Salt Effect Accelerates Site-Selective Cysteine Bioconjugation

Peng Dai[1], Chi Zhang[1], Matthew Welborn[1], James J. Shepherd[1], Tianyu Zhu[1],
Troy Van Voorhis[1] and Bradley L. Pentelute[1]

[1]Department of Chemistry, Massachusetts Institute of Technology,
Cambridge, Massachusetts 02139, United States

Supplementary Information

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Supplementary Experimental Procedures

General Methods. PNGase F was purchased from New England BioLabs Inc. (Ipswich, MA). Ammonium Sulfate (Ultra pure grade) was from Amresco LLC (Solon, Ohio). Recombinant HER2 was from R&D Systems Inc (Minneapolis, MN). Decafluorobiphenyl was purchased from Oakwood Chemicals (West Columbia, SC). *Tris*(2-carboxyethyl)phosphine hydrochloride (TCEP·HCl) was purchased from Hampton Research (Aliso Viejo, CA). 1-[Bis(dimethylamino)methylene]-1H-1,2,3-triazolo[4,5-b]pyridinium 3-oxid hexafluorophosphate) (HATU), Fmoc protected amino acids were purchased from Chem-Impex International (Wood Dale, IL), H-Rink Amide Chemmatrix resin was obtained from PCSS BioMatrix Inc. (Quebec, Canada). *N,N*-Dimethylformamide (DMF), dichloromethane (DCM), diethyl ether, HPLC-grade acetonitrile, and guanidine hydrochloride were obtained from VWR International (Philadelphia, PA). All other reagents were purchased from Sigma-Aldrich and used as received unless otherwise noted.

LC-MS analysis. LC-MS chromatograms and associated mass spectra were acquired using Agilent 6520 ESI-Q-TOF mass spectrometer. Zorbax 300SB C_3 column: 2.1 x 150 mm, 5 μm was used with column temperature set at 40 °C. MS conditions: positive electrospray ionization (ESI) extended dynamic mode in mass range 300 – 3000 *m/z*, temperature of drying gas = 350 ^{o}C, flow rate of drying gas = 11 L/min, pressure of nebulizer gas = 60 psi, the capillary, fragmentor, and octupole rf voltages were set at 4000, 175, and 750, respectively.

Solid phase peptide synthesis and peptide purification. Peptides were synthesized at 0.05 mmol scale on H-Rink Amide Chemmatrix resin. The resin was firstly swelled in dimethylformamide (DMF). General procedure for amino-acid residue coupling: 0.4 mmol Fmoc protected amino acids was dissolved in 1 mL 0.38 M HATU solution in DMF, 200 μL of diisopropylethylamine (DIEA) was added (for coupling of cysteine, 80 μL of DIEA was used to prevent racemization). The mixture was added to resin and react at room temperature for 20 minutes, followed by 3 times wash with DMF, 2 times 8 min deprotection with 20% (v/v) piperidine in DMF and 5 times wash with DMF. The resin was washed thoroughly with DCM and air dried after completion of the stepwise SPPS. The peptide is then simultaneously cleaved from the resin and side-chain deprotected by treatment with 2.5% (v/v) water, 2.5% (v/v) 1,2-ethanedithiol (EDT), and 1% (v/v) triisoproprylsilane in neat trifluoroacetic acid (TFA) for 2 hours at room temperature. Resulting solution containing peptide was evaporated by blowing a stream of nitrogen gas over its surface for 15 minutes, then triturated and washed with cold diethyl ether three times. Obtained gummy-like solid was dissolved in 50% H_2O: 50% acetonitrile containing 0.1% TFA and lyophilized. The crude peptide was dissolved in 95% water with 0.1% TFA (solvent A): 5% acetonitrile with 0.1% TFA (solvent B) with 6 M guanidinium hydrochloride and purified by semi-preparative RP-HPLC (Agilent Zorbax 300SB C_{18} column: 21.2 x 250 mm, 7 μm, linear gradient: 5-50% B over 90 min, flow rate: 5 mL/min). Fractions containing product material were confirmed by LC-MS analysis, combined, and then lyophilized. Peptide sequences and masses are summarized in Table S1.

Expression of proteins 3a and 3b. pET-His_6-SUMO-pGB1 plasmid was constructed as reported previously[1]. The π-clamp was introduced by site-directed mutagenesis using QuickChange Lightning Single Site-directed Mutagenesis Kit (Agilent) following the manufacturer's instructions.
Protein **3a** sequence:

FCPFGGGGGMTYKLILNGKTLKGETTTEAVDAATAEKVFKQYANDNGVDGEWTYDDATKTF
TVTE

Protein **3b** sequence:

GGGGGMTYKLILNGKTLKGETTTEAVDAATAEKVFKQYANDNGVDGEWTYDDATKTFTVTE
GGGG**FCPF**

E. coli BL21(DE3) cells transformed with pET-His_6-SUMO-FCPF-G_5-pGB1 plasmid (for **3a**) or pET-His_6-SUMO-G_5-pGB1-G_4-FCPF (for **3b**) were grown in 1 L of LB medium containing kanamycin (30 μg/mL) at 37 ℃ until OD_{600} = 0.6. Then, expression was induced by addition of 0.5 mM IPTG overnight at 30 ℃. After harvesting the cells by centrifugation (6,000 rpm for 10 min), the cell pellet was lysed by sonication in 25 mL of 50 mM Tris and 150 mM NaCl (pH 7.5) buffer containing 15 mg lysozyme (Calbiochem), 1 mg DNase I (Sigma-Aldrich), and 0.5 tablet of protease inhibitor cocktail (Roche Diagnostics, Germany). The suspension was centrifuged at 17,000 rpm for 30 min to remove cell debris. The supernatant was loaded onto a 5 mL HisTrap FF crude Ni-NTA column (GE Healthcare, UK), which has been pre-washed with water and 40 mL of 20 mM Tris and 150 mM NaCl (pH 8.5). The column was then washed with 40 mL of 20 mM imidazole in 20 mM Tris and 150 mM NaCl (pH 8.5). The protein was eluted from the column with buffer containing 500 mM imidazole in 20 mM Tris and 150 mM NaCl (pH 8.5). Imidazole was removed from protein using a HiPrep 26/10 Desalting column (GE Healthcare, UK), the protein was eluted into 20 mM Tris and 150 mM NaCl (pH 7.5) buffer. SUMO group was cleaved by incubating 1 μg of SUMO protease per mg of protein at 4 °C overnight. The crude reaction mixture was loaded onto a 5 mL HisTrap FF crude Ni-NTA column (GE Healthcare, UK) and the flow through containing protein **3a** and **3b** were collected. The proteins were analyzed by LC-MS confirming sample purity and molecular weight.

Preparation of protein 4a. Protein **4a** was prepared via sortagging reaction between 1C-LF_N-LPSTGGHis$_5$ (**4a-pro**) and G5-TEVsite-π-clamp peptide (**4a-pep**). pET-SUMO-LF_N-LPSTGG-His_5 plasmid was constructed as previously described[2]. N-terminal cysteine was inserted by site-directed mutagenesis using QuickChange Lightning Single Site-directed Mutagenesis Kit (Agilent) following the manufacturer's instructions. The expression of **4a-pro** and sortagging reaction between **4a-pro** and **4a-pep** followed our previous protocols[3].

Protein **4a** sequence:

CGGHGDVGMHVKEKEKNKDENKRKDEERNKTQEEHLKEIMKHIVKIEVKGEEAVKKEAAEK
LLEKVPSDVLEMYKAIGGKIYIVDGDITKHISLEALSEDKKKIKDIYGKDALLHEHYVYAKEGY
EPVLVIQSSEDYVENTEKALNVYYEIGKILSRDILSKINQPYQKFLDVLNTIKNASDSDGQDLLFT
NQLKEHPTDFSVEFLEQNSNEVQEVFAKAFAYYIEPQHRDVLQLYAPEAFNYMDKFNEQEINLS
LEELKDQRLPSTGGGGGNKRENLYFQG**FCPF**

Circular Dichroism

The circular dichroism spectra in 196 nm-260 nm region were collected on an Aviv Model 202 Circular Dichroism Spectrometer. Protein **3a** and **3b** were prepared in 10 mM phosphate, 1 mM TCEP (with or without 2 M ammonium sulfate) at 0.08 mg/mL concentration. CD spectra for 10 mM phosphate, 1 mM TCEP, pH 8.0 and 10 mM phosphate, 1mM TCEP, 2 M ammonium sulfate, pH 8.0 were collected and subtracted as blank.

Secondary structure for protein **3a** and **3b** with or without ammonium sulfate were estimated from CD spectra using K2D3 method[4].

Nuclear magnetic resonance

^{1}H NMR spectra were recorded on a Bruker Avance 400 MHz spectrometer and are reported in ppm using solvent as an internal standard (DMSO-d6 at 2.50 ppm). ^{19}F NMR spectra were recorded on a Bruker Avance 400 MHz (376 MHz) spectrometer and are reported in ppm with trifluoroacetic acid as an internal standard (at -76.6 ppm). ^{1}H NMR and ^{19}F NMR data are reported as (s = singlet, d = doublet, dd = doublet of doublets, t = triplet, q = quartet, m = multiplet, br = broad; coupling constant(s) in Hz; integration). Proton-decoupled ^{13}C NMR spectra were recorded on a Bruker Avance 400 MHz (101 MHz) spectrometer and are reported in ppm using solvent as an internal standard (DMSO-d6 at 39.52 ppm).

Supplementary Tables

Table S1. Summary of the synthesized peptides and probes

Peptide	Sequence	Note	Calc. mass	Obs. mass
1A	NH_2-FCPFGLLKNK-$CONH_2$		1164.65	1164.65
1B	NH_2-XCPXGLLKNK-$CONH_2$	X=3-(2-naphthyl)-L-Ala	1264.68	1264.68
1C	NH_2-XCPXGLLKNK-$CONH_2$	X=p-phenyl-L-Phe	1316.71	1316.71
1D	NH_2-XCPXGLLKNK-$CONH_2$	X=3-(9-anthryl)-L-Ala	1364.71	1364.71
1E	NH_2-XCPXGLLKNK-$CONH_2$	X=3-pyrenyl-L-Ala	1412.71	1412.71
1F	NH_2-XCPXGLLKNK-$CONH_2$	X=4-CF_3-L-Phe	1300.62	1300.62
1G	NH_2-XCPXGLLKNK-$CONH_2$	X=4-Nitro-L-Phe	1254.62	1254.62
1H	NH_2-XCPXGLLKNK-$CONH_2$	X=4-F-L-Phe	1200.63	1200.63
1I	NH_2-XCPXGLLKNK-$CONH_2$	X=4-CN-L-Phe	1214.64	1214.64
1J	NH_2-XCPXGLLKNK-$CONH_2$	X=cyclohexylalanine	1176.74	1176.74
1K	NH_2-XCPXGLLKNK-$CONH_2$	X=cyclopentylalanine	1148.71	1148.71
1L	NH_2-XCPXGLLKNK-$CONH_2$	X=cyclobutylalanine	1120.68	1120.68
1M	NH_2-LCPLGLLKNK-$CONH_2$		1096.68	1096.68
1N	NH_2-GCPGGLLKNK-$CONH_2$		984.55	984.55
2	NH_2-VTLPSTC*GAS-$CONH_2$	C*=Cys($SC_{12}F_9$)	1247.44	1247.44
4a-pep	NH_2-G5NKRENLYFQGFCPF-$CONH_2$		2045.95	2045.95
6A	Biotin-PEG_5-VTLPSTC*GAS-$CONH_2$	C*=Cys($SC_{12}F_9$)	1765.69	1765.72
7	Ac-VTLPSTC*GAS-$CONH_2$	C*=Cys($SC_{14}H_{12}Cl$)	1090.46	1090.46
7-Cys	Ac-VTLPSTCGAS-$CONH_2$		876.40	876.40
9	FFCFFGLLKNK		1361.73	1361.73
10	FKPFGLLKNK		1189.73	1189.73

Table S2. Summary of the rate constants ($M^{-1}s^{-1}$) for reaction between **1A** and **2** with different salt.

	Ammonium Citrate	Ammonium Sulfate	Sodium Chloride	Guanidinium Chloride	Guanidinium Thiocyanate
1 Molar	7.2 ± 0.7	2.9 ± 0.1	1.03 ± 0.01	0.23 ± 0.01	0.035 ± 0.002
2 Molar	74 ± 3.3	43.3 ± 1.6	1.42 ± 0.05	0.074 ± 0.005	0.0043 ± 0.0003
3 Molar	Not measured*	62 ± 3.3	3.95 ± 0.2	0.01 ± 0.001	0.002 ± 0.0002

*The reaction with 3 molar ammonium citrate was not tested due to the solubility limitation of ammonium citrate and the requirement to add phosphate buffer, reducing agent and peptides.

Table S3. Summary of rate constants for reactions between π-clamp variants and probe **2**

Peptide	Rate constant ($M^{-1}s^{-1}$)	Error of Rate constant ($M^{-1}s^{-1}$)
1A	0.63	0.02
1B	2.3	0.2
1C	12.6	0.7
1D	22	0.7
1E	26.8	1.2
1F	4.30	0.07
1G	1.75	0.03
1H	1.05	0.02
1I	0.58	0.03
1J	1.67	0.02
1K	0.72	0.030
1L	0.292	0.007
1M	0.133	0.003
1N	0.00065	0.00005

Table S4. Summary of binding energy between π-clamp variants side chain and perfluoroaryl moiety.

Peptide	Binding energy ΔG (kcal/mol)	Rate constant ($M^{-1}s^{-1}$)	Log (k/k_0)	ΔΔG (kcal/mol)
1A	5.19 (ΔG_0)	0.63 (k_0)	0	0
1B	2.51	2.3	0.562	-2.68
1C	2.28	12.6	1.30	-2.91
1D	0.58	22	1.54	-4.61
1E	0.223	26.8	1.63	-4.97
1F	2.64	4.30	0.834	-2.55
1G	3.69	1.75	0.444	-1.51
1H	5.12	1.05	0.222	-0.0778
1I	4.92	0.58	-0.0359	-0.277
1J	5.22	1.7	0.431	0.0269
1K	6.45	0.72	0.0580	1.26
1L	5.33	0.29	-0.337	0.139
1M	5.88	0.133	-0.675	0.684

Note: To serve as baseline, the term k_0 refers to the rate constant of arylation reaction between peptide **1A** and probe **2**. ΔΔG is defined as $\Delta G - \Delta G_0$, in which ΔG_0 refers to the calculated binding energy in the binding model for **1A** without an ion.

Table S5. Free energy of binding between peptide **1A** side chain and perfluoroaryl moiety in the presence of ions.

Ion	Binding energy ΔG (kcal/mol)
$Citrate^{3-}$	-67.97
SO_4^{2-}	-65.32
NH_4^+	-37.22
Na^+	-53.69 (ΔG_0)
Cl^-	-25.58
$Guanidinium^+$	-32.42
SCN^-	-27.8

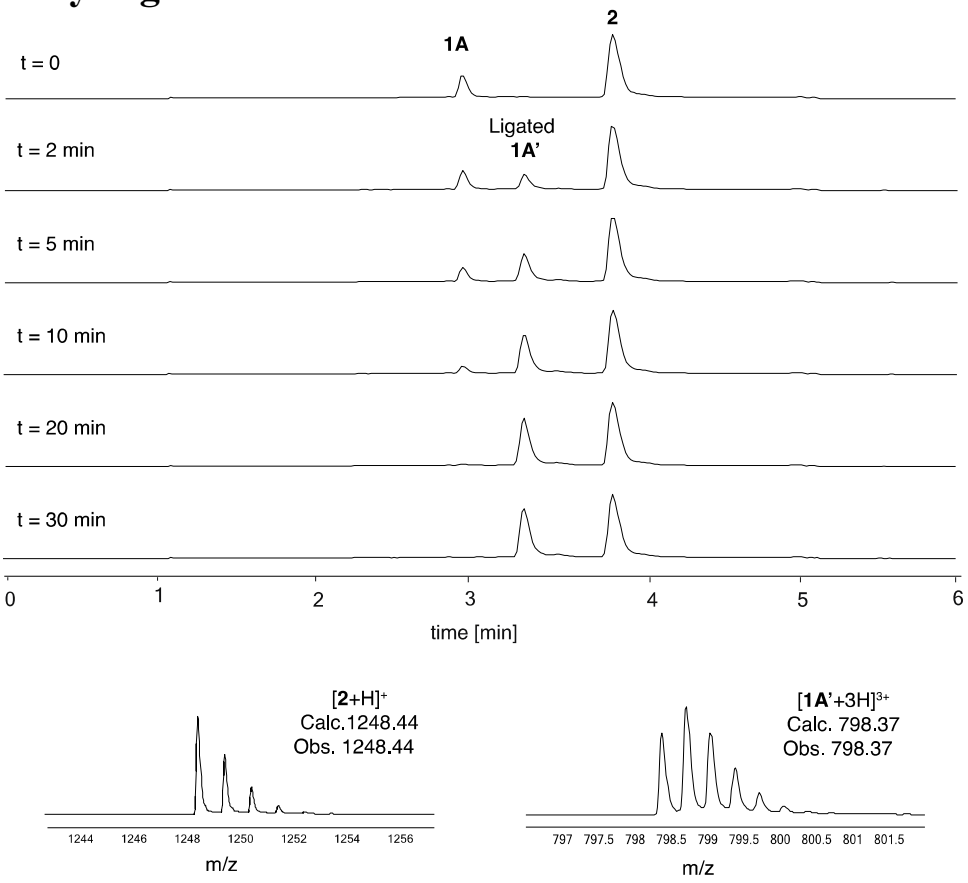
Table S6. Influence of ammonium sulfate on maleimide-cysteine conjugation

Peptide	Electrophile	Yield*	
		No salt	2 M Salt
9	Maleimide	45%	<1%
	N-phenyl Maleimide	32%	<1%
1A	Maleimide	80%[a]	38%[a]
	N-phenyl Maleimide	86%[b]	70%[b]
1N	Maleimide	55%	50%
	N-phenyl Maleimide	70%[b]	55%[b]

Note:
1. Reaction scheme, detailed reaction conditions and the mass spectra for reaction analysis are summarized in Figures S53-55. Yield labeled with [a] and [b] indicated the yield was measured under different reaction conditions. The detailed reaction conditions are reported in the figure captions of Figures S53-55.
2. Due to co-elution of the peptide and conjugated product in LC-MS analysis, total ion current (TIC) spectra of all peptidic species on the chromatogram were integrated, and the yield was calculated as: yield% = $I_{product}/I_{all\ relecant\ species}$, where $I_{product}$ is the peak intensity of conjugated product and $I_{all\ relevant\ species}$ is the sum of the peak intensities of all relevant species (starting material and product) in the mass spectra.

Supplementary Figures

a)



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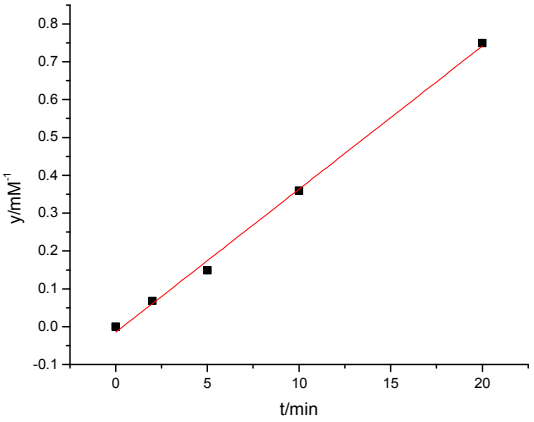
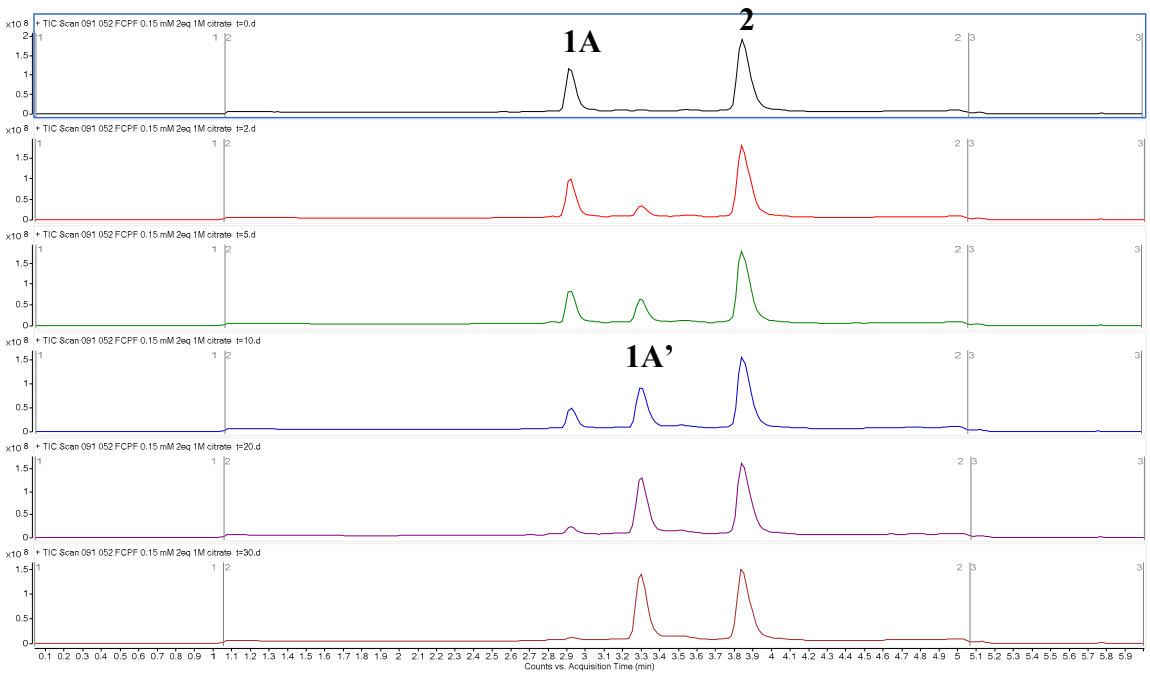


Figure S1. **Kinetics analysis for peptide 1A reacting with probe 2.** a) LC-MS chromatograms for the reaction between peptide **1A** and probe **2** with no additional salts at different time points. Reaction conditions: 1 mM **1A**, 5 mM probe **2**, 200 mM phosphate, 20 mM TCEP, pH 8.0, 37°C. b) Linear fitting of the kinetics data to second-order rate equation. Slope = 0.038, R^2 = 0.996. **1A'** refers to the product of the arylation reaction between **1A** and probe **2**.



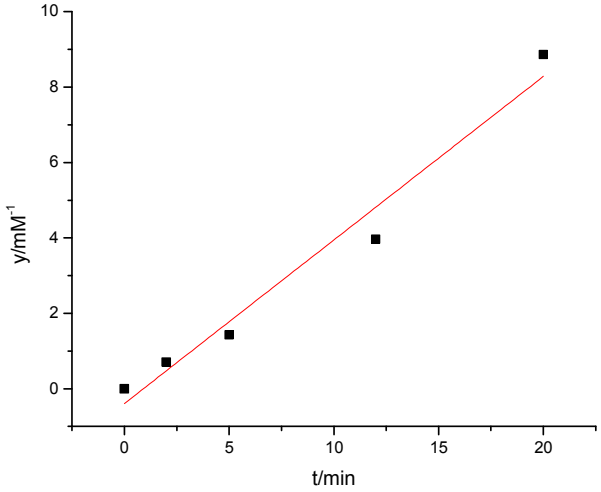
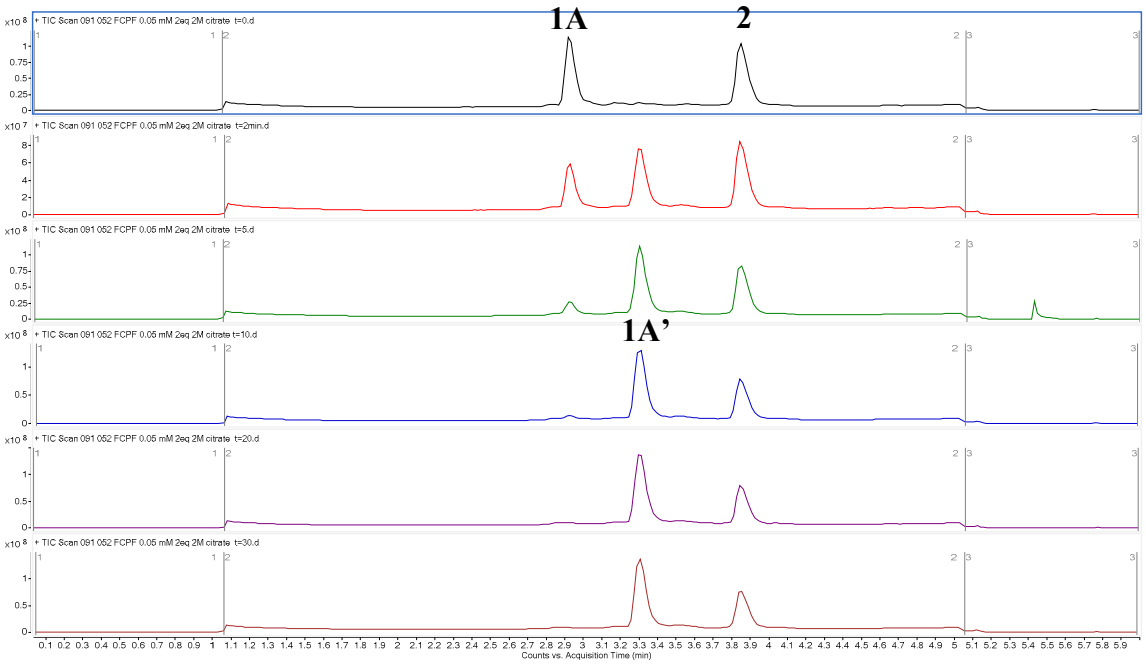


Figure S2. Kinetics analysis for peptide 1A reacting with probe 2 with 1 M ammonium citrate. Top, LC-MS chromatograms for the reaction between peptide **1A** and probe **2** with 1 M ammonium citrate at different time points (minutes). Reaction conditions: 0.15 mM **1A**, 0.3 mM probe **2**, 1 M ammonium citrate, 200 mM phosphate, 20 mM TCEP, pH 8.0, 37°C. Bottom, linear fitting of the kinetics data to second-order rate equation. Slope = 0.43, R^2 = 0.96. **1A'** refers to the product of the arylation reaction between **1A** and probe **2**.



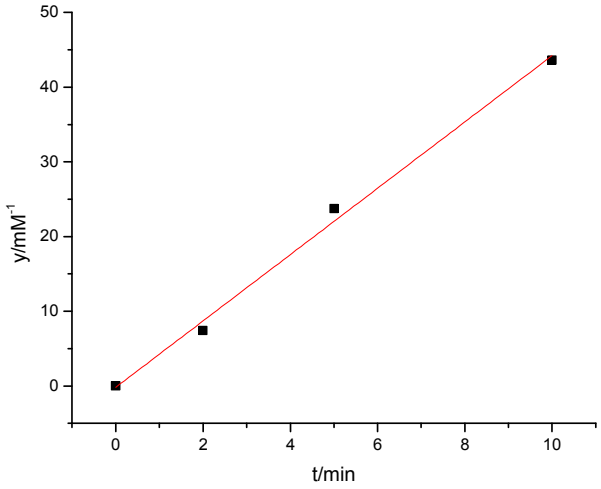
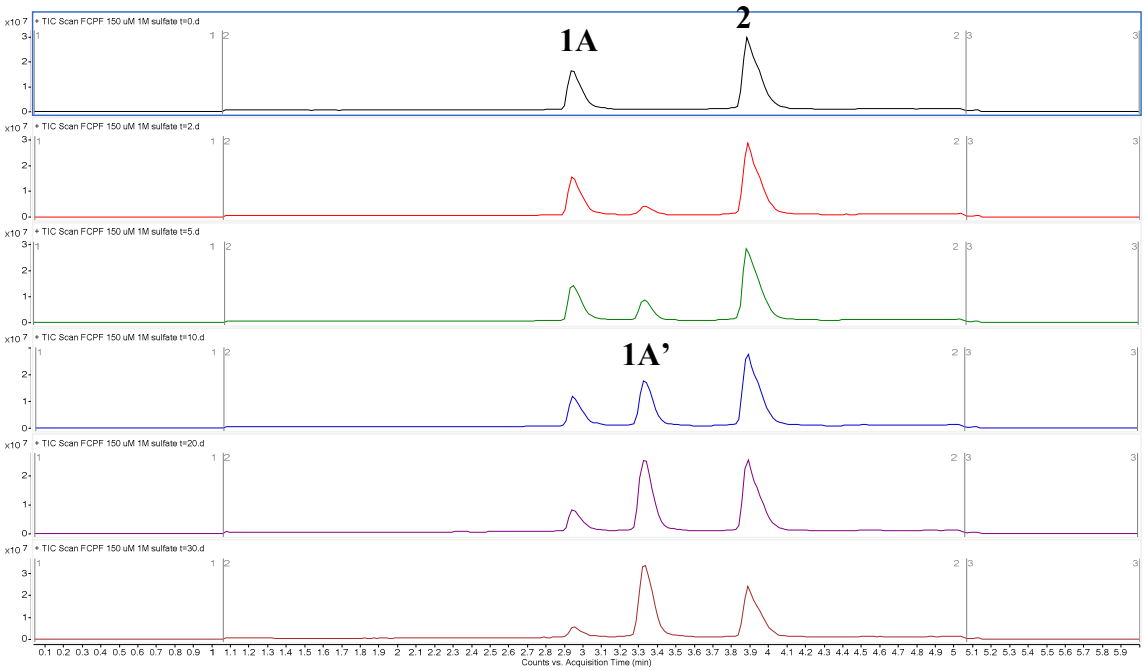


Figure S3. Kinetics analysis for peptide 1A reacting with probe 2 with 2 M ammonium citrate. Top, LC-MS chromatograms for the reaction between peptide **1A** and probe **2** with 2 M ammonium citrate at different time points (minutes). Reaction conditions: 0.05 mM **1A**, 0.1 mM probe **2**, 2 M ammonium citrate, 200 mM phosphate, 20 mM TCEP, pH 8.0, 37°C. Bottom, linear fitting of the kinetics data to second-order rate equation. Slope = 4.44, R^2 = 0.993. **1A'** refers to the product of the arylation reaction between **1A** and probe **2**.



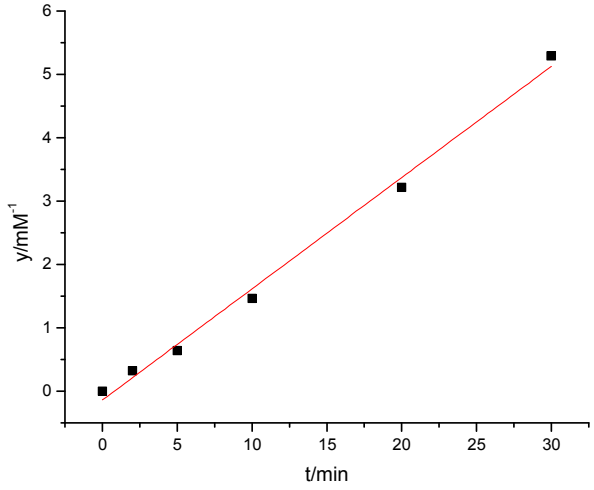


Figure S4. Kinetics analysis for peptide 1A reacting with probe 2 with 1 M ammonium sulfate. Top, LC-MS chromatograms for the reaction between peptide **1A** and probe **2** with 1 M ammonium sulfate at different time points (minutes). Reaction conditions: 0.15 mM **1A**, 0.3 mM probe **2**, 1 M ammonium sulfate, 200 mM phosphate, 20 mM TCEP, pH 8.0, 37°C. Bottom, linear fitting of the kinetics data to second-order rate equation. Slope = 0.15, R^2 = 0.993. **1A'** refers to the product of the arylation reaction between **1A** and probe **2**.

