Nitrogen Arylation for Macrocyclization of Unprotected Peptides

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Supporting Information

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1. Materials

H-Rink Amide-ChemMatrix resin was obtained from PCAS BioMatrix Inc. (St-Jean-sur-Richelieu, Quebec, Canada). 1-[Bis(dimethylamino)methylene]-1H-1,2,3-triazolo[4,5-b]pyridinium-3-oxid-hexafluorophosphate (HATU), Fmoc-L-Arg(Pbf)-OH, Fmoc-L-His(Trt)-OH, Fmoc-L-Lys(Boc)-OH, Fmoc-L-Asp(tBu)-OH, Fmoc-L-Glu(tBu)-OH, Fmoc-L-Ser(tBu)-OH, Fmoc-L-Thr(tBu)-OH, Fmoc-L-Asn(Trt)-OH, Fmoc-L-Gln(Trt)-OH, Fmoc-L-Cys(Trt)-OH, Fmoc-L-Gly-OH, Fmoc-L-Ala-OH, Fmoc-L-Val-OH, Fmoc-L-Leu-OH, Fmoc-L-Met-OH, Fmoc-L-Phe-OH, Fmoc-L-Tyr(tBu)-OH, and Fmoc-L-Trp(Boc)-OH were purchased from Chem-Impex International (Wood Dale, IL). Peptide synthesis-grade N,N-dimethylformamide (DMF), dichloromethane (DCM), diethyl ether, HPLC-grade acetonitrile, were obtained from VWR International (Philadelphia, PA). All reactions were set up on the bench top open to air. Water was deionized and used as is. Anhydrous, oxygen-free dichloromethane and tetrahydrofuran were purchased from J. T. Baker and passed through two activated alumina columns followed by sparging with argon before use. Acetone-d_6 were purchased in sealed ampules from Cambridge Isotopes. All other reagents were purchased from commercial sources and used as received. Purification of products was performed by silica gel column chromatography. Compounds were analyzed by ^{1}H, ^{13}C. New compounds were also analyzed by high resolution ESI-MS in some cases. ^{1}H and ^{13}C NMR spectra were recorded on a Varian XL 300 MHz or Varian Inova 500 MHz spectrometers and were calibrated using residual solvent as an internal reference. The following abbreviations were used to explain multiplicities: s = singlet, d = doublet, t = triplet, brs = broad singlet, q = quartet, m = multiplet. The HRMS spectrum was recorded on a Bruker Daltonics APEXIV 4.7. Tesla Fourier transform ion cyclotron resonance mass spectrometer (FT-ICR-MS).

2. Methods for LC-MS Analysis

LC-MS chromatograms and associated mass spectra were acquired using Agilent 6520 ESI-Q-TOF mass spectrometer equipped with C_3 and C_{18} Zorbax columns. Mobile phases are: 0.1% formic acid in water (solvent A) and 0.1% formic acid in acetonitrile (solvent B). Following LS-MS methods were used:

Method A: LC conditions: Zorbax SB C3 column: 2.1 x 150 mm, 5 μm, column temperature: 40 °C, gradient: 0-2 minutes 1% B, 2-8 minutes 1-30% B, 8-13 minutes 30-95% B, flow rate: 0.8 mL/min.

Method B: LC conditions: Zorbax SB C3 column: 2.1 x 150 mm, 5 μm, column temperature: 40 °C, gradient: 0-2 minutes 1% B, 2-8 minutes 1-30% B, 8-14 minutes 30-60% B, flow rate: 0.8 mL/min.

Method C: LC conditions: Zorbax SB C18 column: 2.1 x 150 mm, 5 μm, column temperature: 40 °C, gradient: 0-3 minutes 1% B, 3-11 minutes 1-31% B, 11-12 minutes 31-70% B, flow rate: 0.8 mL/min.

All data were processed using Agilent MassHunter software package. Y-axis in all chromatograms shown represents total ion current (TIC) unless noted; mass spectrum corresponds to the integration of the TIC peak unless noted. All yields reported were determined by integrating TIC spectra. First, using Agilent MassHunter software package, the peak areas for all relevant peptidic species on the chromatogram were integrated. Then the yield was calculated as following: %yield = S_p/S_{all} where S_p is the peak area of the desired product and S_{all} is sum of the peak areas of all peptidic species.

3. General Method for Peptide Preparation

Fast-flow peptide synthesis

Peptides were synthesized on a 0.1 mmol scale on H-Rink Amide-ChemMatrix resin using manual Fmoc-SPPS (Solid phase peptide synthesis) chemistry under flow using a 3-minute cycle for each amino acid.[1] Specifically, all reagents and solvents are delivered to a stainless steel reactor containing resins at a constant flow rate using an HPLC pump; temperature of the reactor was maintained at 60°C during the synthesis using a water bath. The procedure for each amino acid coupling cycle included a 30 second coupling with 1 mmol Fmoc-protected amino acid, 1 mmol HATU, and 500 µL of N,N-Diisopropylethylamine (DIEA) in 2.5 mL of DMF at a flow rate of 6 mL/min (note that for the coupling of cysteine, tryptophan and histidine, 190 µL of DIEA was used to prevent racemization); 1 min wash with DMF at a flow rate of 20 mL/min; 20 second deprotection with 20% (v/v) piperidine in DMF at a flow rate of 20 mL/min; and 1 minute wash with DMF at a flow rate of 20 mL/min. After completion of the fast-flow synthesis, the resins are washed with DCM (3x) and dried under vacuum.

Peptide cleavage and deprotection

Peptides were cleaved from the resin and the side-chains were simultaneously deprotected by treatment with 2.5% (v/v) 1,2-ethanedithiol (EDT), 5% (v/v) water, 5% (v/v) phenol, 5% (v/v) thioanisole in neat TFA for 8 min at 60°C, 6 ml of cleavage cocktail was used for 0.1 mmol of peptide. The resulting solution was triturated and washed with cold ether (pre-chilled in – 80°C freezer). The trituration was repeated a total of three times. The obtained solids were dissolved in 50% water and 50% acetonitrile and lyophilized.

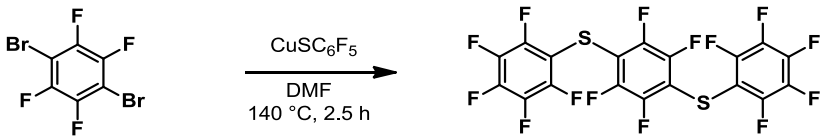
RP-HPLC purification of peptides

The crude peptide was dissolved in a mixture water/acetonitrile and purified by semi-preparative RP-HPLC (Agilent Zorbax SB C3 column: 9.4 x 250 mm, 5 µm or Agilent Zorbax SB C18 column: 9.4 x 250 mm, 5 µm, or Agilent Zorbax SB C3 column: 21.2 x 250 mm, 7 µm). 1 µL of each HPLC fraction was mixed with 1 µL of α-cyano-4-hydroxycinnamic acid (CHCA) matrix in 75% water: 25% acetonitrile, spotted with MALDI, and checked for fractions with desired molecular mass. The purity of fractions was confirmed by analytical RP-HPLC (Agilent Zorbax SB C3 column: 2.1 x

150 mm, 5 μm). HPLC fractions containing only product materials were confirmed by LC-MS analysis, combined, and then lyophilized.

4. Synthetic Procedures

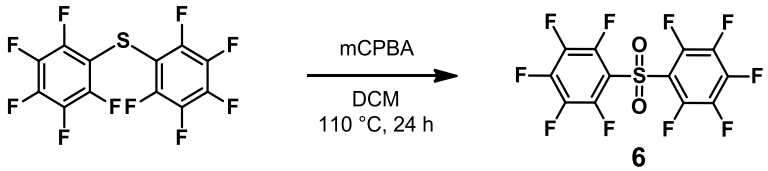
Synthesis of electrophile **5***:*



5

A reaction tube (Fisher scientific 16 × 125 mm, Cat. No. 1495925C) was charged with 1,4-dibromotetrafluorobenzene (167 mg, 0.656 mmol, 1.00 equiv.) and cuprous pentafluorothiophenoxide[2] (500 mg, 1.90 mmol, 2.90 equiv.). The tube was sealed with a Teflon cap (Thermo Scientific SPTA PTFE/SIL F/15-425 10, Cat. No. 03394A) and evacuated. It was backfilled with argon. This process was repeated a total of three times. To the reaction tube was added DMF (2 mL). The reaction tube was placed in a preheated oil bath (140 °C) and vigorously stirred for 2.5 h. At this time, the reaction vessel was cooled to room temperature and quenched with brine (5 mL) and ethyl acetate (5 mL). The organic phase was separated and the aqueous phase was extracted with ethyl acetate (10 mL × 3). The combined organic phases were dried with magnesium sulfate and filtered. After concentration, the crude product was purified by silica gel column chromatography (dichloromethane/hexanes = 1/20), and then recrystallized from hot hexanes to provide the title compound as a white solid (180 mg, 50%). ^{19}F NMR (282 MHz, $CDCl_3$): δ (ppm) –132.5 (d, 23 Hz, 4F), -133.3 (s, 4F), -150.3 (t, 20 Hz, 2F), -160.8 (t, 20 Hz, 4F). Spectrum data match those of the reported.[3] HRMS (DART) m/z calcd. for $C_{18}F_{14}S_2$ $[M]^+$: 545.9212 found 545.9229.

Synthesis of electrophile **6***:*

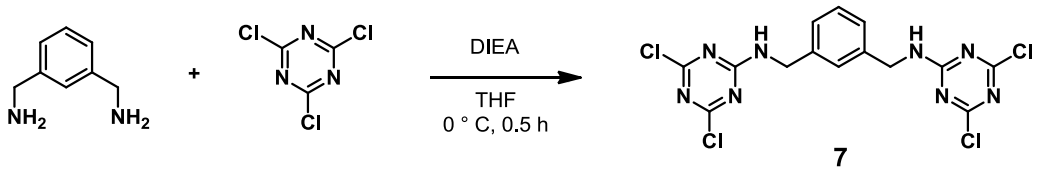


6

A heavy wall pressure vessel (Chemglass CG-1880) was charged with pentafluorophenyl sulfide

(36.6 mg, 0.100 mmol, 1.00 equiv.) and 3-chlorobenzoic acid (<77%, 69.0 mg, 0.400 mmol, 4.00 equiv.). The mixture was suspended in 1 mL of dichloromethane and the tube was sealed with a Teflon cap. The reaction tube was placed in a preheated oil bath (110 °C) behind a blast shield and vigorously stirred for 24 h. At this time, the reaction vessel was cooled to room temperature and concentrated under reduced pressure. After concentration, the crude product was purified by silica gel column chromatography (ethyl acetate/hexanes = 1/20 to 1/10), and then recrystallized from hot hexanes to provide the title compound as a white solid (28.0 mg, 70%). ^{19}F NMR (282 MHz, $CDCl_3$): δ (ppm) –136.4 (d, 20 Hz, 4F), -141.7 (m, 2F), -158.13 (t, 20 Hz, 4F). Spectrum data match those of the reported.[4] HRMS (DART) m/z calcd. for $C_{12}F_{10}O_2S$ $[M+H]^+$: 398.9532 found 398.9545.

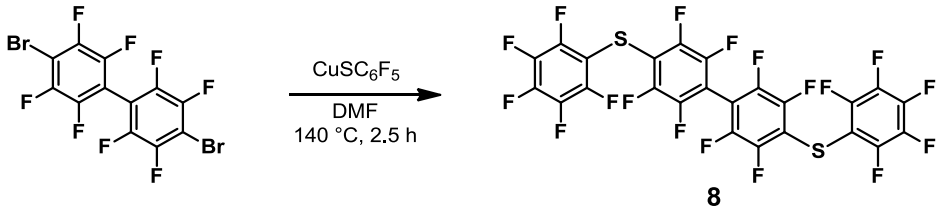
Synthesis of electrophile **7***:*



7

Compound **7** was prepared according to the literature procedure. ^{1}H NMR (500 MHz, acetone-d6): δ (ppm) 8.43 (brs, 2H), 7.42 (s, 1H), 7.37-7.30 (m, 3H), 4.69 (d, J = 10 Hz, 4H). Spectrum data match those of the reported.[5] HRMS (DART) m/z calcd. for $C_{14}H_{10}Cl_4N_8$ $[M+H]^+$: 430.9855 found 430.9864.

Synthesis of electrophile **8***:*

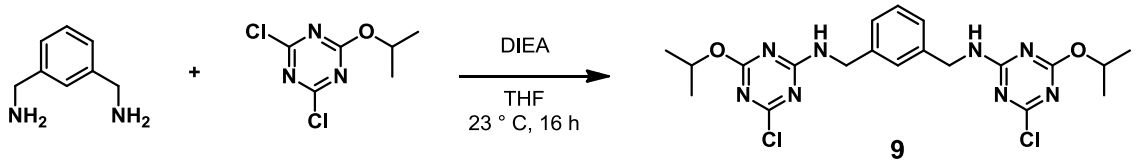


8

A reaction tube (Fisher scientific 16 × 125 mm, Cat. No. 1495925C) was charged with 4,4'-dibromooctafluorobiphenyl (299 mg, 0.656 mmol, 1.00 equiv.) and cuprous pentafluorothiophenoxide[2] (500 mg, 1.90 mmol, 2.90 equiv.). The tube was sealed with a Teflon cap (Thermo Scientific SPTA PTFE/SIL F/15-425 10, Cat. No. 03394A) and evacuated. It was backfilled with argon. This process was repeated a total of three times. To the reaction tube was added DMF (2 mL). The reaction tube was placed in a preheated oil bath (140 °C) and vigorously stirred for 2.5 h. At this time, the reaction vessel was cooled to room temperature and quenched with brine (5 mL) and

ethyl acetate (5 mL). The organic phase was separated and the aqueous phase was extracted with ethyl acetate (10 mL × 3). The combined organic phases were dried with magnesium sulfate and filtered. After concentration, the crude product was purified by silica gel column chromatography (dichloromethane/hexanes = 1/10), and then recrystallized from hot hexanes to provide the title compound as a white solid (174 mg, 38%). ^{19}F NMR (282 MHz, $CDCl_3$): δ (ppm) –132.3 (d, 23 Hz, 4F), -133.5 (brs, 4F), -137.4 (brs, 4F), -150.19 (t, 20 Hz, 2F), -160.7 (t, 20 Hz, 4F). Spectrum data match those of the reported.[6] HRMS (DART) m/z calcd. for $C_{24}F_{18}S_2$ $[M]^+$: 693.9149 found 693.9146.

Synthesis of electrophile **9***:*



9

A round bottom flask was charged with 1,3-phenylenedimethanamine (0.2 mL, 1.47 mmol, 1.00 equiv.) and 2,4-dichloro-6-isopropoxy-1,3,5-triazine (1200 mg, 5.88 mmol, 4 equiv.).[7] The mixture was dissolved in anhydrous THF (50 mL). To the solution was added DIEA (0.74 mL, 5.88 mmol, 4 equiv.) with the aid of a syringe. The reaction mixture was vigorously stirred for 16 hours at ambient temperature. After concentration, the crude product was purified by silica gel column chromatography (ethyl acetate/dichloromethane = 1/4) to provide compound **9** as a white solid (612 mg, 87 %). ^{1}H NMR (500 MHz, acetone-d6): δ (ppm) 7.95-7.80 (m, 2H), 7.39 (brs, 1H), 7.32-7.25 (m, 3H), 5.22 (q, J = 5.5 Hz, 2H), 4.64 (d, J = 6.5 Hz, 4H), 1.28 (d, J = 5.5 Hz, 12H); ^{13}C NMR (125 MHz, acetone-d6): δ (ppm) 171.9, 171.4, 168.4, 140.0, 129.5, 127.3, 127.2, 72.2, 45.1, 21.9. HRMS (ESI) m/z calcd. for $C_{20}H_{24}Cl_2N_8O_2$ $[M+Na]^+$: 501.1291 found 501.1291.

Representative protocol **(A)** *for peptide arylation with electrophiles* ***3*** *or* ***4*** *or* ***5****:*

A 0.6 mL Eppendorf tube was charged with 20 µL of peptide (5 mM stock solution in DMF). 10 µL of Tris base solution (10 equiv, 100 mM stock solution in DMF) was added. The resulting mixture was capped and vortexed for 10 sec. Then 20 µL of electrophile (10 equiv, 50 mM stock solution in DMF) was added. The resulting reaction mixture was capped, vortexed for 10 seconds, and placed in a 37°C water bath for 12 hours. The reaction mixture was diluted with 50% water: 50% acetonitrile and was subjected to LC-MS analysis.

Representative protocol **(B)** *for peptide arylation with electrophiles* **6** *or* **7***:*

A 0.6 mL Eppendorf tube was charged with 20 µL of peptide (2.5 mM stock solution in DMF). 10 µL of DIEA solution (10 equiv, 50 mM stock solution in DMF) was added. The resulting mixture was capped and vortexed for 10 sec. Then 20 µL of electrophile (10 equiv, 25 mM stock

solution in DMF) was added. The resulting reaction mixture was capped, vortexed for 10 seconds, and left at room temperature for 1 hour. The reaction mixture was diluted with 50% water: 50% acetonitrile and was subjected to LC-MS analysis.

Representative protocol **(C)** *for peptide stapling with electrophile* **6***:*

A 0.6 mL Eppendorf tube was charged with 20 µL of peptide (1.25 mM stock solution in DMF). 10 µL of DIEA solution (20 equiv, 50 mM stock solution in DMF) was added. The resulting mixture was capped and vortexed for 10 sec. Then 20 µL of electrophile **6** (1.25 equiv, 1.56 mM stock solution in DMF) was added. The resulting reaction mixture was capped, vortexed for 10 seconds, and left at room temperature for 4 hours. The reaction mixture was diluted with 50% water: 50% acetonitrile and was subjected to LC-MS analysis.

Representative protocol **(D)** *for peptide stapling with electrophiles* **3** *or* **4** *or* **5** *or* **8***:*

A 0.6 mL Eppendorf tube was charged with 20 µL of peptide (5 mM stock solution in DMF). 10 µL of Tris base solution (20 equiv, 200 mM stock solution in DMF) was added. The resulting mixture was capped and vortexed for 10 sec. Then 20 µL of electrophile (6 equiv, 30 mM stock solution in DMF) was added. The resulting reaction mixture was capped, vortexed for 10 seconds, and placed in a 37°C water bath for 24 hours. The reaction mixture was diluted with 50% water: 50% acetonitrile and was subjected to LC-MS analysis.

Representative protocol **(E)** *for peptide stapling with electrophile* **7***:*

A 0.6 mL Eppendorf tube was charged with 20 µL of peptide (2.5 mM stock solution in DMF). 10 µL of DIEA solution (20 equiv, 100 mM stock solution in DMF) was added. The resulting mixture was capped and vortexed for 10 sec. Then 20 µL of electrophile **7** (2 equiv, 5 mM stock solution in DMF) was added. The resulting reaction mixture was capped, vortexed for 10 seconds, and left at room temperature for 4 hours. The reaction mixture was diluted with 50% water: 50% acetonitrile and was subjected to LC-MS analysis.

Representative protocol **(F)** *for peptide stapling with electrophile* **9***:*

A 0.6 mL Eppendorf tube was charged with 20 µL of peptide (5 mM stock solution in DMF). 10 µL of Tris base solution (20 equiv, 200 mM stock solution in DMF) was added. The resulting mixture was capped and vortexed for 10 sec. Then 20 µL of electrophile **9** (5 equiv, 25 mM stock solution in DMF) was added. The resulting reaction mixture was capped, vortexed for 10 seconds, and placed in a 37°C water bath for 24 hours. The reaction mixture was diluted with 50% water: 50% acetonitrile and was subjected to LC-MS analysis.

Representative protocol **(G)** *for peptide labeling with Biotin:*

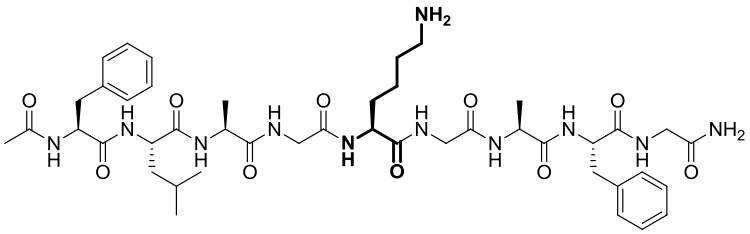
Labeling of peptides with Biotin was performed on the resin bound protected peptides by treating the protected peptide resin with a solution of Biotin-PEG_4-NHS (ChemPep Inc., 2 equiv.) and DIEA (4 equiv.) dissolved in DMF for 6 hours at room temperature.

Representative protocol **(H)** *for peptide labeling with FITC:*

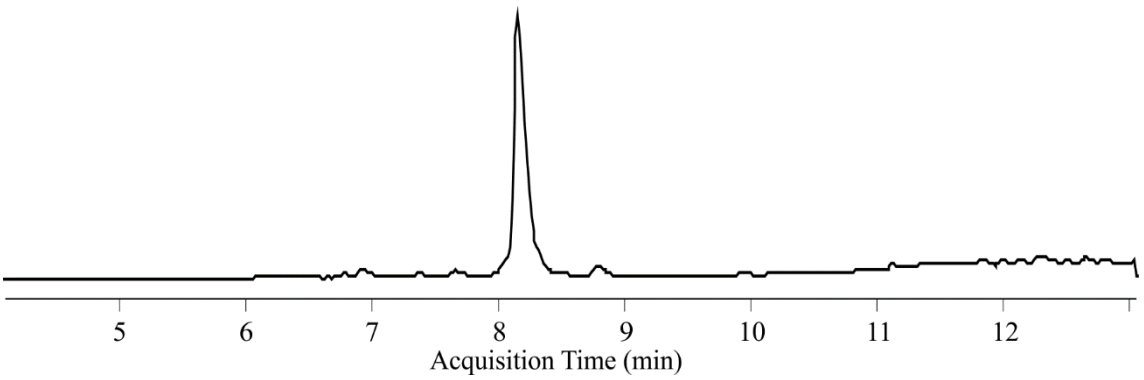
Labeling of peptides with FITC was performed on the resin bound protected peptides by treating the protected peptide resin with a solution of fluorescein isothiocyanate isomer I (Chem-Impex International, 6 equiv.) and DIEA (10 equiv.) dissolved in DMF for 8 hours at room temperature.

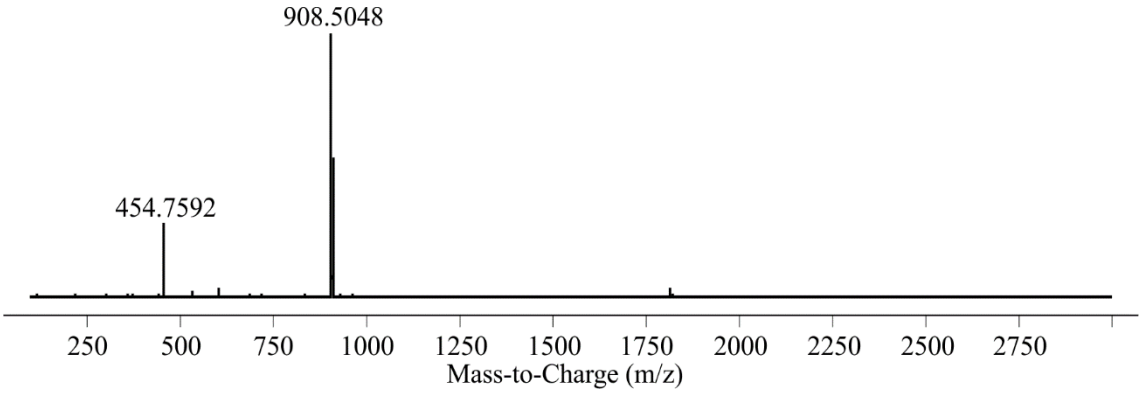
5. Methodology

LC-MS analytical data of purified peptides **1** *and* **2**

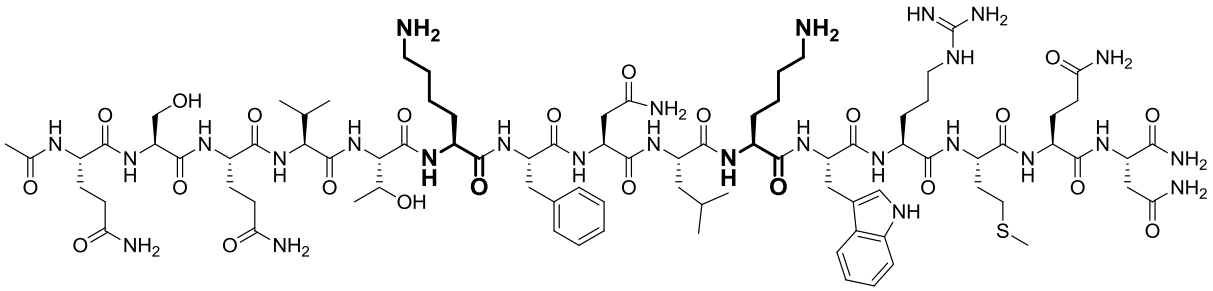


1: AcNH-Phe-Leu-Ala-Gly-**Lys**-Gly-Ala-Phe-Gly-$CONH_2$

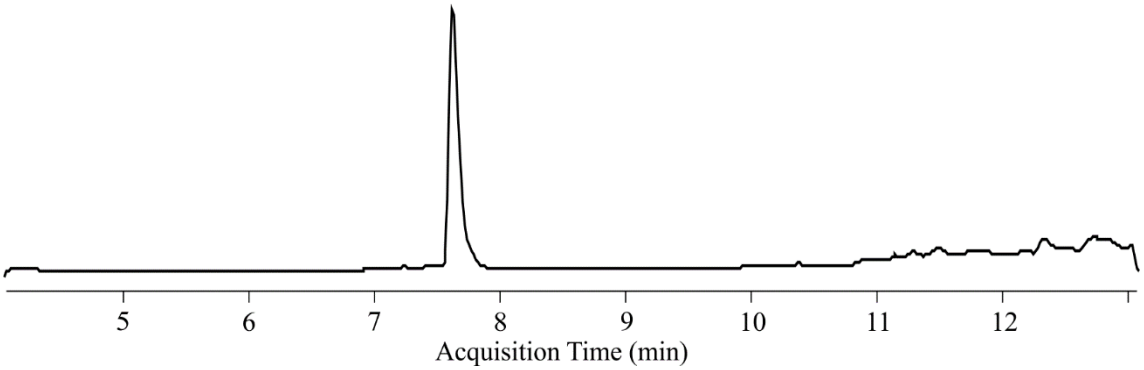


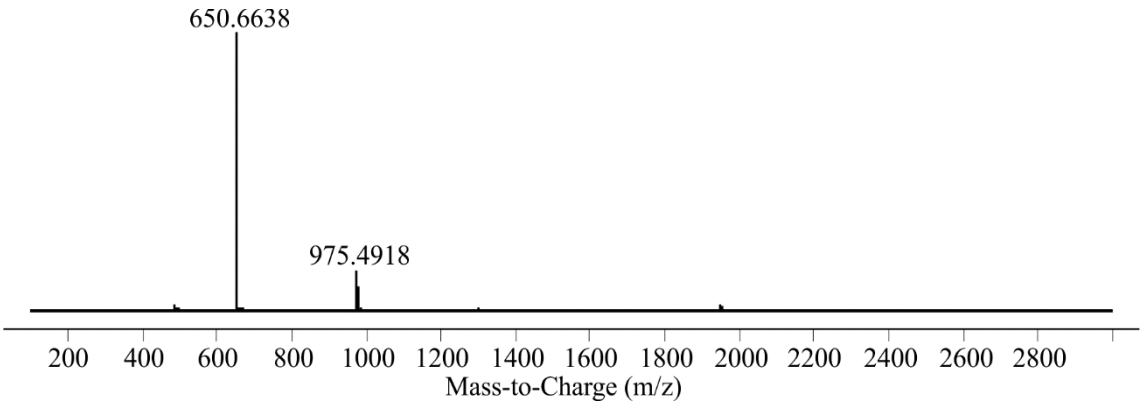


Peptide **1**: LC-MS analysis *Method A*. TIC trace and Mass spectrum of peptide **1**. m/z calcd. for $C_{44}H_{65}N_{11}O_{10}$ $[M+H]^+$: 908.50 found 908.50.



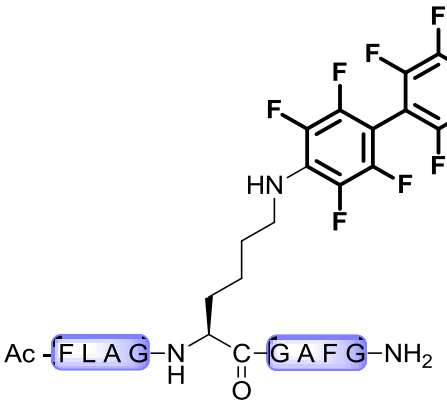
2: AcNH-Gln-Ser-Gln-Val-Thr-**Lys**-Phe-Asn-Leu-**Lys**-Trp-Arg-Met-Gln-Asn-$CONH_2$



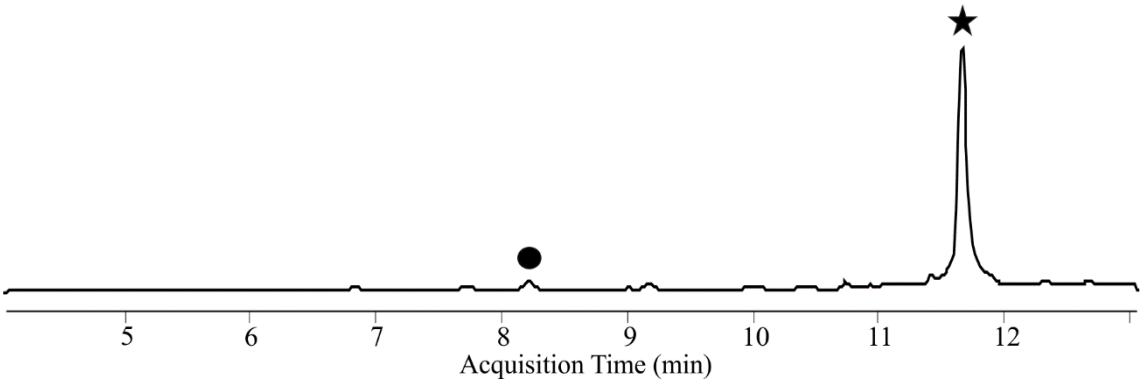


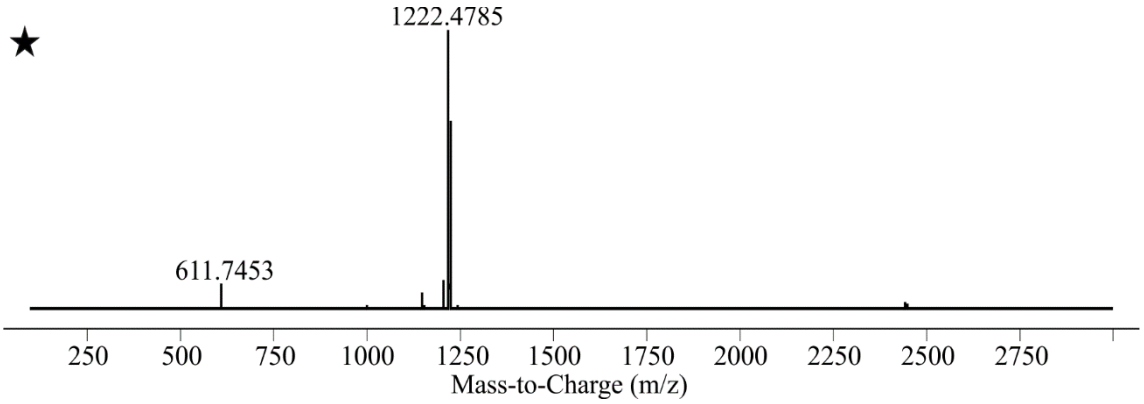
Peptide **2**: LC-MS analysis *Method A*. TIC trace and Mass spectrum of peptide **2**. m/z calcd. for $C_{86}H_{137}N_{27}O_{23}S$ $[M+2H]^{2+}$: 975.01 found 974.99.

LC-MS analytical data of arylation crude reaction with peptide **1**



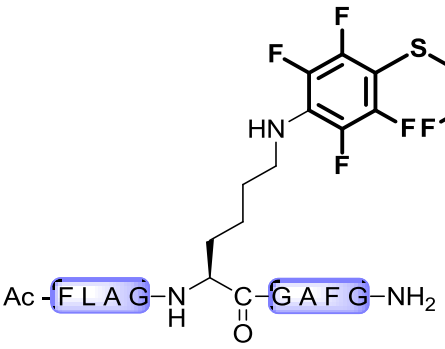
1a



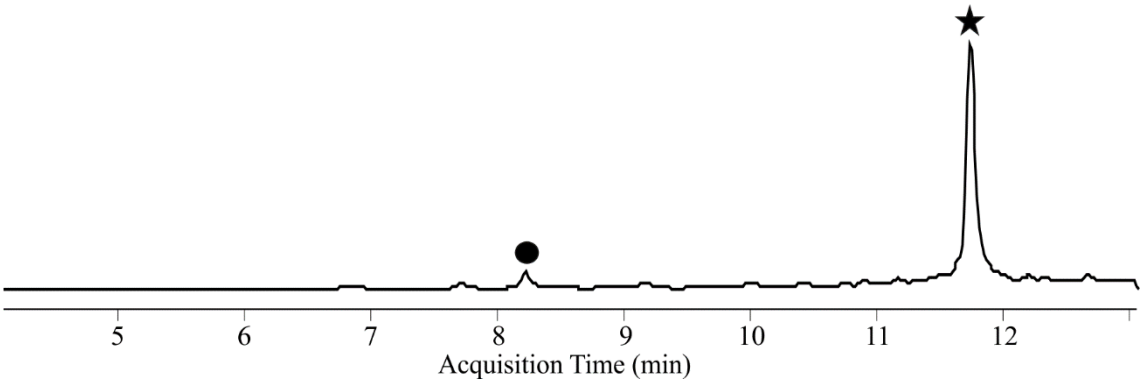


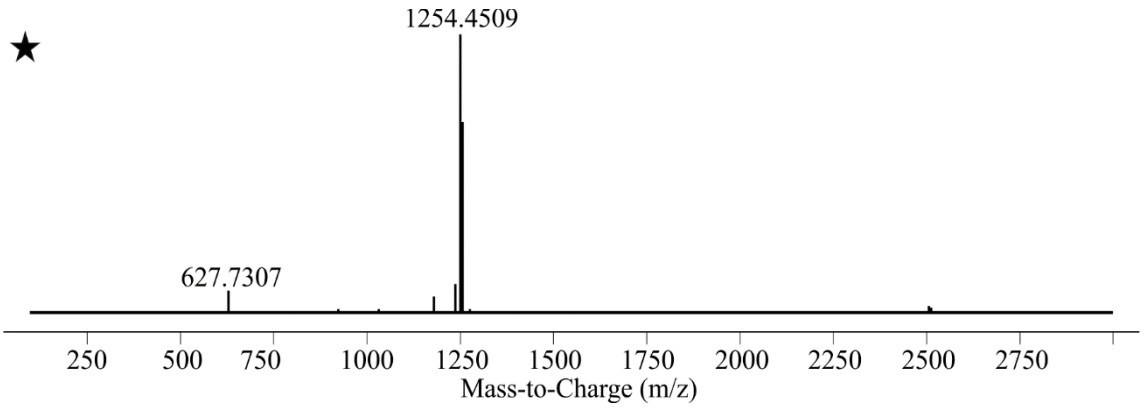
(**1a**) : Prepared according to the representative protocol **(A)** using peptide **1** (2 mM), electrophile **3** (20 mM), and Tris base (20 mM) at 37°C for 12 h. The diluted reaction mixture was analyzed using LC-MS *Method A*. TIC trace of crude reaction and Mass spectrum of product **1a**. Signal of the starting

material **1** (yield = 1 %) is marked with a black circle (●). Signal of the arylation product **1a** (yield = 97 %) is marked with a star (★). Analytical data for **1a** : m/z calcd. for $C_{56}H_{64}F_9N_{11}O_{10}$ $[M+H]^+$: 1222.48 found 1222.48.

