Supplementary Data

An enzymatically cleavable tripeptide linker for maximizing the therapeutic index of antibody–drug conjugates

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Supplementary Figures

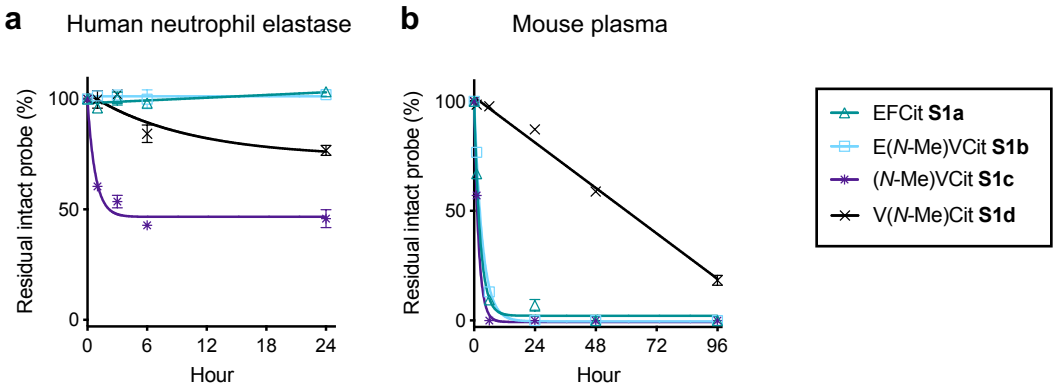


Fig. S1. Stability of probes **S1a**–**d** in the presence of human neutrophil elastase (**a**) or in undiluted BALB/c mouse plasma (**b**) at 37 °C. (**S1a**) green open triangle; (**S1b**) cyan open square; (**S1c**) purple asterisk; (**S1d**) black cross. All assays were performed in duplicate. Data are presented as mean values ± SEM.

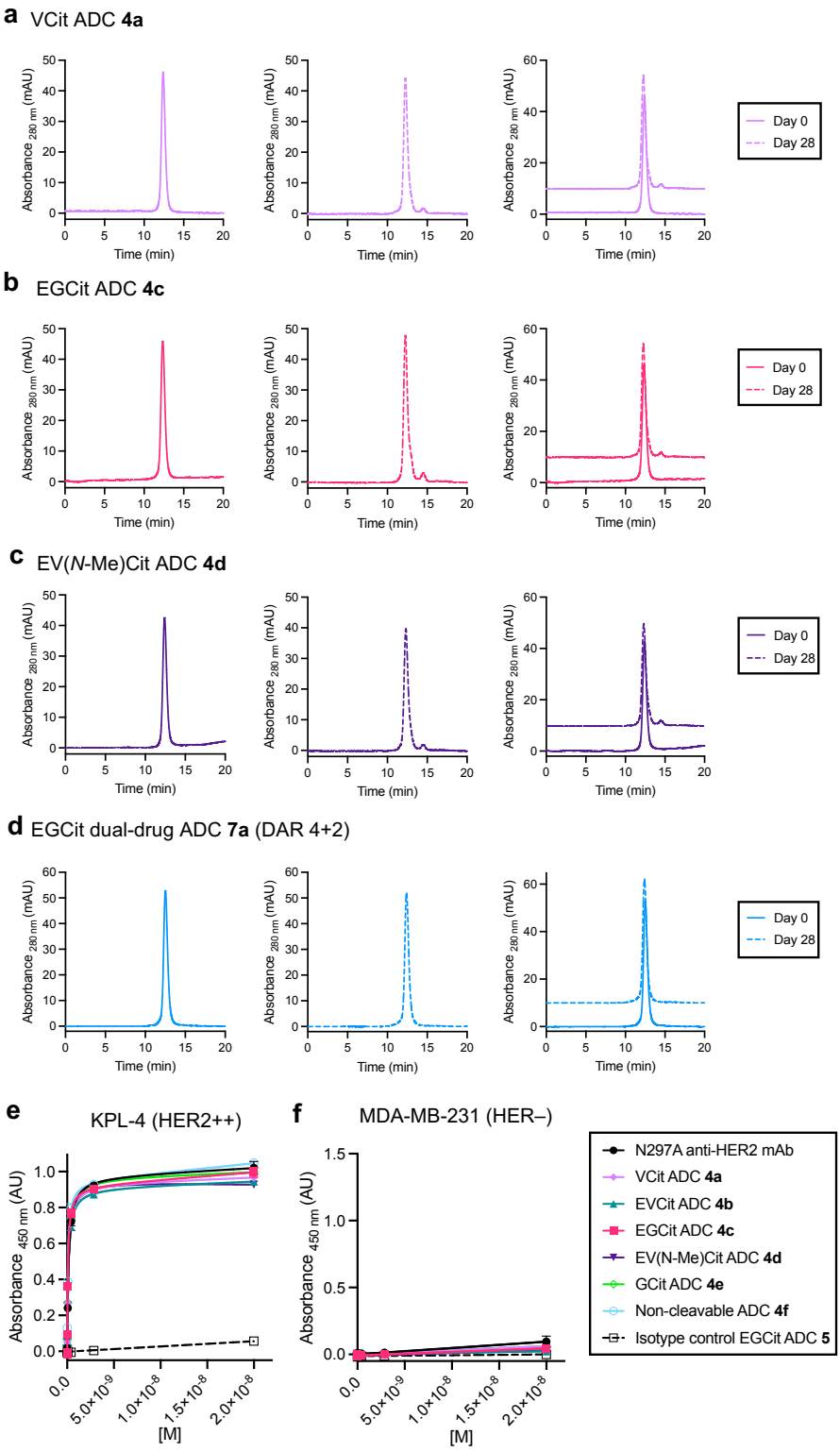


Fig. S2. SEC analysis of ADCs **4a**,**c**,**d**, and **7a** before and after 28-day incubation at 37 ºC in PBS (pH 7.4) and HER2 binding assay. VCit ADC **4a** (**a**), EGCit ADC **4c** (**b**), EV(*N*-Me)Cit ADC **4d** (**c**), and EGCit dual drug ADC **7a** (DAR 4+2) (**d**). Saturation-binding curves obtained by cell-based ELISA. All assays were performed in triplicate. Data are presented as mean values ± SEM (n = 3). The unmodified N297A anti-HER2 mAb and ADC **4a**–**f**, **5** bound to KPL-4 cells (HER2 positive, **e**) with comparable binding affinities but not to MDA-MB-231 cells (HER2 negative, **f**).

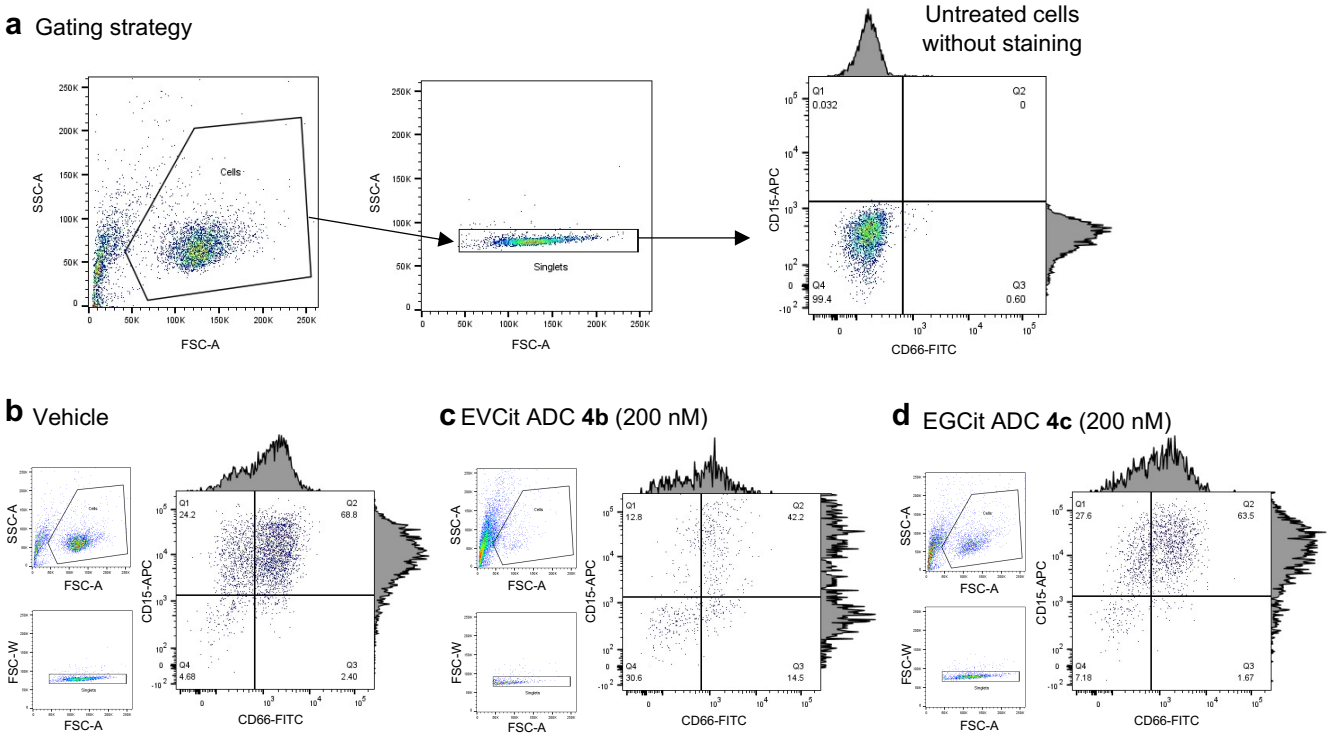


Fig. S3. Gating strategy and CD15/CD66b expression in differentiated hematopoietic cells. a, Gating strategy. Scatter, single cell selection, and CD15/CD66b-negative areas were set up using unstained cells from the vehicle control group. UltraComp eBeads™ Compensation Beads (Invitrogen) labeled with CD15-APC and/or CD66b-FITC were also used to set voltages and gating parameters for obtaining accurate fluorescence signal. Isotype control IgG-APC and -FITC treated cells were used to confirm the specificity of the CD15-APC and CD66b-FITC antibodies. The same gating strategy was used for flow cytometry presented in **b**–**d** in this figure. **b**–**d**, Representative 2D-histograms of differentiated hematopoietic cells after a 7-day treatment with vehicle control (**b**), 200 nM EVCit-MMAE ADC **4b** (**c**), and 200 nM EGCit-MMAE ADC **4c** (**d**). All experiments were performed in triplicate. Data were acquired using a LSR II flow cytometer (BD Biosciences) and Diva acquisition software (version 8.0.1, BD Biosciences) and analyzed using FlowJo analysis software (version 10.8.0, FlowJo, LLC).

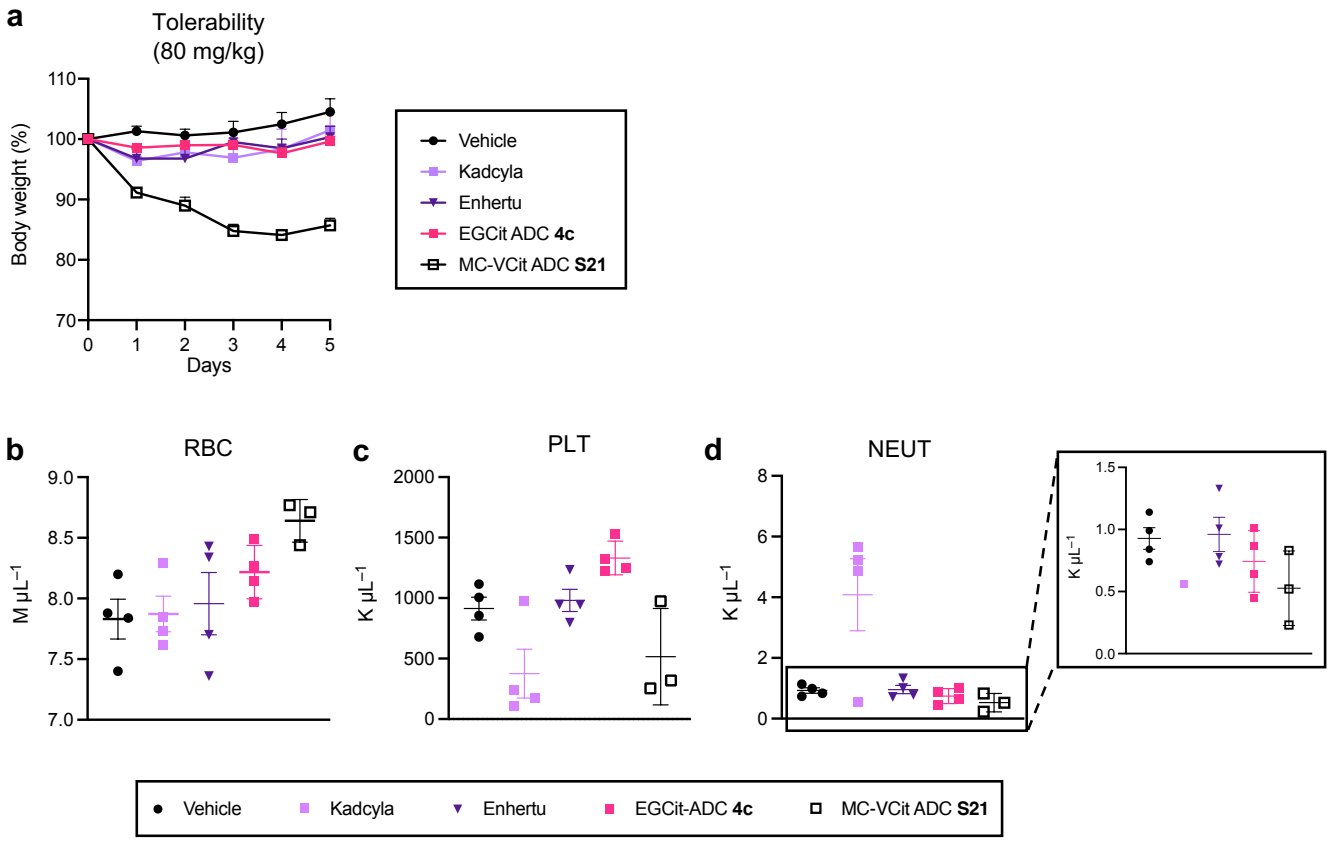


Fig. S4. Tolerability and hematology analysis. a Body weight change after female CD-1® mice were administered with a single dose (80 mg kg^{-1}) of vehicle control (n = 4), Kadcyla® (n = 7), Enhertu® (n = 7), EGCit ADC **4c** (n = 8), or MC-VCit MMAE DAR 4 ADC **S21** (n = 6, one mouse died on Day 3). No mice showed acute symptoms or reached the pre-defined humane endpoint during 5-day monitoring. **b**–**d** Counts of red blood cells (RBC, **b**), platelets (PLT, **c**), and neutrophils (NEUT, **d**) 5 days post injection of a single dose (80 mg kg^{-1}) of vehicle control (n = 4), EGCit ADC **4c** (n = 4), Enhertu® (n = 4), or Kadcyla® (n = 4). Data are presented as mean values (bars) ± SEM. MC, maleimidocaproyl.

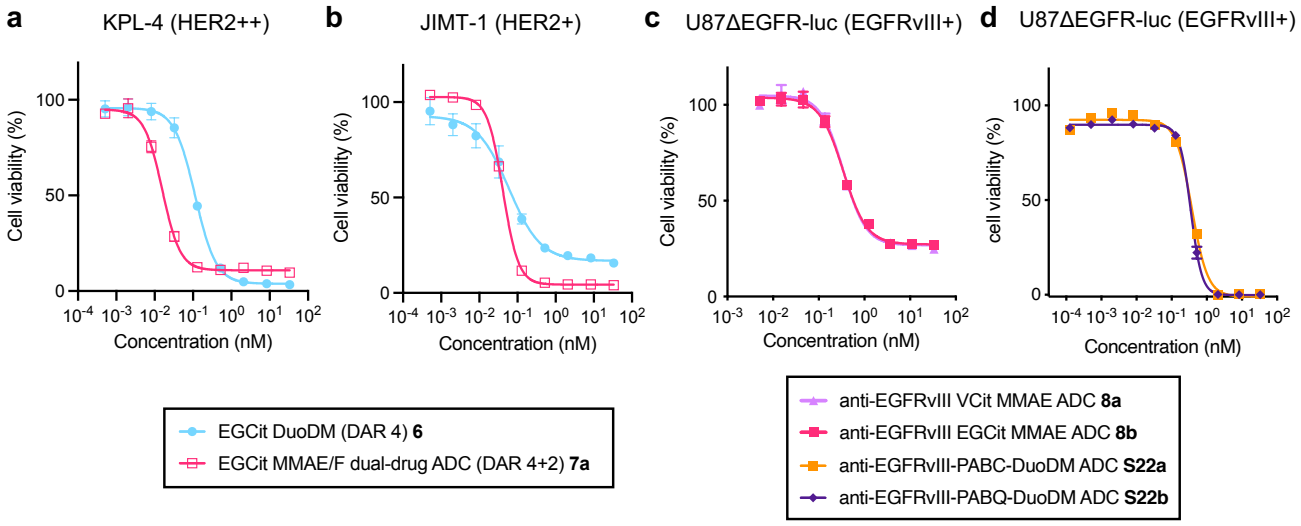


Fig. S5. Additional in vitro cytotoxicity data. **a**,**b** Cytotoxicity of anti-HER2 EGCit-DuoDM ADC (DAR 4, **6**, cyan circle) and anti-HER2 EGCit-MMAE/F dual-drug ADC (DAR 4+2, **7a**, magenta open square) in KPL-4 (**a**) and JIMT-1 (**b**). **c** Cytotoxicity of anti-EGFRvIII VCit-MMAE ADC (DAR 4, **8a**, light purple triangle) and anti-EGFRvIII EGCit-MMAE ADC (DAR 4, **8b**, magenta square) in U87ΔEGFR-luc. **d** Cytotoxicity of anti-EGFRvIII EGCit-PABC-DuoDM ADC (DAR 4, **S22a**, orange square) and anti-EGFRvIII EGCit-PABQ-DuoDM ADC (DAR 4, **S22b**, purple diamond) in U87ΔEGFR-luc. Concentrations (nM) are based on the antibody dose without normalizing to each DAR. All assays were performed in quadruplicate. Data are presented as mean values +/- SEM (n = 3).

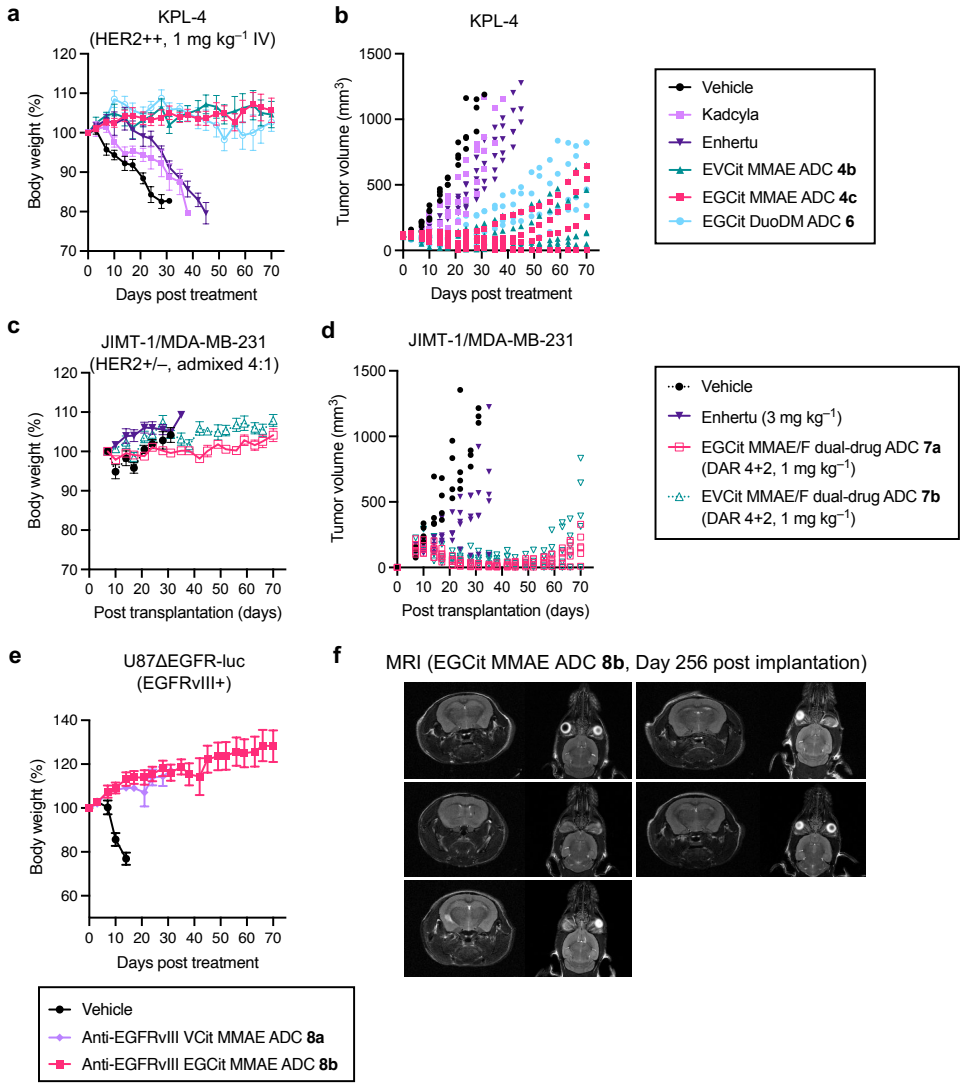


Fig. S6. Body weight change and antitumor activity in various xenograft models. a,**b** Body weight change (**a**) and tumor volume change (**b**) during the treatment of KPL-4 tumor-bearing mice with each ADC at 1 mg kg^{-1}. Mice (n = 5 for all groups) were injected intravenously with a single dose of vehicle control (black circle), Kadcyla® (light purple square), Enhertu® (purple inversed triangle), EVCit-MMAE ADC **4b** (green triangle), EGCit-MMAE ADC **4c** (magenta square) or EGCit-DuoDM ADC **6** (cyan circle) at a tumor volume of ~100 mm^3. **c**,**d** Body weight change (**c**) and tumor volume change (**d**) during the treatment of JIMT-1/MDA-MB-231 admixed tumor-bearing mice. Mice were injected intravenously with a single dose of Enhertu® (3 mg kg^{-1}, purple inversed triangle, n = 5) or EGCit-MMAE/F DAR 4+2 dual-drug ADC **7a**, (1 mg kg^{-1}, magenta open square, n = 6). Note: The tumor volume and survival curve data of vehicle control (black circle with dotted curve, n = 4) and EVCit dual-drug ADC **7b** (1 mg kg^{-1}, green open triangle with dotted curve, n = 5) presented here have been previously generated and reported by us(1). **e** Body weight change during the treatment of orthotopic U87ΔEGFR-luc tumor-bearing mice with each ADC at 5 mg kg^{-1}. Mice were intravenously administered with a single dose of vehicle control, anti-EGFRvIII VCit-MMAE ADC **8a** (light purple), or anti-EGFRvIII EGCit-MMAE ADC **8b** (magenta). n = 6 for vehicle and **8a**; n = 7 for **8b**. Data are presented as mean values +/- SEM. **f** Coronal and sagittal MRI images of the intracranial U87ΔEGFR-luc tumor-implanted mice on Day 256. Brain tumor lesions were not detected in the 5 survivor mice treated with anti-EGFRvIII EGCit-MMAE ADC **8b** (complete remission: 5 out of 7 mice).

Supplementary Tables

Table S1. Stability of pyrene-based peptide probes in the presence of human neutrophil enzymes. Quantification was performed after incubation for 24 h (n = 2). Data are presented as mean values ± SEM.

Probe	% intact probe		
	Neutrophil elastase	Proteinase 3	Cathepsin G
VCit **1**	37.6 ± 1.5	67.9 ± 2.8	83.5 ± 2.8
EVCit **2**	16.1 ± 0.2	6.5 ± 0.1	63.0 ± 0.5
EGCit **3a**	99.2 ± 0.3	98.0 ± 1.1	100.0 ± 1.8
EV(*N*-Me)Cit **3e**	99.0 ± 2.4	84.4 ± 0.9	99.6 ± 0.3

Table S2. Stability of pyrene-based peptide probes in undiluted human, monkey, and mouse plasma. Quantification was performed after incubation for 48 h and 96 h (n = 2). Data are presented as mean values ± SEM.

Probe	% intact probe					
	48 h incubation			96 h incubation		
	Human	Monkey	Mouse	Human	Monkey	Mouse
VCit **1**	97.3 ± 0.2	91.5 ± 1.3	0	86.2 ± 2.5	79.8 ± 3.1	0
EVCit **2**	95.1 ± 0.3	95.8 ± 3.9	13.0 ± 0.5	91.1 ± 1.1	95.8 ± 1.8	0
EGCit **3a**	96.5 ± 1.7	98.7 ± 0.7	67.1 ± 0.9	100.3 ± 5.5	98.8 ± 1.6	45.0 ± 3.8
EV(*N*-Me)Cit **3e**	99.5 ± 0.2	97.8 ± 0.9	39.1 ± 0.6	86.4 ± 3.2	91.9 ± 1.2	15.3 ± 2.9

Table S3. K_D values of unmodified N297A anti-HER2 mAb and ADC **4a**–**f** and **5** in breast cancer lines (n = 3). Calculated based on Fig. S2. Values in parentheses are 95% confidential intervals.

	K_D (nM)	
	KPL-4	MDA-MB-231
N297A anti-HER2 mAb	0.151 (0.124–0.184)	-
VCit ADC **4a**	0.131 (0.122–0.140)	-
EVCit ADC **4b**	0.132 (0.119–0.147)	-
EGCit ADC **4c**	0.084 (0.069–0.102)	-
EV(*N*-Me)Cit **4d**	0.156 (0.133–0.184)	-
GCit ADC **4e**	0.145 (0.126–0.167)	-
Non-cleavable ADC **4f**	0.090 (0.075–0.109)	-
Isotype control EGCit ADC **5**	1507 (not determined)	-

Table S4. EC_{50} values in human breast cancer cell lines and a GBM cell line (n = 3). Calculated based on Fig. 3 and Fig. S5. Values in parentheses are 95% confidential intervals.

	EC_{50} (nM)						
	KPL-4	SK-BR-3	BT-474	JIMT-1	MDA-MB-453	MDA-MB-231	U87ΔEGFR-luc
VCit ADC **4a**	0.084 (0.068–0.102)	0.120 (0.107–0.139)	0.543 (0.300–1.085)	0.102 (0.090–0.117)	0.193 (0.157–0.239)	–	Not tested
EVCit ADC **4b**	0.070 (0.056–0.087)	0.160 (0.148–0.174)	0.470 (0.363–0.607)	0.086 (0.069–0.104)	0.272 (0.197–0.393)	–	Not tested
EGCit ADC **4c**	0.084 (0.078–0.090)	0.167 (0.152–0.183)	0.476 (0.374–0.602)	0.110 (0.100–0.120)	0.260 (0.207–0.307)	–	Not tested
EV(*N*-Me)Cit **4d**	0.234 (0.181–0.300)	0.170 (0.144–0.203)	1.101 (0.796–1.474)	0.394 (0.278–0.583)	1.196 (0.520–9.507)	–	Not tested
GCit ADC **4e**	0.090 (0.084–0.097)	0.154 (0.114–0.221)	0.620 (0.470–0.816)	0.180 (0.153–0.209)	0.619 (0.505–0.775)	–	Not tested
Non-cleavable ADC **4f**	0.476 (0.182–1.180)	Not tested	Not tested	0.644 (Not determined)	Not tested	Not tested	Not tested
EGCit DuoDM ADC **6**	0.114 (0.096–0.135)	Not tested	Not tested	0.060 (0.035–0.094)	Not tested	Not tested	Not tested
EGCit MMAE/F ADC **7a**	0.016 (0.014–0.018)	Not tested	Not tested	0.042 (0.040–0.043)	Not tested	Not tested	Not tested
VCit MMAE ADC **8a**	Not tested	Not tested	Not tested	Not tested	Not tested	Not tested	0.344 (0.295–0.405)
EGCit ADC **8b**	Not tested	Not tested	Not tested	Not tested	Not tested	Not tested	0.344 (0.296–0.401)
PABC-DuoDM ADC **S22a**	Not tested	Not tested	Not tested	Not tested	Not tested	Not tested	0.374 (0.339–0.410)
PABQ-DuoDM ADC **S22b**	Not tested	Not tested	Not tested	Not tested	Not tested	Not tested	0.348 (0.321–0.380)

Table S5. Cell viability at the maximum ADC concentration (n = 3). Values in parentheses are 95% confidential intervals.

	% Cell viability at the maximum ADC concentration						
	KPL-4	SK-BR–3	BT-474	JIMT-1	MDA-MB-453	MDA-MB-231	U87ΔEGFR-luc
VCit ADC **4a**	4.347 (0.7592–7.829)	33.62 (31.35–35.82)	18.02 (2.950–28.41)	15.90 (12.74–18.81)	35.06 (32.08–37.79)	–	Not tested
EVCit ADC **4b**	3.897 (0.3098–7.427)	22.94 (21.61–24.26)	21.02 (16.35–25.18)	14.64 (10.18–18.95)	29.26 (23.80–33.63)	–	Not tested
EGCit ADC **4c**	2.706 (1.335–4.067)	22.84 (21.31–24.36)	20.70 (16.77–24.24)	13.00 (11.33–14.64)	27.80 (24.55–30.93)	–	Not tested
EV(*N*-Me)Cit **4d**	15.86 (11.47–20.19)	29.90 (27.27–32.47)	35.15 (31.02–39.16)	59.28 (55.96–61.73)	56.73 (16.79–64.63)	–	Not tested
GCit ADC **4e**	2.664 (1.022–4.248)	38.74 (34.23–43.08)	14.10 (8.767–18.83)	12.56 (9.912–15.18)	39.13 (35.89–42.05)	–	Not tested
Non-cleavable ADC **4f**	67.65 (61.17–73.01)	Not tested	Not tested	88.99 (Not determined)	Not tested	Not tested	Not tested
EGCit DuoDM ADC **6**	3.842 (0.3194–7.209)	Not tested	Not tested	16.80 (10.13–22.27)	Not tested	Not tested	Not tested
EGCit MMAE/F ADC **7a**	10.86 (8.805–12.90)	Not tested	Not tested	4.346 (3.603–5.087)	Not tested	Not tested	Not tested
VCit MMAE ADC **8a**	Not tested	Not tested	Not tested	Not tested	Not tested	Not tested	26.73 (23.43–29.83)
EGCit ADC **8b**	Not tested	Not tested	Not tested	Not tested	Not tested	Not tested	27.24 (24.18–30.12)
PABC-DuoDM ADC **S22a**	Not tested	Not tested	Not tested	Not tested	Not tested	Not tested	-0.9510 (-3.246–1.302)
PABQ-DuoDM ADC **S22b**	Not tested	Not tested	Not tested	Not tested	Not tested	Not tested	-0.1287 (-1.884–1.619)

Table S6. Stability of ADCs in undiluted human plasma. Quantification was performed after incubation for 7, 14, and 28 days (n = 3). Data are presented as mean values ± SEM.

	Residual ADC (%)			
	Day 0	Day 7	Day 14	Day 28
VCit ADC **4a**	100 ± 0.84	94.8 ± 1.39	94.3 ± 1.68	94.9 ± 1.67
EVCit ADC **4b**	100 ± 2.24	92.0 ± 3.42	91.3 ± 1.58	95.6 ± 1.25
EGCit ADC **4c**	100 ± 1.71	92.2 ± 1.64	91.2 ± 1.95	90.7 ± 0.17

Table S7. Stability of ADCs in undiluted monkey plasma. Quantification was performed after incubation for 7, 14, and 28 days (n = 3). Data are presented as mean values ± SEM.

	Residual ADC (%)			
	Day 0	Day 7	Day 14	Day 28
VCit ADC **4a**	100 ± 1.03	103 ± 3.53	98.7 ± 2.48	98.1 ± 1.40
EVCit ADC **4b**	100 ± 3.77	102 ± 2.74	100 ± 7.83	99 .4± 3.32
EGCit ADC **4c**	100 ± 6.92	103 ± 4.74	101 ± 4.46	103 ± 0.89

Table S8. Stability of ADCs in undiluted mouse plasma. Quantification was performed after incubation for 4, 7, and 14 days (n = 3). Data are presented as mean values ± SEM.

	Residual ADC (%)			
	Day 0	Day 4	Day 7	Day 14
VCit ADC **4a**	100 ± 4.73	29.0 ± 1.95	19.2 ± 1.53	15.8 ± 1.74
EVCit ADC **4b**	100 ± 5.31	96.0 ± 3.92	91.4 ± 9.41	107 ± 13.0
EGCit ADC **4c**	100 ± 2.50	97.6 ± 4.41	96.8 ± 3.48	98.2 ± 9.01

Table S9. Statistical significance.

Main Figures	Method	Comparison	Crude *P* value	Adjusted *P* value[a]	Significance
Fig. 3j	Dunnett's test	EGCit ADC **4c** *vs.* EV(*N*-Me)Cit ADC **4d**	<0.0001	N/A	***
		EGCit ADC **4c** *vs.* VCit ADC **4a**	0.3838	N/A	n.s.
		EGCit ADC **4c** *vs.* EVCit ADC **4b**	0.7436	N/A	n.s.
Fig. 4h	Dunnett's test	EGCit ADC **4c** *vs.* EVCit ADC **4b**	<0.0001	N/A	***
		EGCit ADC **4c** *vs.* Vehicle	0.0619	N/A	n.s.
Fig. 5a	Two-tailed Welch's *t*-test	EGCit ADC **4c** *vs.* Enhertu	0.0159	0.0318	*
		EGCit ADC **4c** *vs.* Kadcyla	0.0630	0.0630	n.s.
Fig. 5b	Two-tailed Welch's *t*-test	EGCit ADC **4c** *vs.* Kadcyla	0.0058	0.0116	*
		EGCit ADC **4c** *vs.* Enhertu	0.0414	0.0414	*
Fig. 5c	Two-tailed Welch's *t*-test	EGCit ADC **4c** *vs.* Kadcyla	0.0789	0.1578	n.s.
		EGCit ADC **4c** *vs.* Enhertu	0.2158	0.2158	n.s.
Fig. 5d	Two-tailed Welch's *t*-test	EGCit ADC **4c** *vs.* Kadcyla	0.0064	0.0128	*
		EGCit ADC **4c** *vs.* Enhertu	0.0159	0.0159	*
Fig. 6a	Two-tailed Welch's *t*-test	**Dose: 1 mg kg^{-1} each**			
		EGCit ADC **4c** *vs.* Enhertu (Day 31)	0.0079	0.0158	*
		EGCit ADC **4c** *vs.* Kadcyla (Day 31)	0.0097	0.0097	**
Fig. 6b	Logrank (Mantel-Cox)	**Dose: 1 mg kg^{-1} each**			
		EGCit ADC **4c** *vs.* Enhertu	0.0019	0.0038	**
		EGCit ADC **4c** *vs.* Kadcyla	0.0021	0.0021	**
Fig. 6c	Two-tailed Welch's *t*-test	1 mg kg^{-1} EGCit MMAE/F ADC **7a** *vs.* 3 mg kg^{-1} Enhertu (Day 31)	0.0065	N/A	**
Fig. 6d	Logrank (Mantel-Cox)	**Dose: 5 mg kg^{-1} (each)** EGCit MMAE/F ADC **7a** *vs.* Enhertu	0.0072	N/A	**
Fig. 6f	Logrank (Mantel-Cox)	**Dose: 5 mg kg^{-1} (each)**			
		EGCit ADC **8b** *vs.* VCit ADC **8a**	0.0002	0.0006	***
		EGCit ADC **8b** *vs.* Vehicle	0.0005	0.0010	***
		VCit ADC **8a** *vs.* Vehicle	0.0010	0.0010	***

[a] Adjusted by the Holm–Bonferroni method. *$P < 0.05$; **$P < 0.01$; ***$P < 0.005$; n.s., not significant.

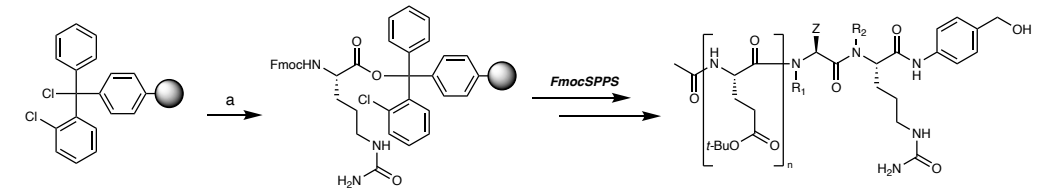
Supplementary Notes

General information

Unless otherwise noted, all materials for chemical synthesis were purchased from commercial suppliers (Acros Organics, AnaSpec, Broadpharm, Chem-Impex International, Fisher Scientific, Levena Biopharma, Sigma Aldrich, TCI America, and other vendors) and used as received. All anhydrous solvents were purchased and stored over activated molecular sieves under argon atmosphere.

Analytical reverse-phase high performance liquid chromatography (RP-HPLC) was performed using a Thermo LC-MS system consisting of a Vanquish UHPLC and a LTQ XL™ linear ion trap mass spectrometer equipped with a C18 reverse-phase column (Accucore™ Vanquish™ C18+ UHPLC column, 2.1 × 50 mm, 1.5 µm, Thermo Scientific). Standard analysis conditions for organic molecules were as follows: flow rate = 0.5 mL min^{-1}; solvent A = water containing 0.1% formic acid; solvent B = acetonitrile containing 0.1% formic acid. Compounds were analyzed using a linear gradient and monitored with UV detection at 210 and 254 nm. Preparative HPLC was performed using a Breeze HPLC system (Waters) equipped with a C18 reverse-phase column (XBridge Peptide BEH C18 OBD Prep Column, 130Å, 5 µm, 19 × 150 mm, Waters). Standard purification conditions were as follows: flow rate = 20 mL min^{-1}; solvent A = water containing 0.05% trifluoroacetic acid (TFA), 0.1% formic acid or 0.1% NH_4OH; solvent B = acetonitrile containing 0.05% TFA (standard conditions), 0.1% formic acid (FA conditions), or 0.1% NH_4OH (basic conditions). Compounds were analyzed using a linear gradient and monitored with UV detection at 210 and 254 nm. In all cases, fractions were analyzed off-line using either of the LC-MS systems for purity confirmation and those containing a desired product were lyophilized using a Labconco Freezone 4.5 Liter Benchtop Freeze Dry System. High-resolution mass spectra were obtained using a Thermo Q Exactive™ Hybrid Quadrupole-Orbitrap™ Mass Spectrometer.

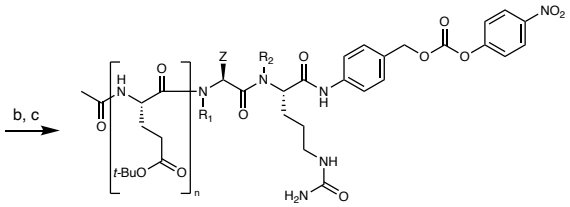
Synthesis



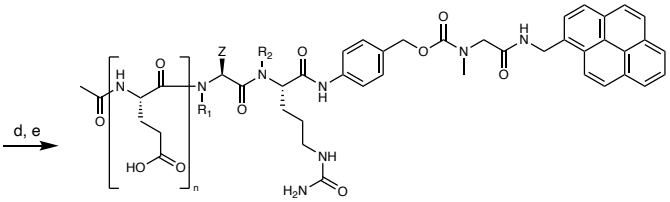
S2a (n = 1, Z = H, R_1 = H, R_2 = H)
S2b (n = 1, Z = CH_3, R_1 = H, R_2 = H)
S2c (n = 1, Z = $CH_2CH(CH_3)_2$, R_1 = H, R_2 = H)
S2d (n = 1, Z = $CH(CH_3)CH_2CH_3$, R_1 = H, R_2 = H)
S2e (n = 1, Z = $CH(CH_3)_2$, R_1 = H, R_2 = CH_3)
S2f (n = 0, Z = H, R_1 = H, R_2 = H)
S2g (n = 1, Z = CH_2-Ph, R_1 = H, R_2 = H)
S2h (n = 1, Z = $CH(CH_3)_2$, R_1 = CH_3, R_2 = H)
S2i (n = 0, Z = $CH(CH_3)_2$, R_1 = CH_3, R_2 = H)
S2j (n = 0, Z = $CH(CH_3)_2$, R_1 = H, R_2 = CH_3)

FmocSPPS

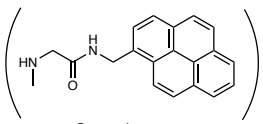
Conditions
Coupling: Fmoc-amino acid (4 equiv.)
HATU (4 equiv.)
DIPEA (6 equiv.)
o-NBS protection: *o*-NBS-Cl (4 equiv.), collidine (10 equiv,)
N-Methylation: DBU (3 equive.), NMP, 3 min; then DMS (10 equiv.), NMP, 2 min
Deprotection: 2-mercaptethanol (10 equiv.), DBU (5 equiv.)
Acetyl capping: Ac_2O (4 equiv.)
DIPEA (6 equiv.)
Fmoc deprotection: 20% piperidine in DMF or 1M oxyma in 20% piperidine in DMF
Resin cleavage: 1% TFA in DCM



S3a (n = 1, Z = H, R_1 = H, R_2 = H)
S3b (n = 1, Z = CH_3, R_1 = H, R_2 = H)
S3c (n = 1, Z = $CH_2CH(CH_3)_2$, R_1 = H, R_2 = H)
S3d (n = 1, Z = $CH(CH_3)CH_2CH_3$, R_1 = H, R_2 = H)
S3e (n = 1, Z = $CH(CH_3)_2$, R_1 = H, R_2 = CH_3)
S3f (n = 0, Z = H, R_1 = H, R_2 = H)
S3g (n = 1, Z = CH_2-Ph, R_1 = H, R_2 = H)
S3h (n = 1, Z = $CH(CH_3)_2$, R_1 = CH_3, R_2 = H)
S3i (n = 0, Z = $CH(CH_3)_2$, R_1 = CH_3, R_2 = H)
S3j (n = 0, Z = $CH(CH_3)_2$, R_1 = H, R_2 = CH_3)



3a (n = 1, Z = H, R_1 = H, R_2 = H)
3b (n = 1, Z = CH_3, R_1 = H, R_2 = H)
3c (n = 1, Z = $CH_2CH(CH_3)_2$, R_1 = H, R_2 = H)
3d (n = 1, Z = $CH(CH_3)CH_2CH_3$, R_1 = H, R_2 = H)
3e (n = 1, Z = $CH(CH_3)_2$, R_1 = H, R_2 = CH_3)
3f (n = 0, Z = H, R_1 = H, R_2 = H)
S1a (n = 1, Z = CH_2-Ph, R_1 = H, R_2 = H)
S1b (n = 1, Z = $CH(CH_3)_2$, R_1 = CH_3, R_2 = H)
S1c (n = 0, Z = $CH(CH_3)_2$, R_1 = CH_3, R_2 = H)
S1d (n = 0, Z = $CH(CH_3)_2$, R_1 = H, R_2 = CH_3)


Sarcosine-pyrene

Synthesis of pyrene probes. Reagents and conditions: (a) Fmoc-citrulline, DIPEA, DMF, room temp, 2 h; (b) *p*-aminobenzyl alcohol, EEDQ, DCM/MeOH = 4:1, room temp, overnight; (c) bis(4-nitrophenyl) carbonate, DMAP, DMF, room temp, 2 h; (d) 20% TFA/DCM, room temp, 40 min for **S3a** or 1N-HCl/ACN, room temp, 1–3 h for **S3b–e** and **S3g**,**h**; (e) sarcosine-pyrene, DMAP, DIPEA, DMF, 37 ºC, 4 h.