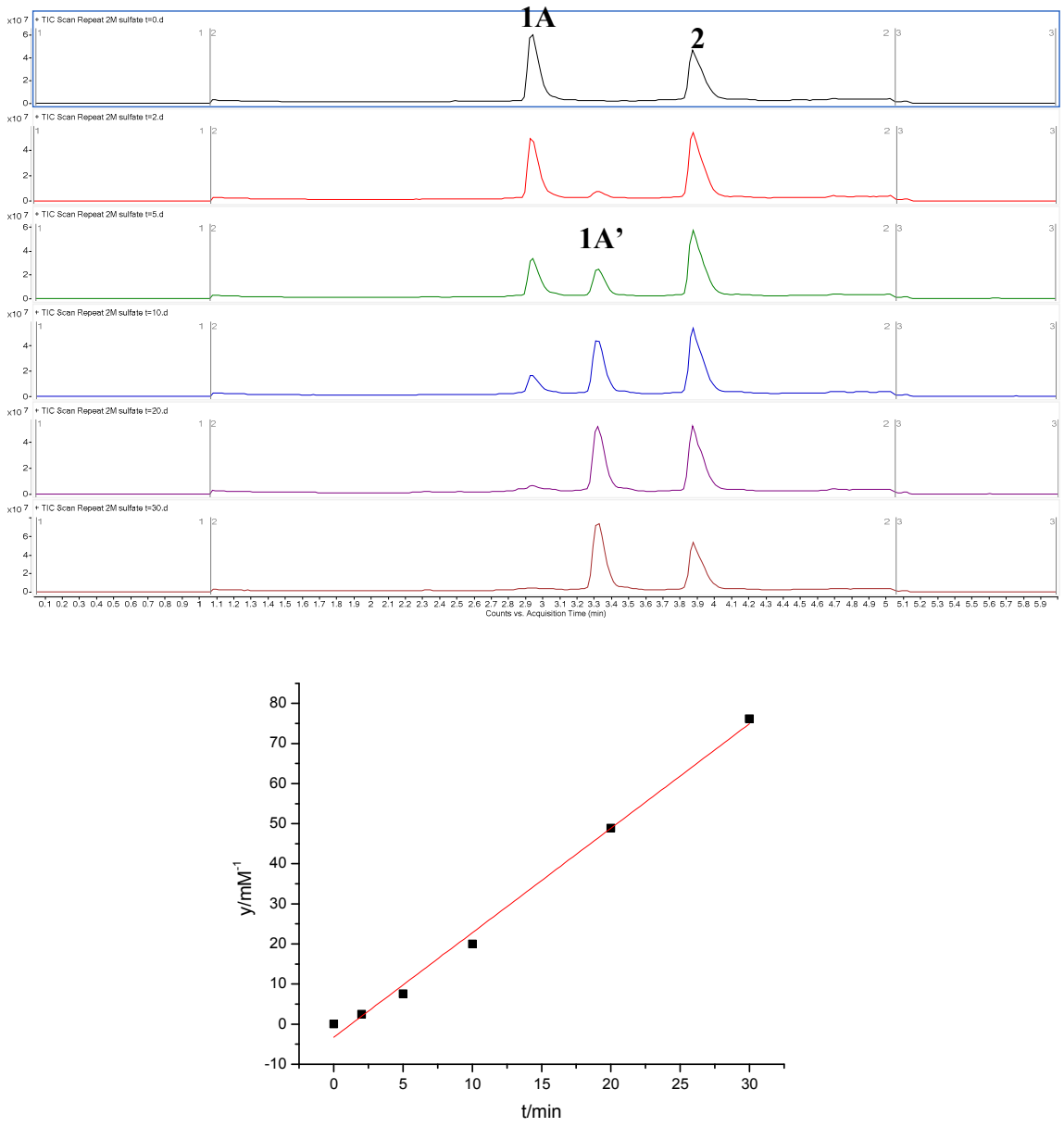
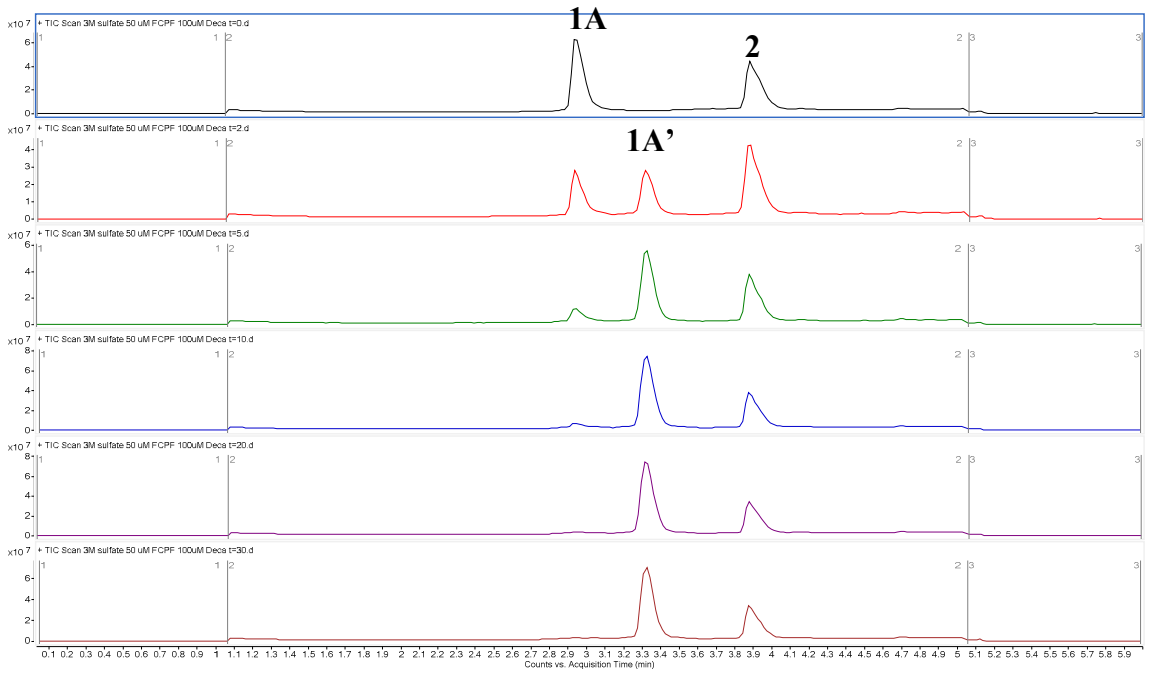


Figure S5. Kinetics analysis for peptide 1A reacting with probe 2 with 2 M ammonium sulfate. Top, LC-MS chromatograms for the reaction between peptide **1A** and probe **2** with 2 M ammonium sulfate at different time points (minutes). Reaction conditions: 0.05 mM **1A**, 0.1 mM probe **2**, 2M ammonium sulfate, 200 mM phosphate, 20 mM TCEP, pH 8.0, 37°C. Bottom, linear fitting of the kinetics data to second-order rate equation. Slope = 2.6, R^2 = 0.993. **1A'** refers to the product of the arylation reaction between **1A** and probe **2**.



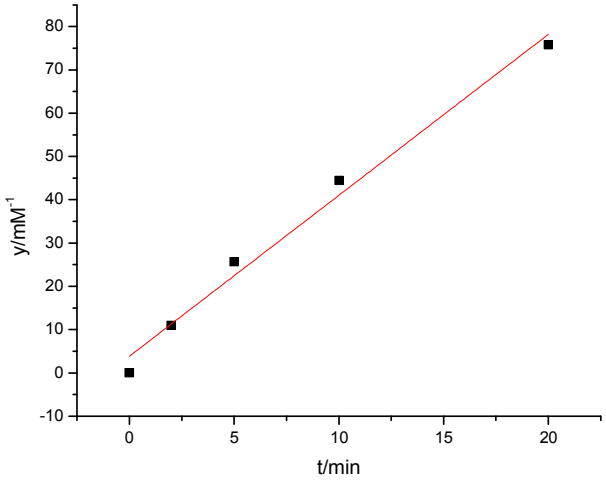
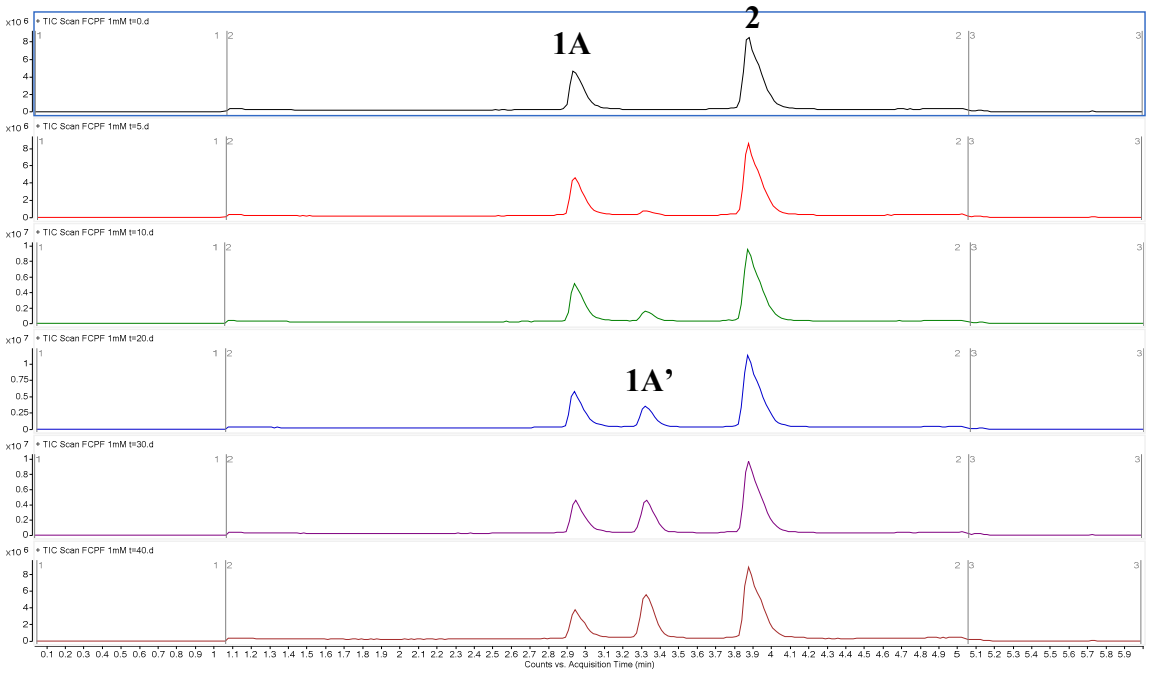


Figure S6. Kinetics analysis for peptide 1A reacting with probe 2 with 3 M ammonium sulfate. Top, LC-MS chromatograms for the reaction between peptide **1A** and probe **2** with 3 M ammonium sulfate at different time points (minutes). Reaction conditions: 0.05 mM **1A**, 0.1 mM probe **2**, 3 M ammonium sulfate, 200 mM phosphate, 20 mM TCEP, pH 8.0, 37°C. Bottom, linear fitting of the kinetics data to second-order rate equation. Slope = 3.72, R^2 = 0.98. **1A'** refers to the product of the arylation reaction between **1A** and probe **2**.



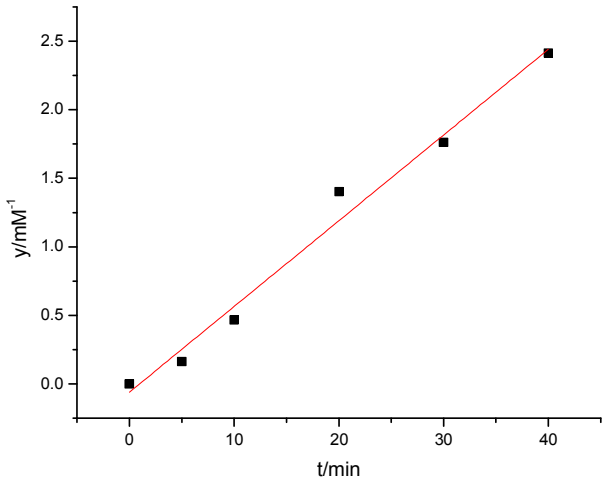


Figure S7. Kinetics analysis for peptide 1A reacting with probe 2 with 1 M sodium chloride. Top, LC-MS chromatograms for the reaction between peptide **1A** and probe **2** with 1 M sodium chloride at different time points (minutes). Reaction conditions: 0.2 mM **1A**, 0.4 mM probe **2**, 1 M sodium chloride, 200 mM phosphate, 20 mM TCEP, pH 8.0, 37°C. Bottom, linear fitting of the kinetics data to second-order rate equation. Slope = 0.062, R^2 = 0.98. **1A'** refers to the product of the arylation reaction between **1A** and probe **2**.

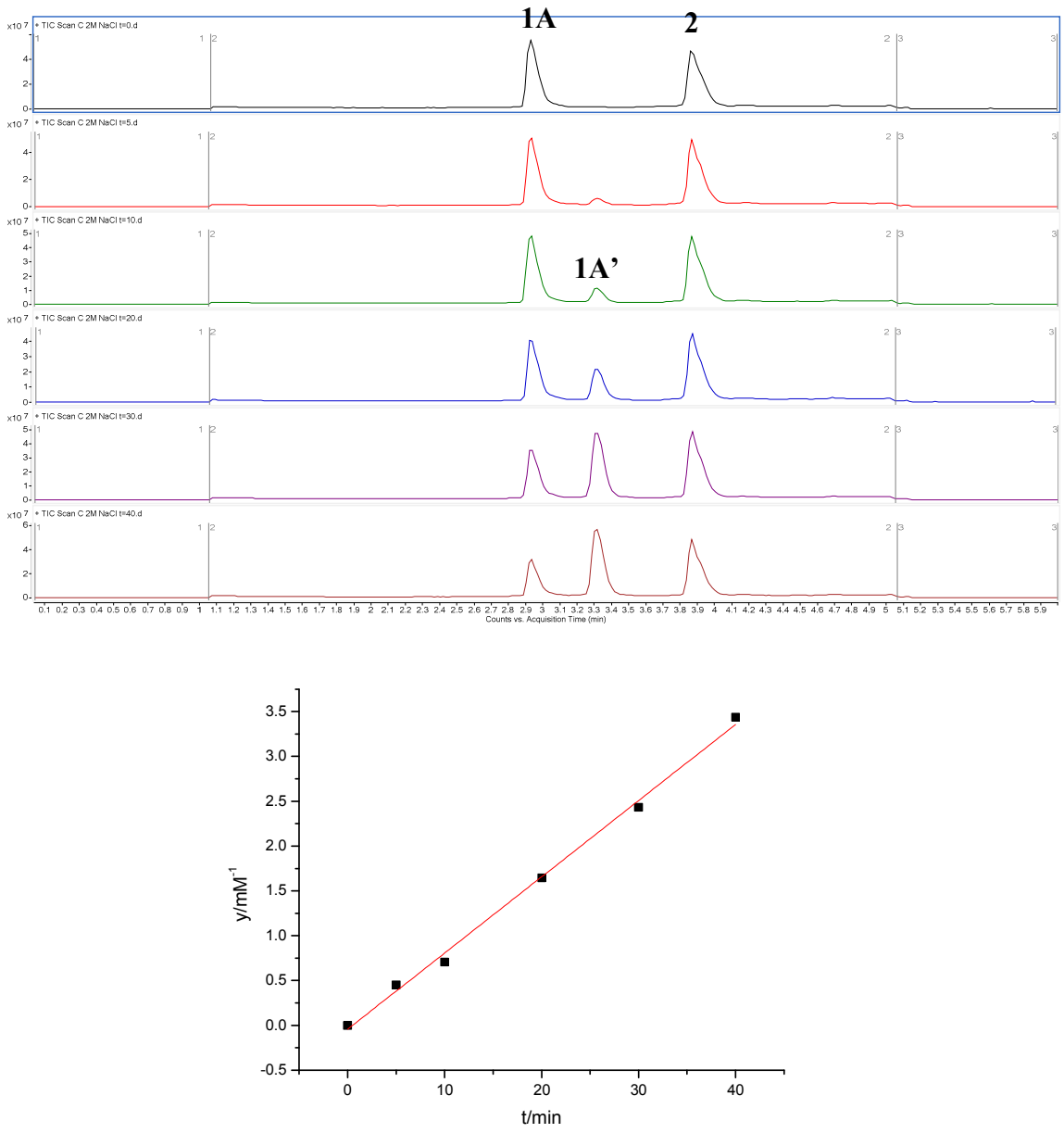
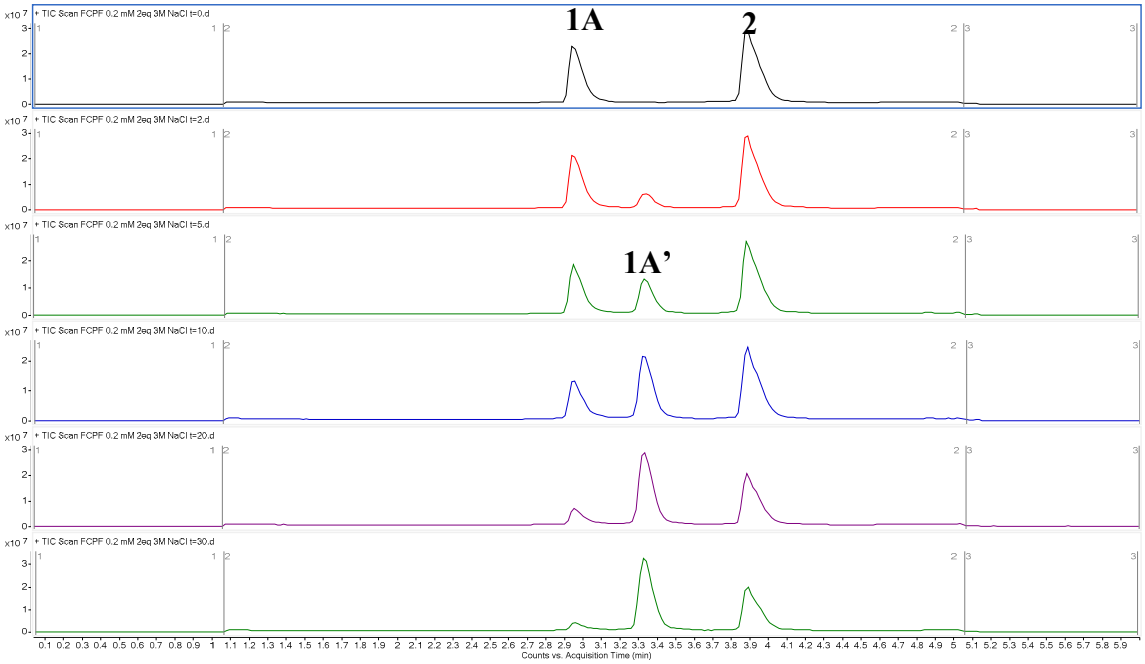


Figure S8. Kinetics analysis for peptide 1A reacting with probe 2 with 2 M sodium chloride. Top, LC-MS chromatograms for the reaction between peptide **1A** and probe **2** with 2 M sodium chloride at different time points (minutes). Reaction conditions: 0.1 mM **1A**, 0.2 mM probe **2**, 2 M sodium chloride, 200 mM phosphate, 20 mM TCEP, pH 8.0, 37°C. Bottom, linear fitting of the kinetics data to second-order rate equation. Slope = 0.085, R^2 = 0.996. **1A'** refers to the product of the arylation reaction between **1A** and probe **2**.



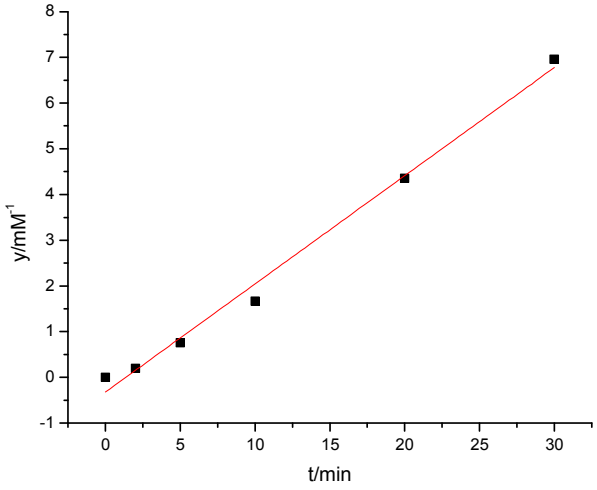
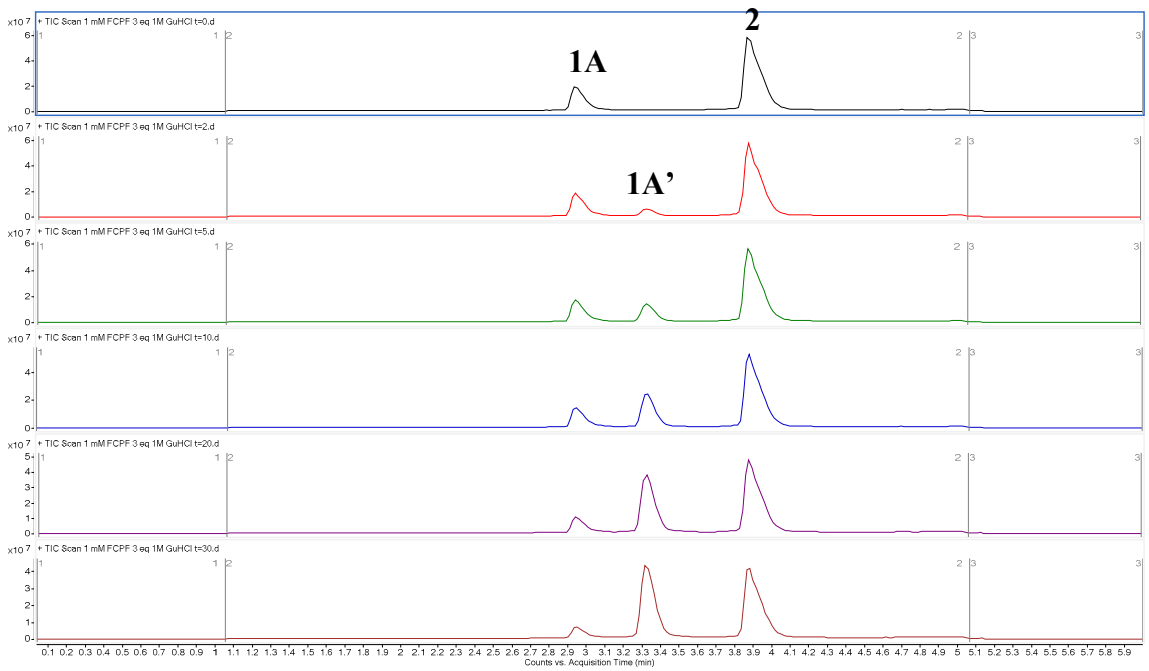


Figure S9. Kinetics analysis for peptide 1A reacting with probe 2 with 3 M sodium chloride. Top, LC-MS chromatograms for the reaction between peptide **1A** and probe **2** with 3 M sodium chloride at different time points (minutes). Reaction conditions: 0.2 mM **1A**, 0.4 mM probe **2**, 3 M sodium chloride, 200 mM phosphate, 20 mM TCEP, pH 8.0, 37°C. Bottom, linear fitting of the kinetics data to second-order rate equation. Slope = 0.237, R^2 = 0.990. **1A'** refers to the product of the arylation reaction between **1A** and probe **2**.



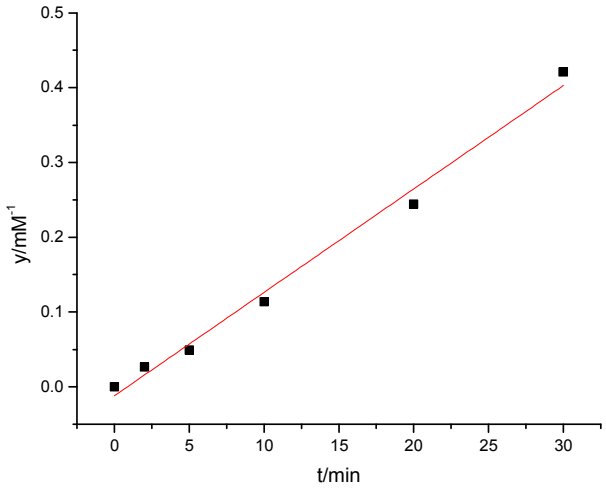


Figure S10. Kinetics analysis for peptide 1A reacting with probe 2 with 1 M guanidinium chloride. Top, LC-MS chromatograms for the reaction between peptide **1A** and probe **2** with 1 M guanidinium chloride at different time points (minutes). Reaction conditions: 1 mM **1A**, 3 mM probe **2**, 1 M guanidinium chloride, 200 mM phosphate, 20 mM TCEP, pH 8.0, 37°C. Bottom, linear fitting of the kinetics data to second-order rate equation. Slope = 0.0138, R^2 = 0.98. **1A'** refers to the product of the arylation reaction between **1A** and probe **2**.

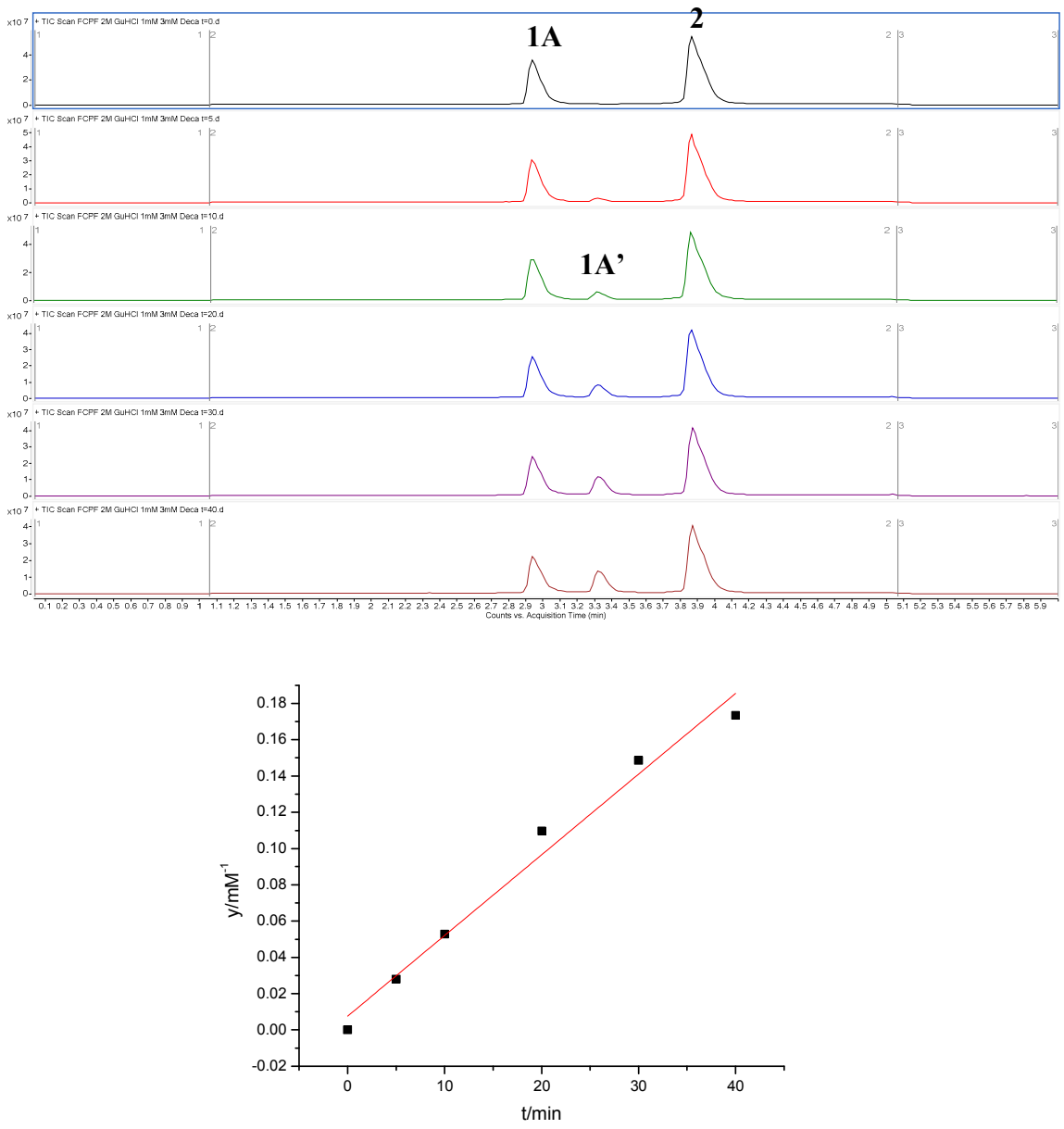


Figure S11. Kinetics analysis for peptide 1A reacting with probe 2 with 2 M guanidinium chloride. Top, LC-MS chromatograms for the reaction between peptide **1A** and probe **2** with 2 M guanidinium chloride at different time points (minutes). Reaction conditions: 1 mM **1A**, 3 mM probe **2**, 2 M guanidinium chloride, 200 mM phosphate, 20 mM TCEP, pH 8.0, 37°C. Bottom, linear fitting of the kinetics data to second-order rate equation. Slope = 0.00445, R^2 = 0.98. **1A'** refers to the product of the arylation reaction between **1A** and probe **2**.

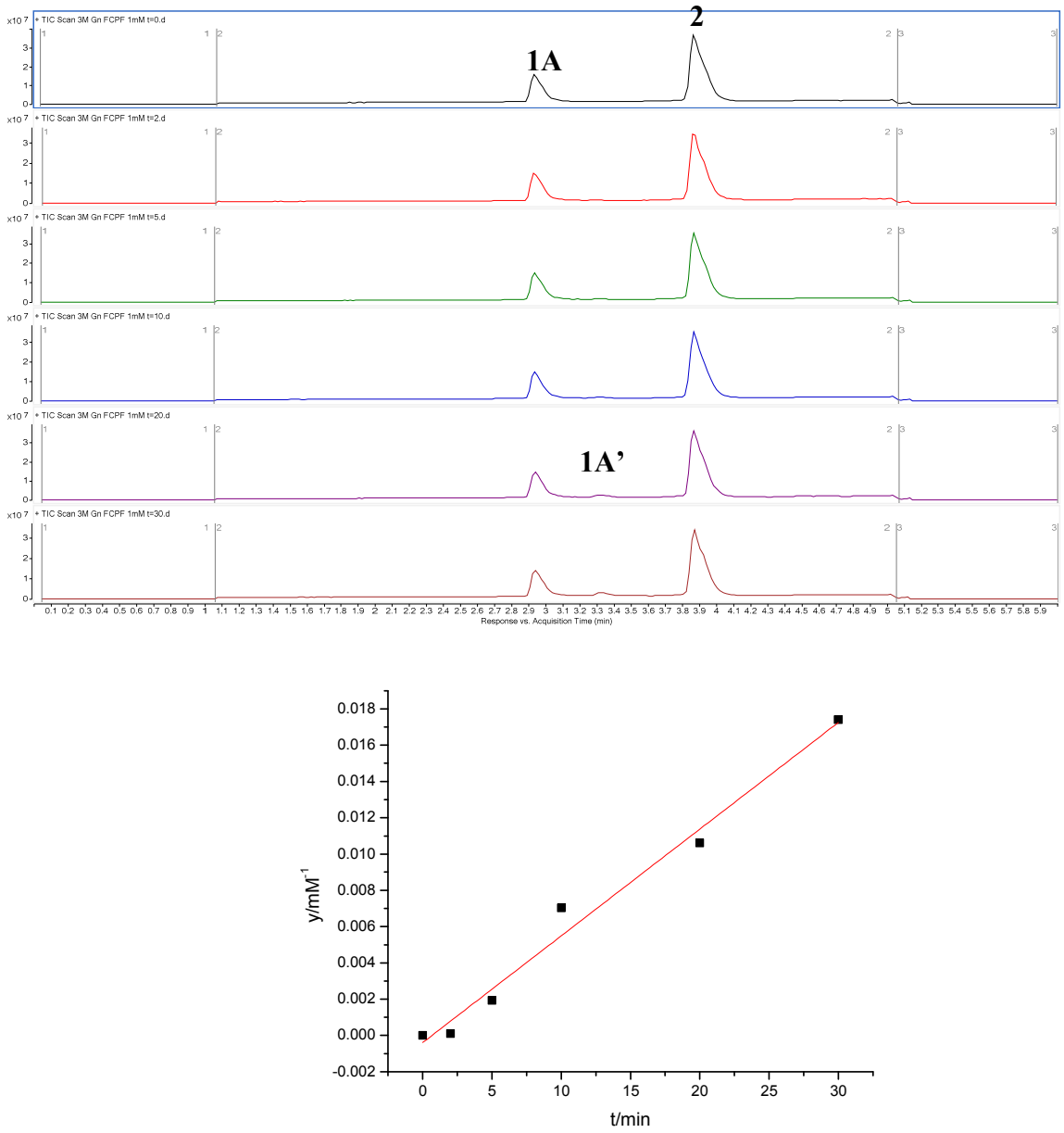
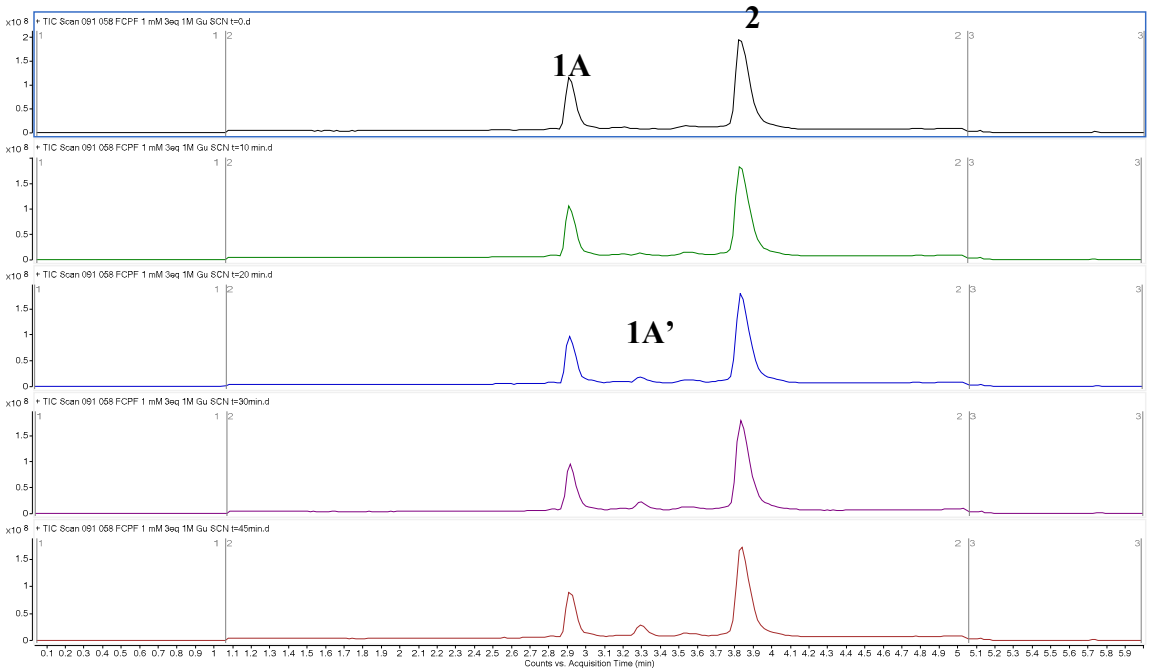


Figure S12. Kinetics analysis for peptide 1A reacting with probe 2 with 3 M guanidinium chloride. Top, LC-MS chromatograms for the reaction between peptide **1A** and probe **2** with 3 M guanidinium chloride at different time points (minutes). Reaction conditions: 1 mM **1A**, 3 mM probe **2**, 3 M guanidinium chloride, 200 mM phosphate, 20 mM TCEP, pH 8.0, 37°C. Bottom, linear fitting of the kinetics data to second-order rate equation. Slope = 0.00059, R^2 = 0.98. **1A'** refers to the product of the arylation reaction between **1A** and probe **2**.