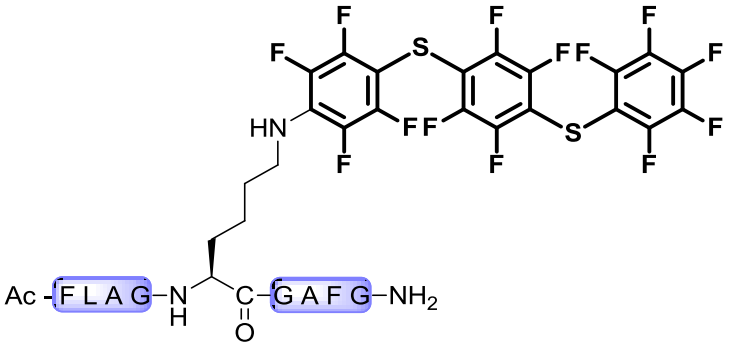


1b

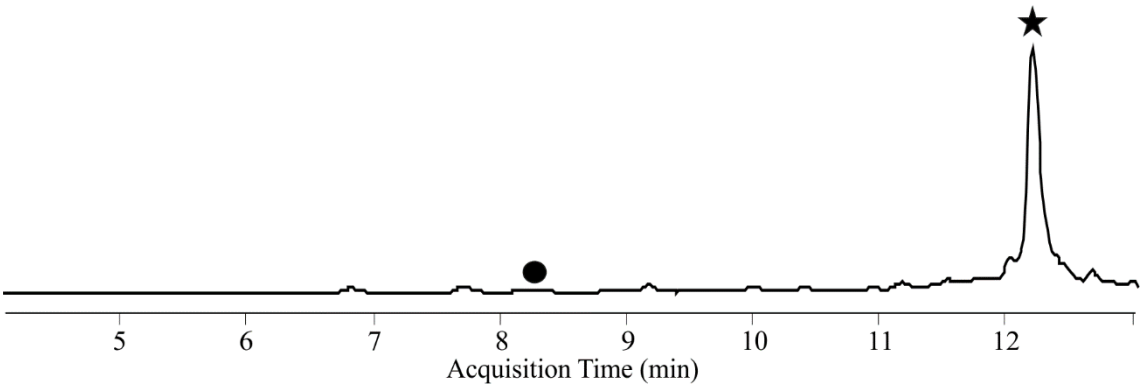


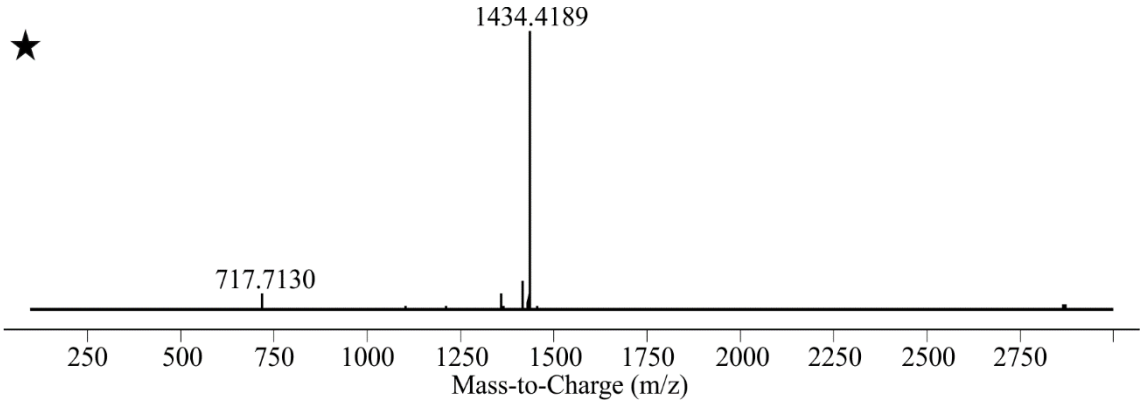


(**1b**) : Prepared according to the representative protocol **(A)** using peptide **1** (2 mM), electrophile **4** (20 mM), and Tris base (20 mM) at 37°C for 12 h. The diluted reaction mixture was analyzed using LC-MS *Method A*. TIC trace of crude reaction and Mass spectrum of product **1b**. Signal of the starting material **1** (yield = 2 %) is marked with a black circle (●). Signal of the arylation product **1b** (yield = 95 %) is marked with a star (★). Analytical data for **1b** : m/z calcd. for $C_{56}H_{64}F_9N_{11}O_{10}S$ $[M+H]^+$: 1254.45 found 1254.45.

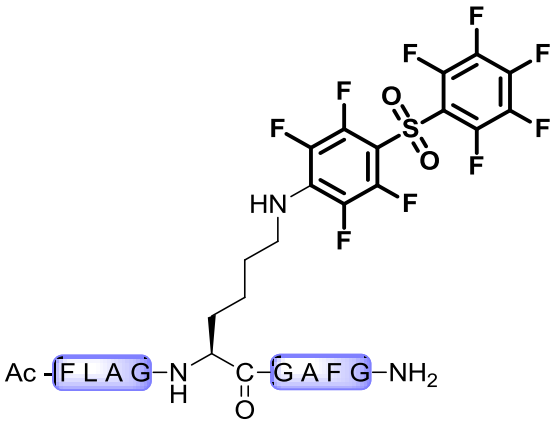


1c

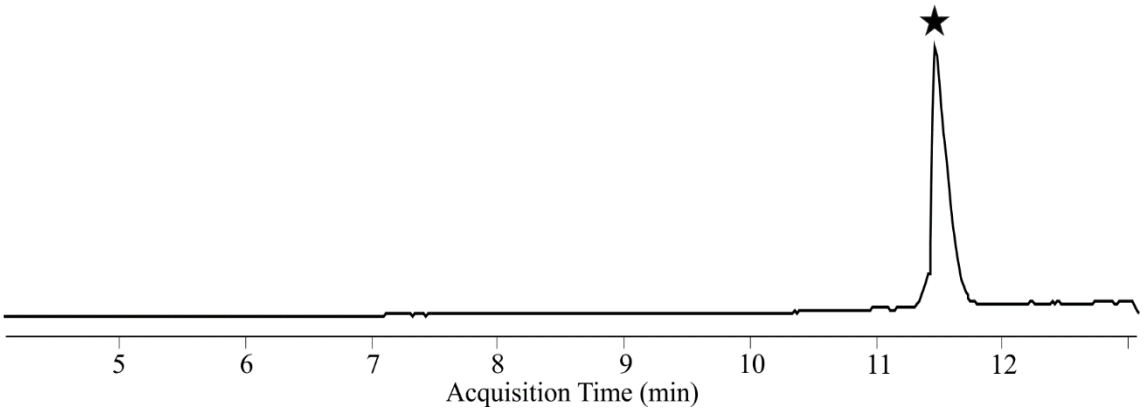


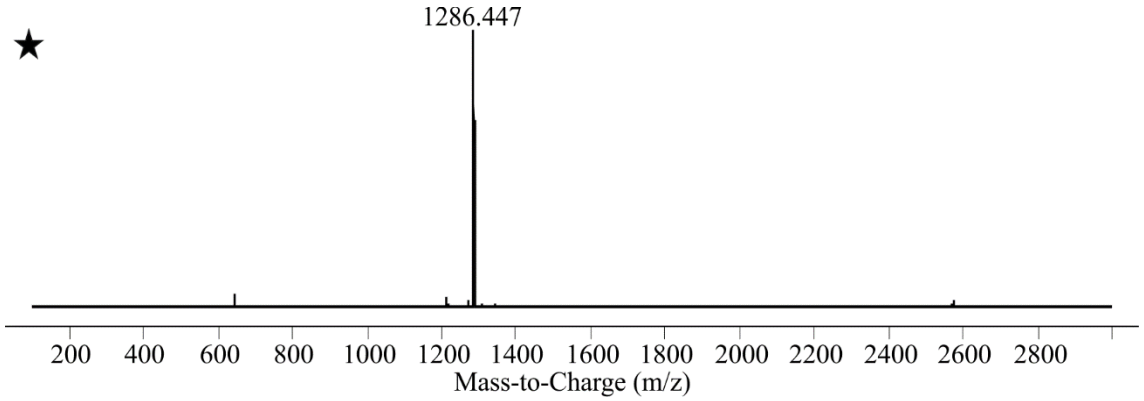


(**1c**) : Prepared according to the representative protocol **(A)** using peptide **1** (2 mM), electrophile **5** (20 mM), and Tris base (20 mM) at 37°C for 12 h. The diluted reaction mixture was analyzed using LC-MS *Method A*. TIC trace of crude reaction and Mass spectrum of product **1c**. Signal of the starting material **1** (yield = 1 %) is marked with a black circle (●). Signal of the arylation product **1c** (yield = 98 %) is marked with a star (★). Analytical data for **1c** : m/z calcd. for $C_{62}H_{64}F_{13}N_{11}O_{10}S_2$ $[M+H]^+$: 1434.42 found 1434.42.

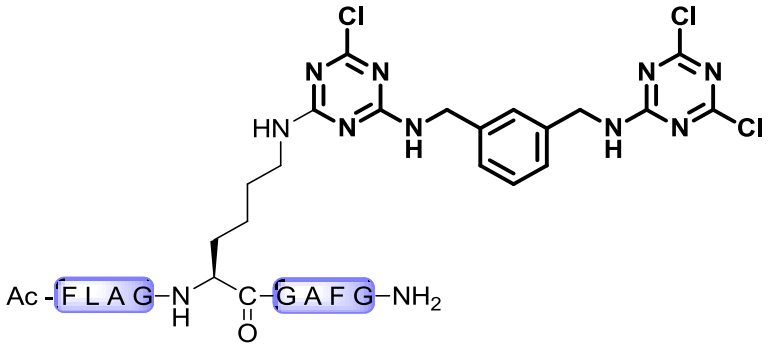


1d

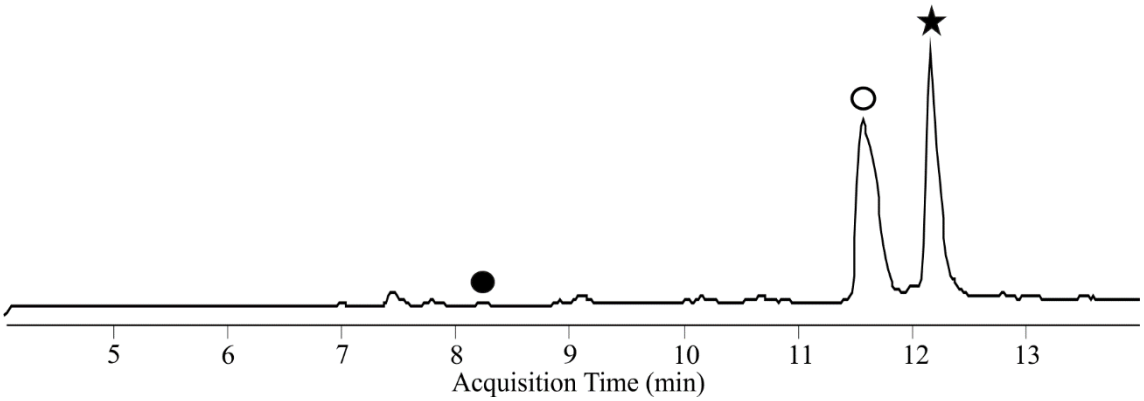


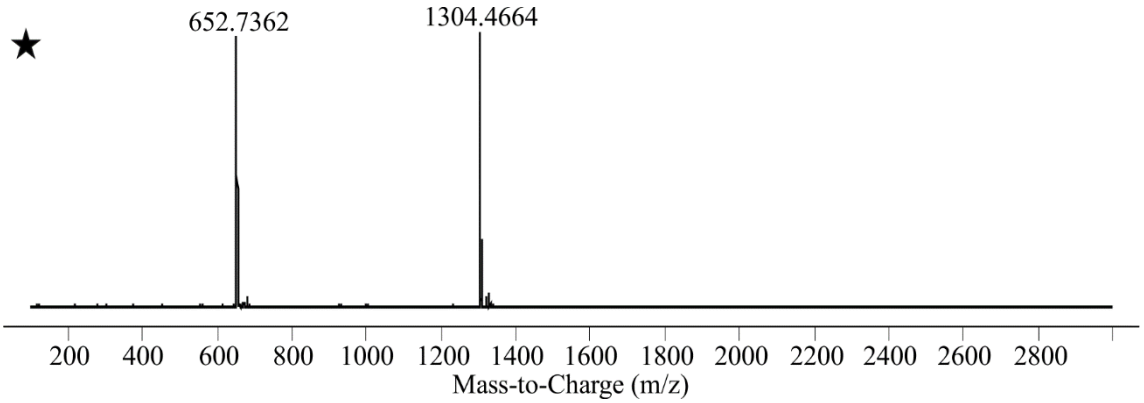


(**1d**) : Prepared according to the representative protocol **(B)** using peptide **1** (1 mM), electrophile **6** (10 mM), and DIEA (10 mM) at room temperature for 1 h. The diluted reaction mixture was analyzed using LC-MS *Method A*. TIC trace of crude reaction and Mass spectrum of product **1d**. Signal of the arylation product **1d** (yield = 99 %) is marked with a star (★). Analytical data for **1d** : m/z calcd. for $C_{56}H_{64}F_9N_{11}O_{12}S$ $[M+H]^+$: 1286.44 found 1286.45.



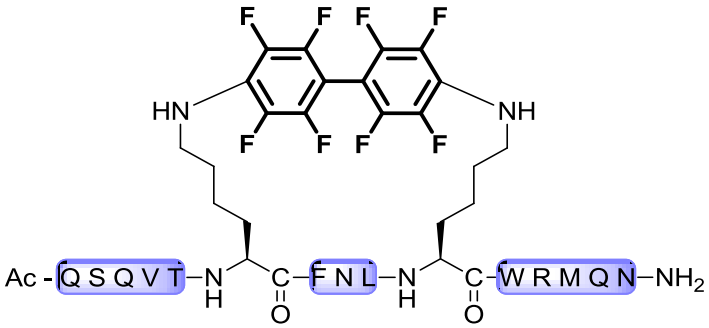
1e



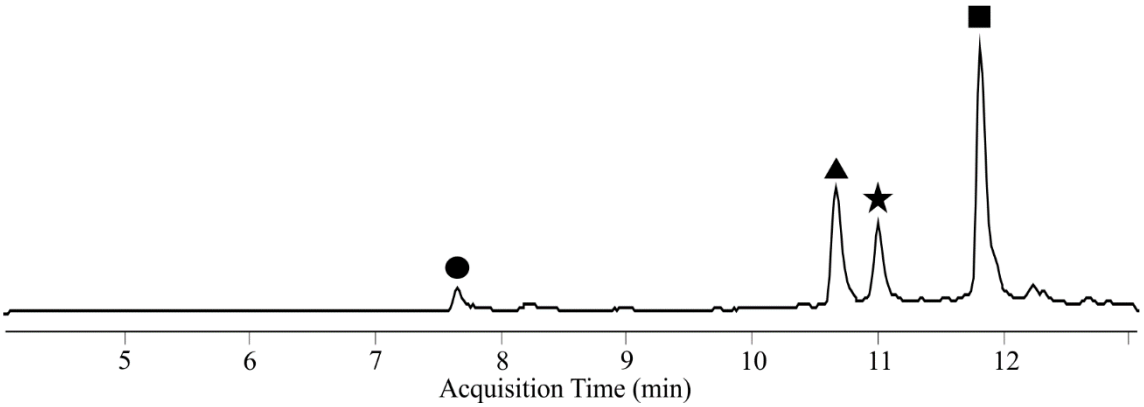


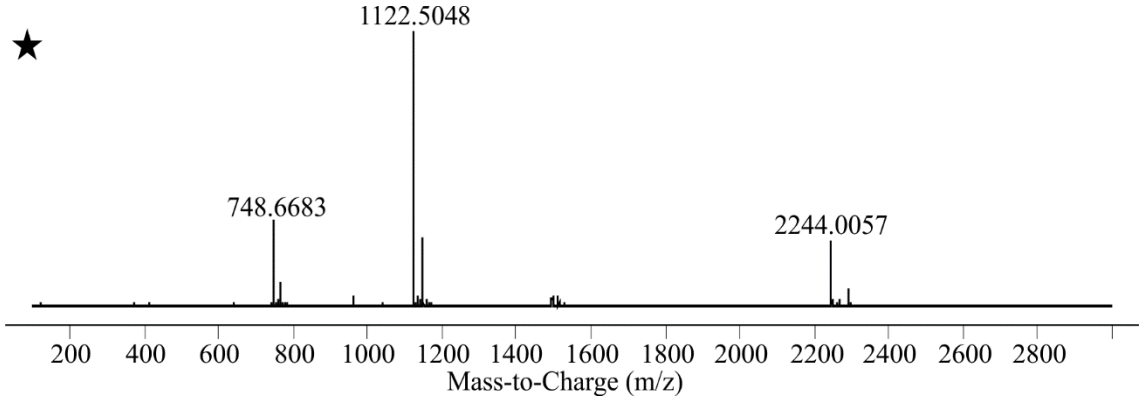
(**1e**) : Prepared according to the representative protocol **(B)** using peptide **1** (1 mM), electrophile **7** (10 mM), and DIEA (10 mM) at room temperature for 1 h. The diluted reaction mixture was analyzed using LC-MS *Method B*. TIC trace of crude reaction and Mass spectrum of product **1e**. Signals of the starting material **1** (yield = 1 %) and **7** are marked with a full black circle (●) and an empty black circle (○), respectively. Signal of the arylation product **1e** (yield = 97 %) is marked with a star (★). Analytical data for **1e** : m/z calcd. for $C_{58}H_{74}Cl_3F_9N_{19}O_{10}$ $[M+H]^+$: 1302.50 found 1302.47.

LC-MS analytical data of stapling crude reaction with peptide **2**



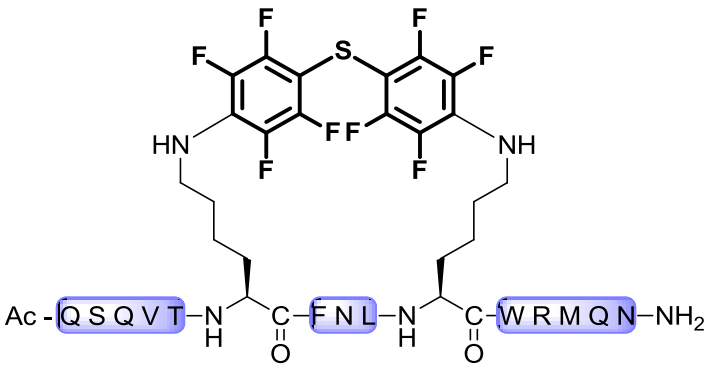
2a



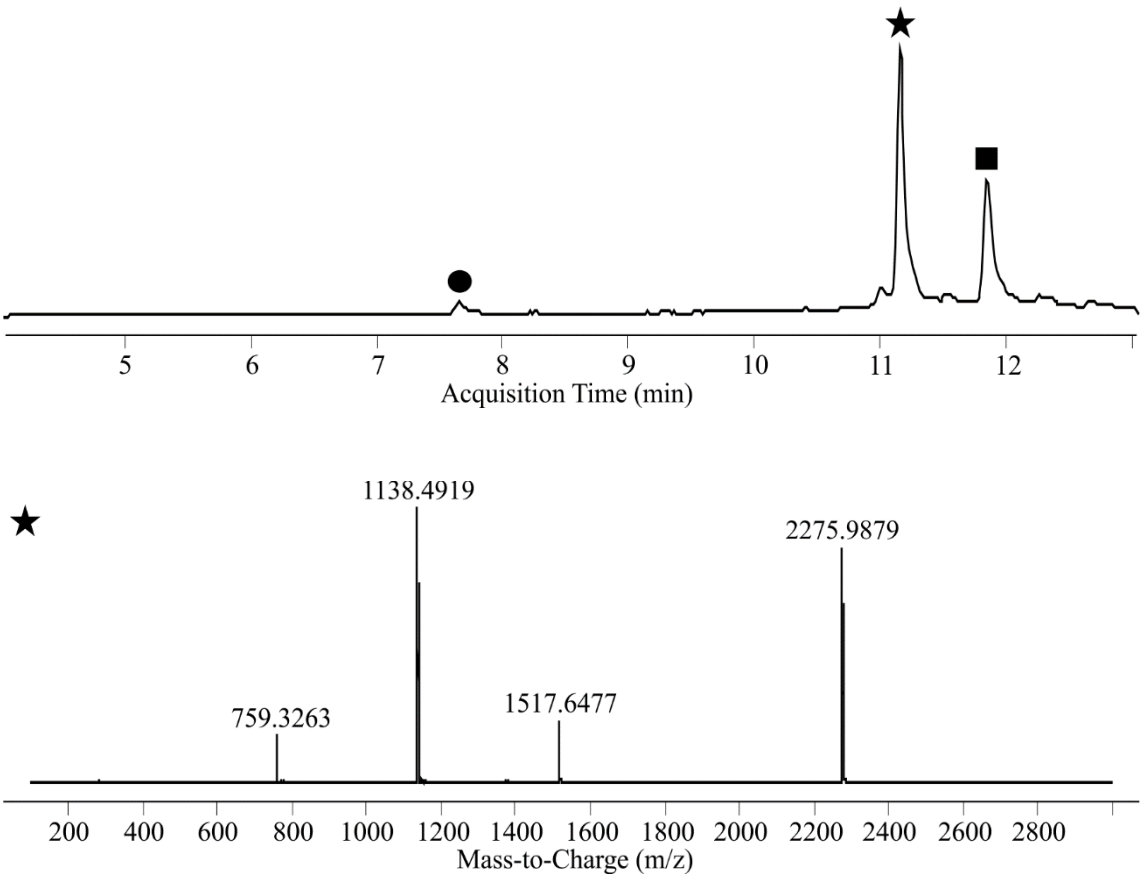


(**2a**) : Prepared according to the representative protocol **(D)** using peptide **2** (2 mM), electrophile **3** (12 mM), and Tris base (40 mM) at 37°C for 24 h. The diluted reaction mixture was analyzed using LC-MS *Method A*. TIC trace of crude reaction and Mass spectrum of product **2a**. Signal of the starting material **2** (yield = 3 %) is marked with a black circle (●). Signal of the mono-arylation product (yield

= 26 %) is marked with a black triangle (▲). Signal of the double-arylation product (yield = 53 %) is marked with a black square (■). Signal of the stapling product **2a** (yield = 17 %) is marked with a star (★). Analytical data for **2a** : m/z calcd. for $C_{98}H_{135}F_8N_{27}O_{23}S$ $[M+2H]^{2+}$: 1122.00 found 1122.00.

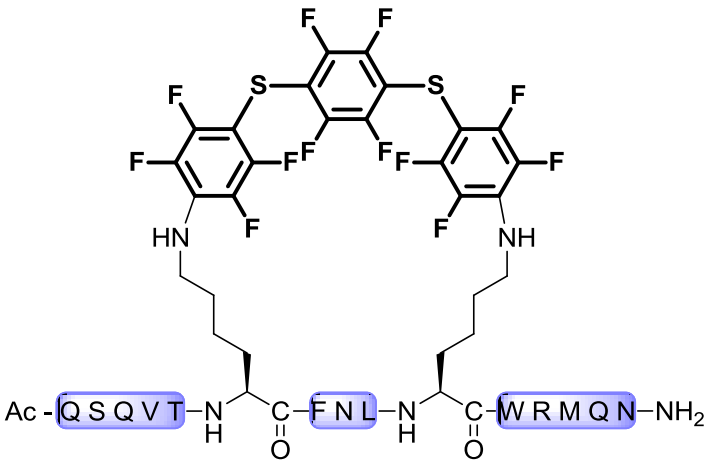


2b

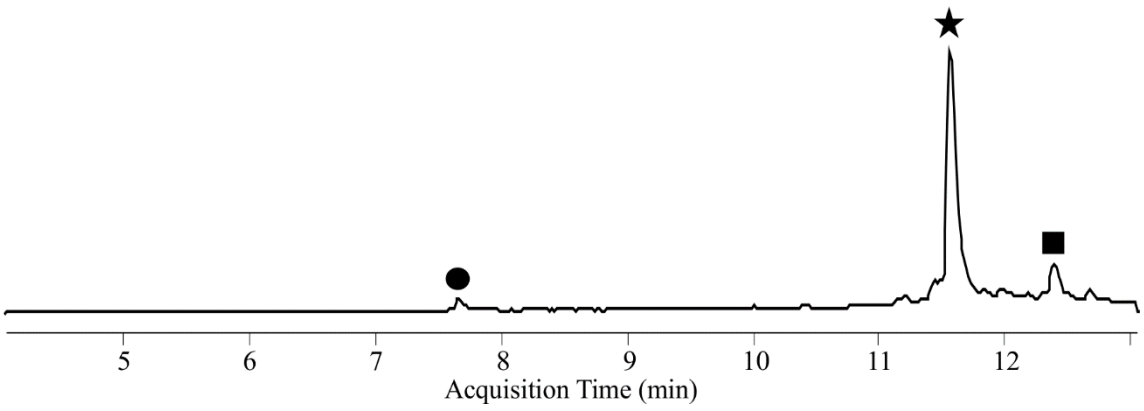


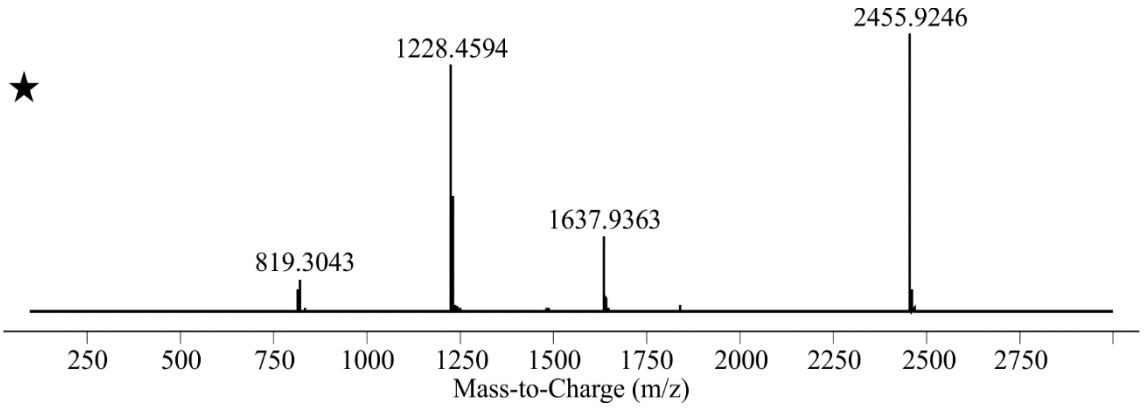
(**2b**) : Prepared according to the representative protocol **(D)** using peptide **2** (2 mM), electrophile **4** (12 mM), and Tris base (40 mM) at 37°C for 24 h. The diluted reaction mixture was analyzed using LC-MS *Method A*. TIC trace of crude reaction and Mass spectrum of product **2b**. Signal of the starting material **2** (yield = 2 %) is marked with a black circle (●). Signal of the double-arylation product (yield = 30 %) is marked with a black square (■). Signal of the stapling product **2b** (yield = 65 %) is

marked with a star (★). Analytical data for **2b** : m/z calcd. for $C_{98}H_{135}F_8N_{27}O_{23}S_2$ $[M+2H]^{2+}$: 1137.98 found 1137.99.



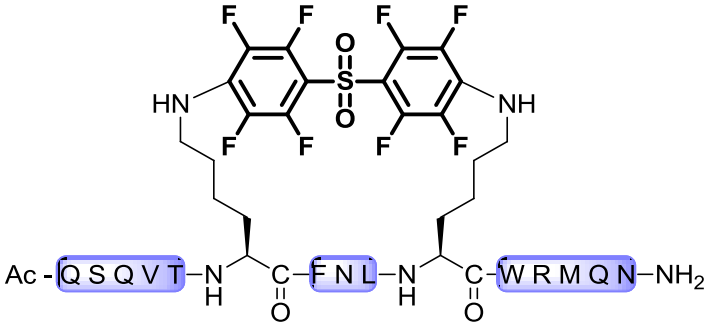
2c



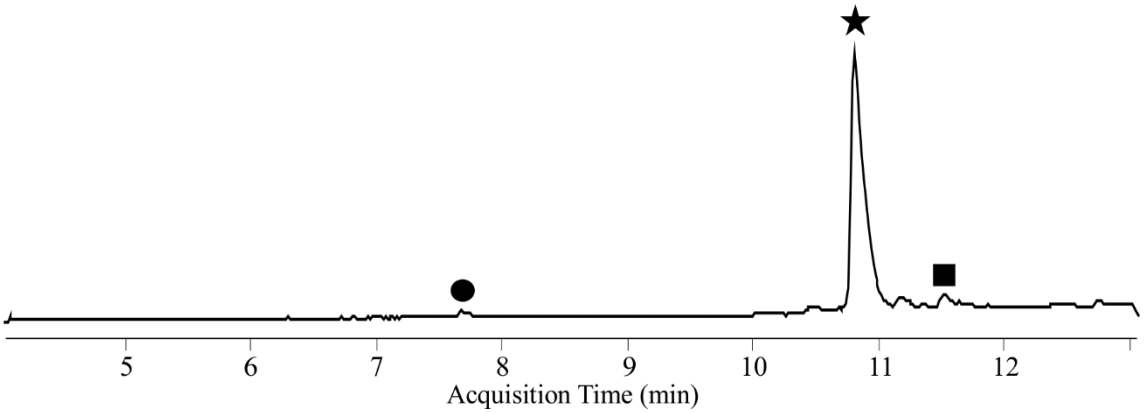


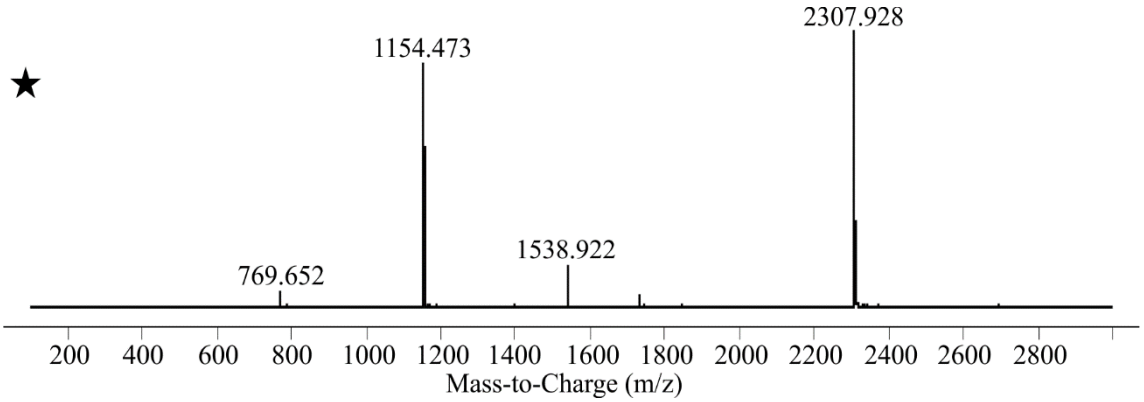
(**2c**) : Prepared according to the representative protocol **(D)** using peptide **2** (2 mM), electrophile **5** (12 mM), and Tris base (40 mM) at 37°C for 24 h. The diluted reaction mixture was analyzed using LC-

MS *Method A*. TIC trace of crude reaction and Mass spectrum of product **2c**. Signal of the starting material **2** (yield = 1 %) is marked with a black circle (●). Signal of the double-arylation product (yield = 7 %) is marked with a black square (■). Signal of the stapling product **2c** (yield = 90 %) is marked with a star (★). Analytical data for **2c** : m/z calcd. for $C_{104}H_{135}F_{12}N_{27}O_{23}S_3$ $[M+2H]^{2+}$: 1227.97 found 1227.96.



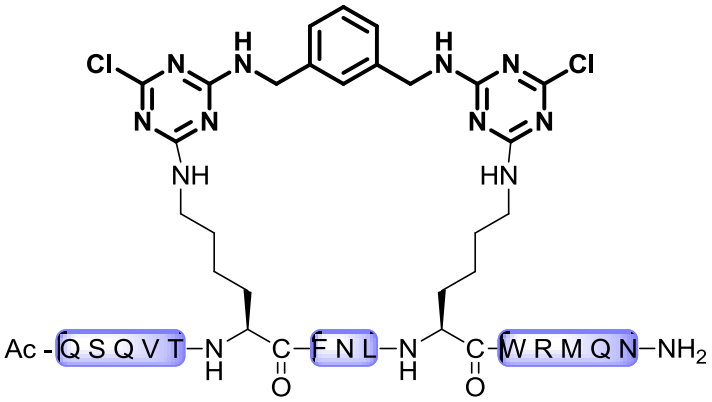
2d



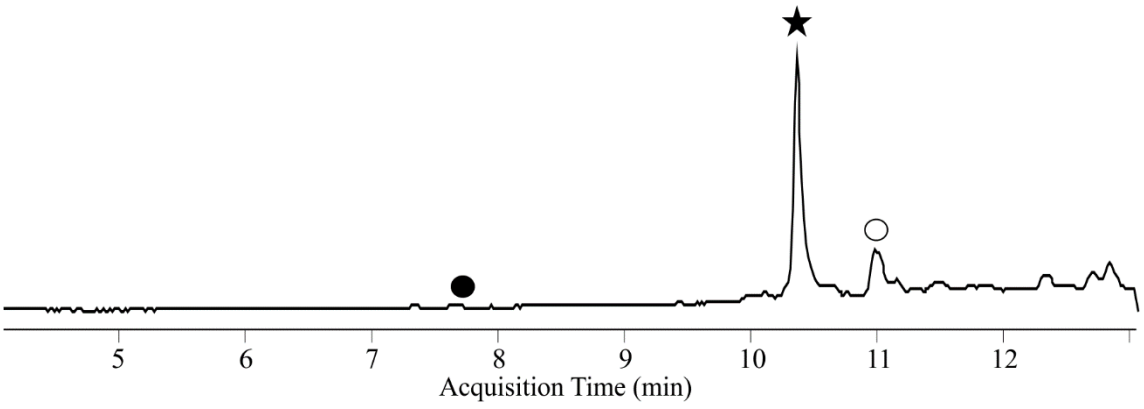


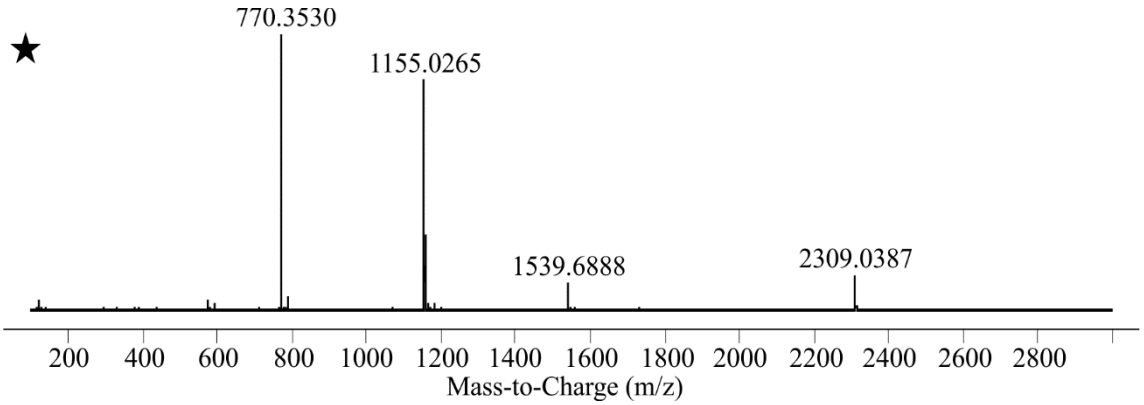
(**2d**) : Prepared according to the representative protocol **(C)** using peptide **2** (0.5 mM), electrophile **6** (0.625 mM), and DIEA (10 mM) at room temperature for 4 h. The diluted reaction mixture was analyzed using LC-MS *Method A*. TIC trace of crude reaction and Mass spectrum of product **2d**.

Signal of the starting material **2** (yield = 1 %) is marked with a black circle (●). Signal of the double-arylation product (yield = 3 %) is marked with a black square (■). Signal of the stapling product **2d** (yield = 95 %) is marked with a star (★). Analytical data for **2d** : m/z calcd. for $C_{98}H_{135}F_8N_{27}O_{25}S_2$ $[M+2H]^{2+}$: 1153.98 found 1153.97.



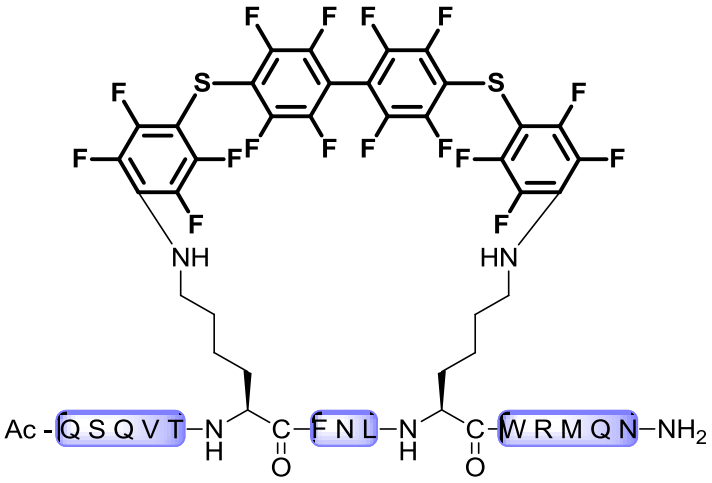
2e



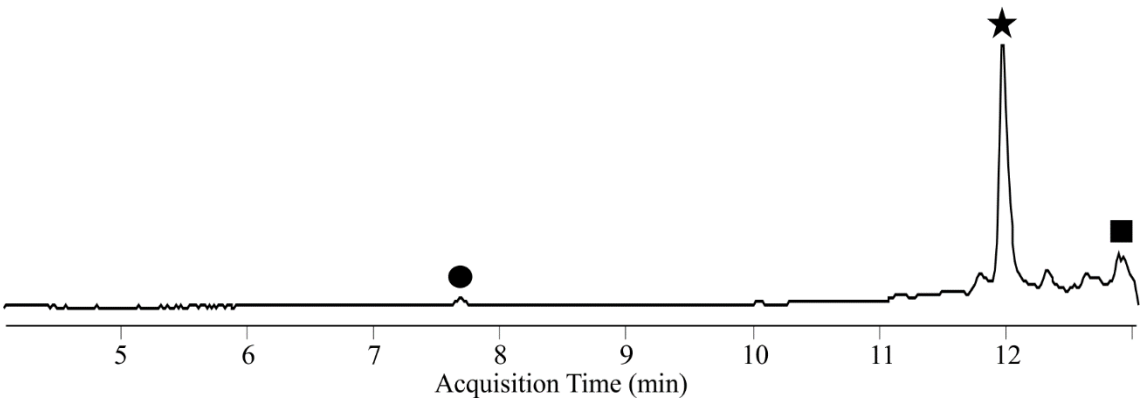


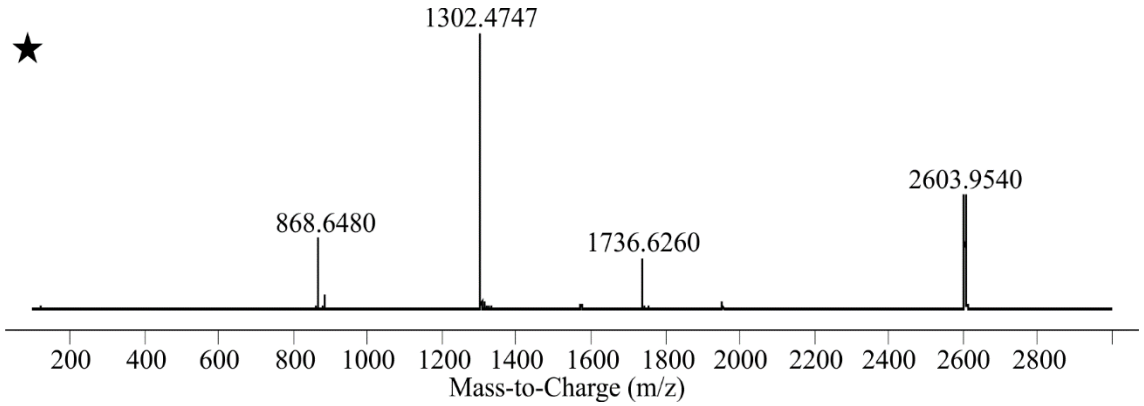
(**2e**) : Prepared according to the representative protocol **(E)** using peptide **2** (1 mM), electrophile **7** (2 mM), and DIEA (20 mM) at room temperature for 4 h. The diluted reaction mixture was analyzed using LC-MS *Method A*. TIC trace of crude reaction and Mass spectrum of product **2e**. Signals of the

starting material **2** (yield = 1 %) and **7** are marked with a full black circle (●) and an empty black circle (○), respectively. Signal of the stapling product **2e** (yield = 96 %) is marked with a star (★). Analytical data for **2e** : m/z calcd. for $C_{100}H_{145}Cl_2N_{35}O_{23}S$ $[M+2H]^{2+}$: 1154.02 found 1154.02.