Fmoc Solid-Phase Peptide Synthesis (Fmoc SPPS) for acetyl-capped compounds (S2a–j)

2-Chlorotrityl chloride resin (600 mg, 0.96 mmol) and Fmoc-citrulline-OH (1.8 equiv.) were taken to a manual solid-phase reactor containing *N*,*N*-diisopropylethylamine (DIPEA, 3 equiv.) and DMF (3 mL) and agitated for 2 h. MeOH (600 μL) was added to the resin and agitated for 20 min. The solution was drained and the resin was washed with DMF (5×3 mL) and DCM (5×3 mL). To remove a Fmoc-protecting group after each coupling, the resin was treated with 20% piperidine/DMF (5 mL) or 1M oxyma in 20% piperidine/DMF (5 mL) for 20 min and washed with DMF (5×3 mL) and DCM (5×3 mL). For *N*-methylation(2), the resin was treated with 2-nitrobenzenesulfonyl chloride (*o*-NBS-Cl, 4 equiv.) and collidine (10 equiv.) in NMP (2 mL) for 15 min and washed with NMP (2×1 mL) to temporally protect amine group with *o*-NBS. After repeating *o*-NBS protection twice, the resin was treated with 1,8-diazabicyclo[5.4.0]undec-7-ene (DBU, 3 equiv.) in NMP (1 mL) for 3 min then treated with dimethyl sulfate (DMS, 10 equiv.) in NMP (1 mL) for 2 min. This step was repeated twice. Then, the resin was treated with 2-mercaptethanol (10 equiv.) and DBU (5 equiv.) in NMP (2 mL) for 5 min twice to deprotect *o*-NBS protecting group. Fmoc-protected amino acid (4 equiv.) was pre-activated using 1-[bis(dimethylamino)methylene]-1H-1,2,3-triazolo[4,5-b]pyridinium 3-oxid hexafluorophosphate (HATU, 4 equiv.) and DIPEA (6 equiv.) in DMF for 3 min, and the cocktail was added to the resin. The resin was agitated for 1 h at room temperature. The completion of the coupling was verified by the Kaiser test. After each coupling step, the coupling cocktail was drained and the resin was washed with DMF (5×3 mL) and DCM (5×3 mL). After elongation of the peptide, the resin was treated with acetic anhydride (4 equiv.) and DIPEA (6 equiv.) in DMF for 1 h and then washed with DMF (5×3 mL) and DCM (5×3 mL). The resulting protected peptide resin was treated with cocktail of 1% trifluoroacetic acid (TFA)/DCM at room temperature for 1 h. The solution was concentrated in vacuo and the crude peptide was precipitated with cold diethyl ether (20 mL) followed by centrifugation at 2,000 ×g for 3 min (3 times). The resulting crude peptide **S2a**–**j** was dried in vacuo and then used immediately in the next step without purification.

Ac-Glu(*t*-Bu)-Gly-Cit-PABC-PNP (S3a)

To a solution of crude **S2a** (9.5 mg, 21 μmol) in DCM/MeOH (4:1, 1.25 mL) were added *p*-aminobenzyl alcohol (5.2 mg, 42 μmol) and EEDQ (10.4 mg, 42 μmol). After being stirred in the dark at room temperature overnight, the solution was concentrated in vacuo and the crude peptide was precipitated with cold diethyl ether (20 mL) followed by centrifugation at 2,000 ×g for 3 min (10 times). The resulting crude peptide was dried in vacuo and then used immediately in the next step without purification. Bis(2,4-dinitrophenyl) carbonate (27.4 mg, 90 μmol) and DMAP (4.4 mg, 36 μmol) were

added to a solution of the crude peptide (10.2 mg, 18 μmol) in DMF (300 μL), and the mixture was stirred at room temperature for 2 h. The reaction was quenched with 3-N HCl/ACN at 0 ºC, then the crude products were purified by preparative RP-HPLC to afford analytically pure peptide **S3a** (6.0 mg, 39% for the 2 steps). Purity was confirmed by LC-MS. White powder. HRMS (ESI) Calcd. For $C_{33}H_{44}N_7O_{12}$ $[M+H]^+$: 730.3043. Found: 730.3033. Peptides **S3b**–**j** were synthesized from **S2b**–**j** in a similar manner.

Ac-Glu(*t*-Bu)-Ala-Cit-PABC-PNP (S3b)

47.9 mg, 80% for the 2 steps. White powder. HRMS (ESI) Calcd. For $C_{34}H_{46}N_7O_{12}$ $[M+H]^+$: 744.3199. Found: 744.3177.

Ac-Glu(*t*-Bu)-Leu-Cit-PABC-PNP (S3c)

8.1 mg, 29% for the 2 steps. White powder. HRMS (ESI) Calcd. For $C_{37}H_{52}N_7O_{12}$ $[M+H]^+$: 786.3669. Found: 786.3647.

Ac-Glu(*t*-Bu)-Ile-Cit-PABC-PNP (S3d)

42 mg, 66% for the 2 steps. White powder. HRMS (ESI) Calcd. For $C_{37}H_{52}N_7O_{12}$ $[M+H]^+$: 786.3669. Found: 786.3644.

Ac-Glu(*t*-Bu)-Val-(*N*-Me)Cit-PABC-PNP (S3e)

6.4 mg, 21% for the 2 steps. White powder. HRMS (ESI) Calcd. For $C_{37}H_{52}N_7O_{12}$ $[M+H]^+$: 786.3669. Found: 786.3643.

Ac-Gly-Cit-PABC-PNP (S3f)

14.2 mg, 31% for the 2 steps. White powder. HRMS (ESI) Calcd. For $C_{24}H_{29}N_6O_9$ $[M+H]^+$: 545.1991. Found: 545.1965.

Ac-Glu(*t*-Bu)-Phe-Cit-PABC-PNP (S3g)

14.7 mg, 44% for the 2 steps. White powder. HRMS (ESI) Calcd. For $C_{40}H_{50}N_7O_{12}$ $[M+H]^+$: 820.3512. Found: 820.3495.

Ac-Glu(*t*-Bu)-(*N*-Me)Val-Cit-PABC-PNP (S3h)

14.1 mg, 57% for the 2 steps. White powder. HRMS (ESI) Calcd. For $C_{37}H_{52}N_7O_{12}$ $[M+H]^+$: 786.3669. Found: 786.3642.

Ac-(*N*-Me)Val-Cit-PABC-PNP (S3i)

9.2 mg, 19% for the 2 steps. White powder. HRMS (ESI) Calcd. For $C_{28}H_{37}N_6O_9$ $[M+H]^+$: 601.2617. Found: 601.2591.

Ac-Val-(*N*-Me)Cit-PABC-PNP (S3j)

6.1 mg, 20% for the 2 steps. White powder. HRMS (ESI) Calcd. For $C_{28}H_{37}N_6O_9$ $[M+H]^+$: 601.2617. Found: 601.2602.

Ac-Glu-Gly-Cit-PABC-sar-pyrene (3a)

Compound **S3a** (6.0 mg, 8.2 μmol) was dissolved in 20% TFA/DCM (200 μL and 800 μL). After being stirred at room temperature for 40 min, the solution was concentrated in vacuo and the crude peptide was precipitated with cold diethyl ether (20 mL) followed by centrifugation at 2,000 ×g for 3 min (3 times). The resulting crude peptide was dried in vacuo and then used immediately in the next step without purification. The crude peptide was dissolved in DMF (300 μL) and to the solution were added sarcosine-pyrene(3) (54.5 μL, 9.0 μmol, 50 mg mL^{-1} in DMSO), DIPEA (2.9 μL, 16.4 μmol), and DMAP (10 μL, 10 mol%, 10 mg mL^{-1} in DMF). The mixture was stirred at 37 ºC for 4 h and purified by preparative RP-HPLC under acidic conditions to afford **3a** (3.3 mg, 48% for the 2 steps). White powder. HRMS (ESI) Calcd. For $C_{43}H_{49}N_8O_{10}$ $[M+H]^+$: 837.3566. Found: 837.3525. Peptides **3b**–**f** and **S1a**–**d** were synthesized from **S3b**–**j** in a similar manner.

Ac-Glu-Ala-Cit-PABC-sar-pyrene (3b)

8.1 mg, 35% for the 2 steps. White powder. HRMS (ESI) Calcd. For $C_{44}H_{51}N_8O_{10}$ $[M+H]^+$: 851.3723. Found: 851.3683.

Ac-Glu-Leu-Cit-PABC-sar-pyrene (3c)

5.0 mg, 59% for the 2 steps. White powder. HRMS (ESI) Calcd. For $C_{47}H_{57}N_8O_{10}$ $[M+H]^+$: 893.4192. Found: 893.4163.

Ac-Glu-Ile-Cit-PABC-sar-pyrene (3d)

0.8 mg, 3.5% for the 2 steps. White powder. HRMS (ESI) Calcd. For $C_{47}H_{57}N_8O_{10}$ $[M+Na]^+$: 893.4192. Found: 893.4178.

Ac-Glu-Val-(*N*-Me)Cit-PABC-sar-pyrene (3e)

0.4 mg, 5.5% for the 2 steps. White powder. HRMS (ESI) Calcd. For $C_{47}H_{57}N_8O_{10}$ $[M+H]^+$: 893.4192. Found: 893.4183.

Ac-Gly-Cit-PABC-sar-pyrene (3f)

4.2 mg, 74%. White powder. HRMS (ESI) Calcd. For $C_{38}H_{42}N_7O_7$ $[M+H]^+$: 708.3140. Found: 708.3128.

Ac-Glu-Phe-Cit-PABC-sar-pyrene (S1a)

6.9 mg, 58% for the 2 steps. White powder. HRMS (ESI) Calcd. For $C_{50}H_{55}N_8O_{10}$ $[M+H]^+$: 927.4036. Found: 927.4027.

Ac-Glu-(*N*-Me)Val-Cit-PABC-sar-pyrene (S1b)

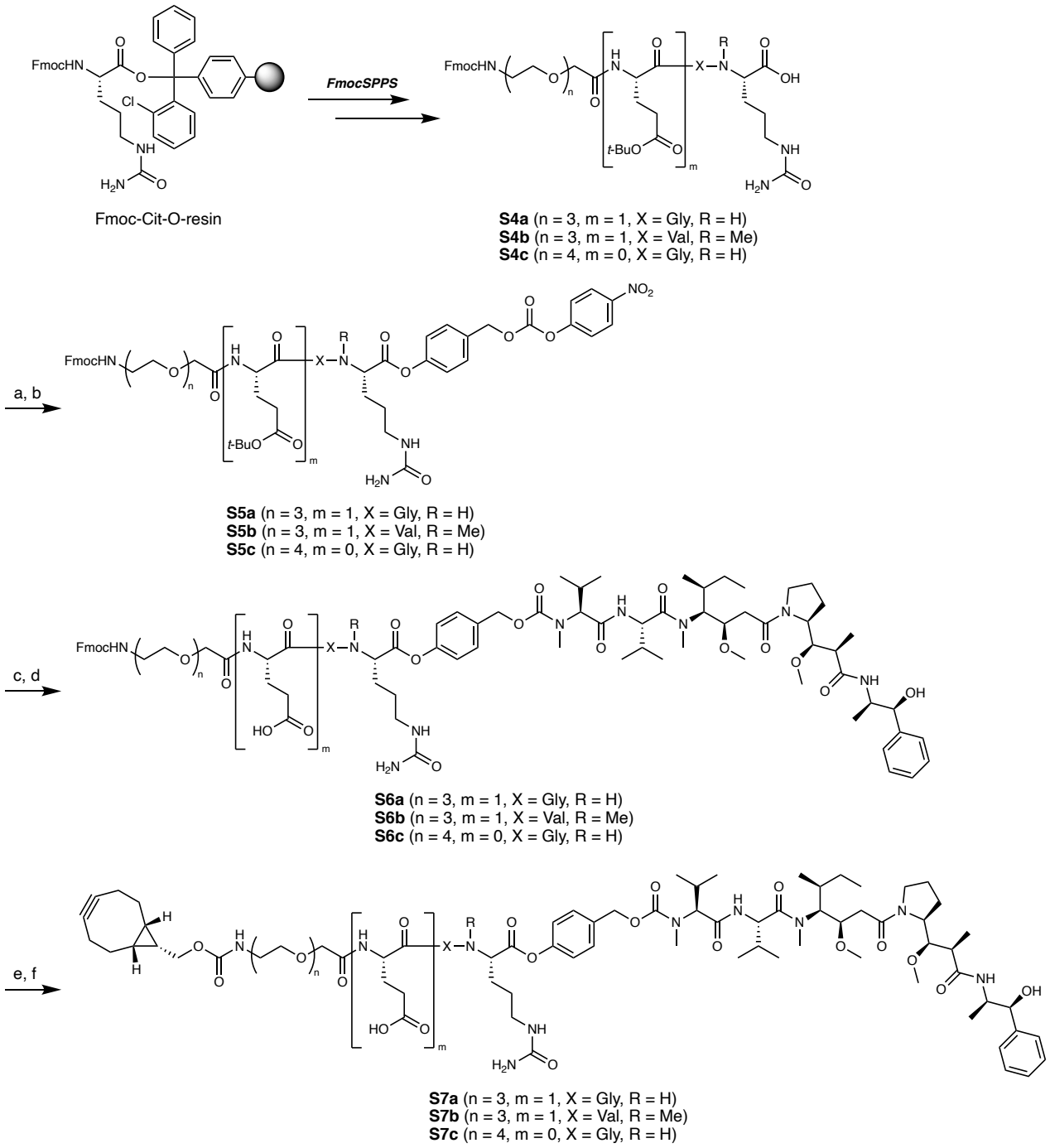
3.1 mg, 45% for the 2 steps. White powder. HRMS (ESI) Calcd. For $C_{47}H_{57}N_8O_{10}$ $[M+H]^+$: 893.4192. Found: 893.4177.

Ac-(*N*-Me)Val-Cit-PABC-sar-pyrene (S1c)

9.0 mg, 79%. White powder. HRMS (ESI) Calcd. For $C_{42}H_{50}N_7O_7$ $[M+H]^+$: 764.3766. Found: 764.3738.

Ac-Val-(*N*-Me)Cit-PABC-sar-pyrene (S1d)

2.5 mg, 33%. White powder. HRMS (ESI) Calcd. For $C_{42}H_{50}N_7O_7$ $[M+H]^+$: 764.3766. Found: 764.3750.



Synthesis of BCN–MMAE modules (**S7a–c**). Reagents and conditions: (a) *p*-aminobenzyl alcohol, EEDQ, DCM/MeOH = 4:1, room temp, overnight; (b) bis(4-nitrophenyl) carbonate, DMAP, DMF, room temp, 2 h; (c) 20% TFA/DCM, room temp, 40 min for **S5a** and **S5b**; (d) MMAE, HOAt, DIPEA, DMF, 37 ºC, overnight; (e) 50% diethylamine/DMF, room temp, 1 h; (f) BCN-NHS, DIPEA, DMF, room temp, 3 h.

Fmoc Solid-Phase Peptide Synthesis (Fmoc SPPS) for Fmoc-protected compounds (S4a–c)

Fmoc-protected peptide compounds **S4a**–**c** were prepared as described above. The resulting protected peptide resin was treated with cocktail of 1% trifluoroacetic acid (TFA)/DCM at room temperature for 1

h. The solution was concentrated in vacuo and the crude peptide was precipitated with cold diethyl ether (20 mL) followed by centrifugation at 2,000 ×g for 3 min (3 times). The resulting crude peptide **S4a**–**c** was dried in vacuo and then used immediately in the next step without purification.

Fmoc-peg3-Glu(*t*-Bu)-Gly-Cit-PABC-PNP (S5a)

To a solution of crude **S4a** (158.6 mg, 192 μmol) in DCM/MeOH (4:1, 2.5 mL) were added *p*-aminobenzyl alcohol (70.8 mg, 576 μmol) and EEDQ (285 mg, 1.15 mmol). After being stirred in the dark at room temperature overnight, the solution was concentrated in vacuo and the crude peptide was precipitated with cold diethyl ether (20 mL) followed by centrifugation at 2,000 ×g for 3 min (10 times). The resulting crude peptide was dried in vacuo and then used immediately in the next step without purification.

Bis(2,4-dinitrophenyl) carbonate (55 mg, 183 μmol) and DMAP (8.9 mg, 73 μmol) were added to a solution of the crude peptide (34.1 mg, 36.5 μmol) in DMF (1 mL), and the mixture was stirred at room temperature for 2 h. The reaction was quenched with 3-N HCl/ACN at 0 ºC, then the crude products were purified by preparative RP-HPLC to afford analytically pure peptide **S5a** (25.1 mg, 63% for the 2 steps). Purity was confirmed by LC-MS. White powder. HRMS (ESI) Calcd. For $C_{54}H_{67}N_8O_{17}$ $[M+H]^+$: 1099.4619. Found: 1099.4602. Peptides **S5b** and **S5c** were synthesized from **S4b** and **S4c** in a similar manner.

Fmoc-peg3-Glu(*t*-Bu)-Val-(*N*-Me)Cit-PABC-PNP (S5b)

8.2 mg, 17% for the 2 steps. White powder. HRMS (ESI) Calcd. For $C_{58}H_{75}N_8O_{17}$ $[M+H]^+$: 1155.5245. Found: 1155.5240.

Fmoc-peg4-Gly-Cit-PABC-PNP (S5c)

7.3 mg, 47% for the 2 steps. White powder. HRMS (ESI) Calcd. For $C_{47}H_{56}N_7O_{15}$ $[M+H]^+$: 958.3829. Found: 958.3811.

Fmoc-peg3-Glu-Gly-Cit-PABC-MMAE (S6a)

Compound **S5a** (25.1 mg, 22.8 μmol) was dissolved in 20% TFA/DCM (200 μL and 800 μL). After being stirred at room temperature for 40 min, the solution was concentrated in vacuo and the crude peptide was precipitated with cold diethyl ether (20 mL) followed by centrifugation at 2,000 ×g for 3 min (3 times). The resulting crude peptide was dried in vacuo and then used immediately in the next step without purification. The crude peptide was dissolved in DMF (500 μL) and to the solution were added MMAE

(18 mg, 25.1 μmol), DIPEA (7.9 μL, 45.6 μmol), and 1-hydroxy-7-azabenzotriazole (HOAt, 6.2 mg, 45.6 μmol). The mixture was stirred at 37 ºC for 4 h and purified by preparative RP-HPLC under acidic conditions to afford **S6a** (20.9 mg, 57% for the 2 steps). White powder. HRMS (ESI) Calcd. For $C_{83}H_{121}N_{12}O_{21}$ $[M+H]^+$: 1621.8764. Found: 1621.8722. Peptides **S6b** and **S6c** were synthesized from **S5b** and **S5c** in a similar manner.

Fmoc-peg3-Glu-Val-(*N*-Me)Cit-PABC-MMAE (S6b)

3.6 mg, 30% for the 2 steps. White powder. HRMS (ESI) Calcd. For $C_{87}H_{129}N_{12}O_{21}$ $[M+H]^+$: 1667.9390. Found: 1667.9362.

Fmoc-peg4-Gly-Cit-PABC-MMAE (S6c)

6.5 mg, 56%. White powder. HRMS (ESI) Calcd. For $C_{80}H_{118}N_{11}O_{19}$ $[M+H]^+$: 1536.8600. Found: 1536.8584.

BCN-peg3-Glu-Gly-Cit-PABC-MMAE (S7a)

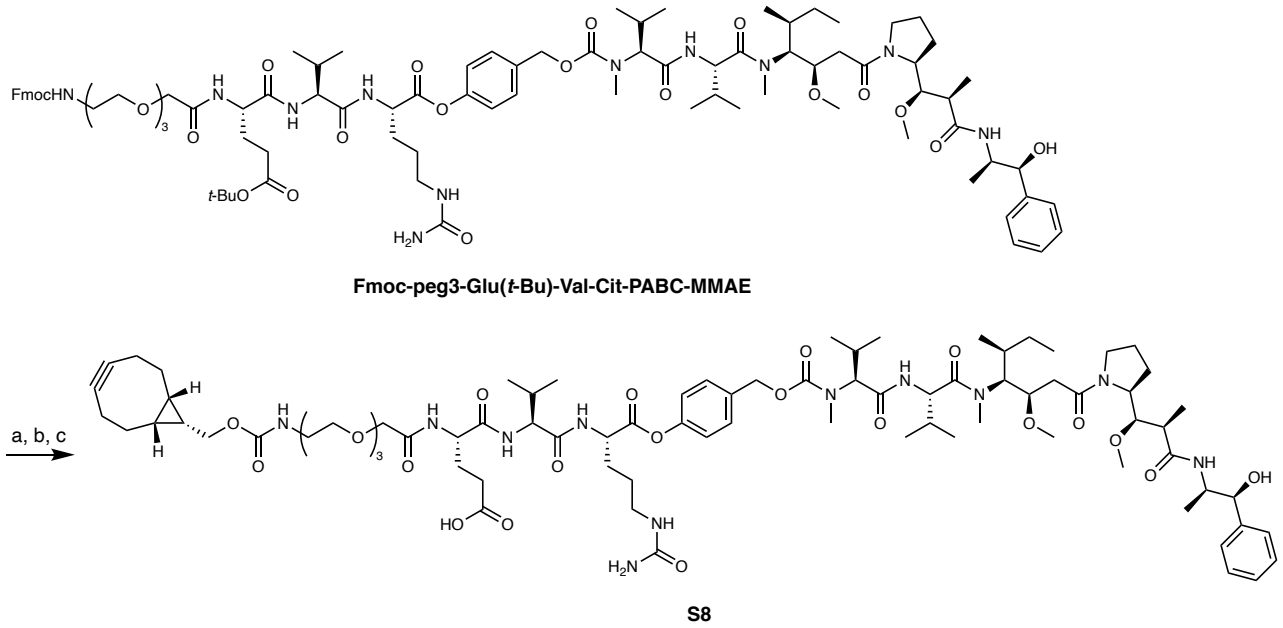
Compound **S6a** (10.5 mg, 6.5 μmol) was dissolved in 50% diethylamine/DMF solution (800 μL) at room temperature. After 1 h, the solution was concentrated in vacuo and used in the next step without further purification. BCN-NHS (2.3 mg, 7.8 μmol, Berry&Associates) and DIPEA (2.3 μL, 13 μmol) were added to a solution of this crude mixture in DMF (400 μL) and the mixture was stirred at room temperature for 3 h. The crude products were purified by preparative RP-HPLC under basic conditions to afford analytically pure peptide **S7a** (7.5 mg, 73% for the 2 steps). Purity was confirmed by LC-MS. White powder. HRMS (ESI) Calcd. For $C_{79}H_{123}N_{12}O_{21}$ $[M+H]^+$: 1575.8920. Found: 1575.8909. Modules **S7b** and **S7c** were synthesized from **S6b** and **S6c** in a similar manner.

BCN-peg3-Glu-Val-(*N*-Me)Cit-PABC-MMAE (S7b)

1.2 mg, 35% for the 2 steps. White powder. HRMS (ESI) Calcd. For $C_{83}H_{131}N_{12}O_{21}$ $[M+H]^+$: 1631.9546. Found: 1631.9529.

BCN-peg4-Gly-Cit-PABC-MMAE (S7c)

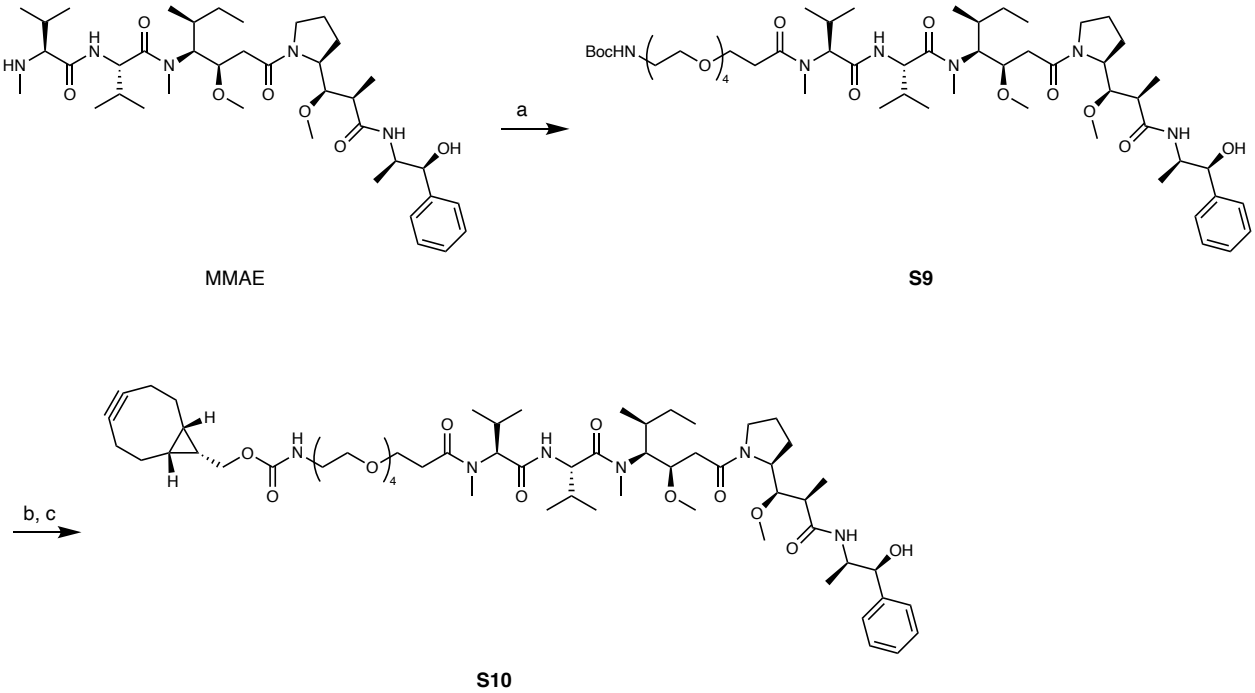
4.1 mg, 65% for the 2 steps. White powder. HRMS (ESI) Calcd. For $C_{76}H_{120}N_{11}O_{19}$ $[M+H]^+$: 1490.8757. Found: 1490.8737.



Synthesis of BCN-EVCit-MMAE module **S8.** Reagents and conditions: (a) 20% TFA/DCM, room temp, 1 h; (b) 50% diethylamine/DMF, room temp, 1 h; (c) BCN-NHS, DIPEA, DMF, room temp, overnight.

BCN-peg3-Glu-Val-Cit-PABC-MMAE (S8)

Fmoc-peg3-Glu(*t*-Bu)-Val-Cit-PABC-MMAE (7.3 mg, 4.2 μmol, prepared as described previously)(1) was added in 20% TFA/DCM solution at room temperature. After 1 h, the solution was concentrated in vacuo and the crude compounds were precipitated with cold diethyl ether (20 mL) followed by centrifugation at 2,000 *g* for 3 min (3 times). The crude products were dissolved in 50% diethylamine/DMF solution. After being stirred at room temperature for 1 h, the solution was concentrated in vacuo and used in the next step without further purification. BCN-NHS (2.5 mg, 8.4 μmol) and DIPEA (2.2 μL, 12.6 μmol) was added to a solution of this crude mixture in DMF and the mixture was stirred at room temperature overnight. The crude products were purified by preparative RP-HPLC under basic conditions to afford **S8** (4.2 mg, 61% for the 3 steps). Purity was confirmed by LC-MS. White powder. HRMS (ESI) Calcd. For $C_{82}H_{128}N_{12}O_{21}Na_2$ $[M+2Na]^{2+}$: 831.4551. Found: 831.4577.



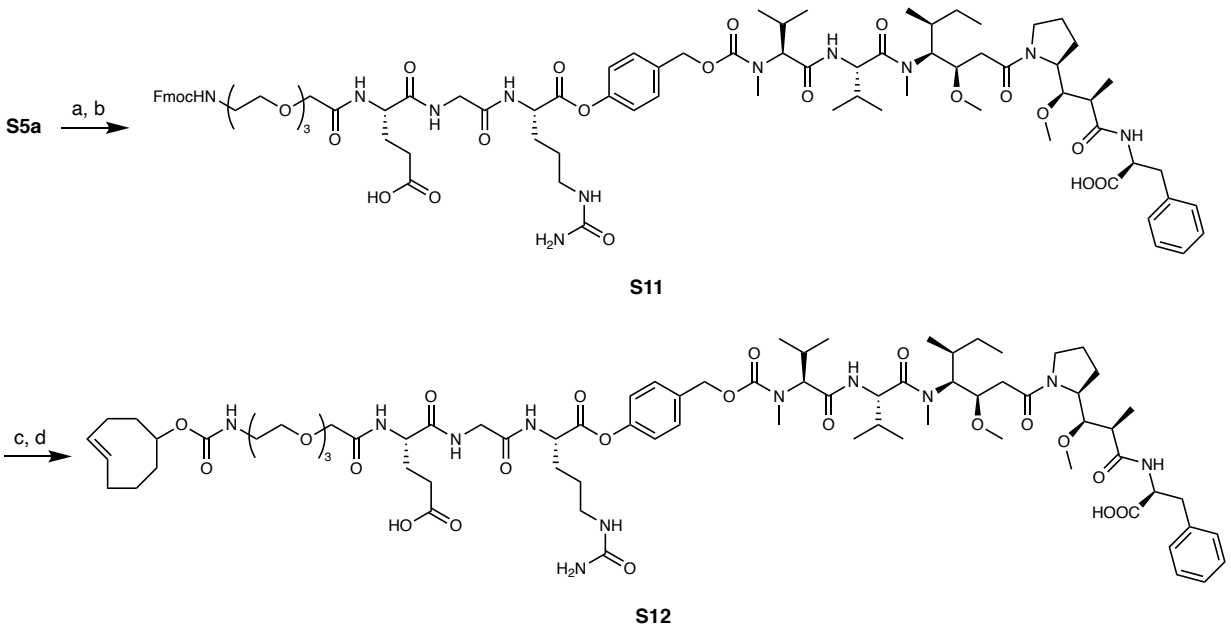
Synthesis of non-cleavable-MMAE module **S10.** Reagents and conditions: (a) Boc-peg4-acid, HATU, DIPEA, DMF, room temp, 1 h; (b) 50% TFA/DCM, room temp, 30 min; (c) BCN-NHS, DIPEA, DMF, room temp, 30 min.

Boc-peg4-MMAE (S9)

Boc-peg4 acid (5.3 mg, 14.6 μmol) in DMF (150 μL) was mixed with HATU (11 mg, 29.1 μmol) and DIPEA (7.6 μL, 43.7 μmol) and stirred for 5 min to activate it. Then the mixture was added to a solution of MMAE (7 mg, 9.7 μmol) in DMF (450 μL) and stirred at room temperature for 1 h. The crude products were purified by preparative RP-HPLC under acidic conditions to afford analytically pure peptide **S9** (13 mg, quant.). Purity was confirmed by LC-MS. White powder. HRMS (ESI) Calcd. For $C_{55}H_{97}N_6O_{14}$ $[M+H]^+$: 1065.7057. Found: 1065.7045.

BCN-peg4-MMAE (S10)

Compound **S9** (13 mg, 12.2 μmol) was dissolved in 50% TFA/DCM solution (1 mL). After being stirred at room temperature for 30 min, the solution was concentrated in vacuo and the crude compounds were precipitated with cold diethyl ether (20 mL) followed by centrifugation at 2,000 *g* for 3 min (3 times). BCN-NHS (0.3 mg, 1.0 μmol) and DIPEA (0.25 μL, 1.4 μmol) was added to a solution of this crude mixture in DMF (500 μL) and the mixture was stirred at room temperature for 30 min. The crude products were purified by preparative RP-HPLC under FA conditions to afford **S10** (1.8 mg, 13% for the 2 steps). Purity was confirmed by LC-MS. White powder. HRMS (ESI) Calcd. For $C_{61}H_{101}N_6O_{14}$ $[M+H]^+$: 1141.7370. Found: 1141.7361.



Synthesis of TCO-EGCit-MMAF module **S12.** Reagents and conditions: (a) 20% TFA/DCM, room temp, 50 min; (b) MMAF, HOAt, DIPEA, DMF, 37 ºC, overnight; (e) 50% diethylamine/DMF, room temp, 30 min; (f) TCO-NHS, DIPEA, DMF, room temp, 3.5 h.

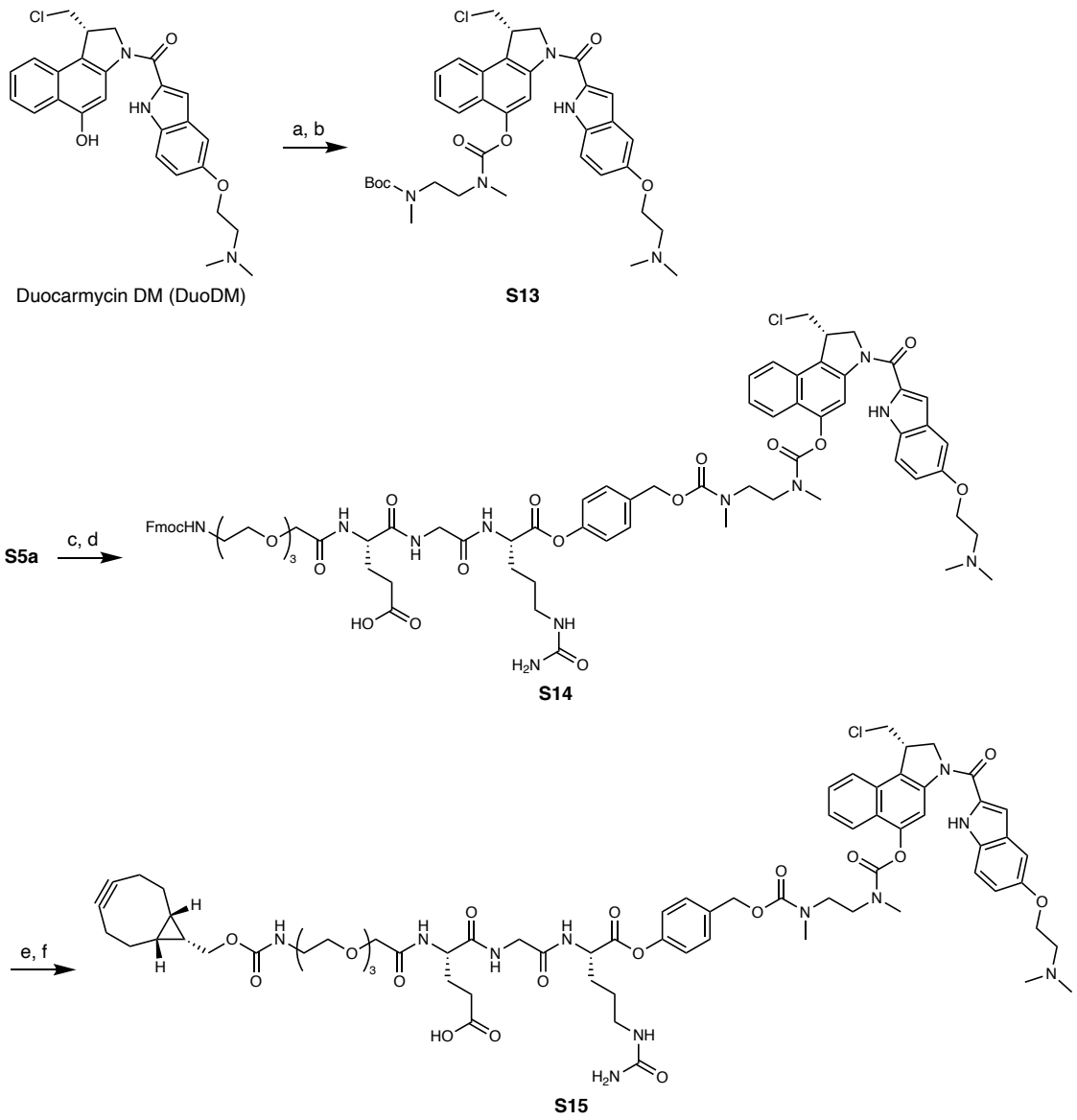
Fmoc-peg3-Glu-Gly-Cit-PABC-MMAF (S11)

Compound **S5a** (7.4 mg, 6.7 μmol) was dissolved in 20% TFA/DCM (200 μL and 800 μL). After being stirred at room temperature for 50 min, the solution was concentrated in vacuo and the crude peptide was precipitated with cold diethyl ether (20 mL) followed by centrifugation at 2,000 ×g for 3 min (3 times). The resulting crude peptide was dried in vacuo and then used immediately in the next step without purification. The crude peptide was dissolved in DMF (350 μL) and to the solution were added MMAF•TFA salt (7.4 mg, 8.7 μmol), DIPEA (2.3 μL, 13.4 μmol), and HOAt (1.8 mg, 13.4 μmol). The mixture was stirred at 37 ºC overnight and purified by preparative RP-HPLC under acidic conditions to afford **S11** (9.4 mg, 85% for the 2 steps). White powder. HRMS (ESI) Calcd. For $C_{83}H_{119}N_{12}O_{22}$ $[M+H]^+$: 1635.8556, Found: 1635.8541.

BCN-peg3-Glu-Gly-Cit-PABC-MMAF (S12)

Compound **S11** (4.7 mg, 2.9 μmol) was dissolved in 50% diethylamine/DMF solution (600 μL) at room temperature. After 30 min, the solution was concentrated in vacuo and used in the next step without further purification. BCN-NHS (1.1 mg, 3.8 μmol) and DIPEA (1 μL, 5.8 μmol) were added to a solution of this crude mixture in DMF (300 μL) and the mixture was stirred at room temperature for 3.5 h. The crude products were purified by preparative RP-HPLC under basic conditions to afford

analytically pure peptide **S12** (1.5 mg, 33% for the 2 steps). Purity was confirmed by LC-MS. White powder. HRMS (ESI) Calcd. For $C_{77}H_{121}N_{12}O_{22}$ $[M+H]^+$: 1565.8713, Found: 1565.8671.



Synthesis of BCN–DuoDM module (**S15**). Reagents and conditions: (a) 4-nitrophenyl chloroformate, DIPEA, ACN, room temp, 30 min; (b) *t*-butyl methyl(2-(methylamino)ethyl)carbamate, room temp, 15 min; (c) 20% TFA/DCM, room temp, 50 min for **S5a**, 50% TFA/DCM, 0 ºC, 30 min for **S13**; (d) Boc-deprotected **S11**, DIPEA, DMF, room temp, 2 h; (e) 50% diethylamine/DMF, room temp, 30 min; (f) BCN-NHS, DIPEA, DMF, room temp, 1 h.

Boc-protected duocarmycin DM (S13)

To a solution of duocarmycin DM (DuoDM, 7.0 mg, 12.1 μmol) in dry ACN (800 μL) were added DIPEA (6.3 μL, 36.3 μmol) and 4-nitrophenyl chloroformate (4.9 mg, 24.2 μmol). After being stirred at room temperature for 30 min, *t*-butyl methyl(2-(methylamino)ethyl)carbamate (12 μL, 60.5 μmol, AK Scientific) was added to the mixture and the mixture was stirred at room temperature for 15 min. The reaction was quenched with 2-N HCl/ACN at 0 ºC and the crude products were purified by preparative RP-HPLC to afford analytically pure compound **S13** (8.0 mg, 84% for the 2 steps). Purity was

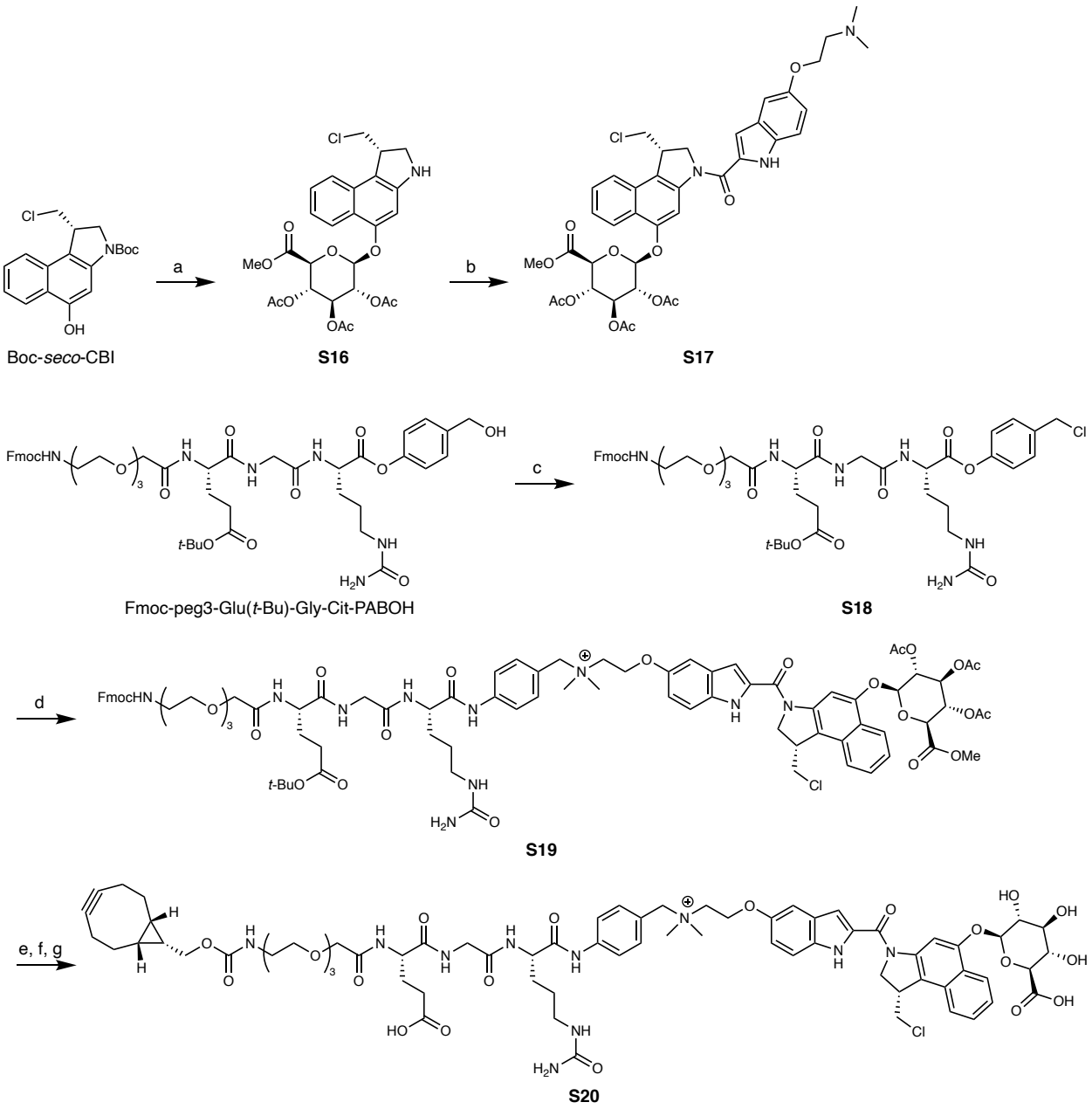
confirmed by LC-MS. White powder. HRMS (ESI) Calcd. For $C_{36}H_{45}N_5O_6Cl$ $[M+H]^+$: 678.3053, Found: 678.3029.