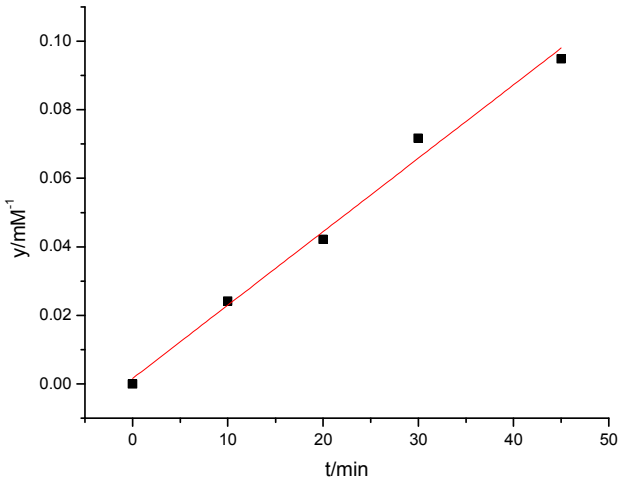
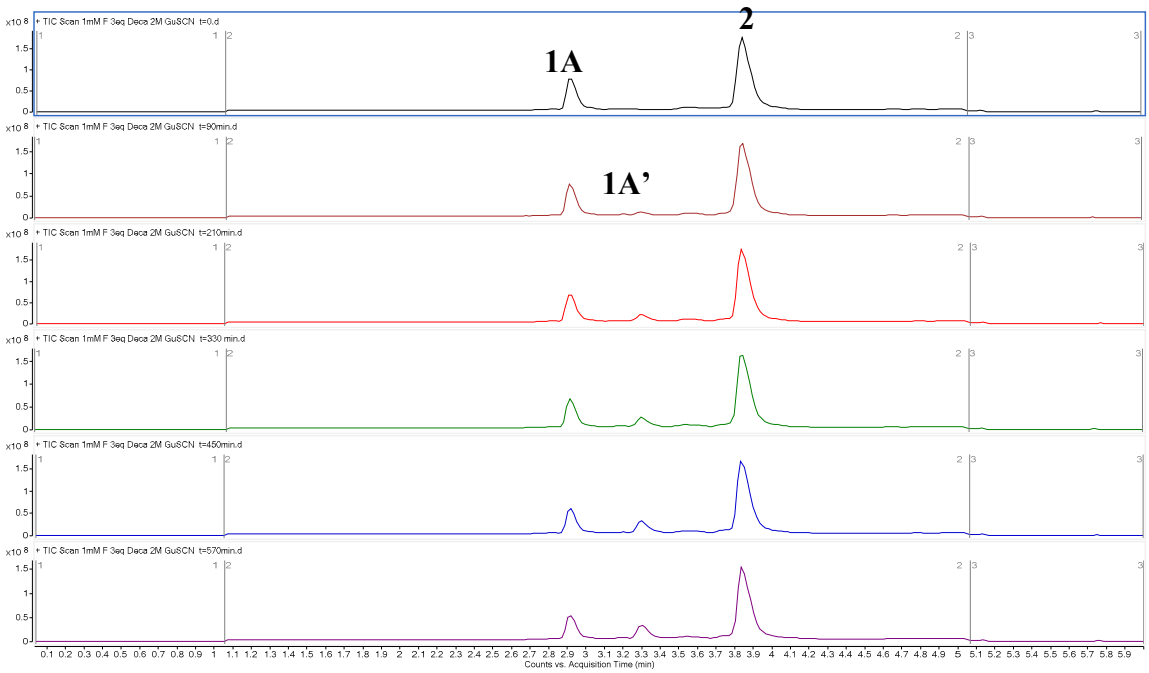


Figure S13. Kinetics analysis for peptide 1A reacting with probe 2 with 1 M guanidinium thiocyanate. Top, LC-MS chromatograms for the reaction between peptide **1A** and probe **2** with 1 M guanidinium thiocyanate at different time points (minutes). Reaction conditions: 1 mM **1A**, 3 mM probe **2**, 1 M guanidinium thiocyanate, 200 mM phosphate, 20 mM TCEP, pH 8.0, 37°C. Bottom, linear fitting of the kinetics data to second-order rate equation. Slope = 0.0021, R^2 = 0.98. **1A'** refers to the product of the arylation reaction between **1A** and probe **2**.



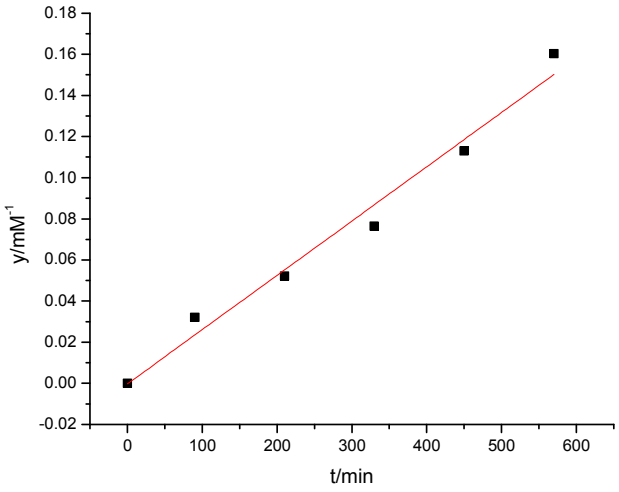
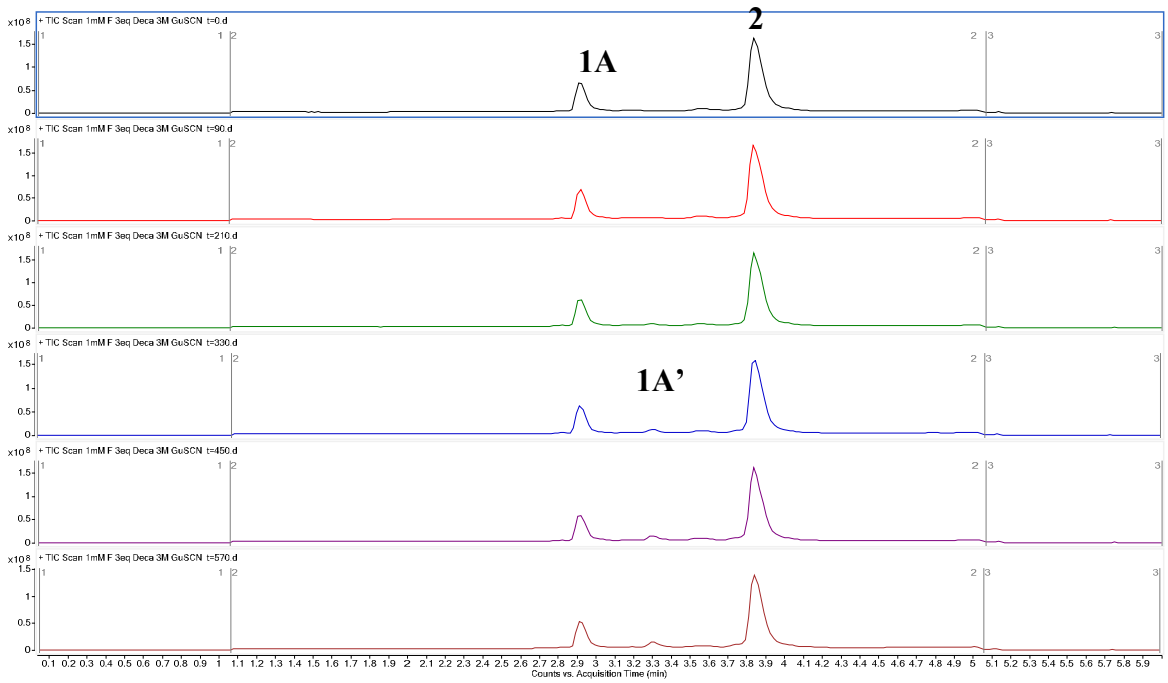


Figure S14. Kinetics analysis for peptide 1A reacting with probe 2 with 2 M guanidinium thiocyanate. Top, LC-MS chromatograms for the reaction between peptide **1A** and probe **2** with 2 M guanidinium thiocyanate at different time points (minutes). Reaction conditions: 1 mM **1A**, 3 mM probe **2**, 2 M guanidinium thiocyanate, 200 mM phosphate, 20 mM TCEP, pH 8.0, 37°C. Bottom, linear fitting of the kinetics data to second-order rate equation. Slope = 0.00026, R^2 = 0.97. **1A'** refers to the product of the arylation reaction between **1A** and probe **2**.



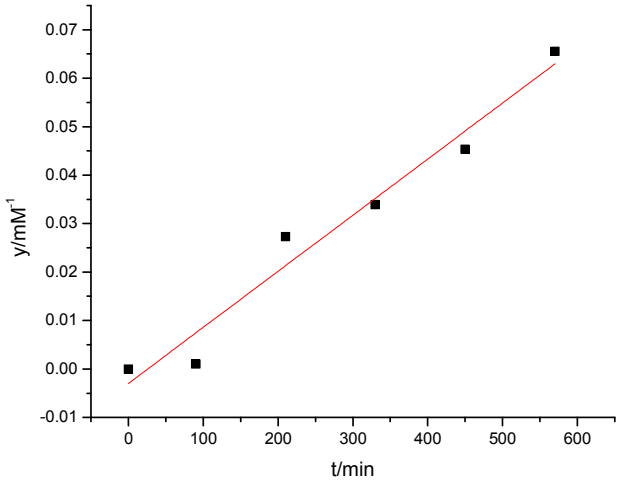
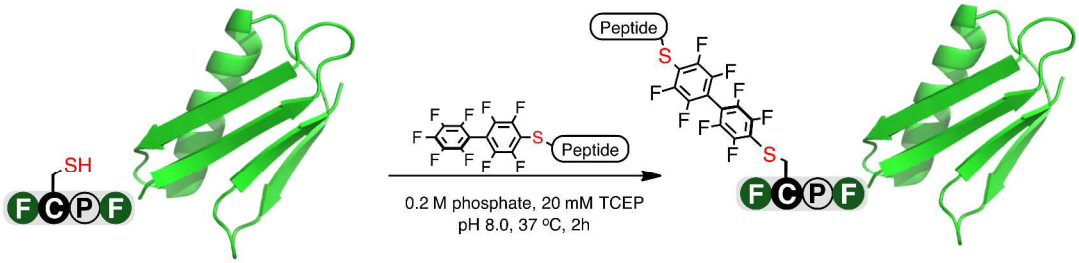


Figure S15. Kinetics analysis for peptide 1A reacting with probe 2 with 3 M guanidinium thiocyanate. Top, LC-MS chromatograms for the reaction between peptide **1A** and probe **2** with 3 M guanidinium thiocyanate at different time points (minutes). Reaction conditions: 1 mM **1A**, 3 mM probe **2**, 2 M guanidinium thiocyanate, 200 mM phosphate, 20 mM TCEP, pH 8.0, 37°C. Bottom, linear fitting of the kinetics data to second-order rate equation. Slope = 0.00012, R^2 = 0.97. **1A'** refers to the product of the arylation reaction between **1A** and probe **2**.

a)



b)

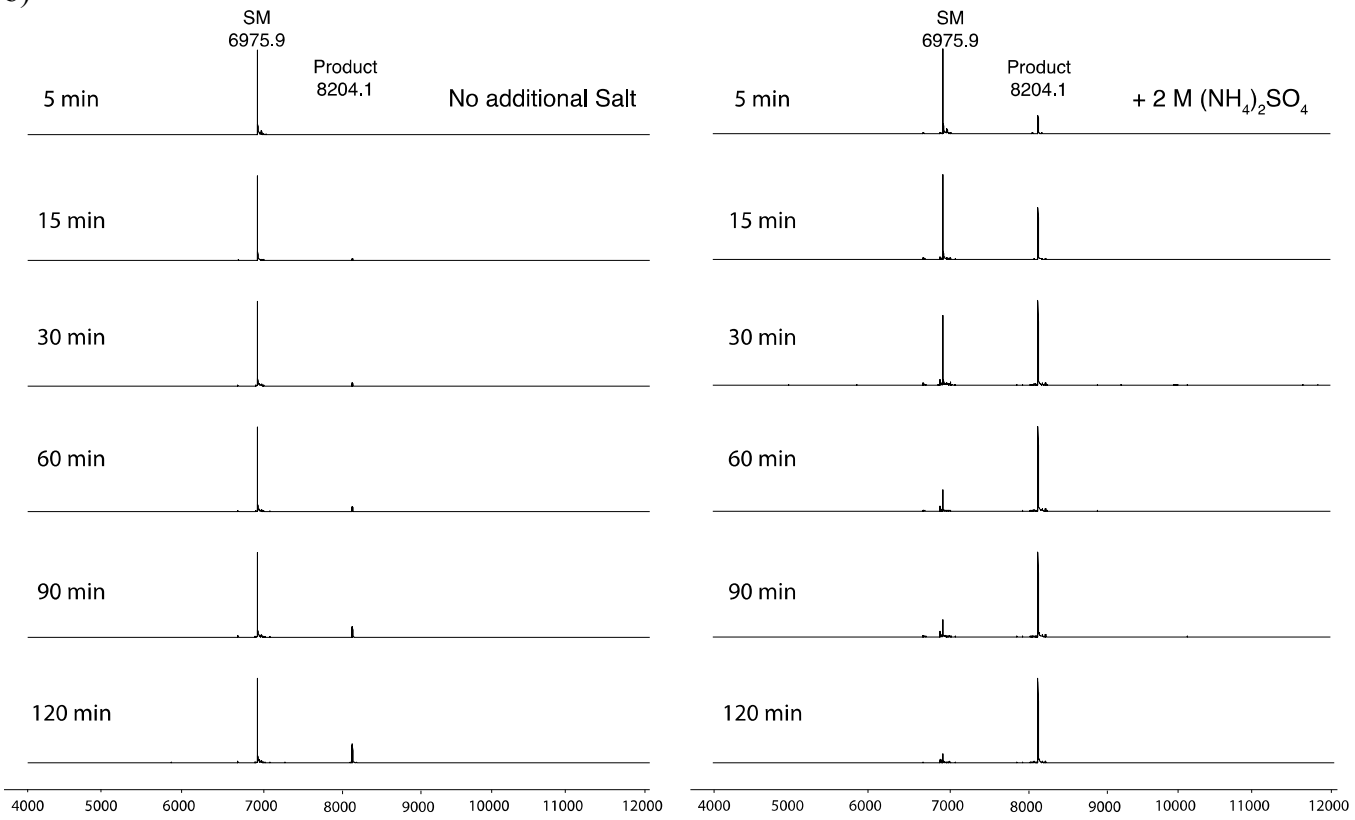


Figure S16. Ammonium sulfate accelerated π-clamp mediated protein 3a labeling. a) The reaction between **3a** and probe **2**. Reaction conditions: 0.1 mM **3a**, 1 mM probe **2**, 200 mM phosphate, 20 mM TCEP, 37 °C. b) Deconvoluted mass spectra of the reaction without or with additional 2 M ammonium sulfate at different time points.

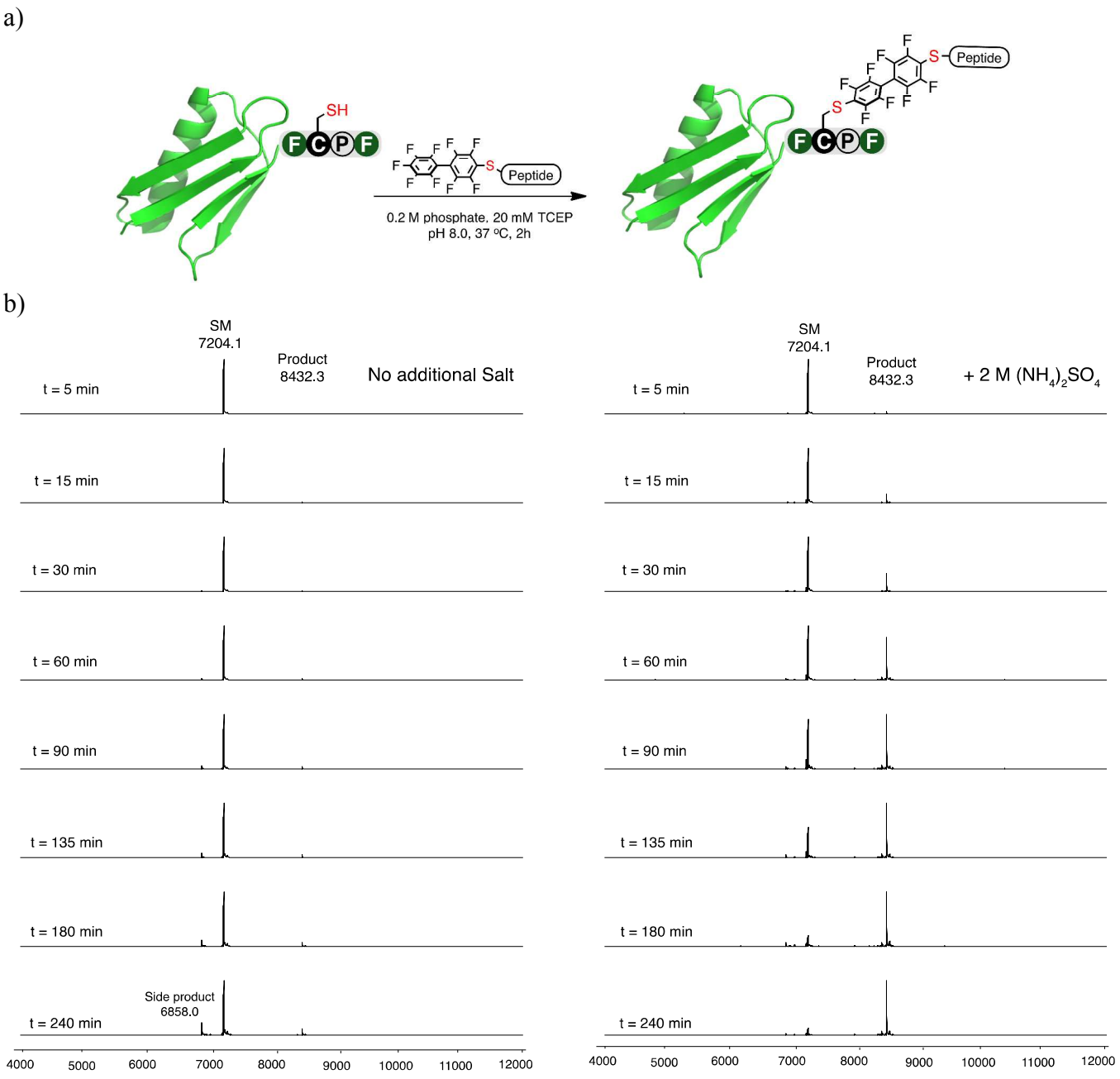
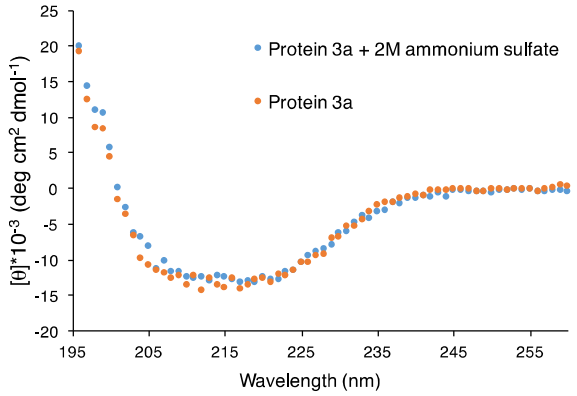
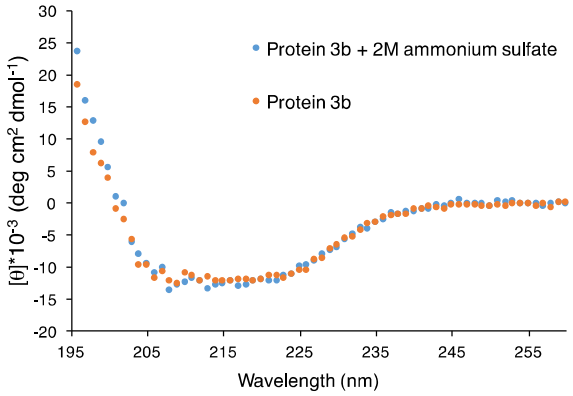


Figure S17. Ammonium sulfate accelerates π-clamp mediated protein 3b labeling. a) The reaction between **3b** and probe **2**. Reaction conditions: 0.1 mM **3b**, 1 mM probe **2**, 200 mM phosphate, 20 mM TCEP, 37 °C, pH 8.0. b) Deconvoluted mass spectra of the reaction without or with additional 2 M ammonium sulfate at different time point.

a)



b)



c)

	Secondary structure	
	In buffer	In buffer + 2M ammonium sulfate
Protein 3a	α helix: 34%, β strand: 18%	α helix: 31%, β strand: 21%
Protein 3b	α helix: 32%, β strand: 19%	α helix: 32%, β strand: 21%

Figure S18. Effect of ammonium sulfate on protein 3a and 3b. a) Circular dichroism spectra of protein **3a** in buffer with or without 2 M ammonium sulfate. Buffer: 10 mM phosphate, 1 mM TCEP, pH 8.0. b) Circular dichroism spectra of protein **3b** in buffer with or without 2 M ammonium sulfate. Buffer: 10 mM phosphate, 1 mM TCEP, pH 8.0. c) Estimated protein secondary structure from CD spectrum.

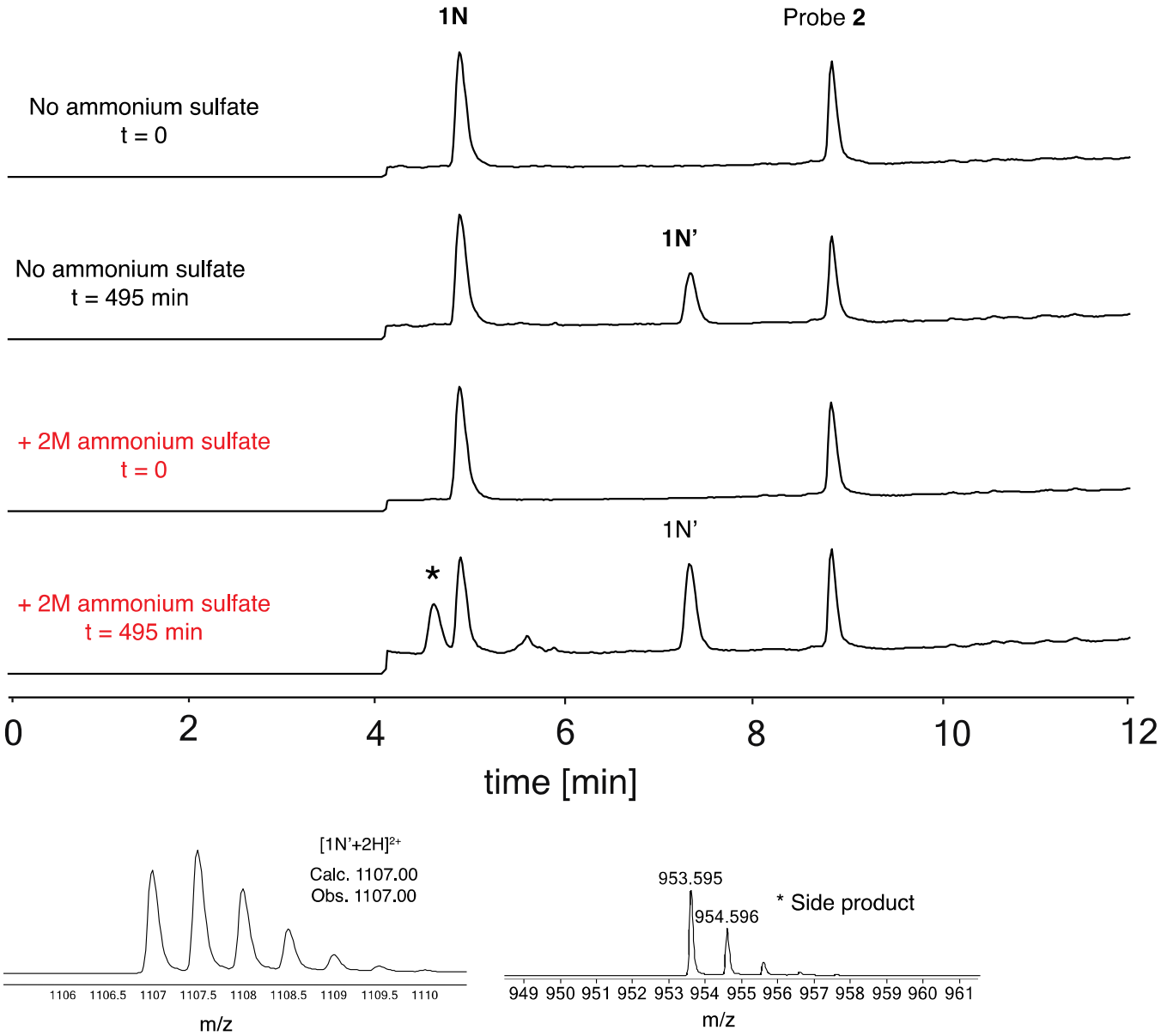
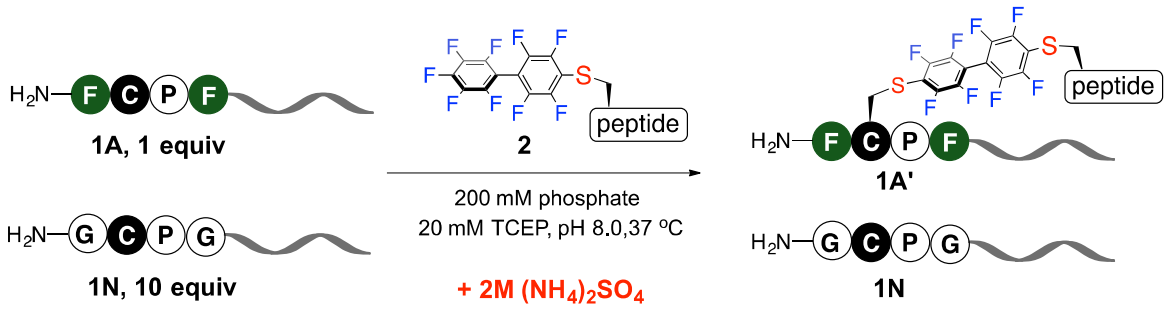


Figure S19. π-clamp double glycine variant (1N) reactivity does not significantly change with addition of 2 M ammonium sulfate. Reaction conditions: 7.4 mM **1N**, 5 mM probe **2**, 200 mM phosphate, 20 mM TCEP, 37 °C, pH 8.0, with or without 2M ammonium sulfate. Reaction time 495 min. The conversion% of probe **2** was 32.0% without salt and 32.4% with 2 M ammonium sulfate. There is a side product (denoted as *) observed with ammonium sulfate at high peptide concentration and long reaction time, which is not observed at lower concentration (**Figure S20**).

a)



b)

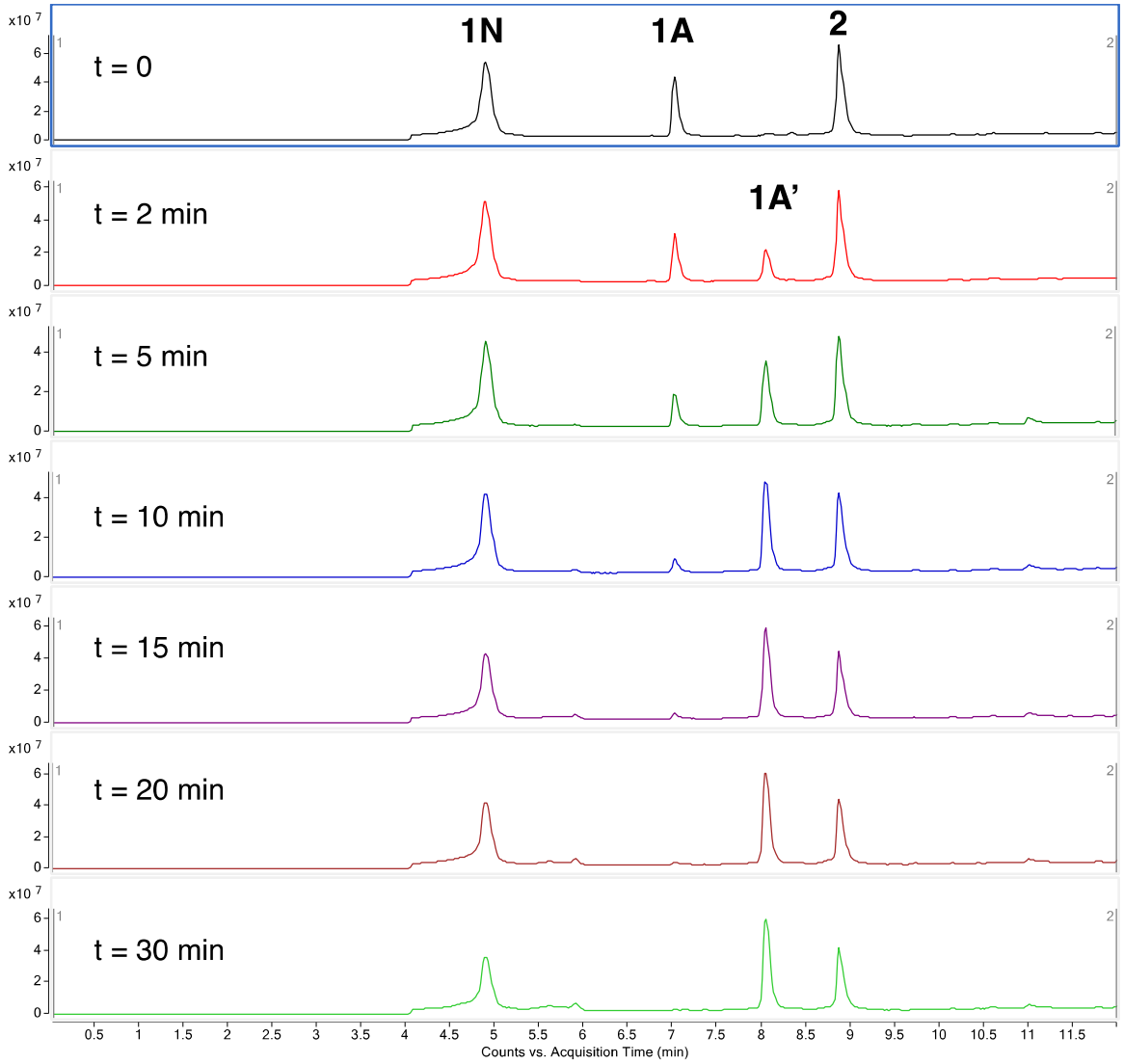
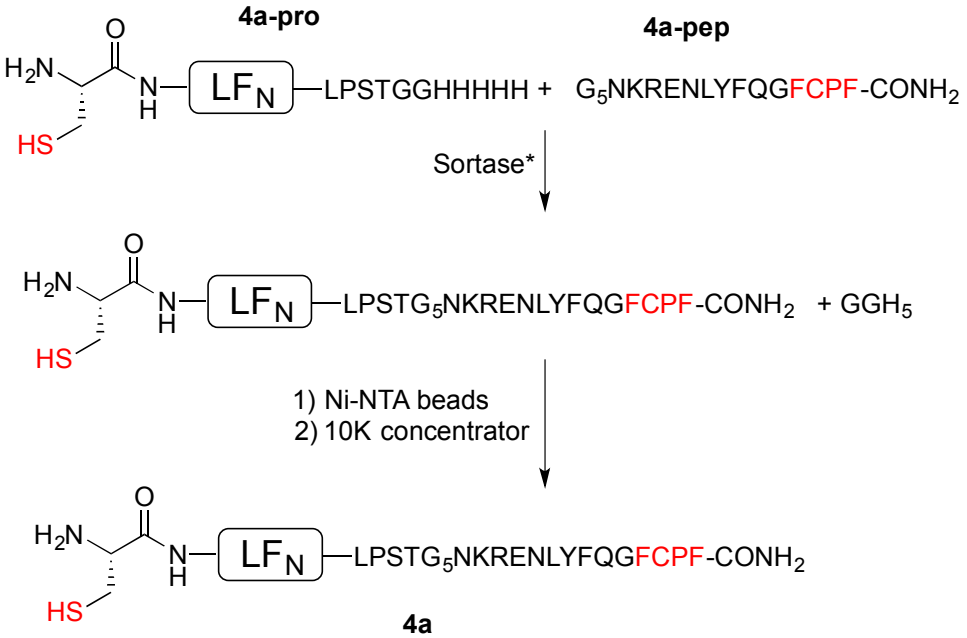