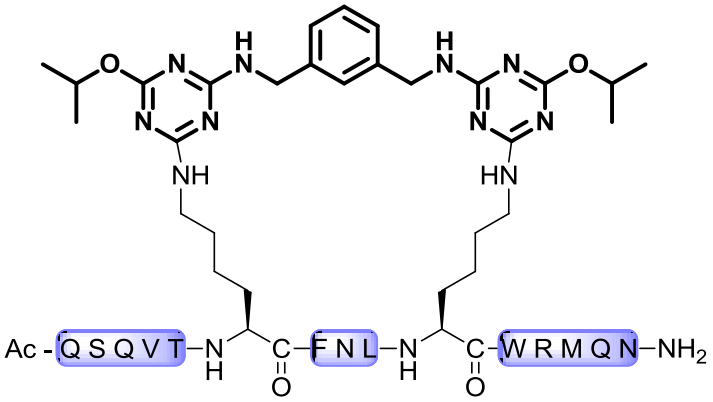
2f



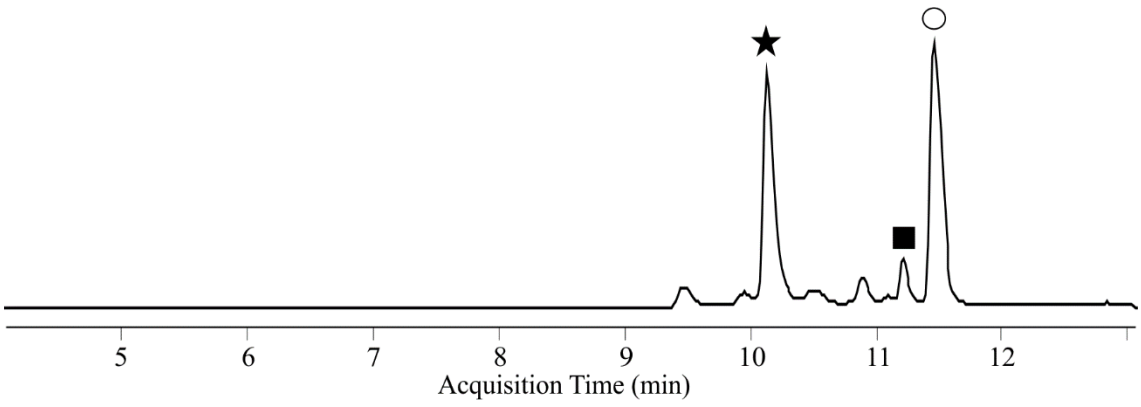


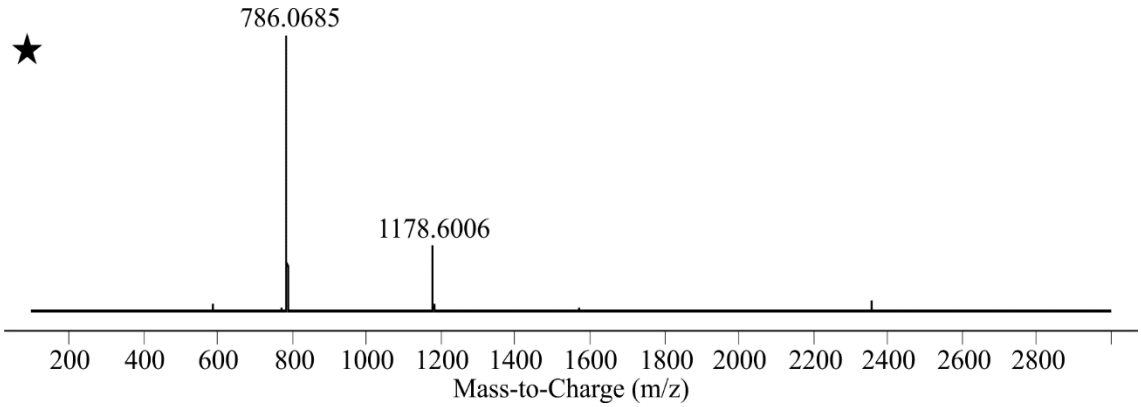
(**2f**) : Prepared according to the representative protocol **(D)** using peptide **2** (2 mM), electrophile (12 mM), and Tris base (40 mM) at 37°C for 24 h. The diluted reaction mixture was analyzed using LC-

MS *Method A*. TIC trace of crude reaction and Mass spectrum of product **2f**. Signal of the starting material **2** (yield = 1 %) is marked with a black circle (●). Signal of the double-arylation product (yield = 8 %) is marked with a black square (■). Signal of the stapling product **2f** (yield = 88 %) is marked with a star (★). Analytical data for **2f** : m/z calcd. for $C_{110}H_{135}F_{16}N_{27}O_{23}S_3$ $[M+2H]^{2+}$: 1301.96 found 1301.97.



2g



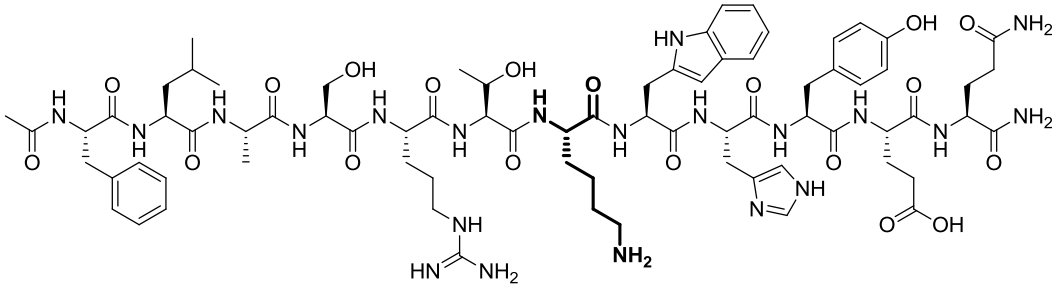


(**2g**) : Prepared according to the representative protocol **(F)** using peptide **2** (2 mM), electrophile **9** (10 mM), and Tris base (40 mM) at 37°C for 24 h. The diluted reaction mixture was analyzed using LC-MS *Method A*. TIC trace of crude reaction and Mass spectrum of product **2g**. Signal of the starting

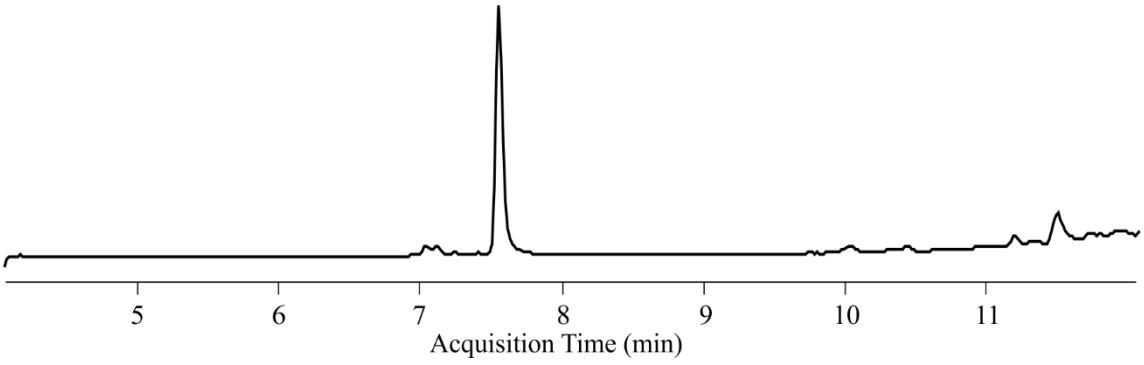
material **9** is marked with an empty black circle (○). Signal of the double-arylation product (yield = 8 %) is marked with a black square (■). Signal of the stapling product **2g** (yield = 88 %) is marked with a star (★). Analytical data for **2g** : m/z calcd. for $C_{110}H_{135}F_{16}N_{27}O_{23}S_3$ $[M+2H]^{2+}$: 1178.11 found 1178.10.

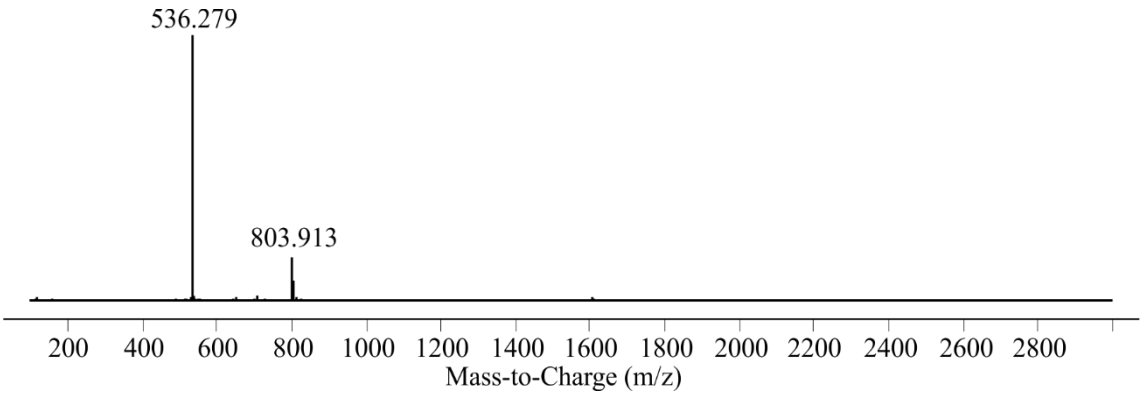
6. Selectivity assay

LC-MS analytical data of purified peptides **16** and **17**

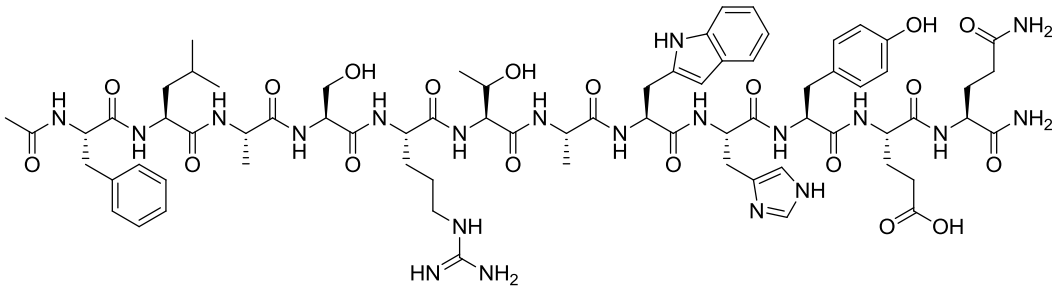


16: AcNH-Phe-Leu-Ala-Ser-Arg-Thr-**Lys**-Trp-His-Tyr-Glu-Gln-$CONH_2$

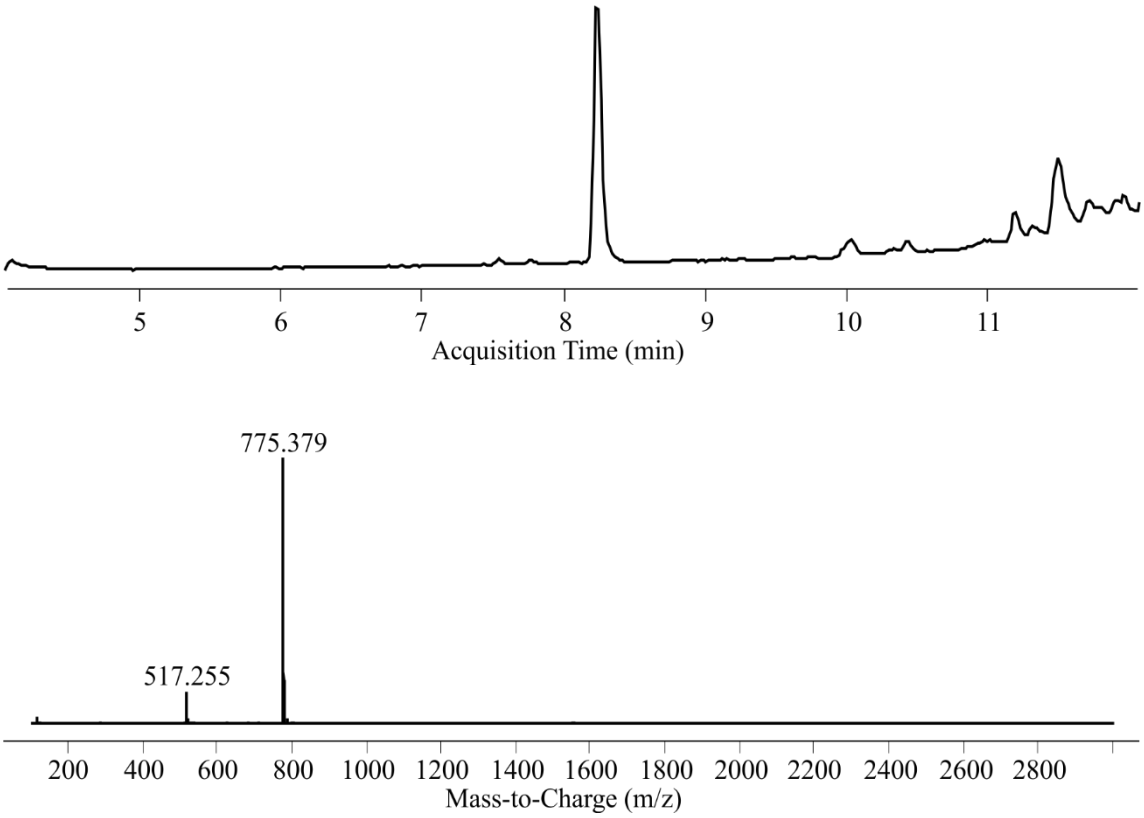




Peptide **16**: LC-MS analysis *Method A*. TIC trace and Mass spectrum of peptide **16**. m/z calcd. for $C_{75}H_{107}N_{21}O_{19}$ $[M+2H]^{2+}$: 803.91 found 803.91.

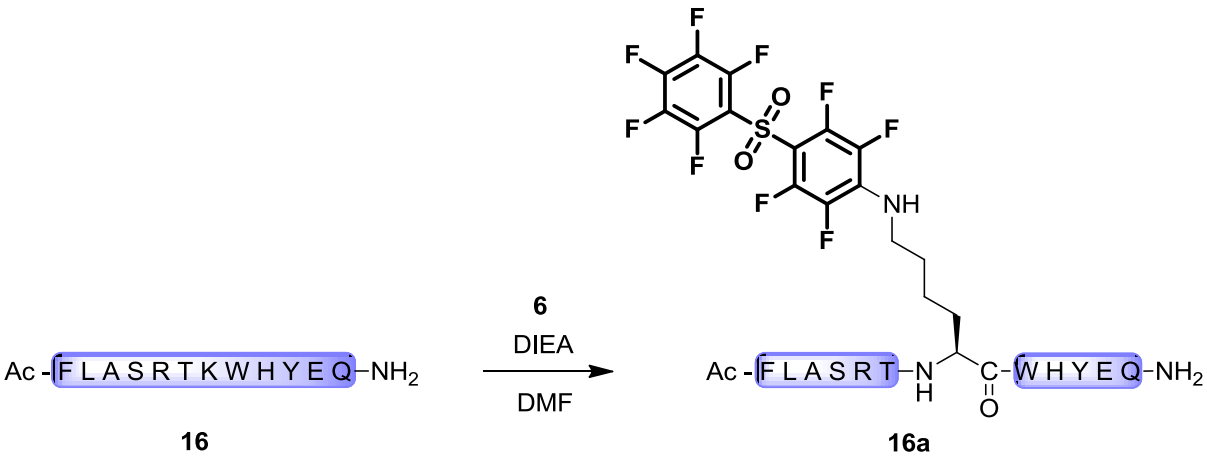


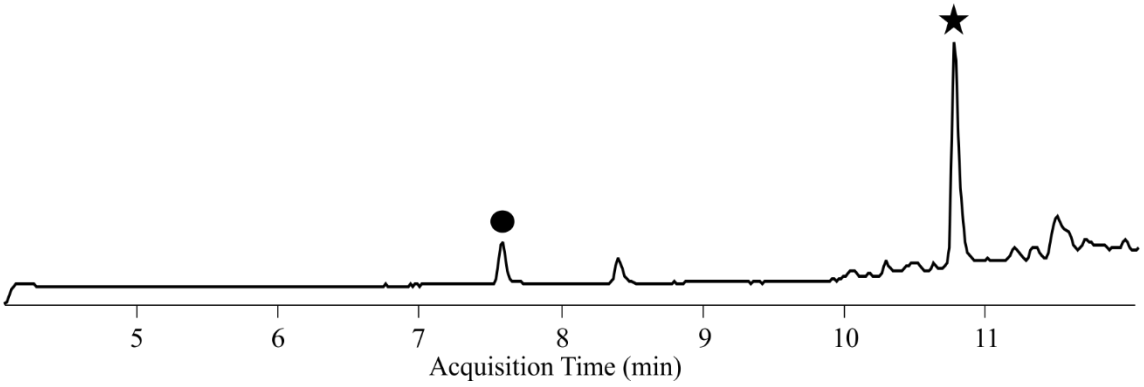
17: AcNH-Phe-Leu-Ala-Ser-Arg-Thr-Ala-Trp-His-Tyr-Glu-Gln-$CONH_2$



Peptide **17**: LC-MS analysis *Method A*. TIC trace and Mass spectrum of peptide **17**. m/z calcd. for $C_{75}H_{107}N_{21}O_{19}$ $[M+2H]^{2+}$: 775.38 found 775.38.

LC-MS analytical data of crude reaction with peptide **16** and **17**





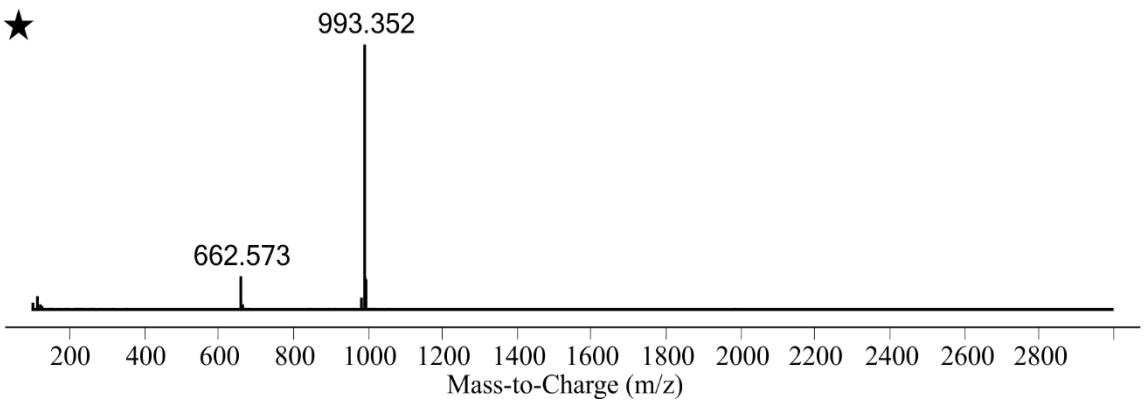


Figure S1. Selectivity assay has been ran under concentrated conditions using peptide **16** (5 mM), electrophile **6** (12.5 mM), and DIEA (50 mM) at room temperature for 30 min. The diluted reaction mixture was analyzed using LC-MS *Method A.* TIC trace of crude reaction and Mass spectrum of product **16a**. Signal of the starting material **16** (yield = 9 %) is marked with a black circle (●). Signal of the arylation product **16a** (yield = 88 %) is marked with a star (★). Analytical data for **16a** : m/z calcd. for $C_{87}H_{106}F_9N_{21}O_{21}S$ $[M+2H]^{2+}$: 992.88 found 992.85.

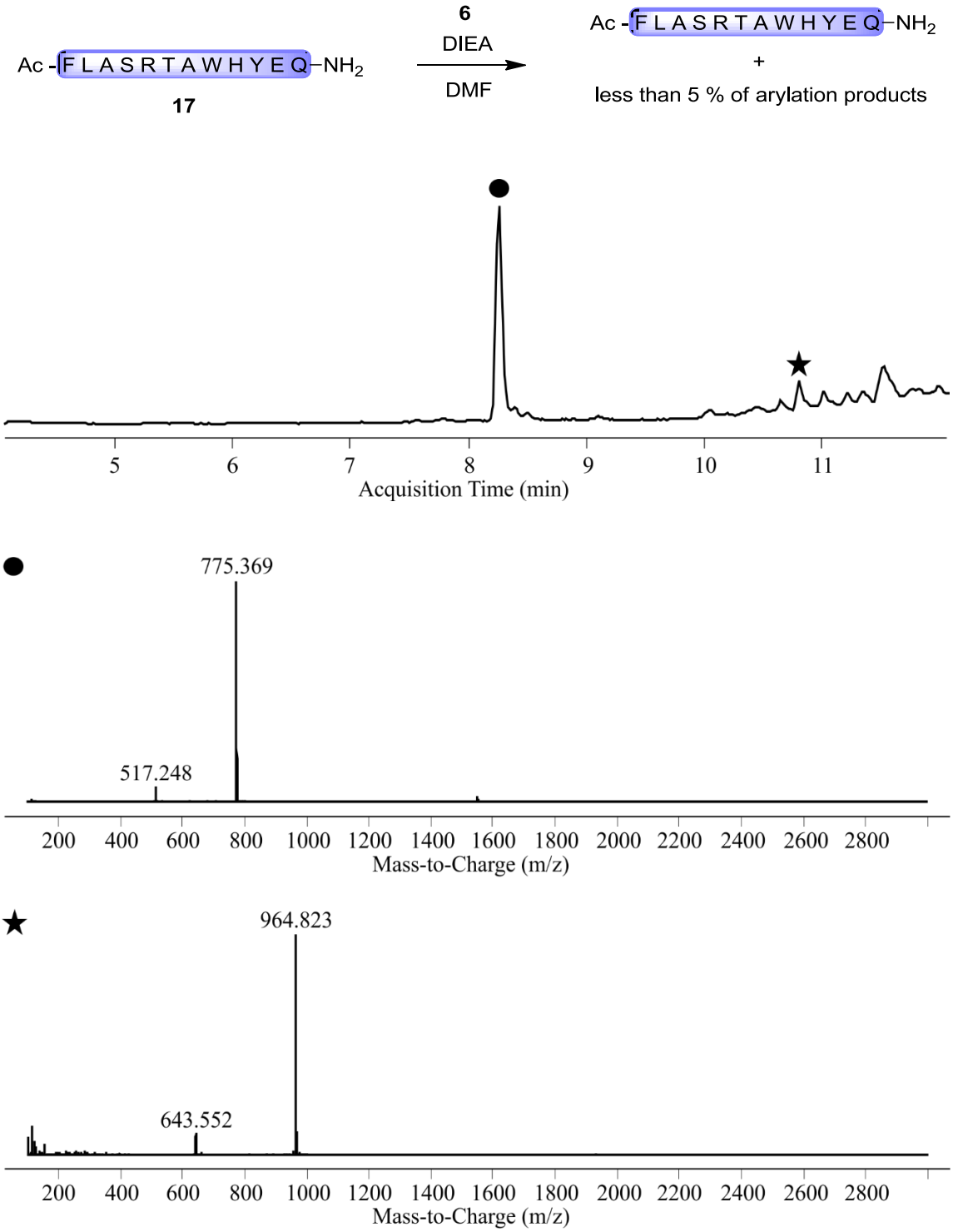


Figure S2. Selectivity assay has been ran under concentrated conditions using peptide **17** (5 mM), electrophile **6** (12.5 mM), and DIEA (50 mM) at room temperature for 30 min. The diluted reaction mixture was analyzed using LC-MS *Method A.* TIC trace of crude reaction and Mass spectrum of peptide **17** and arylation products. Signal of the starting material **17** (yield = 92 %) is marked with a black circle (●). Signal of arylation product (yield ≤ 5 %) is marked with a star (★). Analytical data for arylation product : m/z calcd. for $C_{87}H_{106}F_9N_{21}O_{21}S$ $[M+2H]^{2+}$: 964.35 found 964.32.

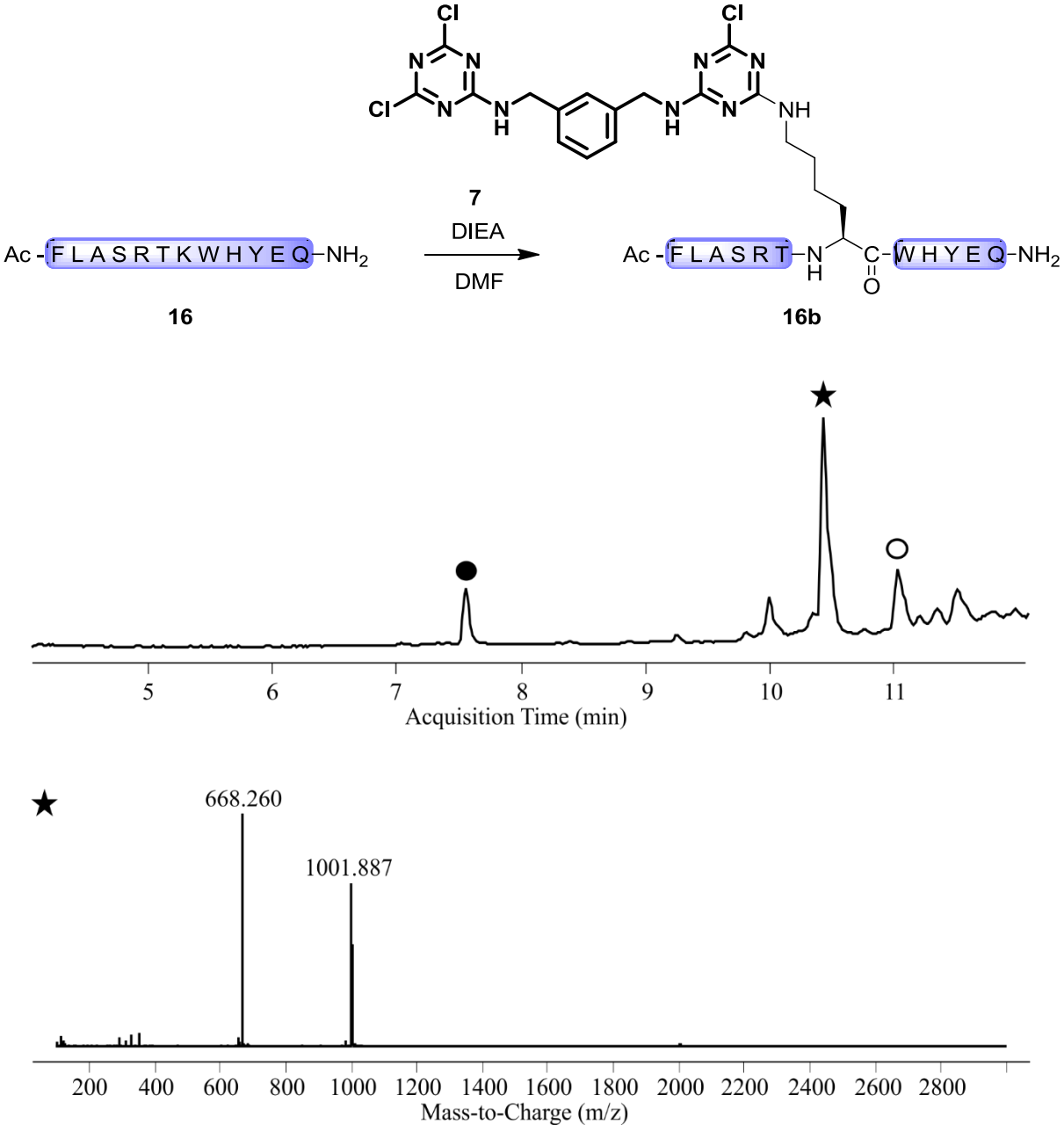


Figure S3. Selectivity assay has been ran under concentrated conditions using peptide **16** (5 mM), electrophile **16b** (25 mM), and DIEA (50 mM) at room temperature for 60 min. The diluted reaction mixture was analyzed using LC-MS *Method A.* TIC trace of crude reaction and Mass spectrum of product **16b**. Signals of the starting material **16** (yield = 15 %) and **7** are marked with a full black

circle (●) and an empty black circle (○), respectively. Signal of the arylation product **16b** (yield = 78 %) is marked with a star (★). Analytical data for **16b** : m/z calcd. for $C_{89}H_{116}Cl_3N_{29}O_{19}$ $[M+2H]^{2+}$: 1000.91 found 1000.89.

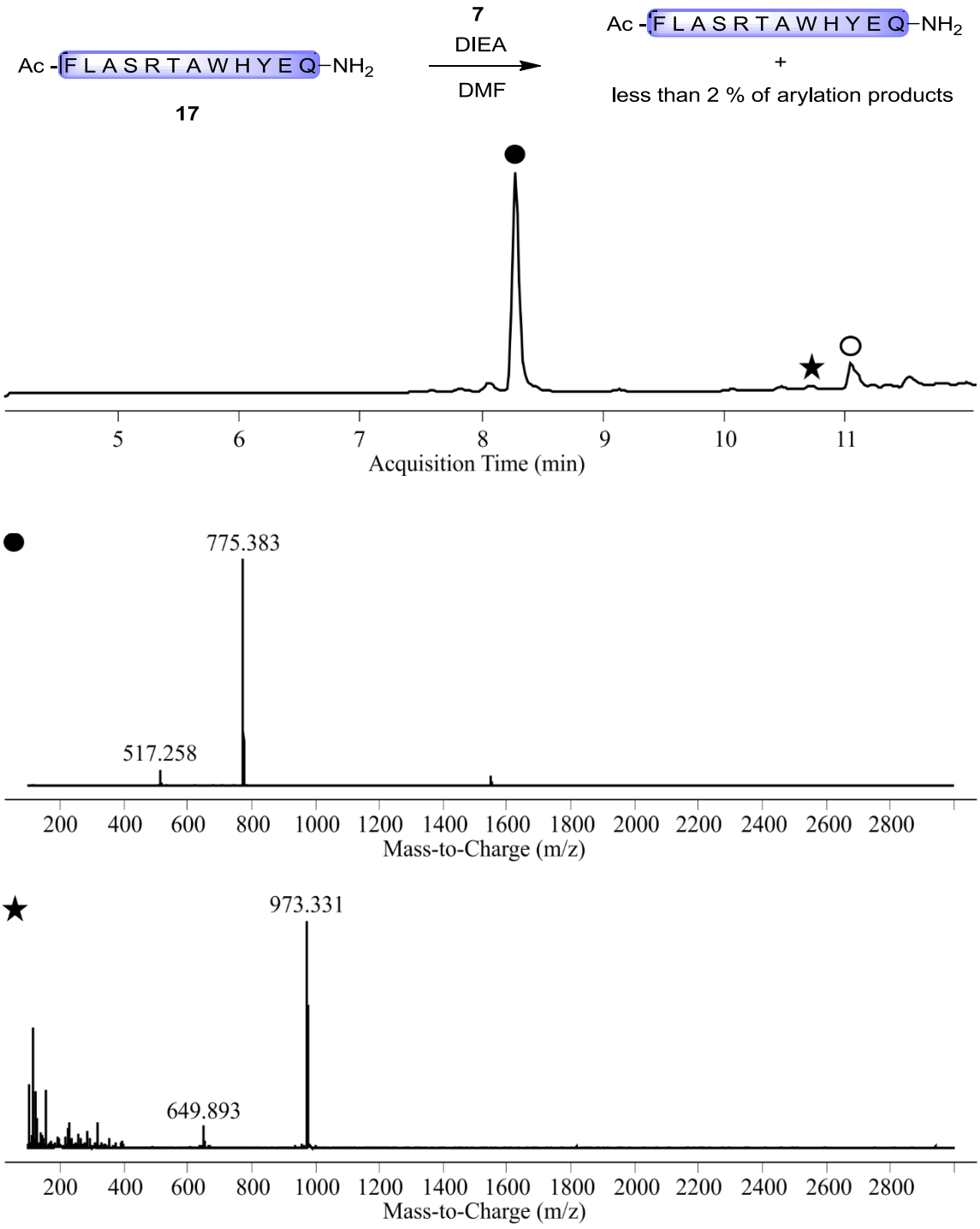
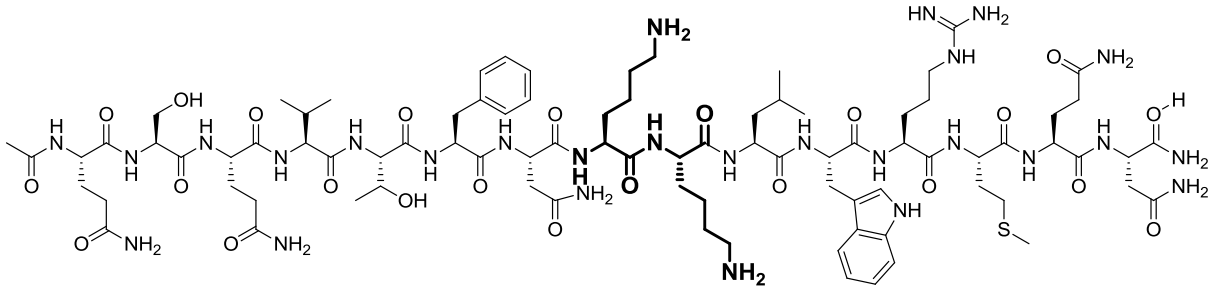


Figure S4. Selectivity assay has been ran under concentrated conditions using peptide **17** (5 mM), electrophile **7** (25 mM), and DIEA (50 mM) at room temperature for 60 min. The diluted reaction

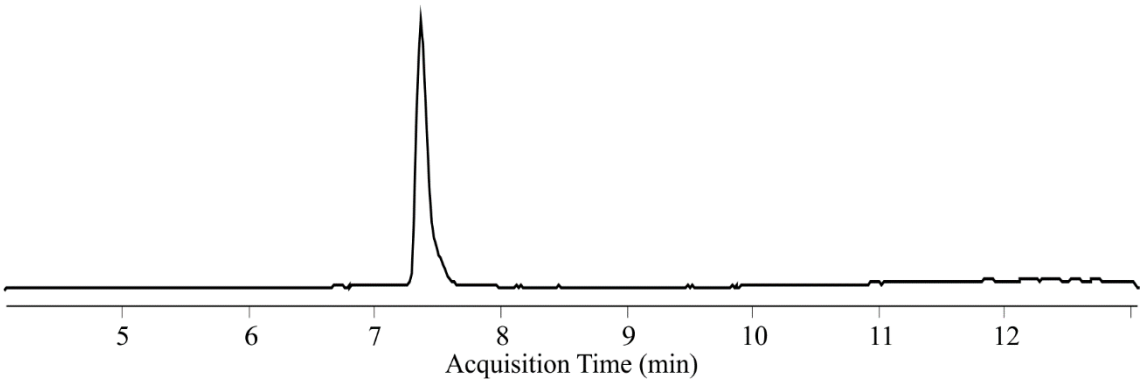
mixture was analyzed using LC-MS *Method A.* TIC trace of crude reaction and Mass spectrum of peptide **17** and arylation products. Signals of the starting material **17** (yield = 96 %) and **7** are marked with a full black circle (●) and an empty black circle (○), respectively. Signal of arylation product (yield ≤ 2 %) is marked with a star (★). Analytical data for arylation product : m/z calcd. for $C_{86}H_{109}Cl_3N_{28}O_{19}$ $[M+2H]^{2+}$: 972.38 found 972.37.