Fmoc-peg3-Glu-Gly-Cit-PABC-DuoDM (S14)

Compound **S5a** (10.4 mg, 9.5 μmol) was dissolved in 20% TFA/DCM (200 μL and 800 μL). After being stirred at room temperature for 50 min, the solution was concentrated in vacuo and the crude peptide was precipitated with cold diethyl ether (20 mL) followed by centrifugation at 2,000 ×g for 3 min (3 times). In parallel, compound **S13** (8.0 mg, 10.1 μmol) was dissolved in 50% TFA/DCM (500 μL and 800 μL) at 0 ºC and the mixture was stirred for 30 min. The solution was concentrated in vacuo and the crude compound was precipitated with cold diethyl ether (20 mL) followed by centrifugation at 2,000 ×g for 3 min (3 times). The crude peptide and Boc-deprotected compound were dissolved in DMF (600 μL) and DIPEA (8.2 μL, 47.5 μmol) was added to the mixture at 0 ºC. The mixture was stirred at room temperature for 2 h and the reaction was quenched with 2-N HCl/ACN at 0 ºC. The crude products were purified by preparative RP-HPLC under acidic conditions to afford **S14** (8.7 mg, 58% for the 2 steps). White powder. HRMS (ESI) Calcd. For $C_{75}H_{90}N_{12}O_{18}Cl$ $[M+H]^+$: 1481.6179, Found: 1481.6165.

BCN-peg3-Glu-Gly-Cit-PABC-DuoDM (S15)

Compound **S14** (8.7 mg, 5.5 μmol) was dissolved in 50% diethylamine/DMF solution (600 μL) at room temperature. After 30 min, the solution was concentrated in vacuo and used in the next step without further purification. BCN-NHS (2.1 mg, 7.2 μmol) and DIPEA (1.9 μL, 11 μmol) were added to a solution of this crude mixture in DMF (300 μL) and the mixture was stirred at room temperature for 1 h. The crude products were purified by preparative RP-HPLC under basic conditions to afford analytically pure peptide **S15** (3.5 mg, 44% for the 2 steps). Purity was confirmed by LC-MS. White powder. HRMS (ESI) Calcd. For $C_{71}H_{92}N_{12}O_{18}Cl$ $[M+H]^+$: 1435.6336, Found: 1435.6329.



Synthesis of BCN–DuoDM-glucuronide module (**S20**). Reagents and conditions: (a) BF_3•Et_2O, methyl-(2,3,4-tri-*O*-acetyl-α-D-glucopyranosyl trichloroacetimidate, DCM, MS (4 Å), –20 ºC, 2 h then BF_3•Et_2O, room temp, 2 h; (b) 5-(2-dimethylaminoethoxy)indole-2-carboxylic acid, EDC•HCl, DMF, room temp, 1.5 h; (c) thionyl chloride, DCM, DMF, 0 ºC, 2.5 h; (d) **S17**, DIPEA, NaI, DMF, room temp, overnight; (e) 20% TFA/DCM, room temp, 1 h; (f) LiOH•H_2O, MeOH, room temp, 1 h; (g) BCN-NHS, DIPEA, DMF, room temp, 1.5 h.

seco-CBI-β-glucuronide (S16)

A suspension of Boc-*seco*-CBI (10 mg, 30 μmol), methyl-(2,3,4-tri-*O*-acetyl-α-D-glucopyranosyl trichloroacetimidate (21.5 mg, 45 μmol, AmBeed), and molecular sieves 4 Å (50 mg) in DCM (1 mL) was stirred at room temperature for 30 min. The mixture was cooled to –20 °C and BF_3•Et_2O (3.3 μL, 15 μmol, ca. 48% BF_3) in DCM (100 μL) was added dropwise. After being stirred at –20 ºC for 2 h,

additional BF_3•Et_2O (20 μL, 90 μmol, ca. 48% BF_3) in DCM (100 μL) was added dropwise, then the mixture was warmed to room temperature and stirred at room temperature for 2 h. The reaction was quenched by filtration over a celite pad. The crude products were purified by preparative RP-HPLC under FA conditions to afford analytically pure compound **S16** (13.5 mg, 82%). Purity was confirmed by LC-MS. Off-white powder. HRMS (ESI) Calcd. For $C_{26}H_{29}NO_{10}Cl$ $[M+H]^+$: 550.1475, Found: 550.1464.

Duocarmycin DM-β-glucuronide (S17)

Compound **S16** (5.4 mg, 9.8 μmol) in DMF (400 μL) was mixed with 5-(2-dimethylaminoethoxy)indole-2-carboxylic acid (7.3 mg, 29.4 μmol, prepared as described previously)(4) and *N*-(3-dimethylaminopropyl)-*N*'-ethylcarbodiimide hydrochloride (EDC•HCl, 11.2 mg, 58.5 μmol) at room temperature, them the reaction mixture was stirred at room temperature for 1.5 h. The crude products were purified by preparative RP-HPLC under FA conditions to afford analytically pure compound **S17** (5.9 mg, 77%). Purity was confirmed by LC-MS. Off-white powder. HRMS (ESI) Calcd. For $C_{39}H_{43}N_3O_{12}Cl$ $[M+H]^+$: 780.2530, Found: 780.2494.

Fmoc-peg3-Glu(*t*-Bu)-Gly-Cit-PAB-Cl (S18)

Fmoc-peg3-Glu(*t*-Bu)-Gly-Cit-PABOH (21.8 mg, 23 μmol, an intermediate of **S5a**) in dry DMF (300 μL) was cooled to 0 ºC and thionyl chloride (1.8 μL, 25 μmol) in dry DCM (100 μL) was added dropwise. After being stirred at 0 ºC for 1 h, additional thionyl chloride (1.8 μL, 25 μmol) in DCM (100 μL) was added dropwise. After 1 h, additional thionyl chloride (1.8 μL, 25 μmol) in DCM (100 μL) was added dropwise, then the mixture was stirred at 0 ºC for further 30 min. The crude products were purified by preparative RP-HPLC to afford peptide **S18** (13.7 mg, ~70% purity). *NOTE: A single peak was collected by preparative RP-HPLC, however, the chloride compound **S18** was hydrolyzed to the starting material during lyophilization. Thus, the purity of **S18** was decreased to ~70% after lyophilization.* White powder. HRMS (ESI) Calcd. For $C_{47}H_{63}N_7O_{12}Cl$ $[M+H]^+$: 952.4218, Found: 952.4213.

Fmoc-peg3-Glu(*t*-Bu)-Gly-Cit-PAB-DuoDM-β-glucuronide (S19)

Compound **S18** (3.4 mg, 1.8 μmol, ~70% purity) in dry DMF (250 μL) was mixed with compound **S17** (0.9 mg, 1.2 μmol), sodium iodide (0.1 mg, 0.6 μmol), and DIPEA (0.4 μL, 2.4 μmol), then the mixture was stirred at room temperature for 6 h. Additional compound **S17** (1.2 mg, 1.5 μmol) and DIPEA (0.4 μL, 2.4 μmol) was added to the mixture. After 2 h, the mixture was added additional compound **S17** (0.6

mg, 0.75 μmol) and stirred at room temperature overnight. The reaction progress was monitored by LC-MS and the starting material **S18** was still remained. The reaction mixture was warmed to 37 ºC and added additional compound **S17** (0.5 mg, 0.64 μmol). After 3 h, the crude products were purified by preparative RP-HPLC under acidic conditions to afford analytically pure compound **S19** (2.9 mg, 95%). Purity was confirmed by LC-MS. White powder. HRMS (ESI) Calcd. For $C_{86}H_{104}N_{10}O_{24}Cl$ $[M]^+$: 1695.6908, Found: 1695.6901.

BCN-peg3-Glu-Gly-Cit-PAB-DuoDM-β-glucuronide (S20)

Compound **S19** (2.7 mg, 1.6 μmol) was dissolved in 20% TFA/DCM (200 μL and 800 μL). After being stirred at room temperature for 1 h, the solution was concentrated in vacuo and the crude peptide was precipitated with cold diethyl ether (20 mL) followed by centrifugation at 2,000 ×g for 3 min (3 times). The crude peptide was dissolved in MeOH (300 μL) and $LiOH•H_2O$ (2 mg, 48 μmol) in water (300 μL) was added to the mixture. After being stirred at room temperature for 1 h, the mixture was cooled to 0 ºC and quenched with formic acid (3 μL). Then, the solution was removed in vacuo and the crude peptide was dissolved in DMF (400 μL). BCN-NHS (0.6 mg, 2.1 μmol) and DIPEA (0.5 μL, 3.2 μmol) were added to the solution and the mixture was stirred at room temperature for 1.5 h. The crude products were purified by preparative RP-HPLC under basic conditions to afford analytically pure peptide **S20** (1.0 mg, 42% for the 3 steps). Purity was confirmed by LC-MS. White powder. HRMS (ESI) Calcd. For $C_{71}H_{90}N_{10}O_{21}Cl$ $[M]^+$: 1453.5965, Found: 1453.5950.

Human neutrophil protease-mediated cleavage assay using pyrene probes

[1] Stability in human neutrophil elastase. Each test compound (10 mM in DMSO, 2 μL) was mixed with 97 μL of tris-buffered saline (TBS, pH 7.4) and 1 μL of 1-pyrenemethylamine (10 mM in DMSO, internal standard). The mixture was incubated at 37 °C for 10 min. Human neutrophil elastase (40 ng μL^{-1}, 20 μL in TBS, MilliporeSigma) was added to the test compound mixture in 1:1 volume ratio respectively, followed by incubation at 37 °C. Aliquots (5 μL) were collected at each time point (0, 1, 3, 6, and 24 h). Cold acetonitrile containing 1% formic acid (25 μL) was added to precipitate proteins. The mixture was then kept at –20 °C for 30 min. Precipitated proteins were pelleted by centrifugation (15,000 × *g*, 4 °C, 30 min) and supernatant of each sample was analyzed for quantification by analytical HPLC (UV absorbance at 342 nm). The amount of each probe was normalized to the peak area of the internal standard. [2] Stability in human proteinase 3. Assays were performed in the same manner using human proteinase 3 (250 ng μL^{-1}, 5 μL in TBS, MilliporeSigma) and aliquots (5 μL) of the sample solution were collected at each time point (0 and 24 h). [3] Stability in human cathepsin G. Assays were performed in the same manner using human cathepsin G (330 ng μL^{-1}, 5 μL in TBS, MilliporeSigma) and aliquots (5 μL) of the sample solution were collected at each time point (0 and 24 h). All assays were performed at least three times in technical duplicate, and data shown are representative of the replicates.

Plasma stability test using pyrene probes

Each test compound (10 mM in DMSO, 2 μL) was mixed with 1 μL of 1-pyrenemethylamine (10 mM in DMSO, internal standard) and incubated at 37 °C for 10 min. Pooled healthy human plasma, monkey cynomolgus plasma or BALB/c mouse plasma (197 μL, Innovative Research) was added to the mixture, followed by incubation at 37 °C. Aliquots (5 μL) were collected at each time point (0, 48, and 96 h for human and monkey cynomolgus plasma; 0, 1, 6, 24, 48, and 96 h for BALB/c mouse plasma) and 25 μL of cold acetonitrile containing 1% formic acid was added to precipitate proteins. The mixture was then kept at –20 °C for 30 min. Precipitated proteins were separated by centrifugation (15,000 × g, 4 °C, 30 min) and supernatant of each sample was obtained and analyzed for quantification by analytical HPLC. All assays were performed at least three times in technical duplicate, and data shown are representative of the replicates.

Antibodies

Anti-HER2, anti-EGFRvIII, and isotype control mAbs with a N297A mutation were expressed in house. Kadcyla® and Enhertu® injectable powders were obtained from the pharmacy division at the M.D.

Anderson Cancer Center. The other antibodies used in this study were purchased from commercial vendors as follows: Mouse anti-MMAE/F mAb (Cat# LEV-MAF3) from Levena Biopharma; goat anti-human IgG Fab antibody (Cat# 109-005-097, RRID:AB_2337540), goat anti-mouse IgG-HRP conjugate (Cat# 115-035-071, RRID:AB_2338506), and donkey anti-human IgG-HRP conjugate (Cat# 709-035-149, RRID:AB_2340495) from Jackson ImmunoResearch; mouse anti-human CD66b FITC conjugate (Cat# 305104, RRID:AB_314496), mouse anti-human CD15 APC conjugate (Cat# 301908, RRID:AB_314200), and mouse anti-human CD34 FITC conjugate (Cat# 343503, RRID:AB_1731923) from BioLegend.

Expression and purification of human monoclonal antibodies

Free style HEK-293 human embryonic kidney cells (Invitrogen) were transfected with a mammalian expression vector encoding for the human IgG1 kappa light chain and full length heavy chain sequences (based on the variable sequences of trastuzumab and depatuxizumab). A mutation of N297A was incorporated into the heavy chain constant region to produce aglycosylated mAbs. The transfected HEK-293 cells were cultured in a humidified cell culture incubator at 37 °C with 8% CO_2 and shaking at 150 rpm for 7 days before harvesting the culture medium. The antibody secreted into the culture medium was purified using Protein A resin (GE Healthcare).

MTGase-mediated antibody–linker conjugation

Anti-HER2 mAb with a N297A mutation (1.06 mL in PBS, 10.5 mg mL^{-1}, 11.1 mg antibody) was incubated with the diazide branched linker developed by us previously (37.1 μL of 100 mM stock in water, 50 equiv.) and Activa TI® (275 μL of 40% solution in PBS, Ajinomoto, purchased from Modernist Pantry) at room temperature for 16–20 h. The reaction was monitored using a Thermo LC-MS system consisting of a Vanquish UHPLC and a Q Exactive™ Hybrid Quadrupole-Orbitrap™ Mass Spectrometer equipped with a MabPac RP column (2.1 × 50 mm, 4 μm, Thermo Scientific). Elution conditions were as follows: mobile phase A = water (0.1% formic acid); mobile phase B = acetonitrile (0.1% formic acid); gradient over 3 min from A:B = 75:25 to 45:55; flow rate = 0.25 mL min^{-1}. The conjugated antibody was purified by SEC (Superdex 200 increase 10/300 GL, GE Healthcare, solvent: PBS, flow rate = 0.6 mL min^{-1}) to afford an antibody–linker conjugate [9.62 mg, 86% yield determined by bicinchoninic acid (BCA) assay]. The other antibody–linker conjugates including tri-armed linker used in this study were prepared in the same manner.

Strain-promoted azide−alkyne cycloaddition for payload installation

BCN–PEG_3–EGCit–PABC–MMAE (18.9 μL of 10 mM stock solution in DMSO, 2 equivalent per azide group) was added to a solution of the mAb–linker conjugate in PBS (1.70 mL, 2.1 mg mL^{-1}), and the mixture was incubated at room temperature for 17 h. The reaction was monitored using a Thermo LC-MS system consisting of a Vanquish UHPLC and a Q Exactive™ Hybrid Quadrupole-Orbitrap™ Mass Spectrometer equipped with a MabPac RP column. The crude products were purified by SEC to afford an ADC [3.0 mg, 85% yield determined by bicinchoninic acid (BCA) assay]. Analysis and purification conditions were the same as described above. Average DAR values were determined based on UV peak areas and ESI-MS analysis. The other conjugates including tri-armed linker used in this study were prepared in a similar manner or according to our previous reports.

HIC analysis

Each ADC (1 mg mL^{-1}, 10 μL in PBS) was analyzed using an Agilent 1100 HPLC system equipped with a MAbPac HIC-Butyl column (4.6 × 100 mm, 5 μm, Thermo Scientific). Elution conditions were as follows: mobile phase A = 50 mM sodium phosphate containing ammonium sulfate (1.5 M) and 5% isopropanol (pH 7.4); mobile phase B = 50 mM sodium phosphate containing 20% isopropanol (pH 7.4); gradient over 30 min from A:B = 99:1 to 1:99; flow rate = 0.5 mL min^{-1}.

Long-term stability test

Each ADC (1 mg mL^{-1}, 100 μL in PBS) was incubated at 37 °C. Aliquots (8 μL) were taken at 28 days and immediately stored at −80 °C until use. Samples were analyzed using an Agilent 1100 HPLC system equipped with a MAbPac SEC analytical column (4.0 × 300 mm, 5 μm, Thermo Scientific). Elution conditions were as follows: flow rate = 0.2 mL min^{-1}; solvent = PBS.

Cell-based ELISA

KPL-4 or MDA-MB-231 cells were seeded in a culture- treated 96-well clear plate (10,000 cells per well in 100 μL culture medium) and incubated at 37 °C under 5% CO_2 for 24 h. Paraformaldehyde (8% in PBS, 100 μL) was added to each well and incubated for 15 min at room temperature. The medium was aspirated and the cells were washed three times with 100 μL of PBS. Cells were treated with 100 μL of blocking buffer [0.2% bovine serum albumin (BSA) in PBS] with agitation at room temperature for 2 h. After the blocking buffer was discarded, serially diluted ADC samples (in 100 μL PBS containing 0.1% BSA) were added and the plate was incubated overnight at 4 °C with agitation. The buffer was discarded and the cells were washed three times with 100 μL of PBS containing 0.25% Tween 20. Cells were then incubated with 100 μL of donkey anti-human IgG–HRP conjugate (diluted 1:10,000 in PBS

containing 0.1% BSA) at room temperature for 1 h. The plate was washed three times with PBS containing 0.25% Tween 20 and 100 μL of 3,3',5,5'-tetramethylbenzidine substrate (0.1 mg mL^{-1}) in phosphate–citrate buffer/30% H_2O_2 (1:0.0003 volume to volume, pH 5) was added. After color was developed for 10–30 min, 25 μL of 3*N*-HCl was added to each well and then the absorbance at 450 nm was recorded using a BioTek Synergy HTX plate reader. K_D values were then calculated using Graph Pad Prism 9 software. All assays were performed in triplicate.

Quantification of intracellularly released MMAE

KPL-4 cells were seeded in a culture-treated 12-well plate (3×10^5 cells per well in 500 μL culture medium) and incubated at 37 °C under 5% CO_2 for 24 h. Each ADC (final conc. 4 μg mL^{-1}, 26.7 nM) was added to each well and the plate was incubated at 37 ºC for 24 h. After the plate was centrifuged (5 min at 500 × g at 4 °C), the supernatant (450 μL) was collected then proteins were precipitated by adding MeOH (600 μL). Cells were lysed with MeOH (500 μL) and transferred to a microtube. Additional 100 μL of MeOH was added to rinse the plate and transferred to the microtube. The supernatant and cell lysate were centrifuged (10 min at 15,000 × *g*), and each supernatant was transferred to microtubes. These supernatants were then dried by air flow at 40 ºC. After drying over, MS grade water (30 μL) was added, and the mixture was centrifuged (5 min at 15,000 × g). Then the supernatant was analyzed by the Q Exactive™ Hybrid Quadrupole-Orbitrap™ Mass Spectrometer. For quantitation, six-point standard curves were made using serial dilutions of free MMAE (0.032–100 nM). Non-treated cell lysates were used for the free MMAE standard curves. All assays were performed in triplicate.

Plasma stability test using ADCs

[1] Stability in mouse plasma. Each ADC (100 μg mL^{-1}, 1.2 μL in PBS) was added to undiluted BALB/c mouse plasma (118.8 μL) to a final concentration of 1 μg mL^{-1}. After incubation at 37 °C for varying time, aliquots (15 μL each) were taken and stored at −80 °C until use. Samples were analyzed by sandwich ELISA assay. A high-binding 96 well plate (Corning) was coated with goat anti-human IgG Fab antibody (500 ng per well). After overnight coating at 4 °C, the plate was blocked with 100 μL of 2% BSA in PBS containing 0.05% Tween 20 (PBS-T) with agitation at room temperature for 1 h. Subsequently, the solution was removed and each ADC sample (100 μL in PBS-T containing 1% BSA) was added to each well, and the plate was incubated at room temperature for 2 h. After each well was washed three times with 100 μL of PBS-T, 100 μL of mouse anti-MMAE/F mAb (1:5,000) was added. After being incubated at room temperature for 1 h, each well was washed three times with 100 μL of

PBS-T, 100 μL of goat anti-mouse IgG-HRP conjugate (1:10,000) was added. After 1 h, the plate was washed three times with 100 μL of PBS-T and 100 μL of 3,3',5,5'-tetramethylbenzidine (TMB) substrate (0.1 mg mL^{-1}) in phosphate–citrate buffer/30% H_2O_2 (1:0.0003 volume to volume, pH 5) was added. After color was developed for 10−30 min, 25 μL of 3*N*-HCl was added to each well and then the absorbance at 450 nm was recorded using a plate reader (Biotek Cytation 5). Concentrations were calculated based on a standard curve. [2] Stability in human plasma and monkey plasma. Assays were performed in the same manner using human HER2 (100 ng per well, ACROBiosystems) for plate coating, mouse anti-MMAE/F mAb (1:5,000) and goat anti-mouse IgG–HRP conjugate (1:10,000) as secondary and tertiary detection antibodies, respectively. All assays were performed in triplicate.

Human neutrophil enzyme-mediated cleavage assay using ADCs

Each ADC (1 mg mL^{-1}, 30 μL) in TBS buffer was incubated at 37 °C for 10 min. To the solution was added pre-warmed human neutrophil elastase (1 ng μL^{-1}, 30 μL, MilliporeSigma), followed by incubation at 37 °C for 24 h. The samples were analyzed using a Thermo LC-MS system consisting of a Vanquish UHPLC and a Q Exactive™ Hybrid Quadrupole-Orbitrap™ Mass Spectrometer equipped with a MabPac RP column (2.1 × 50 mm, 4 μm, Thermo Scientific). Elution conditions were as follows: mobile phase A = water (0.1% formic acid); mobile phase B = acetonitrile (0.1% formic acid); gradient over 3 min from A:B = 75:25 to 45:55; flow rate = 0.25 mL min^{-1}. Note: Hinge cleavage of the anti-HER2 mAb and ADCs was observed in the analysis.

Differentiations of human hematopoietic stem and progenitor cells (HSPC) into neutrophils

CD34-positive HSPCs isolated from the bone marrow were purchased from AllCells (received as a cryopreserved sample and stored in liquid nitrogen until use). CD34 expression in HSPCs was validated by flow cytometry using mouse anti-human CD34 FITC conjugate (see the next section for experimental details). Reagents purchased from StemCell Technologies for this assay are as follows: cell culture medium (StemSpan™ SFEM II, 09655), supplements for expansion (StemSpan™ CC100, 02690), interleukin 3 (IL-3), stem cell factors (SCF), Flt-3 ligands (Flt-3L), granulocyte-macrophage colony-stimulating factors (GM-CSF), and granulocyte colony-stimulating factors (G-CSF). HSCs (2×10^4 cells mL^{-1}) were expanded in StemSpan™ SFEM II supplemented with CC100 for 3 days. Prior to differentiation, the expression levels of granulocyte markers (CD66b and CD15) were measured using a BD LSR II flow cytometer (Day 0, see the next section for details). Expanded HSCs were incubated for 4 days in StemSpan™ SFEM II supplemented with SCF (50 ng mL^{-1}), Flt-3L (100 ng mL^{-1}), IL-3 (5 ng mL^{-1}), GM-CSF (5 ng mL^{-1}), and G-CSF (5 ng mL^{-1}). On Day 4, cell culture medium was replaced with

StemSpan™ SFEM II supplemented with IL-3 (5 ng mL^{-1}) and G-CSF (30 ng mL^{-1}) and cells were further incubated for 3 days. On Day 7, differentiated cells were measured for CD15 and CD66b by flow cytometry.

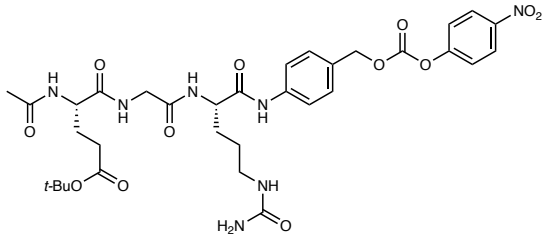
Flow cytometry

Cells were washed twice with cold Stain Buffer (1 mL for microtubes; 200 µL for microwell plates, FBS, BD Bioscience, catalog #: 554656) and harvested by centrifugation (400 × g, 5 min at 4 °C). Then, the cells were resuspended with cold Stain Buffer (100 µL) in microtubes or microwell plates and mixed with 5 µL of mouse anti-human CD34 FITC conjugate (for validation of human HSPCs) or mouse anti-human CD66b FITC conjugate and/or mouse anti-human CD15 APC conjugate (for quantification of the neutrophil population). To set voltages and gating parameters for obtaining accurate fluorescence signal, a drop of UltraComp eBeads™ Compensation Beads (Invitrogen, catalog #: 01-2222-42) was also labeled with 5 µL of the antibodies separately from the cells. Cells and beads were incubated in the dark on ice for 20 min, then washed three times with either 1 mL (for microtubes) or 200 µL (for microwell plates) of cold Stain Buffer to remove unbound antibodies. After centrifugation (400 × g, 5 min at 4 °C), the supernatants were carefully aspirated from cell pellets or beads. Stained cells or beads were resuspended with 1 mL of cold Stain Buffer, transferred to tubes for flow cytometric analysis, and data were acquired with LSR II flow cytometer with Diva acquisition software (version 8.01.1, BD Biosciences) gated for live cells with appropriate isotype-matched controls and unstained cells as negative controls. Data analysis was performed using FlowJo analysis software (version 10.8.0, FlowJo, LLC). Percentage of CD66b/CD15-positive cells or singlet cells generated from flow cytometry was normalized against non-treated samples. All assays were performed in triplicate.

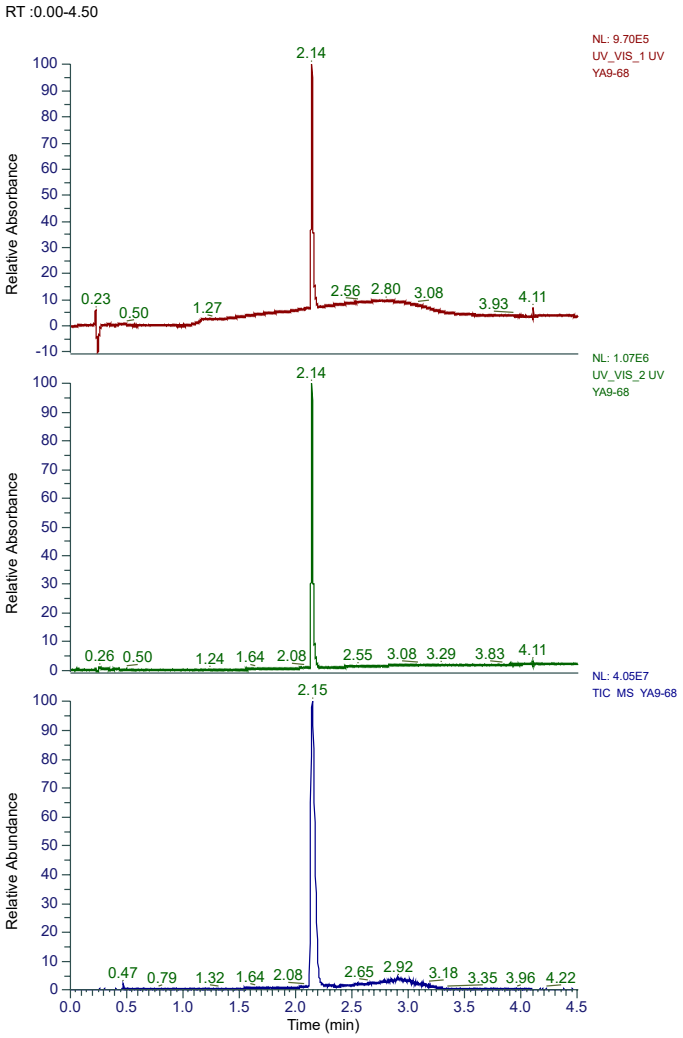
MRI in the U87ΔEGFR-luc model

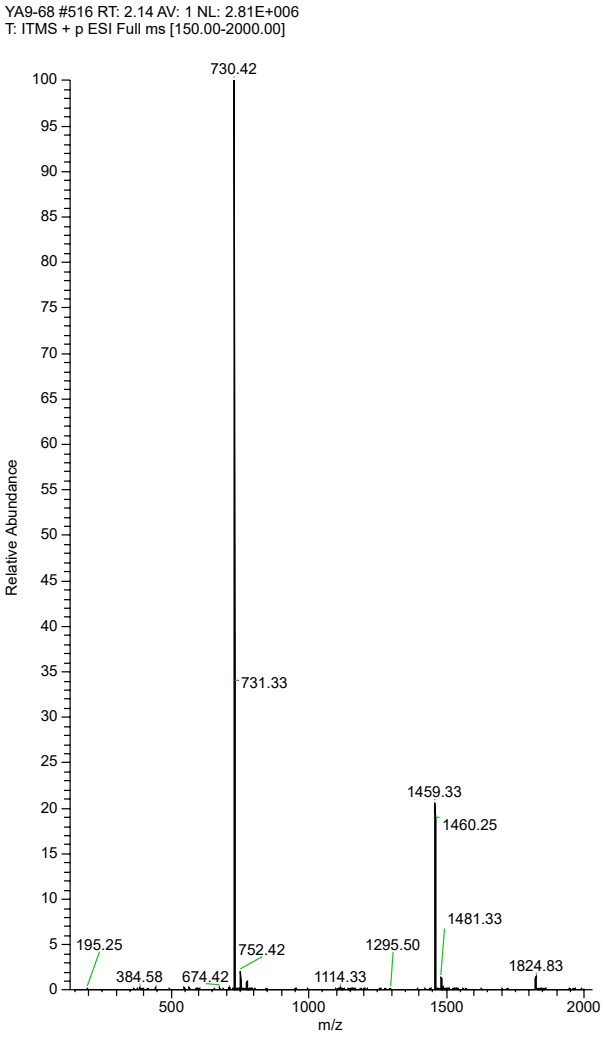
MRI images were taken using a 7 Tesla MRI scanner (Bruker Biospin) 256 days after intracranial implantation of U87ΔEGFR-luc GBM tumors. Five survivor mice treated with anti-EGFRvIII ADC **8b** were anesthetized with 2% isoflurane throughout the imaging procedure. A 35mm ID volume coil (Bruker Biospin) receive setup was used for data acquisition. T2-weighted coronal and axial images were acquired with a Spin Echo RARE sequence. Acquisition parameters were as follows: TR = 3000 ms, TE =57 ms, RARE factor 12, 6 NAV, Slice thickness of 0.75 mm, slice gap 0.25 mm, in plane resolution of 156 µm for coronal and 117 µm for axial.

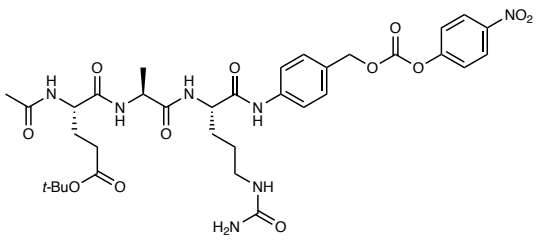
HPLC and deconvoluted ESI-MS Data



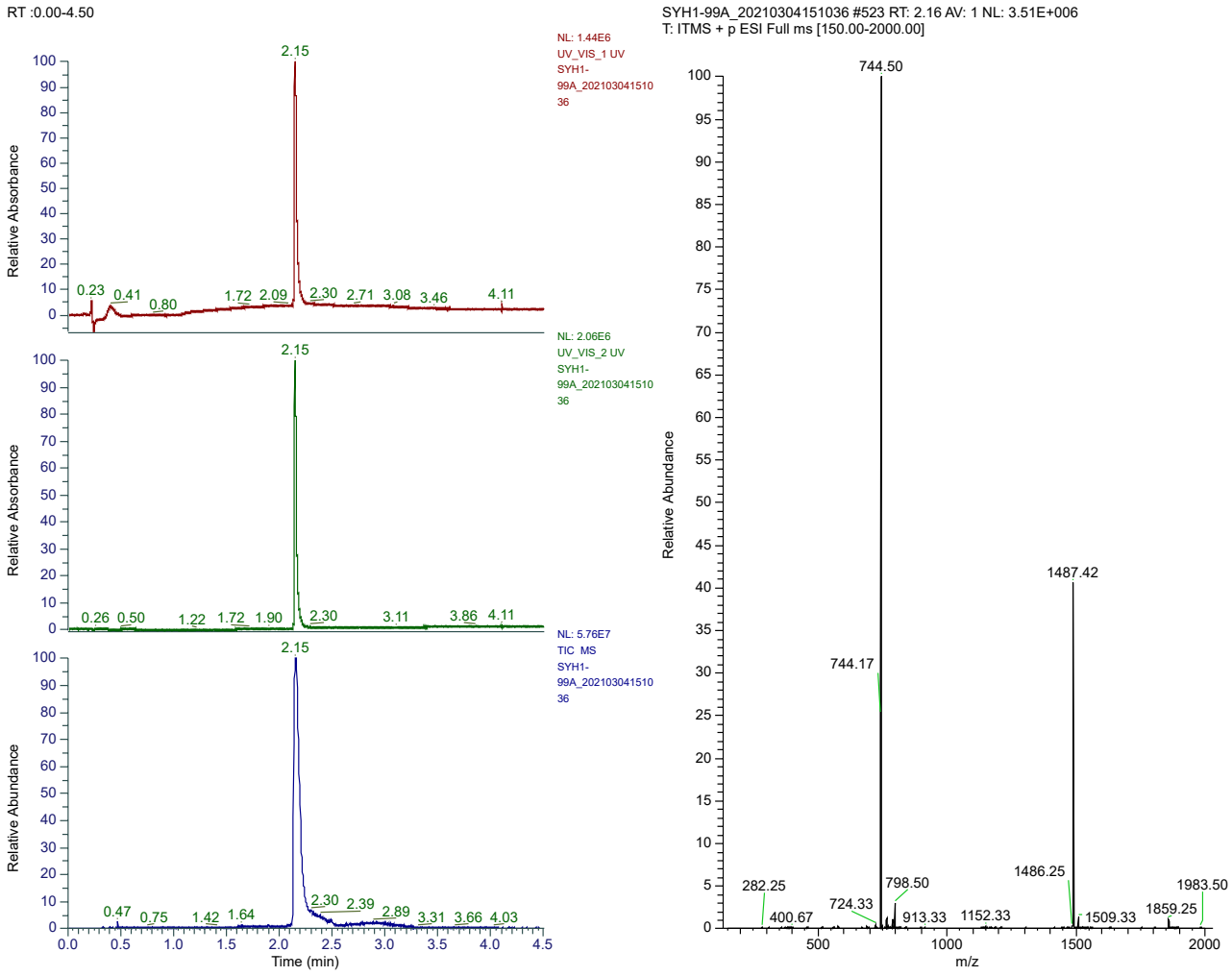
S3a

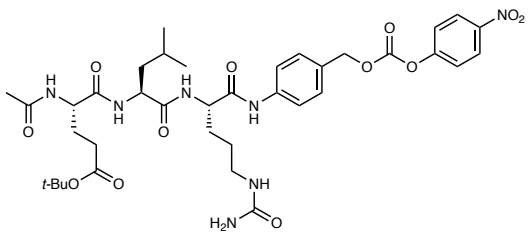




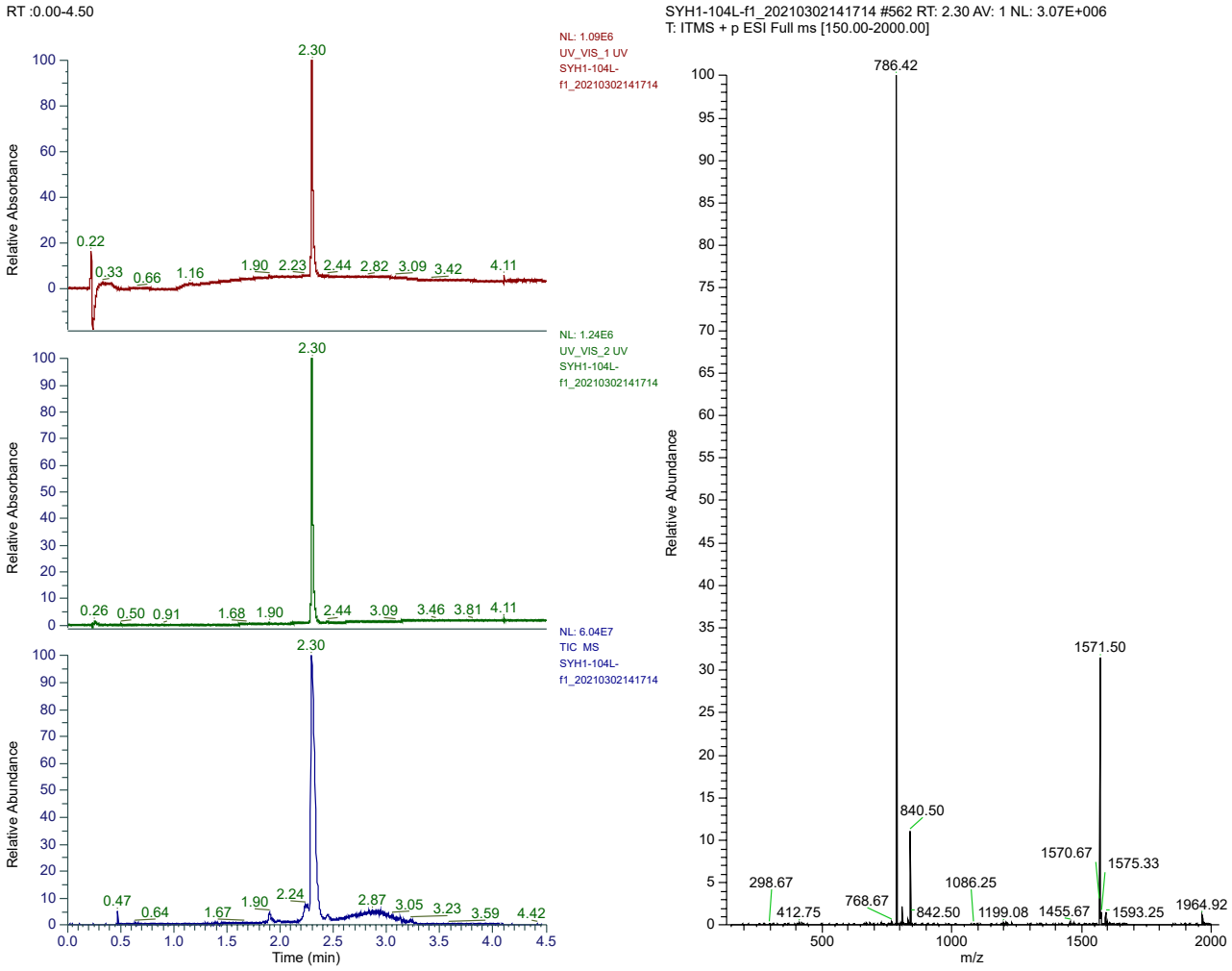


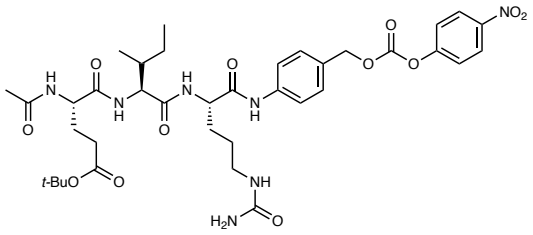
S3b


RT :0.00-4.50
NL: 1.44E6
UV_VIS_1 UV
SYH1-
99A_202103041510
36
NL: 2.06E6
UV_VIS_2 UV
SYH1-
99A_202103041510
36
NL: 5.76E7
TIC MS
SYH1-
99A_202103041510
36
Relative Absorbance
Relative Abundance
Time (min)
SYH1-99A_20210304151036 #523 RT: 2.16 AV: 1 NL: 3.51E+006
T: ITMS + p ESI Full ms [150.00-2000.00]
744.50
1487.42
744.17
1486.25
798.50
1983.50
282.25
724.33
913.33
1152.33
1509.33
1859.25
400.67
m/z