Figure S20. Labeling of the π-clamp peptide 1A in the presence of 10 equivalent of double glycine mutant 1N with ammonium sulfate. a) Scheme for the reaction. Reaction conditions: 0.05 mM **1A**, 0.5 mM **1N**, 0.1 mM probe **2**, 200 mM phosphate, 20 mM TCEP, 2 M ammonium sulfate, 37 °C. 1A' refers to the product of the arylation reaction between 1A and probe 2. b) LC-MS chromatograms for the reaction quenched at different time points.

a)



b)

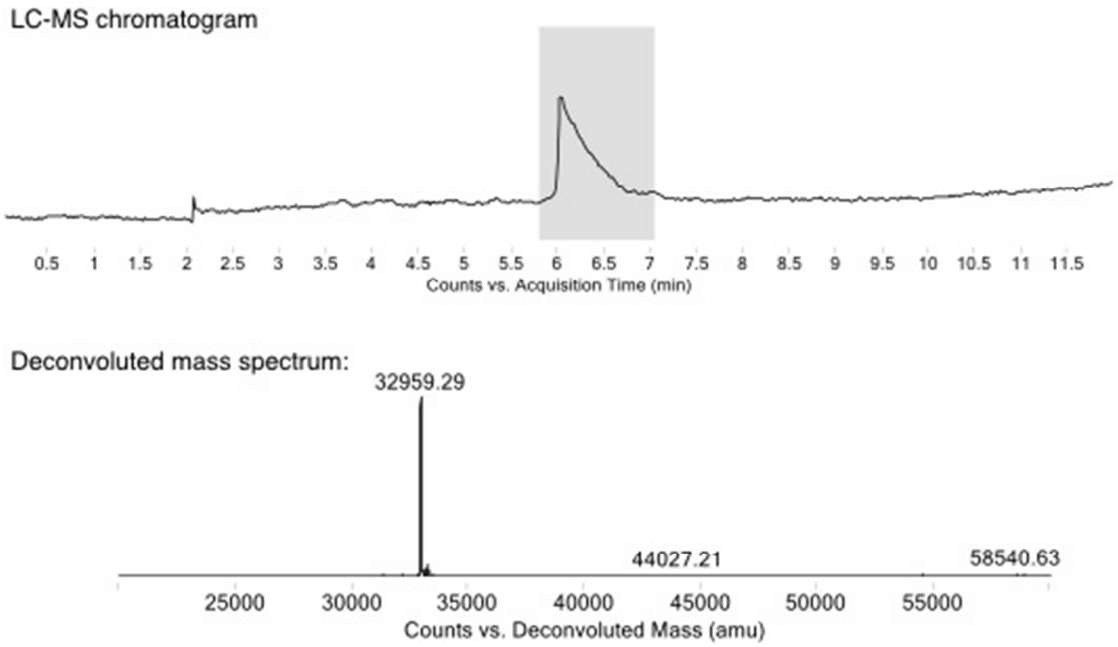
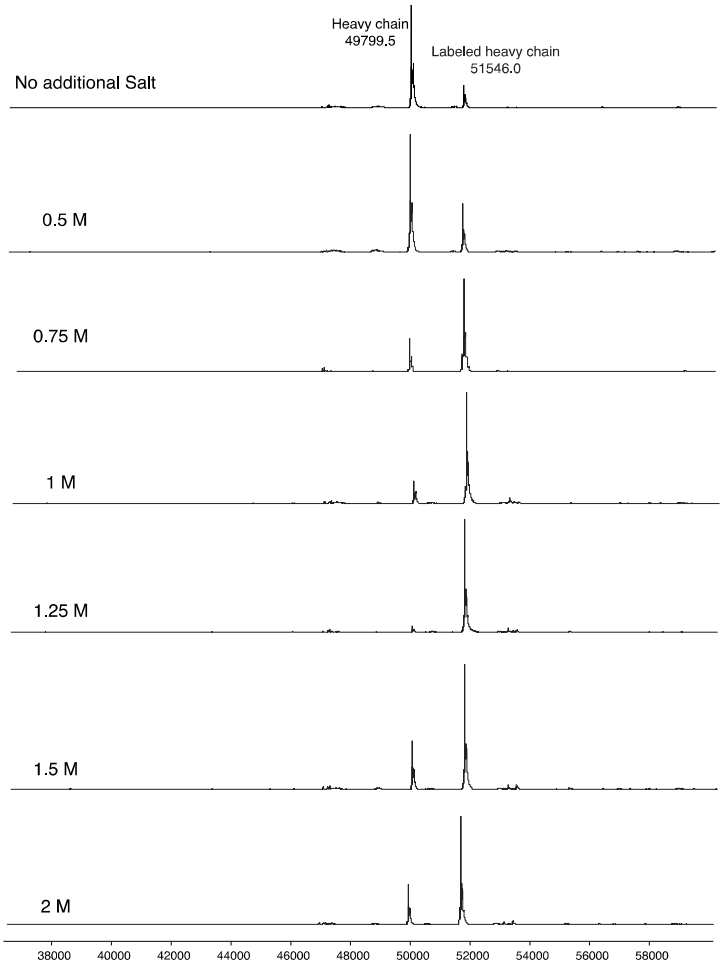


Figure S21. Preparation of protein 4a. (**a**) Scheme for synthesis of protein **4a** via sortagging reaction. (**b**) LC-MS chromatograms and deconvoluted protein mass of protein **4a**.

a)



b)

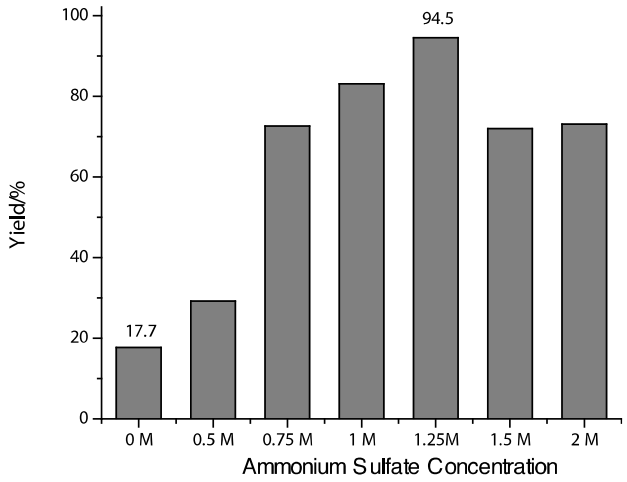


Figure S22. Optimization of ammonium sulfate concentration for antibody 5 labeling. a) Deconvoluted mass spectra of the LC-MS analysis for antibody labeling reaction with different concentration of ammonium sulfate. Reaction conditions: 40 μM π-clamp trastuzumab **5**, 500 μM probe **6**, 100 mM phosphate, 10 mM TCEP, 37 °C, pH 8.0, 150 min, with different amount of ammonium sulfate. b) Yield summary for the reaction. 1.25 M ammonium sulfate was the best salt concentration for this specific condition.

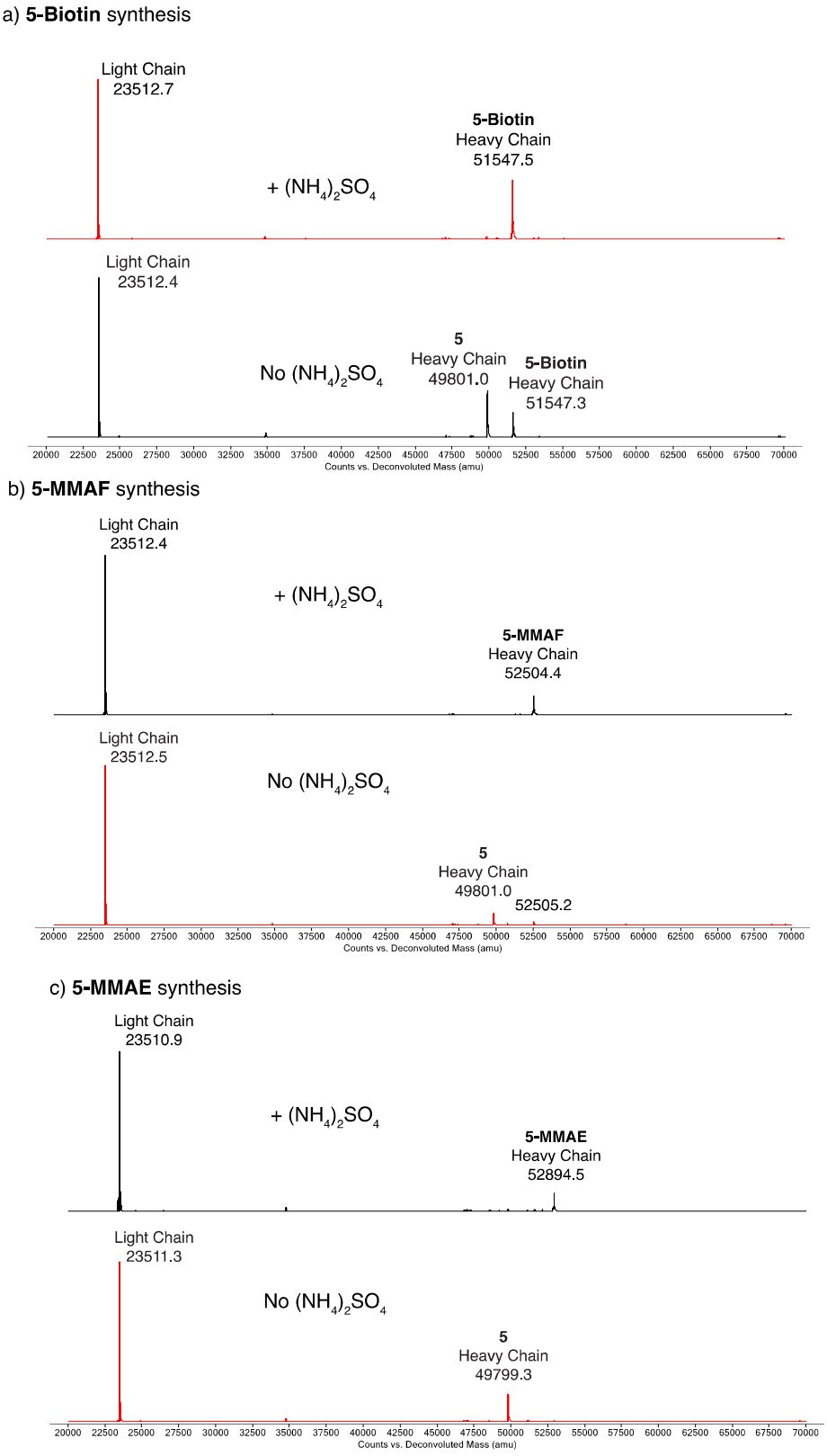


Figure S23. Full deconvoluted mass spectra for the synthesis of 5-biotin, 5-MMAF and 5-MMAE with or without ammonium sulfate. A small peak corresponding to PNGase F was also observed around 35 KDa.

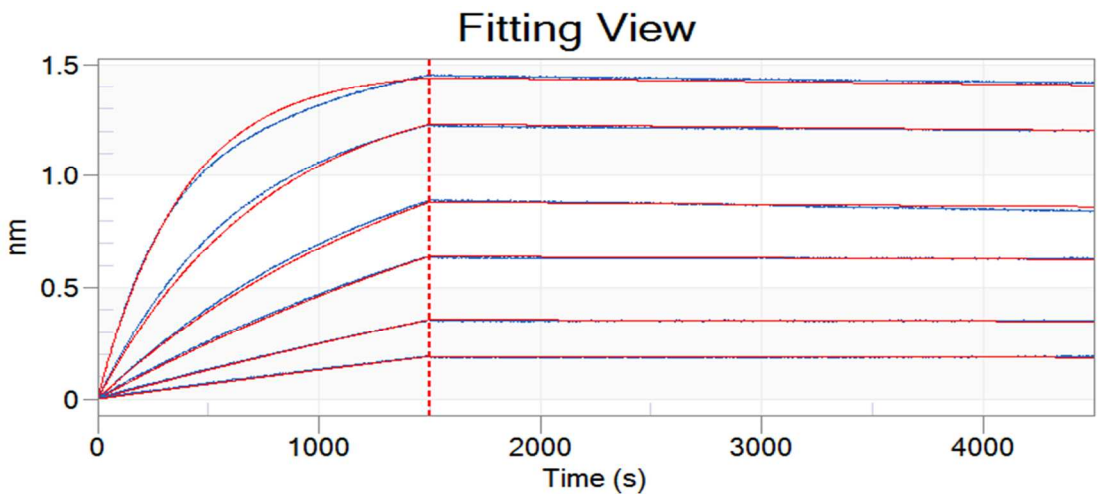


Figure S24. Octet biolayer interferometry assay for binding between 5-biotin and recombinant HER2. Concentration of the recombinant HER2 used in this assay are 40 nM, 20 nM, 10 nM, 5 nM, 2.5 nM and 1.25 nM. The data was fitted with Fortebio Biosystems (global fitting algorism) to obtain the K_D, 122 ± 1.5 pM.

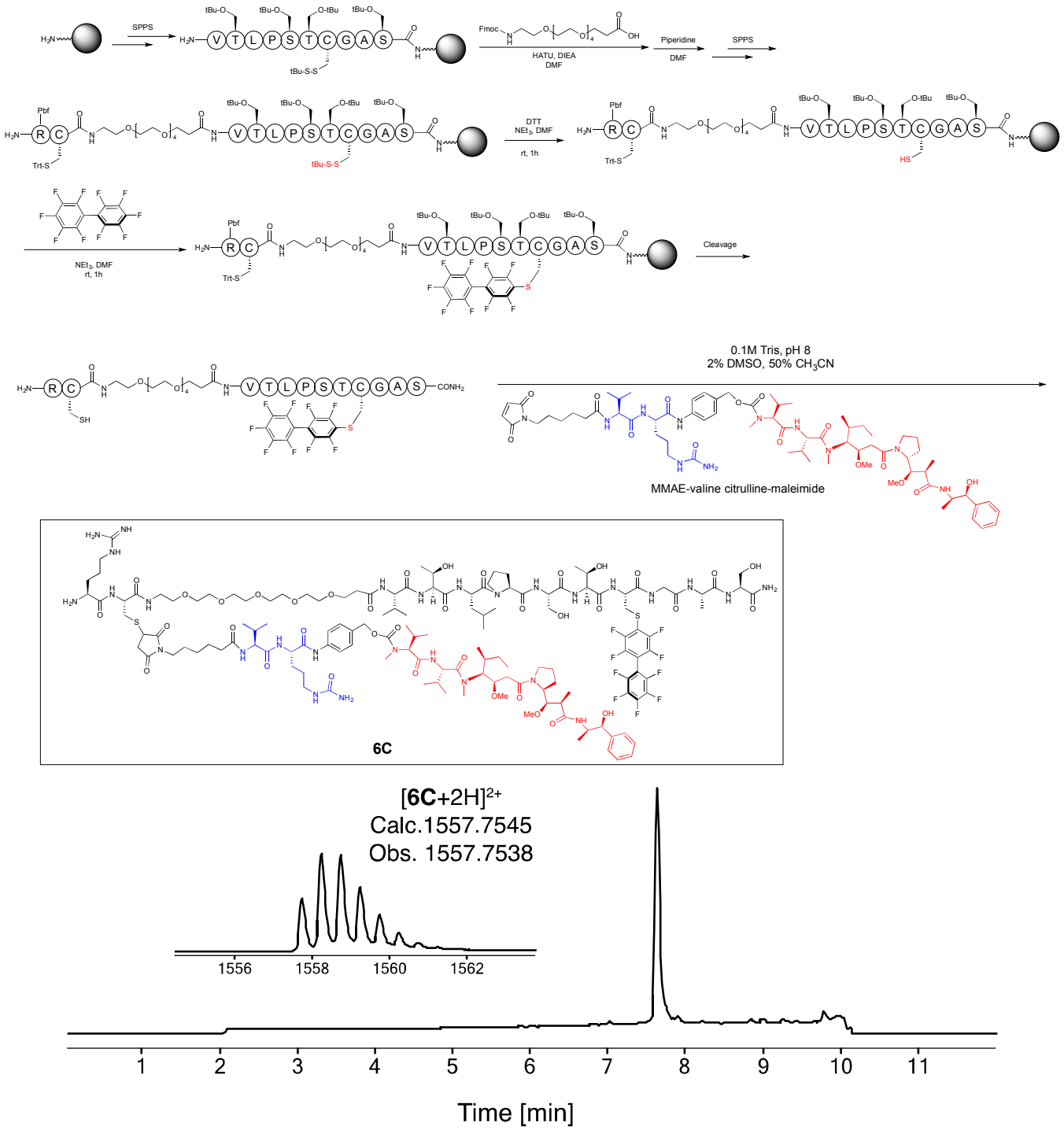
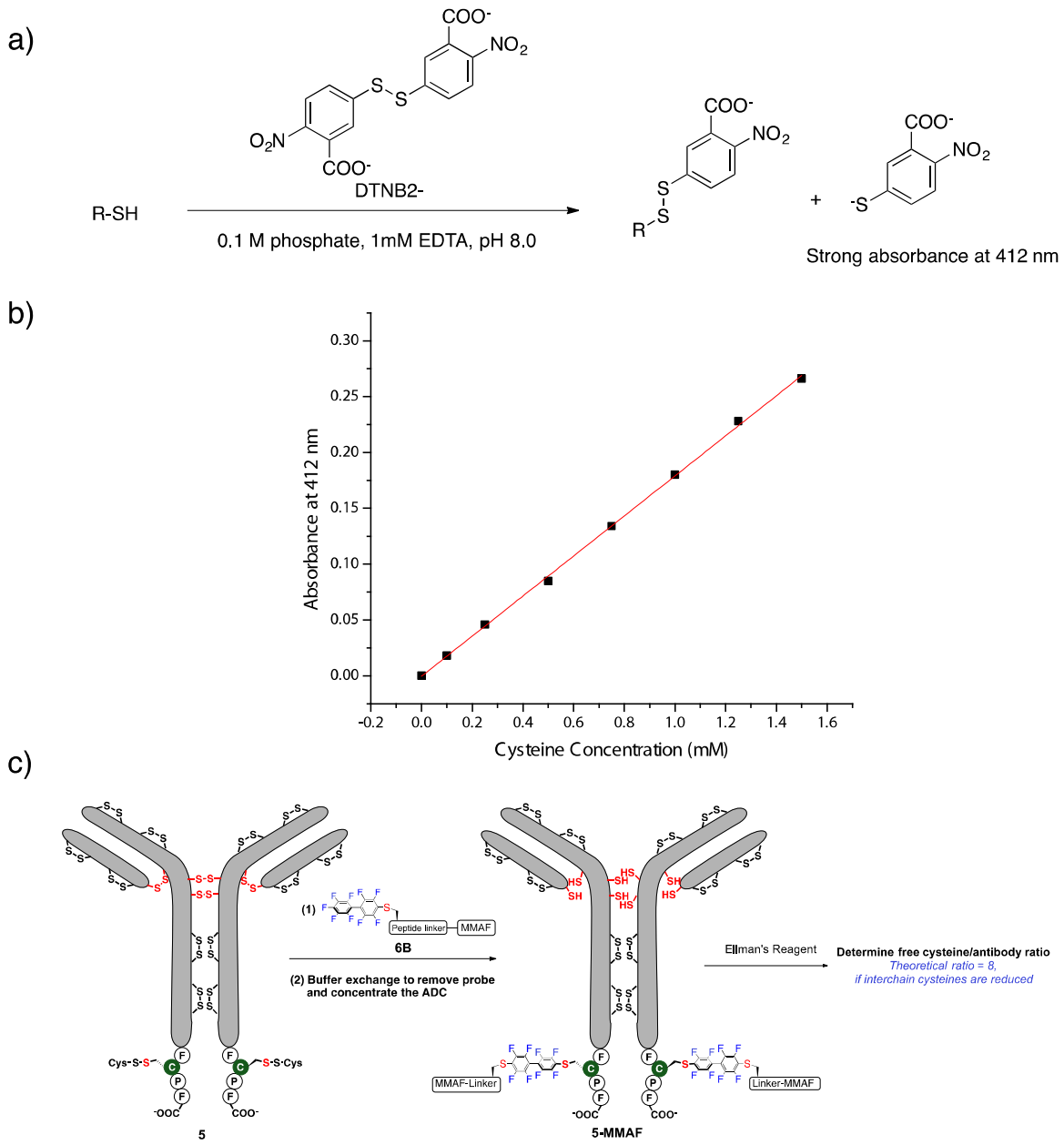


Figure S25. Synthesis of probe 6C (perfluoroaryl-valine-citrulline-MMAE). Synthetic route and LC-HRMS analysis of the purified product. Total ion current (TIC) chromatogram was shown and the mass spectrum for **6C** was shown as insets. The cathepsin-cleavable linker (valine-citrulline) was highlighted in blue and the MMAE was highlighted in red.



Measured free cysteine to antibody ratio:
8.8 ± 1.4

Figure S26. Ellman's reagent to determine the free cysteine-to-antibody ratio of the ADC 5-MMAF which is used for cell assay. a) Reaction of Ellman's reagent with thiol. b) A standard curve generated using various concentrations of L-cysteine. Slope = 0.17942, intercept = -0.00036, R^2 = 0.9992. c) Around 8 free cysteines were present in one **5-MMAF** molecule. In our previous work, it has been shown that the ***interchain*** disulfide bonds were reduced during the site-selective drug conjugation[3], we thought that all the eight inter chain cysteines are not oxidized during storage. Protein concentration is determined by measuring UV absorption at 280 nm; and the free cysteine concentration is determined by reacting with Ellman's reagent and measure the absorption at 412 nm. The measured cysteine-to-antibody ratio is the average of four independent experiments.

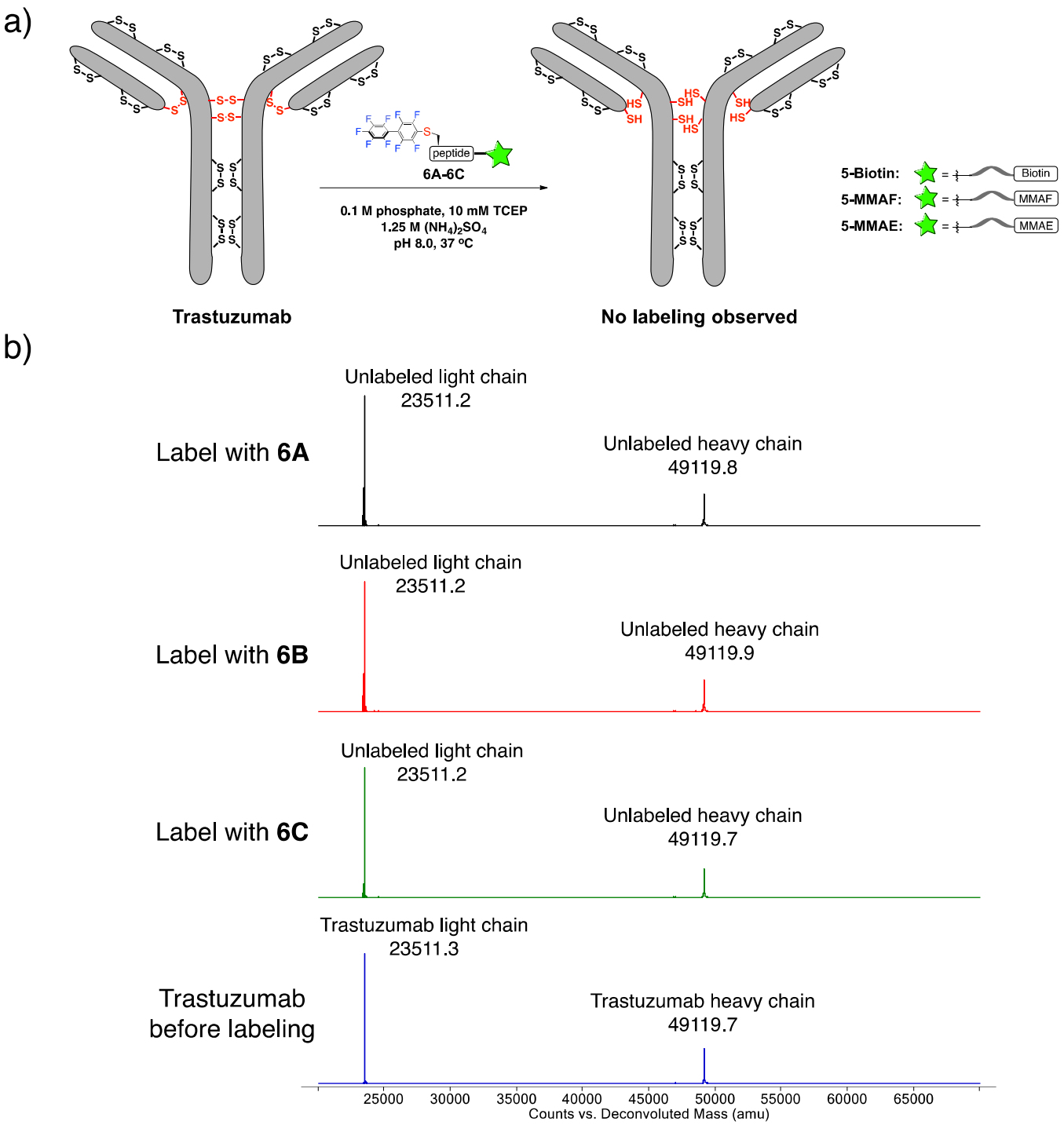
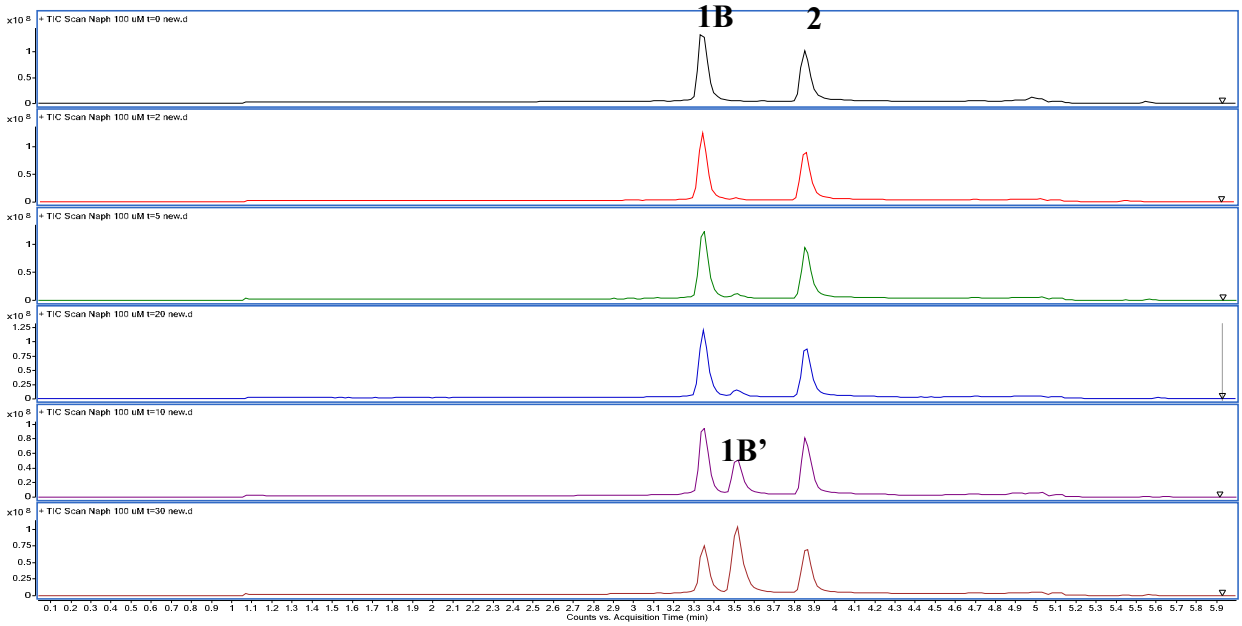


Figure S27. Probe 6A-6C did not react with native trastuzumab (without π-clamp) under the same conditions used to synthesize 5-Biotin, 5-MMAF and 5-MMAE. a) Scheme for the labeling of native trastuzumab with perfluoroaryl probes. b) The deconvoluted mass spectra for the labeling reactions. Antibodies were treated with PNGase F to remove the N-linked glycans before analyzed by LC-MS. Reaction conditions were the same as labeling protein **5**: 40 μM native trastuzumab, 500 μM probe **6A/6B/6C**, 100 mM phosphate, 10 mM TCEP, 1.25 M $(NH_4)_2SO_4$, 37 °C. Reaction time: 3 hours for **6A**, 210 min for **6B**, 16 hours for **6C**.



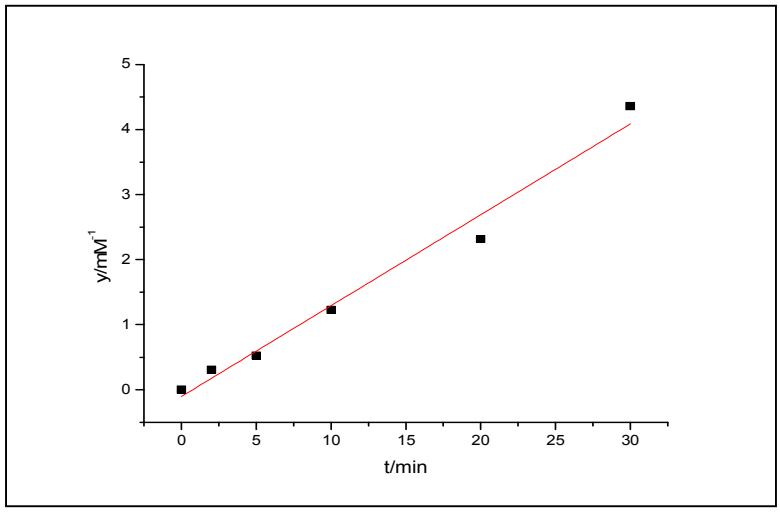
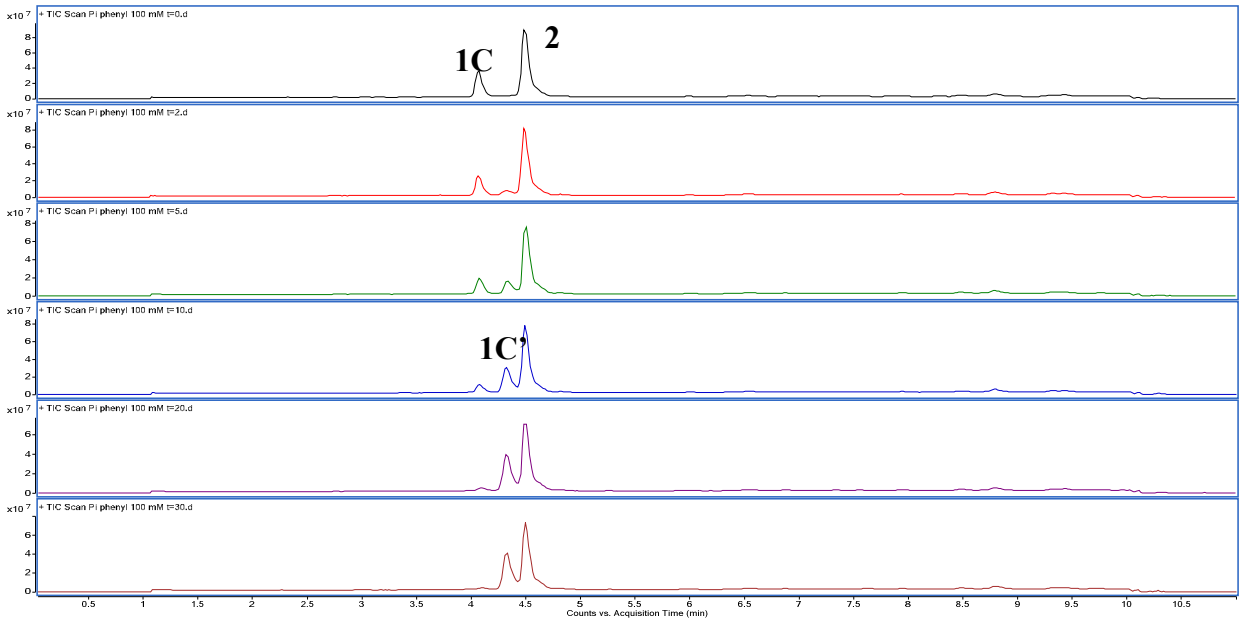


Figure S28. **Kinetics analysis for peptide 1B reacting with probe 2.** Top, LC-MS chromatograms for the reaction between peptide **1B** and probe **2** at different time points (minutes). Reaction conditions: 0.1 mM **1B**, 0.2 mM probe **2**, 200 mM phosphate, 20 mM TCEP, pH 8.0, 37°C. Bottom, linear fitting of the kinetics data to second-order rate equation. Slope = 0.14, R^2 = 0.98. **1B'** refers to the product of the arylation reaction between **1B** and probe **2**.