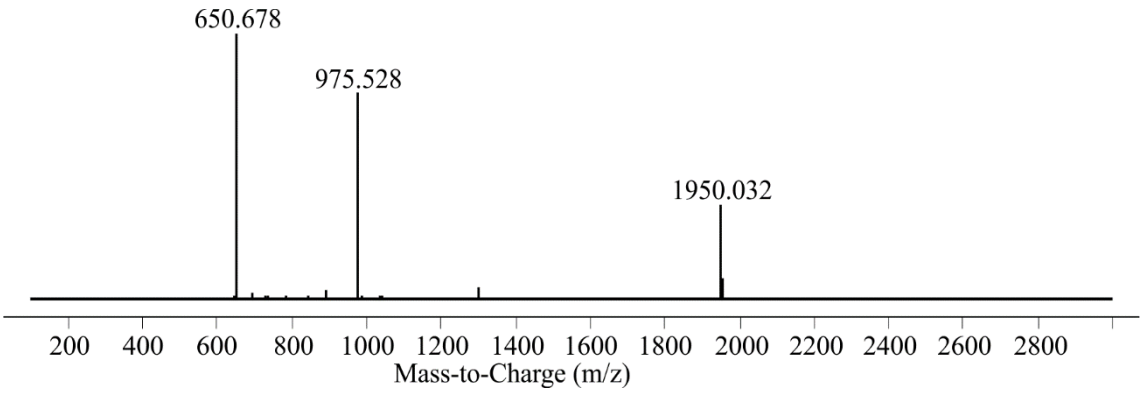
7. Macrocyclization Scan

LC-MS analytical data of purified peptides **18**-**30**

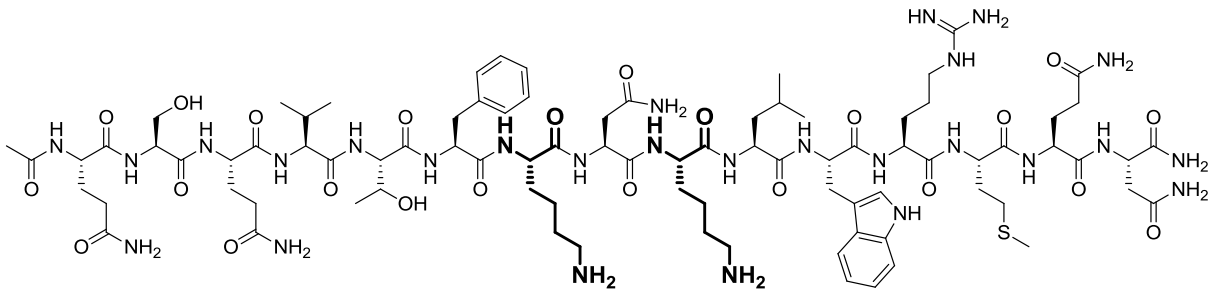


18: AcNH-Gln-Ser-Gln-Val-Thr-Phe-Asn-**Lys**-**Lys**-Leu-Trp-Arg-Met-Gln-Asn-$CONH_2$

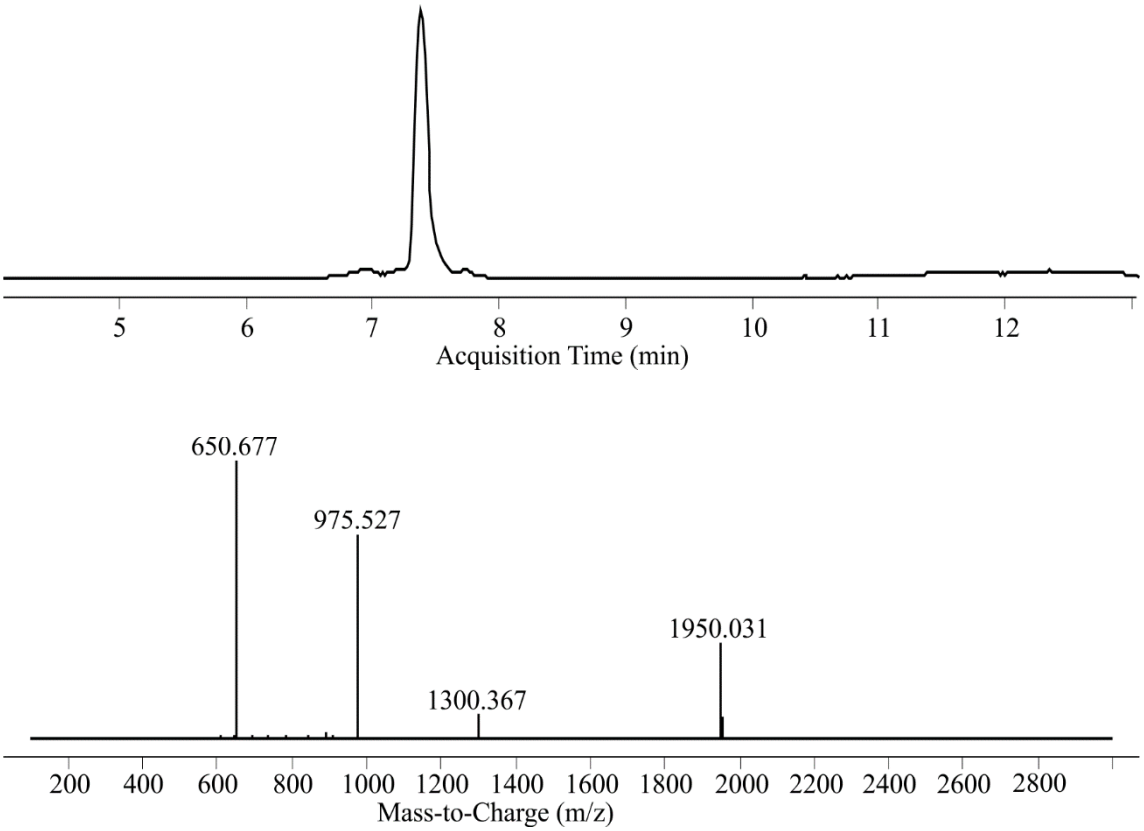




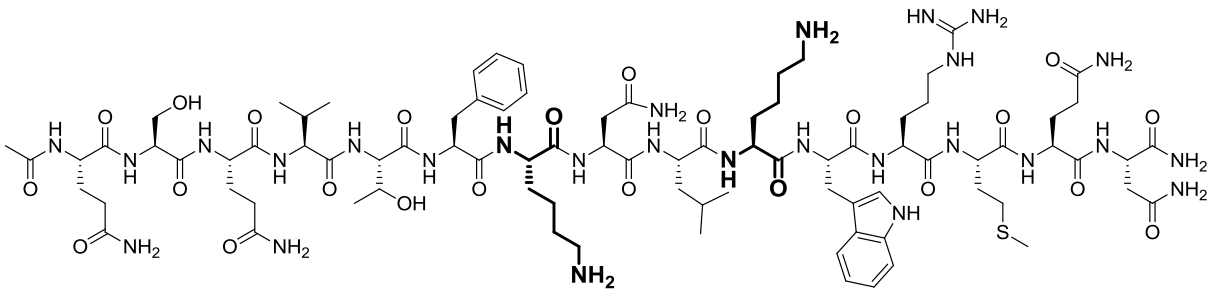
Peptide **18**: LC-MS analysis *Method A*. TIC trace and Mass spectrum of peptide **18**. m/z calcd. for $C_{86}H_{137}N_{27}O_{23}S$ $[M+2H]^{2+}$: 975.01 found 975.03.



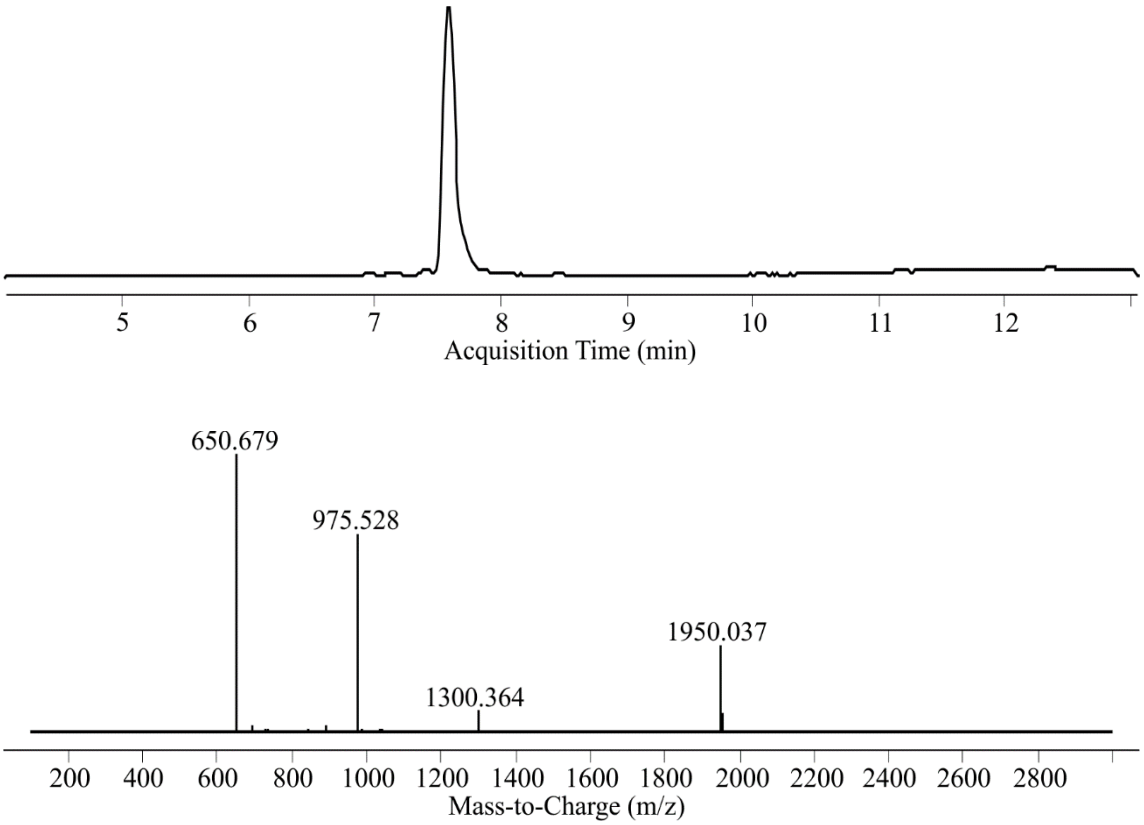
19: AcNH-Gln-Ser-Gln-Val-Thr-Phe-**Lys-**Asn-**Lys-**Leu-Trp-Arg-Met-Gln-Asn-$CONH_2$



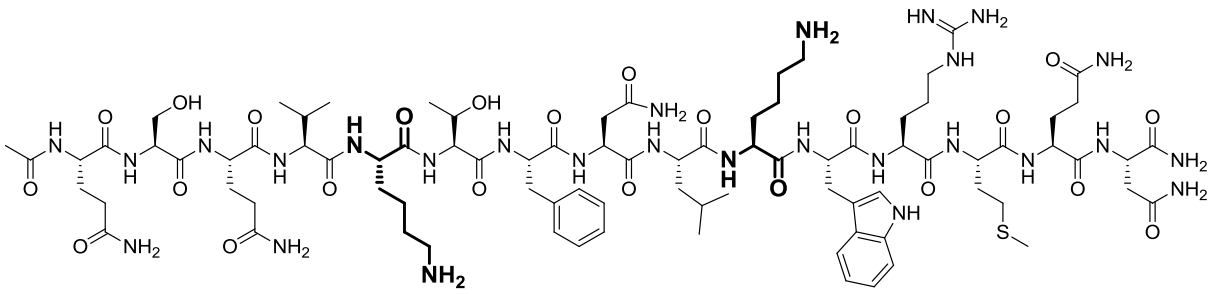
Peptide **19**: LC-MS analysis *Method A*. TIC trace and Mass spectrum of peptide **19**. m/z calcd. for $C_{86}H_{137}N_{27}O_{23}S$ $[M+2H]^{2+}$: 975.01 found 975.03.



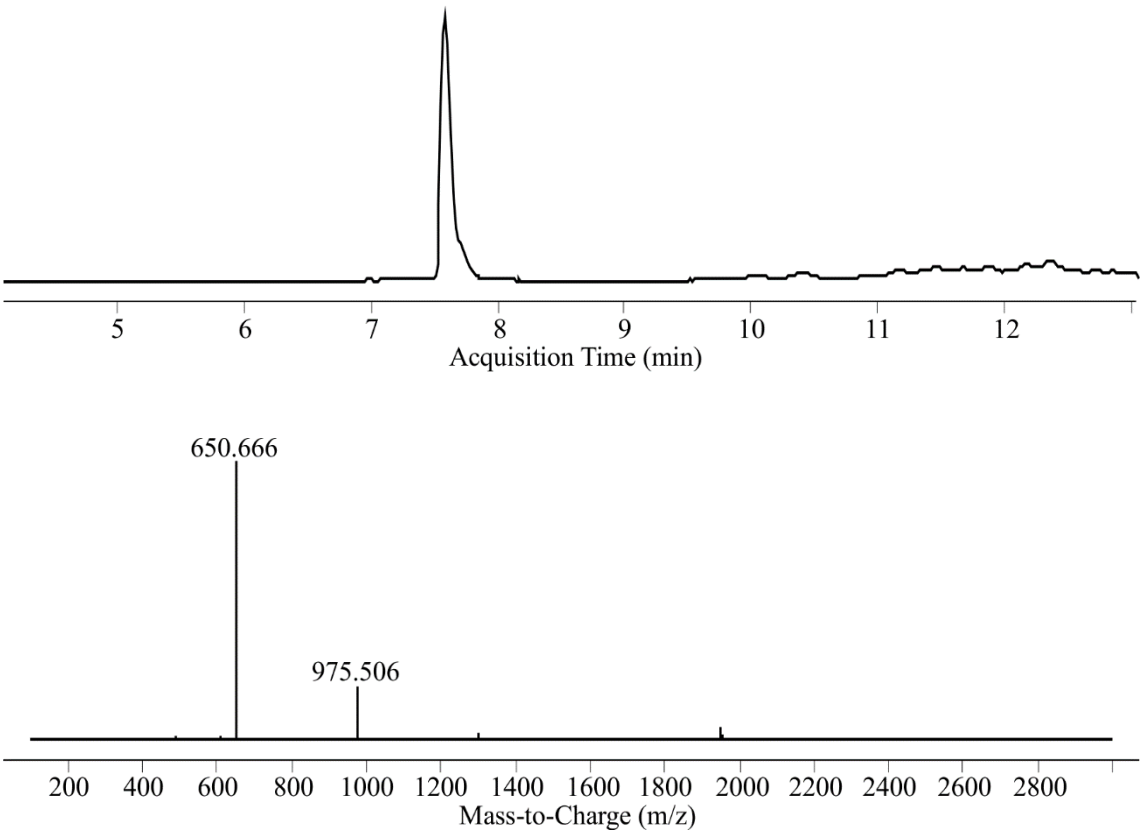
20: AcNH-Gln-Ser-Gln-Val-Thr-Phe-**Lys-**Asn-Leu-**Lys-**Trp-Arg-Met-Gln-Asn-$CONH_2$



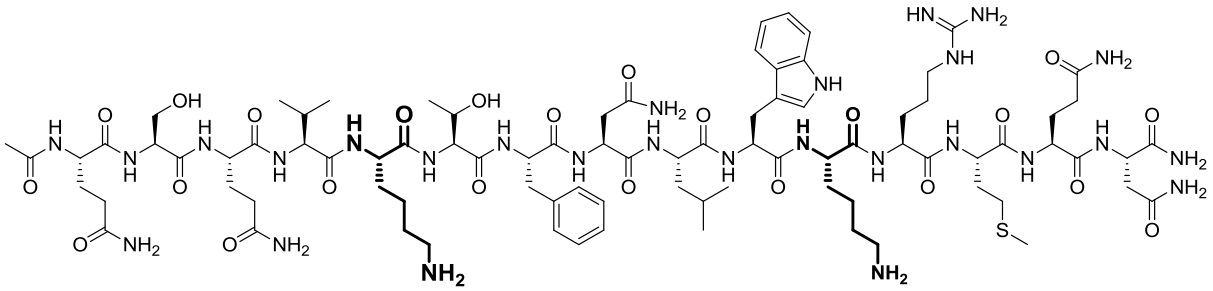
Peptide **20**: LC-MS analysis *Method A*. TIC trace and Mass spectrum of peptide **20**. m/z calcd. for $C_{86}H_{137}N_{27}O_{23}S$ $[M+2H]^{2+}$: 975.01 found 975.03.



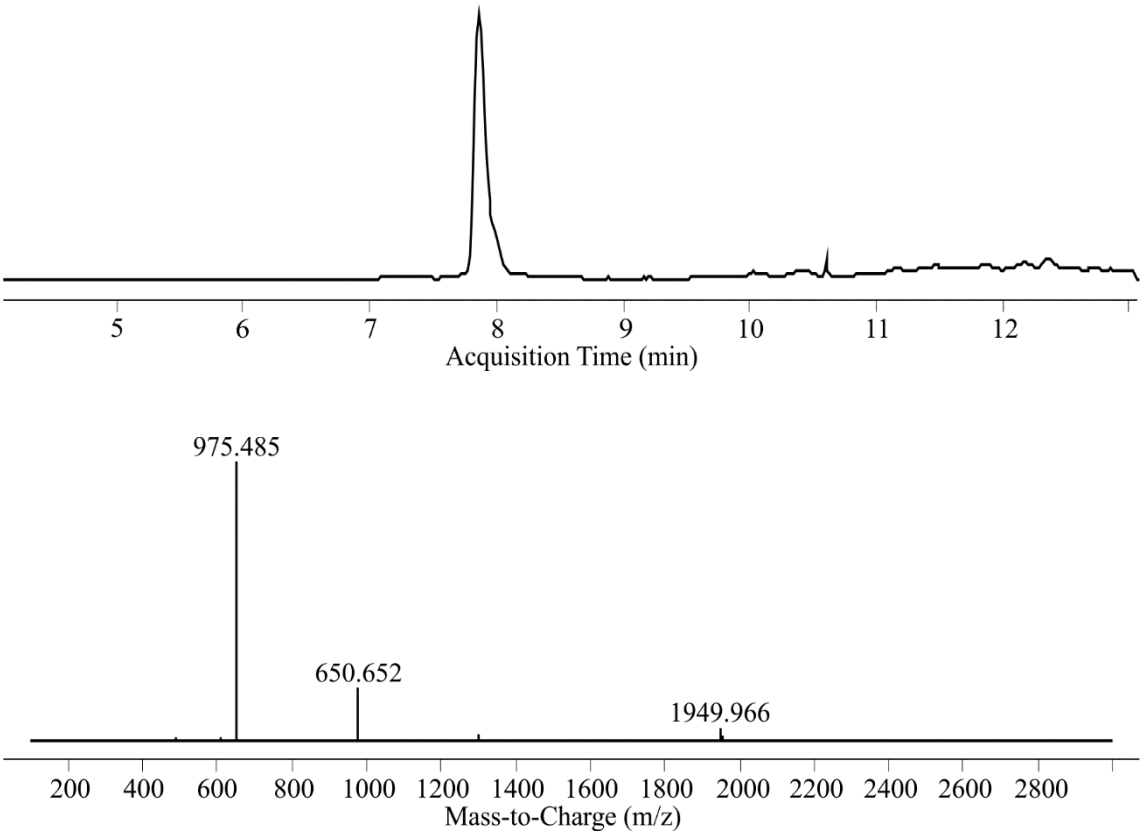
21: AcNH-Gln-Ser-Gln-Val-**Lys-**Thr-Phe-Asn-Leu-**Lys-**Trp-Arg-Met-Gln-Asn-$CONH_2$



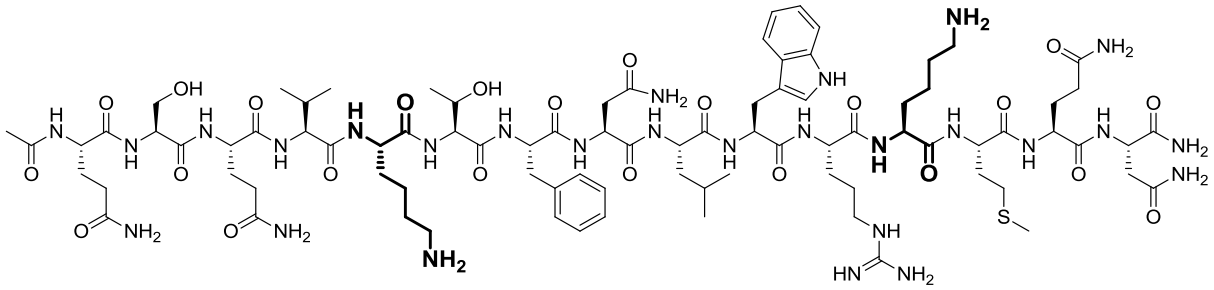
Peptide **21**: LC-MS analysis *Method A*. TIC trace and Mass spectrum of peptide **21**. m/z calcd. for $C_{86}H_{137}N_{27}O_{23}S$ $[M+2H]^{2+}$: 975.01 found 975.01.



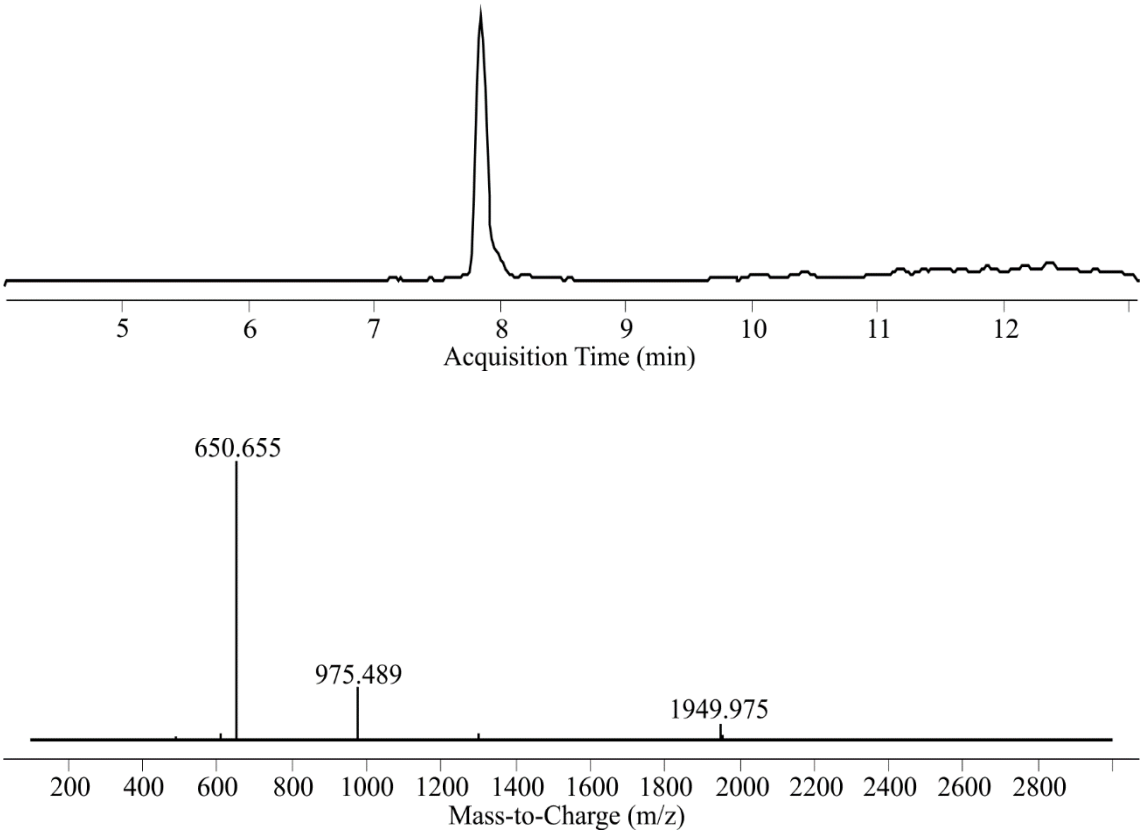
22: AcNH-Gln-Ser-Gln-Val-**Lys-**Thr-Phe-Asn-Leu-Trp-**Lys**-Arg-Met-Gln-Asn-$CONH_2$



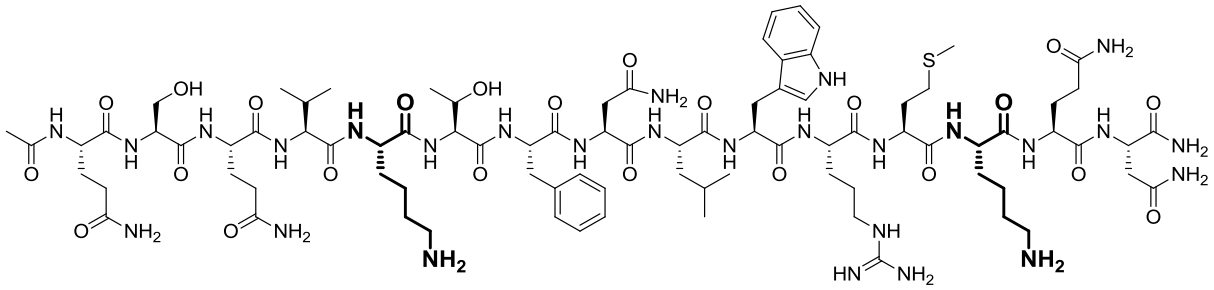
Peptide **22**: LC-MS analysis *Method A*. TIC trace and Mass spectrum of peptide **22**. m/z calcd. for $C_{86}H_{137}N_{27}O_{23}S$ $[M+2H]^{2+}$: 975.01 found 974.98.



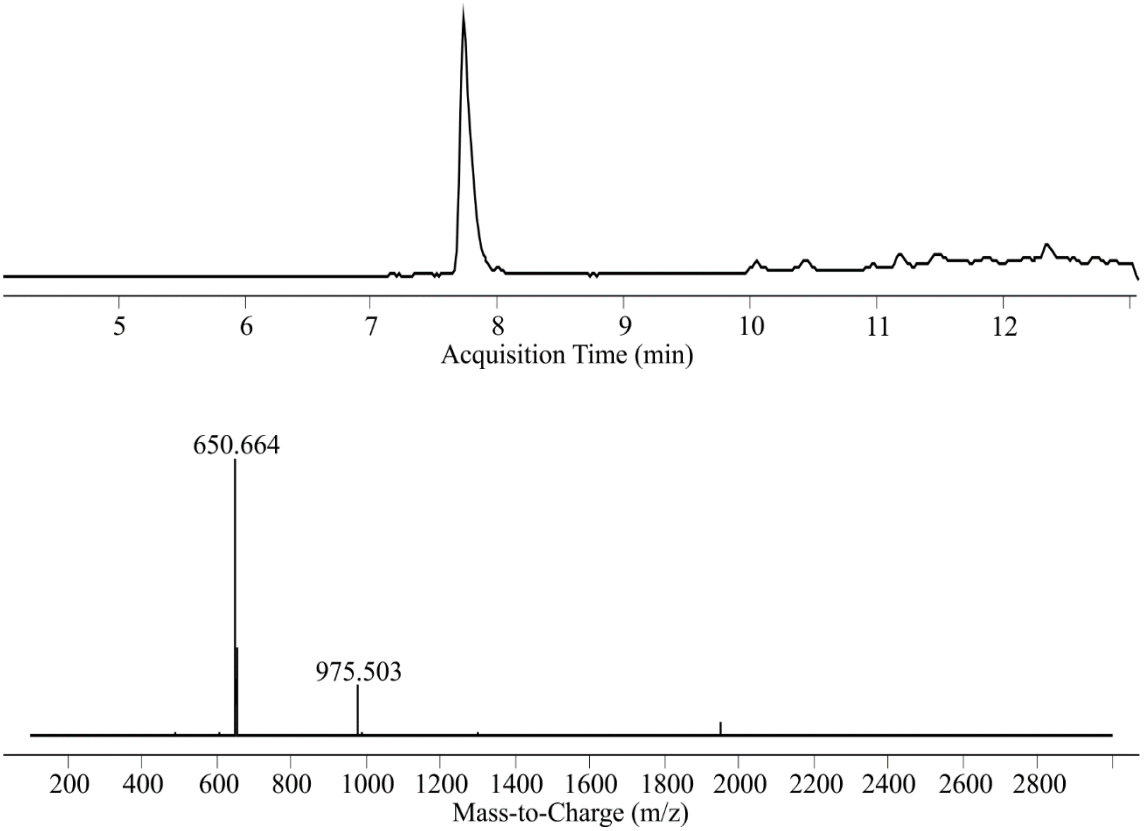
23: AcNH-Gln-Ser-Gln-Val-**Lys**-Thr-Phe-Asn-Leu-Trp-Arg-**Lys**-Met-Gln-Asn-$CONH_2$



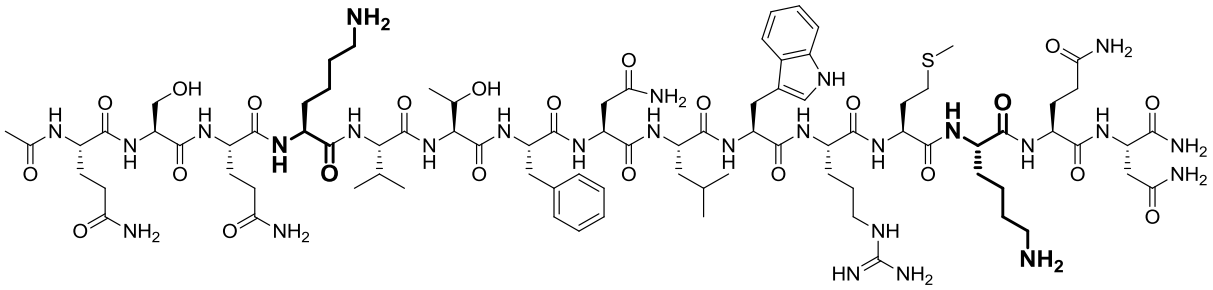
Peptide **23**: LC-MS analysis *Method A*. TIC trace and Mass spectrum of peptide **23**. m/z calcd. for $C_{86}H_{137}N_{27}O_{23}S$ $[M+2H]^{2+}$: 975.01 found 974.99.



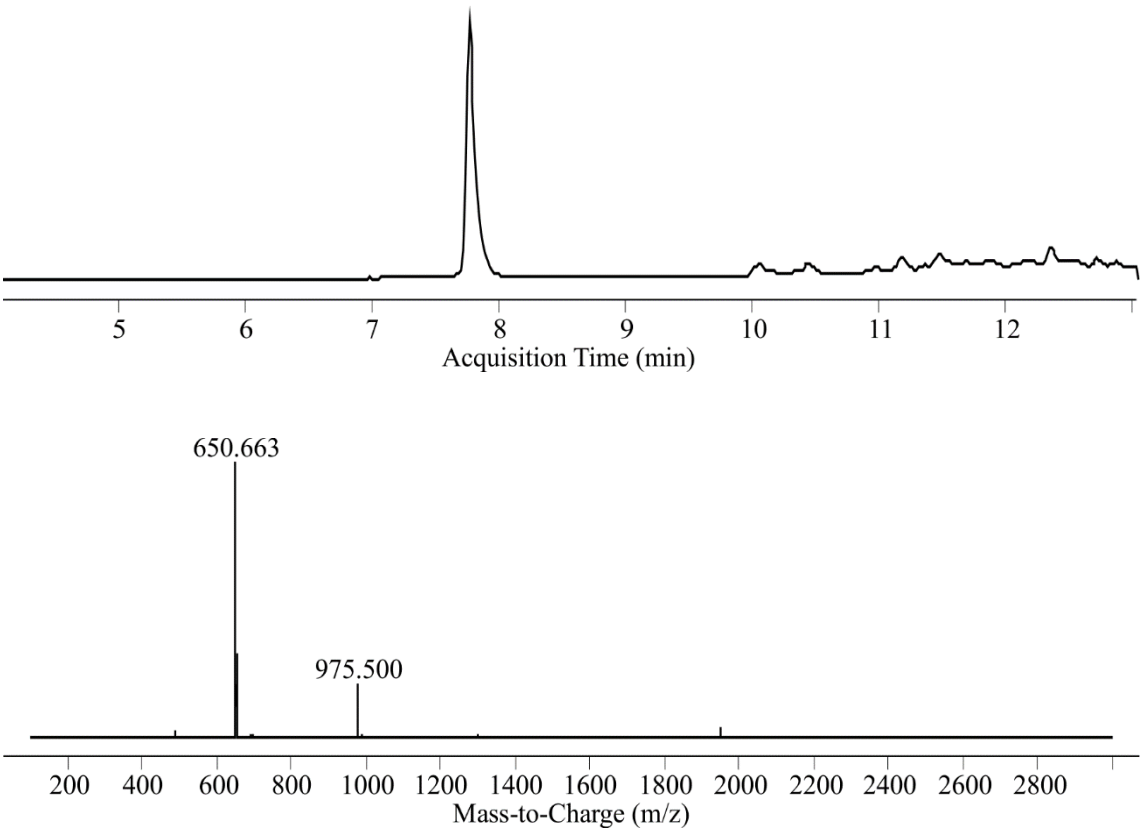
24: AcNH-Gln-Ser-Gln-Val-**Lys-**Thr-Phe-Asn-Leu-Trp-Arg-Met-**Lys**-Gln-Asn-$CONH_2$



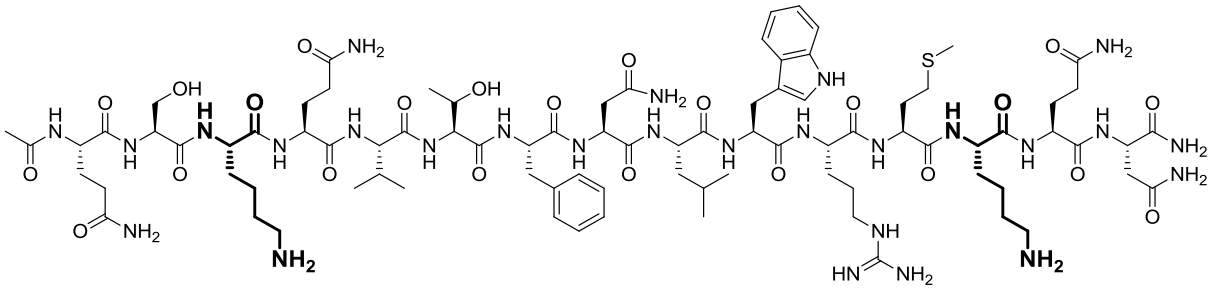
Peptide **24**: LC-MS analysis *Method A*. TIC trace and Mass spectrum of peptide **24**. m/z calcd. for $C_{86}H_{137}N_{27}O_{23}S$ $[M+2H]^{2+}$: 975.01 found 975.00.



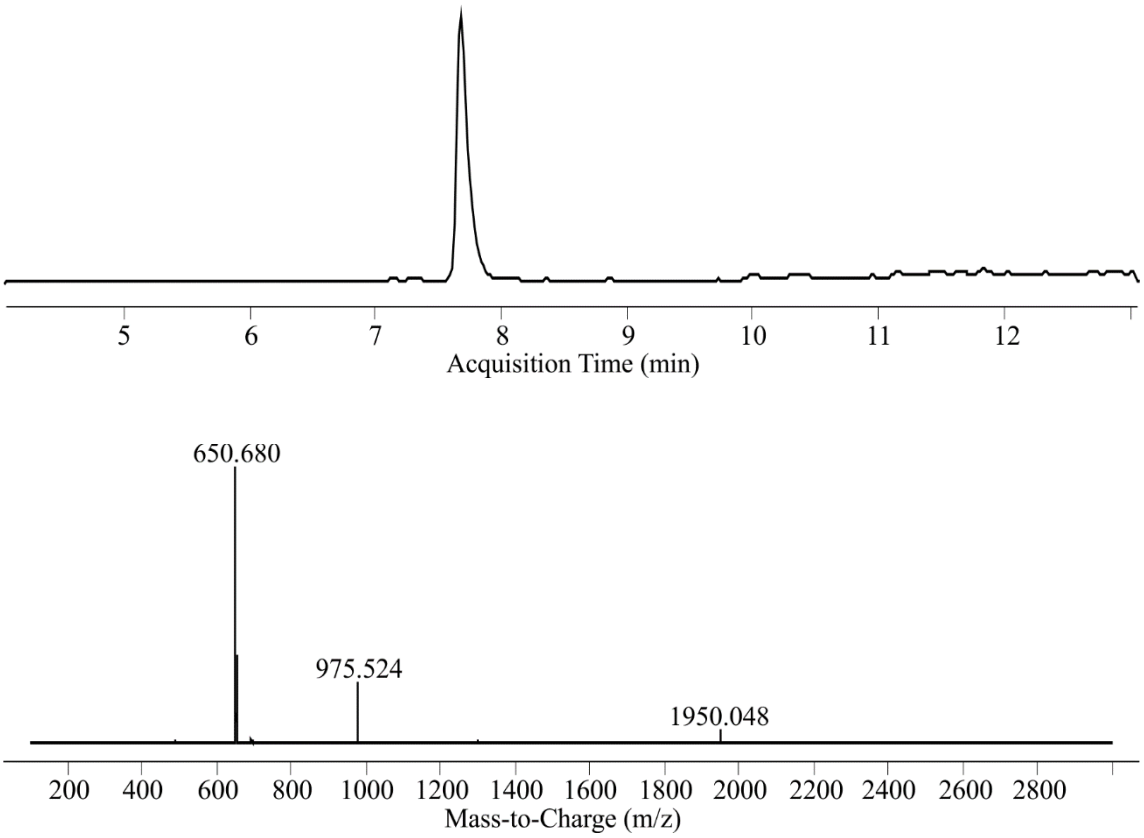
25: AcNH-Gln-Ser-Gln-**Lys**-Val-Thr-Phe-Asn-Leu-Trp-Arg-Met-**Lys**-Gln-Asn-$CONH_2$



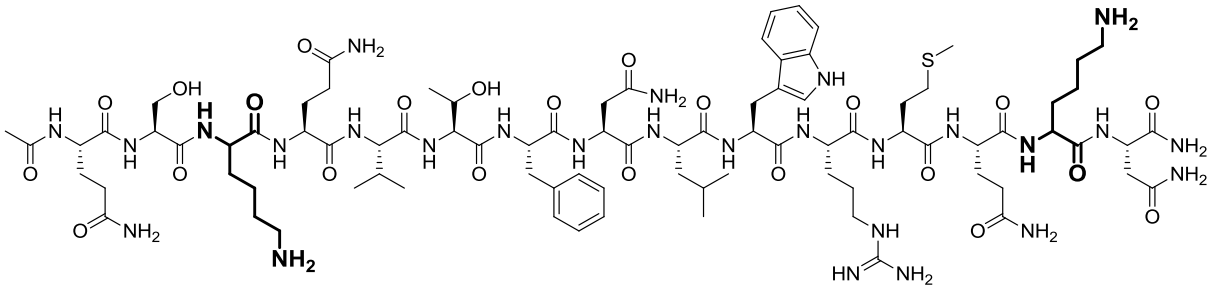
Peptide **25**: LC-MS analysis *Method A*. TIC trace and Mass spectrum of peptide **25**. m/z calcd. for $C_{86}H_{137}N_{27}O_{23}S$ $[M+2H]^{2+}$: 975.01 found 975.00.