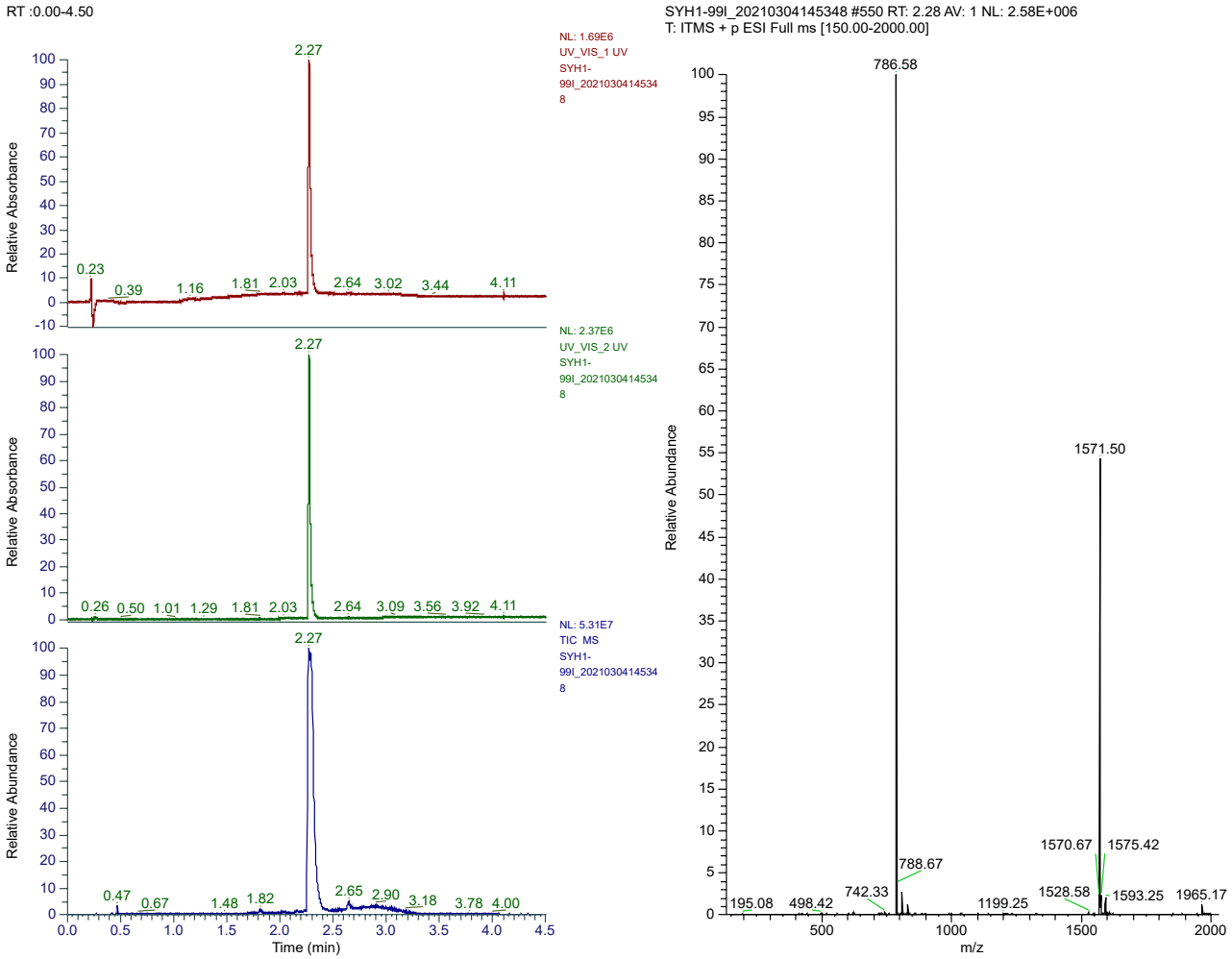


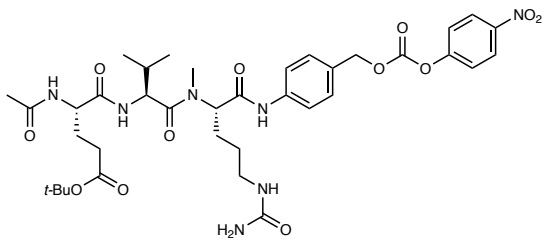
S3c


RT :0.00-4.50
NL: 1.09E6
UV_VIS_1 UV
SYH1-104L-
f1_20210302141714
NL: 1.24E6
UV_VIS_2 UV
SYH1-104L-
f1_20210302141714
NL: 6.04E7
TIC MS
SYH1-104L-
f1_20210302141714
Relative Absorbance
Relative Abundance
Time (min)
SYH1-104L-f1_20210302141714 #562 RT: 2.30 AV: 1 NL: 3.07E+006
T: ITMS + p ESI Full ms [150.00-2000.00]
786.42
1571.50
840.50
1570.67
1575.33
298.67
412.75
768.67
842.50
1086.25
1199.08
1455.67
1593.25
1964.92
m/z

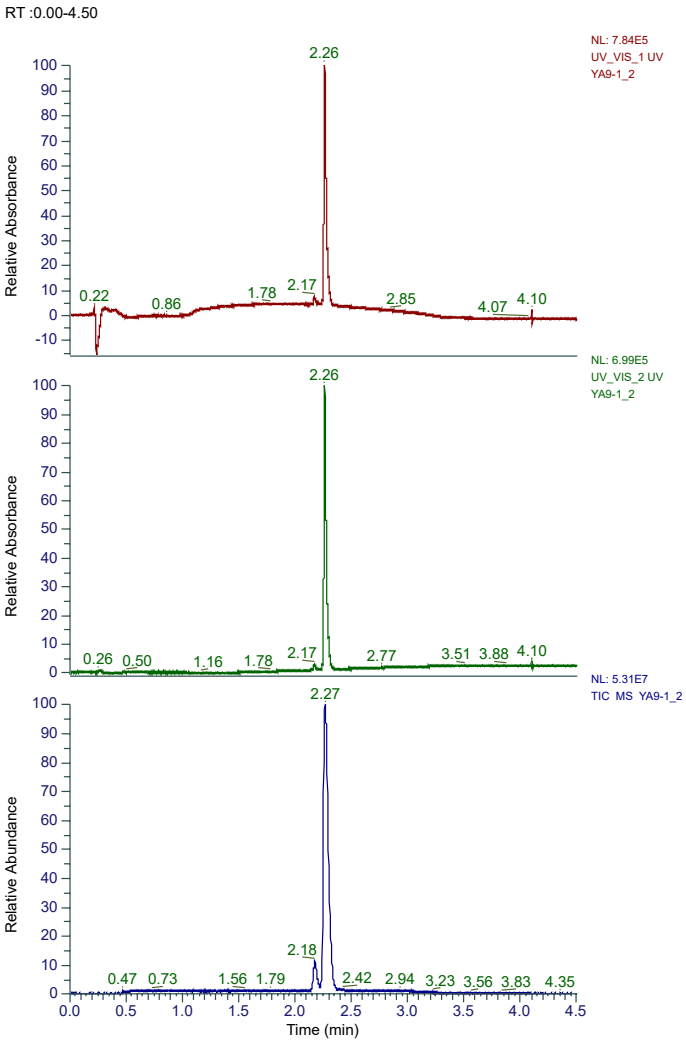


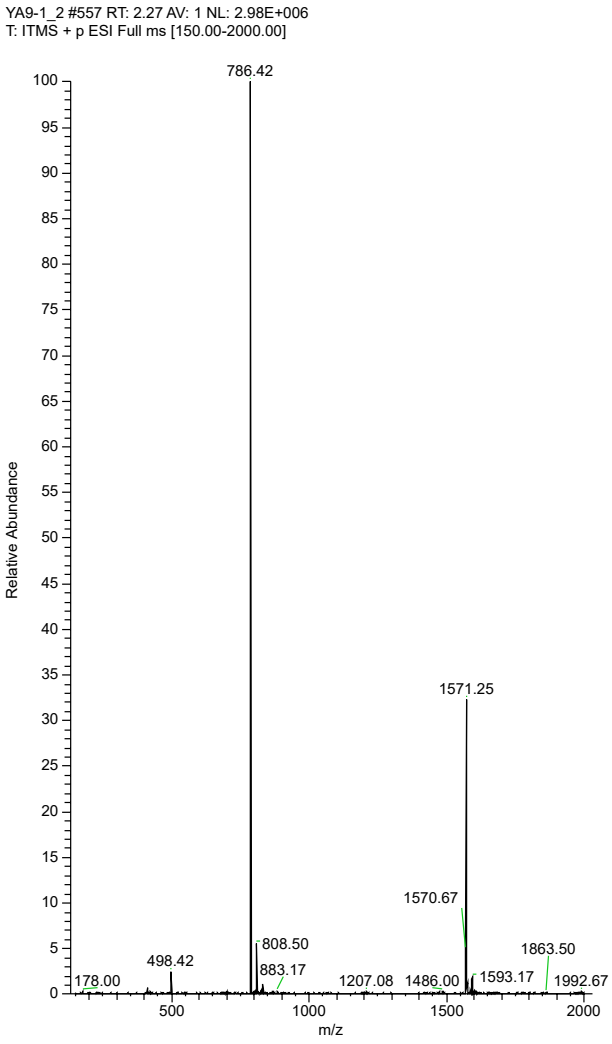
S3d

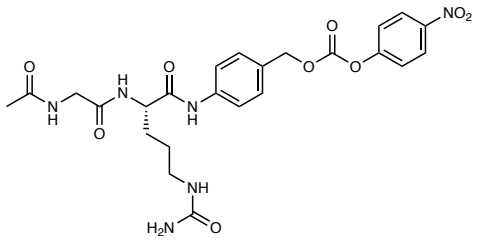

RT :0.00-4.50
NL: 1.69E6
UV_VIS_1 UV
SYH1-
99I_2021030414534
8
NL: 2.37E6
UV_VIS_2 UV
SYH1-
99I_2021030414534
8
NL: 5.31E7
TIC MS
SYH1-
99I_2021030414534
8
Relative Absorbance
Relative Abundance
Time (min)
SYH1-99I_20210304145348 #550 RT: 2.28 AV: 1 NL: 2.58E+006
T: ITMS + p ESI Full ms [150.00-2000.00]
786.58
1571.50
1570.67
1575.42
788.67
742.33
195.08
498.42
1199.25
1528.58
1593.25
1965.17
m/z



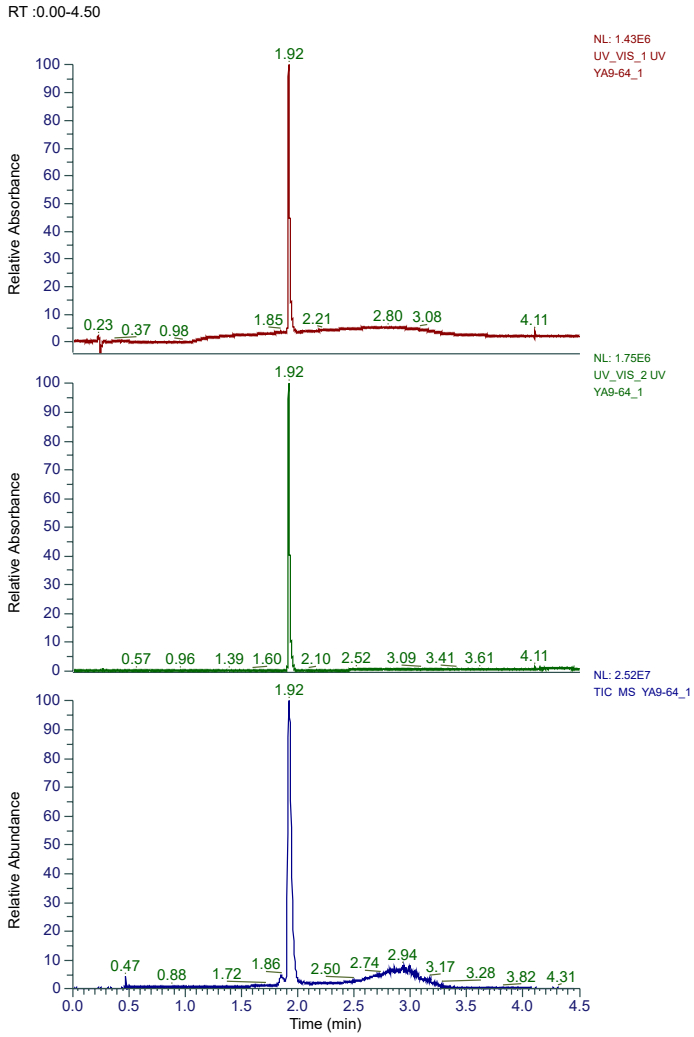
S3e

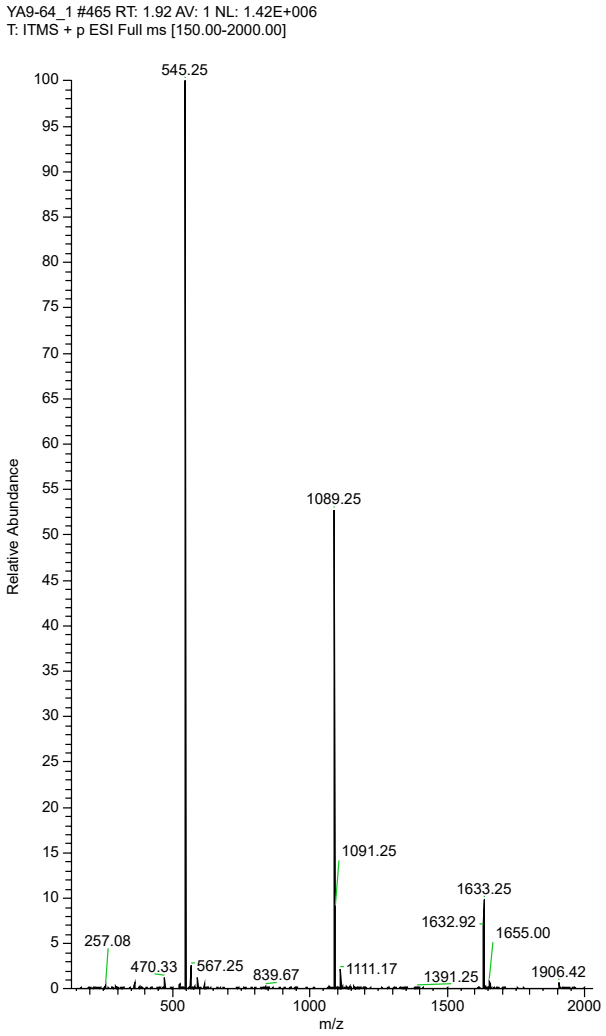

RT :0.00-4.50
NL: 7.84E5
UV_VIS_1 UV
YA9-1_2
NL: 6.99E5
UV_VIS_2 UV
YA9-1_2
NL: 5.31E7
TIC MS YA9-1_2
Relative Absorbance
Relative Abundance
Time (min)

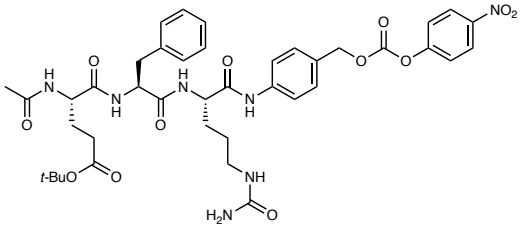

YA9-1_2 #557 RT: 2.27 AV: 1 NL: 2.98E+006
T: ITMS + p ESI Full ms [150.00-2000.00]
Relative Abundance
m/z



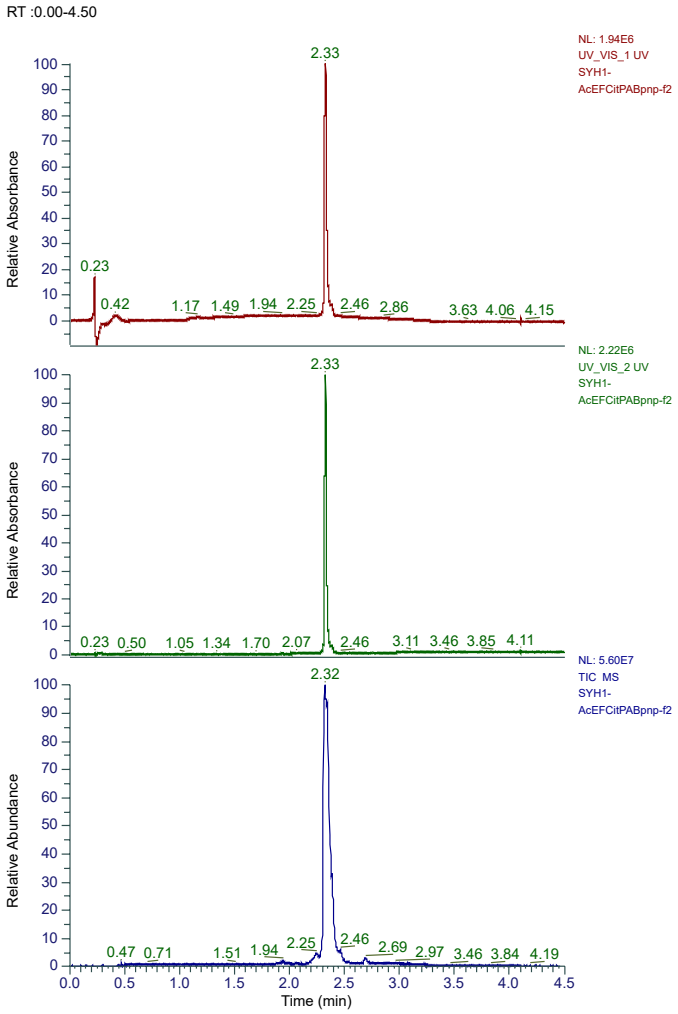
S3f

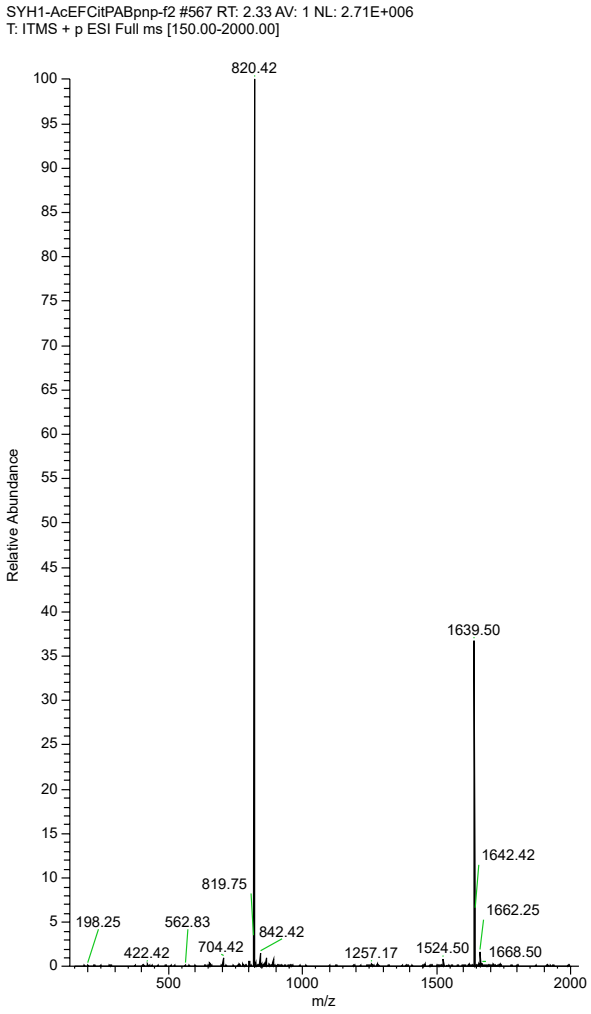

RT :0.00-4.50
NL: 1.43E6
UV_VIS_1 UV
YA9-64_1
1.92
0.23 0.37 0.98 1.85 2.21 2.80 3.08 4.11
Relative Absorbance
NL: 1.75E6
UV_VIS_2 UV
YA9-64_1
1.92
0.57 0.96 1.39 1.60 2.10 2.52 3.09 3.41 3.61 4.11
Relative Absorbance
NL: 2.52E7
TIC MS YA9-64_1
1.92
0.47 0.88 1.72 1.86 2.50 2.74 2.94 3.17 3.28 3.82 4.31
Relative Abundance
Time (min)

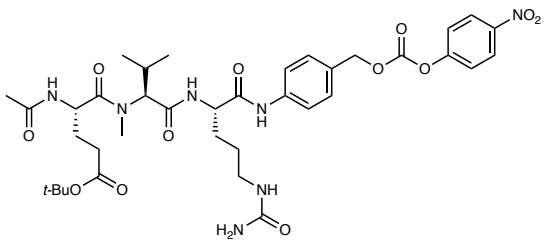

YA9-64_1 #465 RT: 1.92 AV: 1 NL: 1.42E+006
T: ITMS + p ESI Full ms [150.00-2000.00]
545.25
1089.25
1091.25
1633.25
1632.92
1655.00
257.08
470.33
567.25
839.67
1111.17
1391.25
1906.42
Relative Abundance
m/z



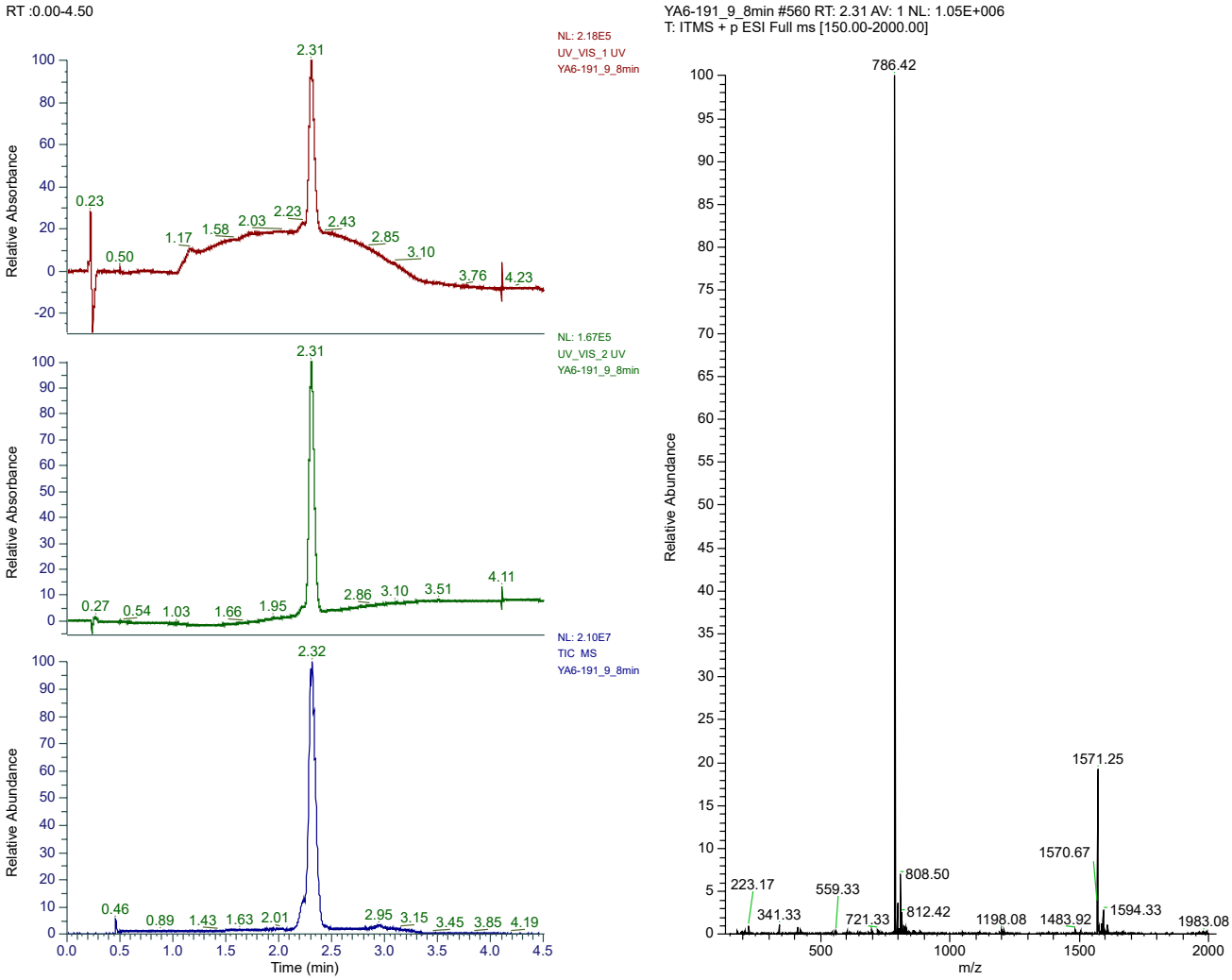
S3g

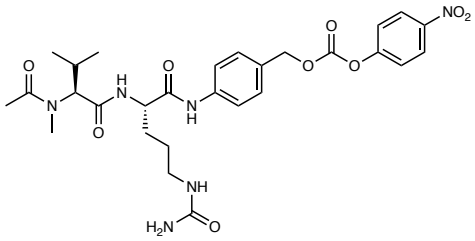

RT :0.00-4.50
NL: 1.94E6
UV_VIS_1 UV
SYH1-
AcEFCitPABpnp-f2
NL: 2.22E6
UV_VIS_2 UV
SYH1-
AcEFCitPABpnp-f2
NL: 5.60E7
TIC MS
SYH1-
AcEFCitPABpnp-f2
Relative Absorbance
Relative Abundance
Time (min)


SYH1-AcEFCitPABpnp-f2 #567 RT: 2.33 AV: 1 NL: 2.71E+006
T: ITMS + p ESI Full ms [150.00-2000.00]
Relative Abundance
m/z

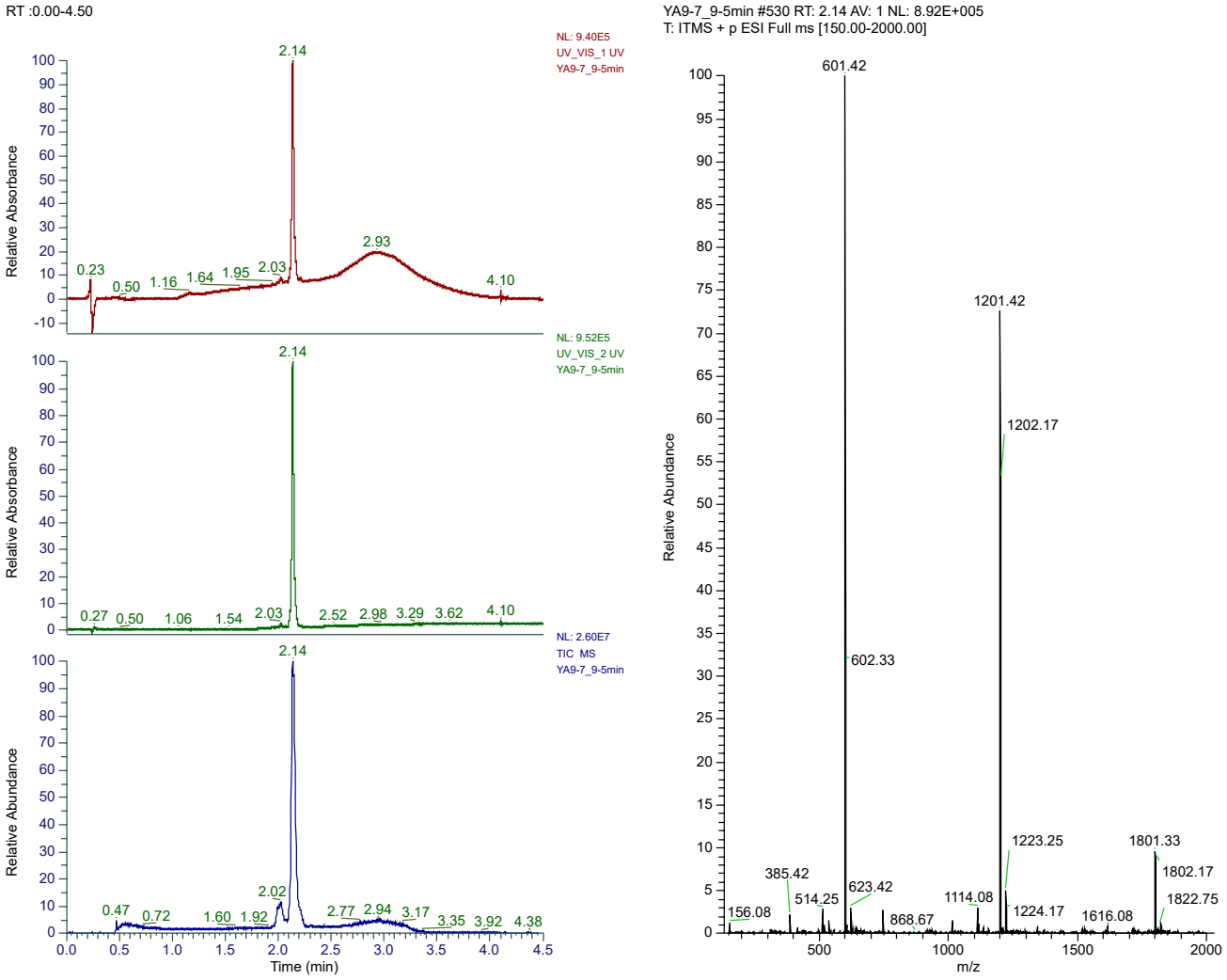


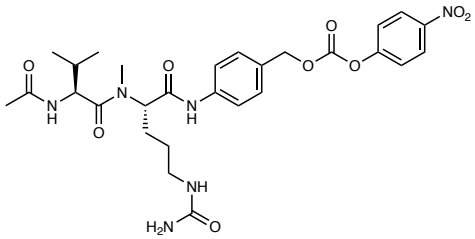
S3h


RT :0.00-4.50
NL: 2.18E5
UV_VIS_1 UV
YA6-191_9_8min
NL: 1.67E5
UV_VIS_2 UV
YA6-191_9_8min
NL: 2.10E7
TIC MS
YA6-191_9_8min
Relative Absorbance
Relative Abundance
Time (min)
YA6-191_9_8min #560 RT: 2.31 AV: 1 NL: 1.05E+006
T: ITMS + p ESI Full ms [150.00-2000.00]
786.42
1571.25
1570.67
808.50
223.17
559.33
341.33
721.33
812.42
1198.08
1483.92
1594.33
1983.08
m/z

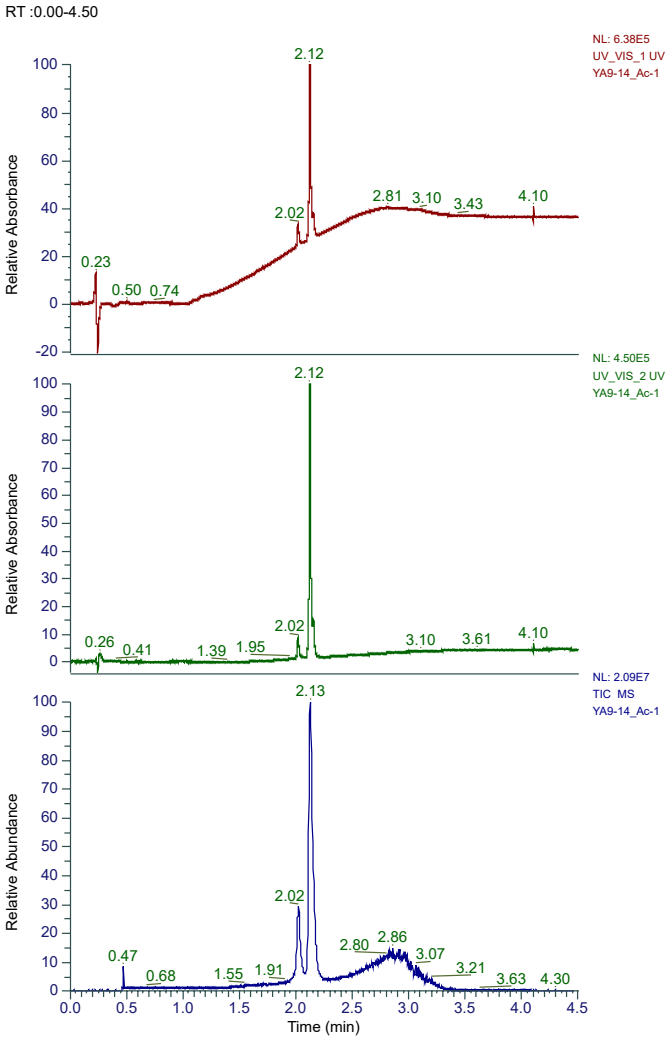


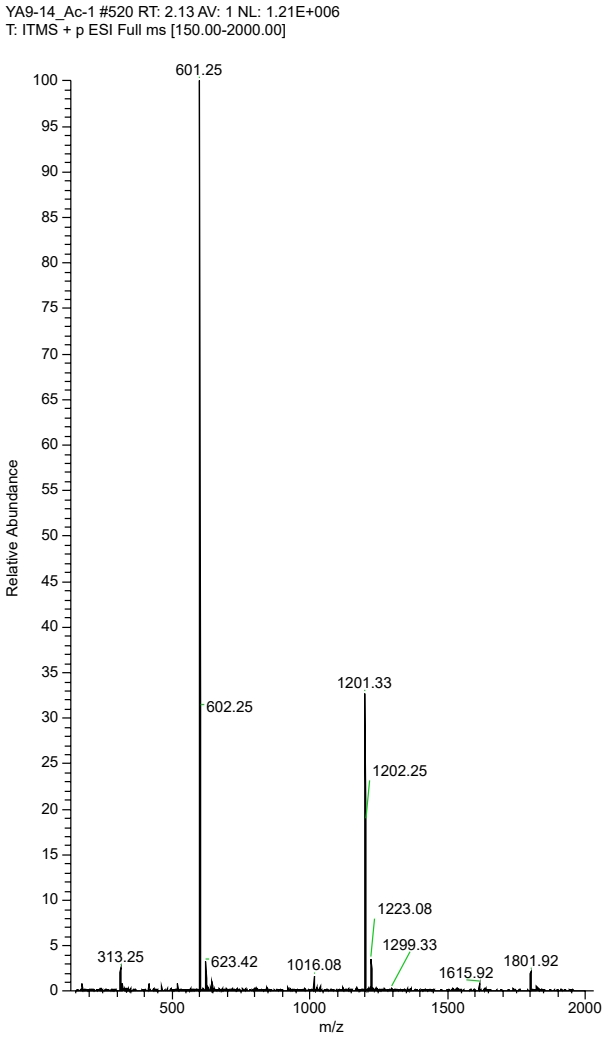
S3i

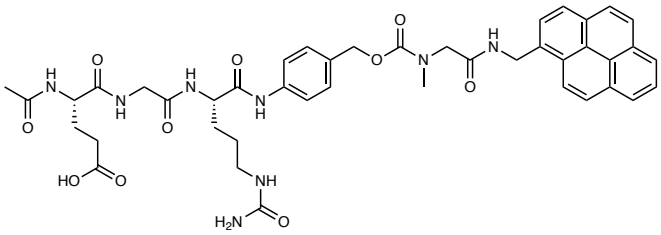

RT :0.00-4.50
NL: 9.40E5
UV_VIS_1 UV
YA9-7_9-5min
NL: 9.52E5
UV_VIS_2 UV
YA9-7_9-5min
NL: 2.60E7
TIC MS
YA9-7_9-5min
Relative Absorbance
Relative Abundance
Time (min)
YA9-7_9-5min #530 RT: 2.14 AV: 1 NL: 8.92E+005
T: ITMS + p ESI Full ms [150.00-2000.00]
Relative Abundance
m/z
601.42
1201.42
1202.17
602.33
1223.25
1801.33
1802.17
1822.75
385.42
514.25
623.42
1114.08
868.67
1224.17
1616.08
156.08



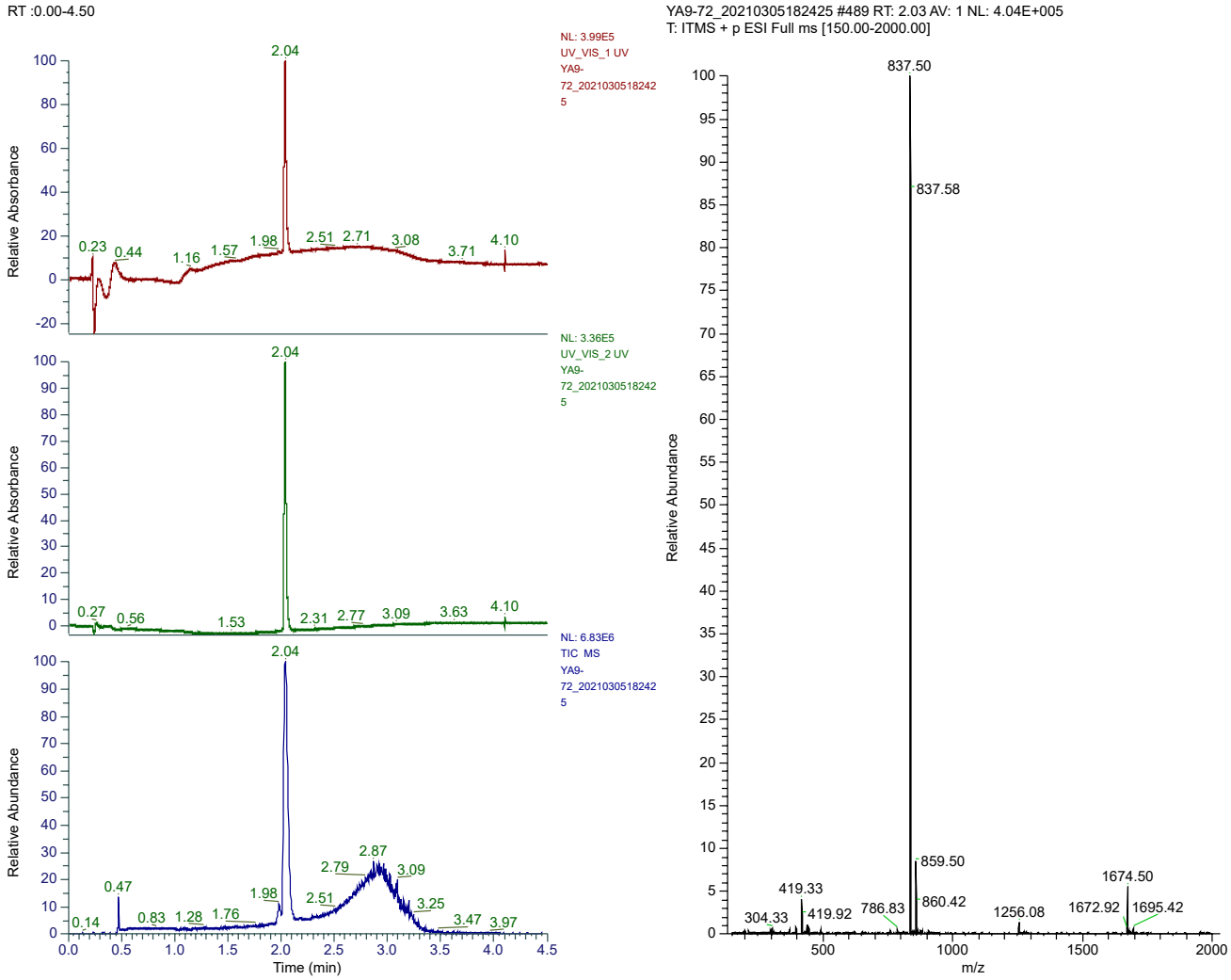
S3j

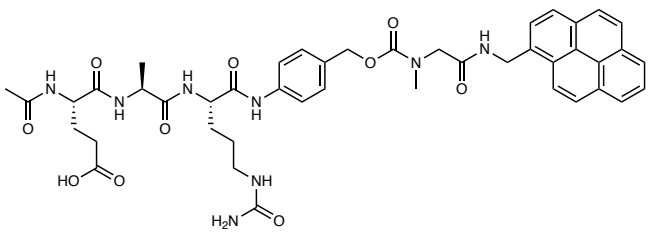

RT :0.00-4.50
NL: 6.38E5
UV_VIS_1 UV
YA9-14_Ac-1
NL: 4.50E5
UV_VIS_2 UV
YA9-14_Ac-1
NL: 2.09E7
TIC MS
YA9-14_Ac-1
Relative Absorbance
Relative Abundance
Time (min)


YA9-14_Ac-1 #520 RT: 2.13 AV: 1 NL: 1.21E+006
T: ITMS + p ESI Full ms [150.00-2000.00]
Relative Abundance
m/z

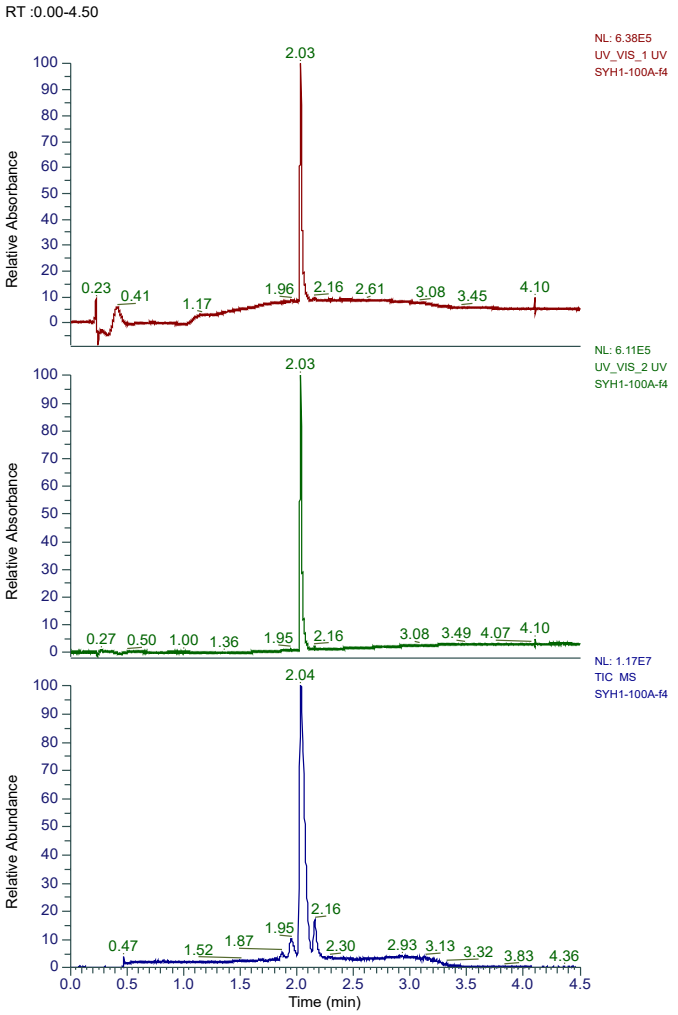


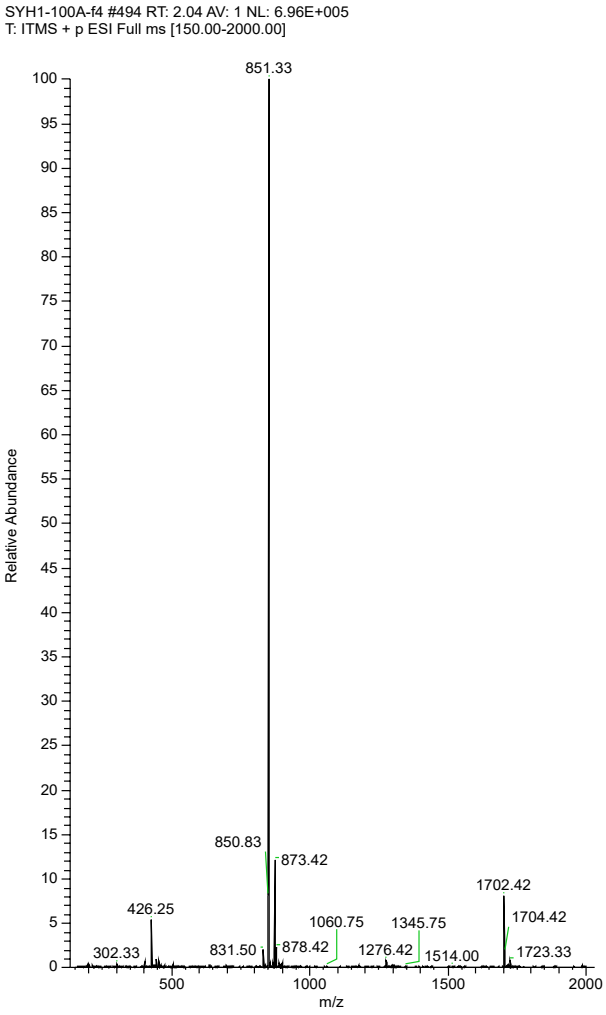
3a

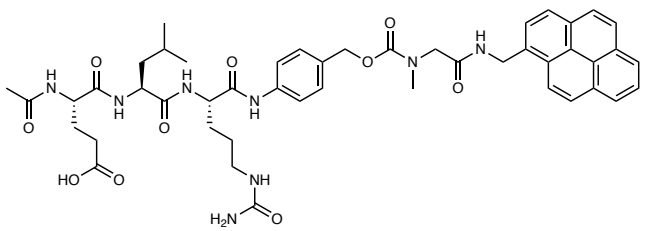

RT :0.00-4.50
NL: 3.99E5
UV_VIS_1 UV
YA9-72_20210305182425
NL: 3.36E5
UV_VIS_2 UV
YA9-72_20210305182425
NL: 6.83E6
TIC MS
YA9-72_20210305182425
Relative Absorbance
Relative Abundance
Time (min)
YA9-72_20210305182425 #489 RT: 2.03 AV: 1 NL: 4.04E+005
T: ITMS + p ESI Full ms [150.00-2000.00]
837.50
837.58
859.50
860.42
1674.50
m/z



