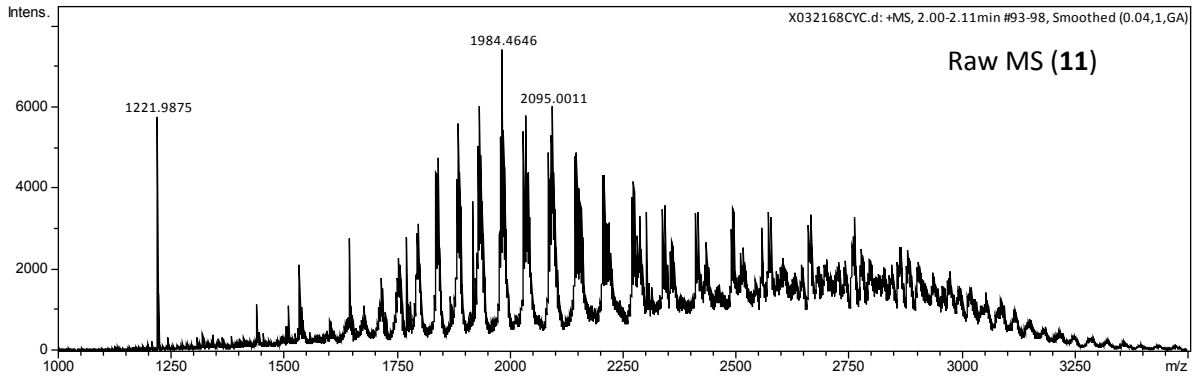
LHH loading: 3.02

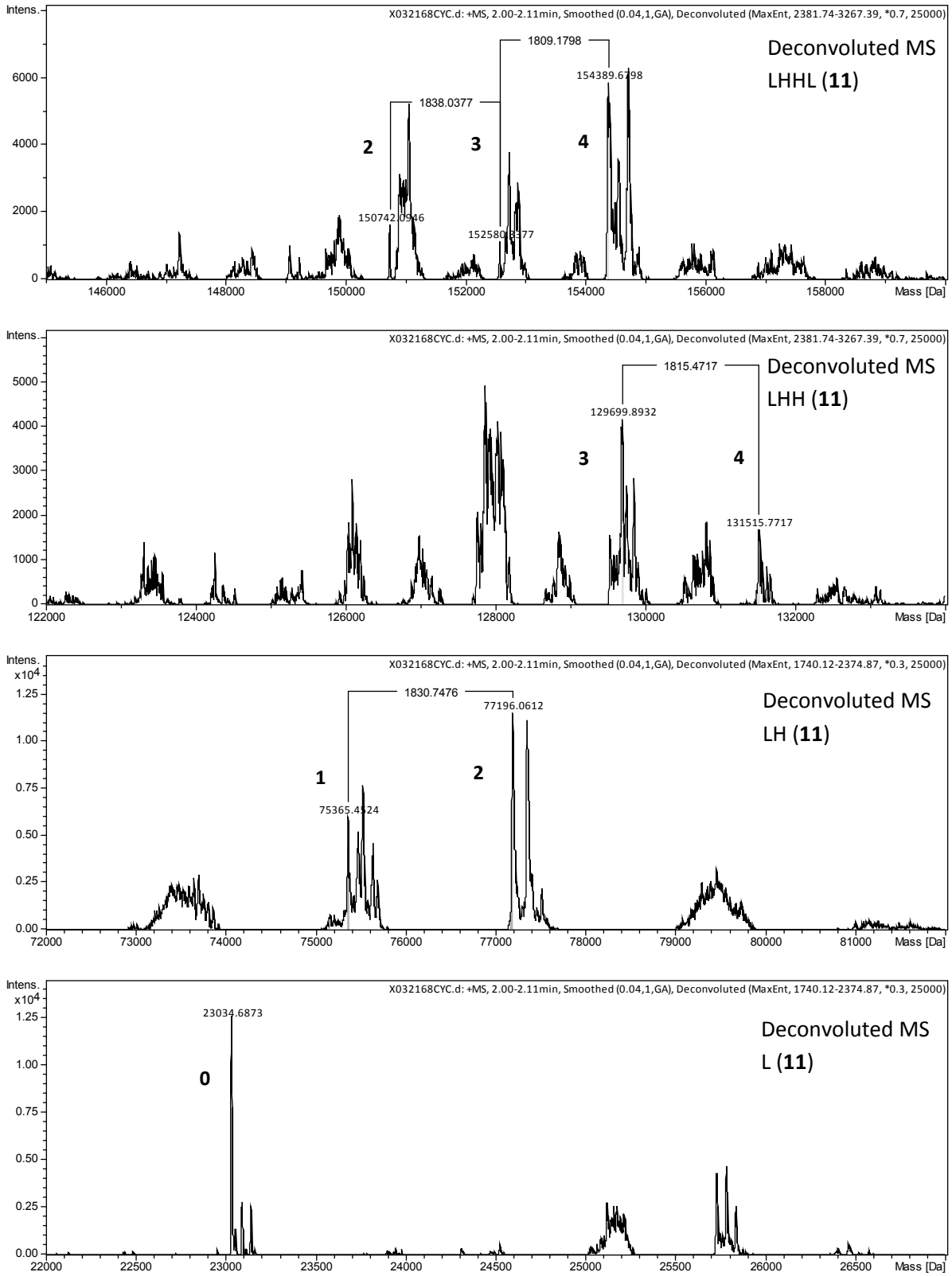
DAR (L+LHH) : 3.02

LH loading : 2.0

DAR (LH+LH) : 4.0

Figure S15: raw and deconvoluted MS for RTX PEG Val-Ala **(11)**





L loading: 0

LHH loading: 3.28

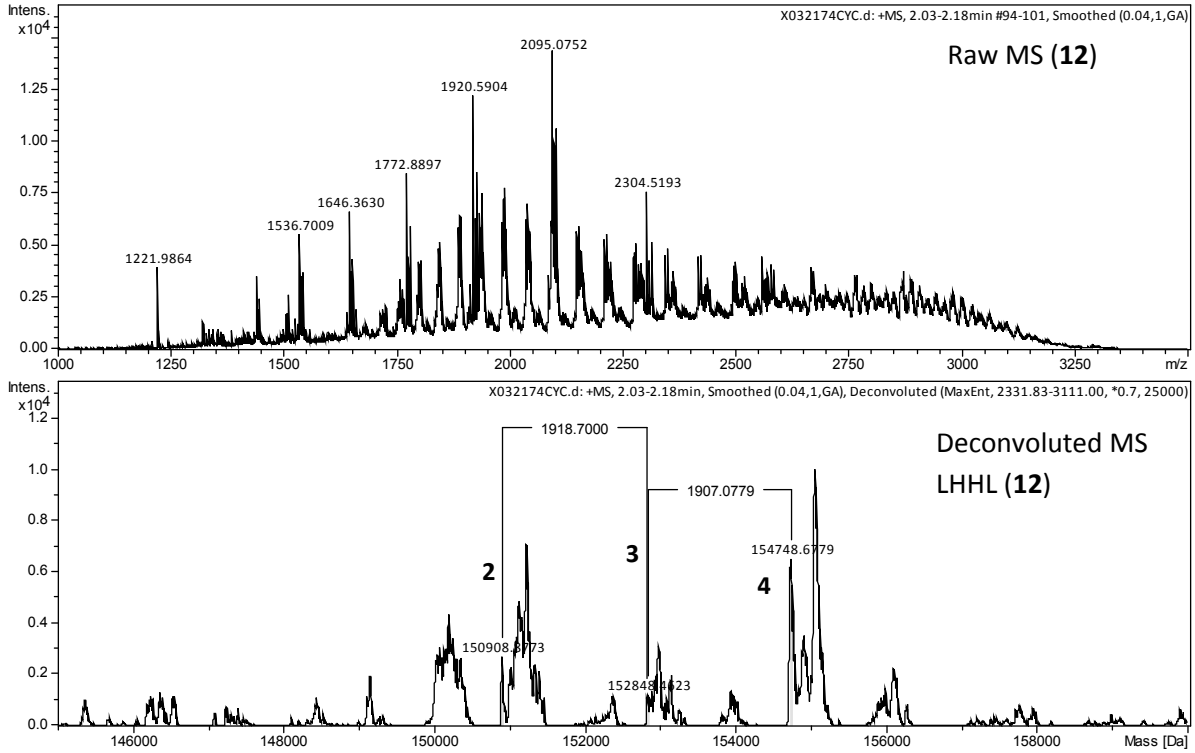
DAR (L+LHH) : 3.28

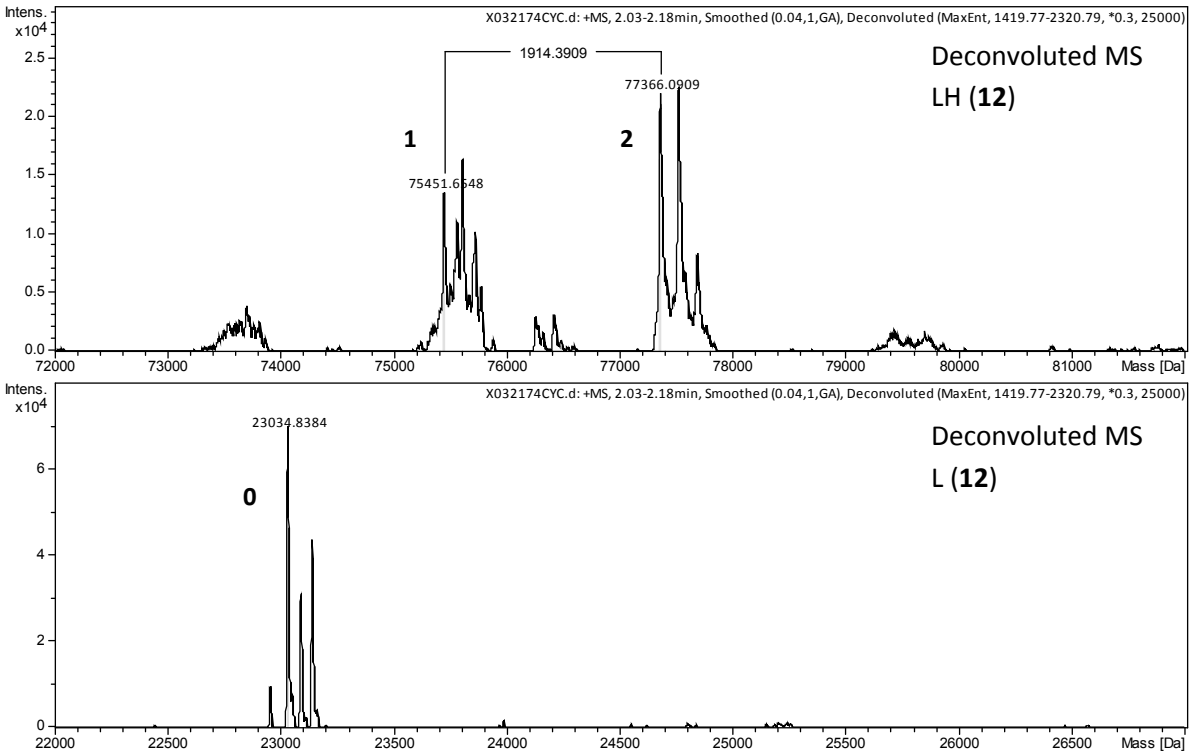
LH loading : 1.65

DAR (LH+LH) : 3.30

DAR LHHL : 3.49

Figure S16: raw and deconvoluted MS for RTX PEG Val-Cit **(12)**





L loading: 0

LH loading : 1.62

DAR (LH+LH) : 3.24

DAR LHHL : 3.36

HER2 binding by ELISA

The functionality of ADCs was checked by indirect enzyme-linked immunosorbent assays (ELISA) using the HER2 protein (Sino Biologicals, Beijing, P. R. China.) as a target. The samples were detected by protein L-peroxydase (Thermo Scientific Pierce) in the presence of a chromatic substrate, 3,3',5,5'-tetramethylbenzidine (TMB; Sigma, St Louis, USA). Briefly, HER2 was coated in a 96-well plates at 1 µg/mL and incubated overnight at 4 °C. The wells were then saturated with 3% BSA-PBS for 1 h