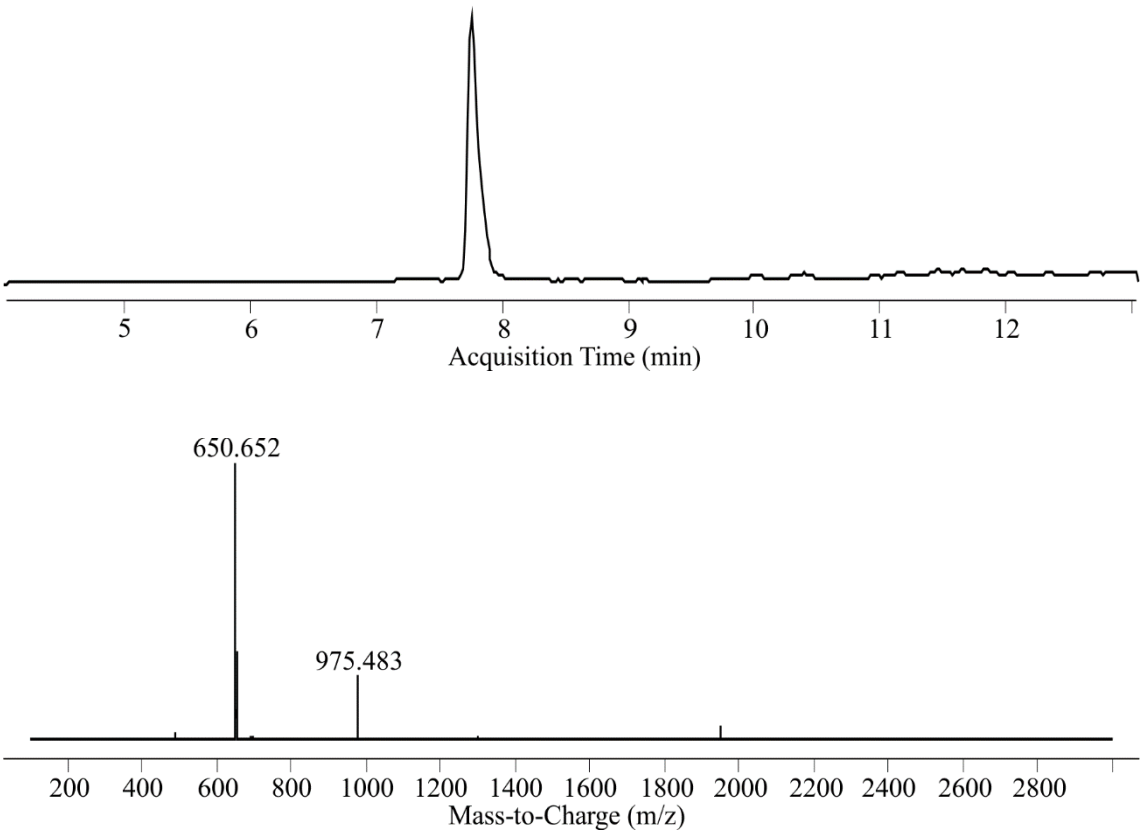
26: AcNH-Gln-Ser-**Lys**-Gln-Val-Thr-Phe-Asn-Leu-Trp-Arg-Met-**Lys**-Gln-Asn-$CONH_2$



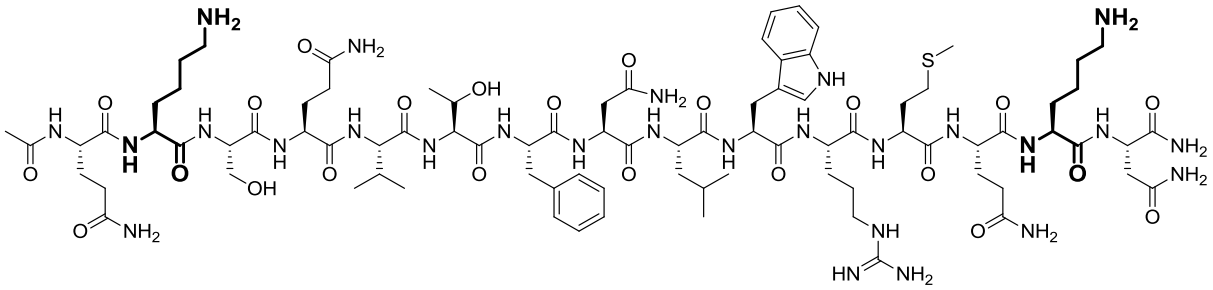
Peptide **26**: LC-MS analysis *Method A*. TIC trace and Mass spectrum of peptide **26**. m/z calcd. for $C_{86}H_{137}N_{27}O_{23}S$ $[M+2H]^{2+}$: 975.01 found 975.02.



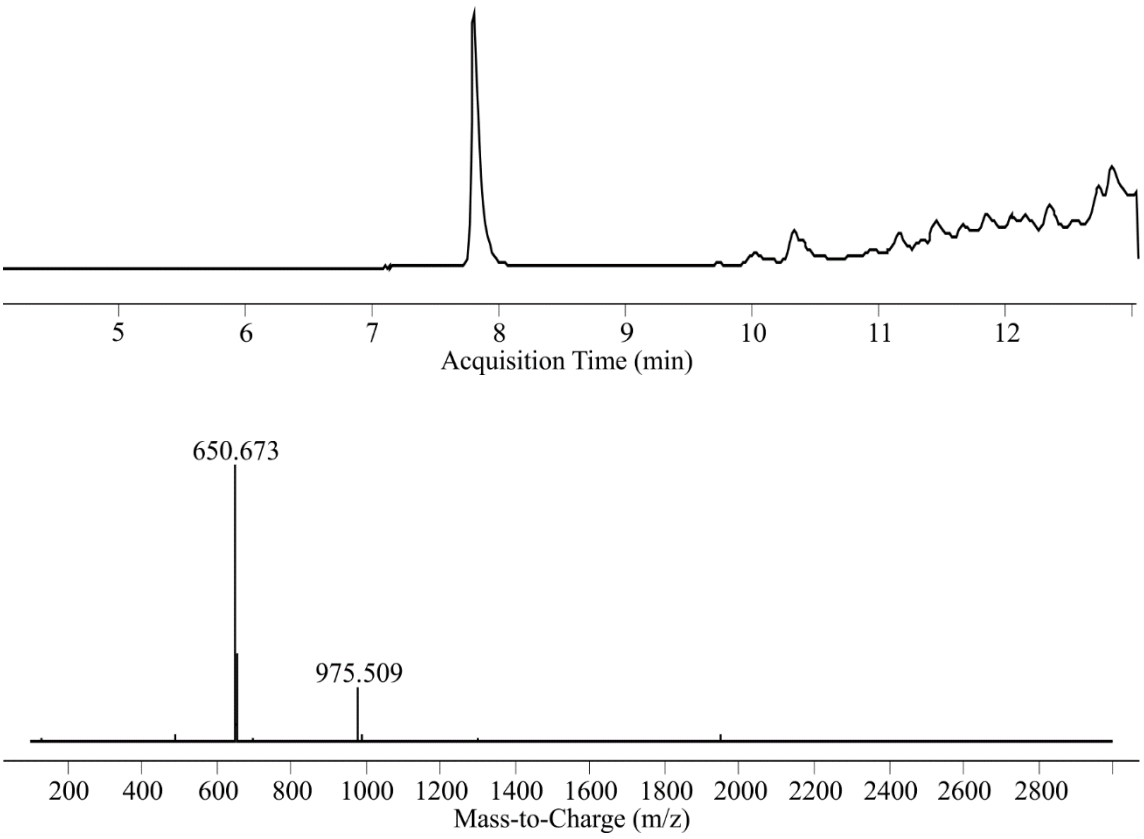
27: AcNH-Gln-Ser-**Lys**-Gln-Val-Thr-Phe-Asn-Leu-Trp-Arg-Met-Gln-**Lys**-Asn-$CONH_2$



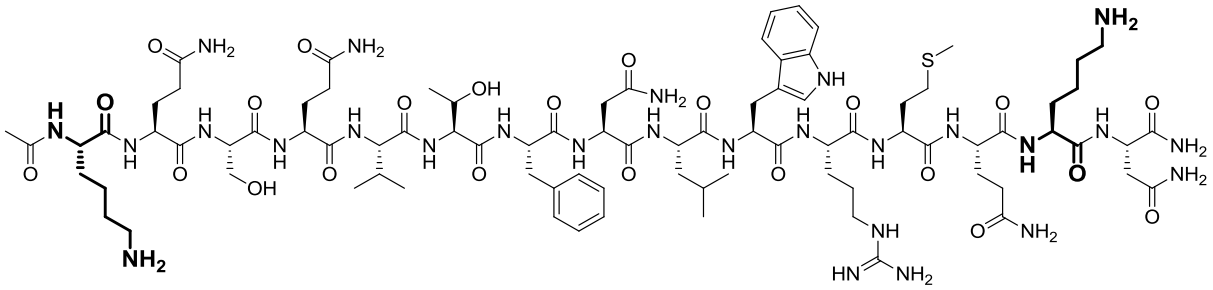
Peptide **27**: LC-MS analysis *Method A*. TIC trace and Mass spectrum of peptide **27**. m/z calcd. for $C_{86}H_{137}N_{27}O_{23}S$ $[M+2H]^{2+}$: 975.01 found 974.98.



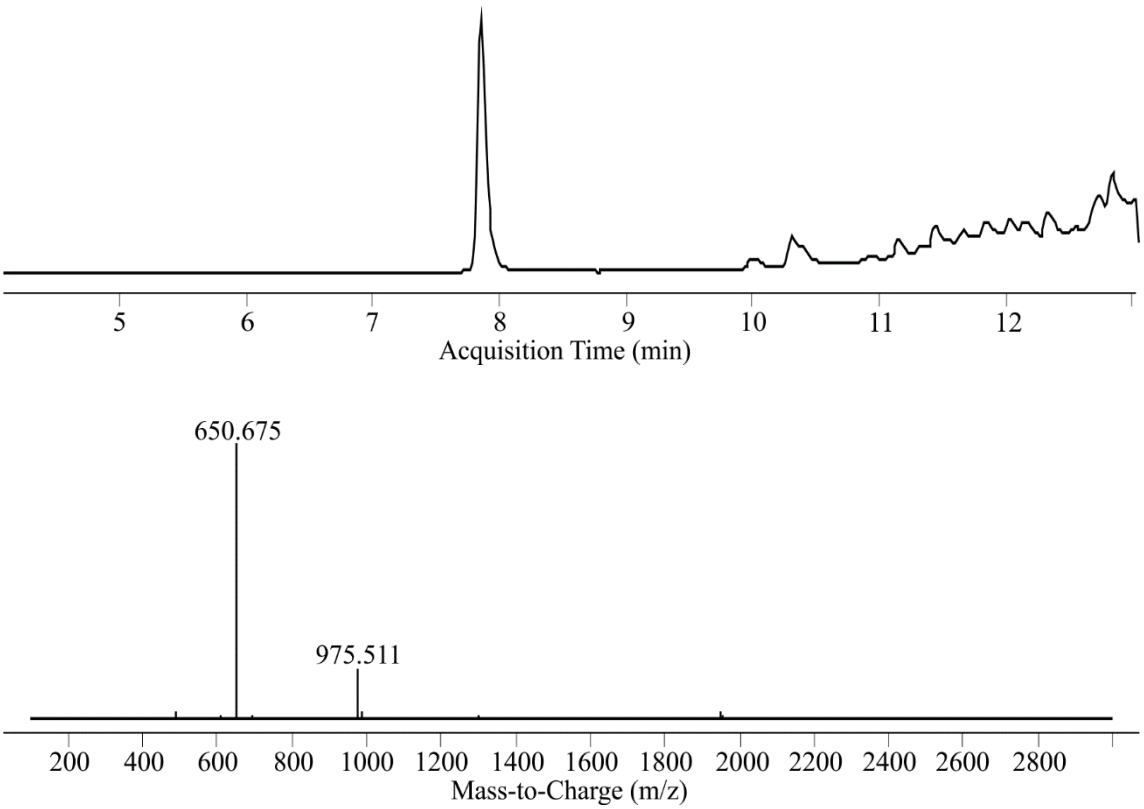
28: AcNH-Gln-**Lys**-Ser-Gln-Val-Thr-Phe-Asn-Leu-Trp-Arg-Met-Gln-**Lys**-Asn-$CONH_2$



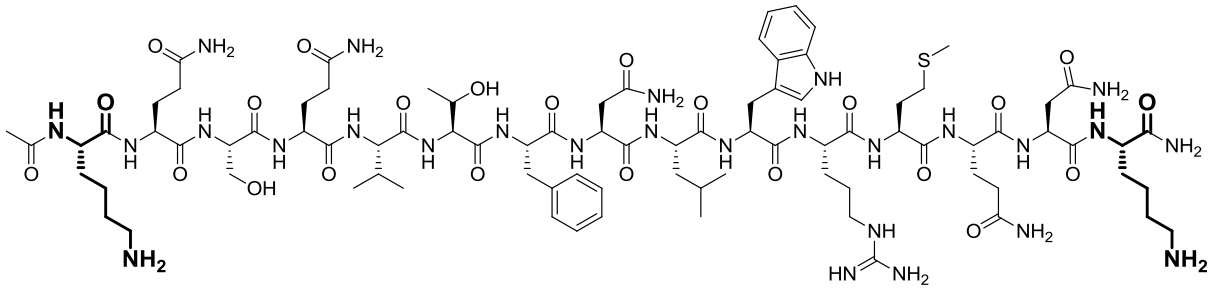
Peptide **28**: LC-MS analysis *Method A*. TIC trace and Mass spectrum of peptide **28**. m/z calcd. for $C_{86}H_{137}N_{27}O_{23}S$ $[M+2H]^{2+}$: 975.01 found 975.00.



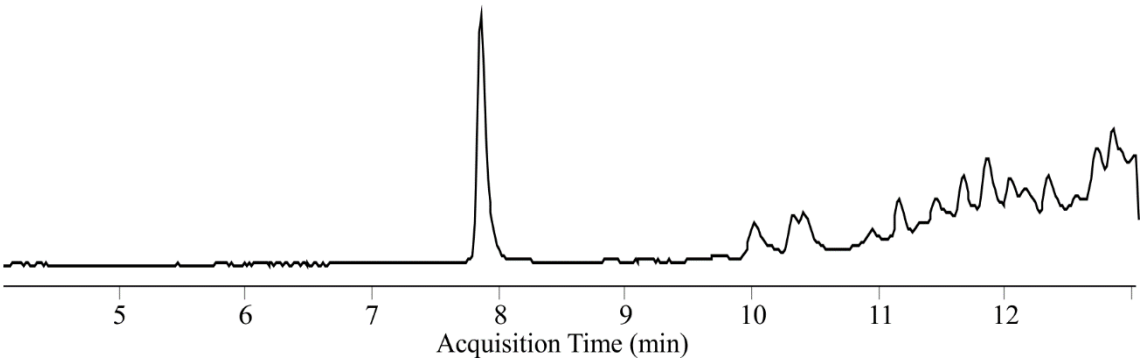
29: AcNH-**Lys**-Gln-Ser-Gln-Val-Thr-Phe-Asn-Leu-Trp-Arg-Met-Gln-**Lys**-Asn-$CONH_2$

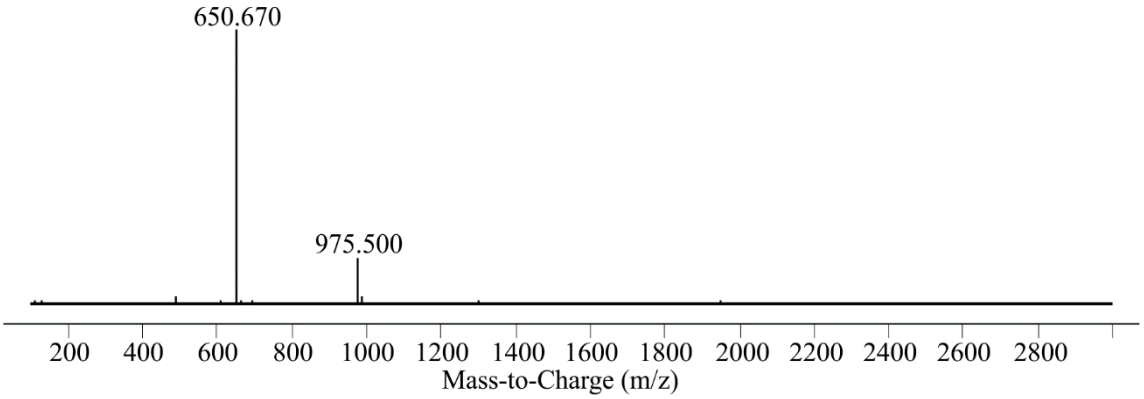


Peptide **29**: LC-MS analysis *Method A*. TIC trace and Mass spectrum of peptide **29**. m/z calcd. for $C_{86}H_{137}N_{27}O_{23}S$ $[M+2H]^{2+}$: 975.01 found 975.01.



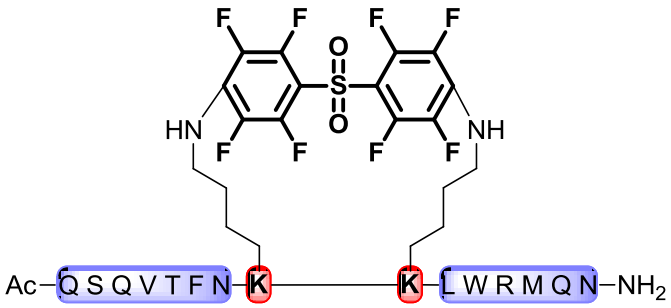
30: AcNH-**Lys**-Gln-Ser-Gln-Val-Thr-Phe-Asn-Leu-Trp-Arg-Met-Gln-Asn-**Lys**-$CONH_2$



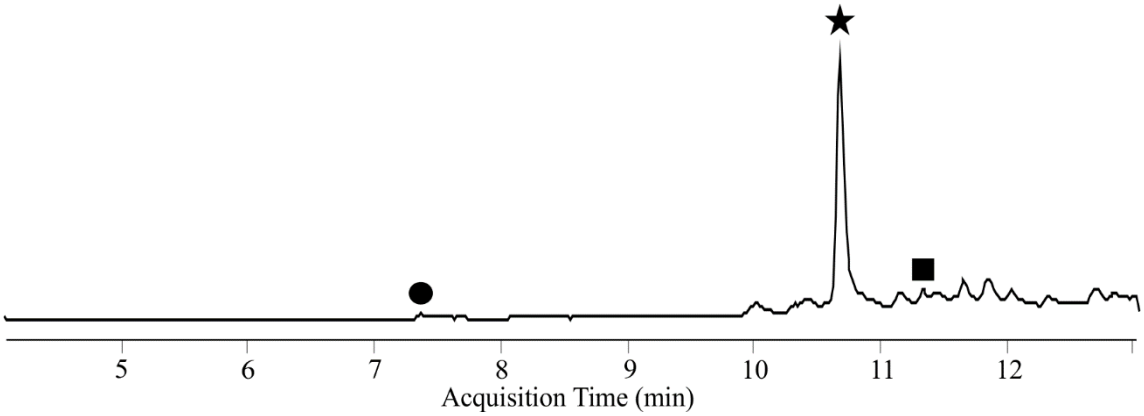


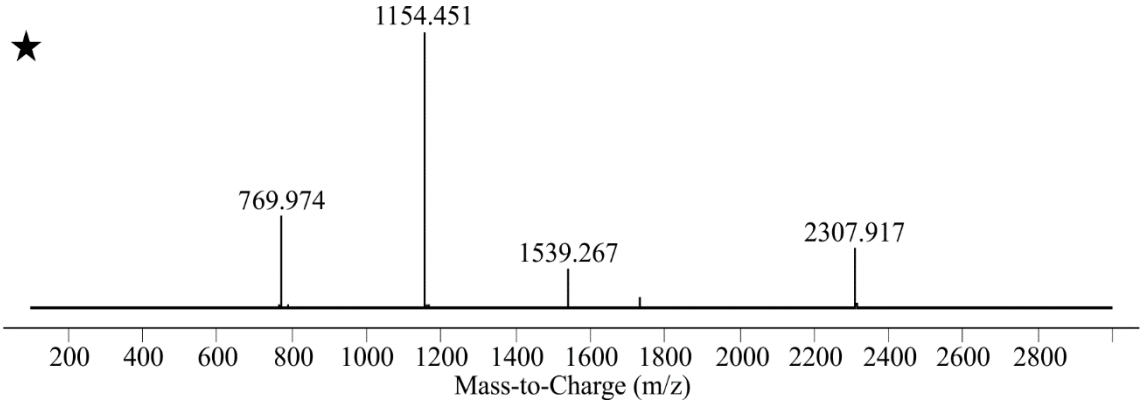
Peptide **30**: LC-MS analysis *Method A*. TIC trace and Mass spectrum of peptide **30**. m/z calcd. for $C_{86}H_{137}N_{27}O_{23}S$ $[M+2H]^{2+}$: 975.01 found 975.00.

LC-MS analytical data of stapling crude reaction for macrocyclization scan

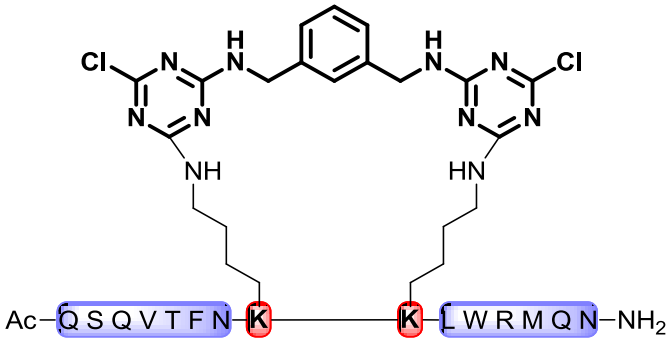


(18a): spacing: i, i+1

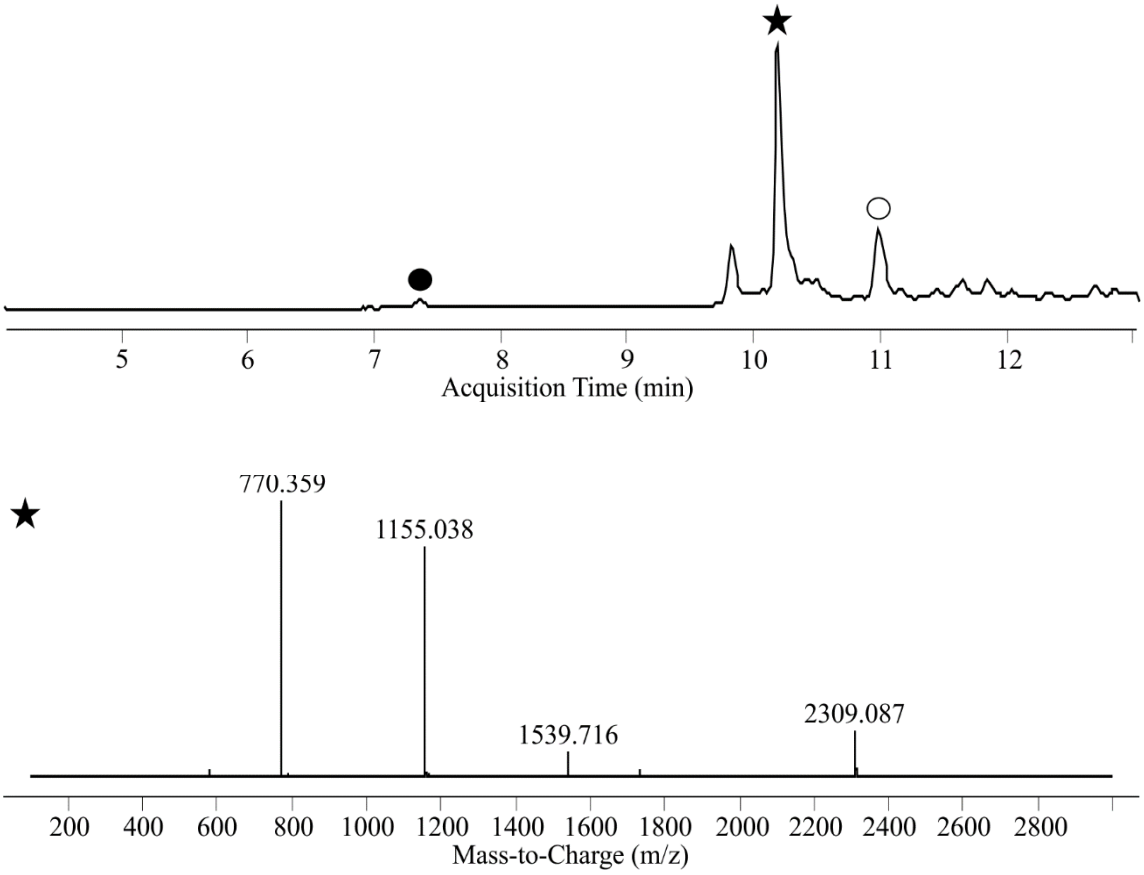




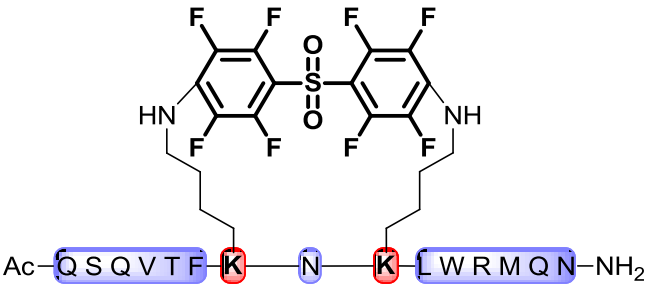
i, i+1 (**18a**): Prepared according to the representative protocol **(C)** using peptide **18** (0.5 mM), electrophile **6** (0.625 mM), and DIEA (10 mM) at room temperature for 4 h. The diluted reaction mixture was analyzed using LC-MS *Method A*. TIC trace of crude reaction and Mass spectrum of product **18a**. Signal of the starting material **18** (yield = 1 %) is marked with a black circle (●). Signal of the double-arylation product (yield = 3 %) is marked with a black square (■). Signal of the stapling product **18a** (yield = 95 %) is marked with a star (★). Analytical data for **18a** : m/z calcd. for $C_{98}H_{135}F_8N_{27}O_{25}S_2$ $[M+2H]^{2+}$: 1153.98 found 1153.95.



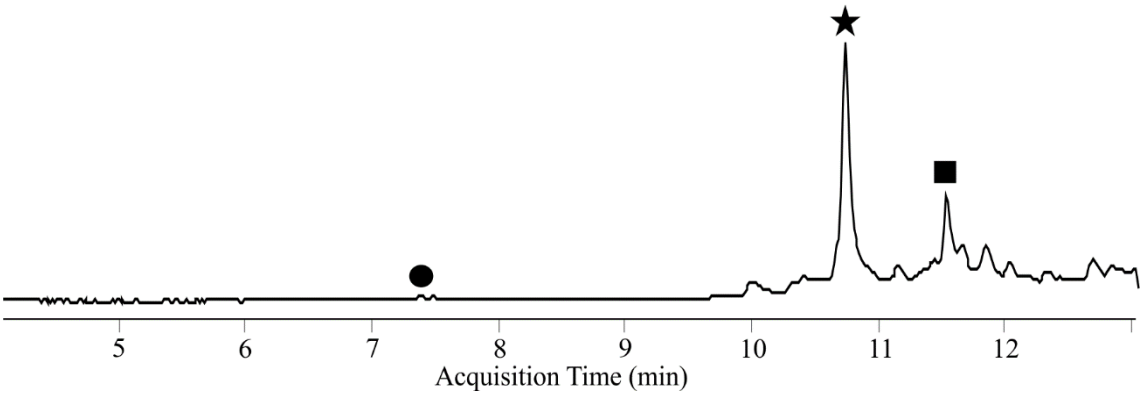
(18b): spacing: i, i+1

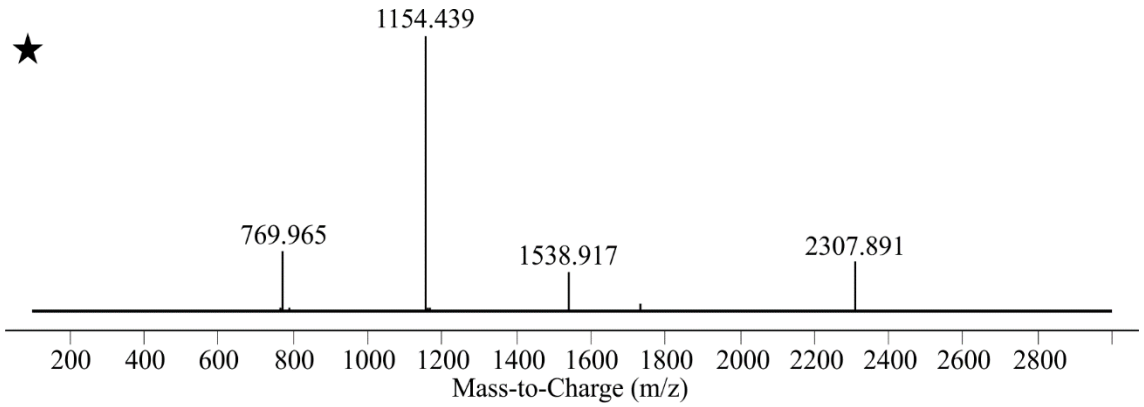


i, i+1 (**18b**): Prepared according to the representative protocol **(E)** using peptide **18** (1 mM), electrophile **7** (2 mM), and DIEA (20 mM) at room temperature for 4 h. The diluted reaction mixture was analyzed using LC-MS *Method A*. TIC trace of crude reaction and Mass spectrum of product **18b**. Signals of the starting material **18** (yield = 1 %) and **7** are marked with a full black circle (●) and an empty black circle (○), respectively. Signal of the stapling product **18b** (yield = 90 %) is marked with a star (★). Analytical data for **18b** : m/z calcd. for $C_{100}H_{145}Cl_2N_{35}O_{23}S$ $[M+2H]^{2+}$: 1154.03 found 1154.04.

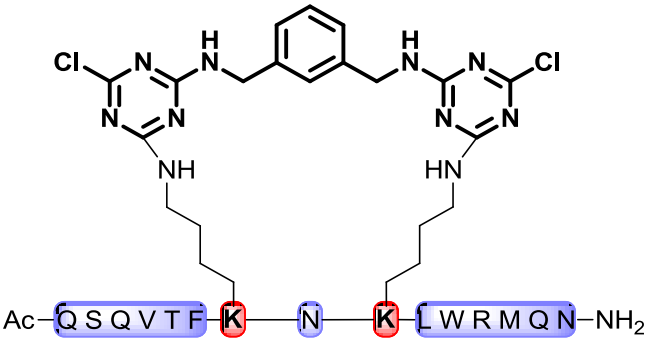


(19a): spacing: i, i+2

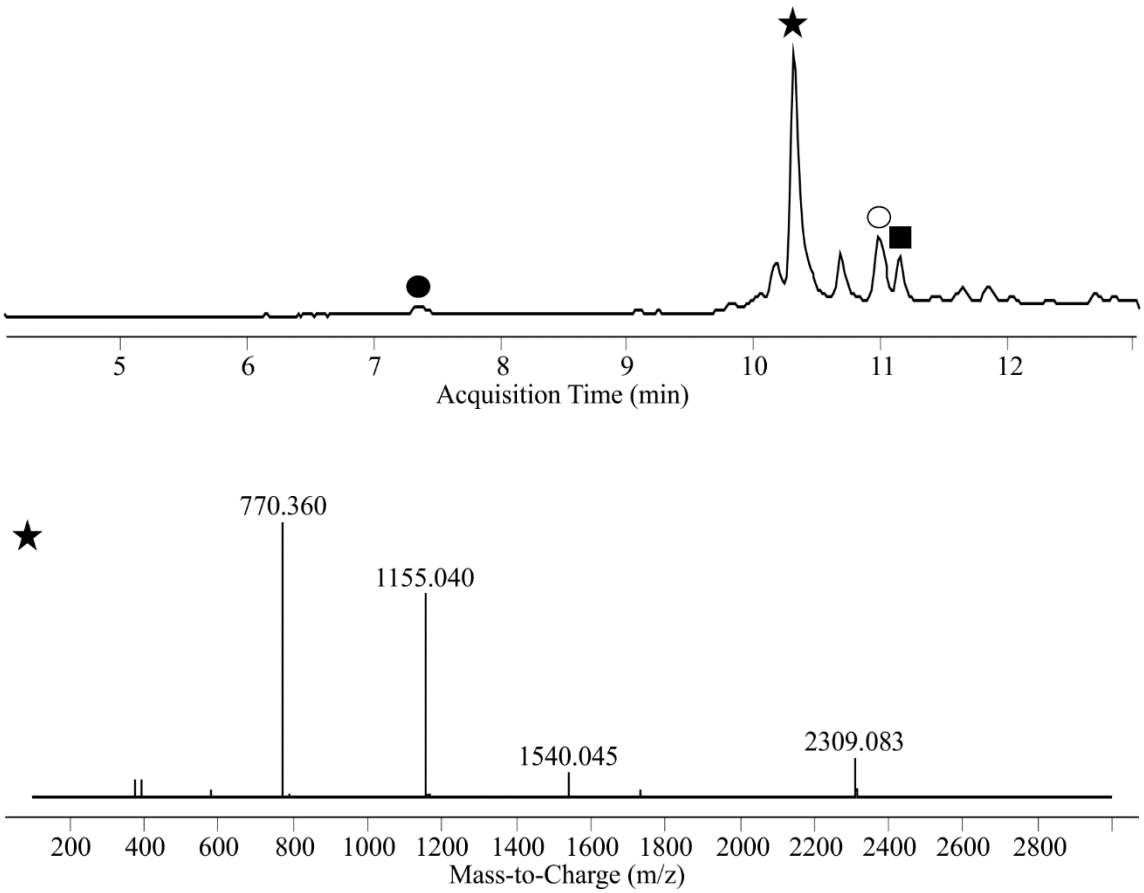




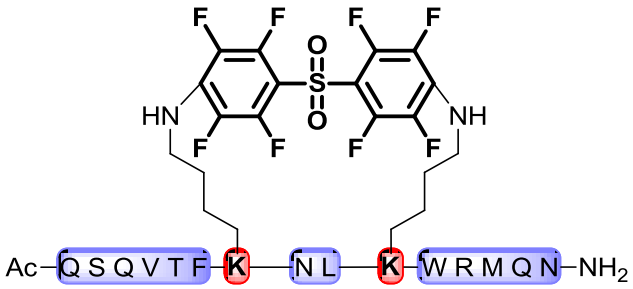
i, i+2 (**19a**): Prepared according to the representative protocol **(C)** using peptide **19** (0.5 mM), electrophile **6** (0.625 mM), and DIEA (10 mM) at room temperature for 4 h. The diluted reaction mixture was analyzed using LC-MS *Method A*. TIC trace of crude reaction and Mass spectrum of product **19a**. Signal of the starting material **19** (yield = 1 %) is marked with a black circle (●). Signal of the double-arylation product (yield = 11 %) is marked with a black square (■). Signal of the stapling product **19a** (yield = 87 %) is marked with a star (★). Analytical data for **19a** : m/z calcd. for $C_{98}H_{135}F_8N_{27}O_{25}S_2$ $[M+2H]^{2+}$: 1153.98 found 1153.94.



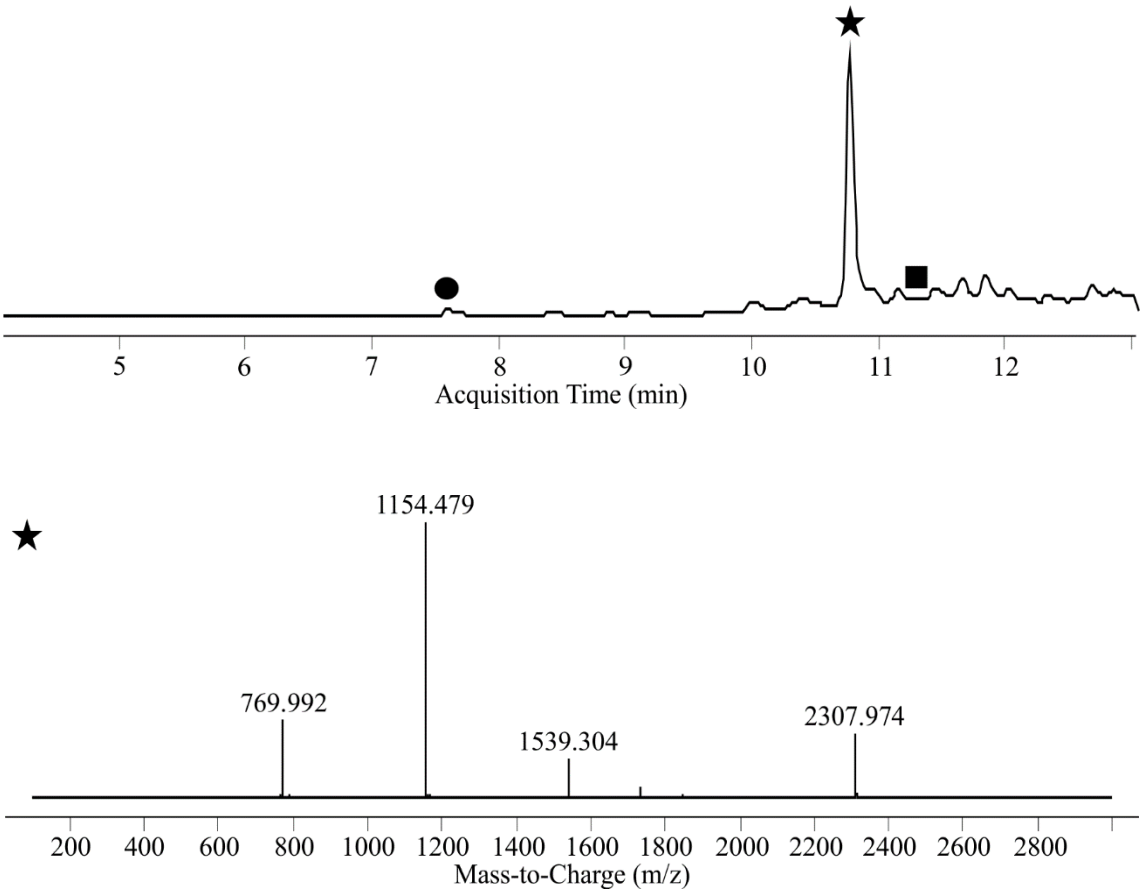
(19b): spacing: i, i+2



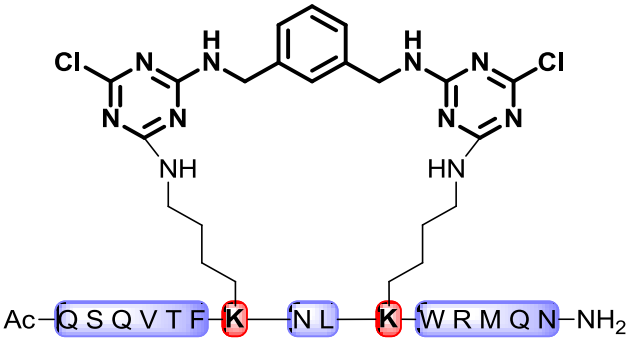
i, i+2 (**19b**): Prepared according to the representative protocol **(E)** using peptide **19** (1 mM), electrophile **7** (2 mM), and DIEA (20 mM) at room temperature for 4 h. The diluted reaction mixture was analyzed using LC-MS *Method A*. TIC trace of crude reaction and Mass spectrum of product **19b**. Signals of the starting material **19** (yield = 2 %) and **7** are marked with a full black circle (●) and an empty black circle (○), respectively. Signal of the double-arylation product (yield = 8 %) is marked with a black square (■). Signal of the stapling product **19b** (yield = 85 %) is marked with a star (★). Analytical data for **19b** : m/z calcd. for $C_{100}H_{145}Cl_2N_{35}O_{23}S$ $[M+2H]^{2+}$: 1154.03 found 1154.04.