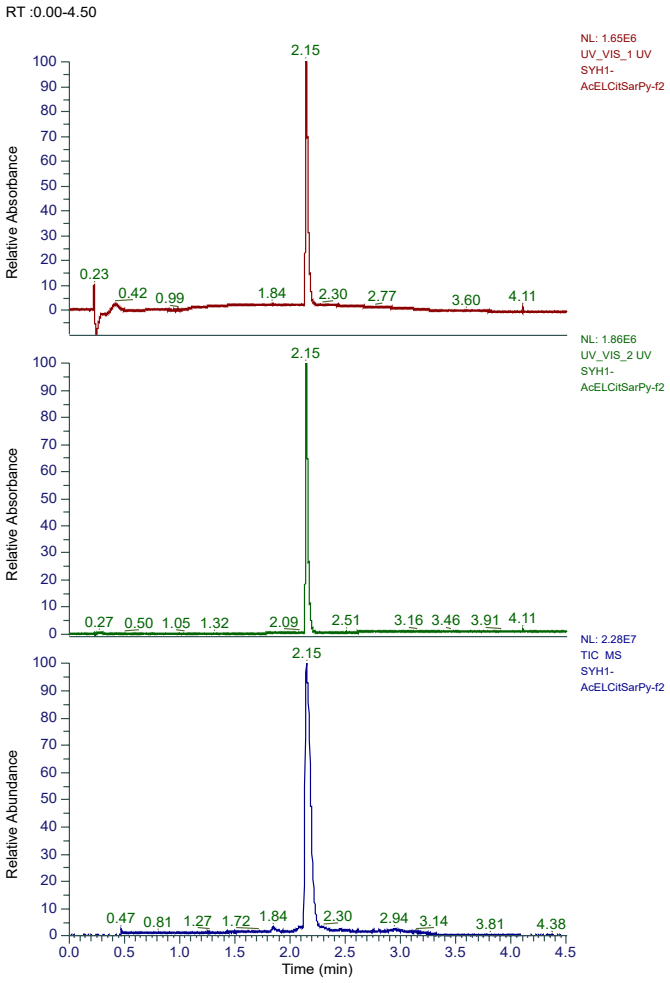
3b

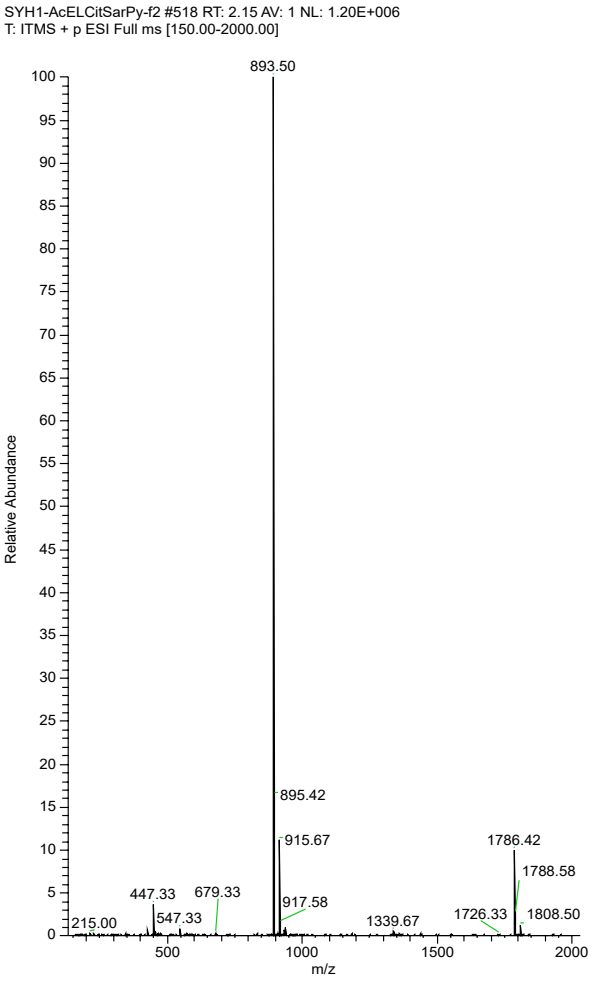

RT :0.00-4.50
NL: 6.38E5
UV_VIS_1 UV
SYH1-100A-f4
NL: 6.11E5
UV_VIS_2 UV
SYH1-100A-f4
NL: 1.17E7
TIC MS
SYH1-100A-f4
Relative Absorbance
Relative Abundance
Time (min)

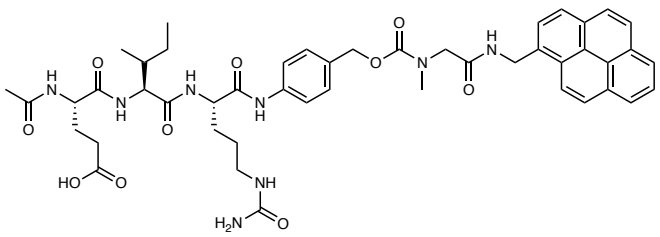

SYH1-100A-f4 #494 RT: 2.04 AV: 1 NL: 6.96E+005
T: ITMS + p ESI Full ms [150.00-2000.00]
Relative Abundance
m/z



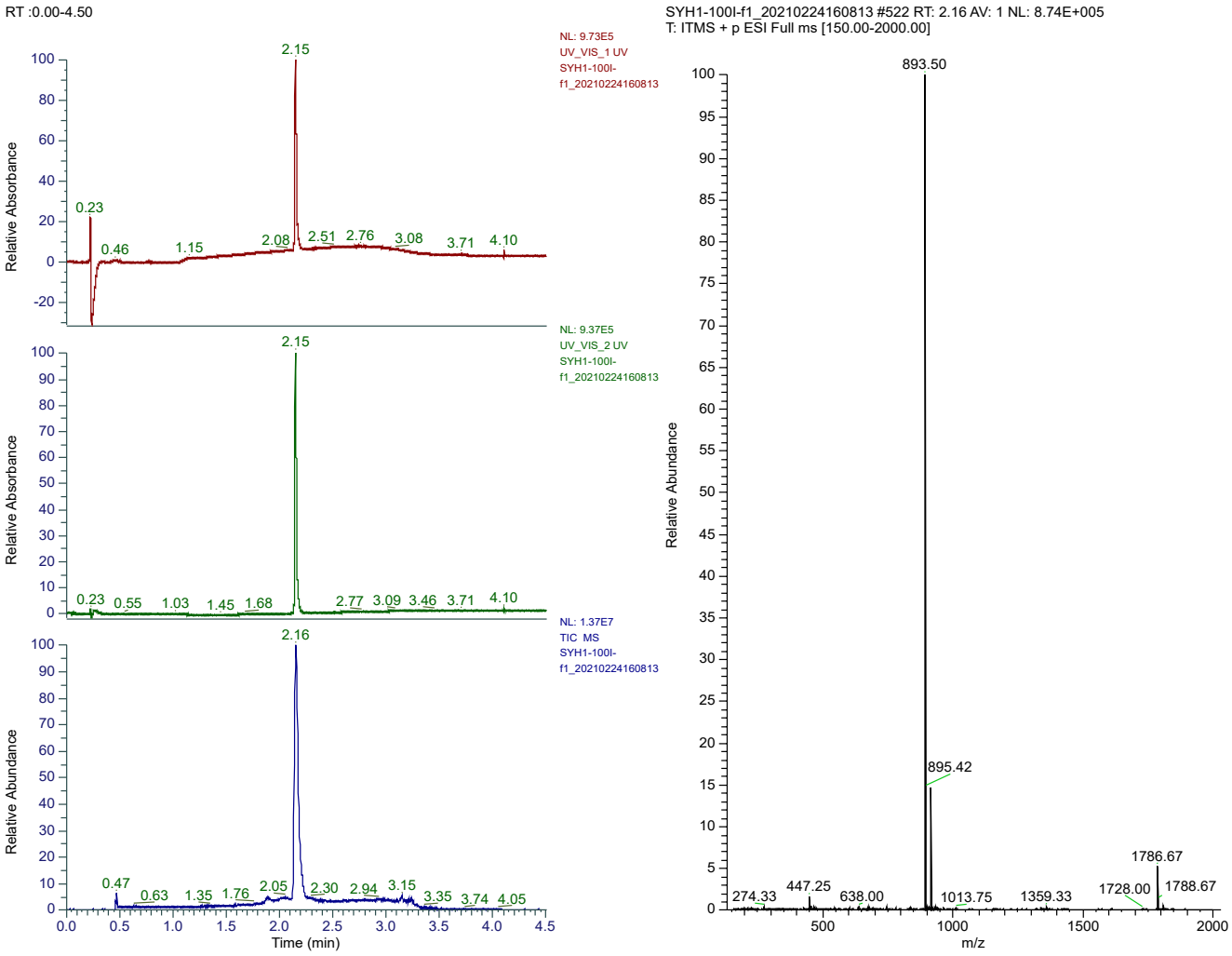
3c

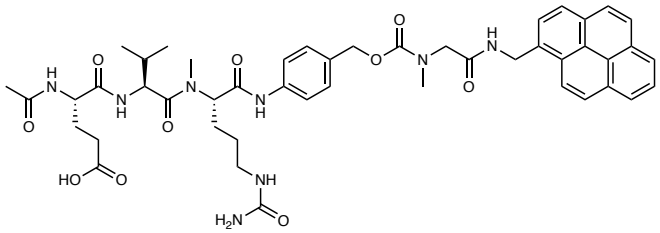

RT :0.00-4.50
NL: 1.65E6
UV_VIS_1 UV
SYH1-
AcELCitSarPy-f2
NL: 1.86E6
UV_VIS_2 UV
SYH1-
AcELCitSarPy-f2
NL: 2.28E7
TIC MS
SYH1-
AcELCitSarPy-f2
Relative Absorbance
Relative Abundance
Time (min)


SYH1-AcELCitSarPy-f2 #518 RT: 2.15 AV: 1 NL: 1.20E+006
T: ITMS + p ESI Full ms [150.00-2000.00]
Relative Abundance
m/z

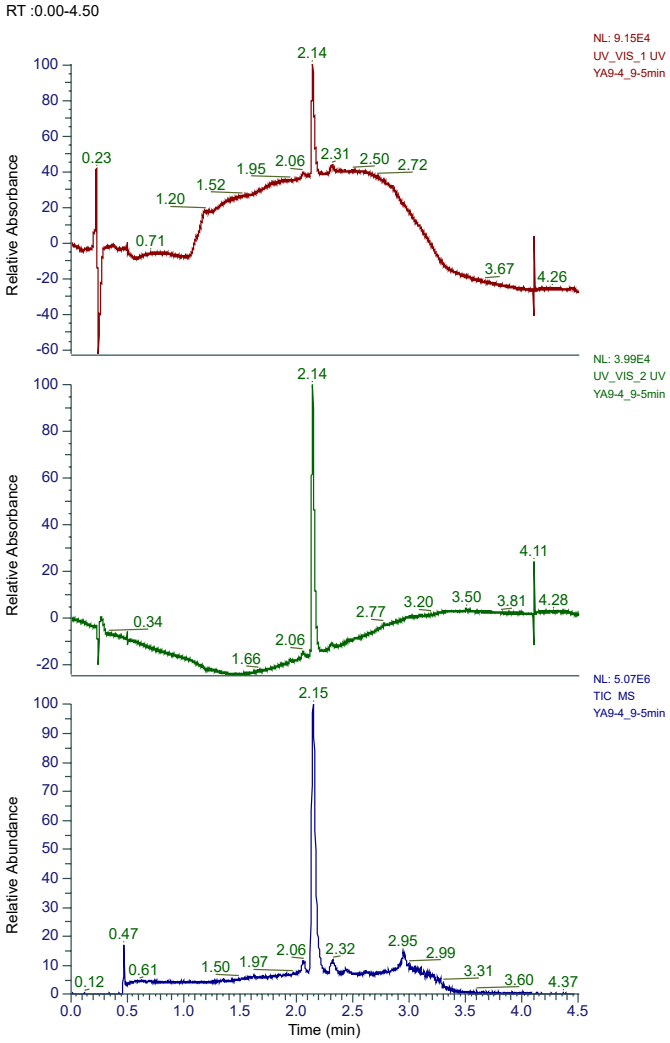


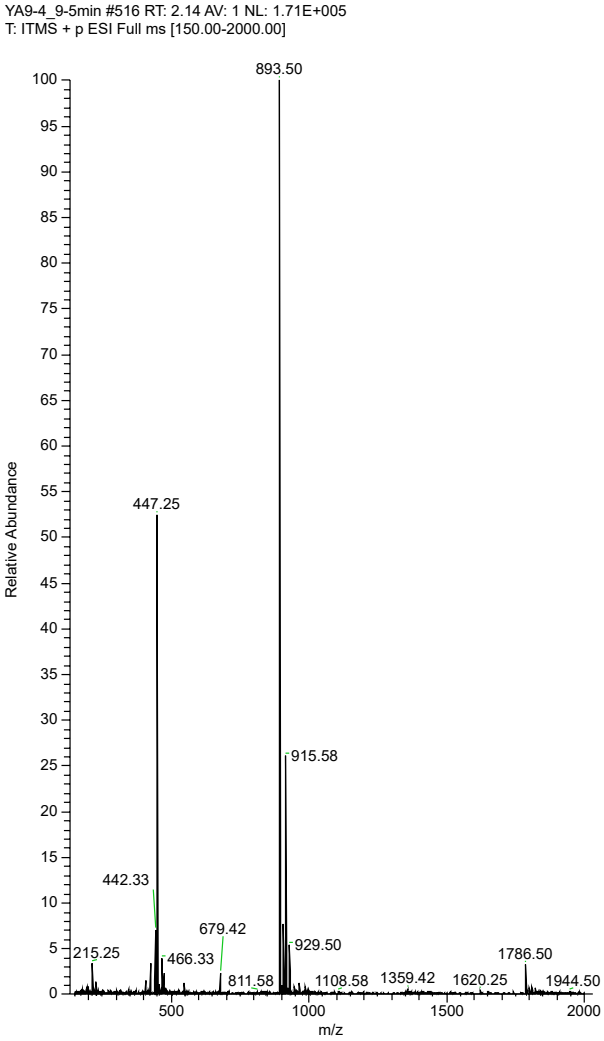
3d

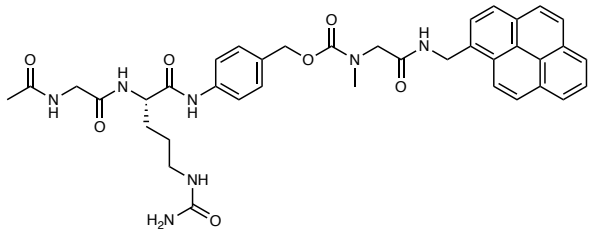

RT :0.00-4.50
NL: 9.73E5
UV_VIS_1 UV
SYH1-100I-
f1_20210224160813
NL: 9.37E5
UV_VIS_2 UV
SYH1-100I-
f1_20210224160813
NL: 1.37E7
TIC MS
SYH1-100I-
f1_20210224160813
Relative Absorbance
Relative Abundance
Time (min)
SYH1-100I-f1_20210224160813 #522 RT: 2.16 AV: 1 NL: 8.74E+005
T: ITMS + p ESI Full ms [150.00-2000.00]
893.50
895.42
1786.67
1788.67
m/z



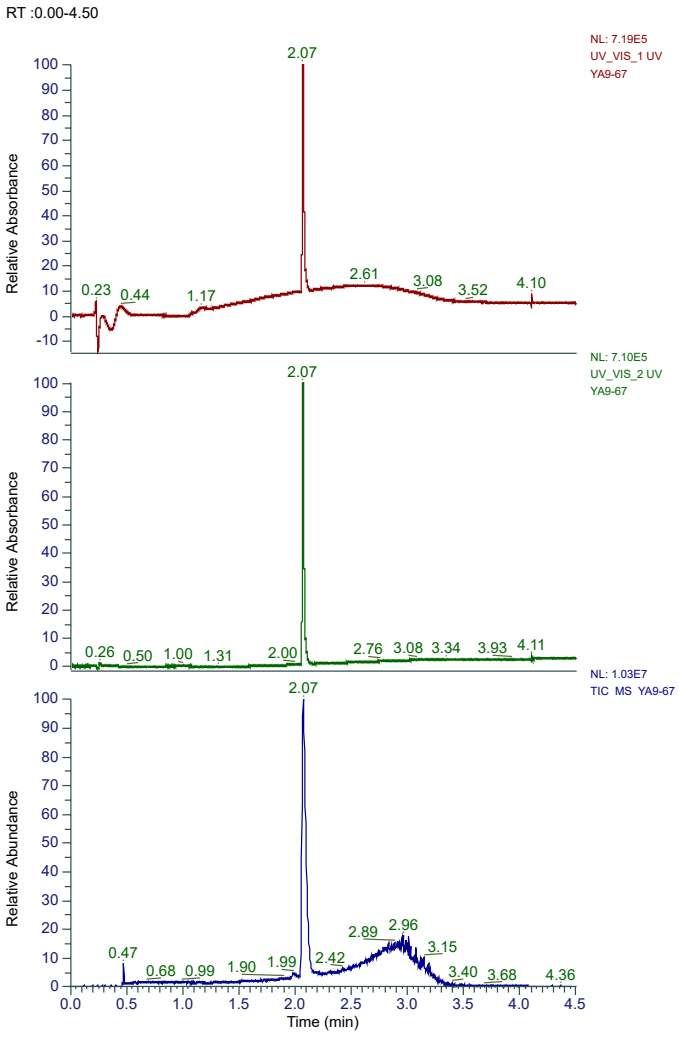
3e

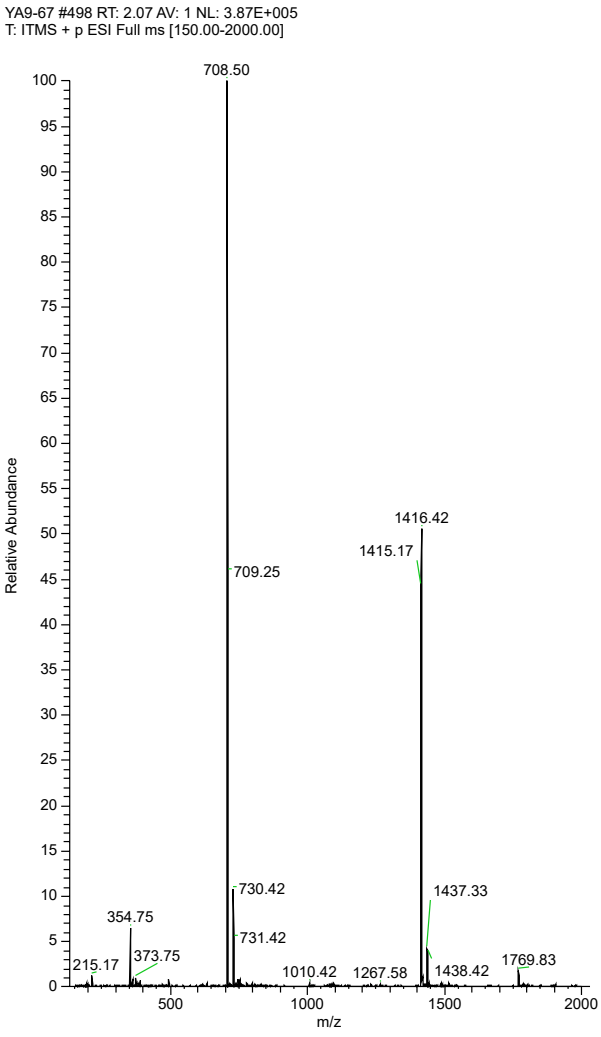

RT :0.00-4.50
NL: 9.15E4
UV_VIS_1 UV
YA9-4_9-5min
NL: 3.99E4
UV_VIS_2 UV
YA9-4_9-5min
NL: 5.07E6
TIC MS
YA9-4_9-5min
Relative Absorbance
Relative Abundance
Time (min)

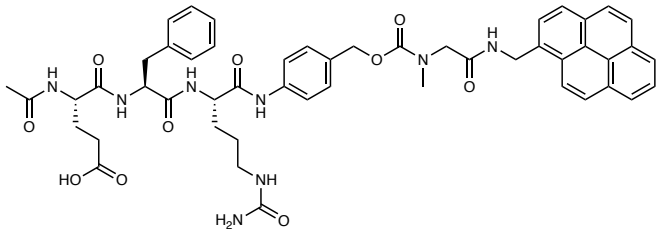

YA9-4_9-5min #516 RT: 2.14 AV: 1 NL: 1.71E+005
T: ITMS + p ESI Full ms [150.00-2000.00]
Relative Abundance
m/z



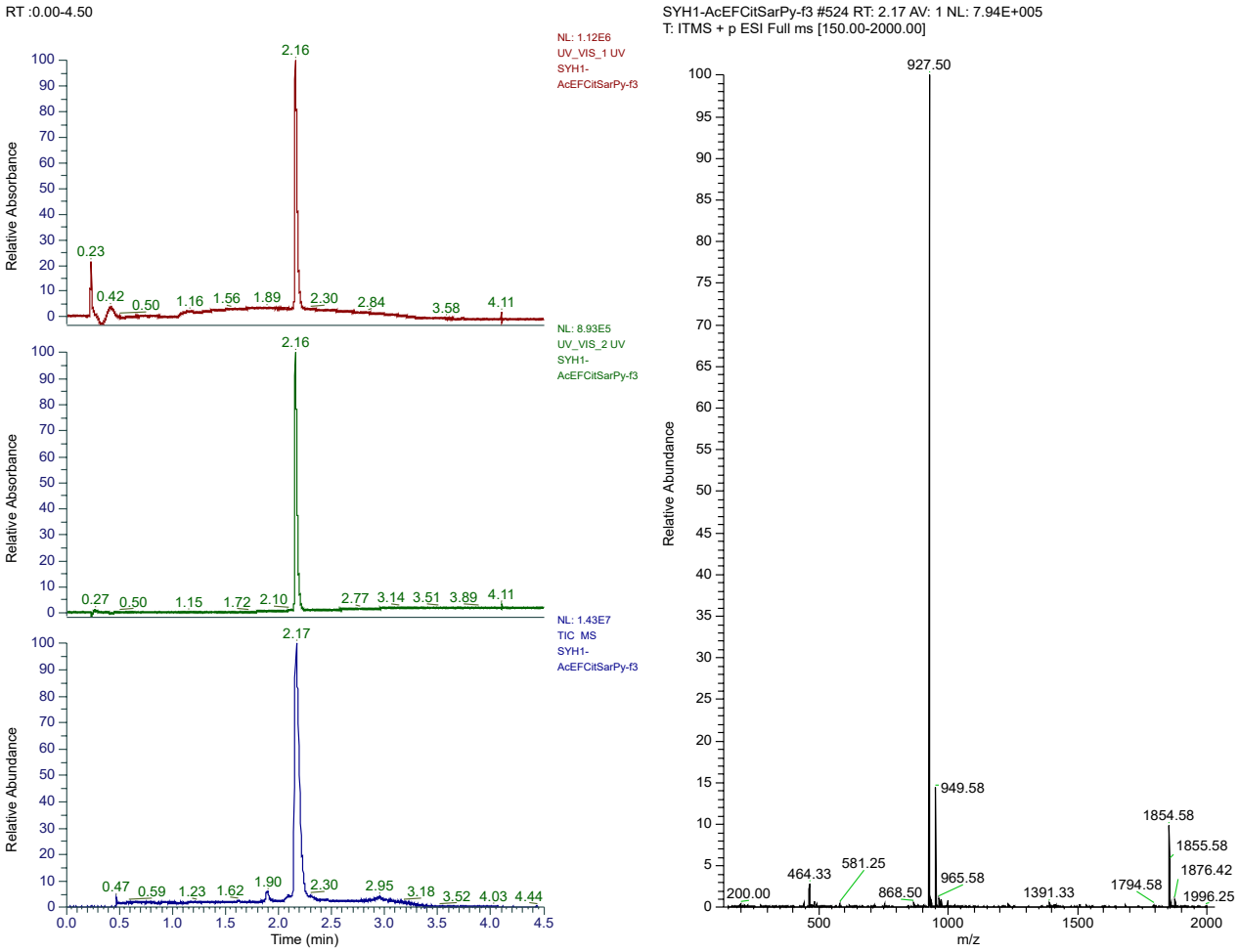
3f

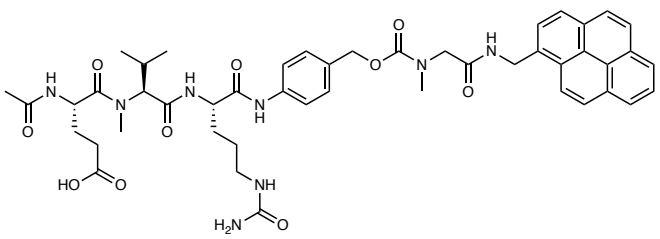

RT :0.00-4.50
NL: 7.19E5
UV_VIS_1 UV
YA9-67
NL: 7.10E5
UV_VIS_2 UV
YA9-67
NL: 1.03E7
TIC MS YA9-67
Relative Absorbance
Relative Abundance
Time (min)


YA9-67 #498 RT: 2.07 AV: 1 NL: 3.87E+005
T: ITMS + p ESI Full ms [150.00-2000.00]
Relative Abundance
m/z

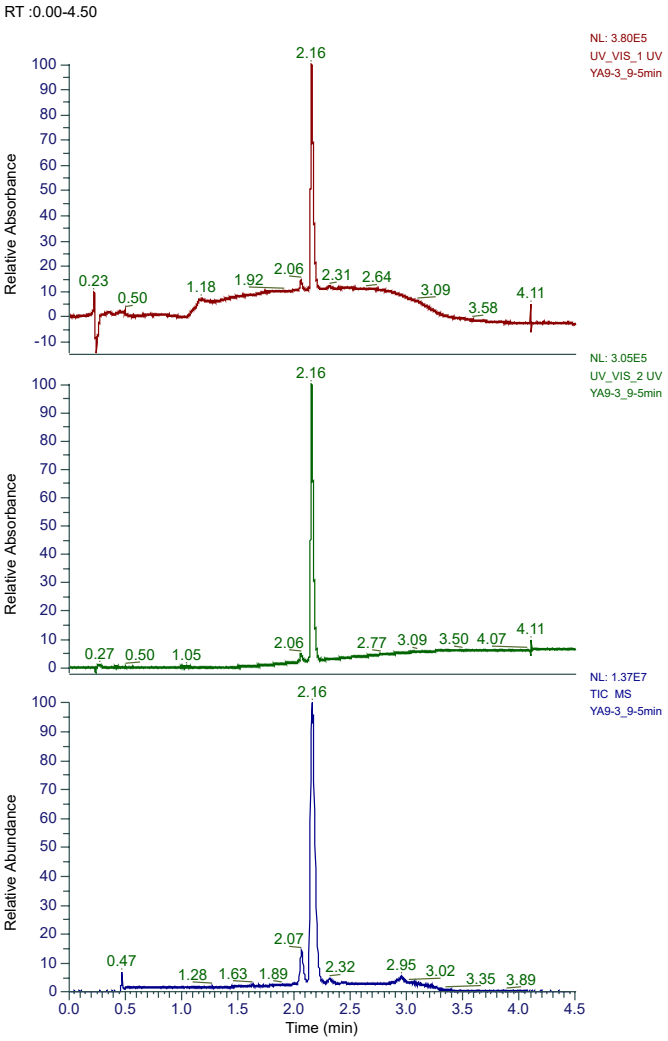


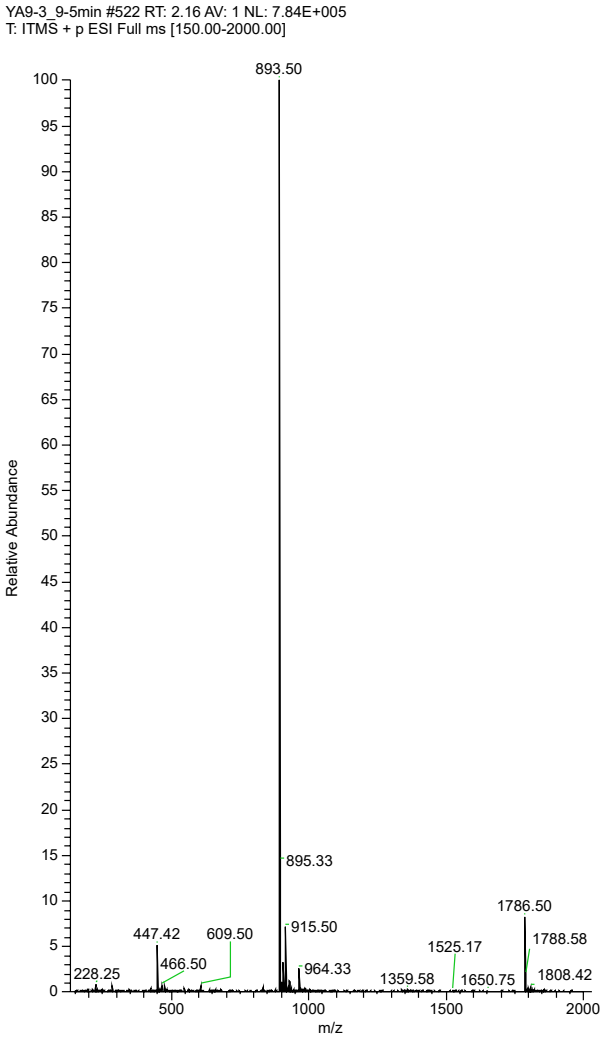
S1a

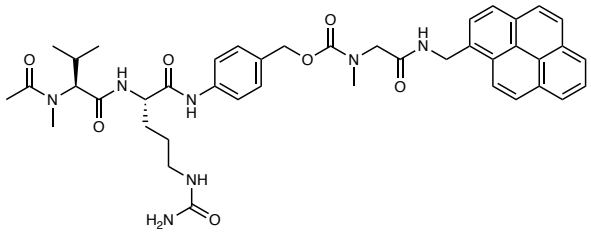

RT :0.00-4.50
NL: 1.12E6
UV_VIS_1 UV
SYH1-
AcEFCitSarPy-f3
NL: 8.93E5
UV_VIS_2 UV
SYH1-
AcEFCitSarPy-f3
NL: 1.43E7
TIC MS
SYH1-
AcEFCitSarPy-f3
Relative Absorbance
Relative Abundance
Time (min)
SYH1-AcEFCitSarPy-f3 #524 RT: 2.17 AV: 1 NL: 7.94E+005
T: ITMS + p ESI Full ms [150.00-2000.00]
927.50
949.58
1854.58
1855.58
1876.42
1996.25
581.25
464.33
965.58
868.50
1391.33
1794.58
200.00
m/z



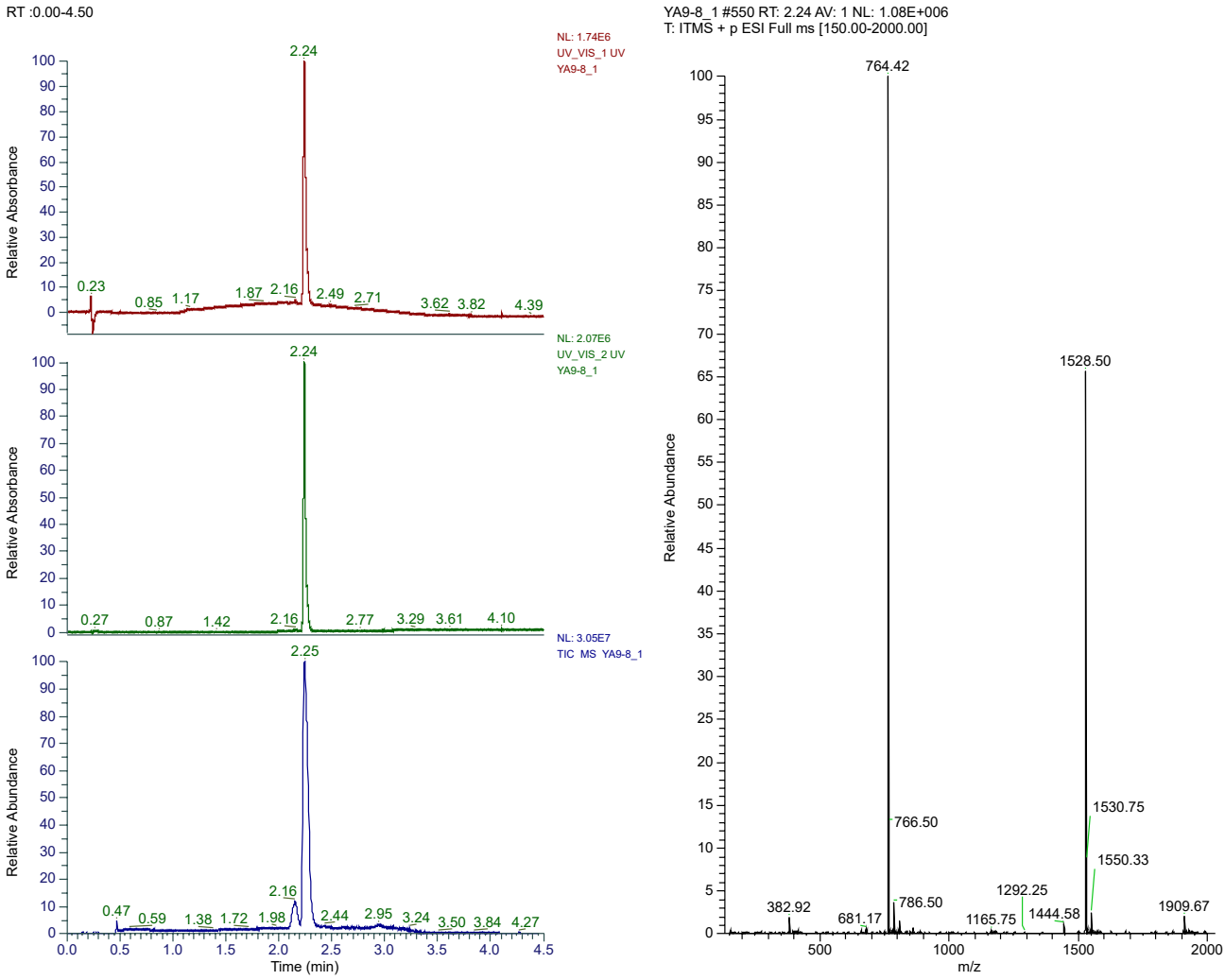
S1b

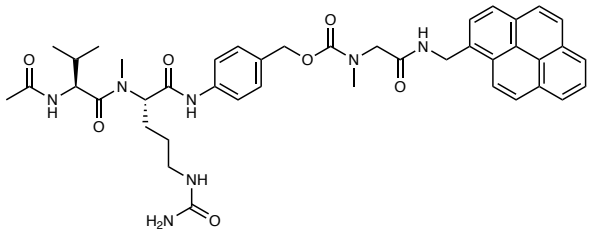

RT :0.00-4.50
NL: 3.80E5
UV_VIS_1 UV
YA9-3_9-5min
NL: 3.05E5
UV_VIS_2 UV
YA9-3_9-5min
NL: 1.37E7
TIC MS
YA9-3_9-5min
Relative Absorbance
Relative Abundance
Time (min)


YA9-3_9-5min #522 RT: 2.16 AV: 1 NL: 7.84E+005
T: ITMS + p ESI Full ms [150.00-2000.00]
Relative Abundance
m/z

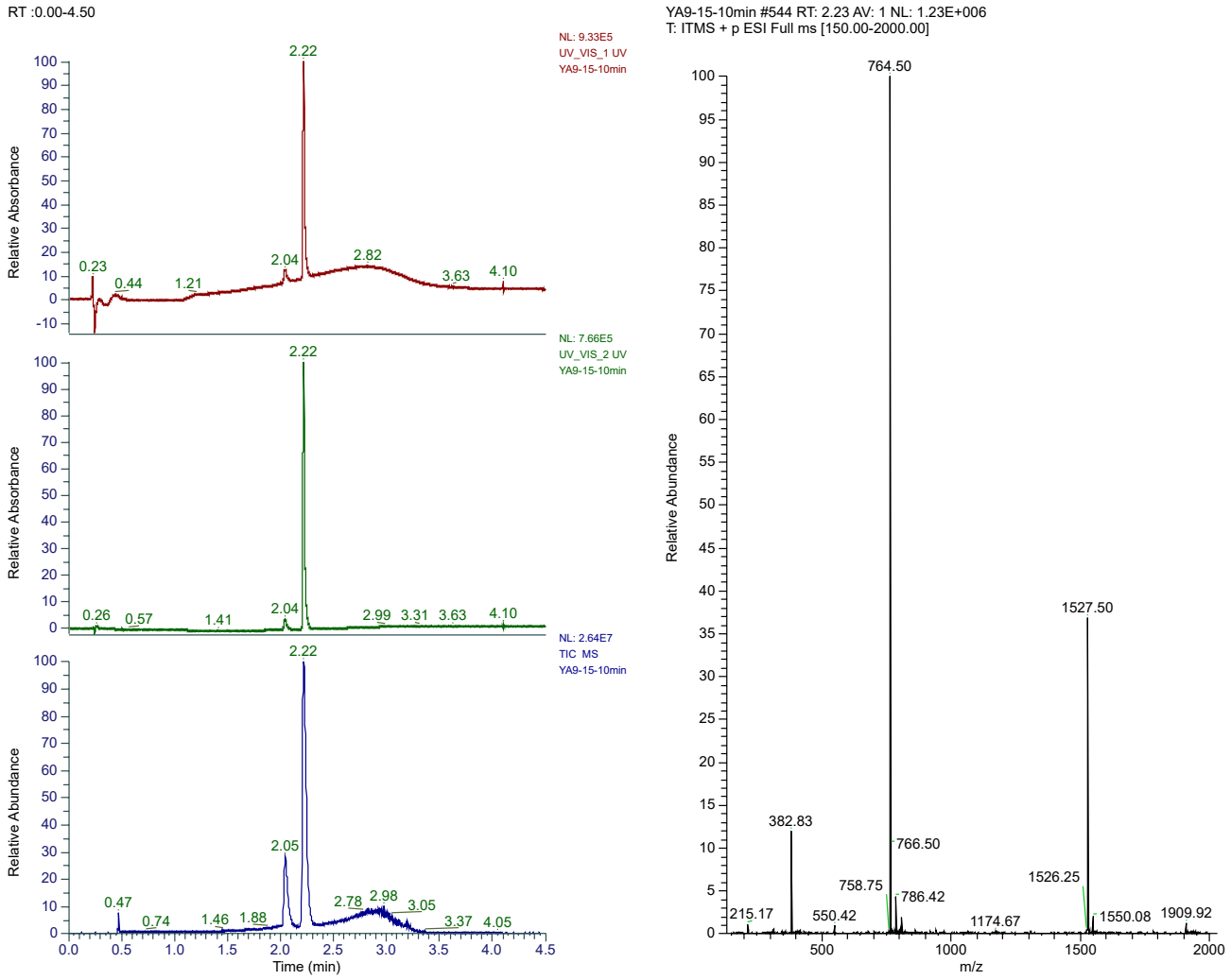


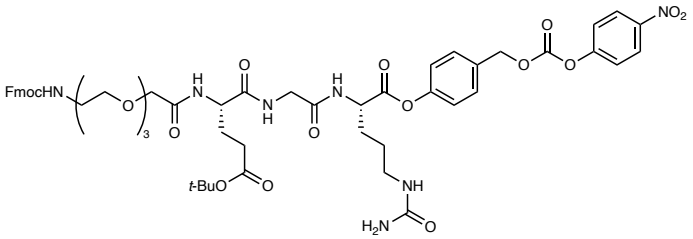
S1c


RT :0.00-4.50
NL: 1.74E6
UV_VIS_1 UV
YA9-8_1
NL: 2.07E6
UV_VIS_2 UV
YA9-8_1
NL: 3.05E7
TIC MS YA9-8_1
Relative Absorbance
Relative Abundance
Time (min)
YA9-8_1 #550 RT: 2.24 AV: 1 NL: 1.08E+006
T: ITMS + p ESI Full ms [150.00-2000.00]
764.42
1528.50
766.50
1530.75
1550.33
382.92
681.17
786.50
1165.75
1292.25
1444.58
1909.67
m/z