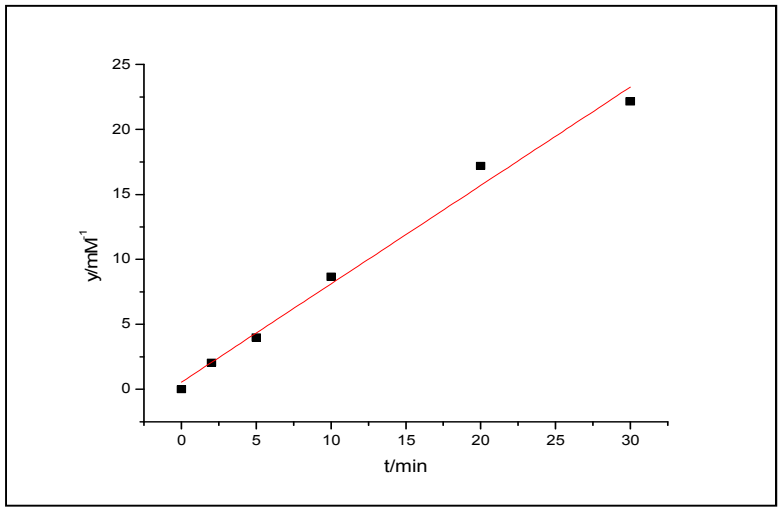
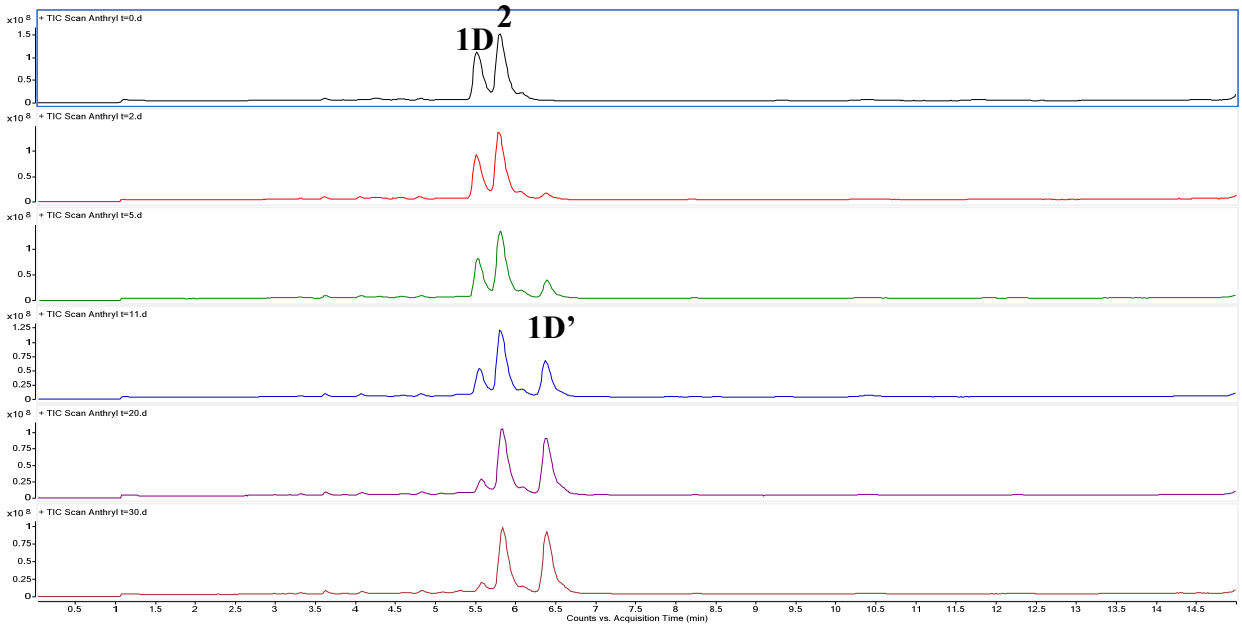


Figure S29. **Kinetics analysis for peptide 1C reacting with probe 2.** Top, LC-MS chromatograms for the reaction between peptide **1C** and probe **2** at different time points (minutes). Reaction conditions: 0.1 mM **1C**, 0.2 mM probe **2**, 200 mM phosphate, 20 mM TCEP, pH 8.0, 37°C. Bottom, linear fitting of the kinetics data to second-order rate equation. Slope = 0.76, R^2 = 0.98. **1C'** refers to the product of the arylation reaction between **1C** and probe **2**.



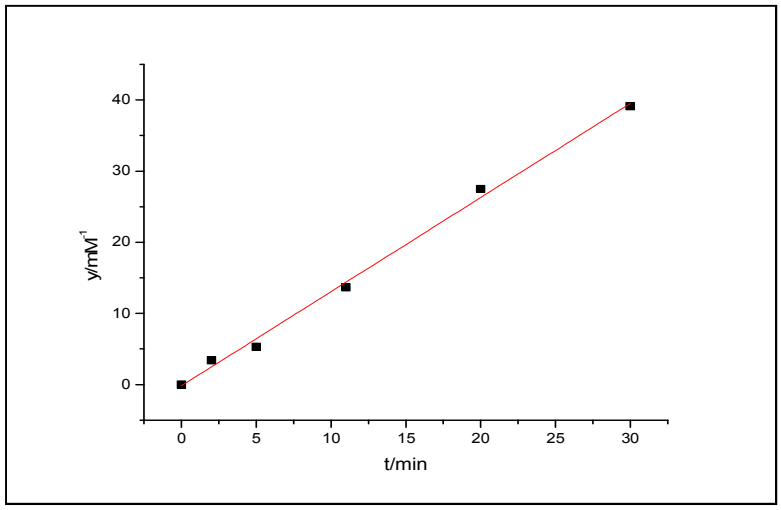
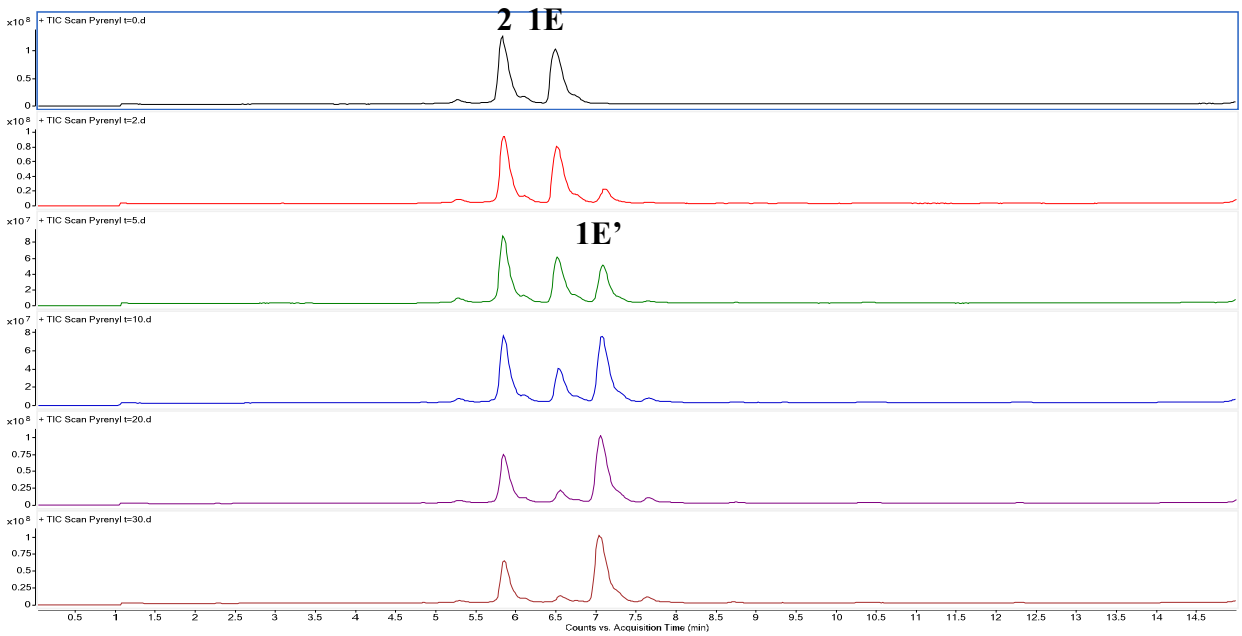


Figure S30. **Kinetics analysis for peptide 1D reacting with probe 2.** Top, LC-MS chromatograms for the reaction between peptide **1D** and probe **2** at different time points (minutes). Reaction conditions: 0.05 mM **1D**, 0.1 mM probe **2**, 200 mM phosphate, 20 mM TCEP, pH 8.0, 37°C. Bottom, linear fitting of the kinetics data to second-order rate equation. Slope = 1.32, R^2 = 0.995. **1D'** refers to the product of the arylation reaction between **1D** and probe **2**.



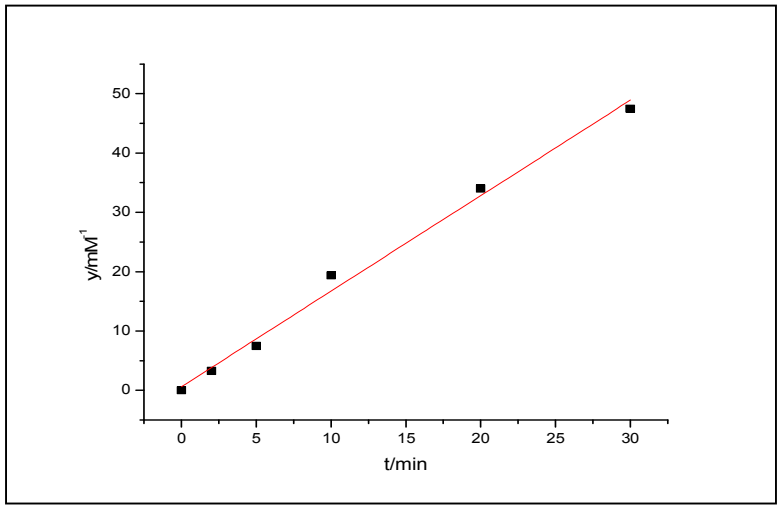


Figure S31. **Kinetics analysis for peptide 1E reacting with probe 2.** Top, LC-MS chromatograms for the reaction between peptide **1E** and probe **2** at different time points (minutes). Reaction conditions: 0.05 mM **1E**, 0.1 mM probe **2**, 200 mM phosphate, 20 mM TCEP, pH 8.0, 37°C. Bottom, linear fitting of the kinetics data to second-order rate equation. Slope = 1.61, R^2 = 0.991. **1E'** refers to the product of the arylation reaction between **1E** and probe **2**.

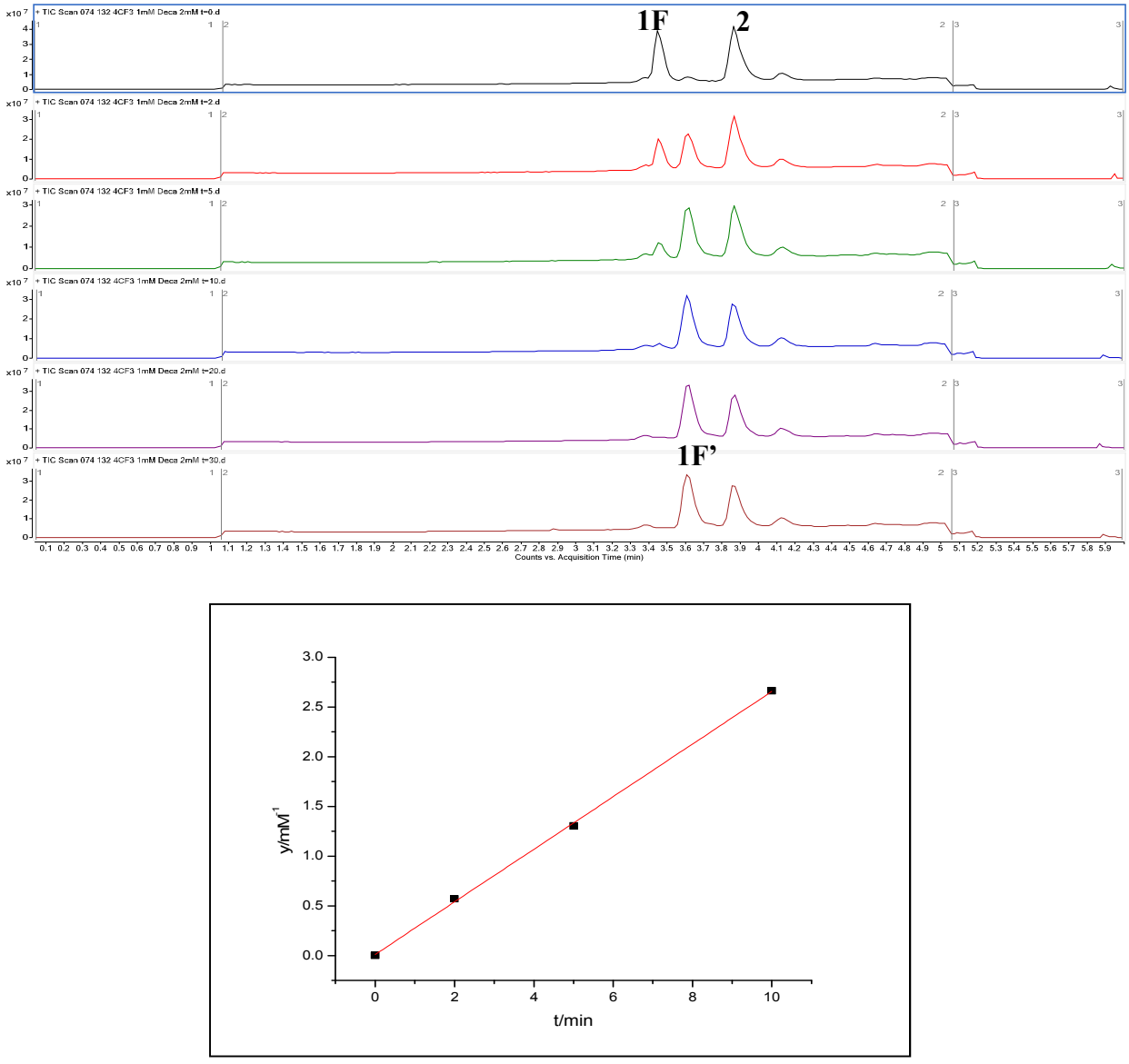


Figure S32. Kinetics analysis for peptide 1F reacting with probe 2. Top, LC-MS chromatograms for the reactions between peptide **1F** and probe **2** at different time points (minutes). Reaction conditions: 1 mM **1F**, 2 mM probe **2**, 200 mM phosphate, 20 mM TCEP, 37 °C. Bottom, linear fitting of the kinetics data to second-order rate equation. Slope = 0.26, R^2 = 0.9990. **1F'** refers to the product of the arylation reaction between **1F** and probe **2**.

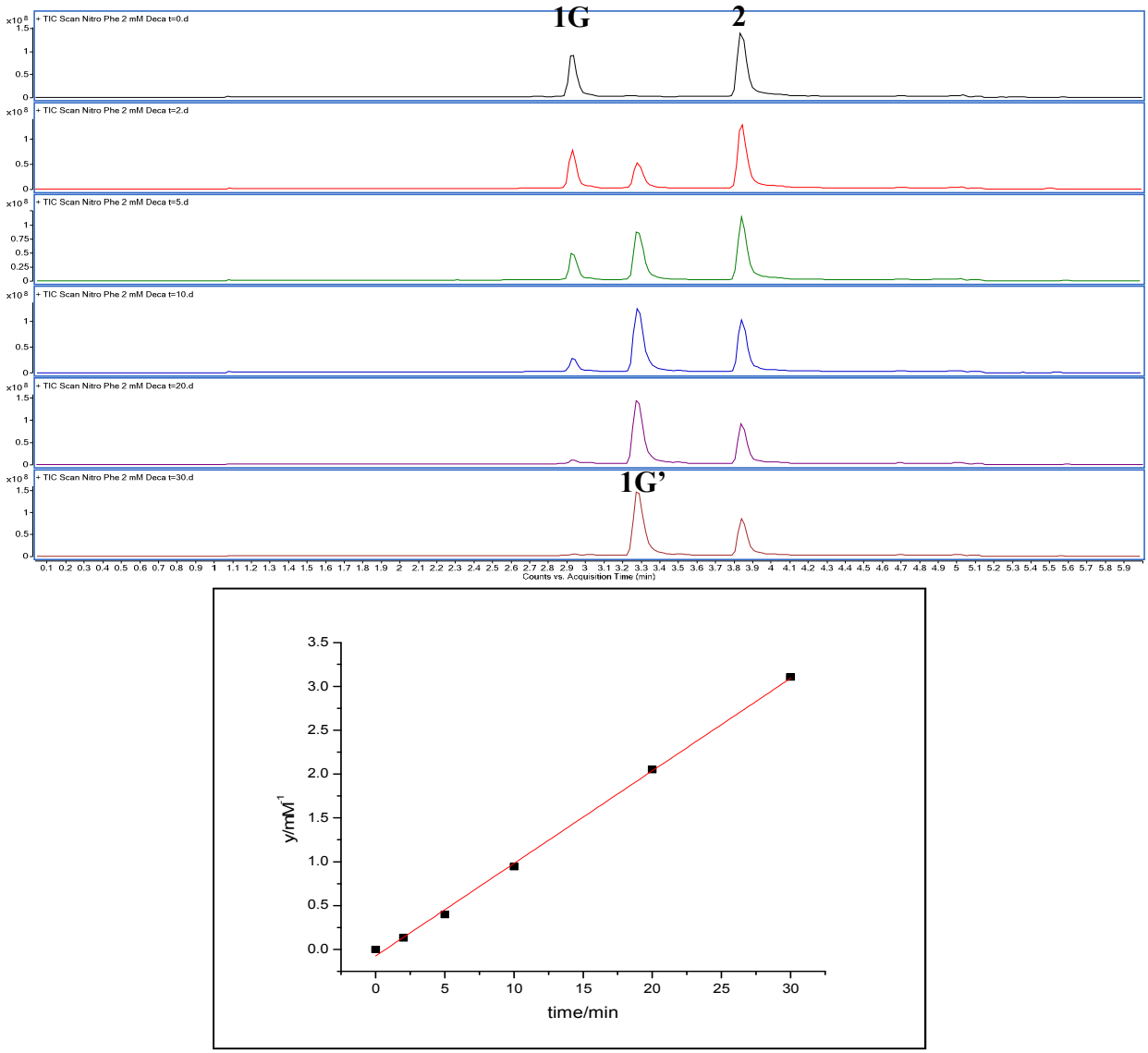
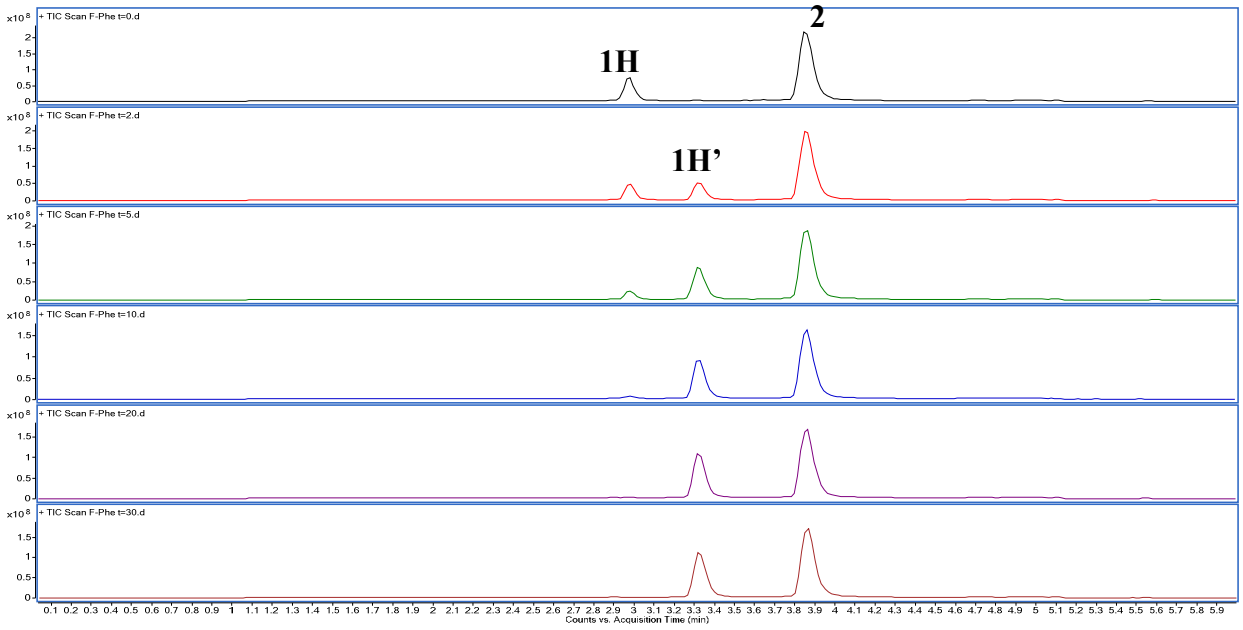


Figure S33. Kinetics analysis for peptide 1G reacting with probe 2. Top, LC-MS chromatograms for the reactions between peptide **1G** and probe **2** at different time points (minutes). Reaction conditions: 1 mM **1G**, 2 mM probe **2**, 200 mM phosphate, 20 mM TCEP, 37 °C. Bottom, linear fitting of the kinetics data to second-order rate equation. Slope = 0.105, R^2 = 0.998. **1G'** refers to the product of the arylation reaction between **1G** and probe **2**.



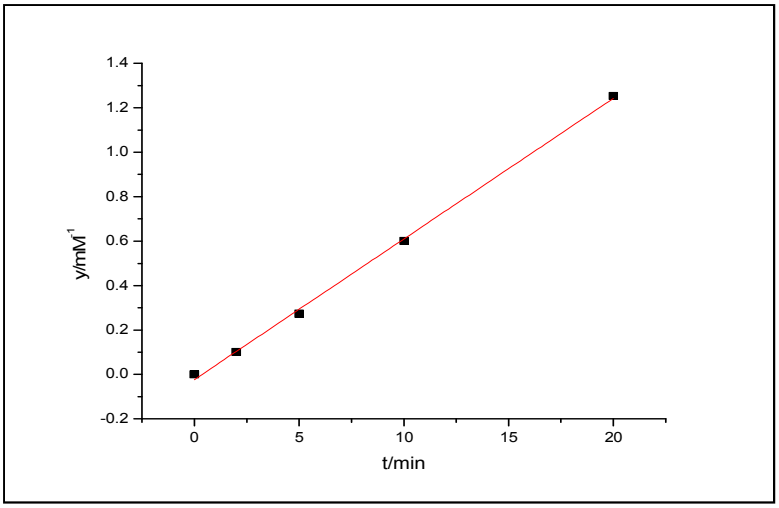
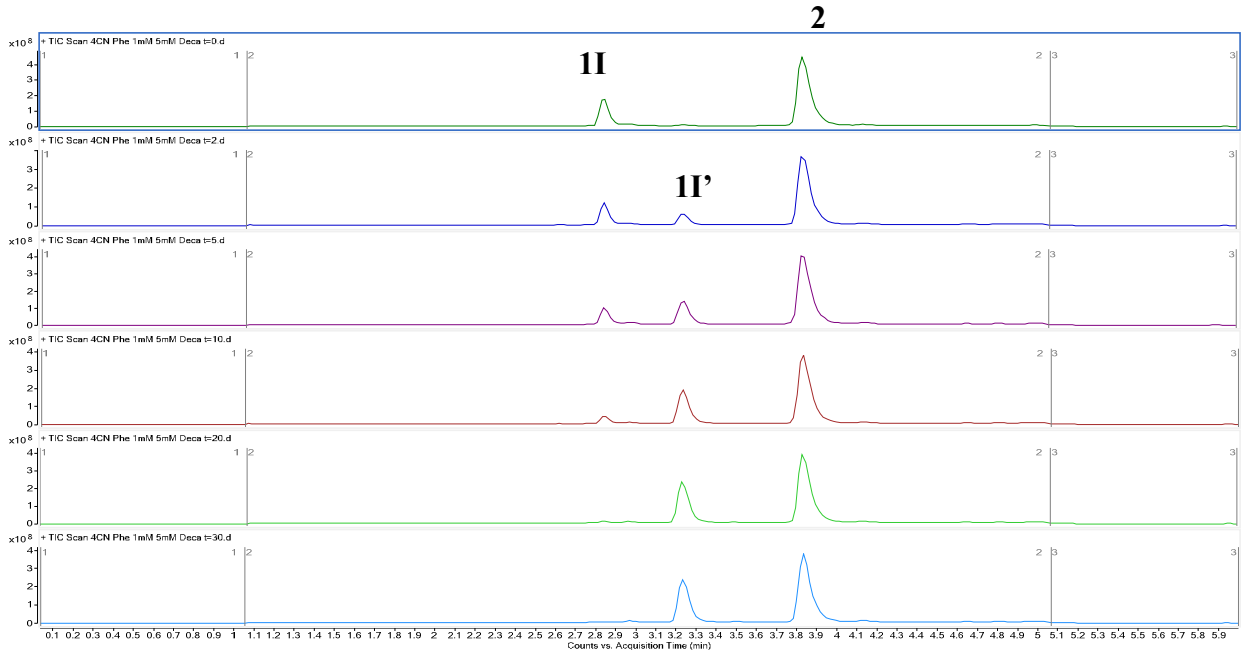


Figure S34. Kinetics analysis for peptide 1H reacting with probe 2. Top, LC-MS chromatograms for the reactions between peptide **1H** and probe **2** at different time points (minutes). Reaction conditions: 1 mM **1H**, 2 mM probe **2**, 200 mM phosphate, 20 mM TCEP, 37 °C. Bottom, linear fitting of the kinetics data to second-order rate equation. Slope = 0.063, R^2 = 0.998. **1H'** refers to the product of the arylation reaction between **1H** and probe **2**.



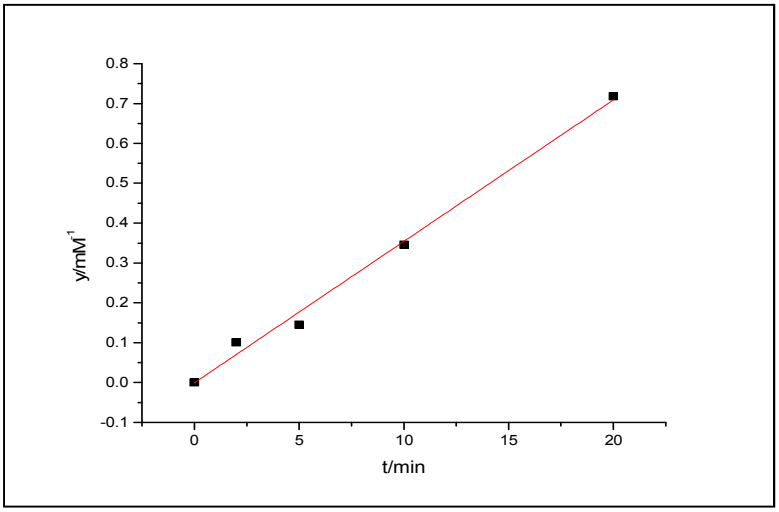


Figure S35. Kinetics analysis for peptide 1I reacting with probe 2. Top, LC-MS chromatograms for the reactions between peptide **1I** and probe **2** at different time points (minutes). Reaction conditions: 1 mM **1I**, 2 mM probe **2**, 200 mM phosphate, 20 mM TCEP, 37 °C. Bottom, linear fitting of the kinetics data to second-order rate equation. Slope = 0.035, R^2 = 0.991. **1I'** refers to the product of the arylation reaction between **1I** and probe **2**.

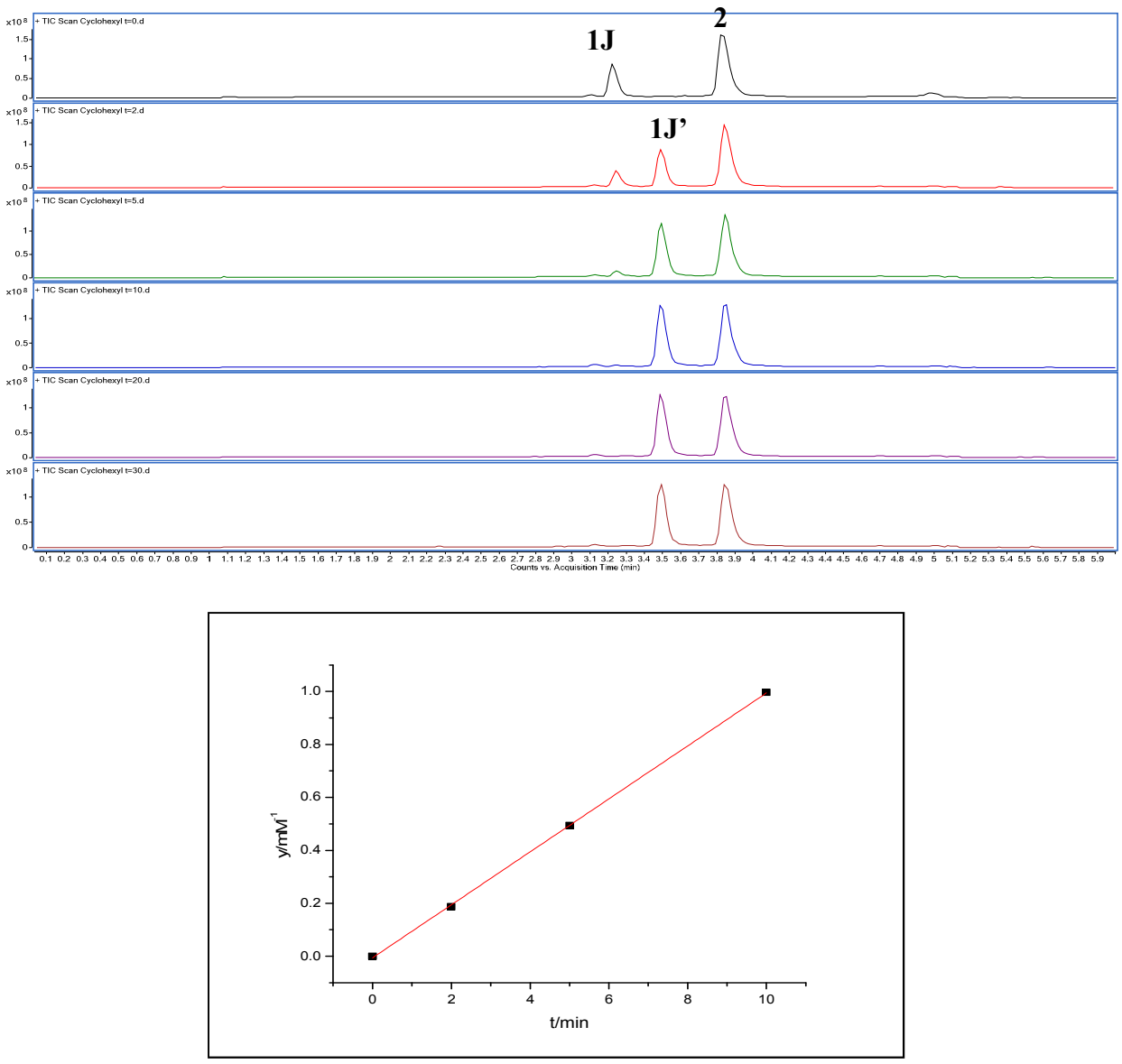
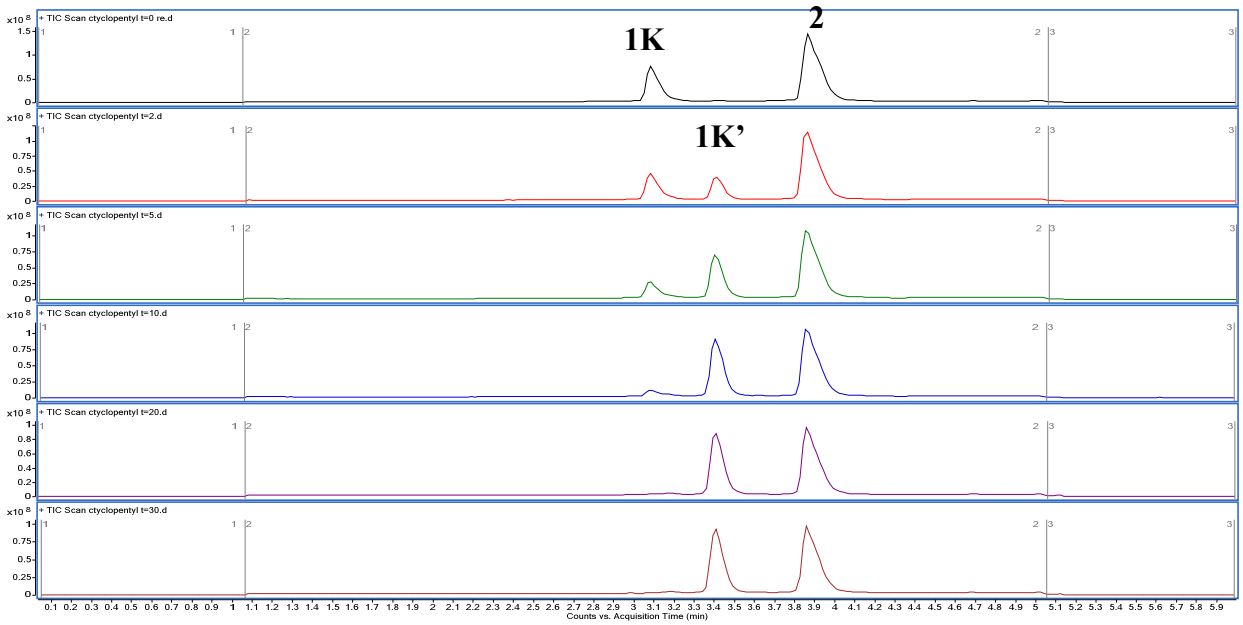


Figure S36. **Kinetics analysis for peptide 1J reacting with probe 2.** Top, LC-MS chromatograms for the reaction between peptide **1J** and probe **2** at different time points (minutes). Reaction conditions: 1 mM **1J**, 5 mM probe **2**, 200 mM phosphate, 20 mM TCEP, pH 8.0, 37°C. Bottom, linear fitting of the kinetics data to second-order rate equation. Slope = 0.10, R^2 = 0.990. **1J'** refers to the product of the arylation reaction between **1J** and probe **2**.