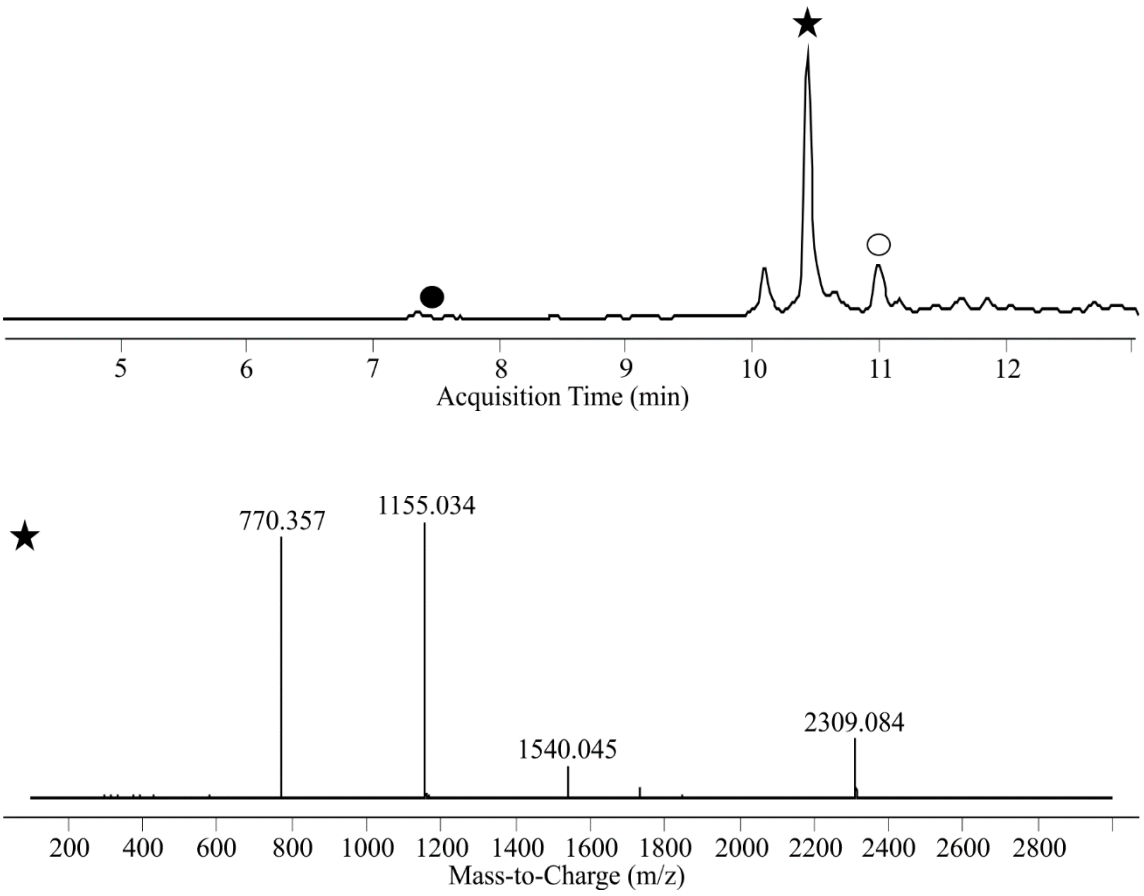
(20a): spacing: i, i+3



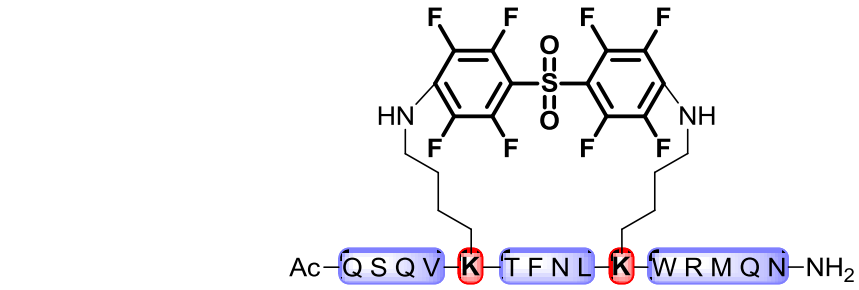
i, i+3 (**20a**): Prepared according to the representative protocol **(C)** using peptide **20** (0.5 mM), electrophile **6** (0.625 mM), and DIEA (10 mM) at room temperature for 4 h. The diluted reaction mixture was analyzed using LC-MS *Method A*. TIC trace of crude reaction and Mass spectrum of product **20a**. Signal of the starting material **20** (yield = 1 %) is marked with a black circle (●). Signal of the double-arylation product (yield = 1 %) is marked with a black square (■). Signal of the stapling product **20a** (yield = 97 %) is marked with a star (★). Analytical data for **20a** : m/z calcd. for $C_{98}H_{135}F_8N_{27}O_{25}S_2$ $[M+2H]^{2+}$: 1153.98 found 1153.98.



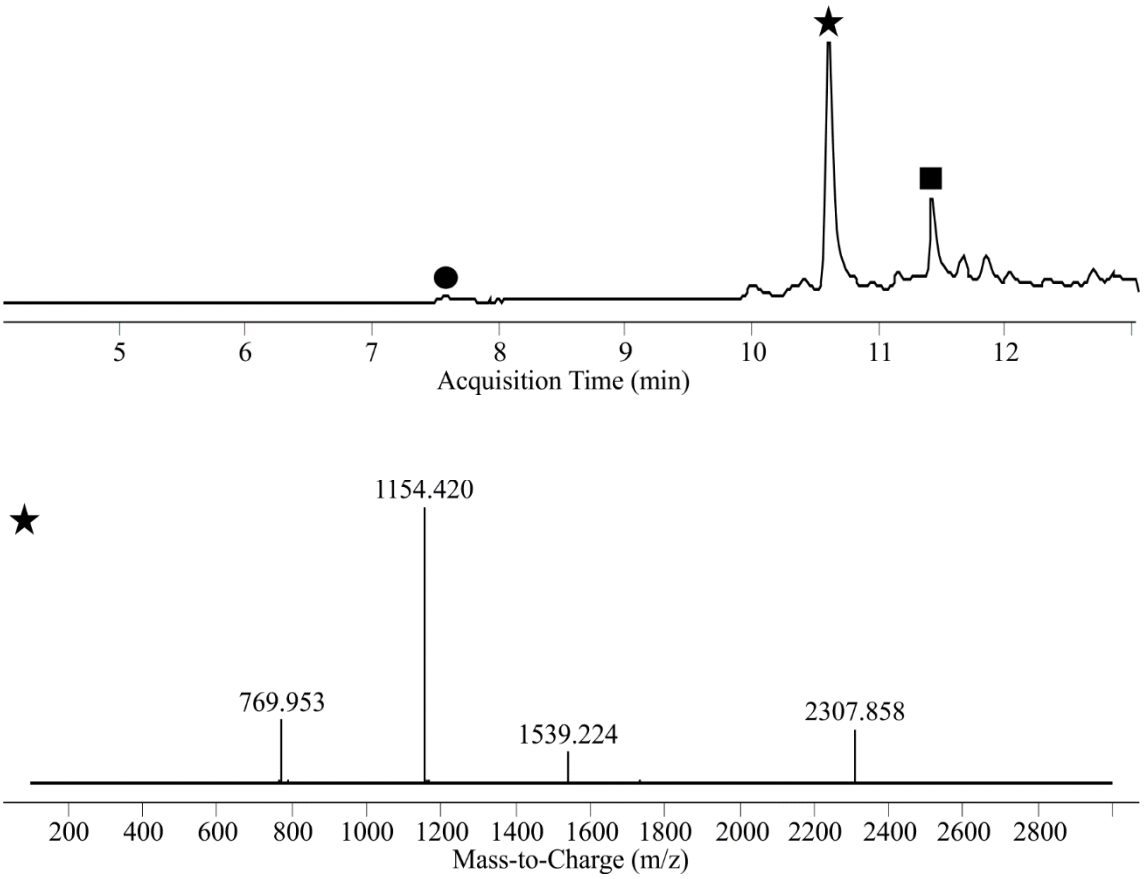
(20b): spacing: i, i+3



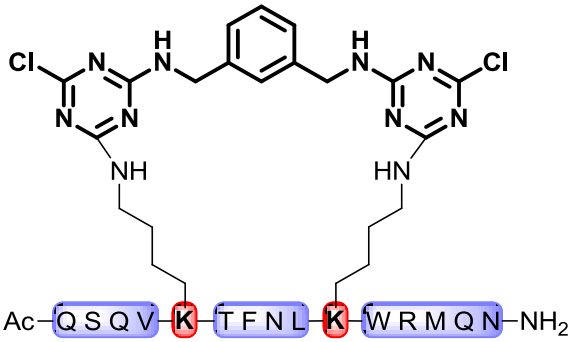
i, i+3 (**20b**): Prepared according to the representative protocol **(E)** using peptide **20** (1 mM), electrophile **7** (2 mM), and DIEA (20 mM) at room temperature for 4 h. The diluted reaction mixture was analyzed using LC-MS *Method A*. TIC trace of crude reaction and Mass spectrum of product **20b**. Signals of the starting material **20** (yield = 1 %) and **7** are marked with a full black circle (●) and an empty black circle (○), respectively. Signal of the stapling product **20b** (yield = 94 %) is marked with a star (★). Analytical data for **20b** : m/z calcd. for $C_{100}H_{145}Cl_2N_{35}O_{23}S$ $[M+2H]^{2+}$: 1154.03 found 1154.04.



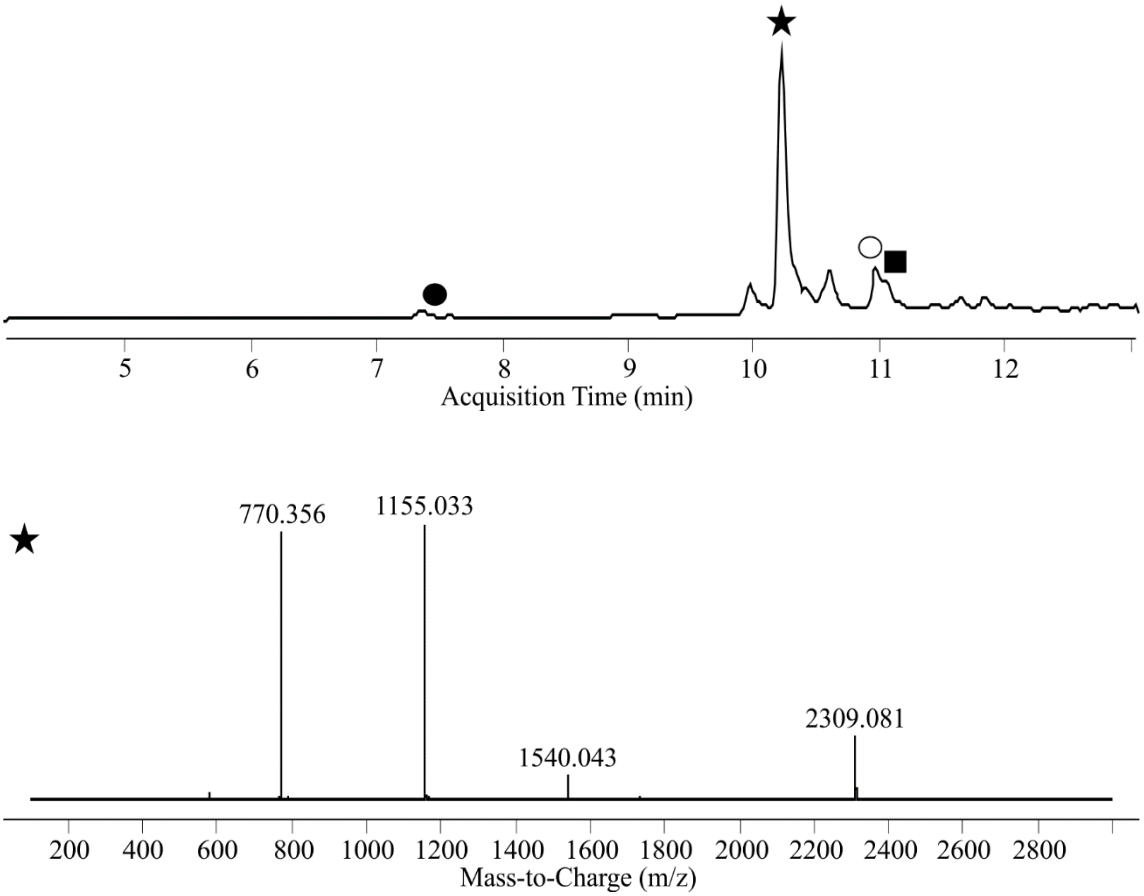
(21a): spacing: i, i+5



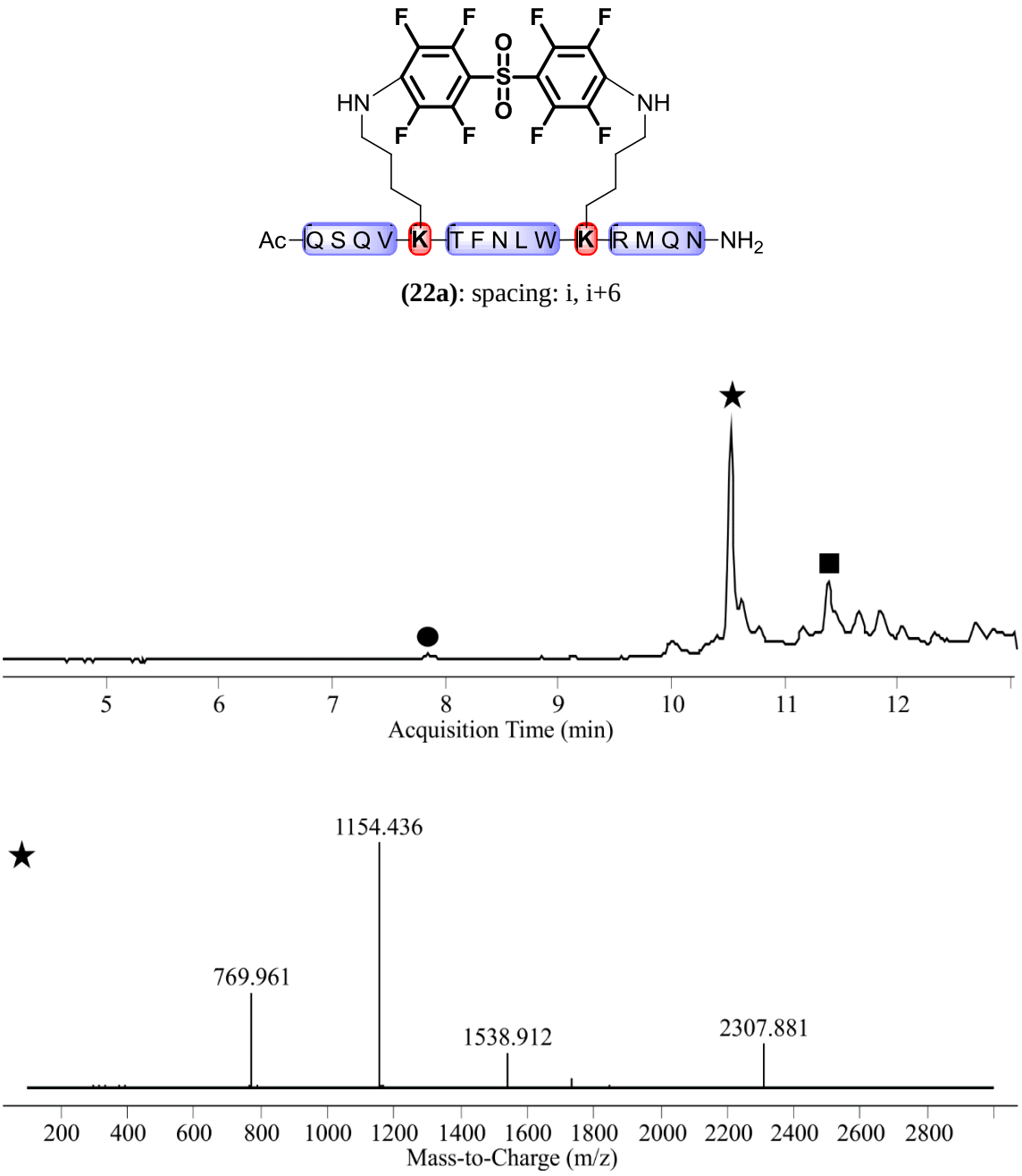
i, i+5 (**21a**): Prepared according to the representative protocol **(C)** using peptide **21** (0.5 mM), electrophile **6** (0.625 mM), and DIEA (10 mM) at room temperature for 4 h. The diluted reaction mixture was analyzed using LC-MS *Method A*. TIC trace of crude reaction and Mass spectrum of product **21a**. Signal of the starting material **21** (yield = 1 %) is marked with a black circle (●). Signal of the double-arylation product (yield = 16 %) is marked with a black square (■). Signal of the stapling product **21a** (yield = 81 %) is marked with a star (★). Analytical data for **21a** : m/z calcd. for $C_{98}H_{135}F_8N_{27}O_{25}S_2$ $[M+2H]^{2+}$: 1153.98 found 1153.92.



(21b): spacing: i, i+5

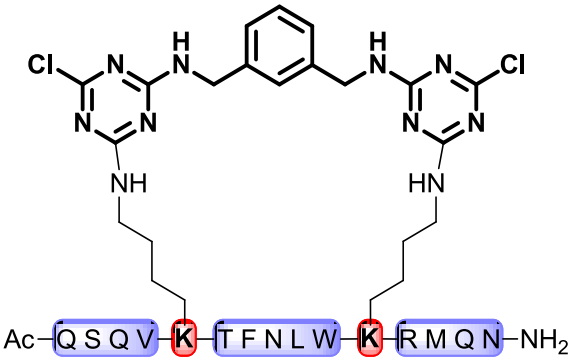


i, i+5 (**21b**): Prepared according to the representative protocol **(E)** using peptide **21** (1 mM), electrophile **7** (2 mM), and DIEA (20 mM) at room temperature for 4 h. The diluted reaction mixture was analyzed using LC-MS *Method A*. TIC trace of crude reaction and Mass spectrum of product **21b**. Signals of the starting material **21** (yield = 1 %) and **7** are marked with a full black circle (●) and an empty black circle (○), respectively. Signal of the double-arylation product (yield = 6 %) is marked with a black square (■). Signal of the stapling product **21b** (yield = 86 %) is marked with a star (★). Analytical data for **21b** : m/z calcd. for $C_{100}H_{145}Cl_2N_{35}O_{23}S$ $[M+2H]^{2+}$: 1154.03 found 1154.04.

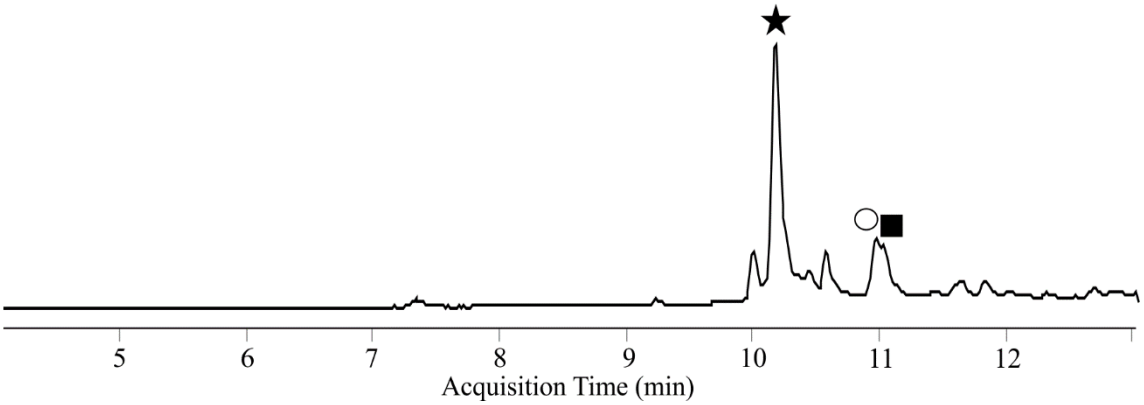


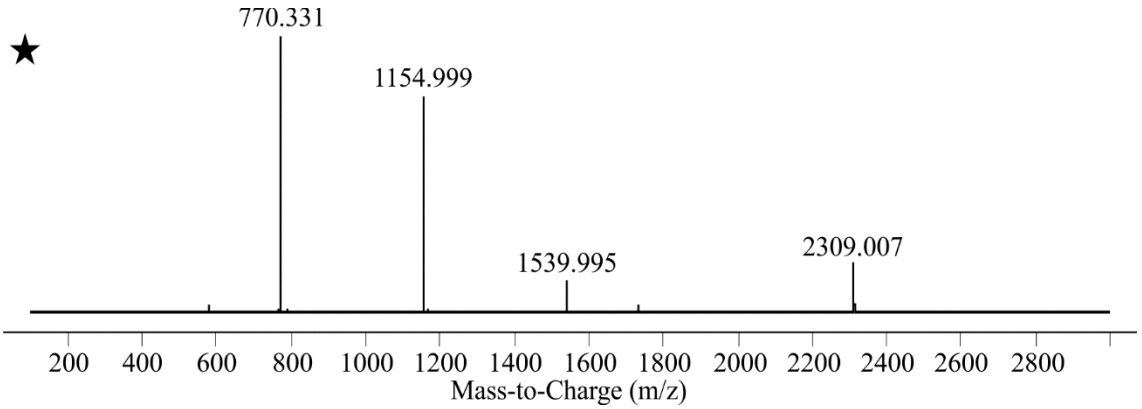
(22a): spacing: i, i+6

i, i+6 (**22a**): Prepared according to the representative protocol **(C)** using peptide **22** (0.5 mM), electrophile **6** (0.625 mM), and DIEA (10 mM) at room temperature for 4 h. The diluted reaction mixture was analyzed using LC-MS *Method A*. TIC trace of crude reaction and Mass spectrum of product **22a**. Signal of the starting material **22** (yield = 1 %) is marked with a black circle (●). Signal of the double-arylation product (yield = 13 %) is marked with a black square (■). Signal of the stapling product **22a** (yield = 84 %) is marked with a star (★). Analytical data for **22a** : m/z calcd. for $C_{98}H_{135}F_8N_{27}O_{25}S_2$ $[M+2H]^{2+}$: 1153.98 found 1153.94.

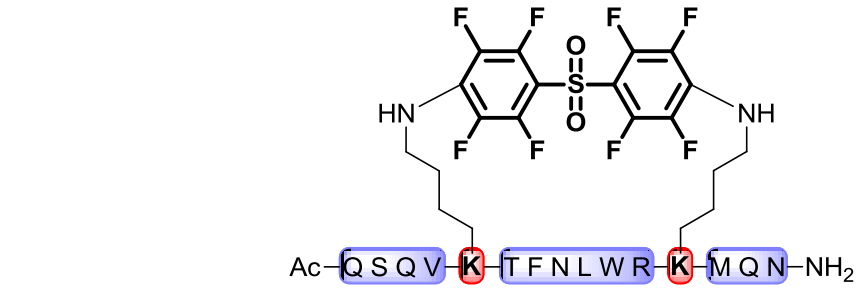


(22b): spacing: i, i+6

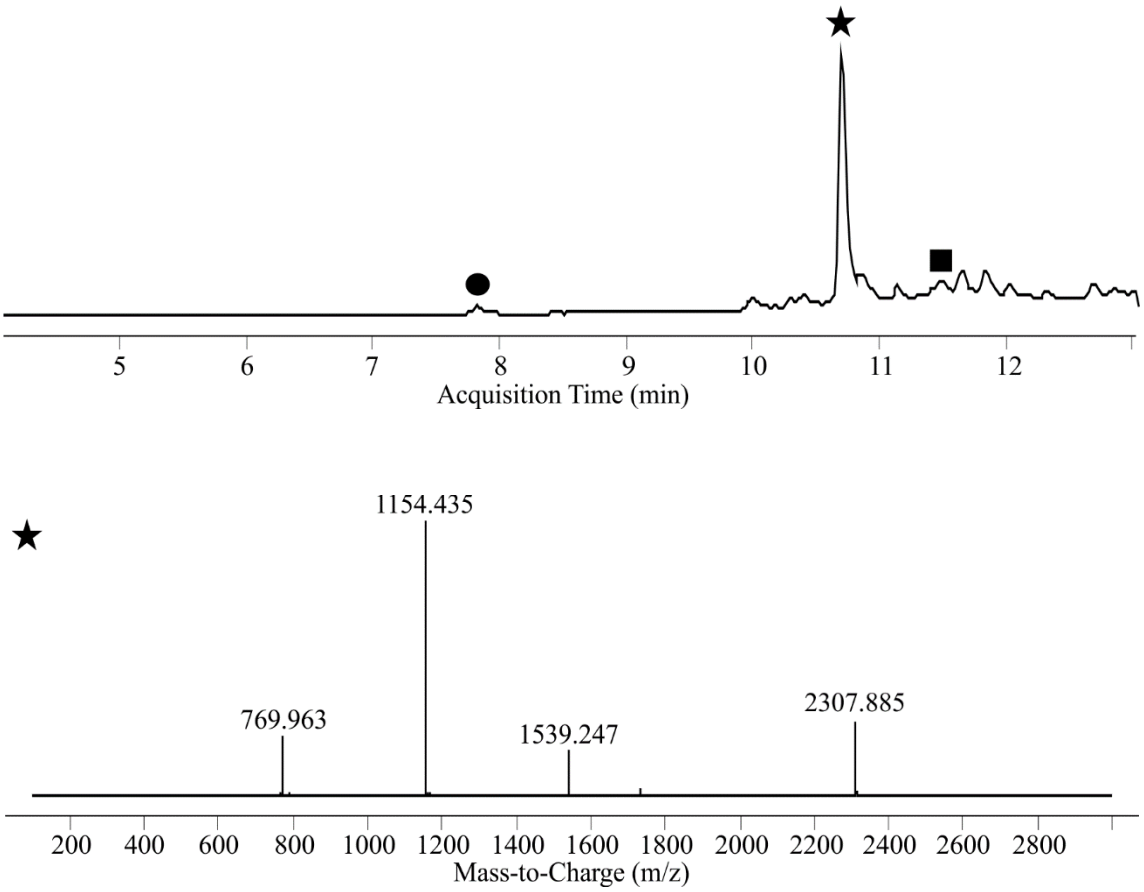




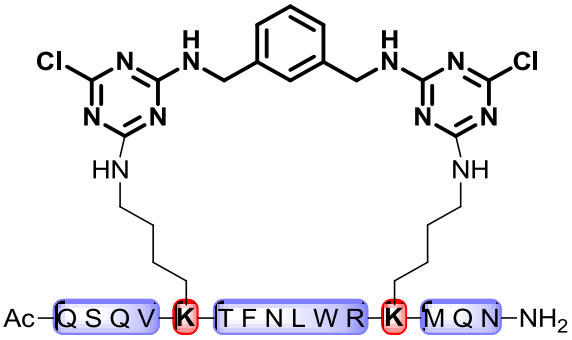
i, i+6 (**22b**): Prepared according to the representative protocol **(E)** using peptide **22** (1 mM), electrophile **7** (2 mM), and DIEA (20 mM) at room temperature for 4 h. The diluted reaction mixture was analyzed using LC-MS *Method A*. TIC trace of crude reaction and Mass spectrum of product **22b**. Signal of the starting material **7** is marked with an empty black circle (○). Signal of the double-arylation product (yield = 8 %) is marked with a black square (■). Signal of the stapling product **22b** (yield = 89 %) is marked with a star (★). Analytical data for **22b** : m/z calcd. for $C_{100}H_{145}Cl_2N_{35}O_{23}S$ $[M+2H]^{2+}$: 1154.03 found 1154.00.



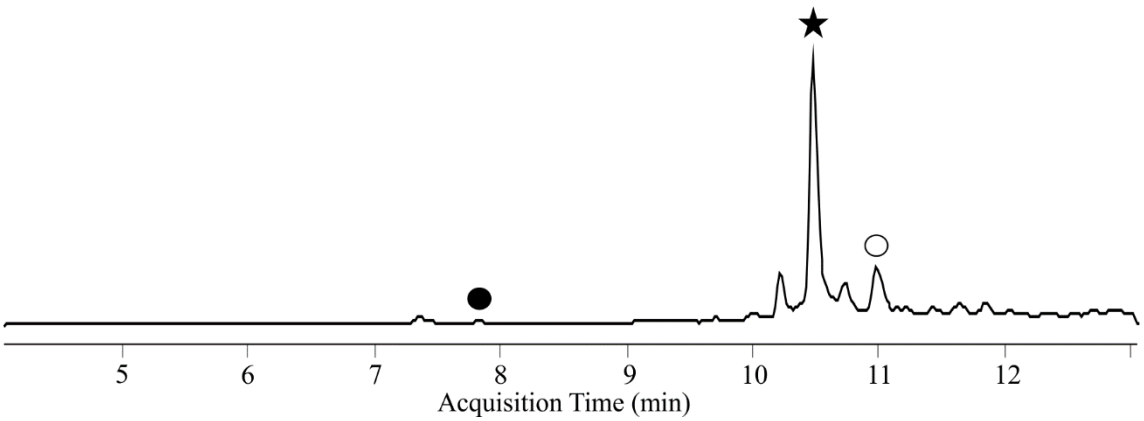
(23a): spacing: i, i+7

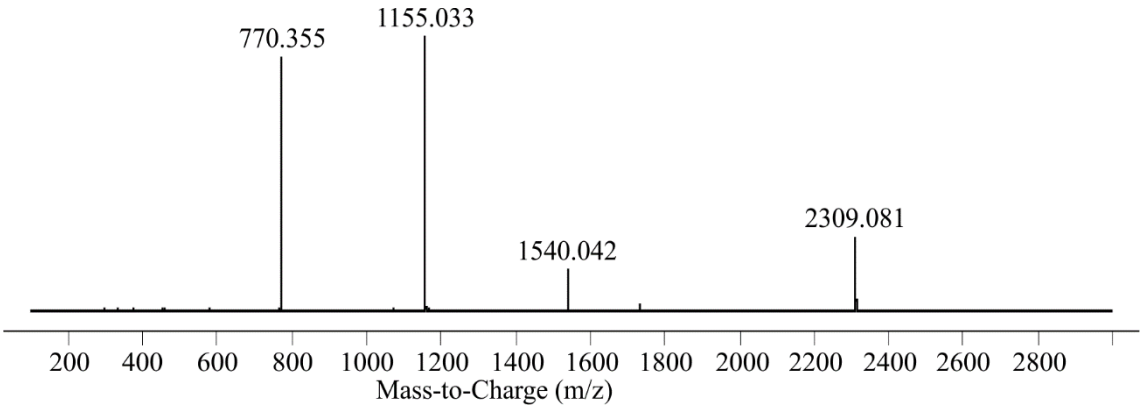


i, i+7 (**23a**): Prepared according to the representative protocol **(C)** using peptide **23** (0.5 mM), electrophile **6** (0.625 mM), and DIEA (10 mM) at room temperature for 4 h. The diluted reaction mixture was analyzed using LC-MS *Method A*. TIC trace of crude reaction and Mass spectrum of product **23a**. Signal of the starting material **23** (yield = 1 %) is marked with a black circle (●). Signal of the double-arylation product (yield = 4 %) is marked with a black square (■). Signal of the stapling product **23a** (yield = 94 %) is marked with a star (★). Analytical data for **23a** : m/z calcd. for $C_{98}H_{135}F_8N_{27}O_{25}S_2$ $[M+2H]^{2+}$: 1153.98 found 1153.94.

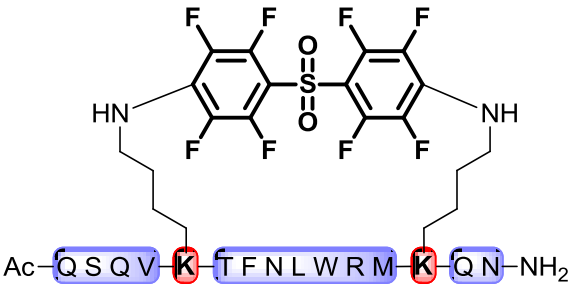


(23b): spacing: i, i+7

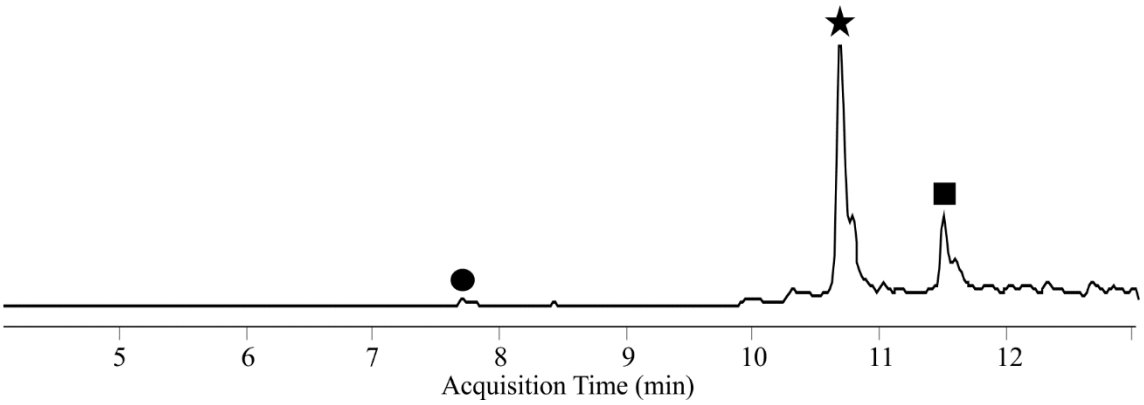


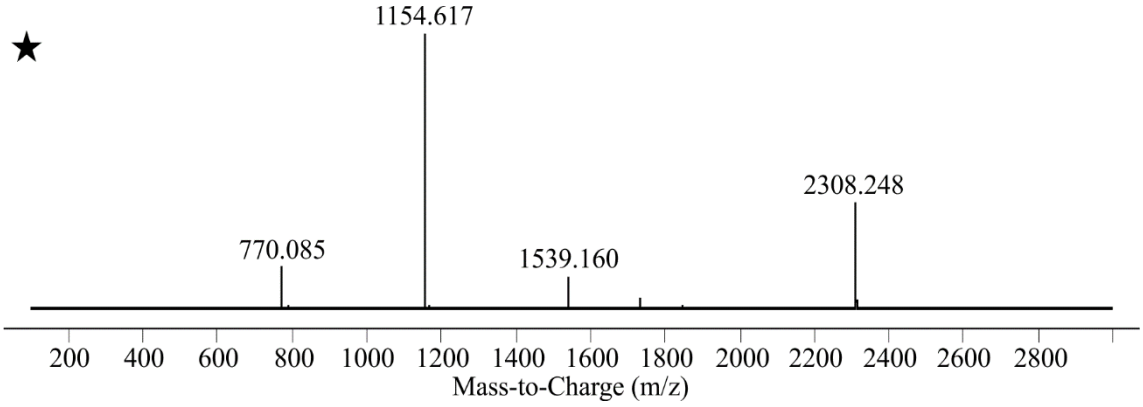


i, i+7 (**23b**): Prepared according to the representative protocol **(E)** using peptide **23** (1 mM), electrophile **7** (2 mM), and DIEA (20 mM) at room temperature for 4 h. The diluted reaction mixture was analyzed using LC-MS *Method A*. TIC trace of crude reaction and Mass spectrum of product **23b**. Signals of the starting material **23** (yield = 1 %) and **7** are marked with a full black circle (●) and an empty black circle (○), respectively. Signal of the stapling product **23b** (yield = 92 %) is marked with a star (★). Analytical data for **23b** : m/z calcd. for $C_{100}H_{145}Cl_2N_{35}O_{23}S$ $[M+2H]^{2+}$: 1154.03 found 1154.04.