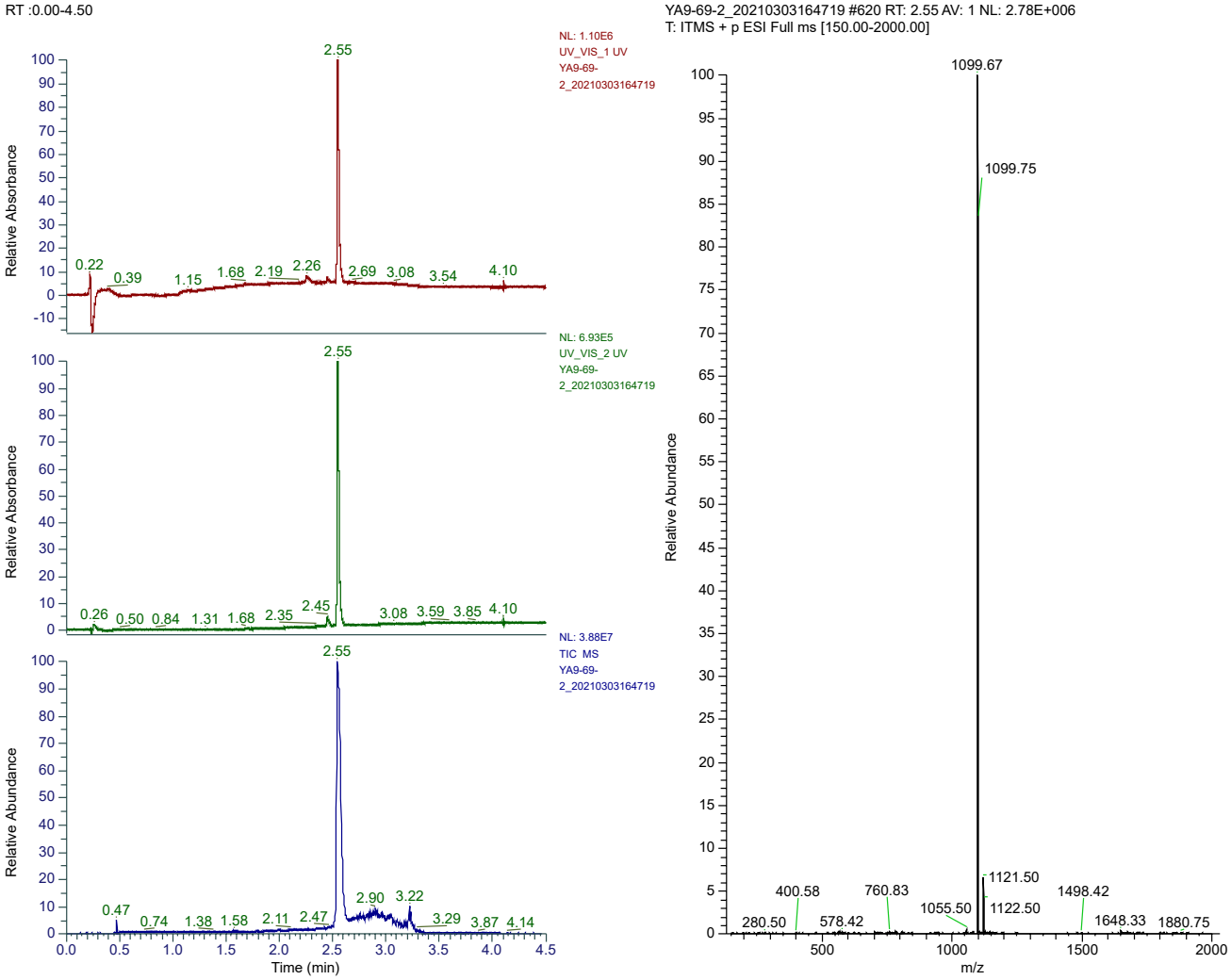


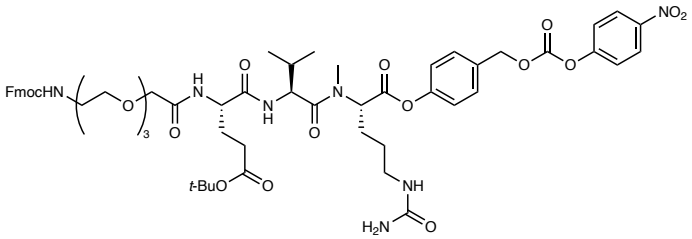
S1d


RT :0.00-4.50
NL: 9.33E5
UV_VIS_1 UV
YA9-15-10min
NL: 7.66E5
UV_VIS_2 UV
YA9-15-10min
NL: 2.64E7
TIC MS
YA9-15-10min
Relative Absorbance
Relative Abundance
Time (min)
YA9-15-10min #544 RT: 2.23 AV: 1 NL: 1.23E+006
T: ITMS + p ESI Full ms [150.00-2000.00]
Relative Abundance
m/z
764.50
1527.50
382.83
766.50
758.75
786.42
1526.25
215.17
550.42
1174.67
1550.08
1909.92

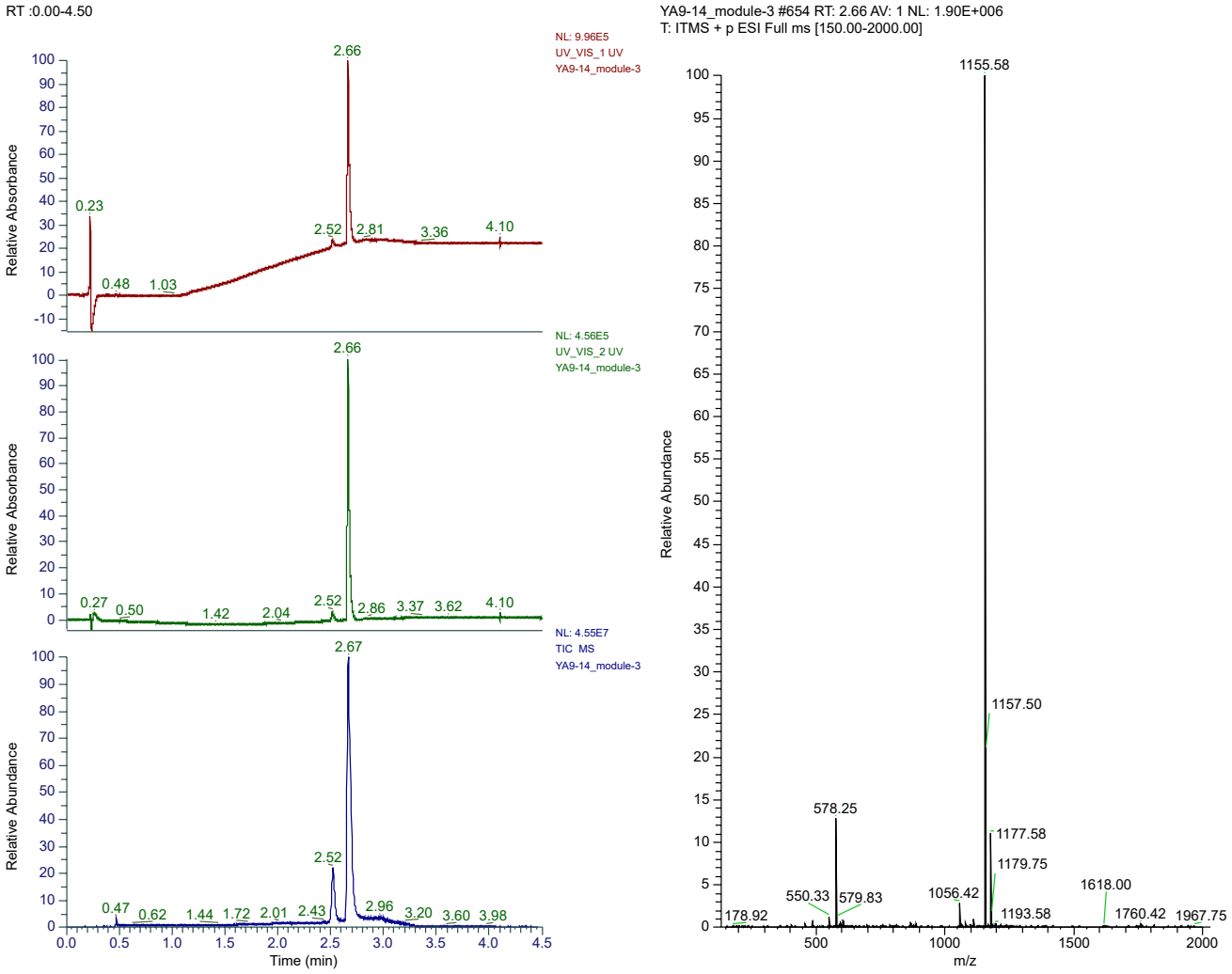

FmocHN
O
H
N
O
N
H
O
H
N
O
O
O
O
O
O
O
NO2
3
t-BuO
O
NH
H2N
O

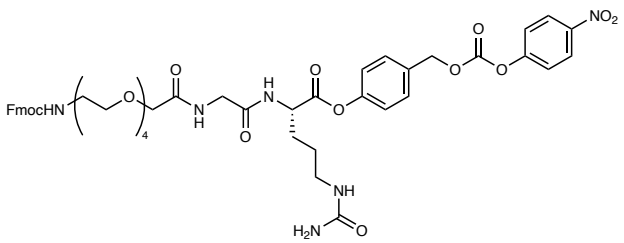
S5a


RT :0.00-4.50
NL: 1.10E6
UV_VIS_1 UV
YA9-69-
2_20210303164719
Relative Absorbance
2.55
0.22
0.39
1.15
1.68
2.19
2.26
2.69
3.08
3.54
4.10
NL: 6.93E5
UV_VIS_2 UV
YA9-69-
2_20210303164719
Relative Absorbance
2.55
0.26
0.50
0.84
1.31
1.68
2.35
2.45
3.08
3.59
3.85
4.10
NL: 3.88E7
TIC MS
YA9-69-
2_20210303164719
Relative Abundance
2.55
0.47
0.74
1.38
1.58
2.11
2.47
2.90
3.22
3.29
3.87
4.14
Time (min)
YA9-69-2_20210303164719 #620 RT: 2.55 AV: 1 NL: 2.78E+006
T: ITMS + p ESI Full ms [150.00-2000.00]
Relative Abundance
1099.67
1099.75
400.58
760.83
1055.50
1121.50
1122.50
1498.42
280.50
578.42
1648.33
1880.75
m/z

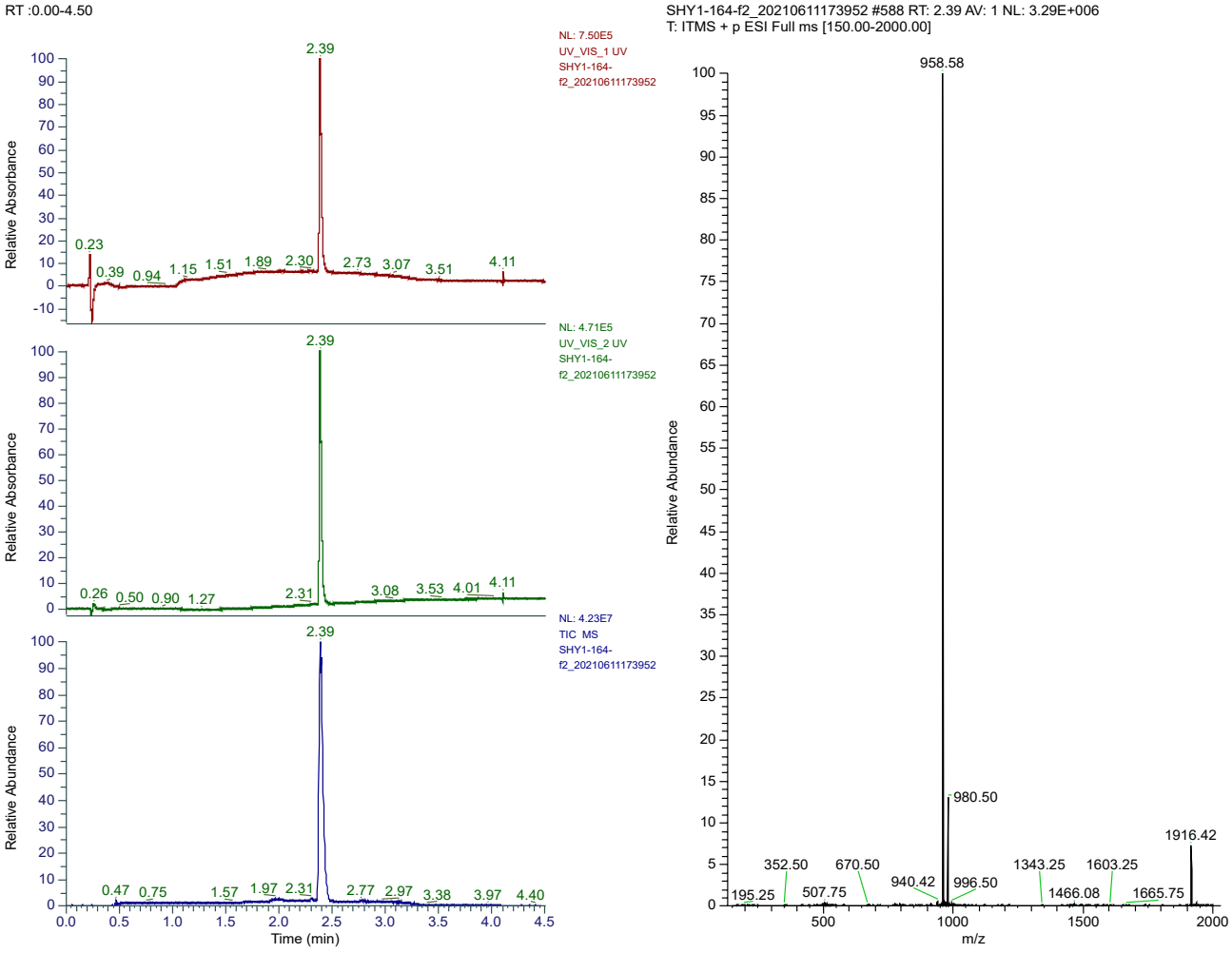

FmocHN
O
3
H
N
O
O
N
H
N
O
O
O
O
O
O
NO2
t-BuO
O
NH
H2N
O

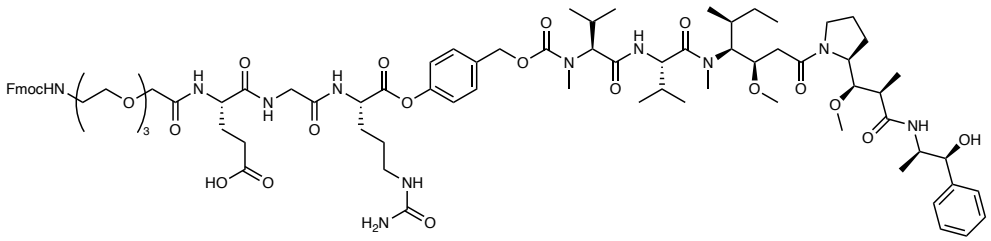
S5b


RT :0.00-4.50
NL: 9.96E5
UV_VIS_1 UV
YA9-14_module-3
Relative Absorbance
2.66
0.23
2.52
2.81
3.36
4.10
0.48
1.03
NL: 4.56E5
UV_VIS_2 UV
YA9-14_module-3
Relative Absorbance
2.66
0.27
0.50
1.42
2.04
2.52
2.86
3.37
3.62
4.10
NL: 4.55E7
TIC MS
YA9-14_module-3
Relative Abundance
2.67
2.52
0.47
0.62
1.44
1.72
2.01
2.43
2.96
3.20
3.60
3.98
Time (min)
YA9-14_module-3 #654 RT: 2.66 AV: 1 NL: 1.90E+006
T: ITMS + p ESI Full ms [150.00-2000.00]
Relative Abundance
1155.58
1157.50
578.25
1177.58
1179.75
1618.00
550.33
579.83
1056.42
178.92
1193.58
1760.42
1967.75
m/z

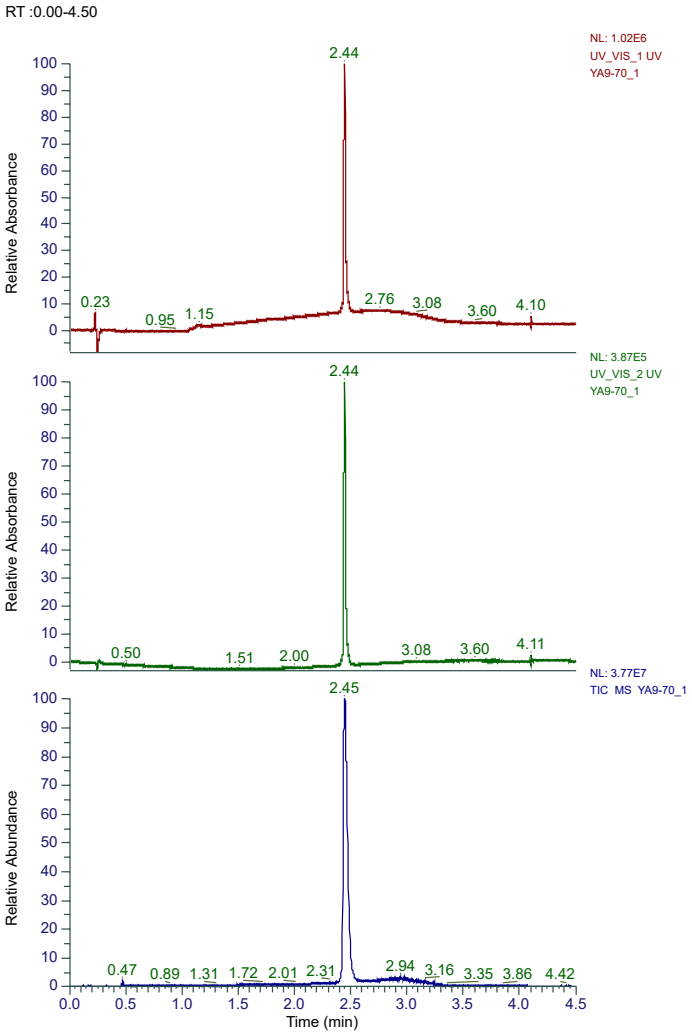

FmocHN
O
4
H
N
NH
O
NO2
NH
H2N

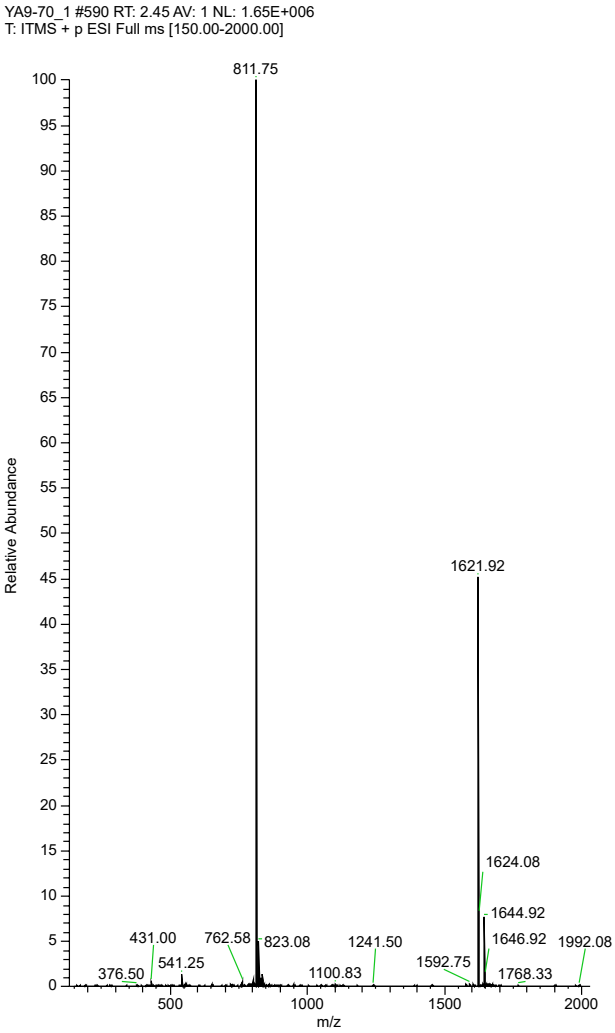
S5c

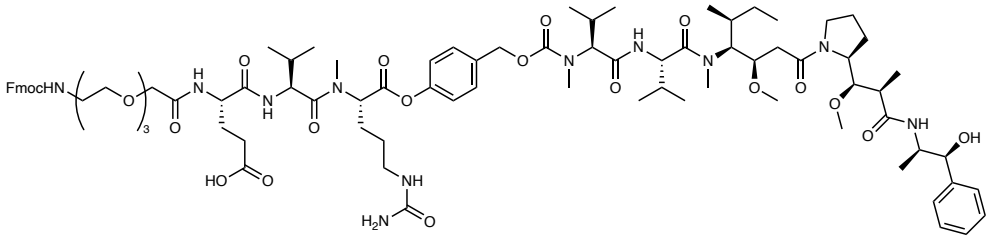

RT :0.00-4.50
NL: 7.50E5
UV_VIS_1 UV
SHY1-164-
f2_20210611173952
NL: 4.71E5
UV_VIS_2 UV
SHY1-164-
f2_20210611173952
NL: 4.23E7
TIC MS
SHY1-164-
f2_20210611173952
Relative Absorbance
Relative Abundance
Time (min)
SHY1-164-f2_20210611173952 #588 RT: 2.39 AV: 1 NL: 3.29E+006
T: ITMS + p ESI Full ms [150.00-2000.00]
958.58
980.50
1916.42
352.50
670.50
940.42
996.50
1343.25
1603.25
195.25
507.75
1466.08
1665.75
m/z



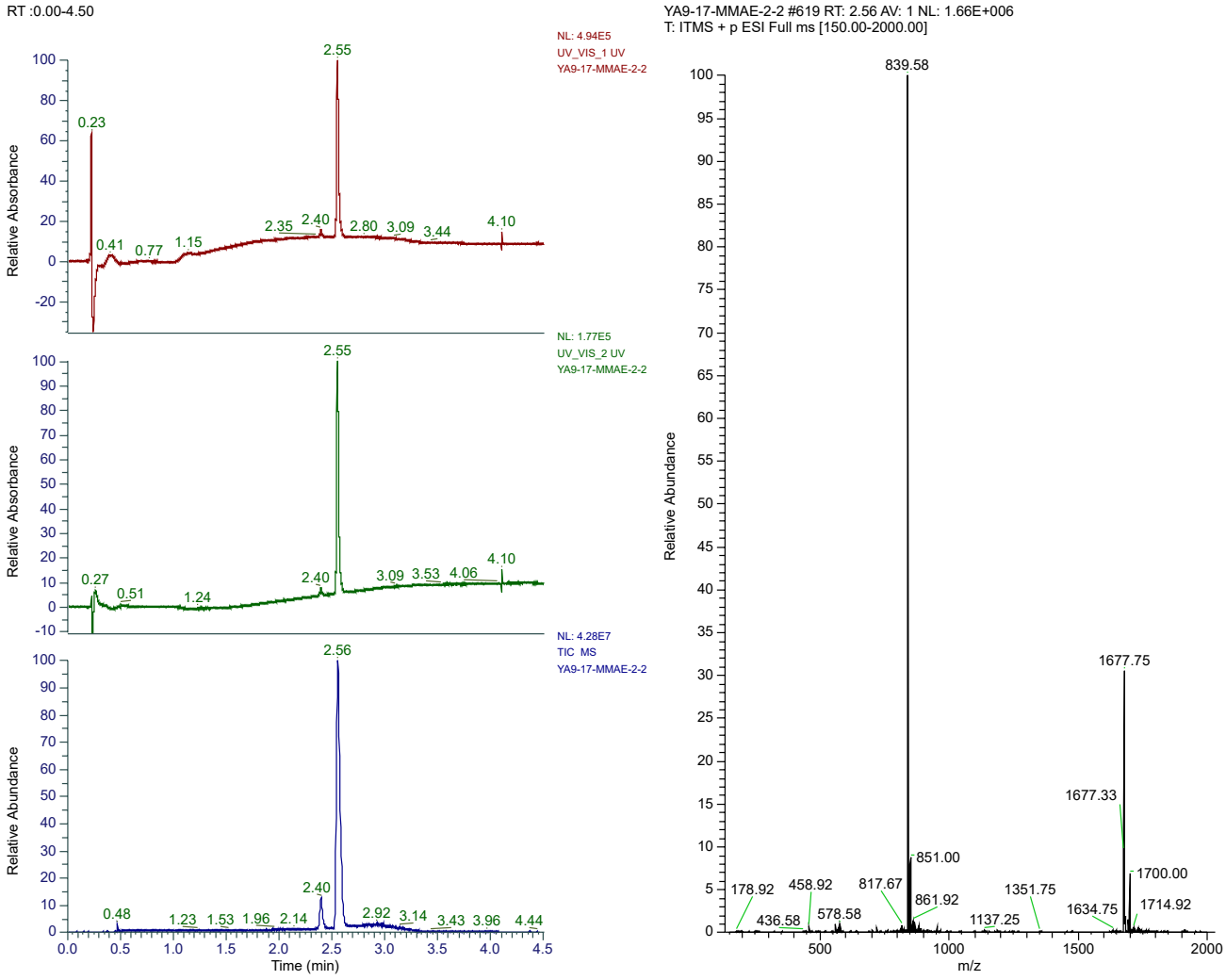
S6a

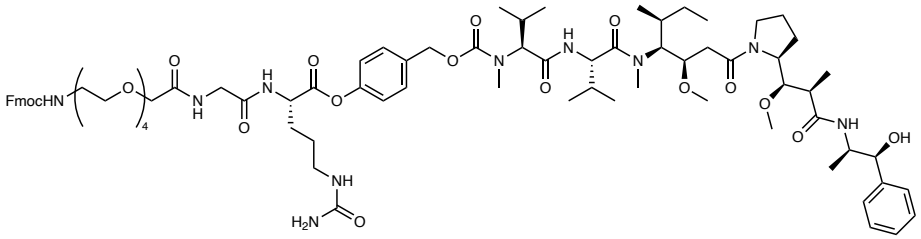

RT :0.00-4.50
NL: 1.02E6
UV_VIS_1 UV
YA9-70_1
NL: 3.87E5
UV_VIS_2 UV
YA9-70_1
NL: 3.77E7
TIC MS YA9-70_1
Relative Absorbance
Relative Abundance
Time (min)


YA9-70_1 #590 RT: 2.45 AV: 1 NL: 1.65E+006
T: ITMS + p ESI Full ms [150.00-2000.00]
Relative Abundance
m/z

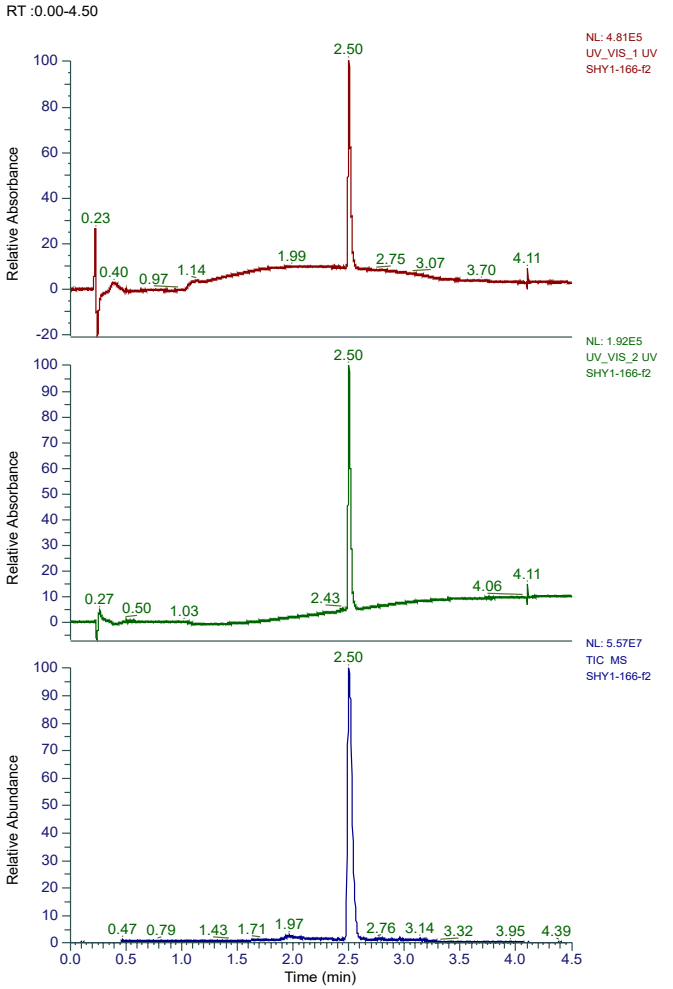


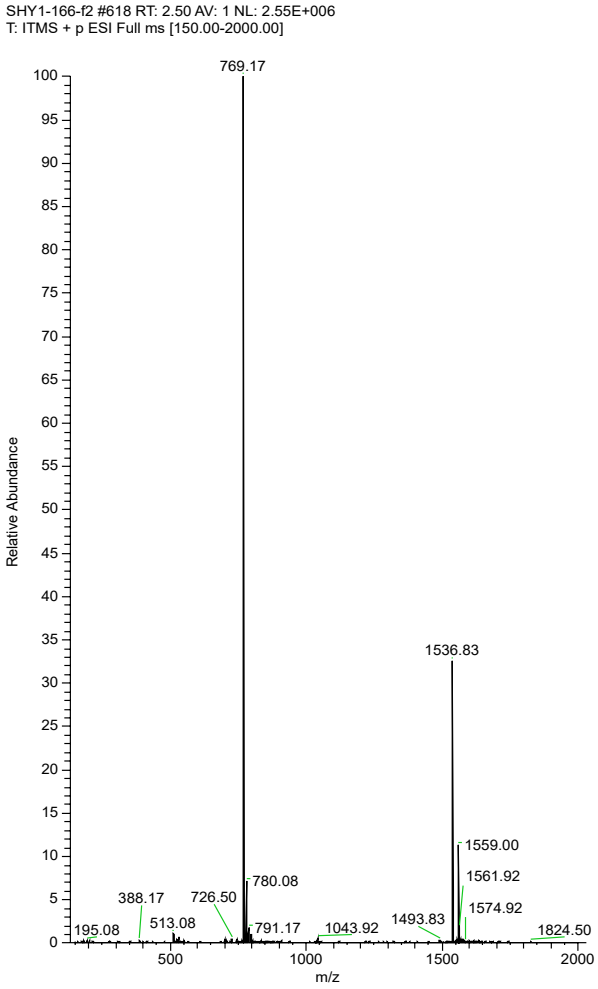
S6b

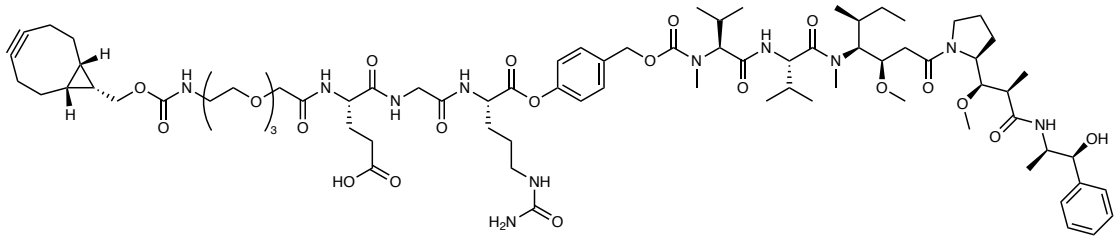

RT :0.00-4.50
NL: 4.94E5
UV_VIS_1 UV
YA9-17-MMAE-2-2
NL: 1.77E5
UV_VIS_2 UV
YA9-17-MMAE-2-2
NL: 4.28E7
TIC MS
YA9-17-MMAE-2-2
Relative Absorbance
Relative Abundance
Time (min)
YA9-17-MMAE-2-2 #619 RT: 2.56 AV: 1 NL: 1.66E+006
T: ITMS + p ESI Full ms [150.00-2000.00]
839.58
1677.75
1677.33
851.00
1700.00
1714.92
m/z



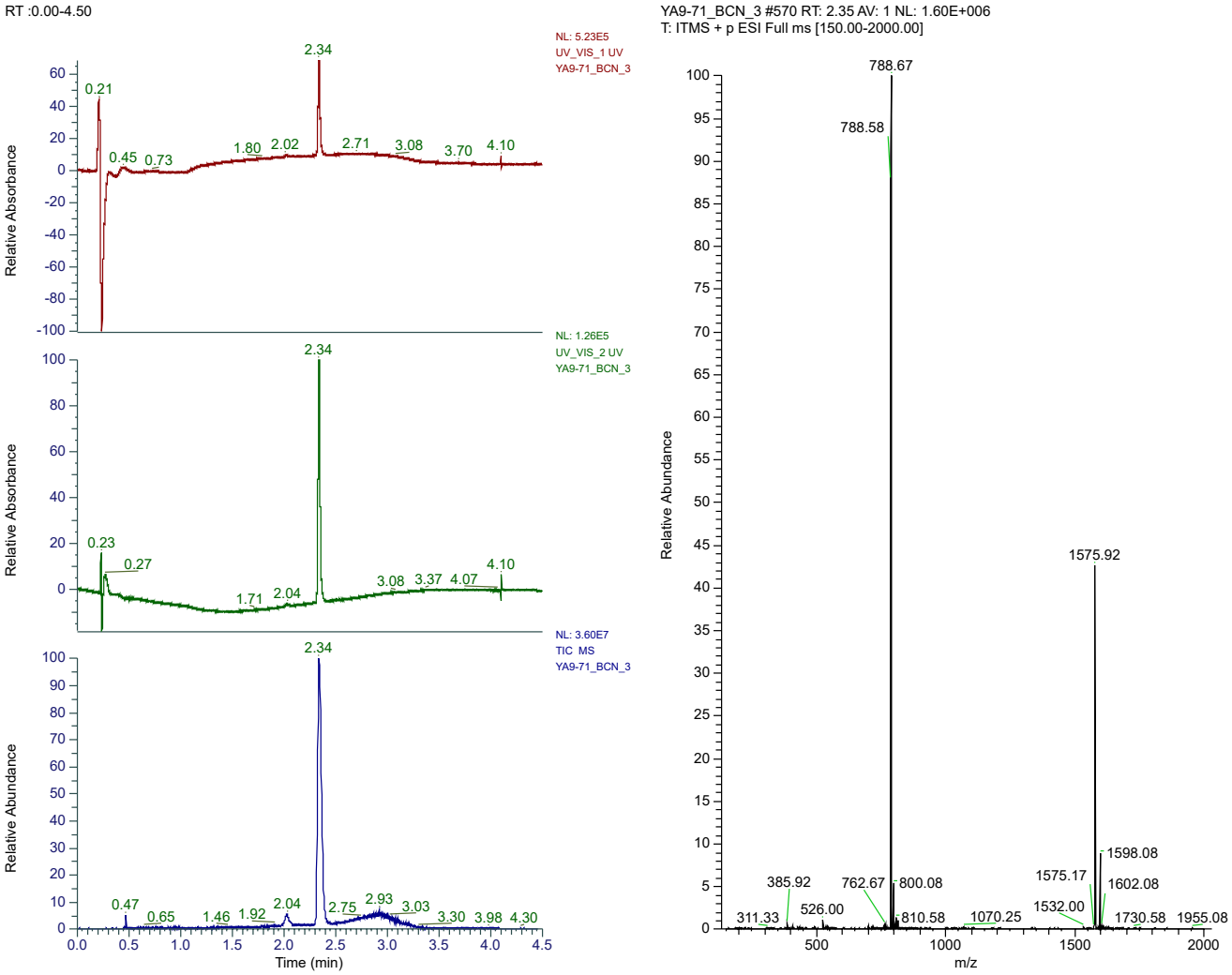
S6c

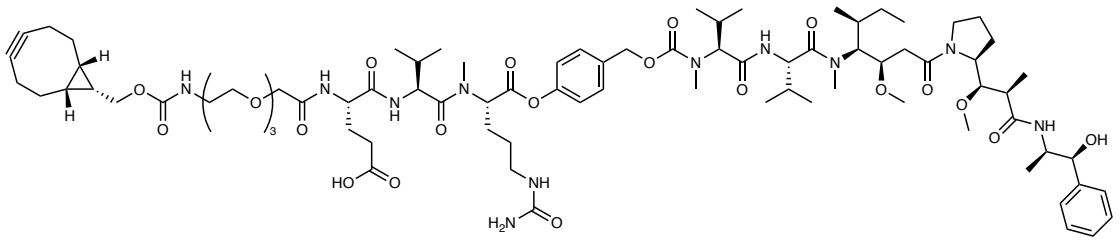

RT :0.00-4.50
NL: 4.81E5
UV_VIS_1 UV
SHY1-166-f2
NL: 1.92E5
UV_VIS_2 UV
SHY1-166-f2
NL: 5.57E7
TIC MS
SHY1-166-f2
Relative Absorbance
Relative Abundance
Time (min)


SHY1-166-f2 #618 RT: 2.50 AV: 1 NL: 2.55E+006
T: ITMS + p ESI Full ms [150.00-2000.00]
Relative Abundance
m/z

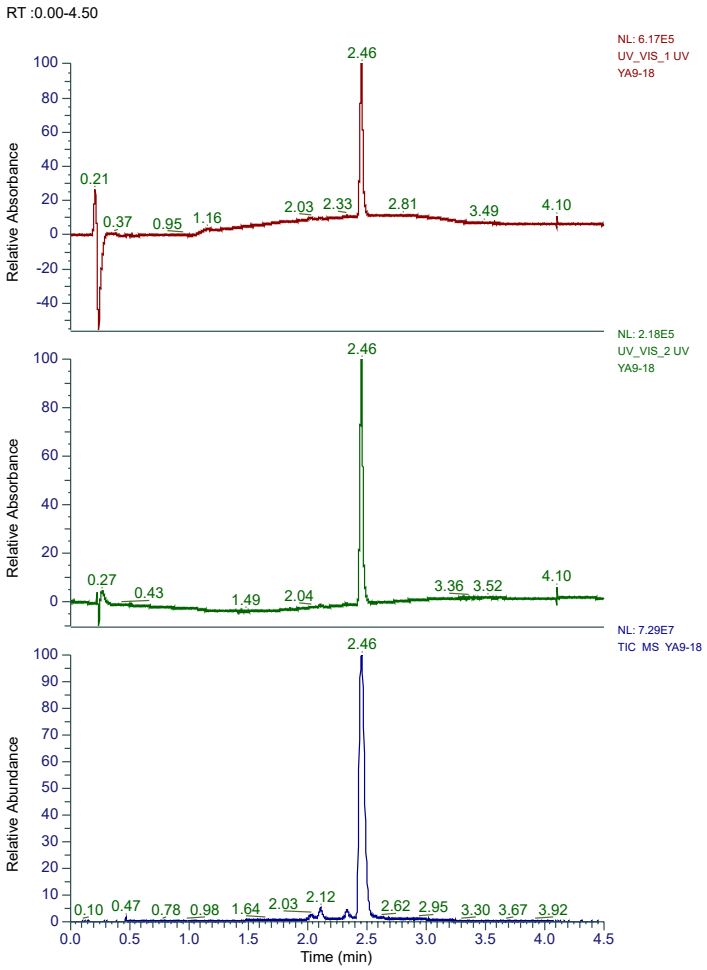


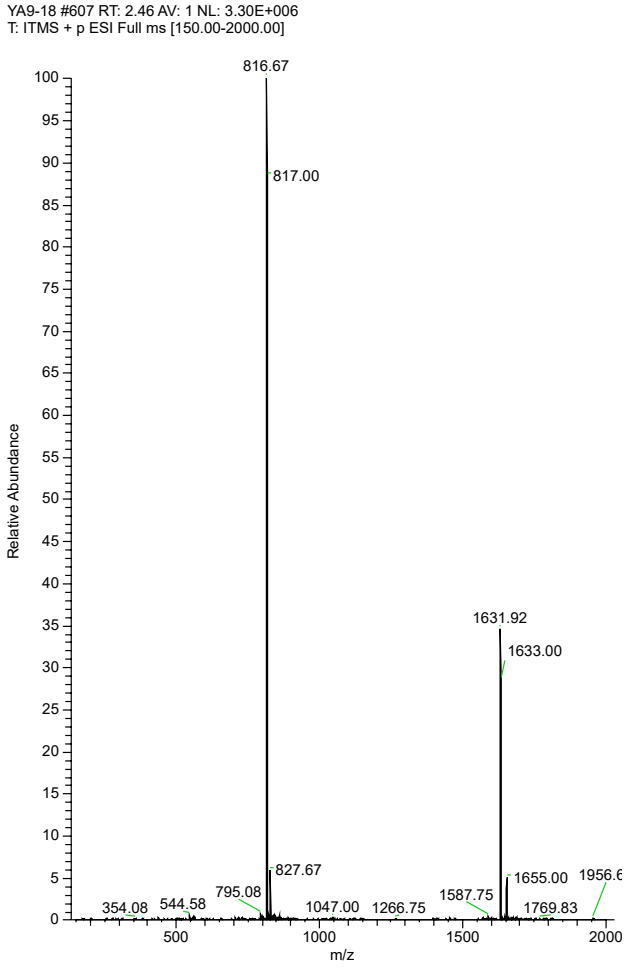
S7a

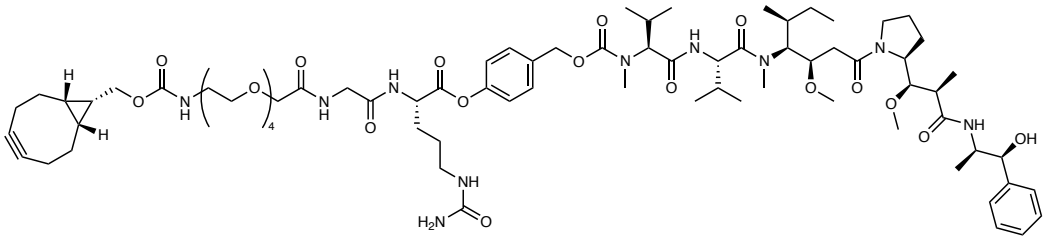

RT :0.00-4.50
NL: 5.23E5
UV_VIS_1 UV
YA9-71_BCN_3
NL: 1.26E5
UV_VIS_2 UV
YA9-71_BCN_3
NL: 3.60E7
TIC MS
YA9-71_BCN_3
Relative Absorbance
Relative Abundance
Time (min)
YA9-71_BCN_3 #570 RT: 2.35 AV: 1 NL: 1.60E+006
T: ITMS + p ESI Full ms [150.00-2000.00]
Relative Abundance
m/z
788.67
788.58
1575.92
1598.08
1575.17
1602.08
385.92
762.67
800.08
526.00
311.33
810.58
1070.25
1532.00
1730.58
1955.08