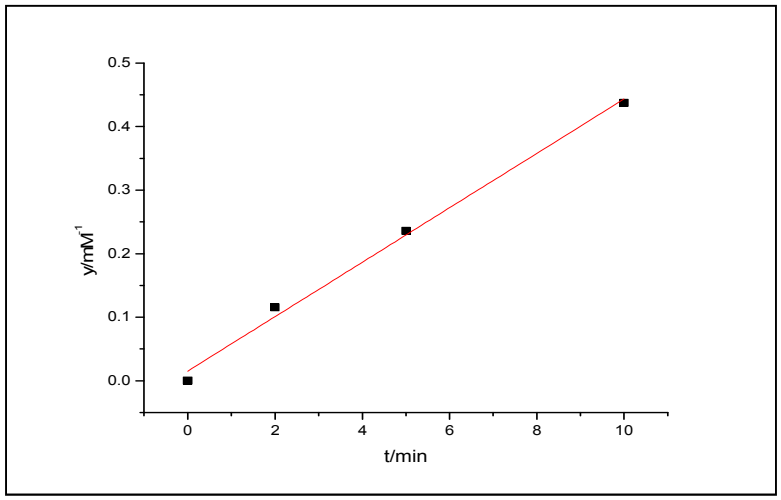
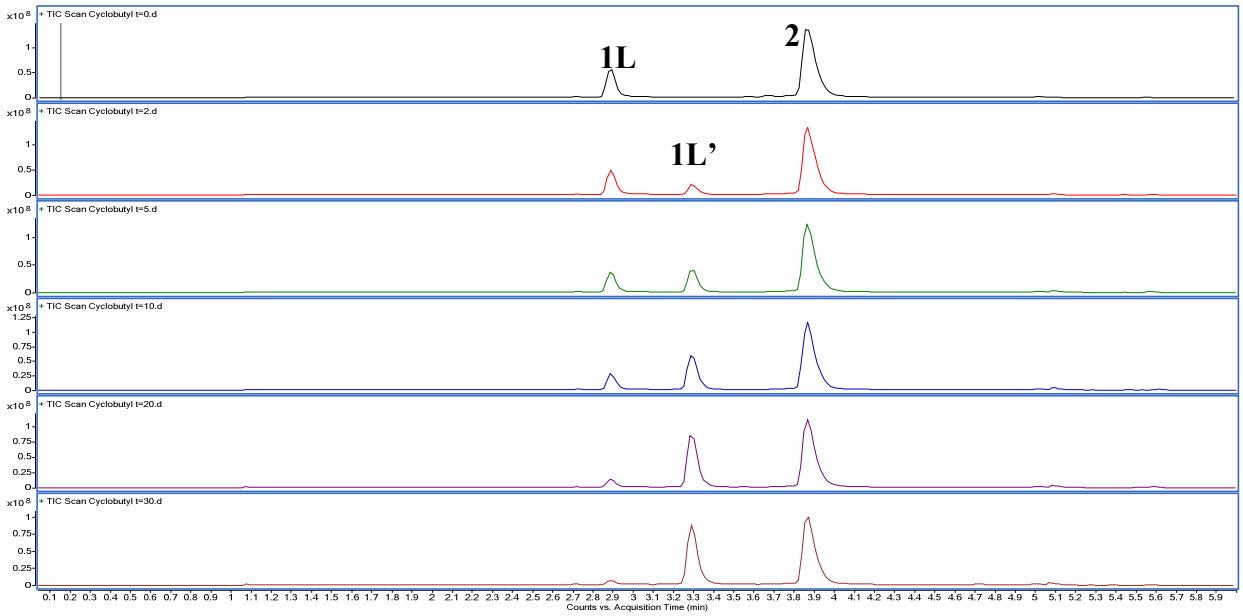


Figure S37. **Kinetics analysis for peptide 1K reacting with probe 2.** Top, LC-MS chromatograms for the reaction between peptide **1K** and probe **2** at different time points (minutes). Reaction conditions: 1 mM **1K**, 5 mM probe **2**, 200 mM phosphate, 20 mM TCEP, pH 8.0, 37°C. Bottom, linear fitting of the kinetics data to second-order rate equation. Slope = 0.043, R^2 = 0.992. **1K'** refers to the product of the arylation reaction between **1K** and probe **2**.



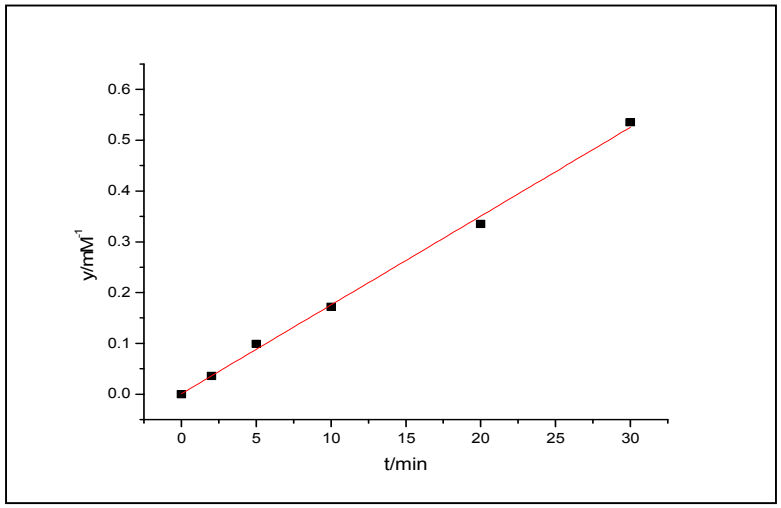
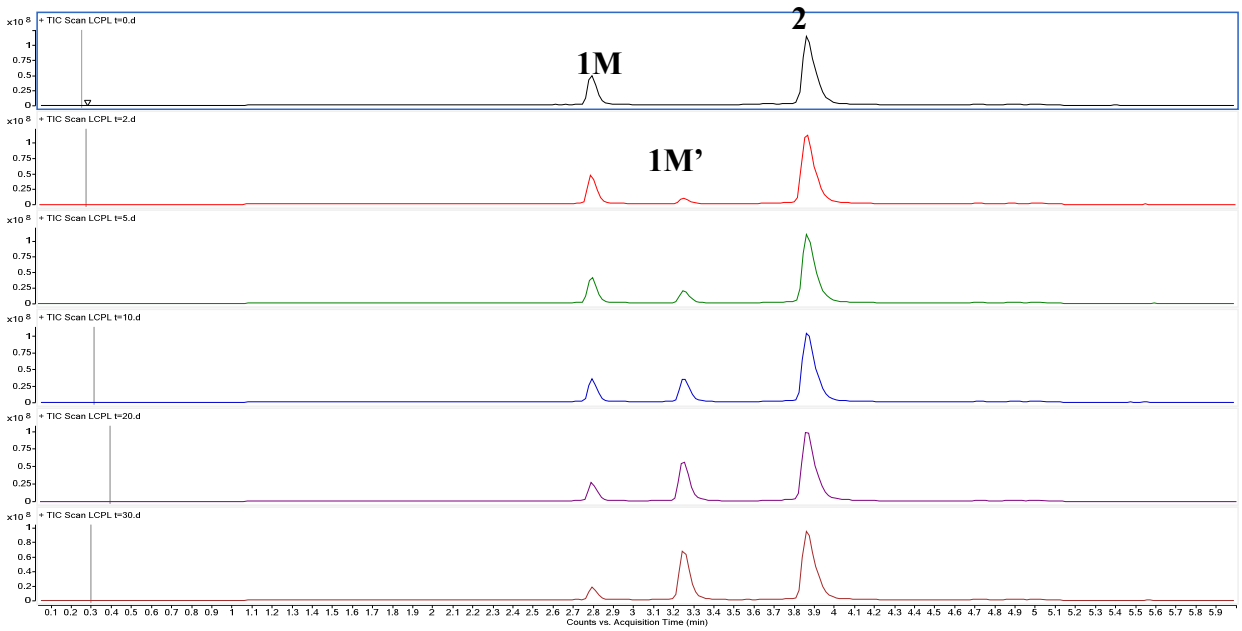


Figure S38. **Kinetics analysis for peptide 1L reacting with probe 2.** Top, LC-MS chromatograms for the reaction between peptide **1L** and probe **2** at different time points (minutes). Reaction conditions: 1 mM **1L**, 5 mM probe **2**, 200 mM phosphate, 20 mM TCEP, pH 8.0, 37°C. Bottom, linear fitting of the kinetics data to second-order rate equation. Slope = 0.017, R^2 = 0.997. **1L'** refers to the product of the arylation reaction between **1L** and probe **2**.



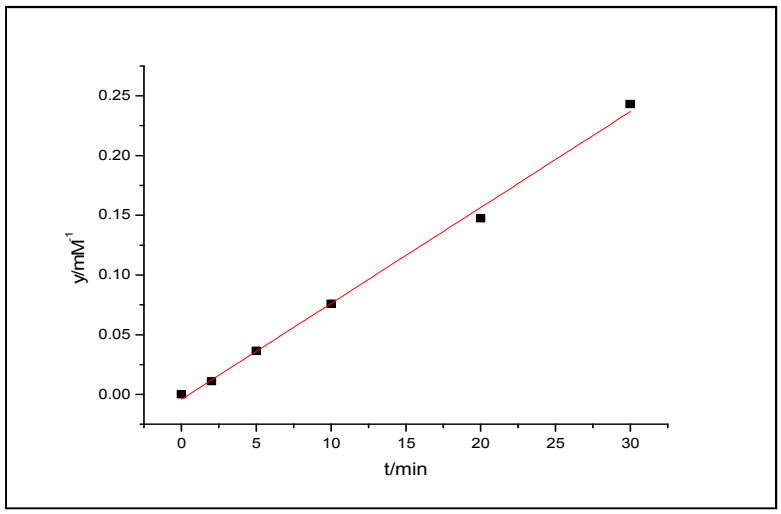
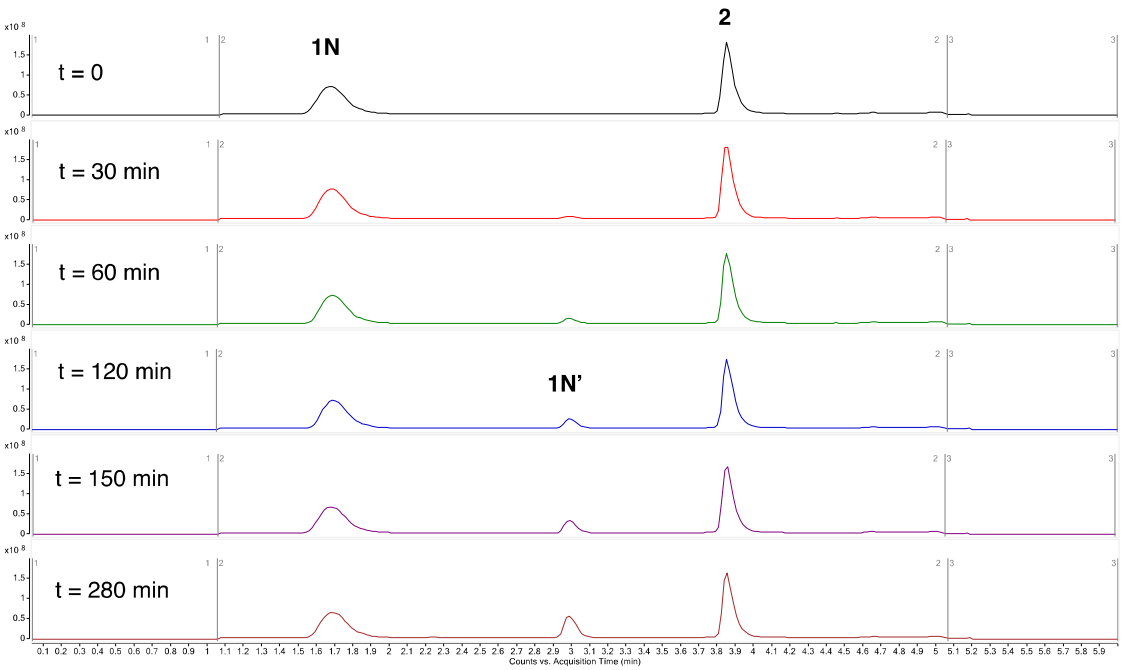


Figure S39. **Kinetics analysis for peptide 1M reacting with probe 2.** Top, LC-MS chromatograms for the reaction between peptide **1M** and probe **2** at different time points (minutes). Reaction conditions: 1 mM **1M**, 5 mM probe **2**, 200 mM phosphate, 20 mM TCEP, pH 8.0, 37°C. Bottom, linear fitting of the kinetics data to second-order rate equation. Slope = 0.008, R^2 = 0.996. **1M'** refers to the product of the arylation reaction between **1M** and probe **2**.



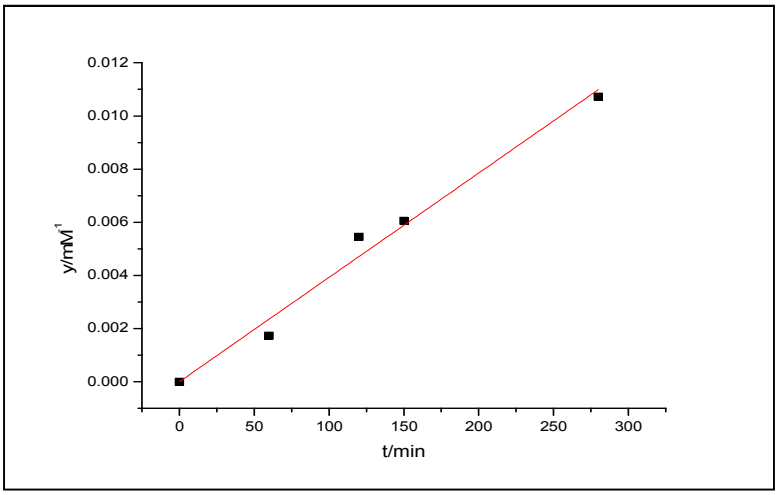


Figure S40. **Kinetics analysis for peptide 1N reacting with probe 2.** Top, LC-MS chromatograms for the reaction between peptide **1N** and probe **2** at different time points (0, 30, 60, 120, 150, 280 min). Reaction conditions: 16.7 mM **1N**, 11.1 mM probe **2**, 200 mM phosphate, 20 mM TCEP, pH 8.0, 37°C. Bottom, linear fitting of the kinetics data to second-order rate equation. Slope = 0.00004, R^2 = 0.98. **1N'** refers to the product of the arylation reaction between **1N** and probe **2**.

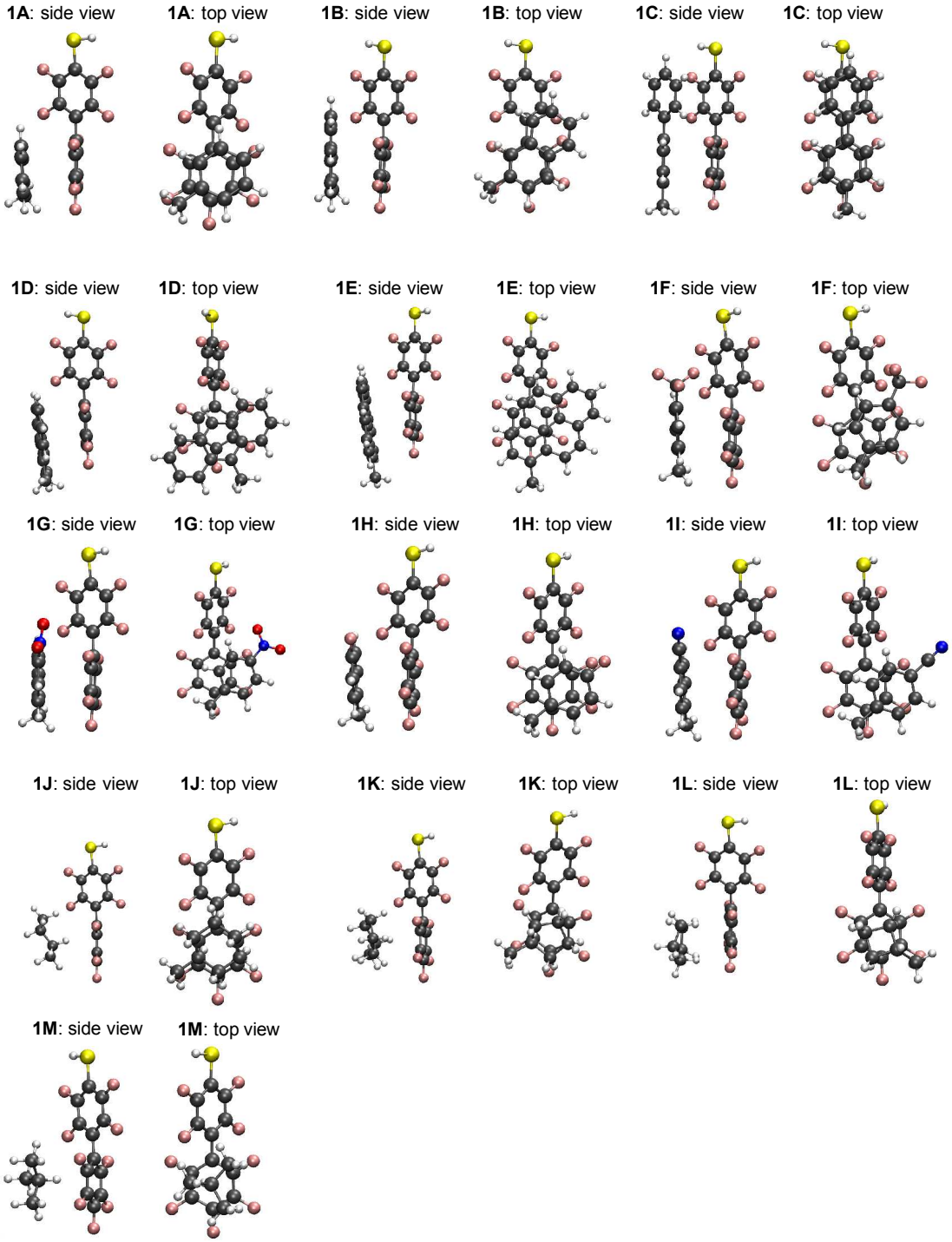


Figure S41. The lowest energy structures for π-clamp/π-clamp variants (1A-1M) side chain-perfluoroaryl interaction. Optimization was initialized from the six possible stacked geometries, and from these calculations one optimal structure was selected (shown above). Details of the initial geometries and of this selection are given in the Methods Section. These optimized structures were used in the following DFT calculation to obtain the binding energy between side chain and perfluoroaryl moiety.

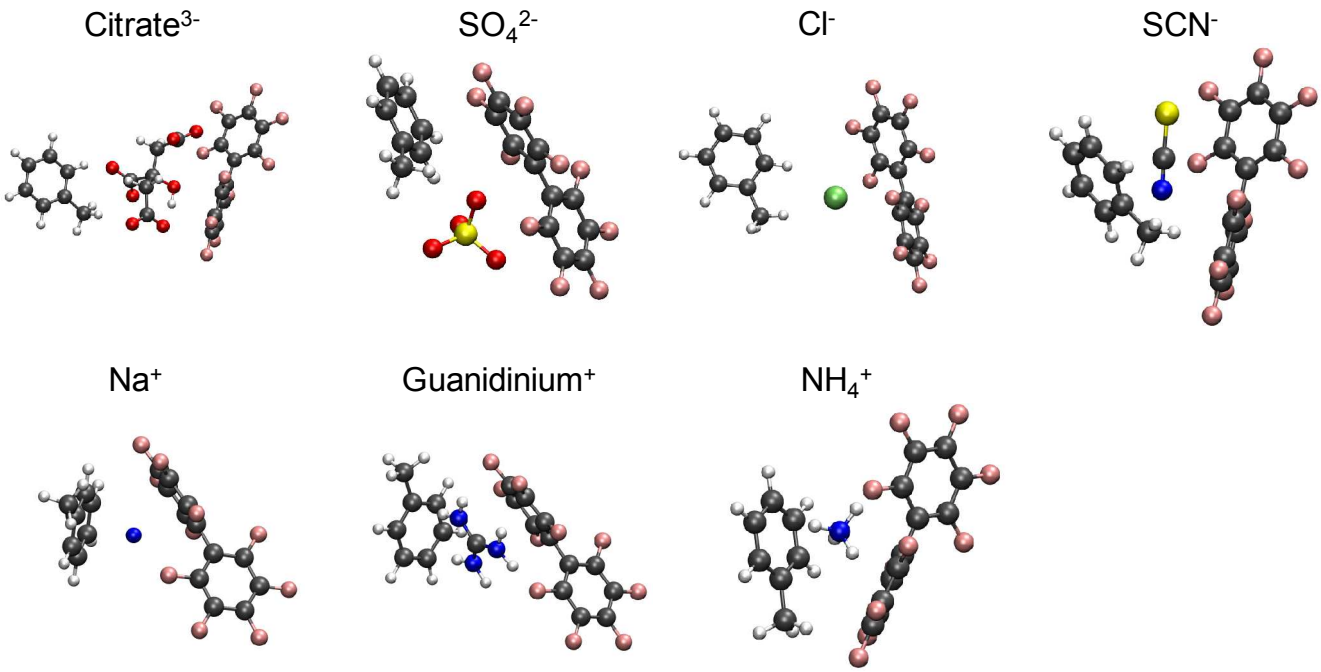
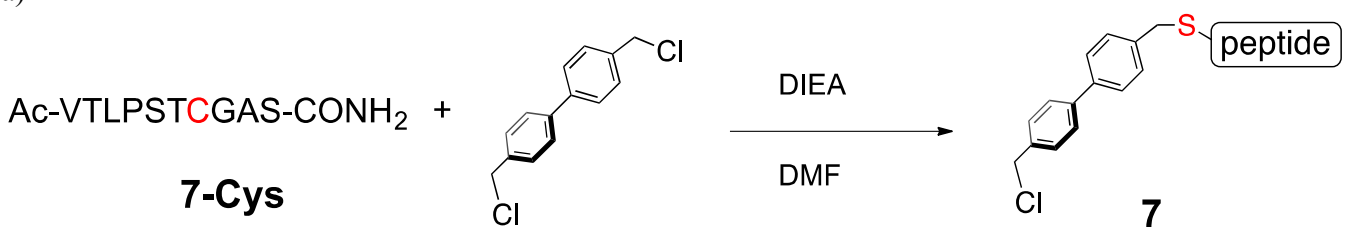


Figure S42. The lowest energy structure for 1A side chain-perfluoroaryl binding with ion. Optimization with ion started with one initial structure: a sandwich of the ion between the phenylalanine side chain and perfluoroaryl moiety, the details of which were described in Methods Section. The optimized structures are shown above.

a)



b)

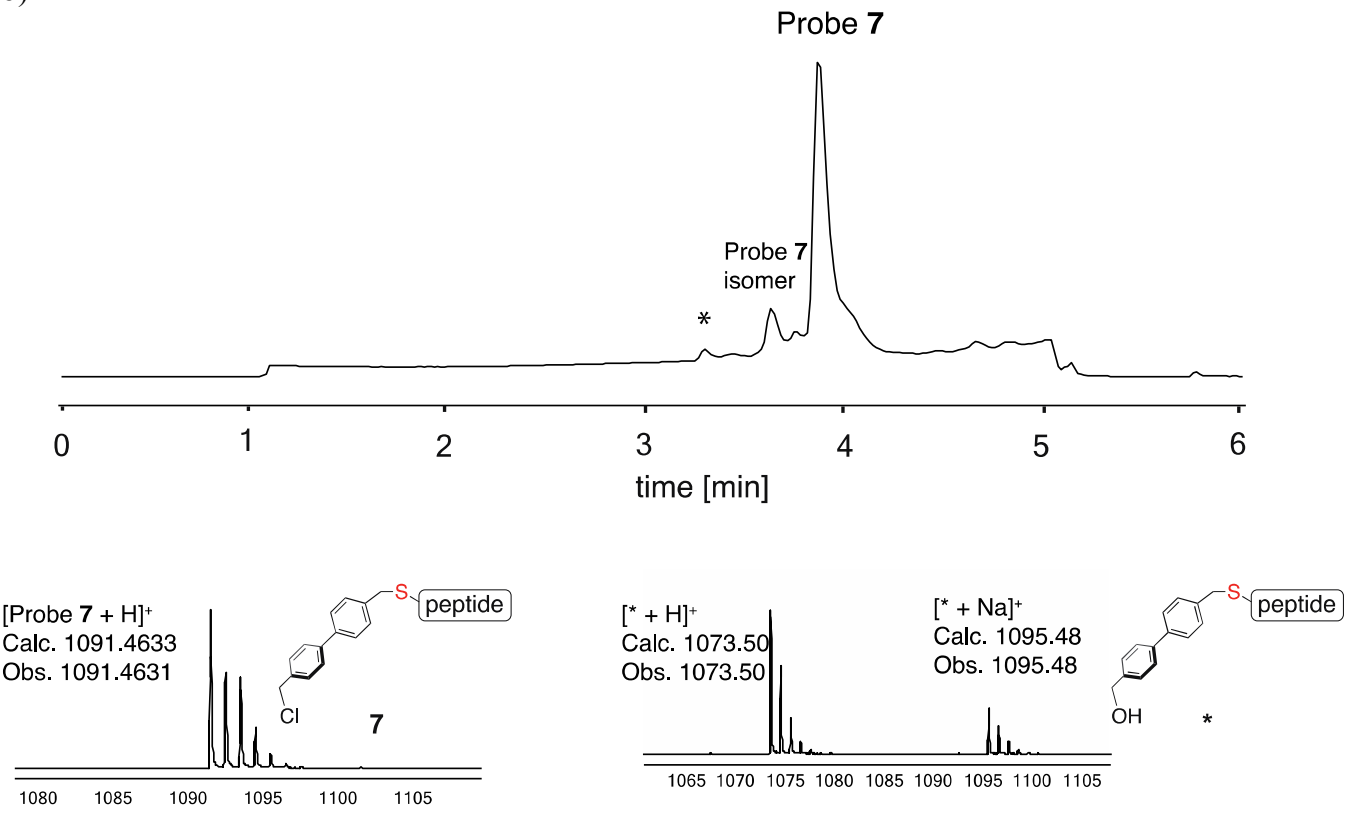


Figure S43. Preparation of probe 7. a) Scheme for the reaction. b) LC-HRMS analysis of the purified probe **7**. * indicates the hydrolyzed probe **7**.

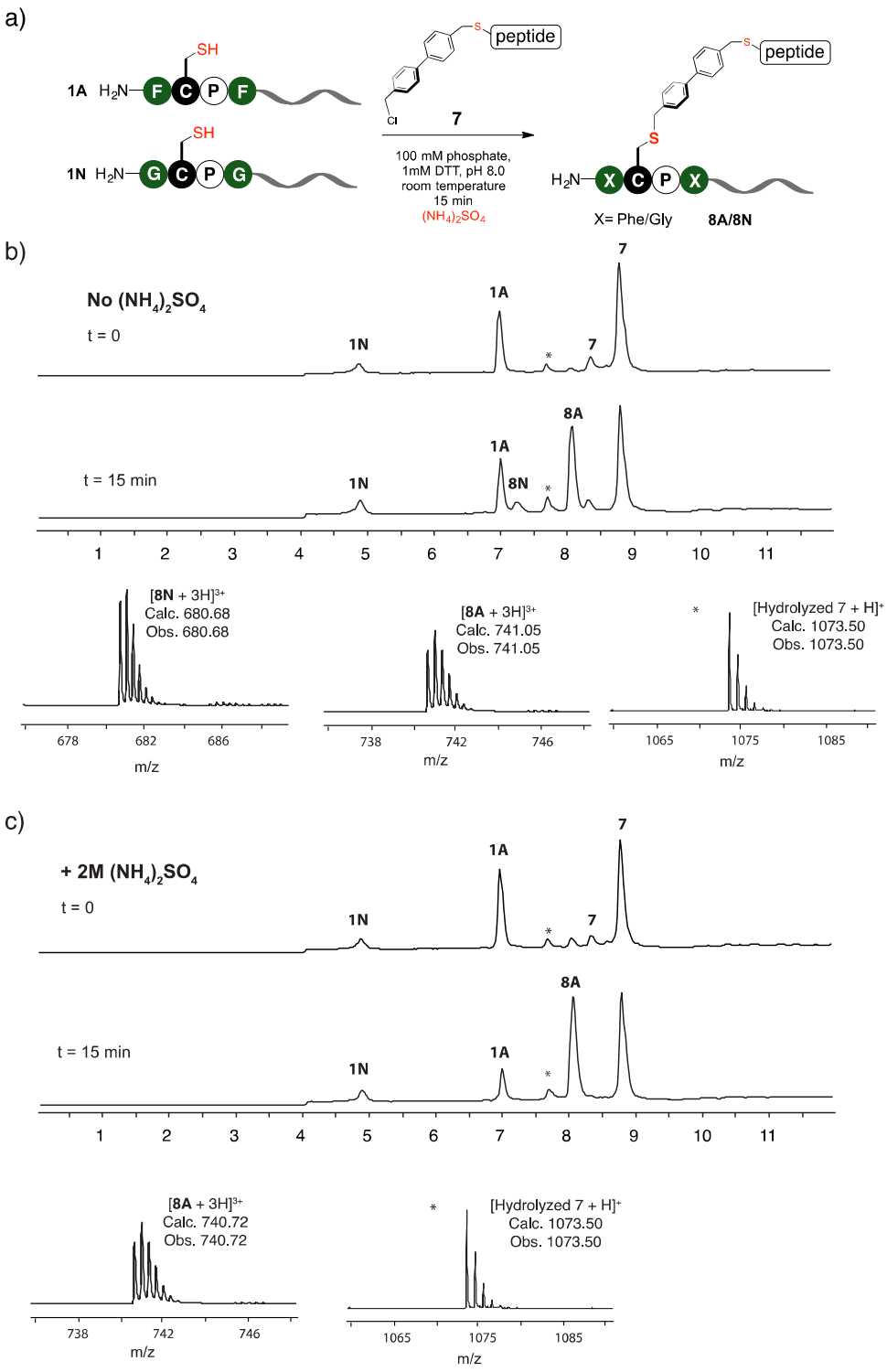


Figure S44. Competing alkylation reaction between 1A and 1N using probe 7 revealed selectivity. a) Reaction scheme. Reaction conditions: 0.1 mM **1A**, 0.1 mM **1N**, 1 mM DTT, 0.5 mM probe **7**, 100 mM phosphate, 0.25% DMSO, pH 8.0, with or without 2 M ammonium sulfate, room temperature, 15 min. b) LC-MS analysis of the reaction mixture without the addition of ammonium sulfate. c) LC-MS analysis of the reaction mixture with the addition of 2 M ammonium sulfate.

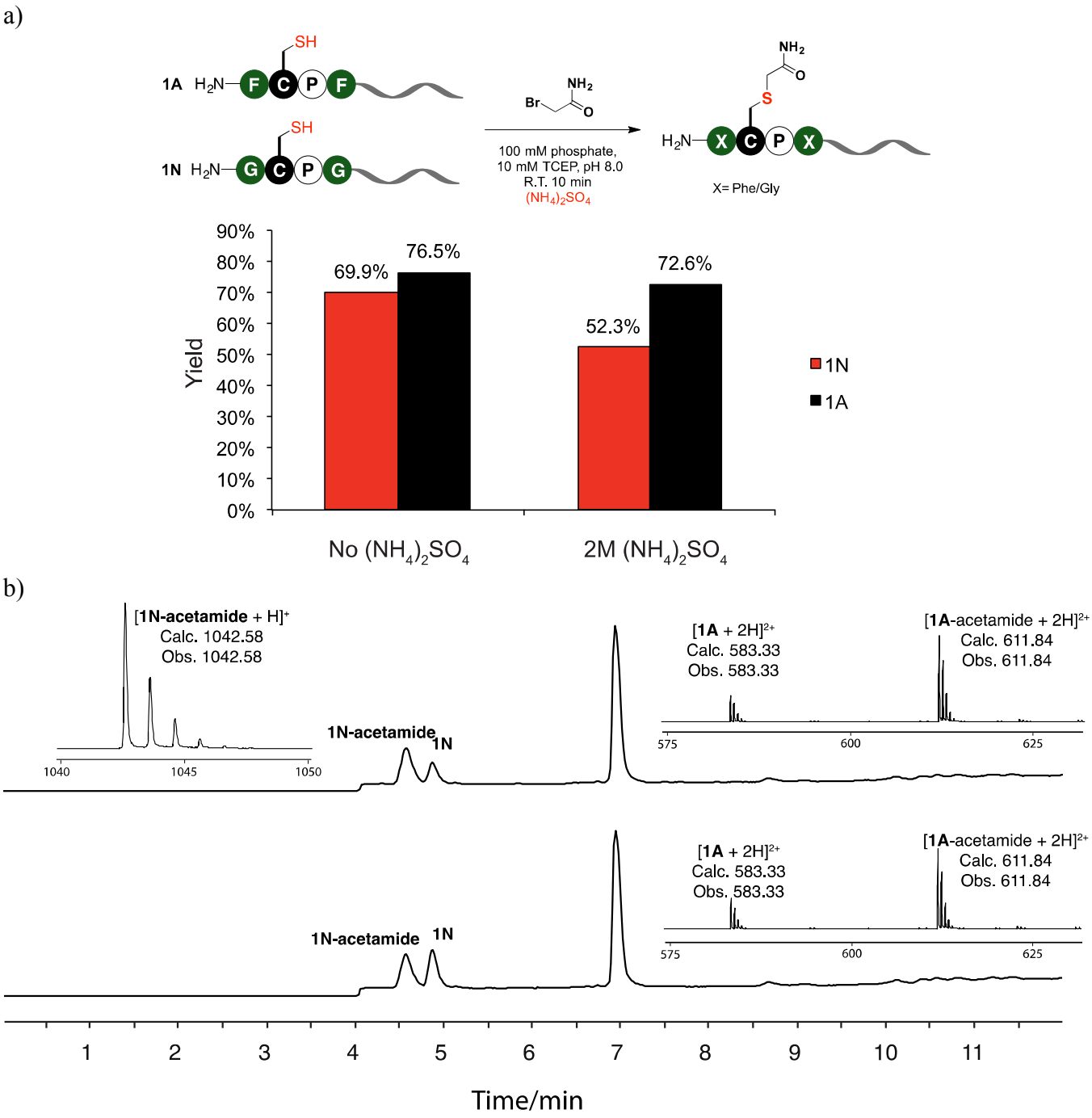


Figure S45. Competing alkylation reaction between 1A and 1N using bromoacetamide. a) Reaction scheme. Reaction conditions: 0.5 mM **1A**, 0.5 mM **1N**, 10 mM TCEP, 2.5 mM bromoacetamide, 100 mM phosphate, pH 8.0, room temperature, 10 min. Since **1A** and **1A-acetamide** coeluted in LC-MS analysis, the yield was calculated based on the relative intensities of **1A** and **1A-acetamide** in mass spectra. The yield of the reaction with or without 2 M ammonium sulfate was summarized in the bar graph. b) LC-MS analysis of the reaction mixture with or without the addition of ammonium sulfate.