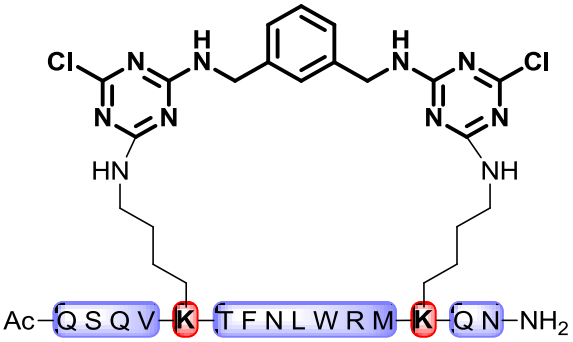


(24a): spacing: i, i+8

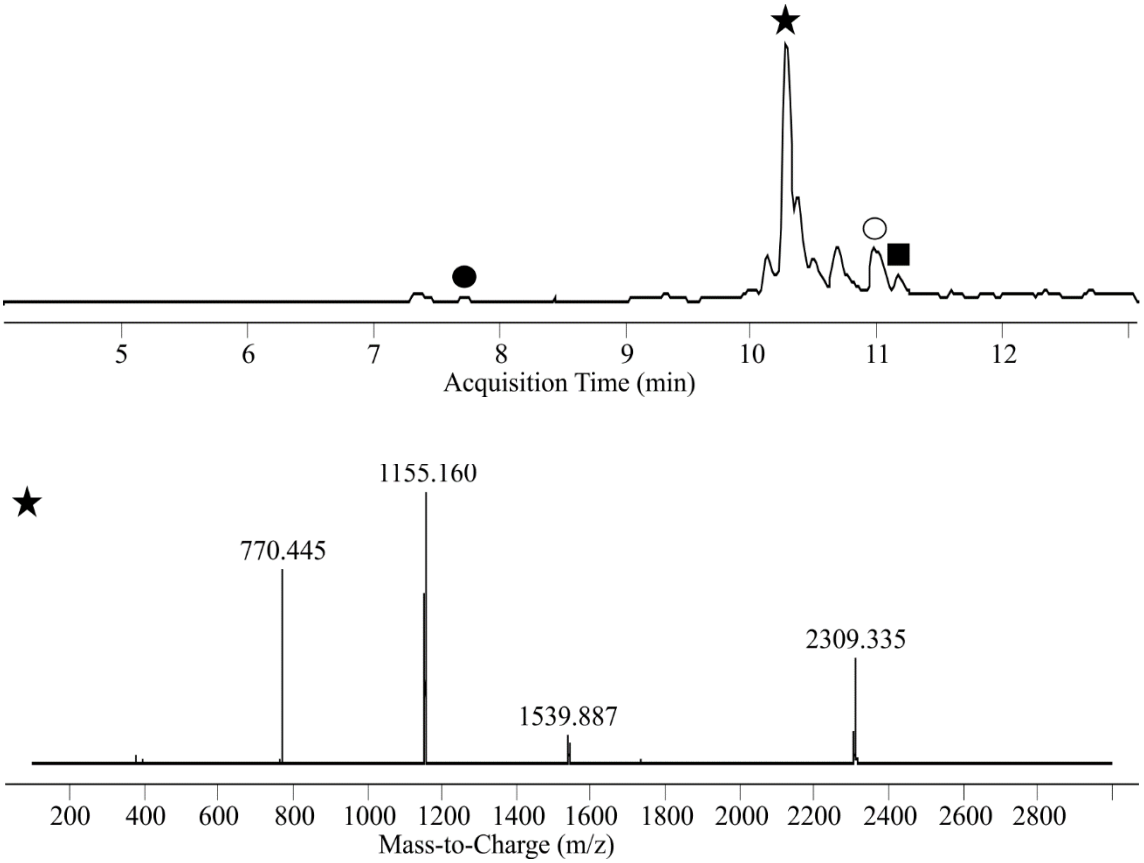




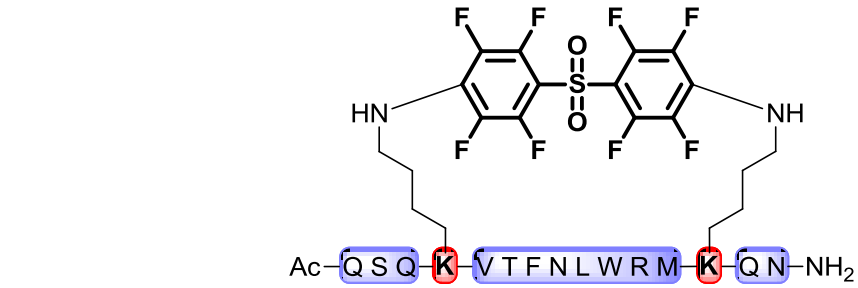
i, i+8 (**24a**): Prepared according to the representative protocol **(C)** using peptide **24** (0.5 mM), electrophile **6** (0.625 mM), and DIEA (10 mM) at room temperature for 4 h. The diluted reaction mixture was analyzed using LC-MS *Method A*. TIC trace of crude reaction and Mass spectrum of product **24a**. Signal of the starting material **24** (yield = 1 %) is marked with a black circle (●). Signal of the double-arylation product (yield = 18 %) is marked with a black square (■). Signal of the stapling product **24a** (yield = 78 %) is marked with a star (★). Analytical data for **24a** : m/z calcd. for $C_{98}H_{135}F_8N_{27}O_{25}S_2$ $[M+2H]^{2+}$: 1153.98 found 1154.12.



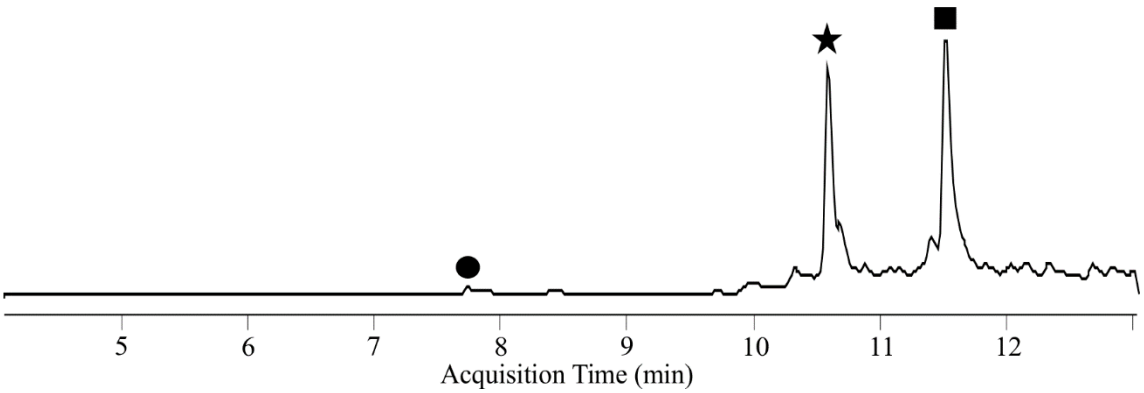
(24b): spacing: i, i+8

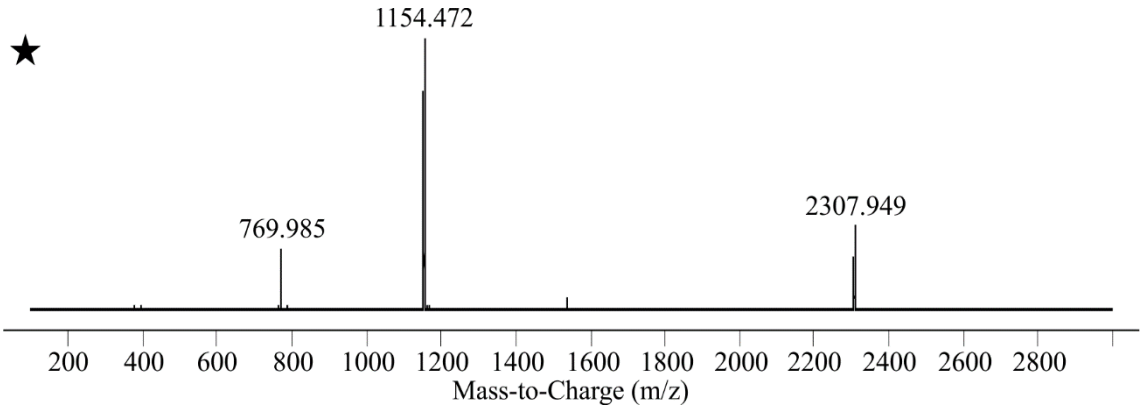


i, i+8 (**24b**): Prepared according to the representative protocol **(E)** using peptide **24** (1 mM), electrophile **7** (2 mM), and DIEA (20 mM) at room temperature for 4 h. The diluted reaction mixture was analyzed using LC-MS *Method A*. TIC trace of crude reaction and Mass spectrum of product **24b**. Signals of the starting material **24** (yield = 1 %) and **7** are marked with a full black circle (●) and an empty black circle (○), respectively. Signal of the double-arylation product (yield = 7 %) is marked with a black square (■). Signal of the stapling product **24b** (yield = 80 %) is marked with a star (★). Analytical data for **24b** : m/z calcd. for $C_{100}H_{145}Cl_2N_{35}O_{23}S$ $[M+2H]^{2+}$: 1154.03 found 1154.17.

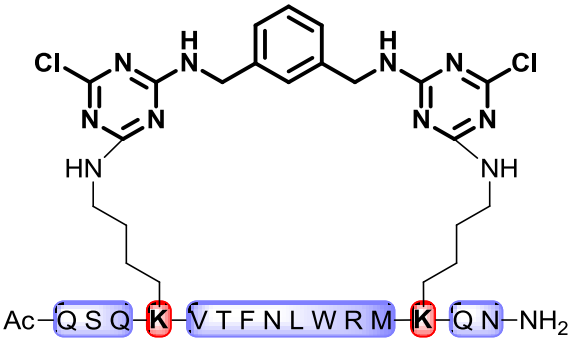


(25a): spacing: i, i+9

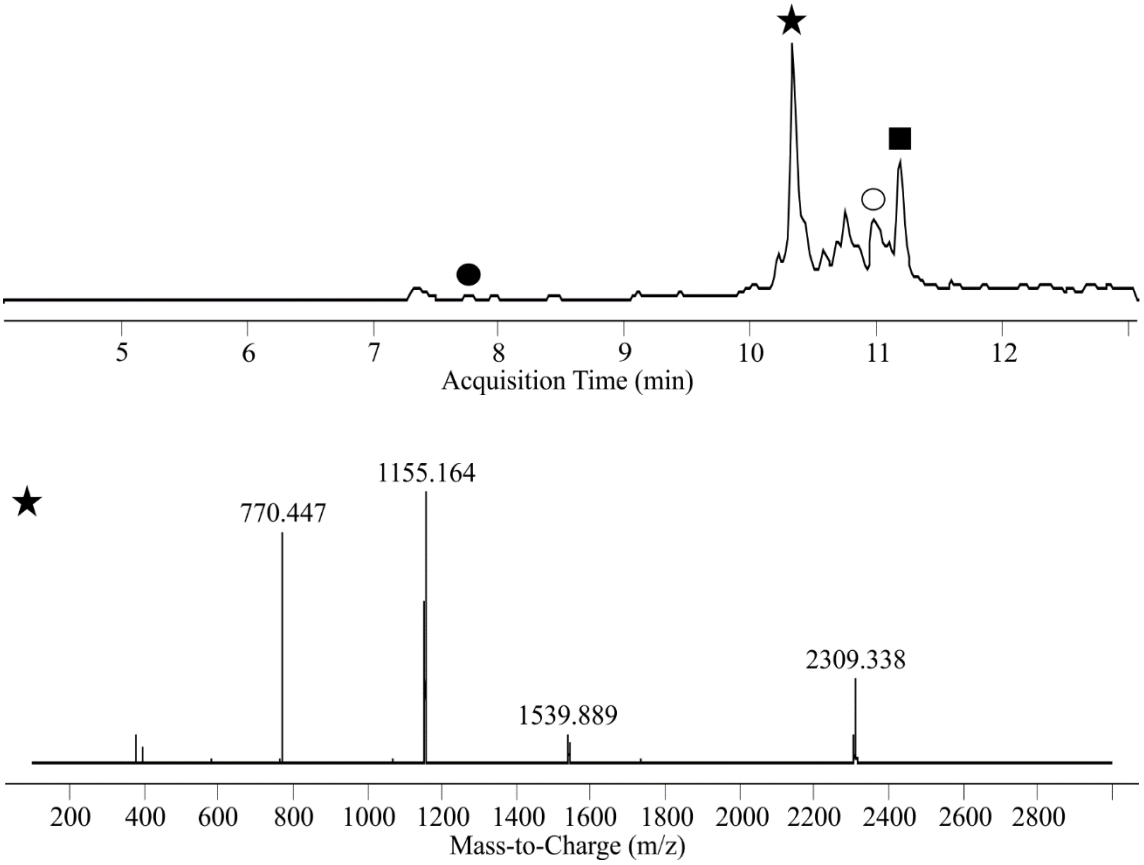




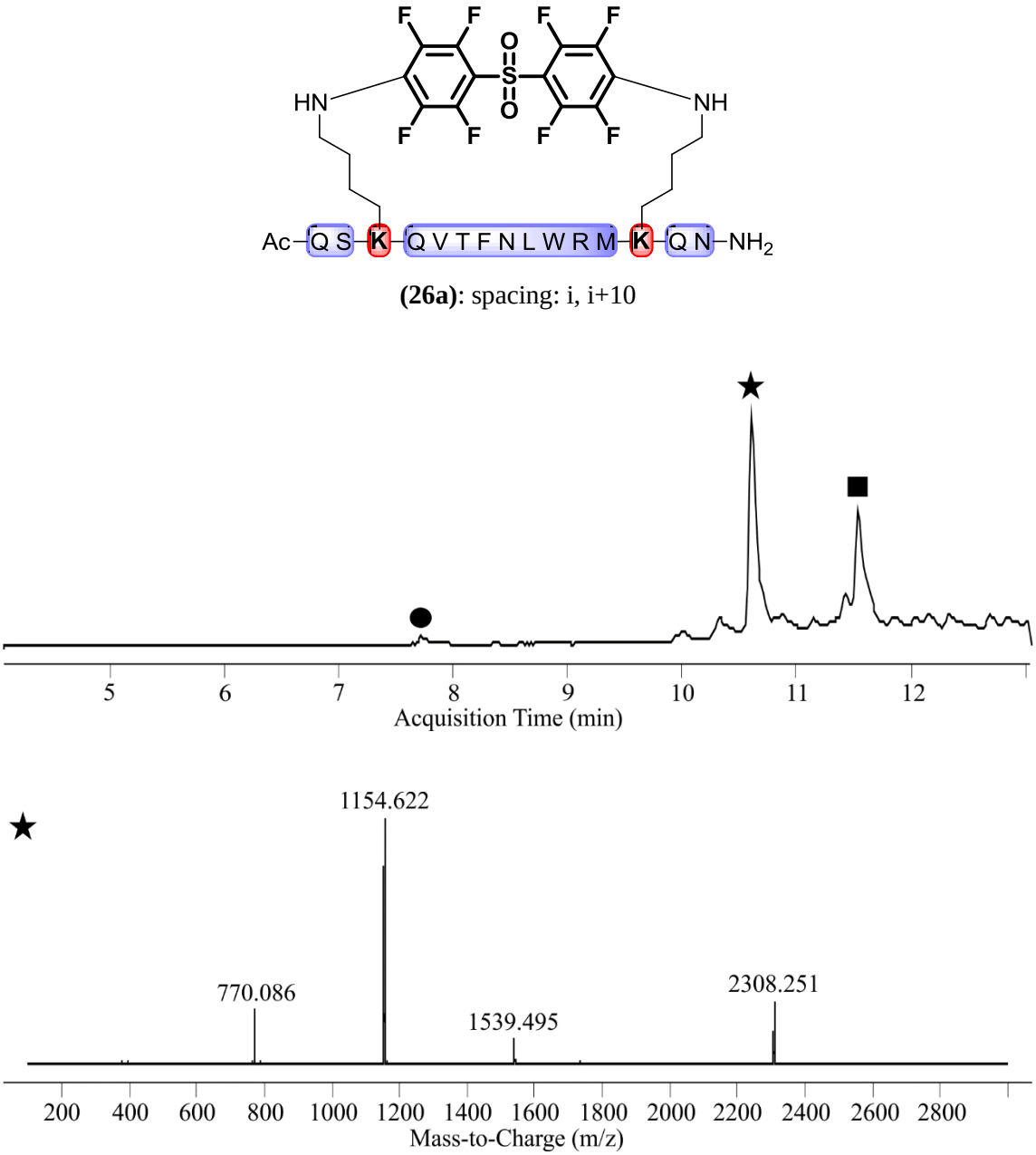
i, i+9 (**25a**): Prepared according to the representative protocol **(C)** using peptide **25** (0.5 mM), electrophile **6** (0.625 mM), and DIEA (10 mM) at room temperature for 4 h. The diluted reaction mixture was analyzed using LC-MS *Method A*. TIC trace of crude reaction and Mass spectrum of product **25a**. Signal of the starting material **25** (yield = 1 %) is marked with a black circle (●). Signal of the double-arylation product (yield = 50 %) is marked with a black square (■). Signal of the stapling product **25a** (yield = 47 %) is marked with a star (★). Analytical data for **25a** : m/z calcd. for $C_{98}H_{135}F_8N_{27}O_{25}S_2$ $[M+2H]^{2+}$: 1153.98 found 1153.97.



(25b): spacing: i, i+9

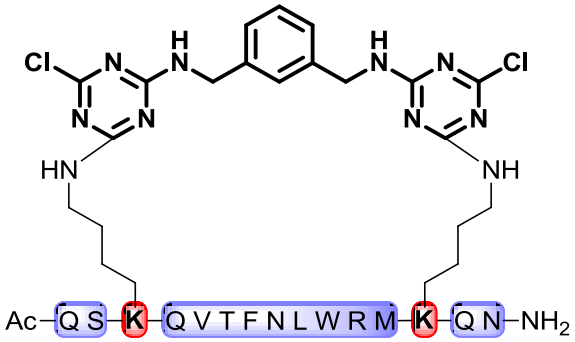


i, i+9 (**25b**): Prepared according to the representative protocol **(E)** using peptide **25** (1 mM), electrophile **7** (2 mM), and DIEA (20 mM) at room temperature for 4 h. The diluted reaction mixture was analyzed using LC-MS *Method A*. TIC trace of crude reaction and Mass spectrum of product **25b**. Signals of the starting material **25** (yield = 1 %) and **7** are marked with a full black circle (●) and an empty black circle (○), respectively. Signal of the double-arylation product (yield = 21 %) is marked with a black square (■). Signal of the stapling product **25b** (yield = 66 %) is marked with a star (★). Analytical data for **25b** : m/z calcd. for $C_{100}H_{145}Cl_2N_{35}O_{23}S$ $[M+2H]^{2+}$: 1154.03 found 1154.17.

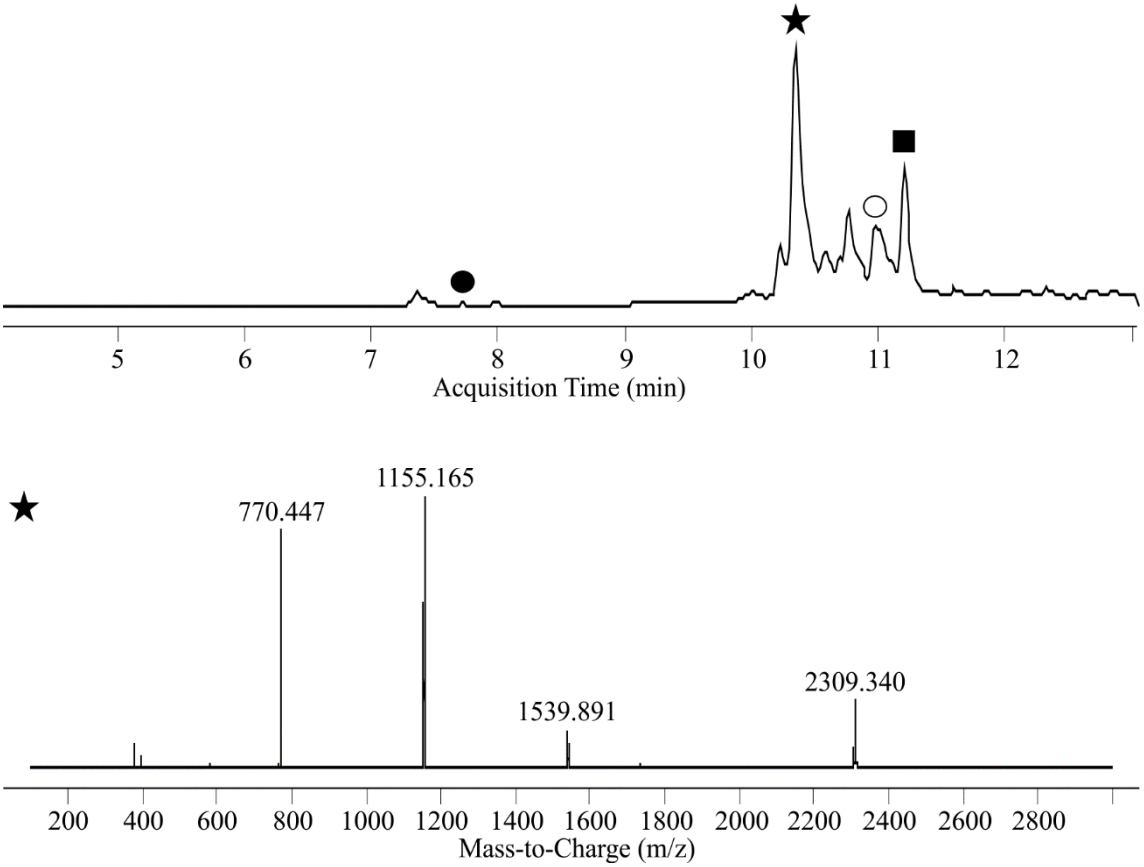


(26a): spacing: i, i+10

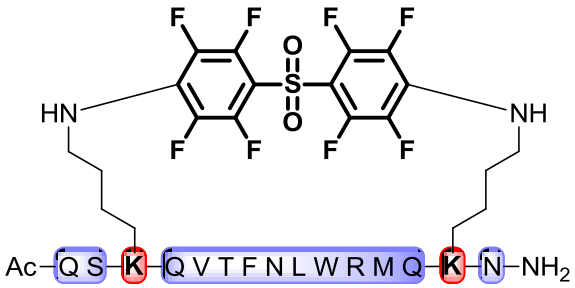
i, i+10 (**26a**): Prepared according to the representative protocol **(C)** using peptide **26** (0.5 mM), electrophile **6** (0.625 mM), and DIEA (10 mM) at room temperature for 4 h. The diluted reaction mixture was analyzed using LC-MS *Method A*. TIC trace of crude reaction and Mass spectrum of product **26a**. Signal of the starting material **26** (yield = 1 %) is marked with a black circle (●). Signal of the double-arylation product (yield = 29 %) is marked with a black square (■). Signal of the stapling product **26a** (yield = 68 %) is marked with a star (★). Analytical data for **26a** : m/z calcd. for $C_{98}H_{135}F_8N_{27}O_{25}S_2$ $[M+2H]^{2+}$: 1153.98 found 1154.12.



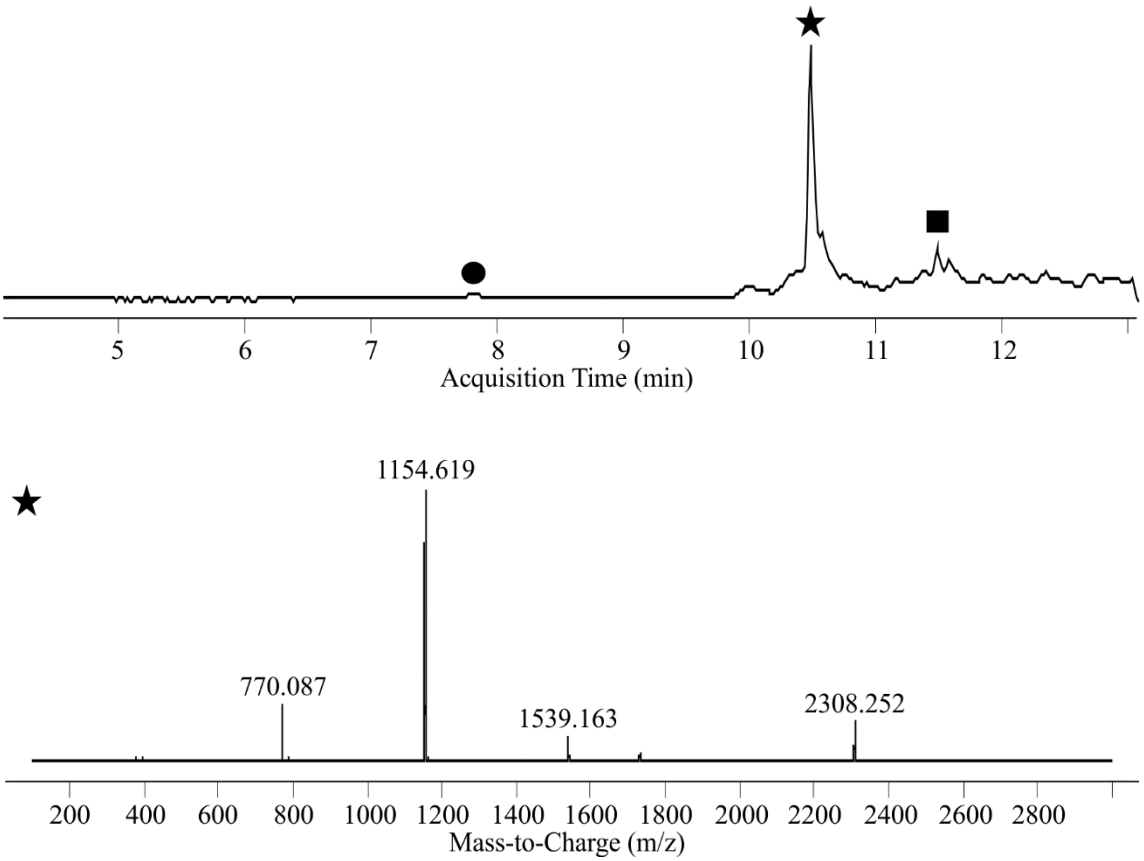
(26b): spacing: i, i+10



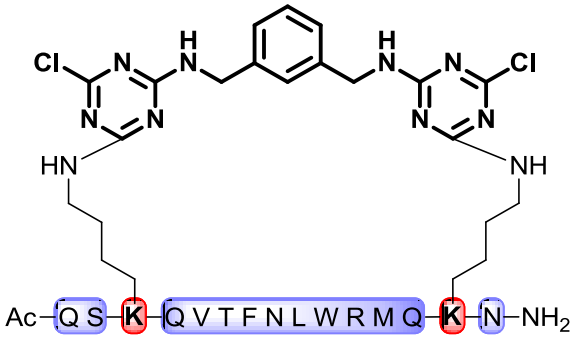
i, i+10 (**26b**): Prepared according to the representative protocol **(E)** using peptide **26** (1 mM), electrophile **7** (2 mM), and DIEA (20 mM) at room temperature for 4 h. The diluted reaction mixture was analyzed using LC-MS *Method A*. TIC trace of crude reaction and Mass spectrum of product **26b**. Signals of the starting material **26** (yield = 1 %) and **7** are marked with a full black circle (●) and an empty black circle (○), respectively. Signal of the double-arylation product (yield = 25 %) is marked with a black square (■). Signal of the stapling product **26b** (yield = 61 %) is marked with a star (★). Analytical data for **26b** : m/z calcd. for $C_{100}H_{145}Cl_2N_{35}O_{23}S$ $[M+2H]^{2+}$: 1154.03 found 1154.17.



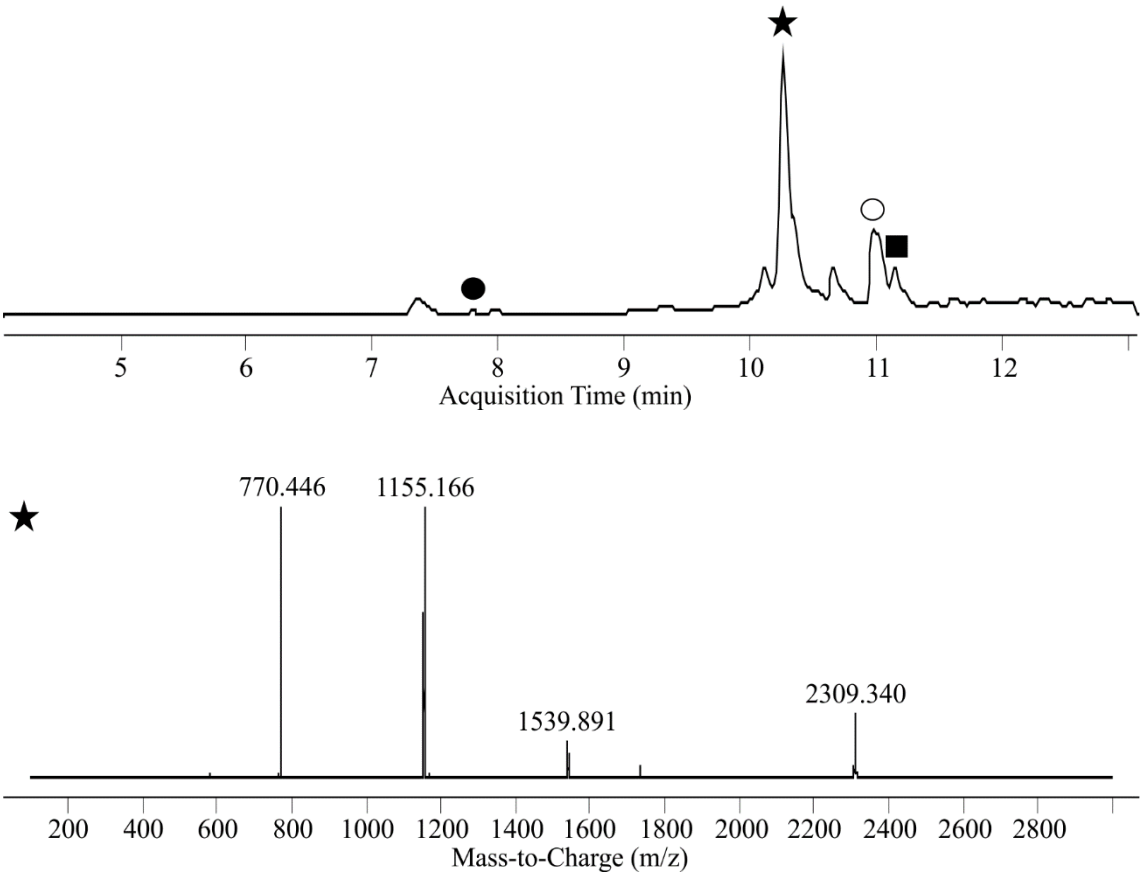
(27a): spacing: i, i+11



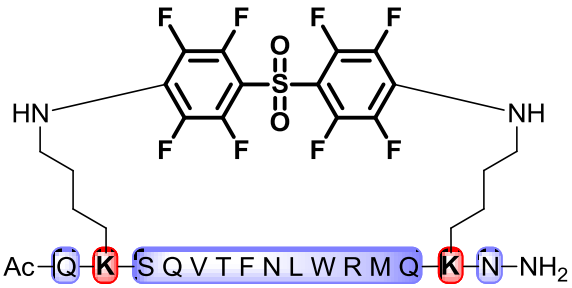
i, i+11 (**27a**): Prepared according to the representative protocol **(C)** using peptide **27** (0.5 mM), electrophile **6** (0.625 mM), and DIEA (10 mM) at room temperature for 4 h. The diluted reaction mixture was analyzed using LC-MS *Method A*. TIC trace of crude reaction and Mass spectrum of product **27a**. Signal of the starting material **27** (yield = 1 %) is marked with a black circle (●). Signal of the double-arylation product (yield = 6 %) is marked with a black square (■). Signal of the stapling product **27a** (yield = 88 %) is marked with a star (★). Analytical data for **27a** : m/z calcd. for $C_{98}H_{135}F_8N_{27}O_{25}S_2$ $[M+2H]^{2+}$: 1153.98 found 1154.12.



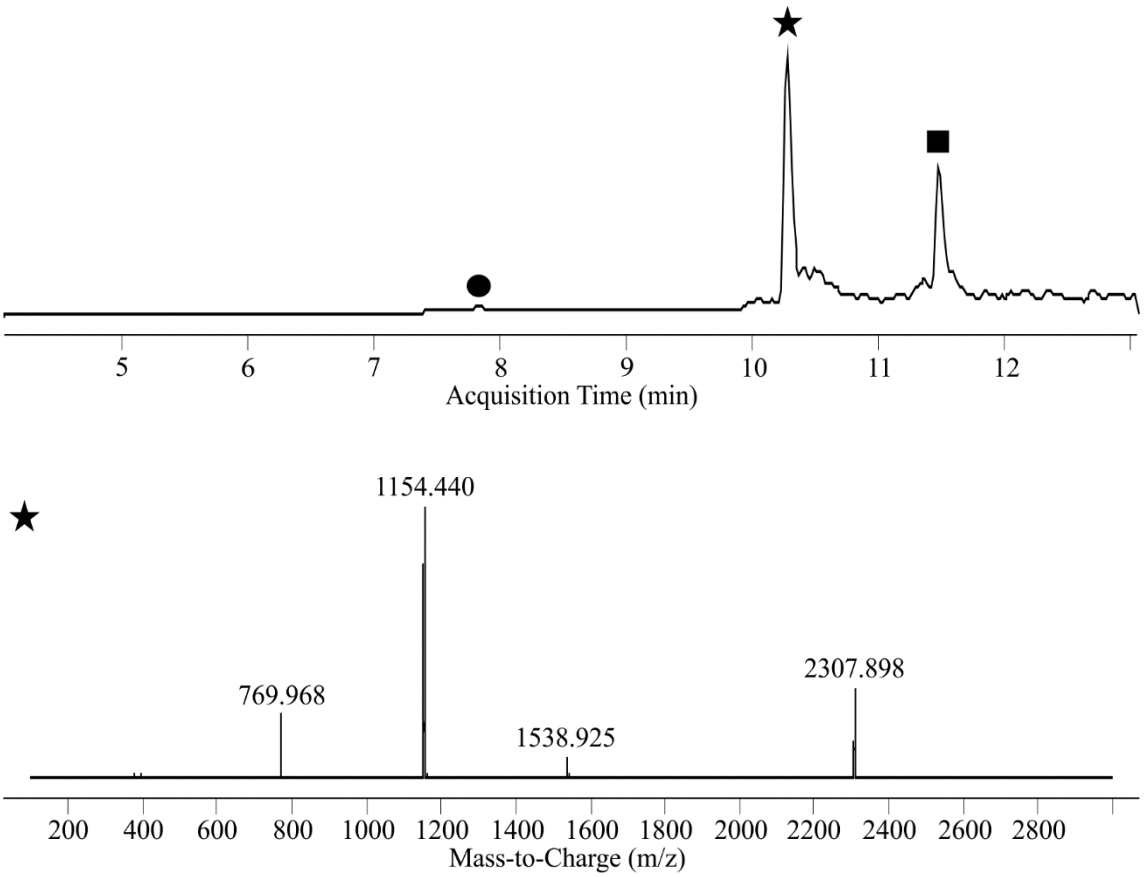
(27b): spacing: i, i+11



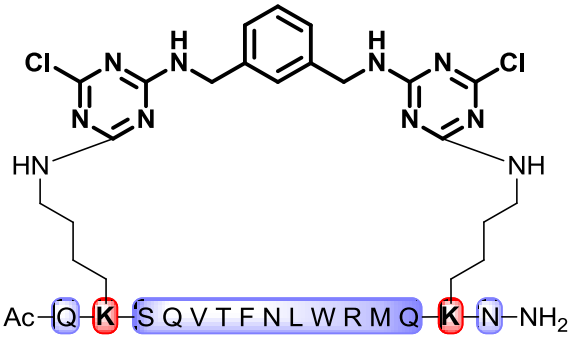
i, i+11 (**27b**): Prepared according to the representative protocol **(E)** using peptide **27b** (1 mM), electrophile **7** (2 mM), and DIEA (20 mM) at room temperature for 4 h. The diluted reaction mixture was analyzed using LC-MS *Method A*. TIC trace of crude reaction and Mass spectrum of product **27b**. Signals of the starting material **27** (yield = 1 %) and **7** are marked with a full black circle (●) and an empty black circle (○), respectively. Signal of the double-arylation product (yield = 5 %) is marked with a black square (■). Signal of the stapling product **27b** (yield = 89 %) is marked with a star (★). Analytical data for **27b** : m/z calcd. for $C_{100}H_{145}Cl_2N_{35}O_{23}S$ $[M+2H]^{2+}$: 1154.03 found 1154.17.



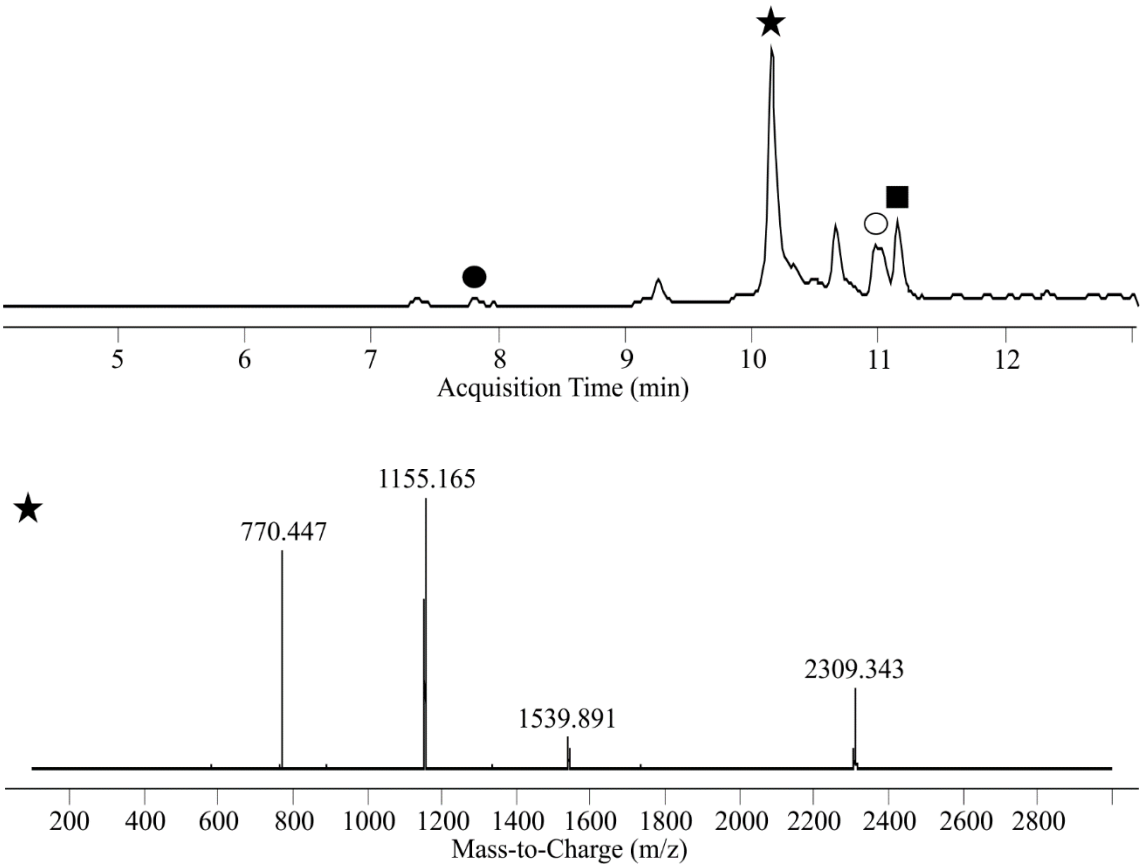
(28a): spacing: i, i+12



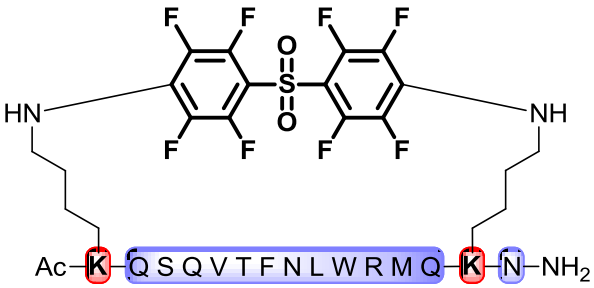
i, i+12 (**28a**): Prepared according to the representative protocol **(C)** using peptide **28** (0.5 mM), electrophile **6** (0.625 mM), and DIEA (10 mM) at room temperature for 4 h. The diluted reaction mixture was analyzed using LC-MS *Method A*. TIC trace of crude reaction and Mass spectrum of product **28a**. Signal of the starting material **28** (yield = 1 %) is marked with a black circle (●). Signal of the double-arylation product (yield = 25 %) is marked with a black square (■). Signal of the stapling product **28a** (yield = 71 %) is marked with a star (★). Analytical data for **28a** : m/z calcd. for $C_{98}H_{135}F_8N_{27}O_{25}S_2$ $[M+2H]^{2+}$: 1153.98 found 1154.17.