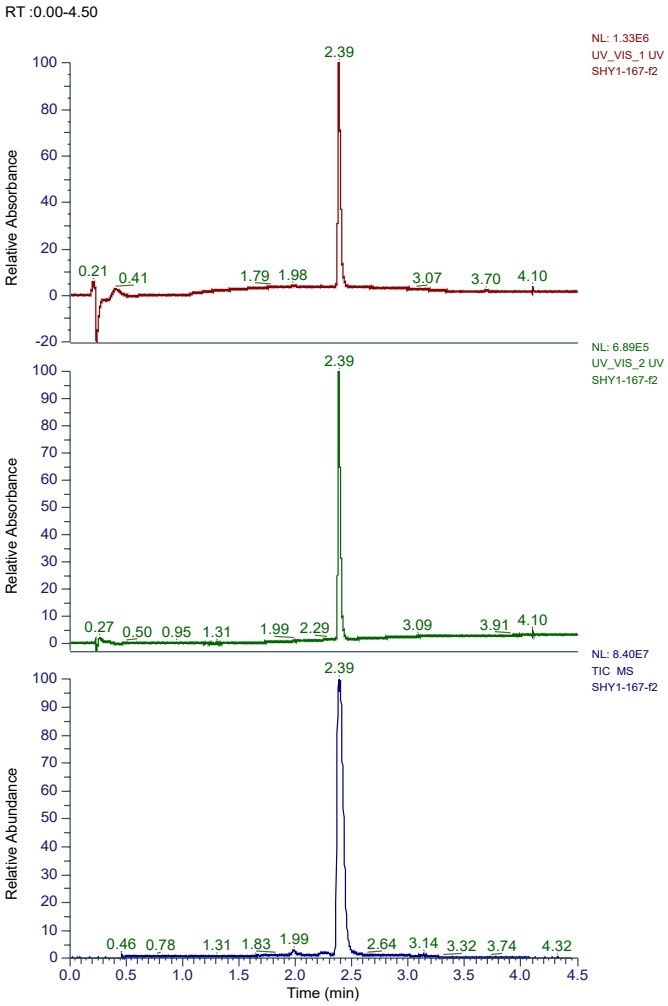
S7b

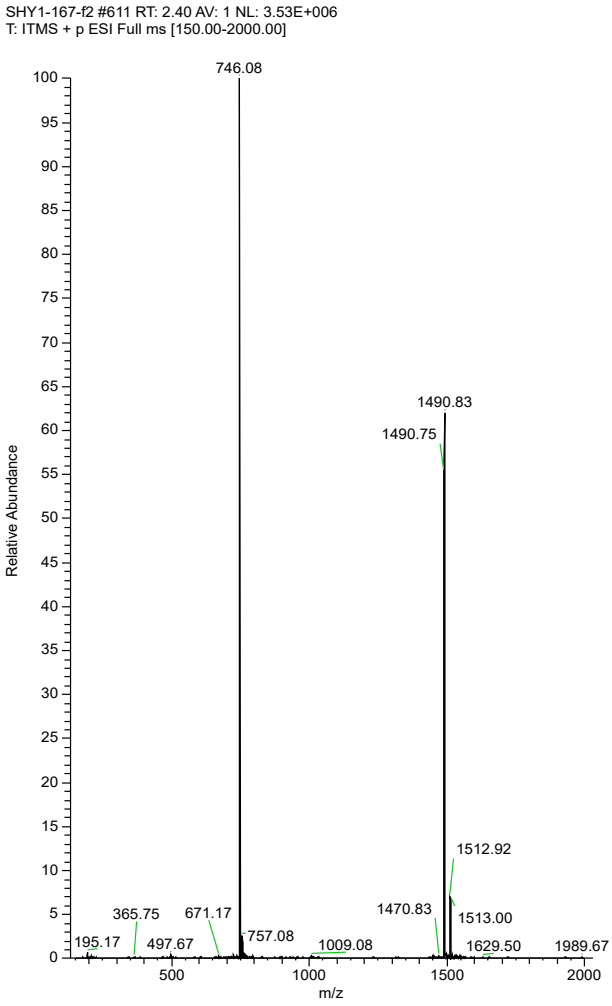

RT :0.00-4.50
NL: 6.17E5
UV_VIS_1 UV
YA9-18
Relative Absorbance
2.46
0.21
0.37
0.95
1.16
2.03
2.33
2.81
3.49
4.10
NL: 2.18E5
UV_VIS_2 UV
YA9-18
Relative Absorbance
2.46
0.27
0.43
1.49
2.04
3.36
3.52
4.10
NL: 7.29E7
TIC MS YA9-18
Relative Abundance
2.46
0.10
0.47
0.78
0.98
1.64
2.03
2.12
2.62
2.95
3.30
3.67
3.92
Time (min)

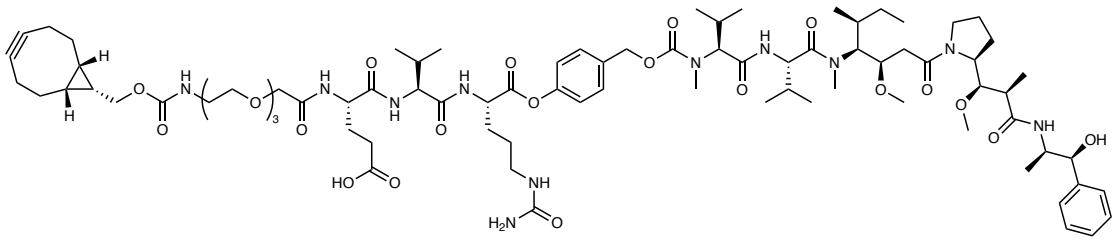

YA9-18 #607 RT: 2.46 AV: 1 NL: 3.30E+006
T: ITMS + p ESI Full ms [150.00-2000.00]
Relative Abundance
816.67
817.00
827.67
795.08
354.08
544.58
1047.00
1266.75
1631.92
1633.00
1587.75
1655.00
1769.83
m/z



S7c

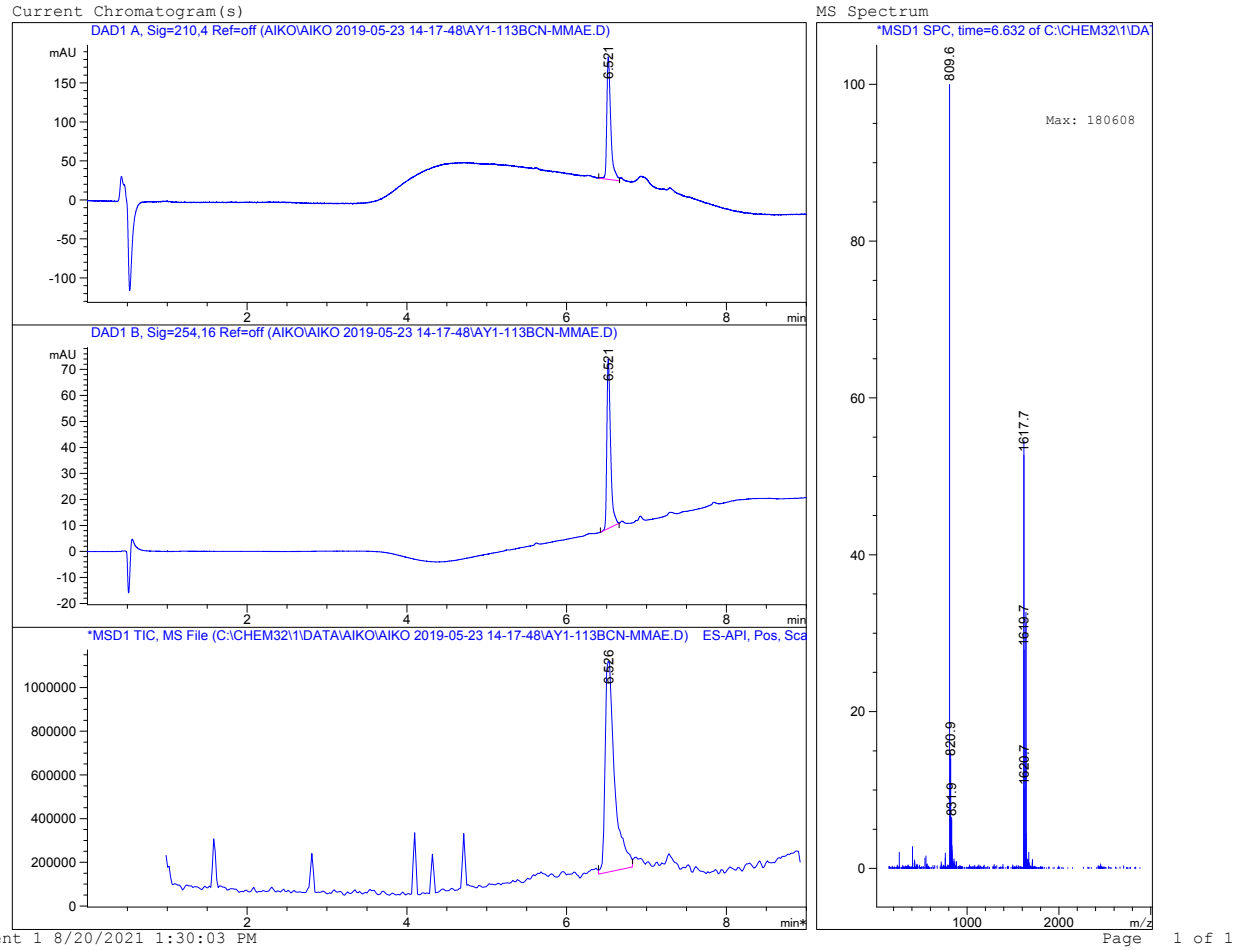

RT :0.00-4.50
NL: 1.33E6
UV_VIS_1 UV
SHY1-167-f2
NL: 6.89E5
UV_VIS_2 UV
SHY1-167-f2
NL: 8.40E7
TIC MS
SHY1-167-f2
Relative Absorbance
Relative Abundance
Time (min)


SHY1-167-f2 #611 RT: 2.40 AV: 1 NL: 3.53E+006
T: ITMS + p ESI Full ms [150.00-2000.00]
Relative Abundance
m/z

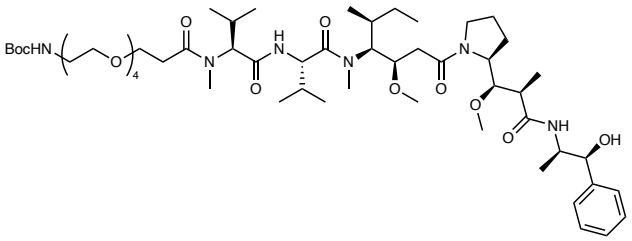


S8

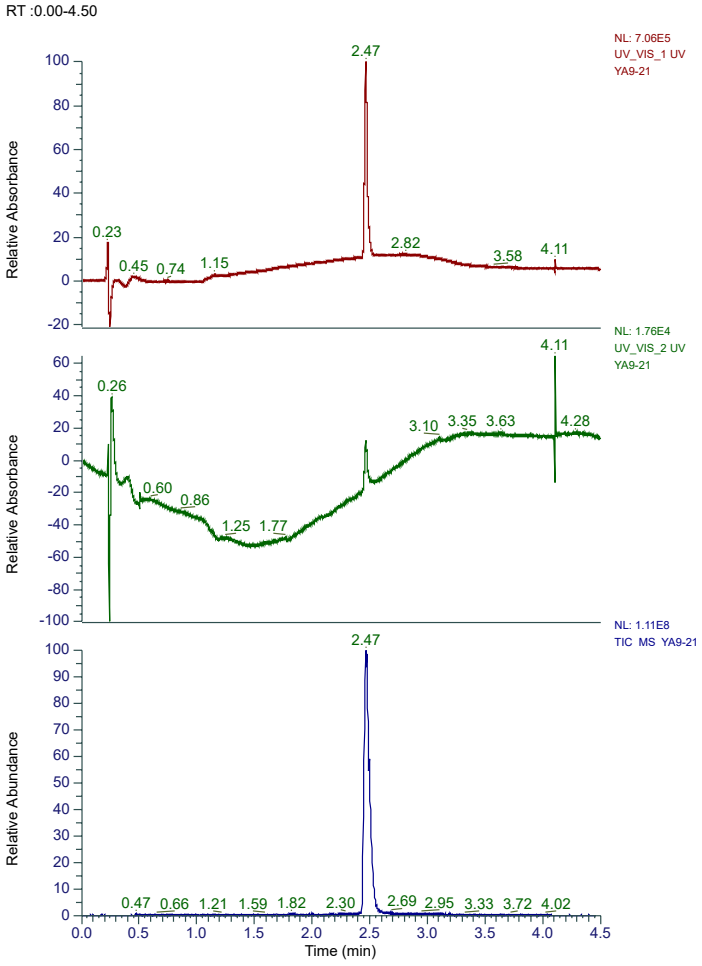
Print of all graphic windows

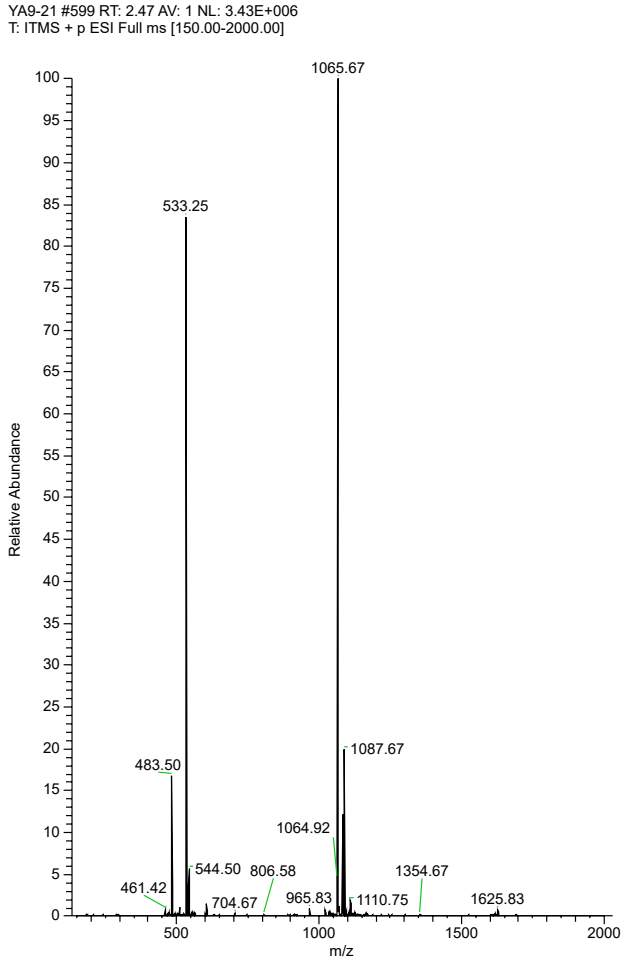


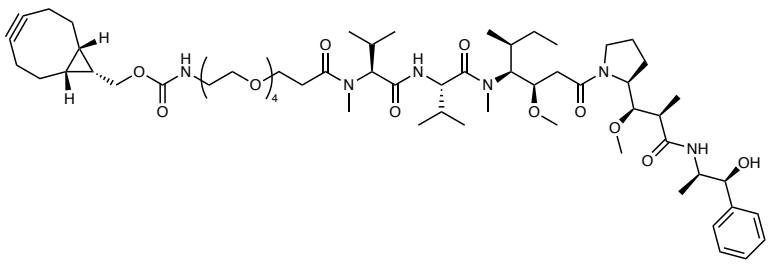
Instrument 1 8/20/2021 1:30:03 PM


BocHN
O
O
4
N
H
N
O
O
O
O
O
N
O
NH
OH

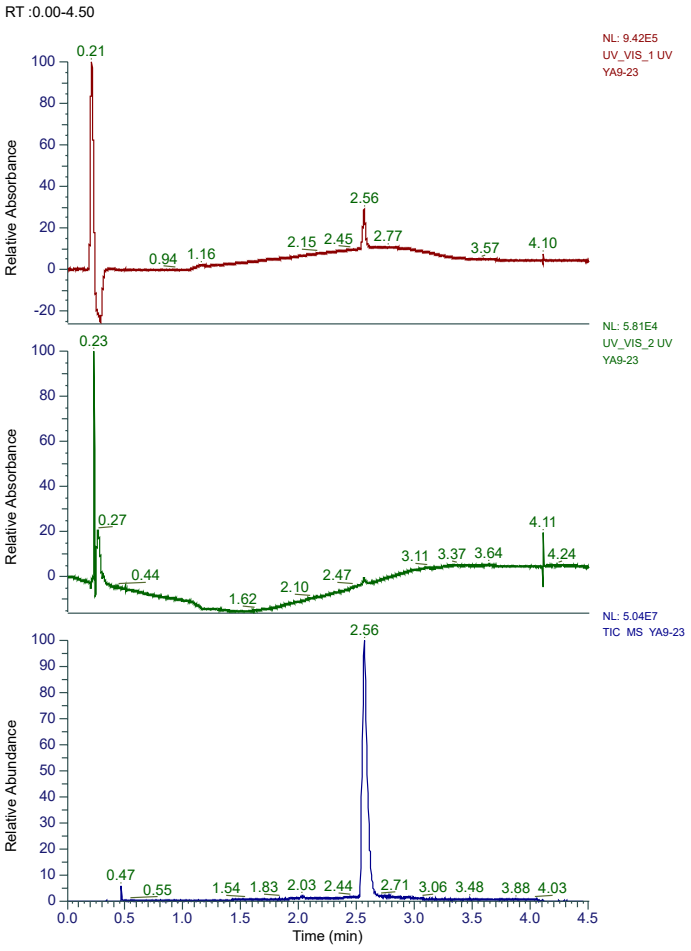
S9

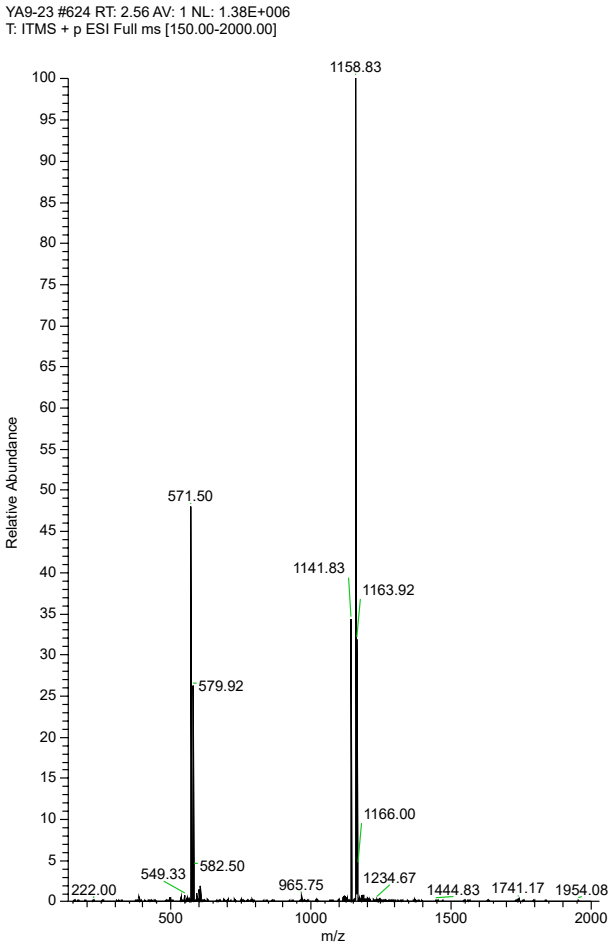

RT :0.00-4.50
NL: 7.06E5
UV_VIS_1 UV
YA9-21
Relative Absorbance
2.47
0.23
0.45
0.74
1.15
2.82
3.58
4.11
NL: 1.76E4
UV_VIS_2 UV
YA9-21
Relative Absorbance
4.11
0.26
3.10
3.35
3.63
4.28
0.60
0.86
1.25
1.77
NL: 1.11E8
TIC MS YA9-21
Relative Abundance
2.47
0.47
0.66
1.21
1.59
1.82
2.30
2.69
2.95
3.33
3.72
4.02
Time (min)

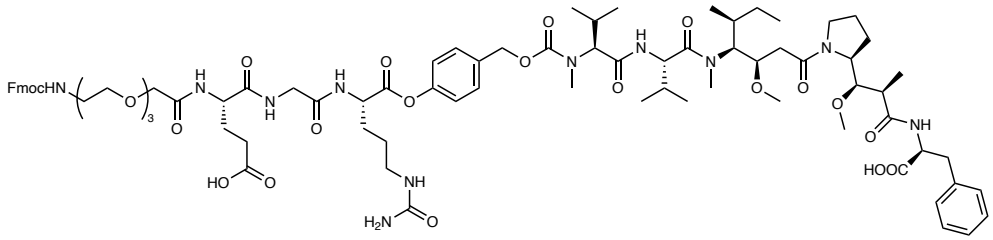

YA9-21 #599 RT: 2.47 AV: 1 NL: 3.43E+006
T: ITMS + p ESI Full ms [150.00-2000.00]
Relative Abundance
1065.67
533.25
1087.67
483.50
1064.92
544.50
806.58
1354.67
461.42
704.67
965.83
1110.75
1625.83
m/z



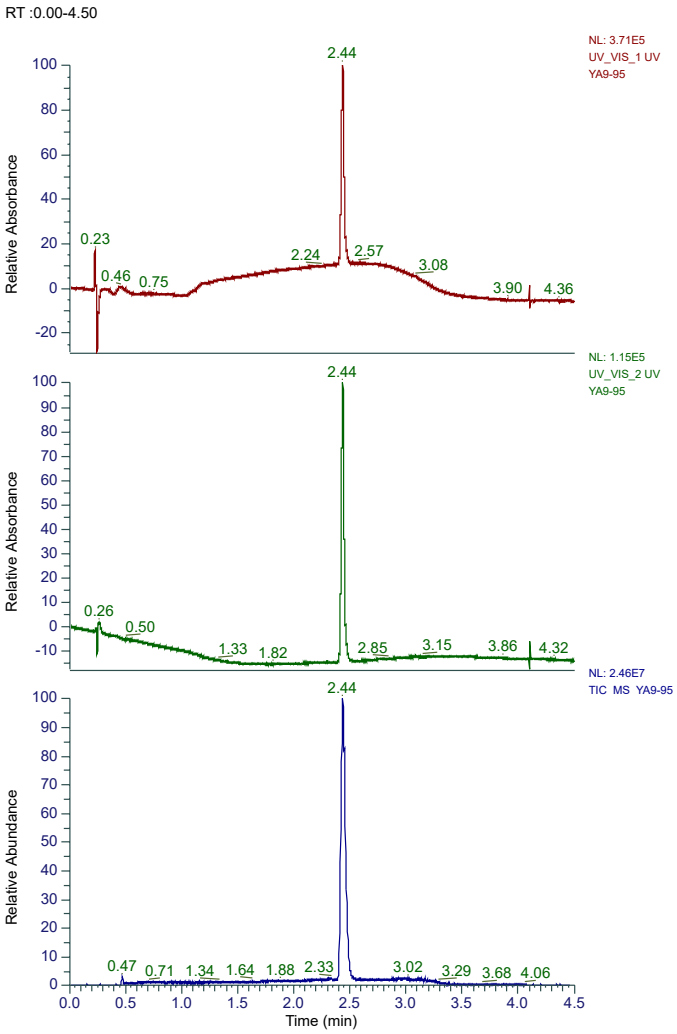
S10

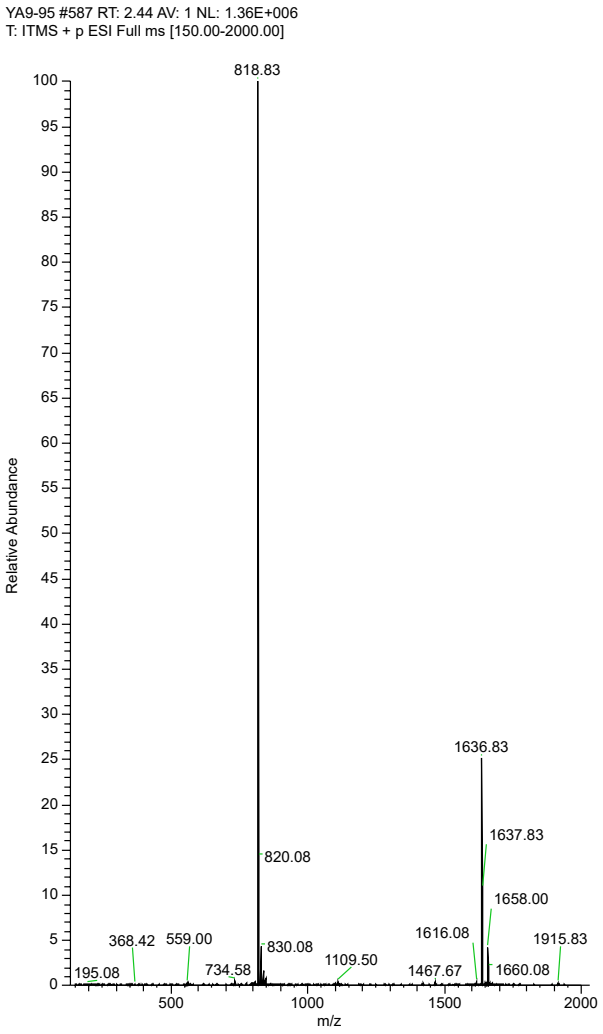

RT :0.00-4.50
NL: 9.42E5
UV_VIS_1 UV
YA9-23
Relative Absorbance
0.21
0.94
1.16
2.15
2.45
2.56
2.77
3.57
4.10
NL: 5.81E4
UV_VIS_2 UV
YA9-23
Relative Absorbance
0.23
0.27
0.44
1.62
2.10
2.47
3.11
3.37
3.64
4.11
4.24
NL: 5.04E7
TIC MS YA9-23
Relative Abundance
0.47
0.55
1.54
1.83
2.03
2.44
2.56
2.71
3.06
3.48
3.88
4.03
Time (min)

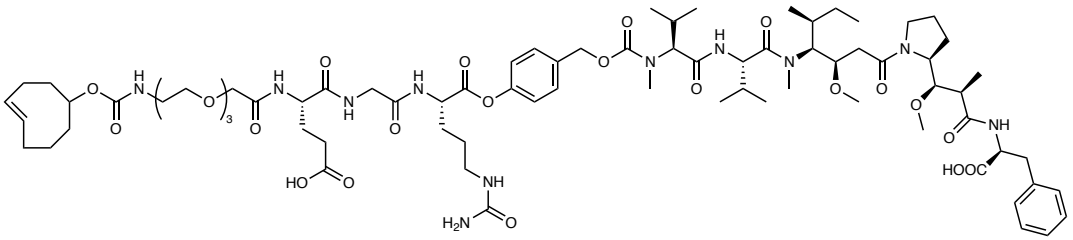

YA9-23 #624 RT: 2.56 AV: 1 NL: 1.38E+006
T: ITMS + p ESI Full ms [150.00-2000.00]
Relative Abundance
1158.83
571.50
1141.83
1163.92
579.92
1166.00
549.33
582.50
965.75
1234.67
222.00
1444.83
1741.17
1954.08
m/z


FmocHN
HO
NH
H_2N
HOOC

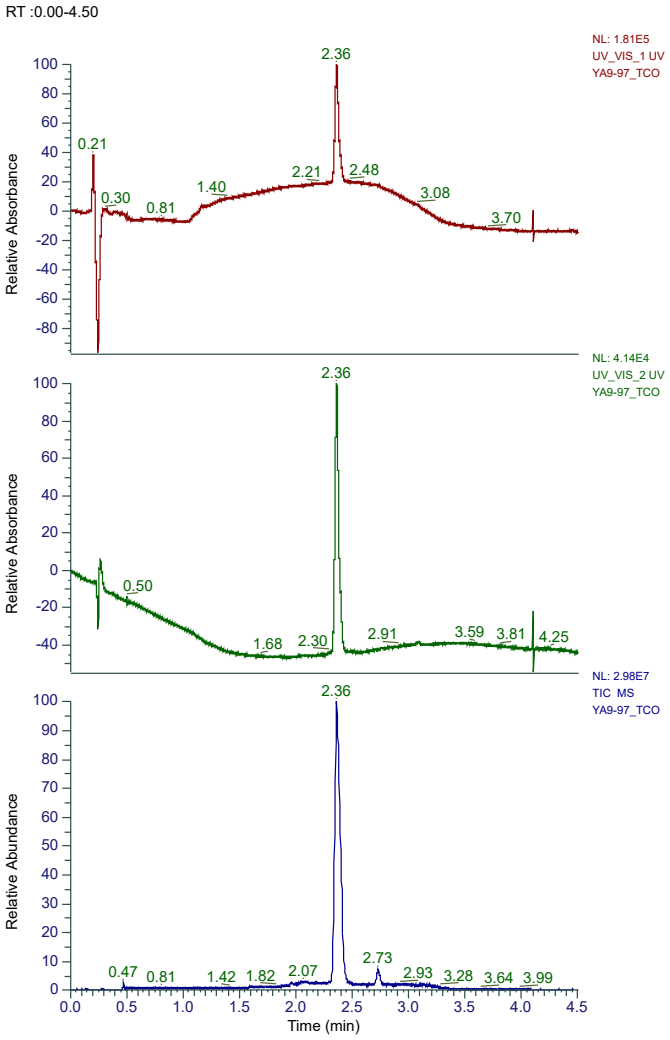
S11

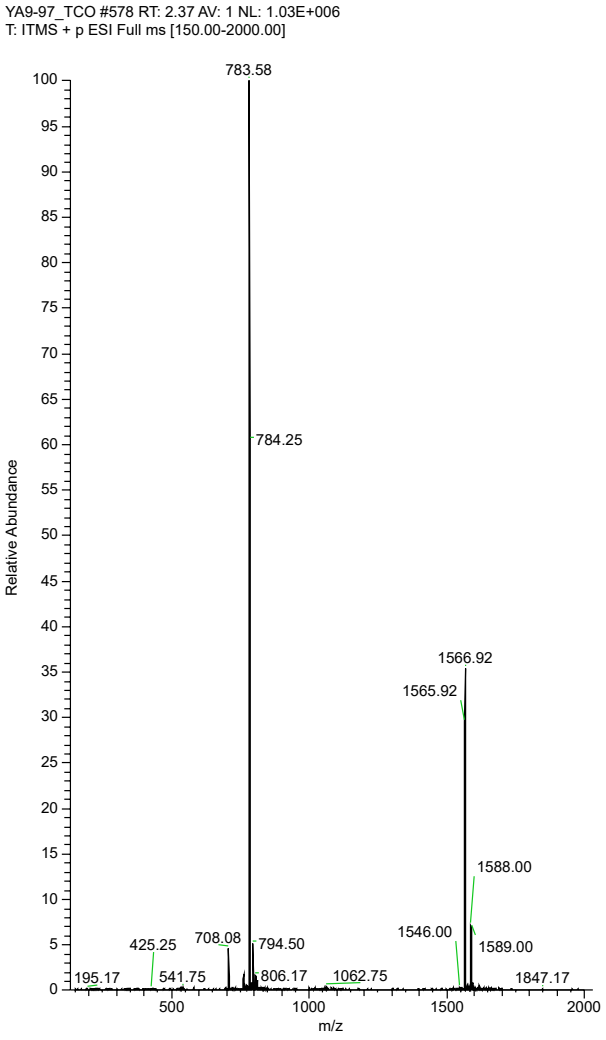

RT :0.00-4.50
NL: 3.71E5
UV_VIS_1 UV
YA9-95
NL: 1.15E5
UV_VIS_2 UV
YA9-95
NL: 2.46E7
TIC MS YA9-95
Relative Absorbance
Relative Abundance
Time (min)

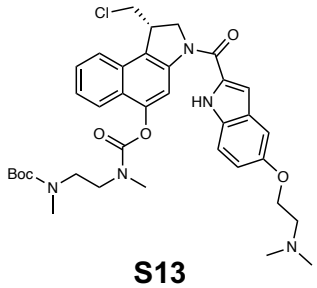

YA9-95 #587 RT: 2.44 AV: 1 NL: 1.36E+006
T: ITMS + p ESI Full ms [150.00-2000.00]
Relative Abundance
m/z



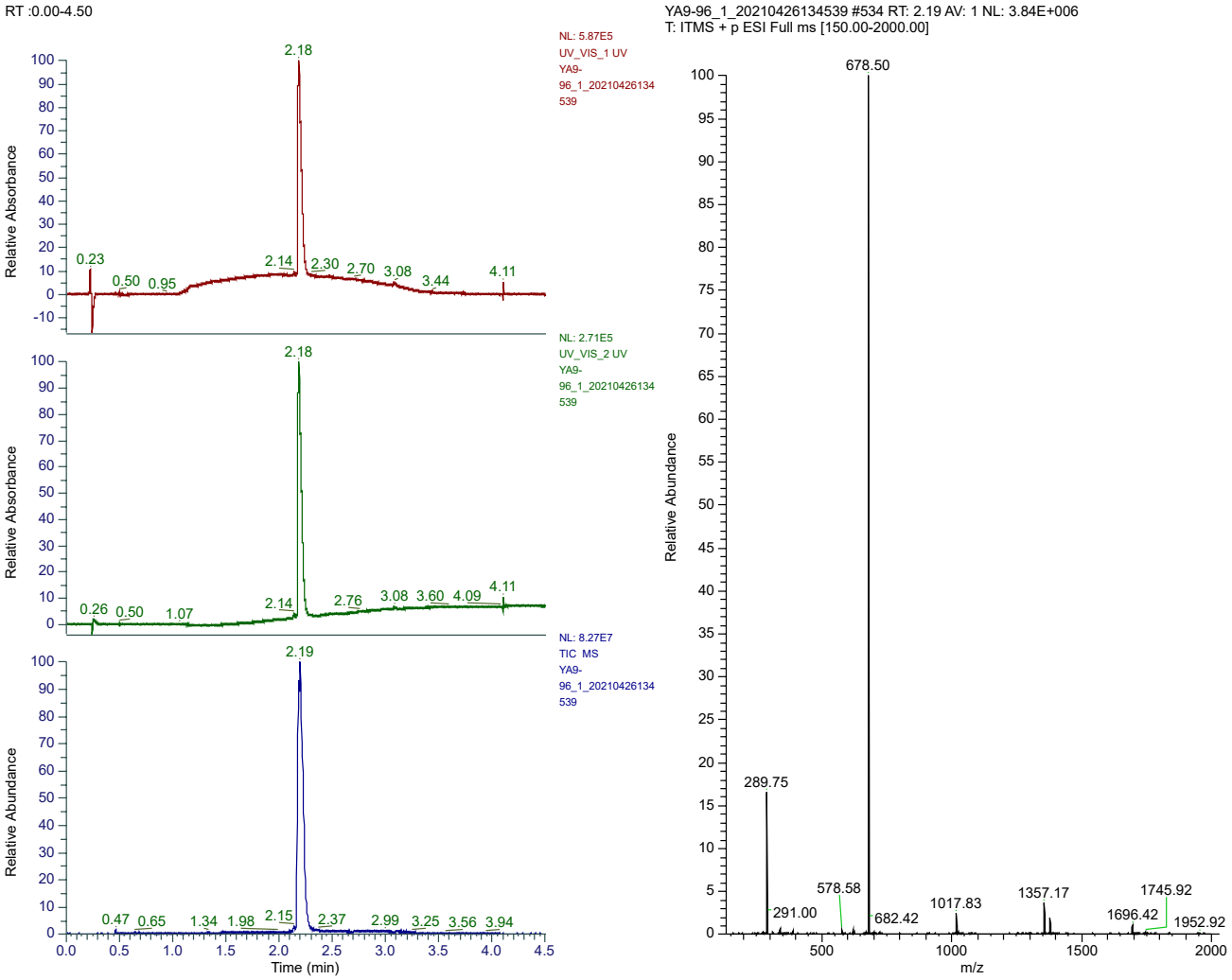
S12

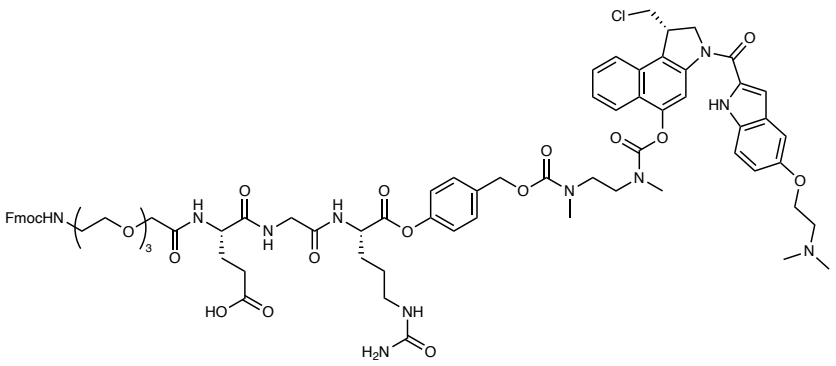

RT :0.00-4.50
NL: 1.81E5
UV_VIS_1 UV
YA9-97_TCO
Relative Absorbance
2.36
0.21
0.30
0.81
1.40
2.21
2.48
3.08
3.70
NL: 4.14E4
UV_VIS_2 UV
YA9-97_TCO
Relative Absorbance
2.36
0.50
1.68
2.30
2.91
3.59
3.81
4.25
NL: 2.98E7
TIC MS
YA9-97_TCO
Relative Abundance
2.36
0.47
0.81
1.42
1.82
2.07
2.73
2.93
3.28
3.64
3.99
Time (min)


YA9-97_TCO #578 RT: 2.37 AV: 1 NL: 1.03E+006
T: ITMS + p ESI Full ms [150.00-2000.00]
Relative Abundance
783.58
784.25
1566.92
1565.92
1588.00
1546.00
1589.00
425.25
708.08
794.50
195.17
541.75
806.17
1062.75
1847.17
m/z

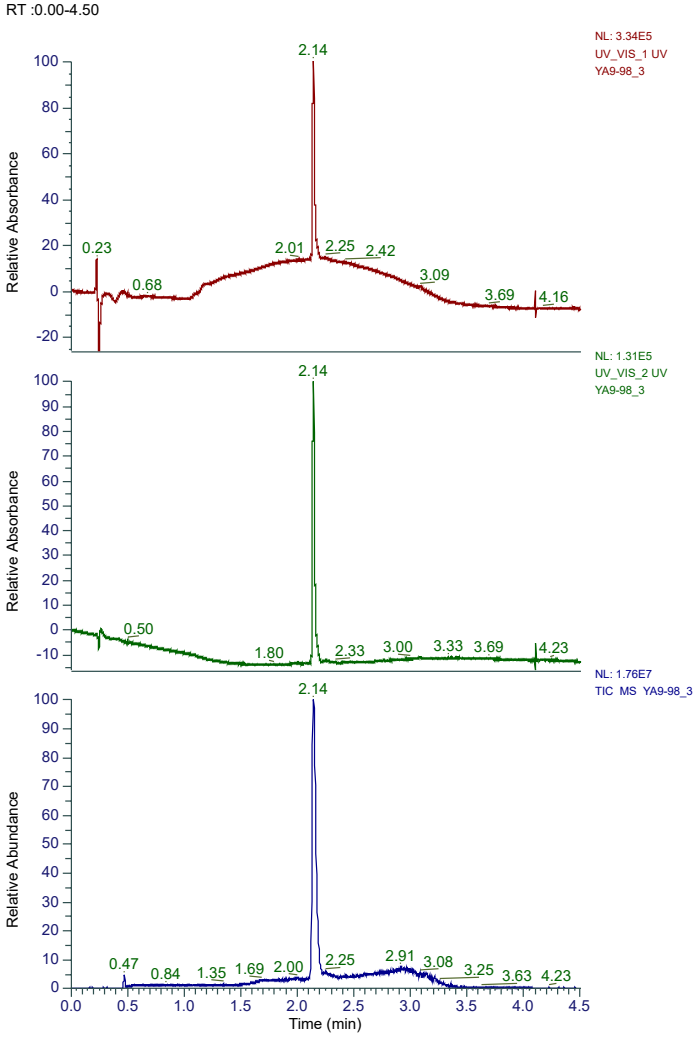

Cl
N
O
HN
O
O
Boc
N
N
O
N

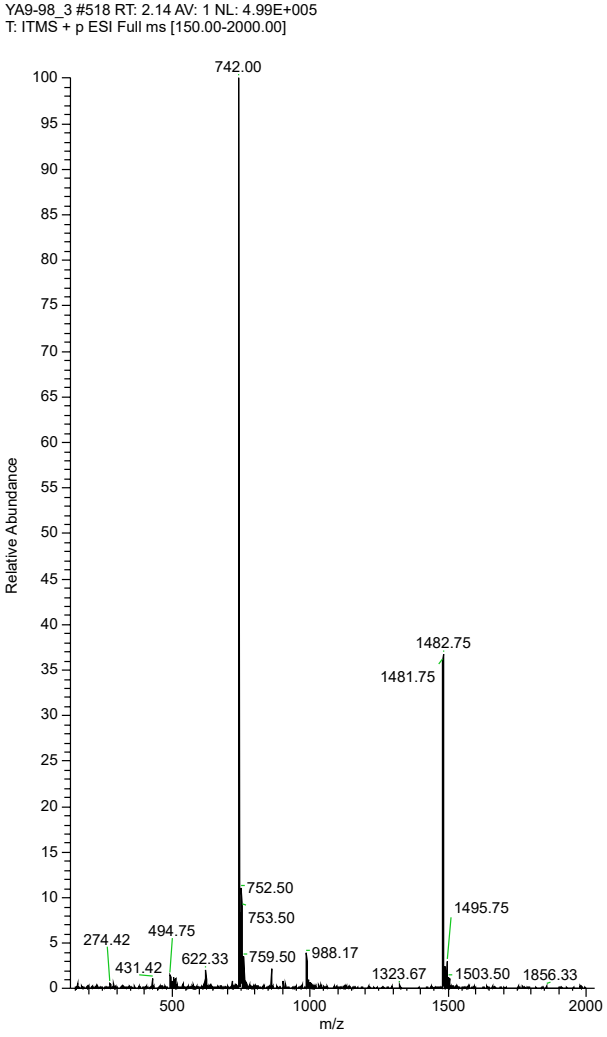
S13

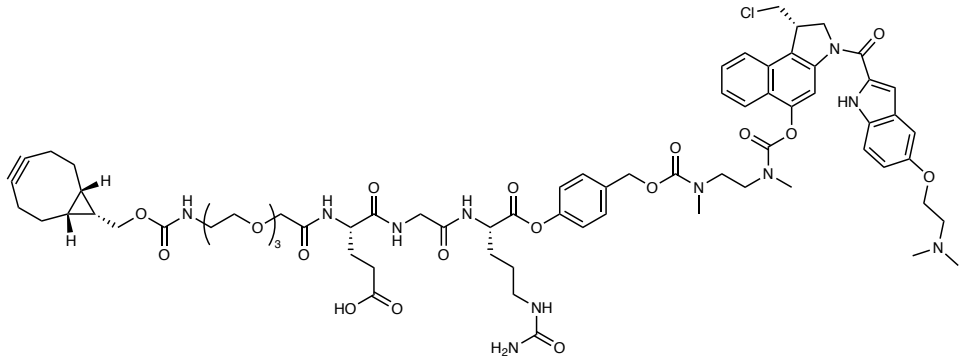

RT :0.00-4.50
NL: 5.87E5
UV_VIS_1 UV
YA9-
96_1_20210426134
539
Relative Absorbance
2.18
0.23
0.50
0.95
2.14
2.30
2.70
3.08
3.44
4.11
NL: 2.71E5
UV_VIS_2 UV
YA9-
96_1_20210426134
539
Relative Absorbance
2.18
0.26
0.50
1.07
2.14
2.76
3.08
3.60
4.09
4.11
NL: 8.27E7
TIC MS
YA9-
96_1_20210426134
539
Relative Abundance
2.19
0.47
0.65
1.34
1.98
2.15
2.37
2.99
3.25
3.56
3.94
Time (min)
YA9-96_1_20210426134539 #534 RT: 2.19 AV: 1 NL: 3.84E+006
T: ITMS + p ESI Full ms [150.00-2000.00]
Relative Abundance
678.50
289.75
291.00
578.58
682.42
1017.83
1357.17
1696.42
1745.92
1952.92
m/z