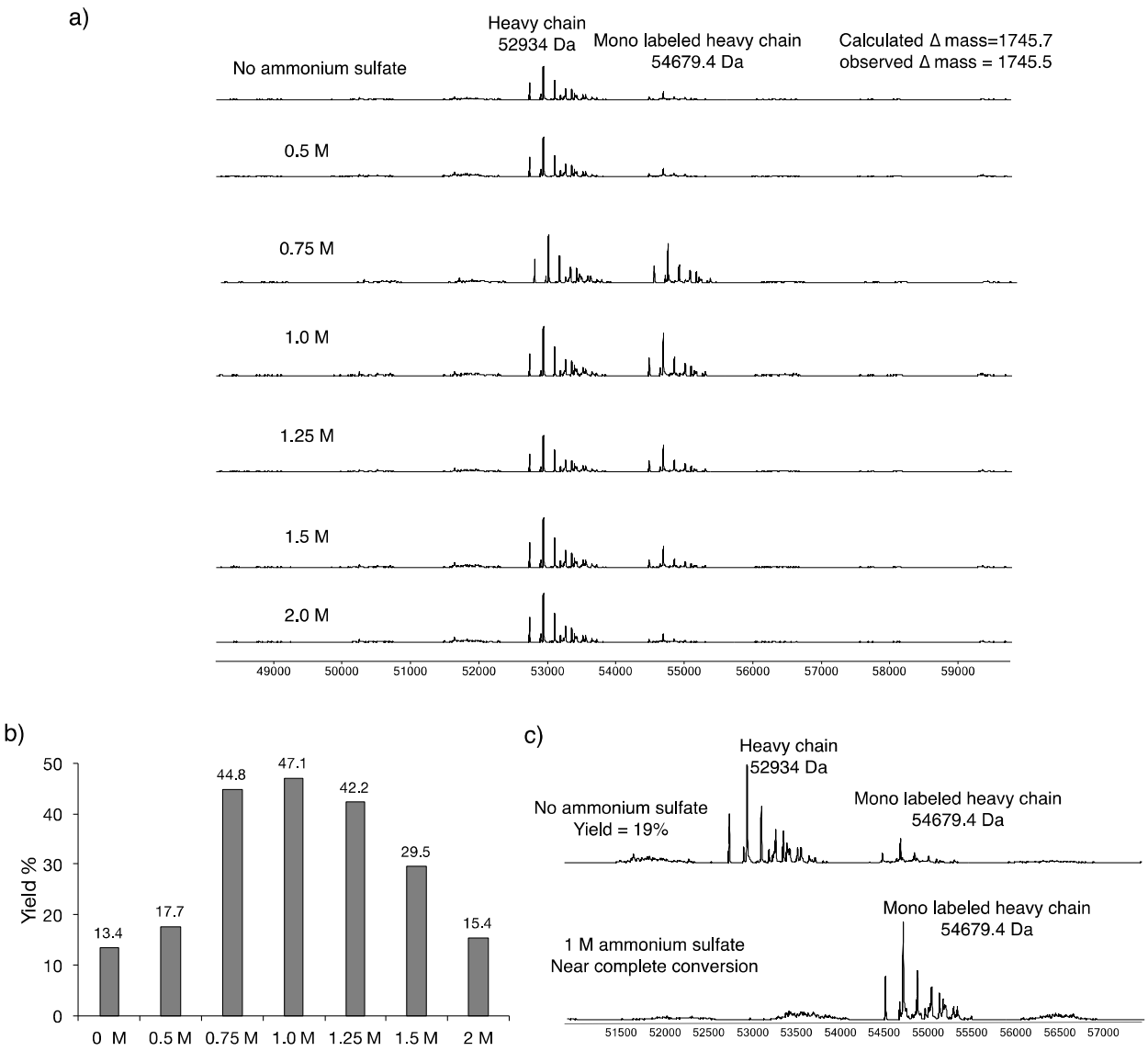
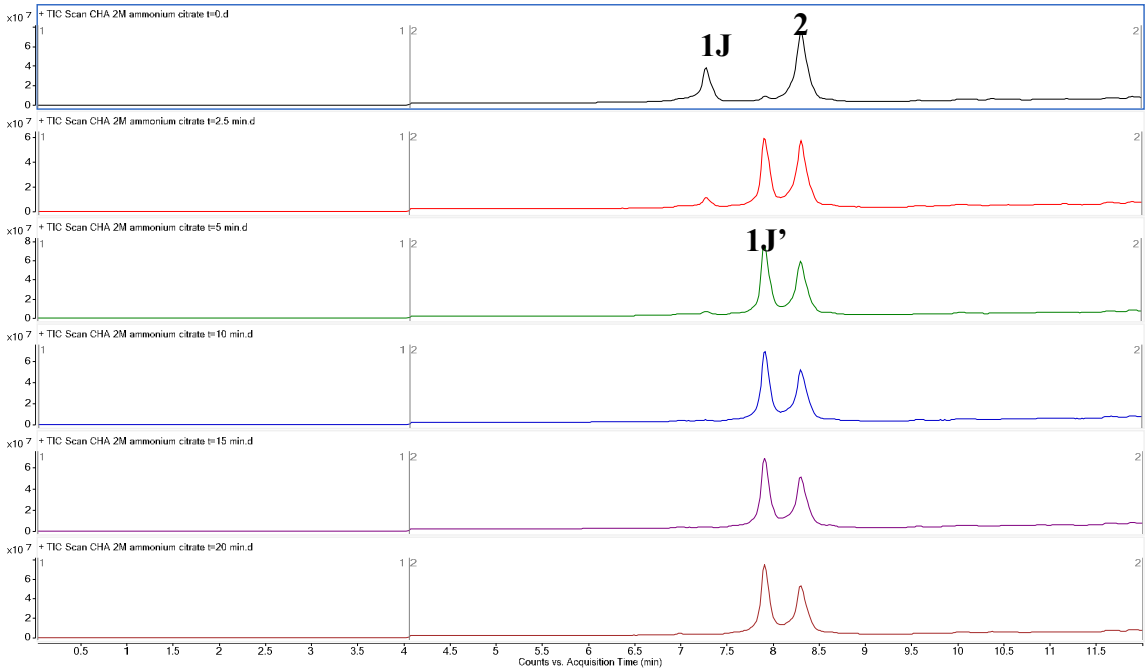
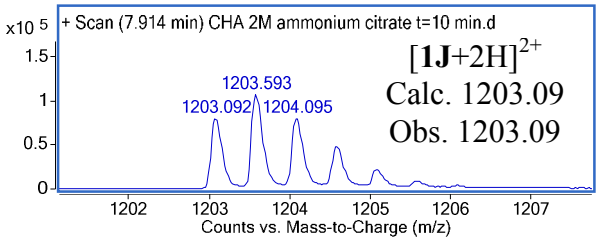


Figure S46. Ammonium sulfate promoted site-selective labeling of π-clamp C225 antibody. a) Ammonium sulfate concentration optimization. Reaction conditions: 40 μM π-clamp C225, 500 μM probe **6**, 100 mM phosphate, 10 mM TCEP, 37 °C, pH 8.0, with different amount of ammonium sulfate, 80 min. Previous study observed heterogeneous C225 heavy chain even after removing N-glycosylation with PNGase F[3]. So here, the reactions were quenched by adding 20-fold volume of H_2O containing 0.1% TFA and subjected to LC-MS analysis without treatment with PNGase F. b) Yield summary for the reactions. 1 M ammonium sulfate was the best salt concentration for this specific reaction condition. c) Deconvoluted mass spectra for π-clamp C225 antibody labeling. Reaction conditions: 40 μM π-clamp C225, 500 μM probe **6**, 100 mM phosphate, 10 mM TCEP, 37 °C, pH 8.0, 1 M ammonium sulfate, 3 hours.

a)





b)

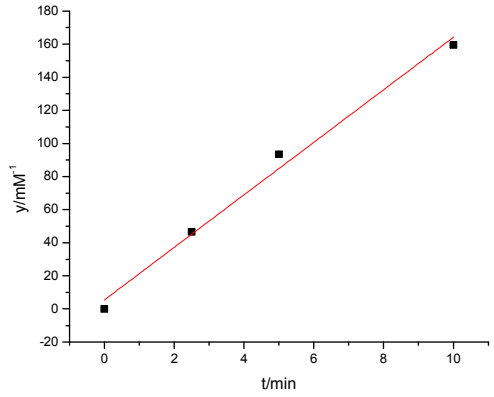


Figure S47. Kinetics analysis for peptide 1J reacting with probe 2 with 2M ammonium citrate. a) LC-MS chromatograms for the reaction between peptide **1J** and probe **2** with 2 M ammonium citrate at different time points (minutes). Reaction conditions: 25 μM **1J**, 50 μM probe **2**, 200 mM phosphate, 20 mM TCEP, pH 8.0, 37°C. b) Linear fitting of the kinetics data to second-order rate equation. Slope = 15.9, R^2 = 0.98. **1J'** refers to the product of the arylation reaction between **1J** and probe **2**.

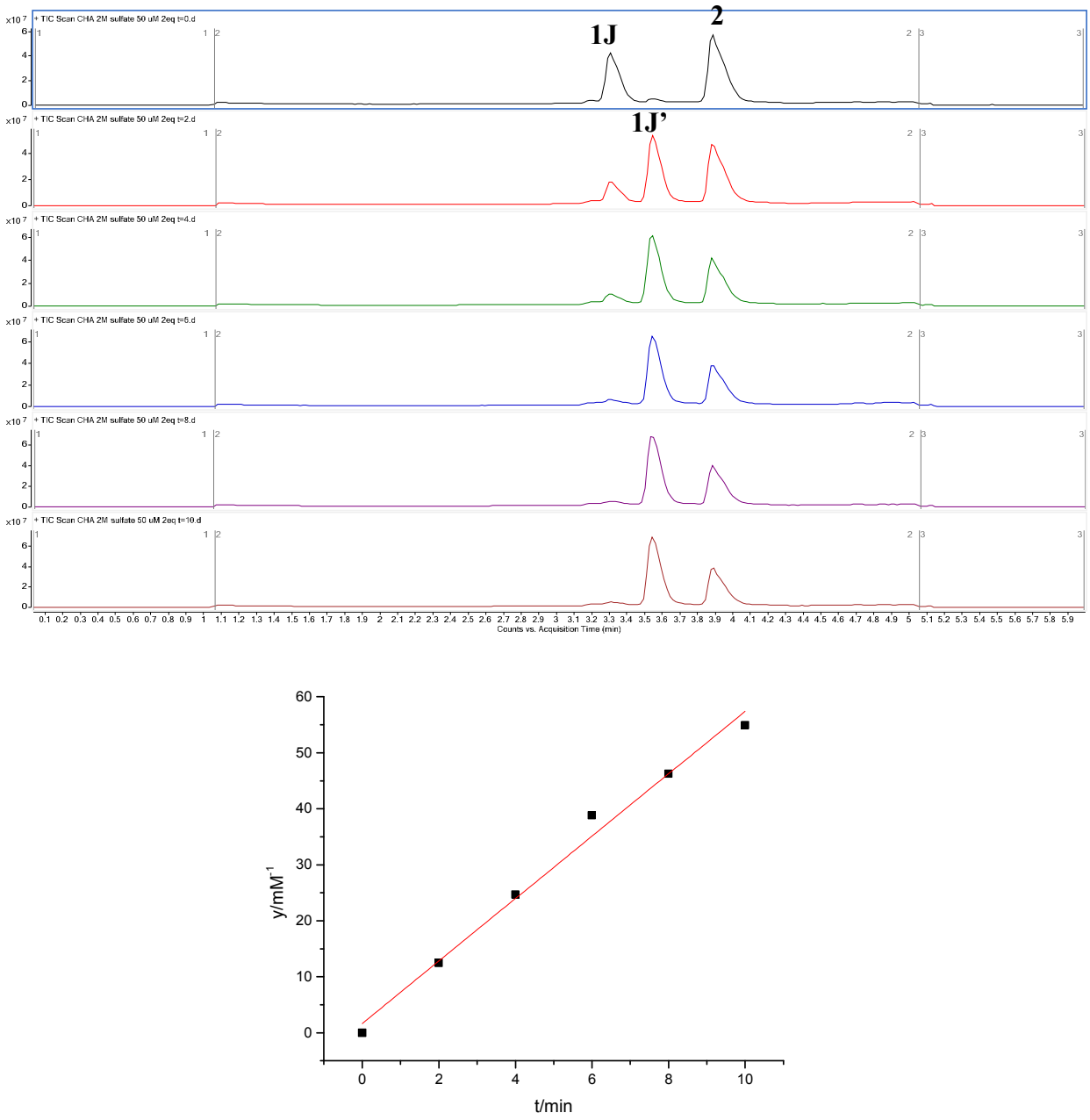


Figure S48. **Kinetics analysis for peptide 1J reacting with probe 2 with 2M ammonium sulfate.** Top, LC-MS chromatograms for the reaction between peptide **1J** and probe **2** with 2 M ammonium sulfate at different time points (minutes). Reaction conditions: 50 μM **1J**, 100 μM probe **2**, 200 mM phosphate, 20 mM TCEP, pH 8.0, 37°C. Bottom, linear fitting of the kinetics data to second-order rate equation. Slope = 5.57, R^2 = 0.98. **1J'** refers to the product of the arylation reaction between **1J** and probe **2**.

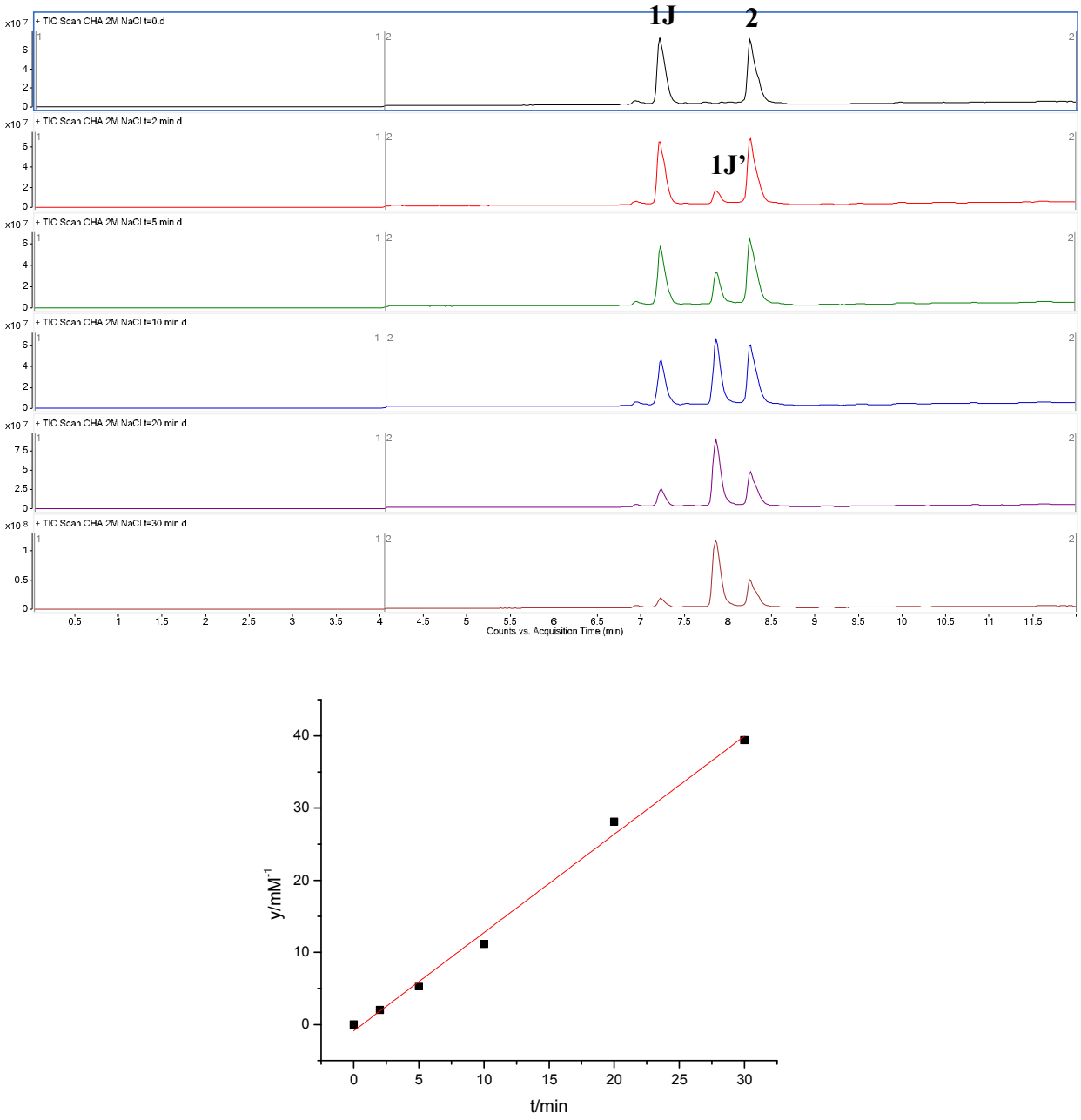


Figure S49. **Kinetics analysis for peptide 1J reacting with probe 2 with 2M NaCl.** Top, LC-MS chromatograms for the reaction between peptide **1J** and probe **2** with 2 M NaCl at different time points (minutes). Reaction conditions: 100 μM **1J**, 200 μM probe **2**, 200 mM phosphate, 20 mM TCEP, pH 8.0, 37°C. Bottom, linear fitting of the kinetics data to second-order rate equation. Slope = 1.36, R^2 = 0.993. **1J'** refers to the product of the arylation reaction between **1J** and probe **2**.

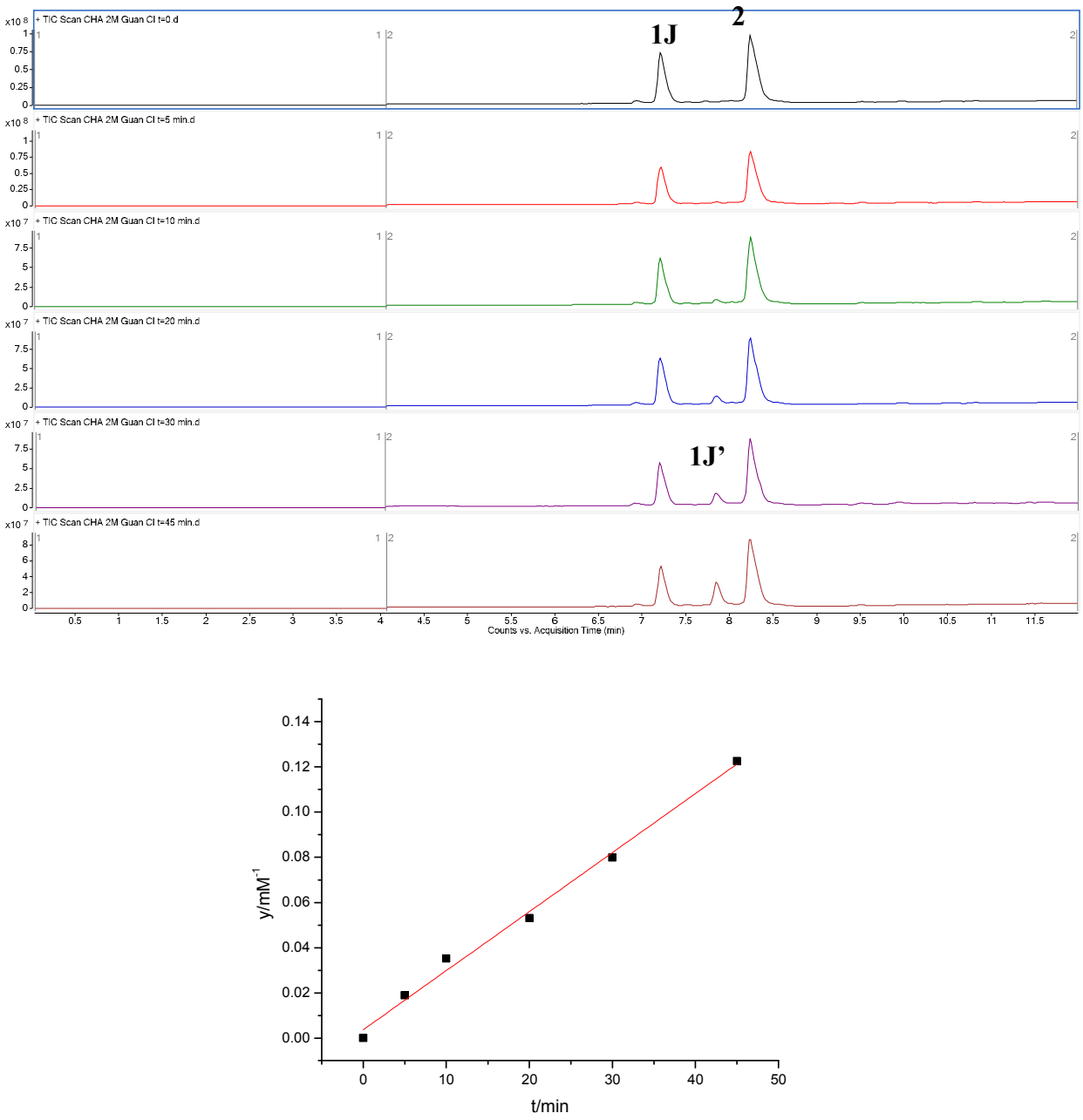
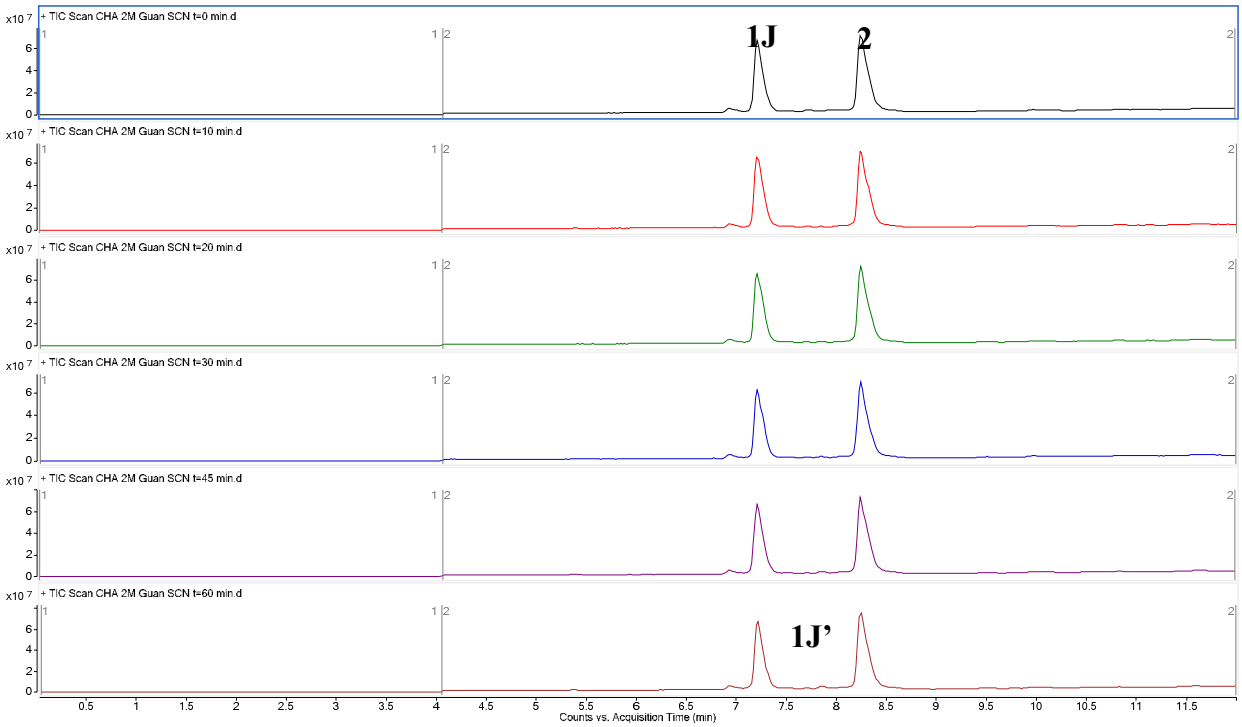


Figure S50. **Kinetics analysis for peptide 1J reacting with probe 2 with 2M guanidinium chloride.** Top, LC-MS chromatograms for the reaction between peptide **1J** and probe **2** with 2 M guanidinium chloride at different time points (minutes). Reaction conditions: 1 mM **1J**, 3 mM probe **2**, 200 mM phosphate, 20 mM TCEP, pH 8.0, 37°C. Bottom, linear fitting of the kinetics data to second-order rate equation. Slope = 0.0026, R^2 = 0.992. **1J'** refers to the product of the arylation reaction between **1J** and probe **2**.



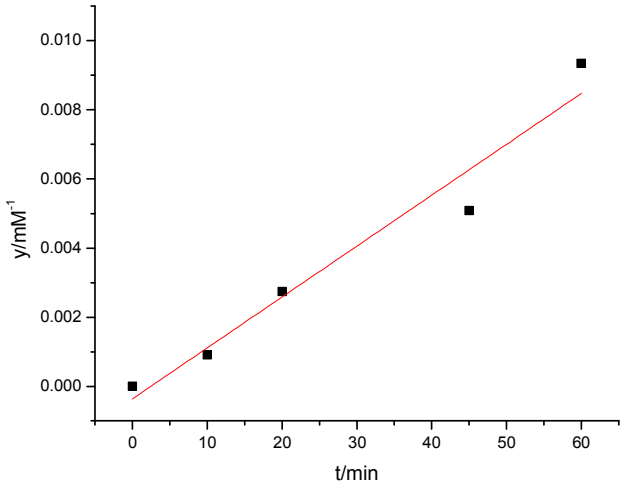


Figure S51. **Kinetics analysis for peptide 1J reacting with probe 2 with 2M guanidinium thiocyanate.** Top, LC-MS chromatograms for the reaction between peptide **1J** and probe **2** with 2 M guanidinium thiocyanate at different time points (minutes). Reaction conditions: 1 mM **1J**, 3 mM probe **2**, 200 mM phosphate, 20 mM TCEP, pH 8.0, 37°C. Bottom, linear fitting of the kinetics data to second-order rate equation. Slope = 0.00015, R^2 = 0.94. **1J'** refers to the product of the arylation reaction between **1J** and probe **2**.

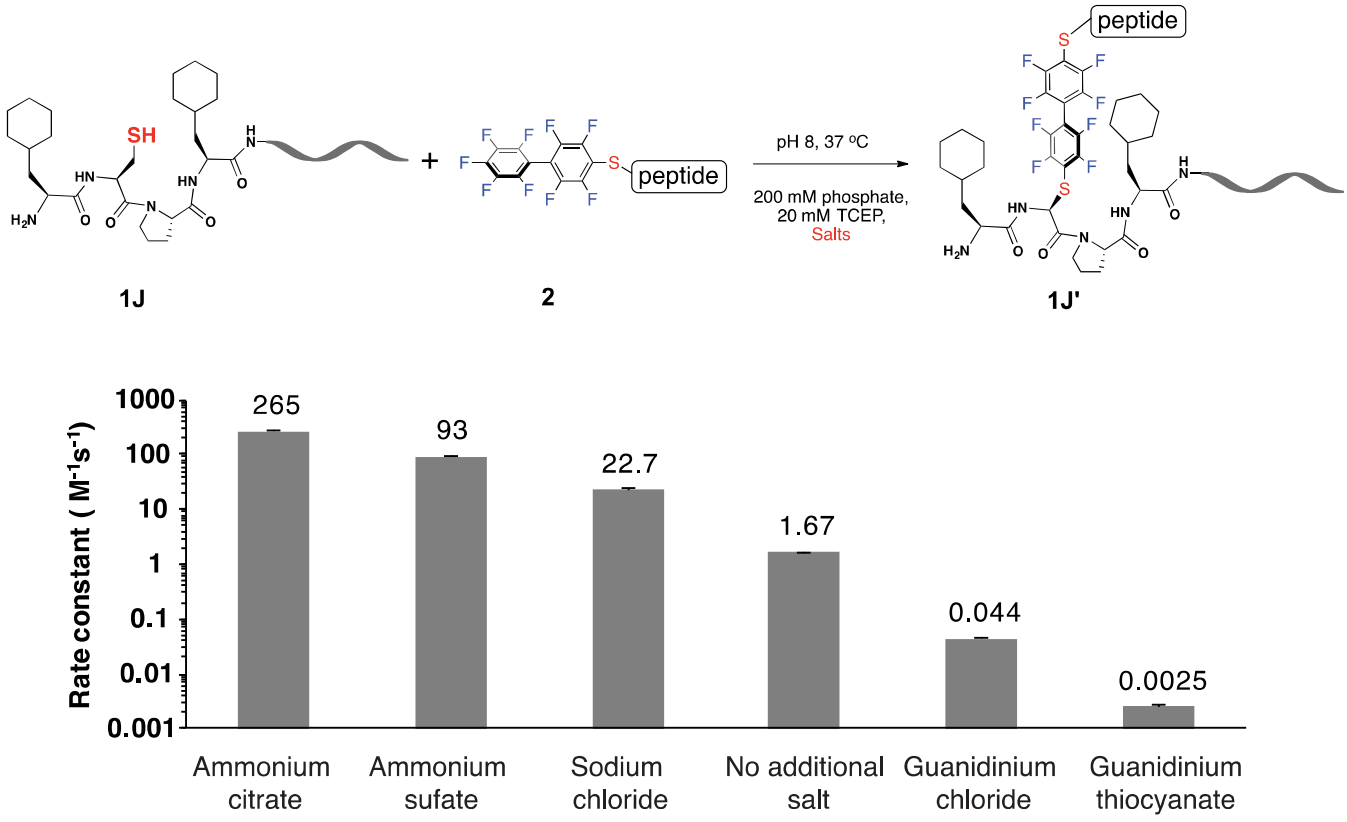


Figure S52. 2 M different salts tune the arylation reaction rate between the π-clamp variant peptide 1J and perfluoroaryl probe 2.