at 37 °C and washed with PBS prior to incubation with AFC from 0.01 nM to 31.00 nM during 1 h at 37 °C. Wells were then washed with PBS-tween 20 (0.05%) and incubated with 100 µL of protein-L-peroxydase (1.25 µg/mL) for 1 h at 37 °C added to 100 µL of TMB substrate (Sigma-Aldrich). Enzymatic reactions were stopped with the addition of 50 µL of 1M H_2SO_4 and the absorbance was measured at 450 nm using a microplate reader (Biotek).

Cytotoxicity

Cells lines:

The SK-BR-3 and T-47D cancer cell lines were obtained from ATCC (Rockville, MD, USA). Routine characterization was done by morphological observation, and mycoplasma testing was performed using the MycoAlert™. Mycoplasma Detection Kit (Lonza). Cells were authenticated by short tandem repeat profiling using the Promega Power Plex 21 System. All cell lines were cultured following the ATCC recommendations.

Viability assay:

The effect of trastuzumab, MMAE, T-DM1 and ADCs on cell proliferation was evaluated after 5 days using 3-[4,5-dimethylthiazol-2-yl]-5-[3-carboxymethoxyphenyl]-2-[4-sulfophenyl]-2H-tetrazolium (MTS) and the electron coupling reagent phenazine methosulfate (PMS). the OD (490 nm) was read using a microplane reader. Growth viability was calculated based on the percentage of proliferating cells in treated samples relative to untreated cultures.

Statistical Analysis

ANOVA non-parametric test was used to compare EC_{50} of TTZ and ADCs on SK-BR-3 cells (n=3). All EC_{50} and P-Values are present in the following tables.

EC_{50} of TTZ and ADCs

Treatment	T-DM1	Val-Ala (5)	Val-Cit (6)	PEG-Val-Ala(7)	PEG-Val-Cit (8)	TTZ
EC_{50} (µg/mL)	0.002189	0.003048	0.002626	0.004068	0.004401	0.02263
Standard Error	0.0007528	0.0008949	0.0005912	0.001139	0.001657	0.007464

P Value data and degrees of significance

Comparison	Adjusted P Values	Summary
T-DM1 vs. Val-Ala (5)	0.9984	ns
T-DM1 vs. Val-Cit (6)	0.9998	ns
T-DM1 vs. TTZ	0.0214	*
Val-Ala (5) vs. Val-Cit (6)	0.9998	ns
Val-Ala (5) vs. TTZ	0.0267	*
Val-Cit (6) vs. TTZ	0.024	*
PEG-Val-Ala (7) vs. PEG-Val-Cit (8)	0.9995	ns
PEG-Val-Ala (7) vs. TTZ	0.0017	**
PEG-Val-Cit (8) vs. TTZ	0.0019	**

ns : not significant