FmocHN
HO
NH
H_2N
Cl
HN

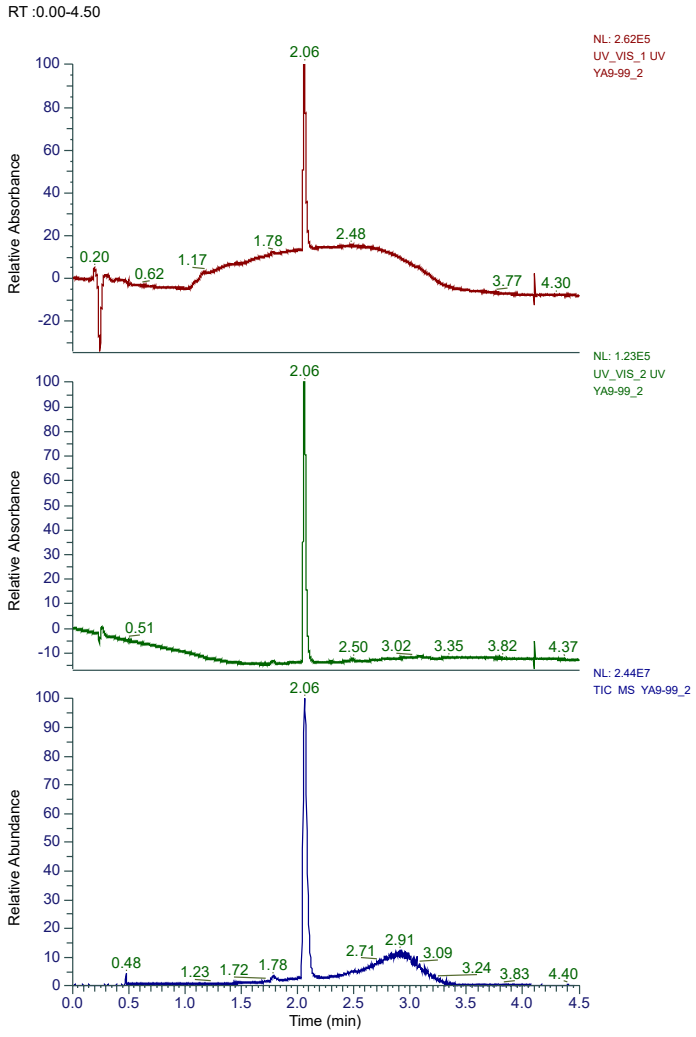
S14

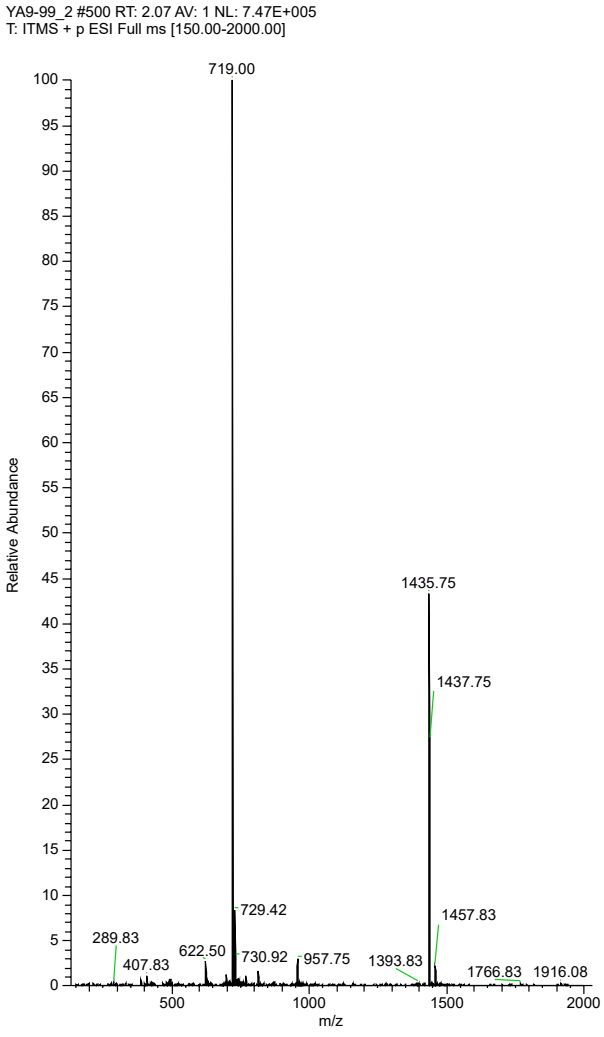

RT :0.00-4.50
NL: 3.34E5
UV_VIS_1 UV
YA9-98_3
NL: 1.31E5
UV_VIS_2 UV
YA9-98_3
NL: 1.76E7
TIC MS YA9-98_3
Relative Absorbance
Relative Abundance
Time (min)

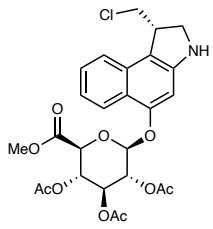

YA9-98_3 #518 RT: 2.14 AV: 1 NL: 4.99E+005
T: ITMS + p ESI Full ms [150.00-2000.00]
Relative Abundance
m/z



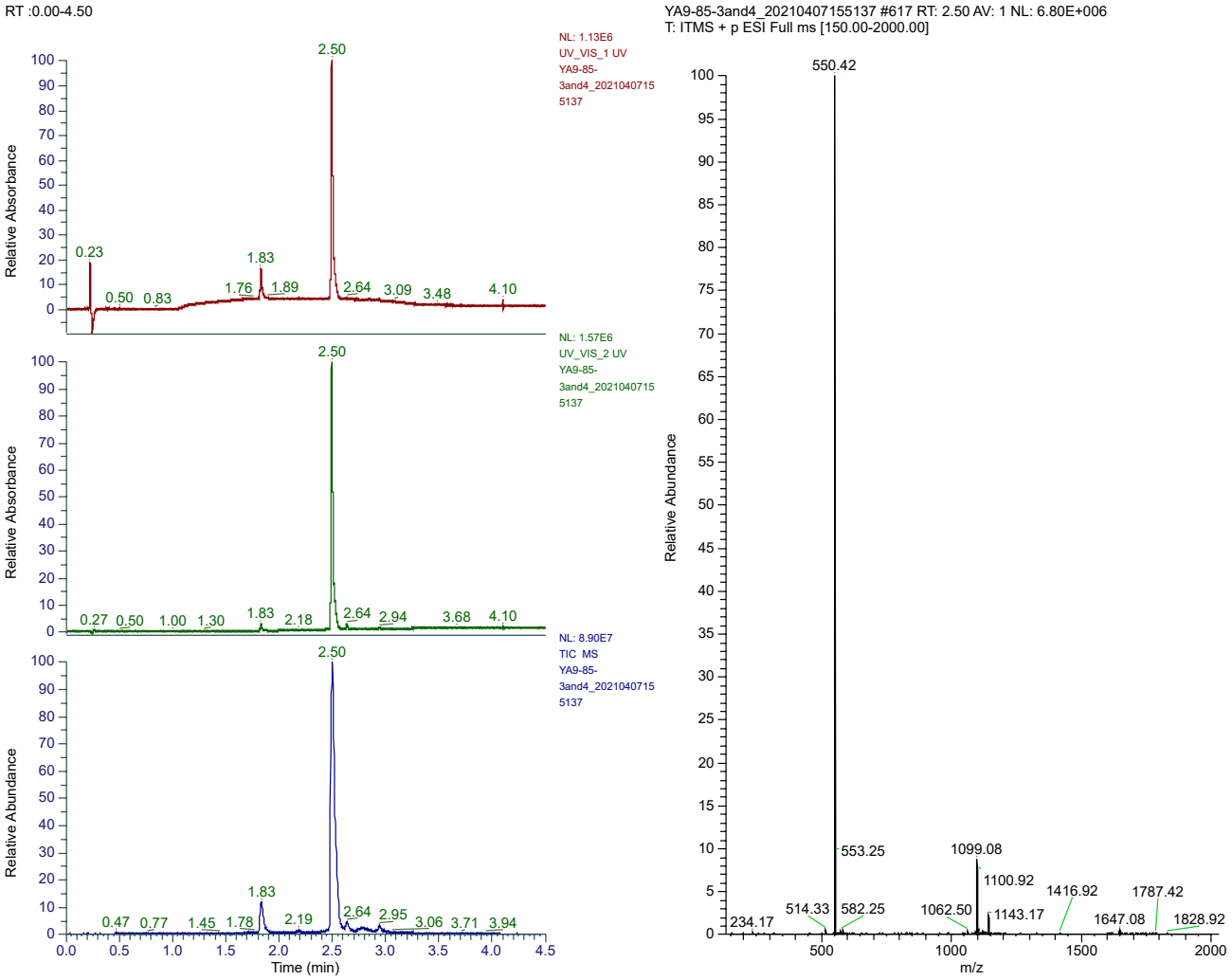
S15

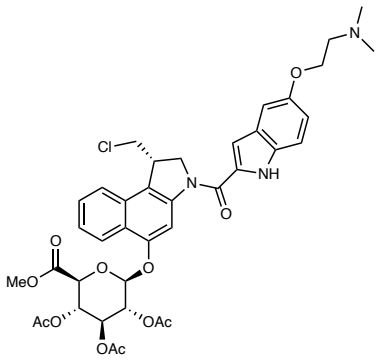

RT :0.00-4.50
NL: 2.62E5
UV_VIS_1 UV
YA9-99_2
Relative Absorbance
2.06
0.20
0.62
1.17
1.78
2.48
3.77
4.30
NL: 1.23E5
UV_VIS_2 UV
YA9-99_2
Relative Absorbance
2.06
0.51
2.50
3.02
3.35
3.82
4.37
NL: 2.44E7
TIC MS YA9-99_2
Relative Abundance
2.06
0.48
1.23
1.72
1.78
2.71
2.91
3.09
3.24
3.83
4.40
Time (min)


YA9-99_2 #500 RT: 2.07 AV: 1 NL: 7.47E+005
T: ITMS + p ESI Full ms [150.00-2000.00]
Relative Abundance
719.00
1435.75
1437.75
729.42
1457.83
289.83
407.83
622.50
730.92
957.75
1393.83
1766.83
1916.08
m/z

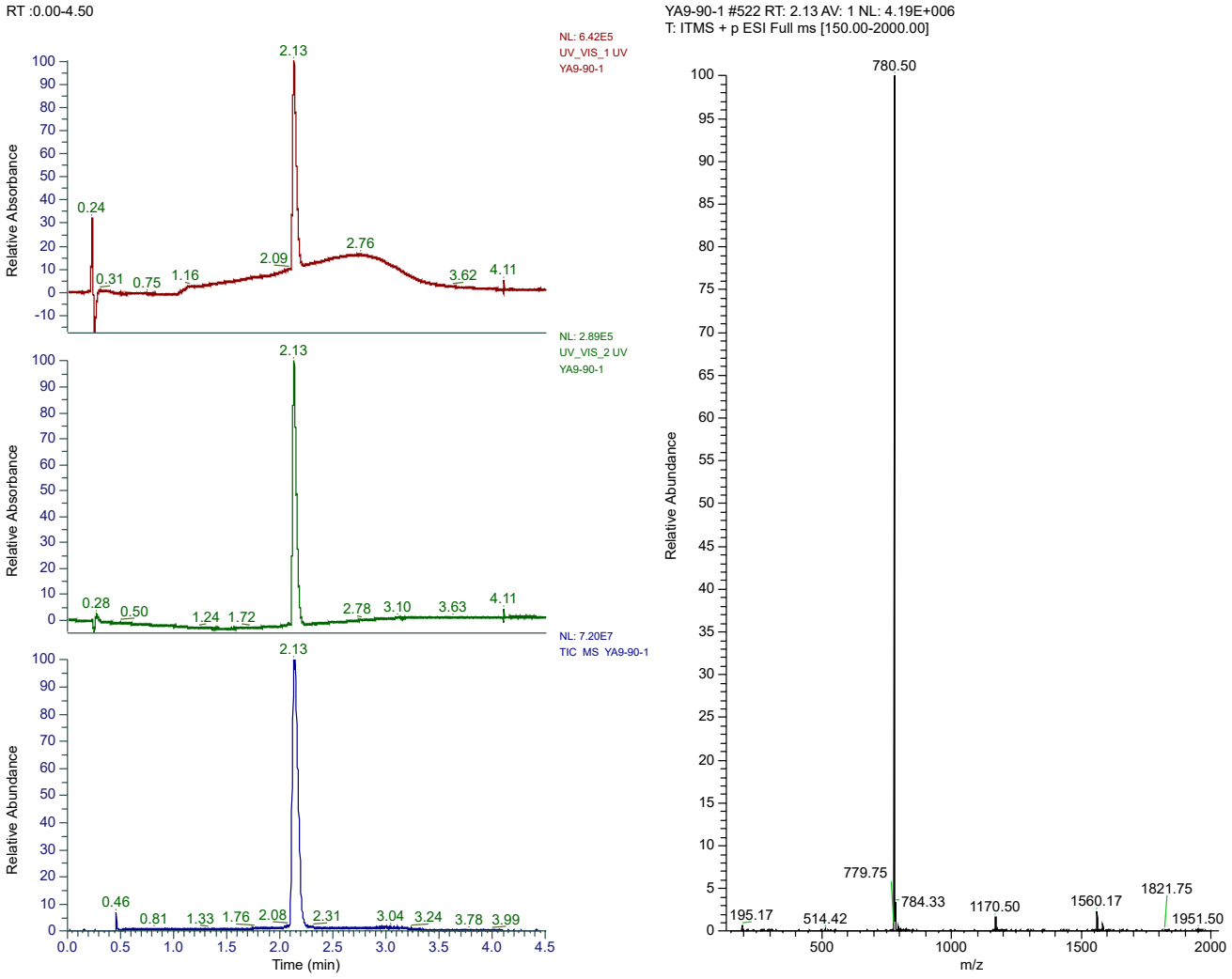

Cl
NH
O
MeO
O
O
AcO
OAc
OAc

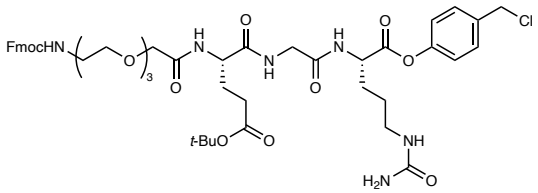
S16


RT :0.00-4.50
NL: 1.13E6
UV_VIS_1 UV
YA9-85-
3and4_2021040715
5137
NL: 1.57E6
UV_VIS_2 UV
YA9-85-
3and4_2021040715
5137
NL: 8.90E7
TIC MS
YA9-85-
3and4_2021040715
5137
Relative Absorbance
Relative Abundance
Time (min)
YA9-85-3and4_20210407155137 #617 RT: 2.50 AV: 1 NL: 6.80E+006
T: ITMS + p ESI Full ms [150.00-2000.00]
Relative Abundance
m/z
550.42
553.25
1099.08
1100.92
1416.92
1787.42
514.33
582.25
1062.50
1143.17
1647.08
1828.92
234.17

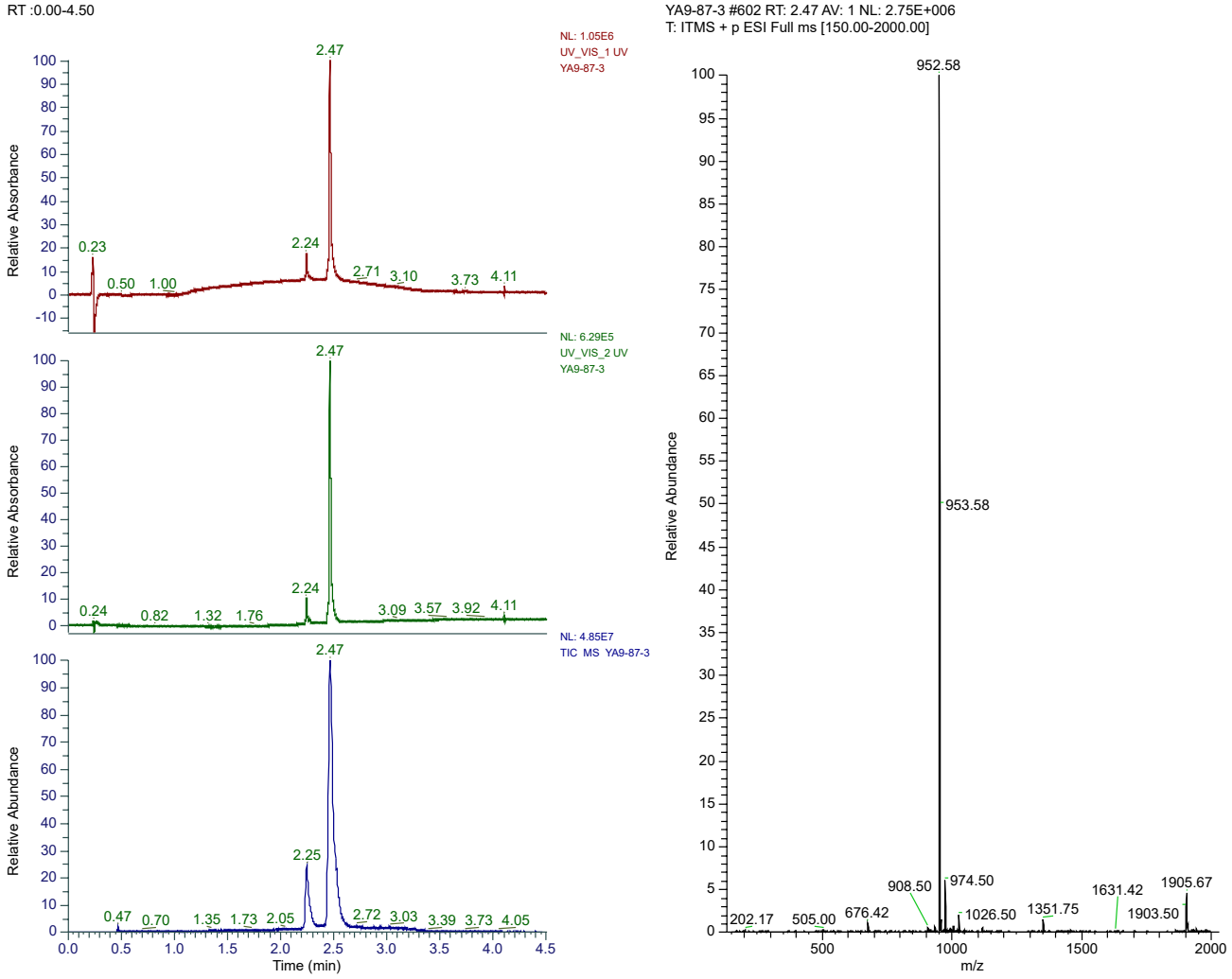

N
O
Cl
NH
N
O
O
O
MeO
O
AcO
OAc
OAc

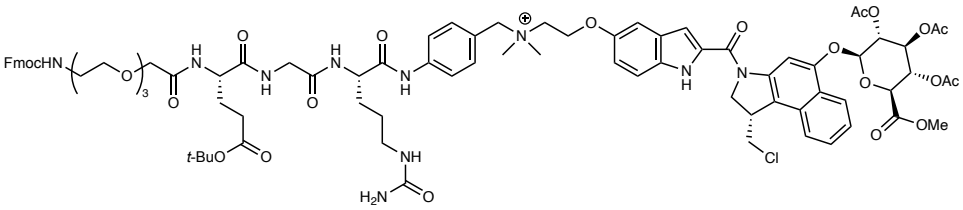
S17


RT :0.00-4.50
NL: 6.42E5
UV_VIS_1 UV
YA9-90-1
NL: 2.89E5
UV_VIS_2 UV
YA9-90-1
NL: 7.20E7
TIC MS YA9-90-1
Relative Absorbance
Relative Abundance
Time (min)
YA9-90-1 #522 RT: 2.13 AV: 1 NL: 4.19E+006
T: ITMS + p ESI Full ms [150.00-2000.00]
780.50
779.75
784.33
1170.50
1560.17
1821.75
1951.50
195.17
514.42
m/z

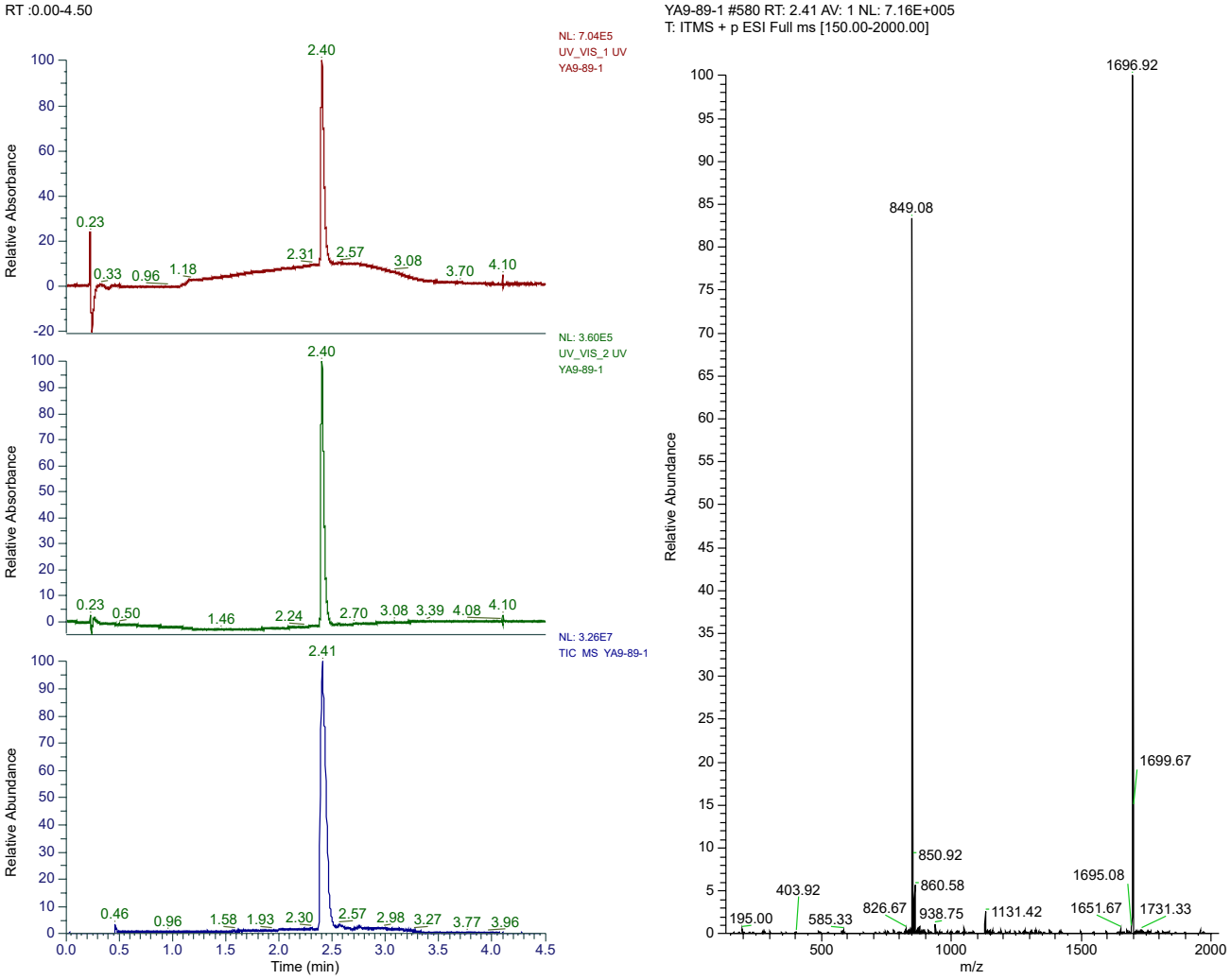

FmocHN
O
3
H
N
O
O
N
H
H
N
O
O
O
Cl
t-BuO
O
NH
H2N
O

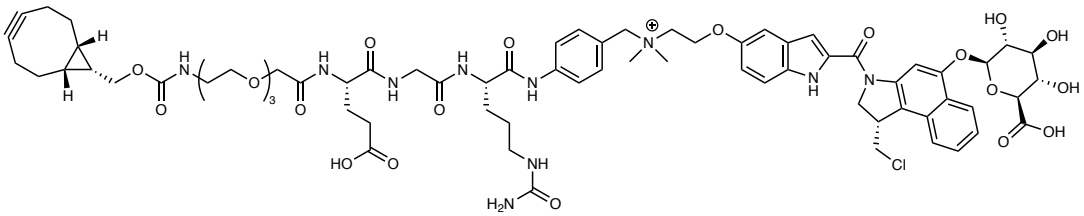
S18


RT :0.00-4.50
NL: 1.05E6
UV_VIS_1 UV
YA9-87-3
Relative Absorbance
2.47
0.23
0.50
1.00
2.24
2.71
3.10
3.73
4.11
NL: 6.29E5
UV_VIS_2 UV
YA9-87-3
Relative Absorbance
2.47
0.24
0.82
1.32
1.76
2.24
3.09
3.57
3.92
4.11
NL: 4.85E7
TIC MS YA9-87-3
Relative Abundance
2.47
2.25
0.47
0.70
1.35
1.73
2.05
2.72
3.03
3.39
3.73
4.05
Time (min)
YA9-87-3 #602 RT: 2.47 AV: 1 NL: 2.75E+006
T: ITMS + p ESI Full ms [150.00-2000.00]
Relative Abundance
952.58
953.58
908.50
974.50
1026.50
1351.75
1631.42
1905.67
1903.50
676.42
505.00
202.17
m/z

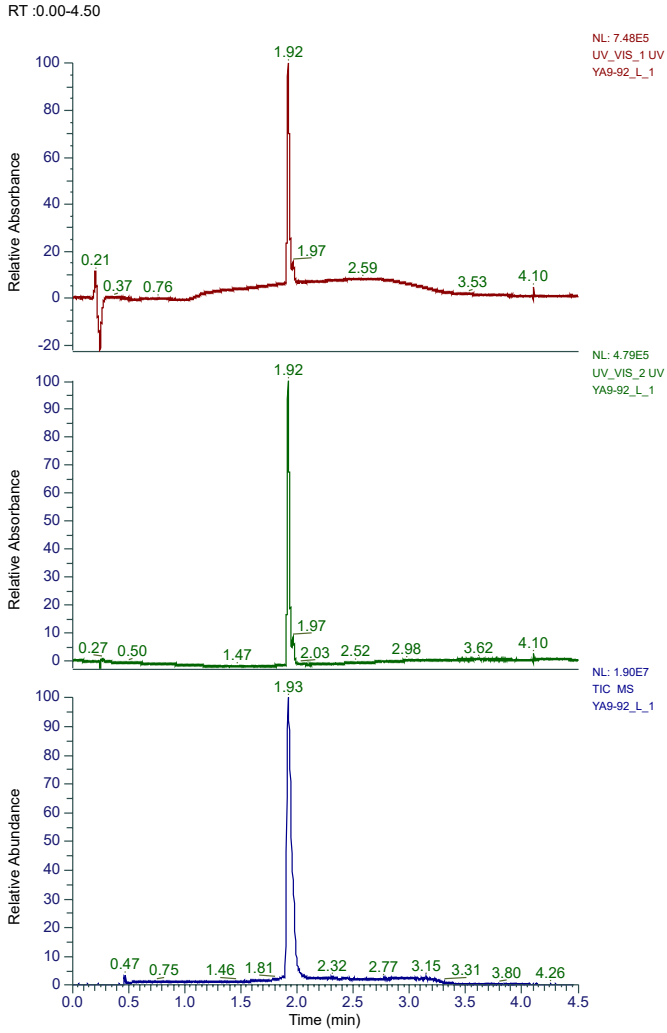

FmocHN
t-BuO
AcO
OAc
OAc
OMe
Cl
NH
H2N

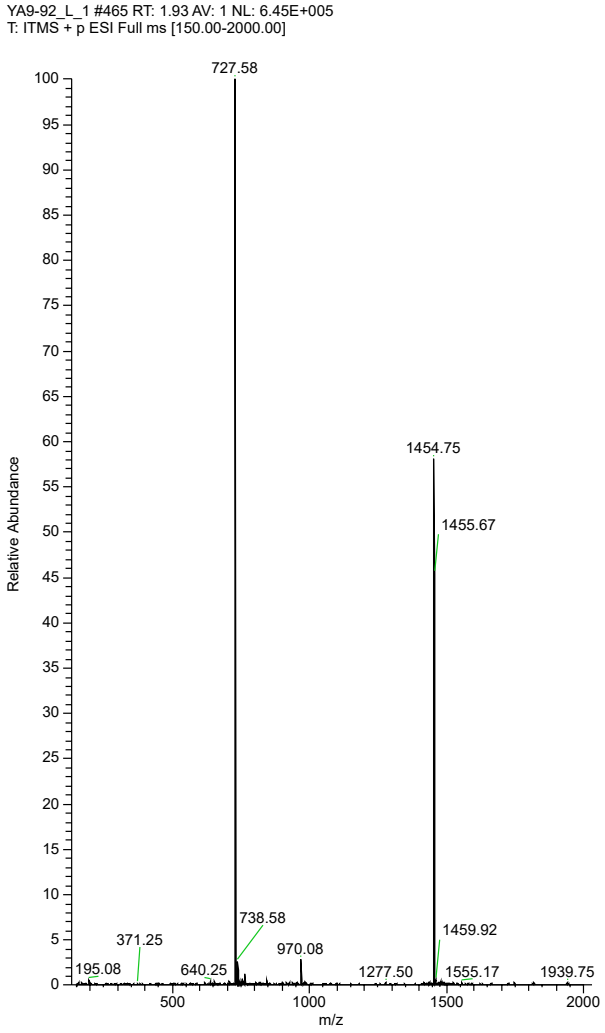
S19

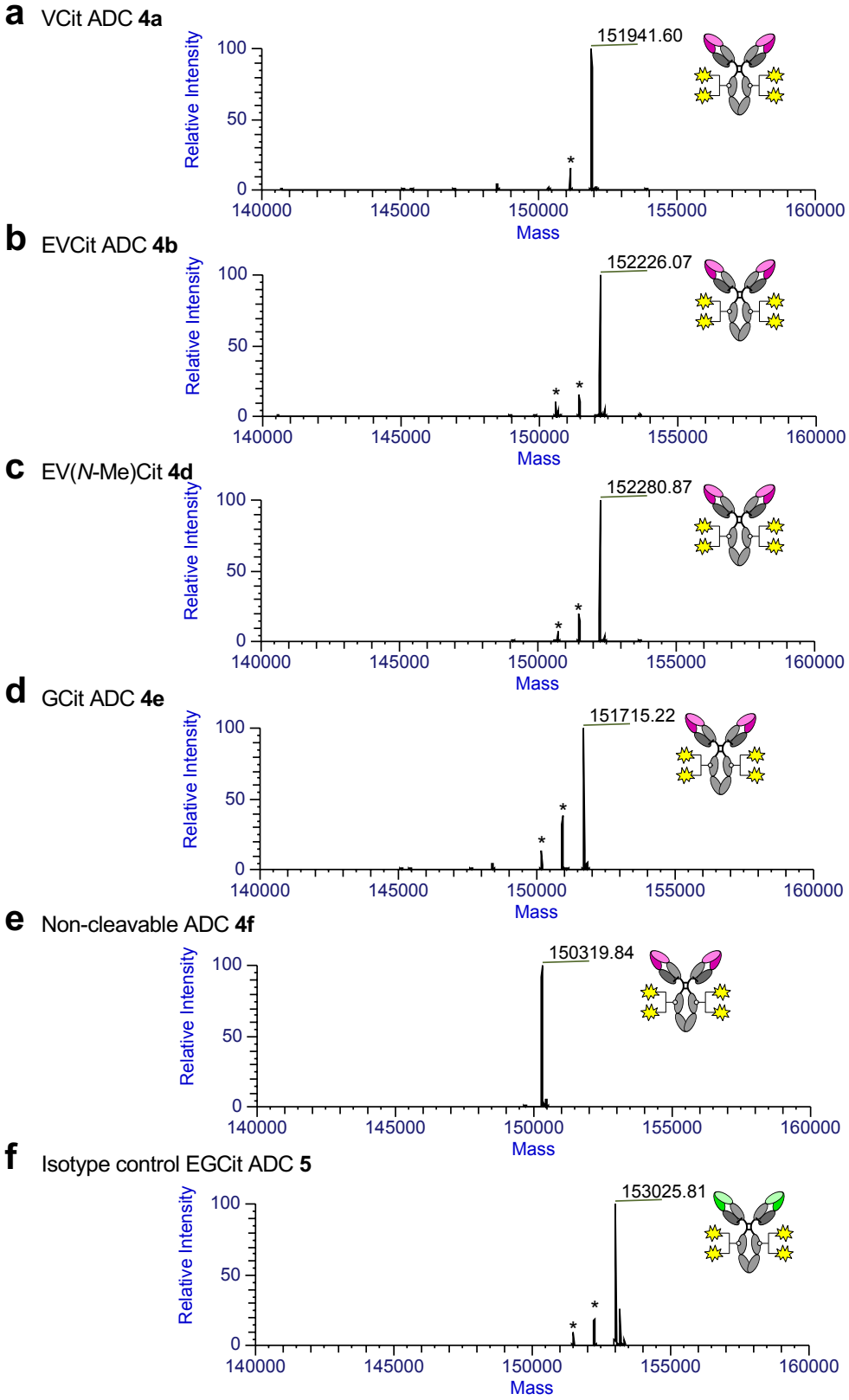

RT :0.00-4.50
NL: 7.04E5
UV_VIS_1 UV
YA9-89-1
NL: 3.60E5
UV_VIS_2 UV
YA9-89-1
NL: 3.26E7
TIC MS YA9-89-1
Relative Absorbance
Relative Abundance
Time (min)
YA9-89-1 #580 RT: 2.41 AV: 1 NL: 7.16E+005
T: ITMS + p ESI Full ms [150.00-2000.00]
Relative Abundance
m/z
1696.92
849.08
1699.67
850.92
860.58
1695.08
403.92
826.67
938.75
1131.42
1651.67
1731.33
195.00
585.33



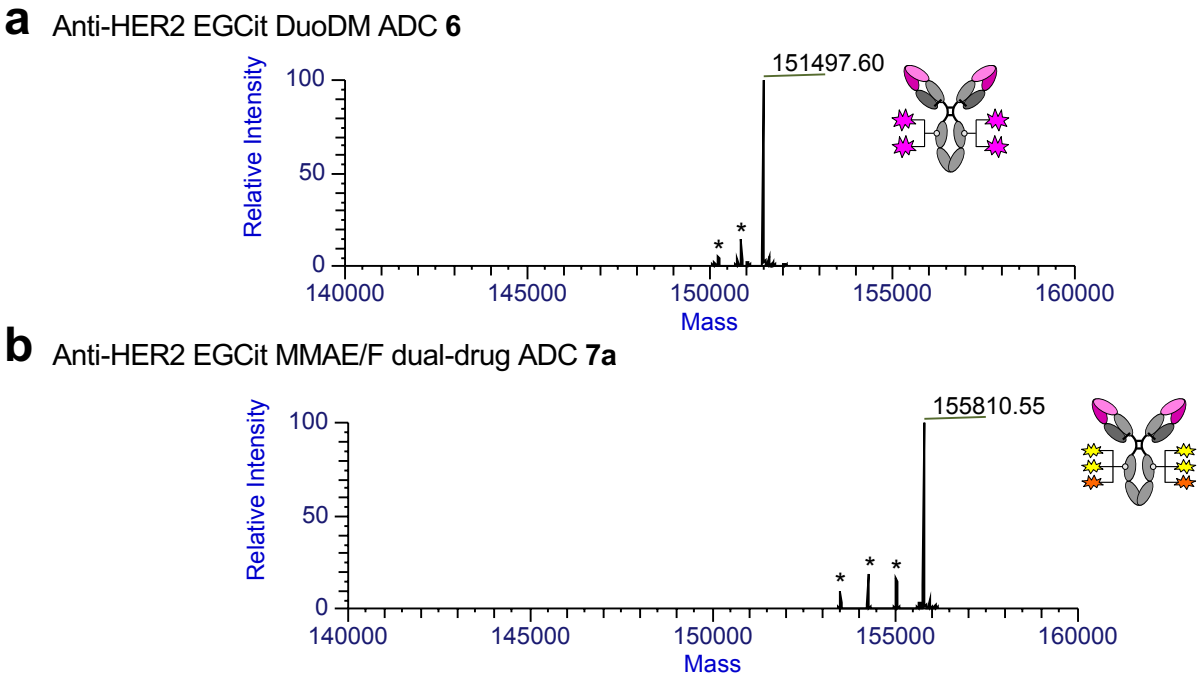
S20


RT :0.00-4.50
NL: 7.48E5
UV_VIS_1 UV
YA9-92_L_1
1.92
0.21
0.37
0.76
1.97
2.59
3.53
4.10
Relative Absorbance
NL: 4.79E5
UV_VIS_2 UV
YA9-92_L_1
1.92
0.27
0.50
1.47
1.97
2.03
2.52
2.98
3.62
4.10
Relative Absorbance
NL: 1.90E7
TIC MS
YA9-92_L_1
1.93
0.47
0.75
1.46
1.81
2.32
2.77
3.15
3.31
3.80
4.26
Relative Abundance
Time (min)

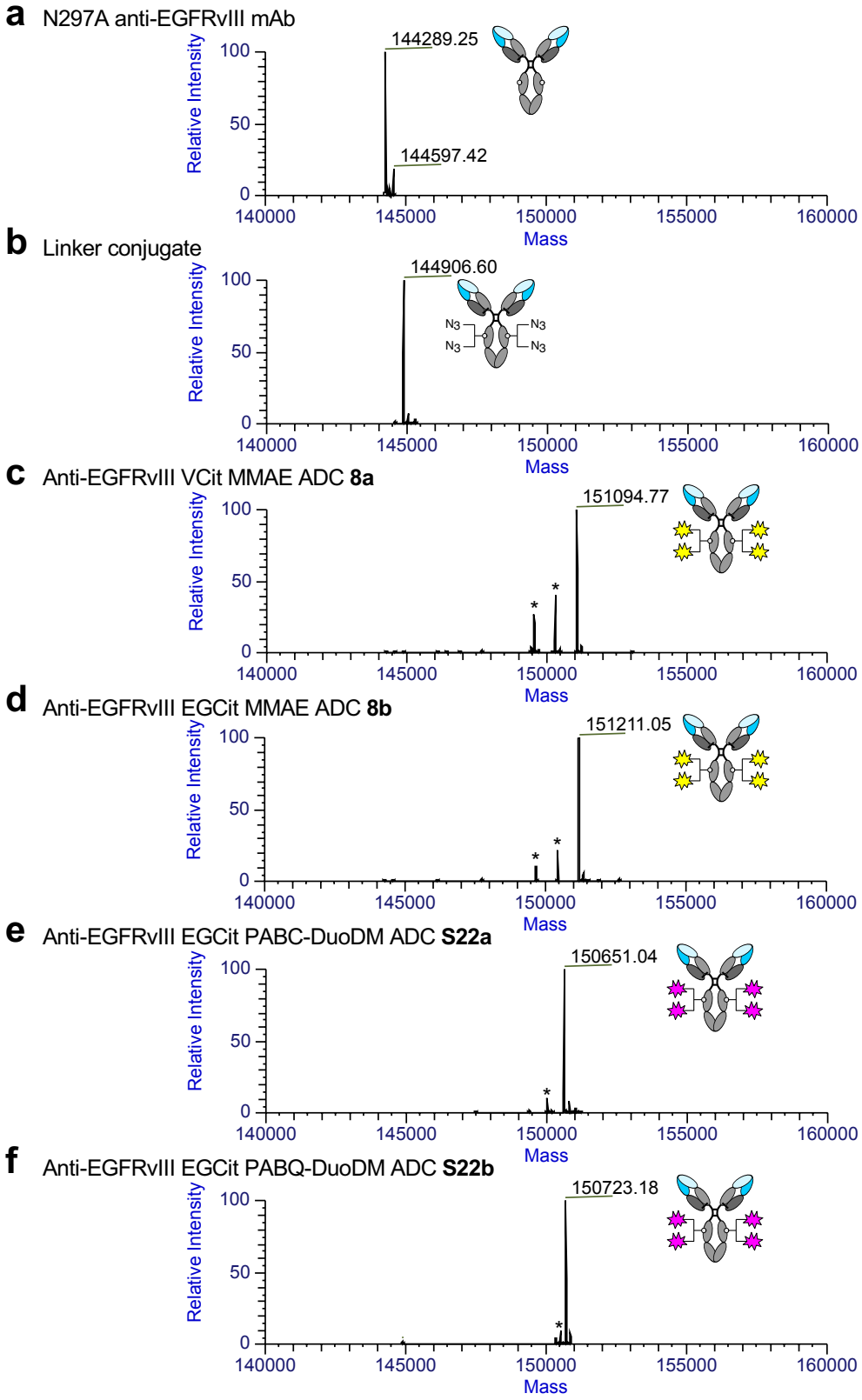

YA9-92_L_1 #465 RT: 1.93 AV: 1 NL: 6.45E+005
T: ITMS + p ESI Full ms [150.00-2000.00]
727.58
1454.75
1455.67
738.58
1459.92
371.25
970.08
195.08
640.25
1277.50
1555.17
1939.75
Relative Abundance
m/z



Deconvoluted ESI-mass spectra of VCit ADC **4a** (**a**), EVCit ADC **4b** (**b**), EV(*N*-Me)Cit **4d** (**c**), GCit ADC **4e** (**d**), non-cleavable ADC **4f** (**e**), Isotype control EGCit ADC **5** (**f**). Asterisk (*) indicates fragment ion(s) detected in ESI-MS analysis.



Deconvoluted ESI-mass spectra of anti-HER2 EGCit DuoDM ADC **6** (**a**) and anti-HER2 EGCit MMAE/F dual-drug ADC **7a** (**b**). Asterisk (*) indicates fragment ion(s) detected in ESI-MS analysis.



Deconvoluted ESI-mass spectra of aglycosylated N297A anti-EGFRvIII mAb (**a**), mAb–linker conjugate (**b**), anti-EGFRvIII VCit MMAE ADC **8a** (**c**), anti-EGFRvIII EGCit MMAE ADC **8b** (**d**), anti-EGFRvIII EGCit PABC-DuoDM ADC **S22a** (**e**), and anti-EGFRvIII EGCit PABQ-DuoDM ADC **S22b** (**f**). Asterisk (*) indicates fragment ion(s) detected in ESI-MS analysis.

Supplementary References

1. Yamazaki CM, Yamaguchi A, Anami Y, Xiong W, Otani Y, Lee J, et al. Antibody-drug conjugates with dual payloads for combating breast tumor heterogeneity and drug resistance. Nat Commun. 2021;12:3528.

2. Chatterjee J, Laufer B, Kessler H. Synthesis of N-methylated cyclic peptides. Nat Protoc. Springer Science and Business Media LLC; 2012;7:432–44.

3. Anami Y, Yamazaki CM, Xiong W, Gui X, Zhang N, An Z, et al. Glutamic acid-valine-citrulline linkers ensure stability and efficacy of antibody-drug conjugates in mice. Nat Commun. Nature Publishing Group; 2018;9:2512.

4. Twum EA, Nathubhai A, Wood PJ, Lloyd MD, Thompson AS, Threadgill MD. Initial development of a cytotoxic amino-seco-CBI warhead for delivery by prodrug systems. Bioorg Med Chem. Elsevier Ltd; 2015;23:3481–9.