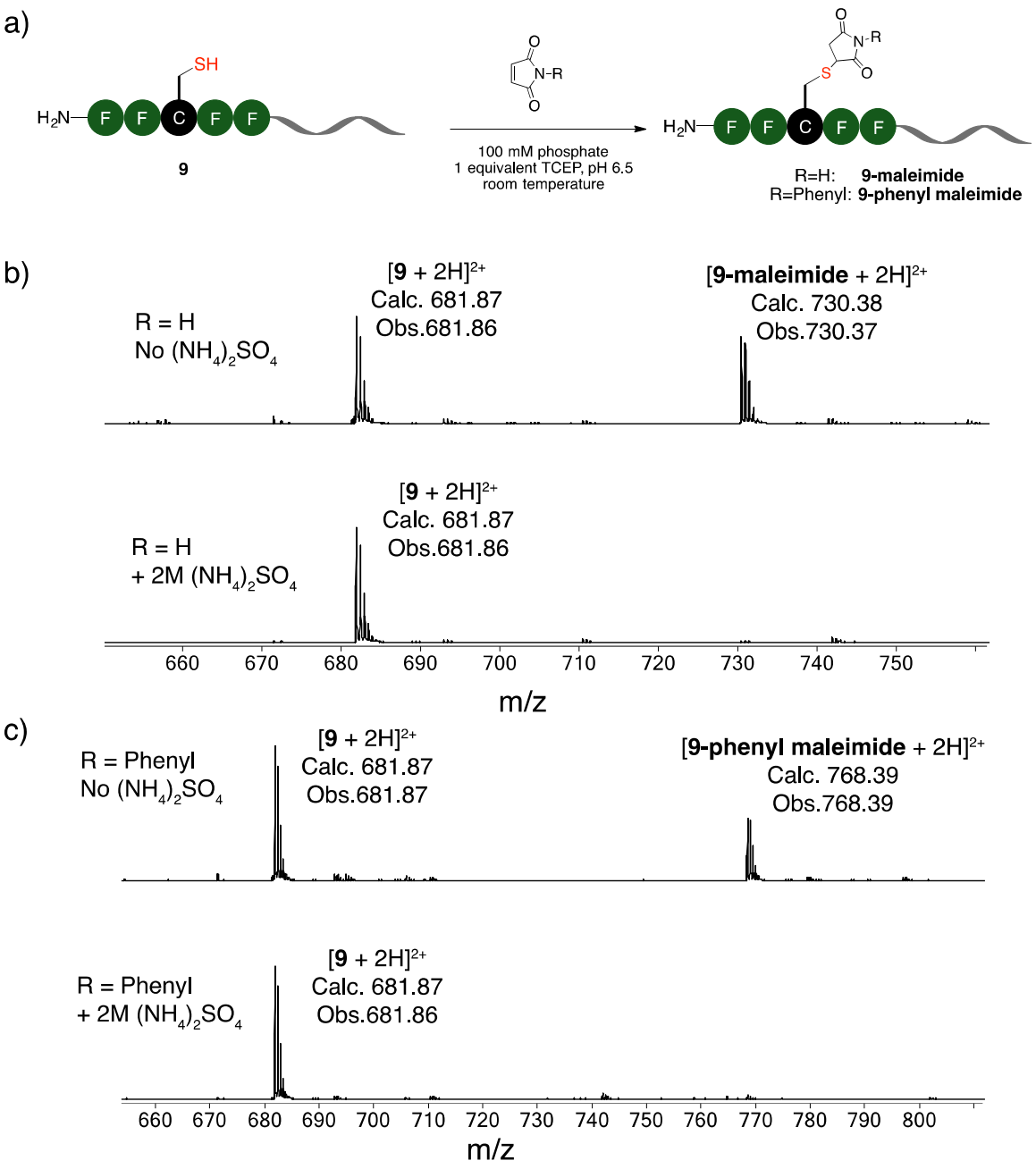


Figure S53. Influence of ammonium sulfate on the reaction between maleimide/N-phenyl maleimide and peptide 9. a) Reaction scheme. Reaction conditions: 4 µM peptide, 4 µM TCEP, 10 µM maleimide/N-phenyl maleimide, 100 mM phosphate, pH 6.5, room temperature, 2 minutes. b) Mass spectra of the reaction mixture with or without the addition of ammonium sulfate (maleimide as the electrophile). c) Mass spectra of the reaction mixture with or without the addition of ammonium sulfate (N-phenyl maleimide as the electrophile).

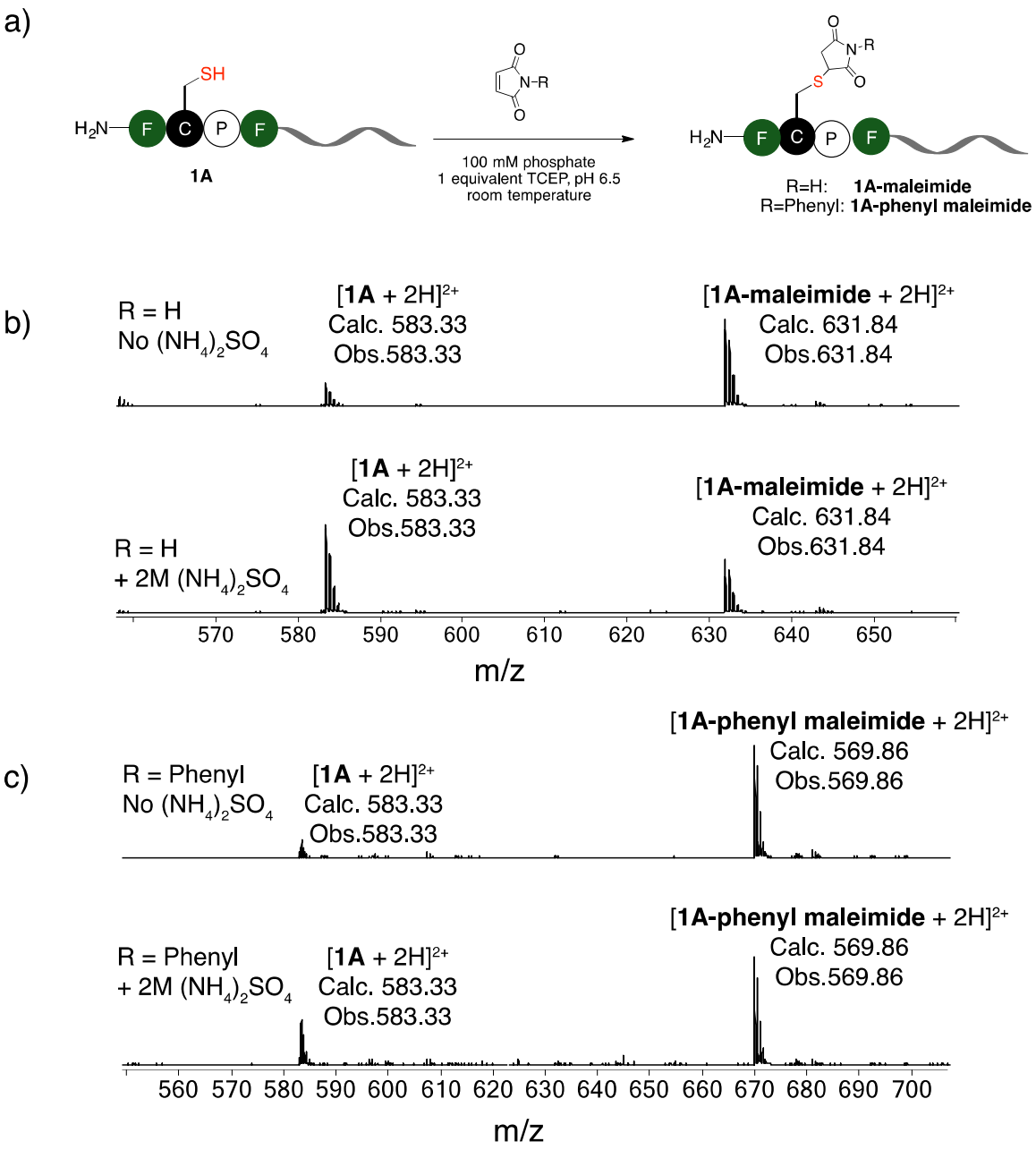


Figure S54. Influence of ammonium sulfate on the reaction between maleimide/N-phenyl maleimide and peptide 1A. a) Reaction scheme. b) Mass spectra of the reaction mixture with or without the addition of ammonium sulfate (maleimide as the electrophile). Reaction conditions: 4 µM peptide, 4 µM TCEP, 10 µM maleimide, 100 mM phosphate, pH 6.5, room temperature, 30 seconds. c) Mass spectra of the reaction mixture with or without the addition of ammonium sulfate (N-phenyl maleimide as the electrophile). Reaction conditions: 2 µM peptide, 2 µM TCEP, 5 µM N-phenyl maleimide, 100 mM phosphate, pH 6.5, room temperature, 20 seconds.

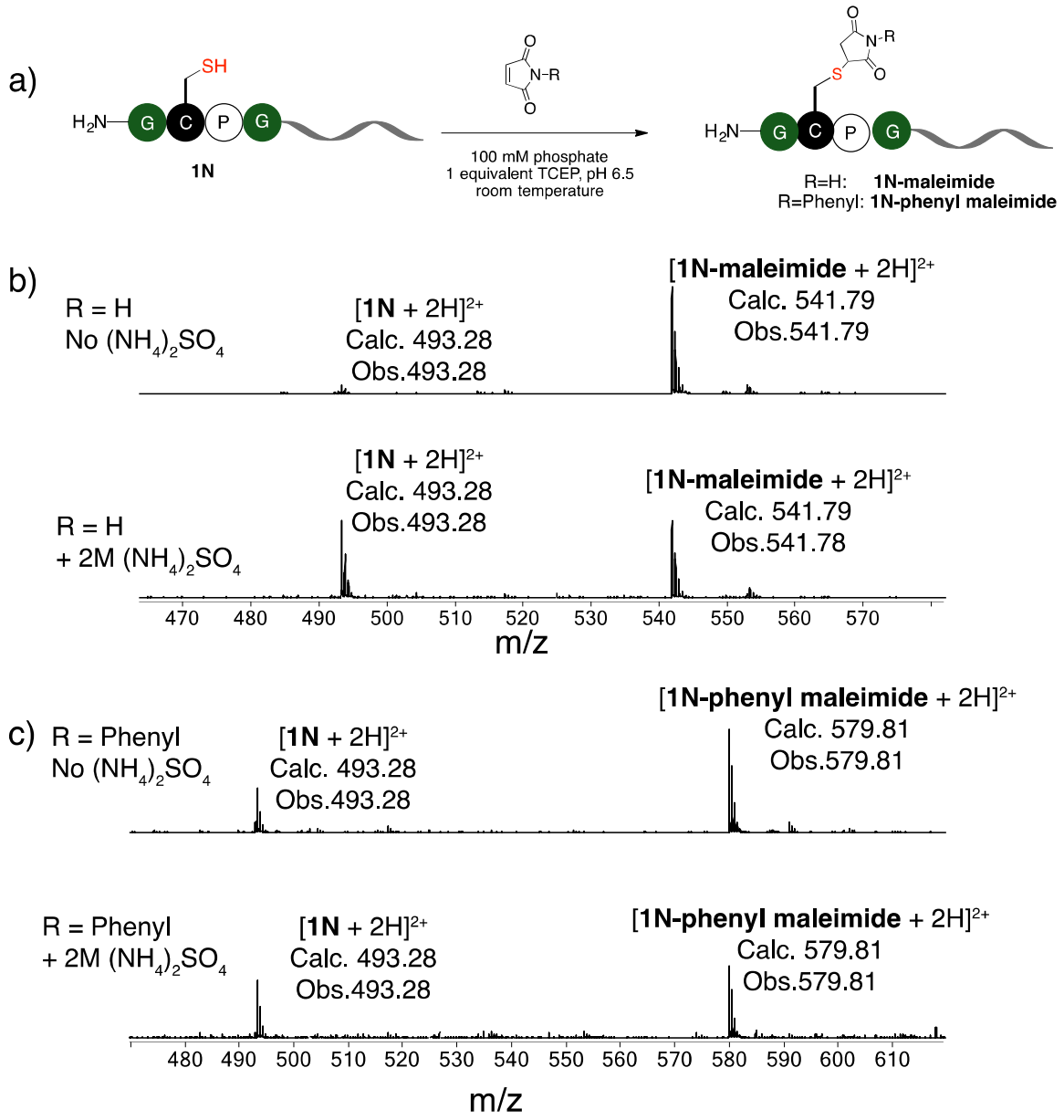


Figure S55. Influence of ammonium sulfate on the reaction between maleimide/N-phenyl maleimide and peptide 1N. a) Reaction scheme. b) Mass spectra of the reaction mixture with or without the addition of ammonium sulfate (maleimide as the electrophile). Reaction conditions: 4 µM peptide, 4 µM TCEP, 10 µM maleimide, 100 mM phosphate, pH 6.5, room temperature, 2 minutes. c) Mass spectra of the reaction mixture with or without the addition of ammonium sulfate (N-phenyl maleimide as the electrophile). Reaction conditions: 2 µM peptide, 2 µM TCEP, 5 µM N-phenyl maleimide, 100 mM phosphate, pH 6.5, room temperature, 20 seconds.

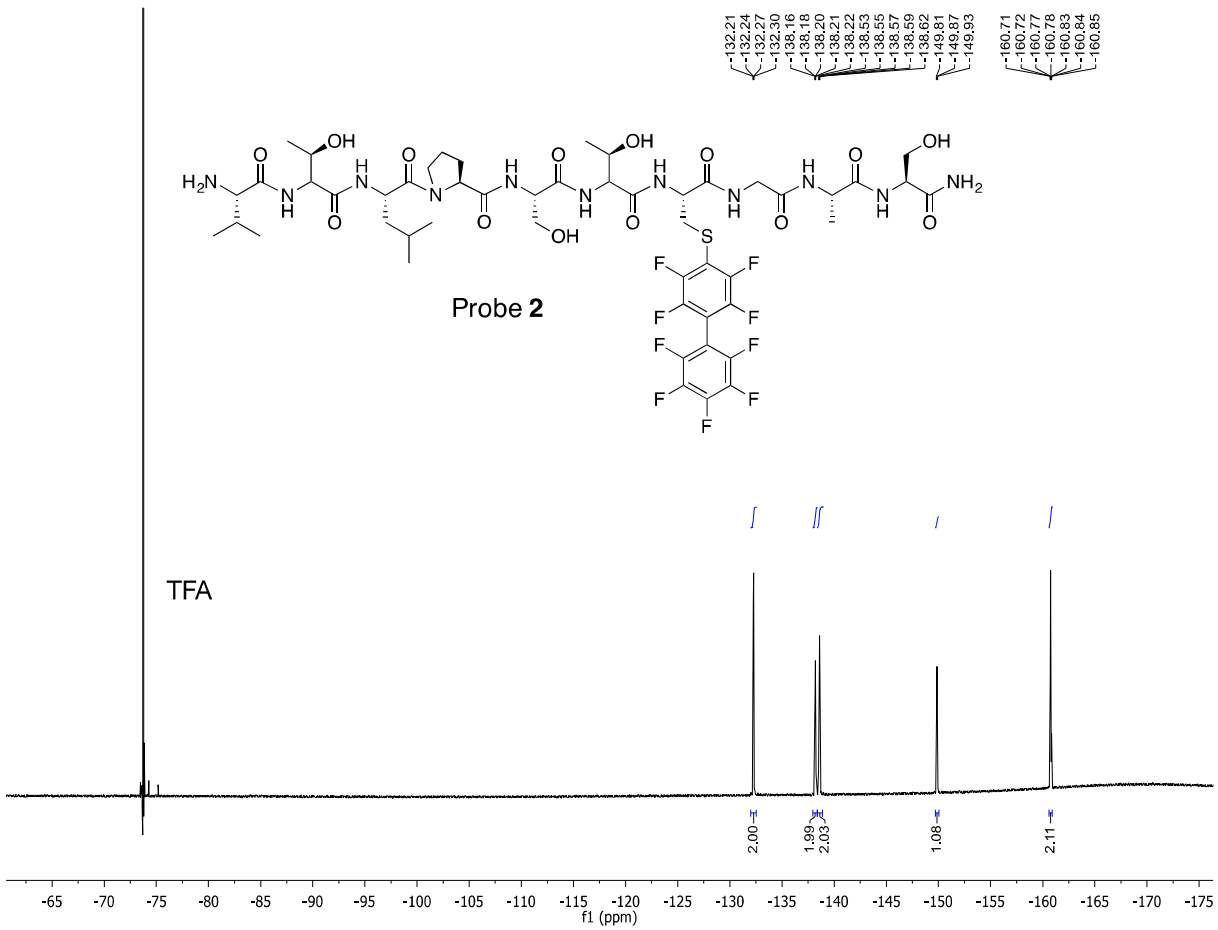


Figure S56. ^{19}F NMR (376 MHz, DMSO-d6) spectrum of probe 2. ^{19}F NMR (376 MHz, DMSO-d6): δ -132.25 (dd, J = 22.6, 11.3 Hz, 2F), -137.95 – -138.39 (m, 2F), -138.40 – -138.88 (m, 2F), -149.87 (t, J = 22.6 Hz, 1F), -160.77 (td, J = 22.6, 3.8 Hz, 2F).

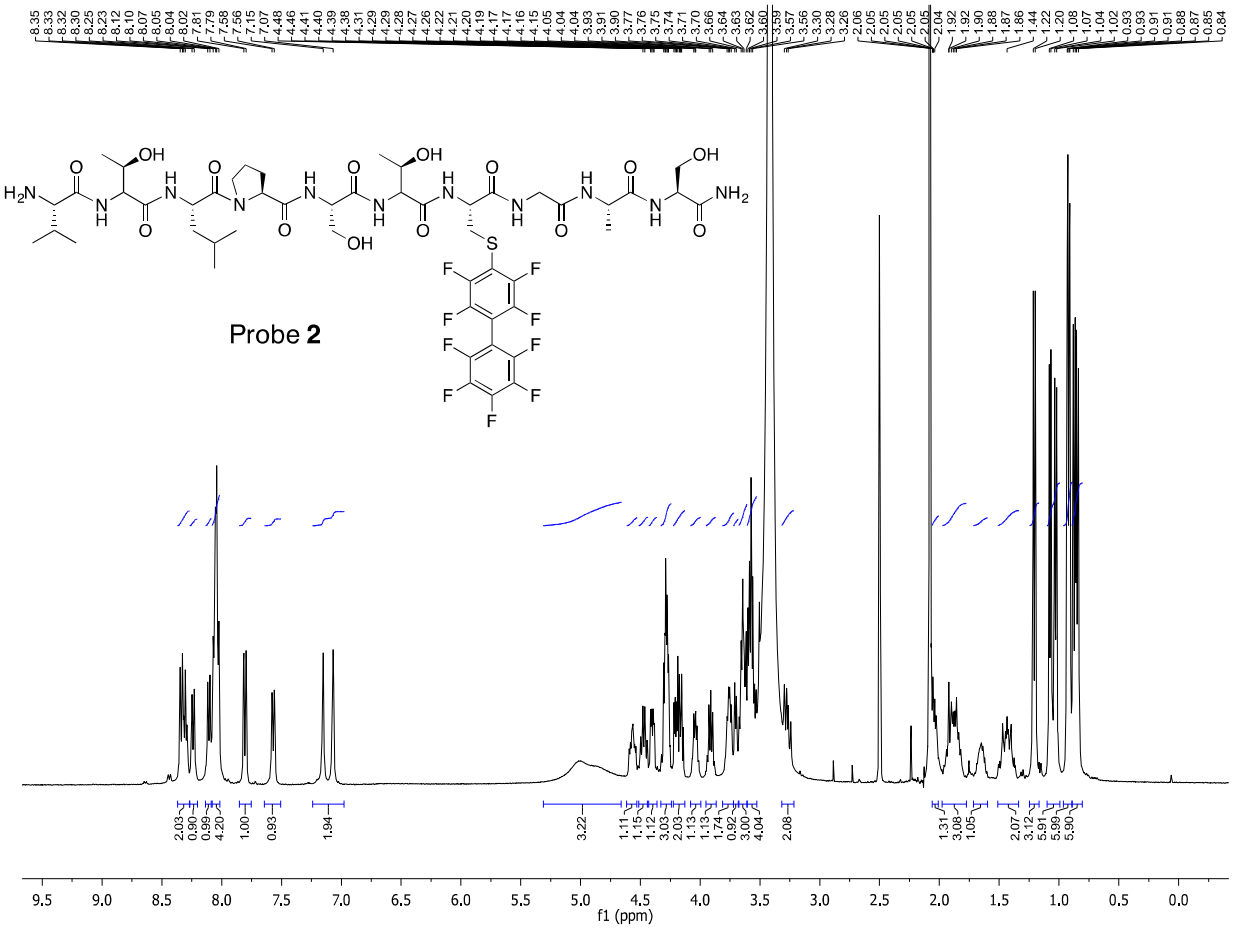


Figure S57. ^{1}H NMR (400 MHz, DMSO-d6) spectrum of probe 2. ^{1}H NMR (400 MHz, DMSO-d6): δ 8.37 – 8.27 (m, 2H), 8.24 (d, J = 7.7 Hz, 1H), 8.11 (d, J = 7.3 Hz, 1H), 8.05 (dd, J = 11.1, 7.5 Hz, 4H), 7.80 (d, J = 8.0 Hz, 1H), 7.57 (d, J = 8.0 Hz, 1H), 7.11 (d, J = 33.4 Hz, 2H), 5.00 (br, 3H), 4.61 – 4.53 (m, 1H), 4.47 (dd, J = 13.8, 7.9 Hz, 1H), 4.40 (dd, J = 8.1, 3.7 Hz, 1H), 4.33 – 4.24 (m, 3H), 4.22 – 4.13 (m, 2H), 4.04 (dd, J = 6.2, 4.0 Hz, 1H), 3.95 – 3.87 (m, 1H), 3.76 (dd, J = 8.7, 5.5 Hz, 2H), 3.70 (d, J = 5.7 Hz, 1H), 3.64 (dd, J = 10.6, 6.4 Hz, 3H), 3.58 (dd, J = 11.2, 5.2 Hz, 4H), 3.27 (dd, J = 13.5, 8.3 Hz, 2H), 2.06 – 2.01 (m, 1H), 1.98 – 1.77 (m, 3H), 1.72 – 1.60 (m, 1H), 1.51 – 1.34 (m, 2H), 1.21 (d, J = 7.1 Hz, 3H), 1.05 (dd, J = 18.3, 6.3 Hz, 6H), 0.92 (dd, J = 6.9, 2.3 Hz, 6H), 0.86 (dd, J = 11.0, 6.6 Hz, 6H).

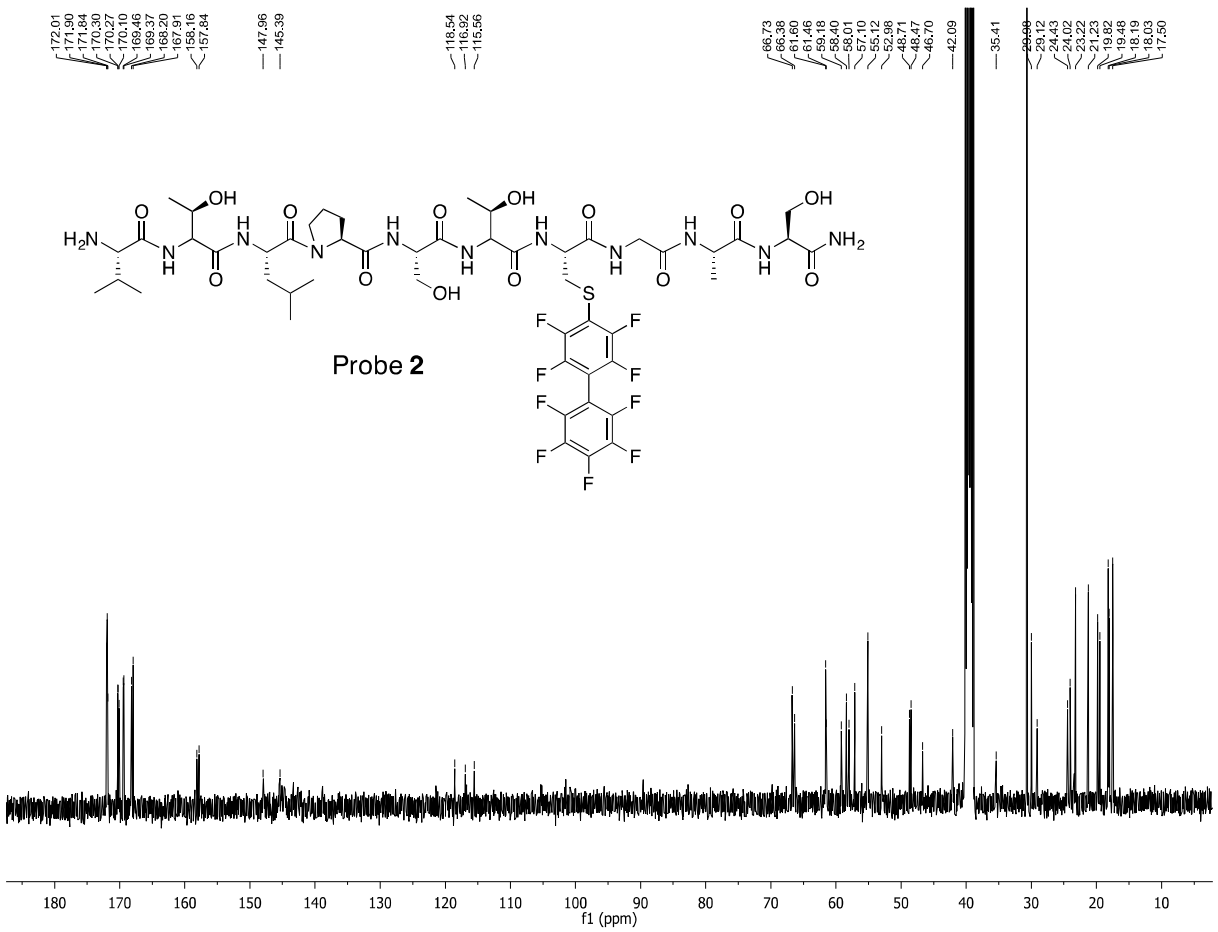


Figure S58. ^{13}C NMR (101 MHz, DMSO-d6) spectrum of probe 2. ^{13}C NMR (101 MHz, DMSO-d6): δ 172.01, 171.90, 171.84, 170.30, 170.27, 170.10, 169.46, 169.37, 168.20, 167.91, 147.96, 145.39, 66.73, 66.38, 61.60, 61.46, 59.18, 58.40, 58.01, 57.10, 55.12, 52.98, 48.71, 48.47, 46.70, 42.09, 35.41, 29.98, 29.12, 24.43, 24.02, 23.22, 21.23, 19.82, 19.48, 18.19, 18.03, 17.50.

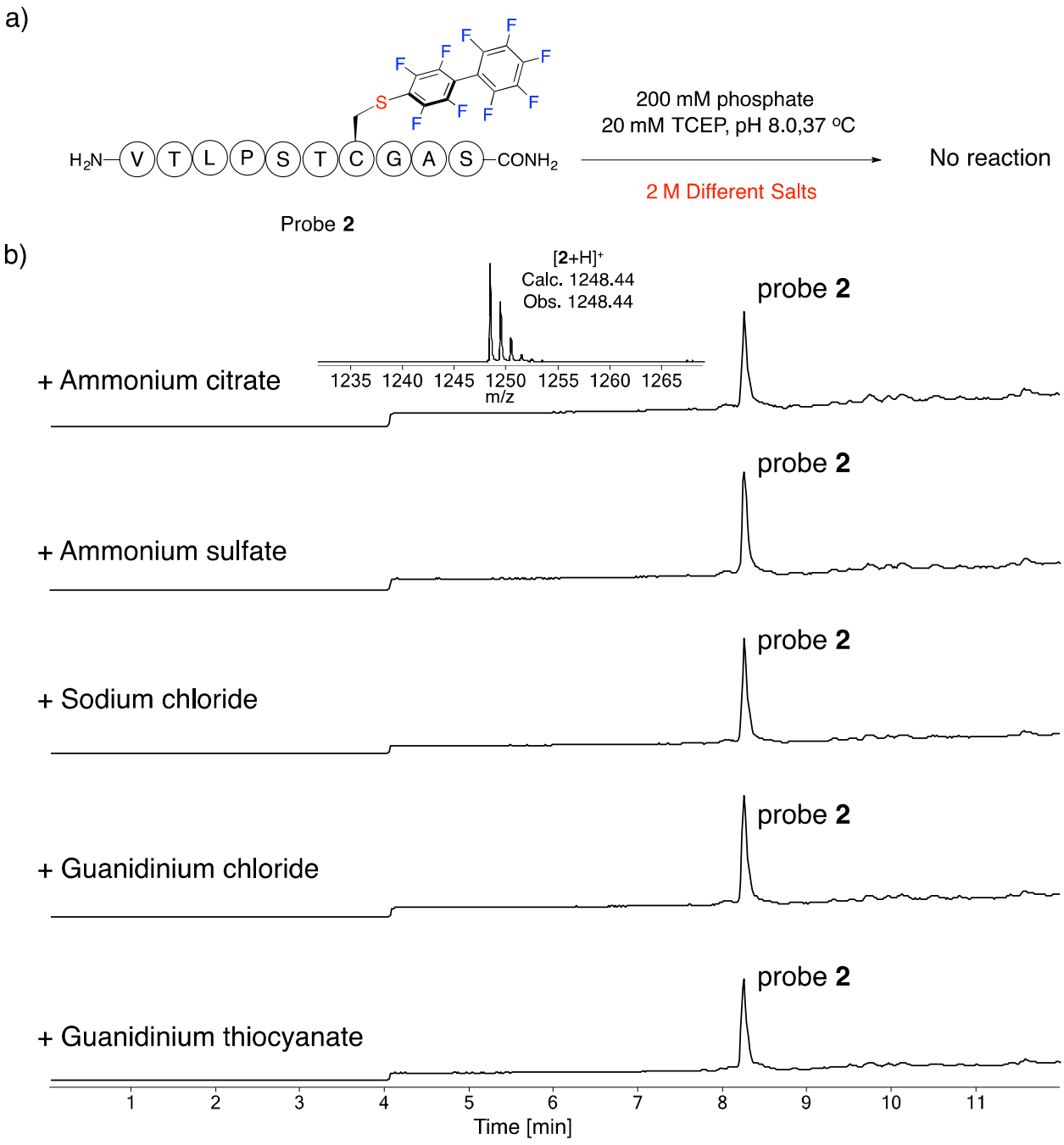


Figure S59. The arylation probe 2 used in this study is stable under the developed reaction conditions with different salts. a) Reaction scheme. b) LC-MS chromatograms for the reaction of probe **2** under different salt conditions. Reaction conditions: 0.5 mM probe **2**, 200 mM phosphate, 20 mM TCEP, pH 8.0, 37°C, with 2 M different salts, 2 hours. Probe **2** is the only peptidic species observed after the reaction.

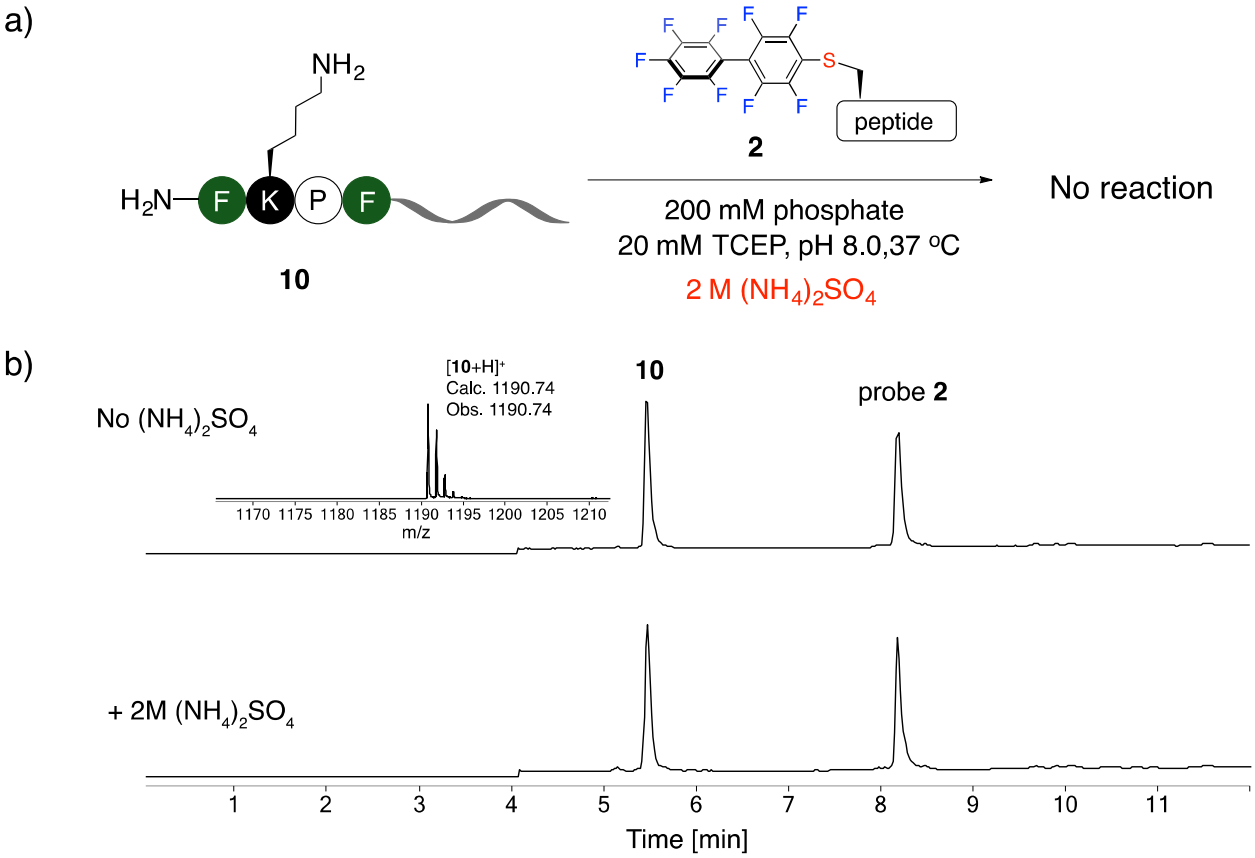


Figure S60. No arylation reaction observed between probe 2 and "π-clamp lysine variant" peptide 10. a) Reaction scheme. b) LC-MS chromatograms for the reaction between probe **2** and peptide **10**. Reaction conditions: 1 mM peptide **10**, 1 mM probe **2**, 200 mM phosphate, 20 mM TCEP, pH 8.0, 37°C, with or without 2 M ammonium sulfate, 2 hours.

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