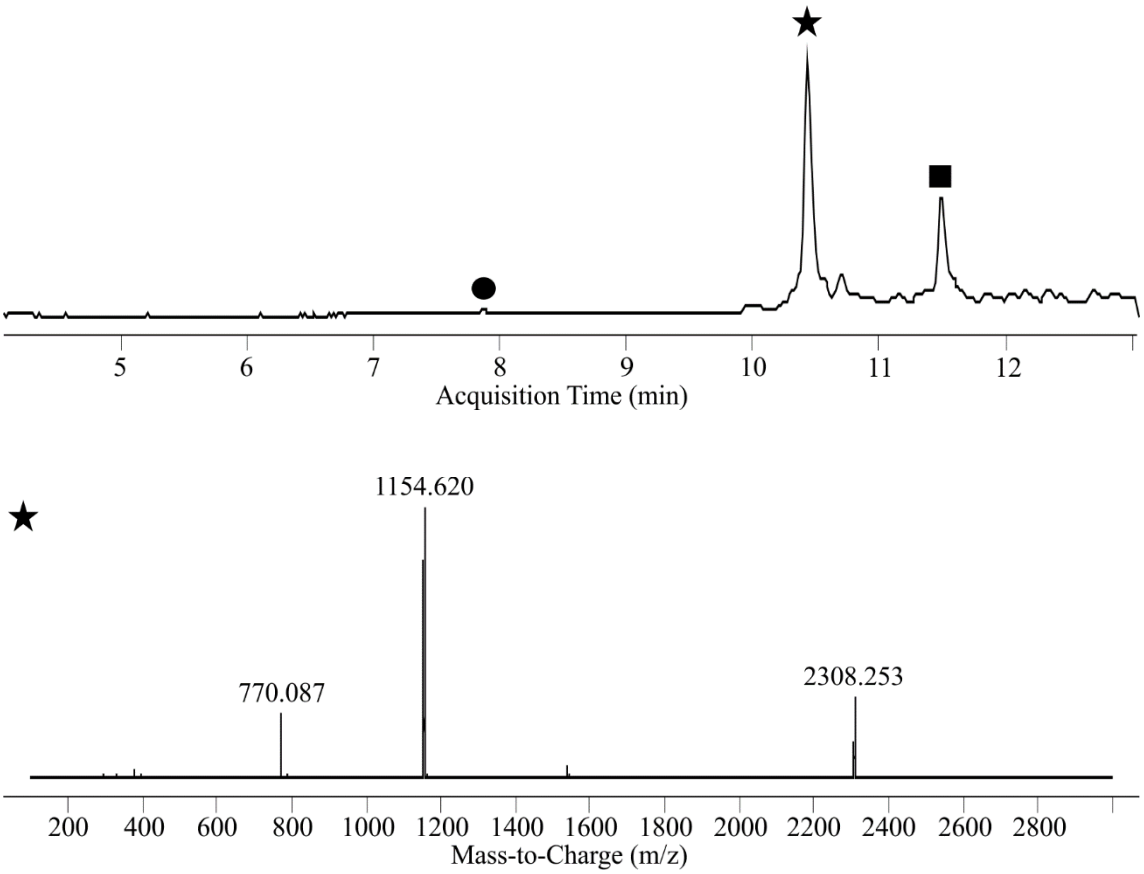
(28b): spacing: i, i+12



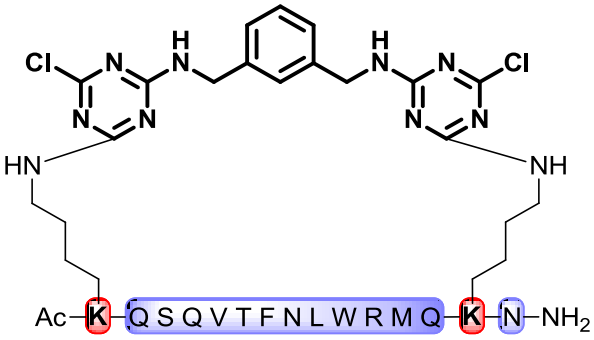
i, i+12 (**28b**): Prepared according to the representative protocol **(E)** using peptide **28** (1 mM), electrophile **7** (2 mM), and DIEA (20 mM) at room temperature for 4 h. The diluted reaction mixture was analyzed using LC-MS *Method A*. TIC trace of crude reaction and Mass spectrum of product **28b**. Signals of the starting material **28** (yield = 1 %) and **7** are marked with a full black circle (●) and an empty black circle (○), respectively. Signal of the double-arylation product (yield = 14 %) is marked with a black square (■). Signal of the stapling product **28b** (yield = 78 %) is marked with a star (★). Analytical data for **28b** : m/z calcd. for $C_{100}H_{145}Cl_2N_{35}O_{23}S$ $[M+2H]^{2+}$: 1154.03 found 1154.17.



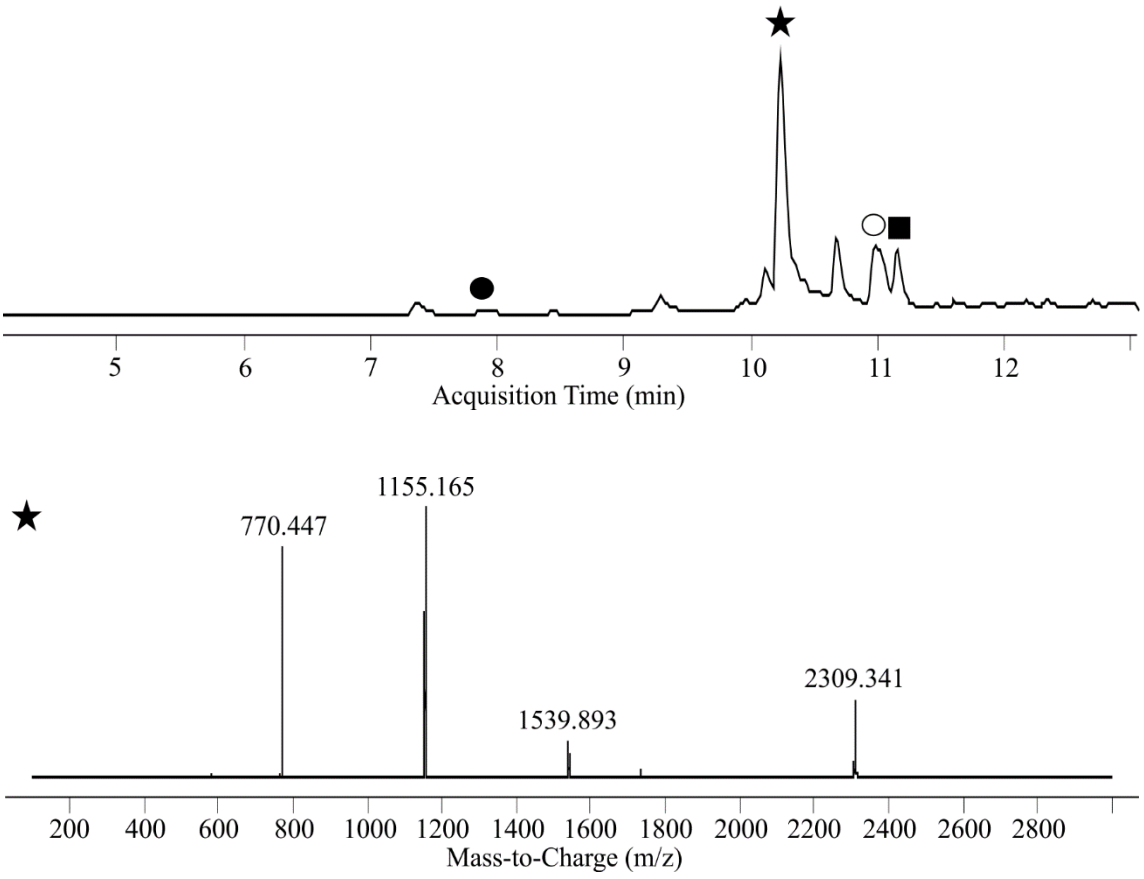
(29a): spacing: i, i+13



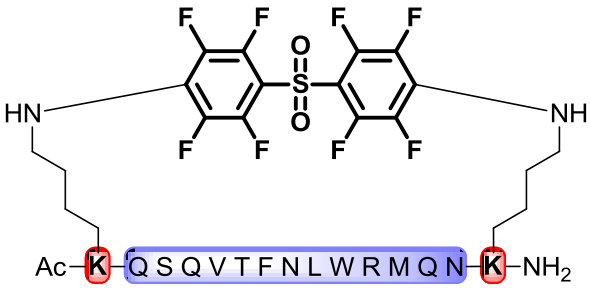
i, i+13 (**29a**): Prepared according to the representative protocol **(C)** using peptide **29** (0.5 mM), electrophile **6** (0.625 mM), and DIEA (10 mM) at room temperature for 4 h. The diluted reaction mixture was analyzed using LC-MS *Method A*. TIC trace of crude reaction and Mass spectrum of product **29a**. Signal of the starting material **29** (yield = 1 %) is marked with a black circle (●). Signal of the double-arylation product (yield = 26 %) is marked with a black square (■). Signal of the stapling product **29a** (yield = 72 %) is marked with a star (★). Analytical data for **29a** : m/z calcd. for $C_{98}H_{135}F_8N_{27}O_{25}S_2$ $[M+2H]^{2+}$: 1153.98 found 1154.12.



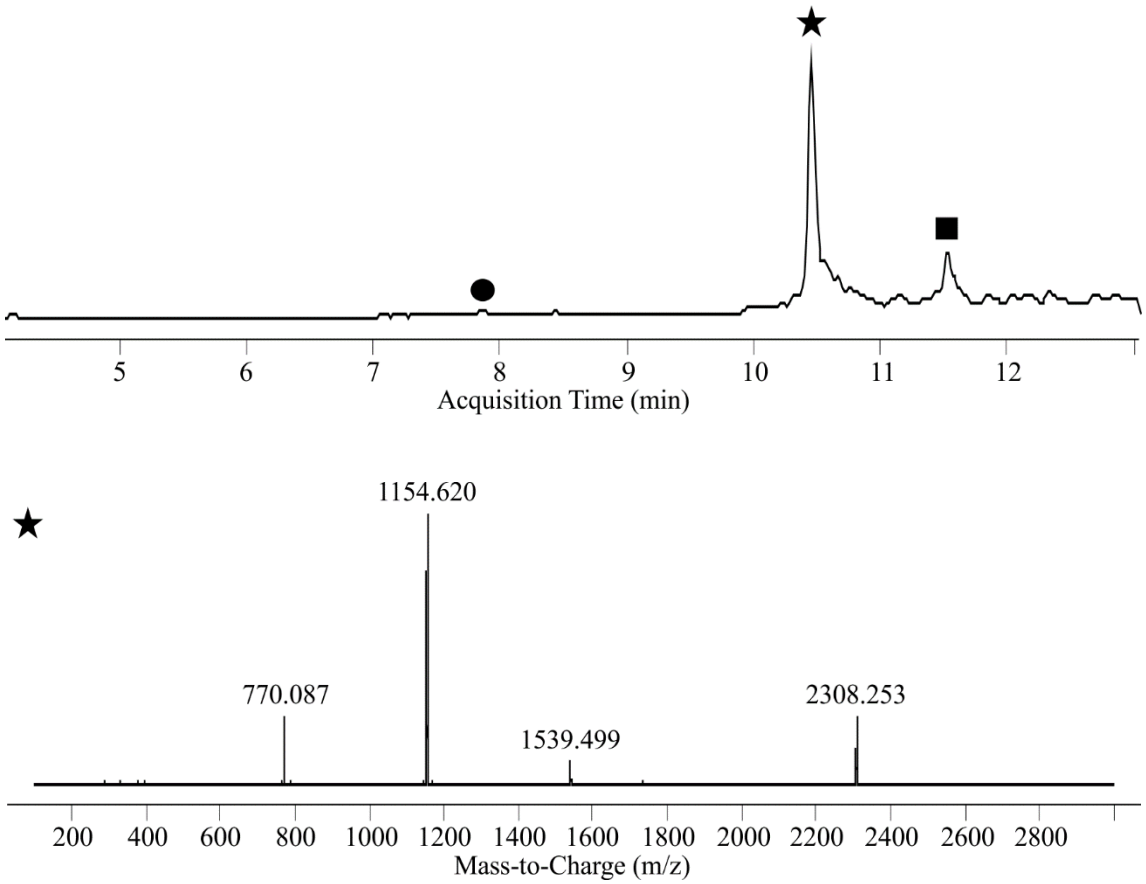
(29b): spacing: i, i+13



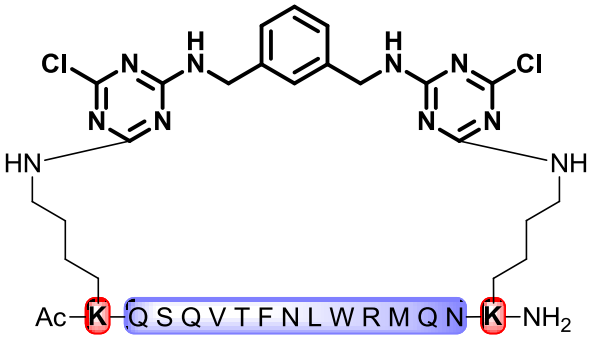
i, i+13 (**29b**): Prepared according to the representative protocol **(E)** using peptide **29** (1 mM), electrophile **7** (2 mM), and DIEA (20 mM) at room temperature for 4 h. The diluted reaction mixture was analyzed using LC-MS *Method A*. TIC trace of crude reaction and Mass spectrum of product **29b**. Signals of the starting material **29** (yield = 1 %) and **7** are marked with a full black circle (●) and an empty black circle (○), respectively. Signal of the double-arylation product (yield = 14 %) is marked with a black square (■). Signal of the stapling product **29b** (yield = 80 %) is marked with a star (★). Analytical data for **29b** : m/z calcd. for $C_{100}H_{145}Cl_2N_{35}O_{23}S$ $[M+2H]^{2+}$: 1154.03 found 1154.17.



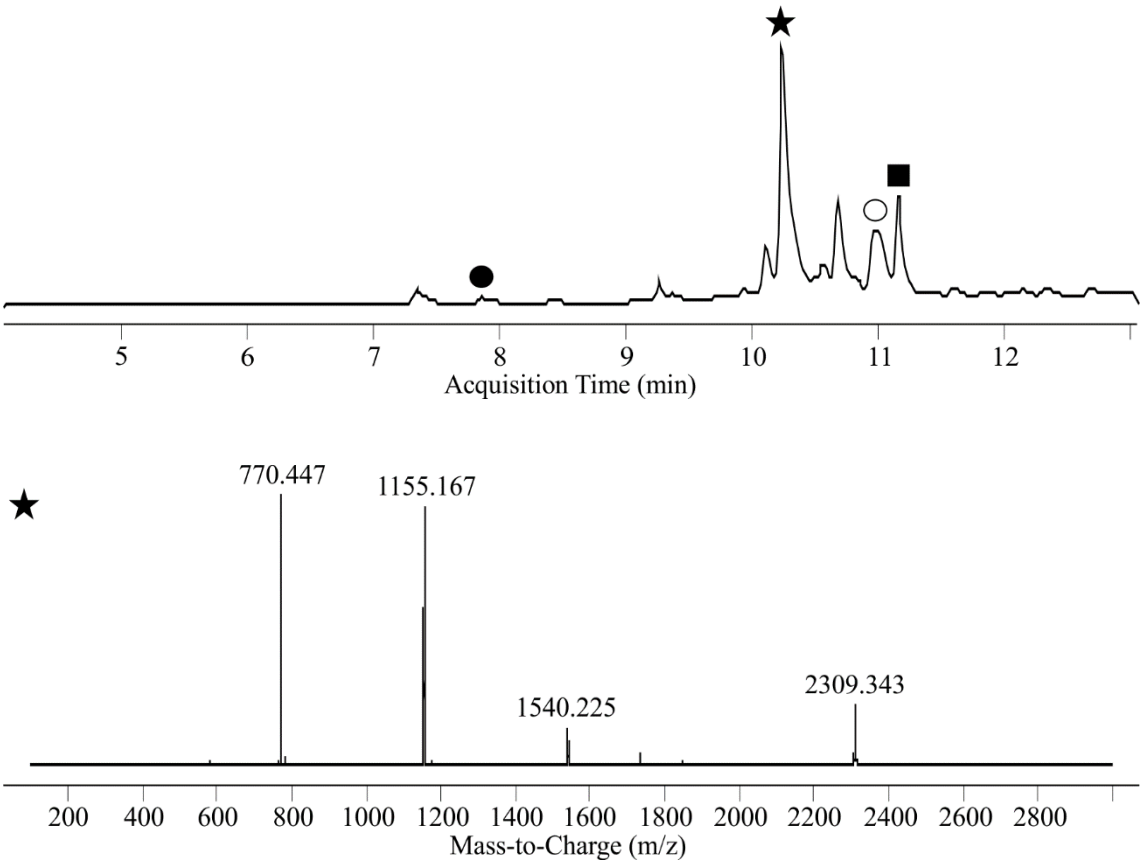
(30a): spacing: i, i+14



i, i+14 (**30a**): Prepared according to the representative protocol **(C)** using peptide **30** (0.5 mM), electrophile **6** (0.625 mM), and DIEA (10 mM) at room temperature for 4 h. The diluted reaction mixture was analyzed using LC-MS *Method A*. TIC trace of crude reaction and Mass spectrum of product **30a**. Signal of the starting material **30** (yield = 1 %) is marked with a black circle (●). Signal of the double-arylation product (yield = 18 %) is marked with a black square (■). Signal of the stapling product **30a** (yield = 79 %) is marked with a star (★). Analytical data for **30a** : m/z calcd. for $C_{98}H_{135}F_8N_{27}O_{25}S_2$ $[M+2H]^{2+}$: 1153.98 found 1154.12.



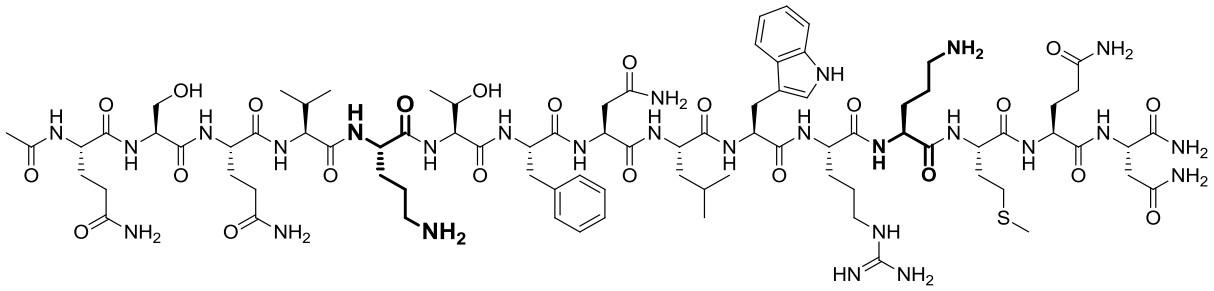
(30b): spacing: i, i+14



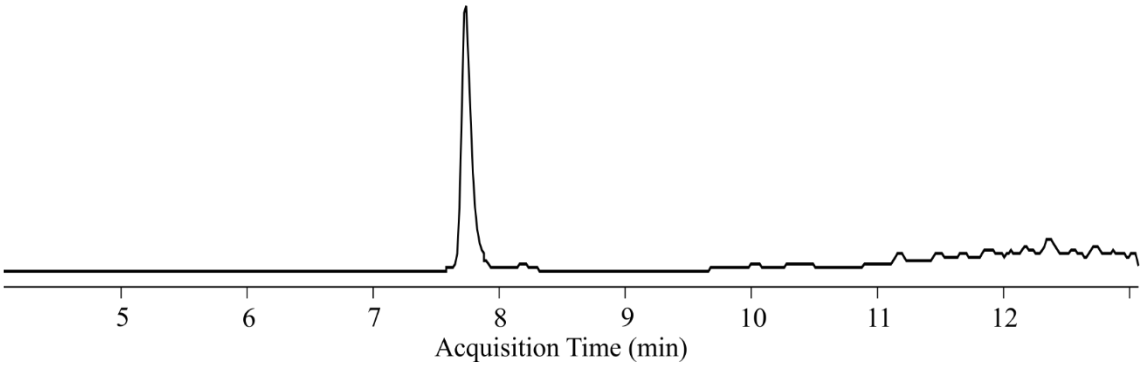
i, i+14 (**30b**):Prepared according to the representative protocol **(E)** using peptide **30** (1 mM), electrophile **7** (2 mM), and DIEA (20 mM) at room temperature for 4 h. The diluted reaction mixture was analyzed using LC-MS *Method A*. TIC trace of crude reaction and Mass spectrum of product **30b**. Signals of the starting material **30** (yield = 1 %) and **7** are marked with a full black circle (●) and an empty black circle (○), respectively. Signal of the double-arylation product (yield = 22 %) is marked with a black square (■). Signal of the stapling product **30b** (yield = 70 %) is marked with a star (★). Analytical data for **30b** : m/z calcd. for $C_{100}H_{145}Cl_2N_{35}O_{23}S$ $[M+2H]^{2+}$: 1154.03 found 1154.17.

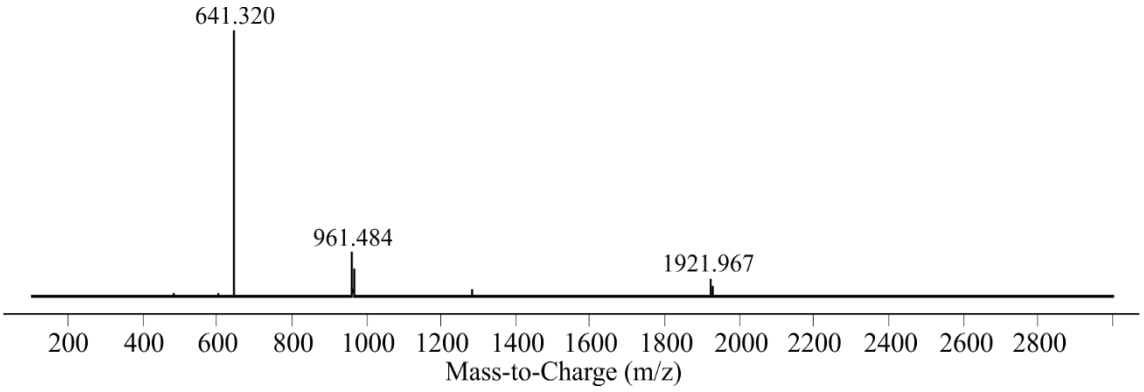
8. Tuning of Macrocycle Rigidity

LC-MS analytical data of purified peptides **31**, **32** *and* **33**

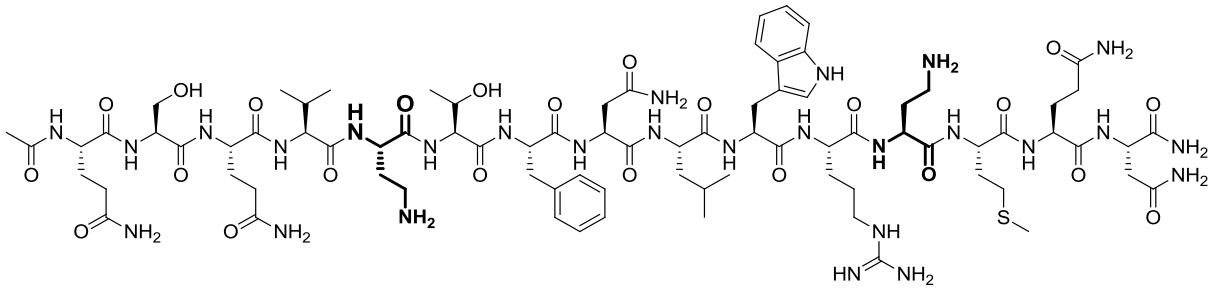


31: AcNH-Gln-Ser-Gln-Val-**Orn**-Thr-Phe-Asn-Leu-Trp-Arg-**Orn**-Met-Gln-Asn-$CONH_2$

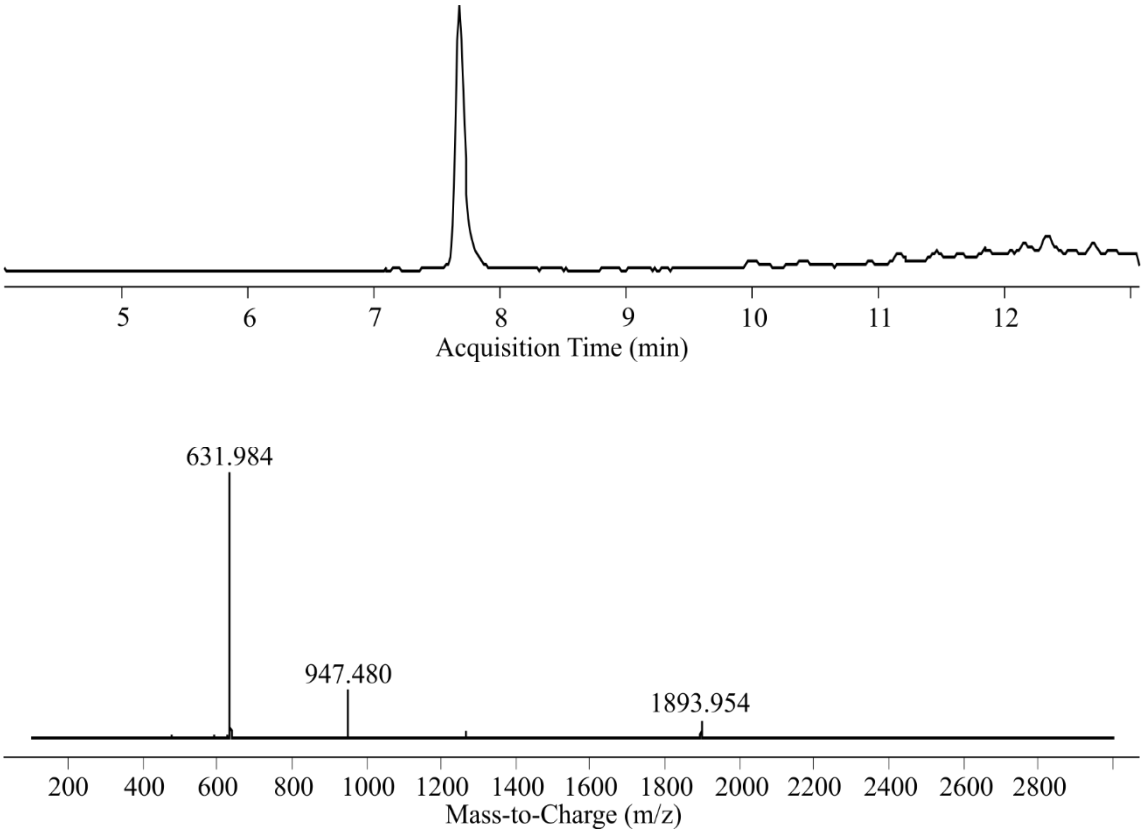




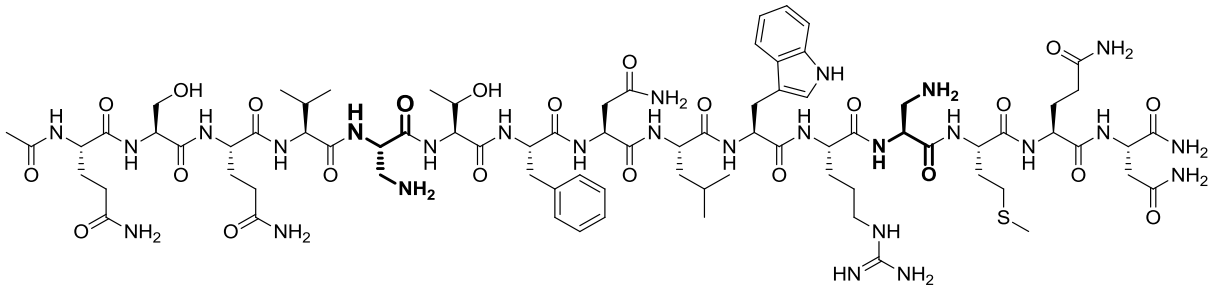
Peptide **31**: LC-MS analysis *Method A*. TIC trace and Mass spectrum of peptide **31**. m/z calcd. for $C_{84}H_{133}N_{27}O_{23}S$ $[M+2H]^{2+}$: 961.00 found 960.98.



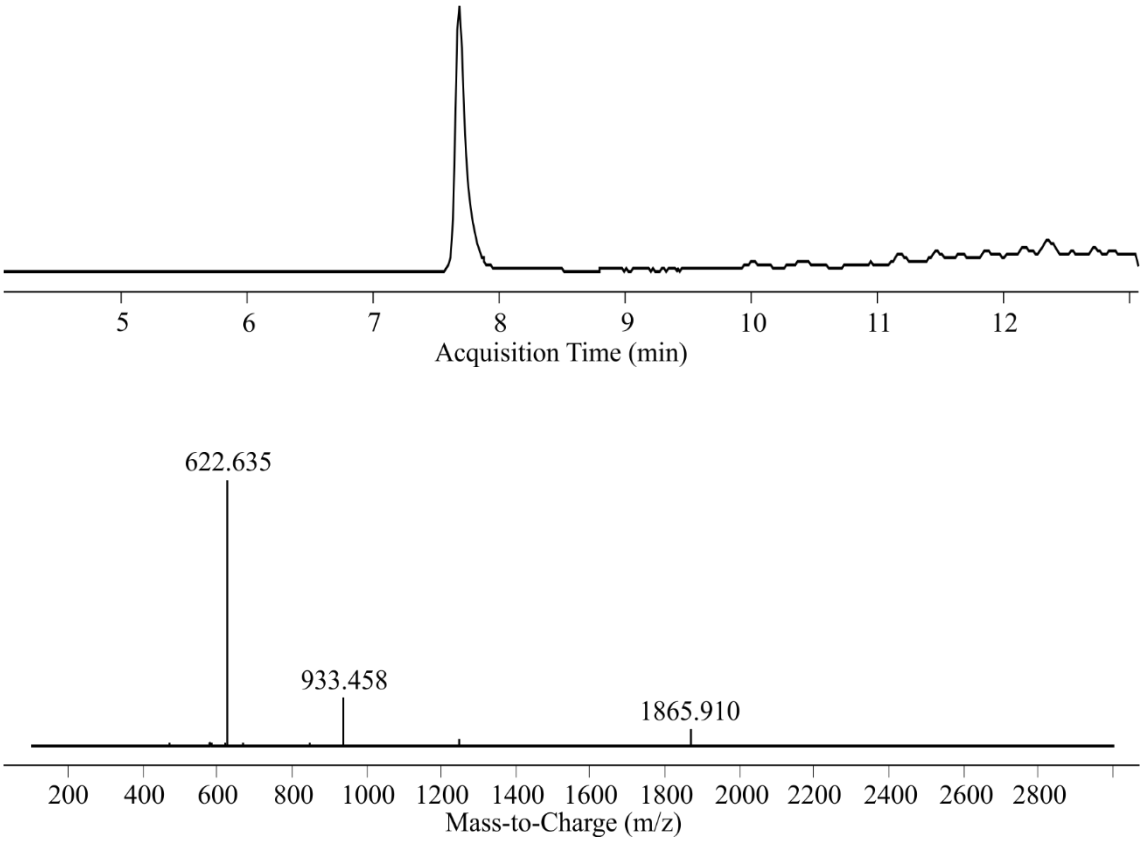
32: AcNH-Gln-Ser-Gln-Val-**Dab-**Thr-Phe-Asn-Leu-Trp-Arg-**Dab**-Met-Gln-Asn-$CONH_2$



Peptide **32:** LC-MS analysis *Method A*. TIC trace and Mass spectrum of peptide **32**. m/z calcd. for $C_{82}H_{129}N_{27}O_{23}S$ $[M+2H]^{2+}$: 946.98 found 946.98.

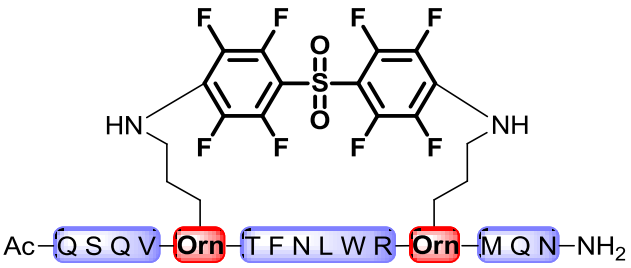


33: AcNH-Gln-Ser-Gln-Val-**Dap-**Thr-Phe-Asn-Leu-Trp-Arg-**Dap**-Met-Gln-Asn-$CONH_2$

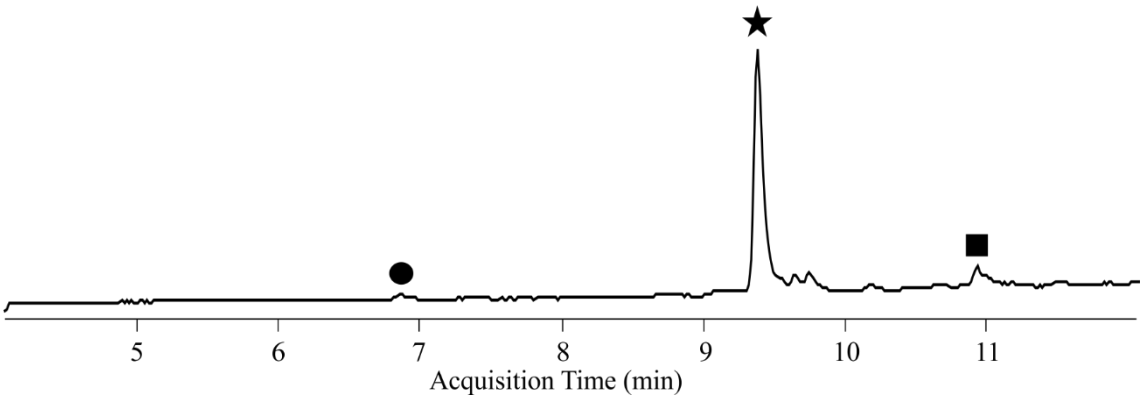


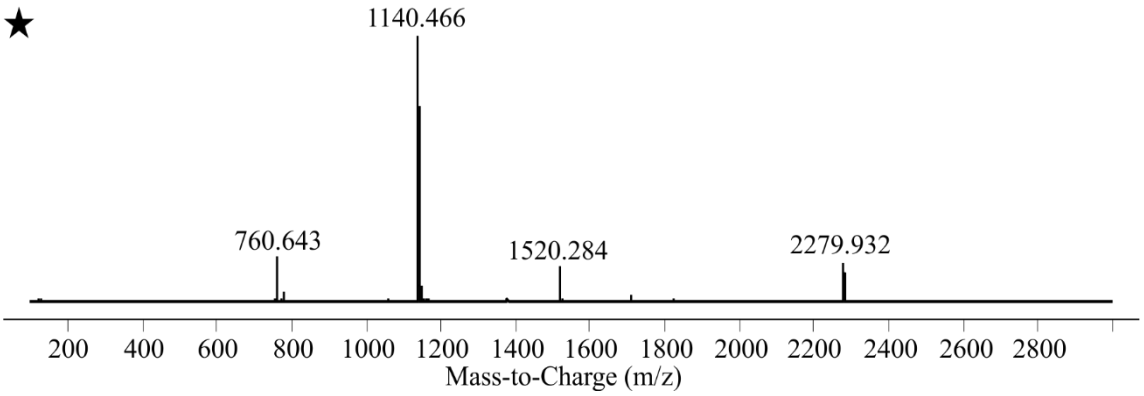
Peptide **33:** LC-MS analysis *Method A*. TIC trace and Mass spectrum of peptide **33**. m/z calcd. for $C_{80}H_{125}N_{27}O_{23}S$ $[M+2H]^{2+}$: 932.97found 932.96.

LC-MS analytical data of stapling crude reaction with peptides **31**, **32** *and* **33**

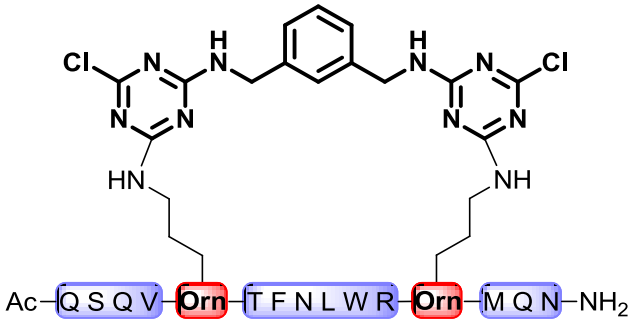


31a

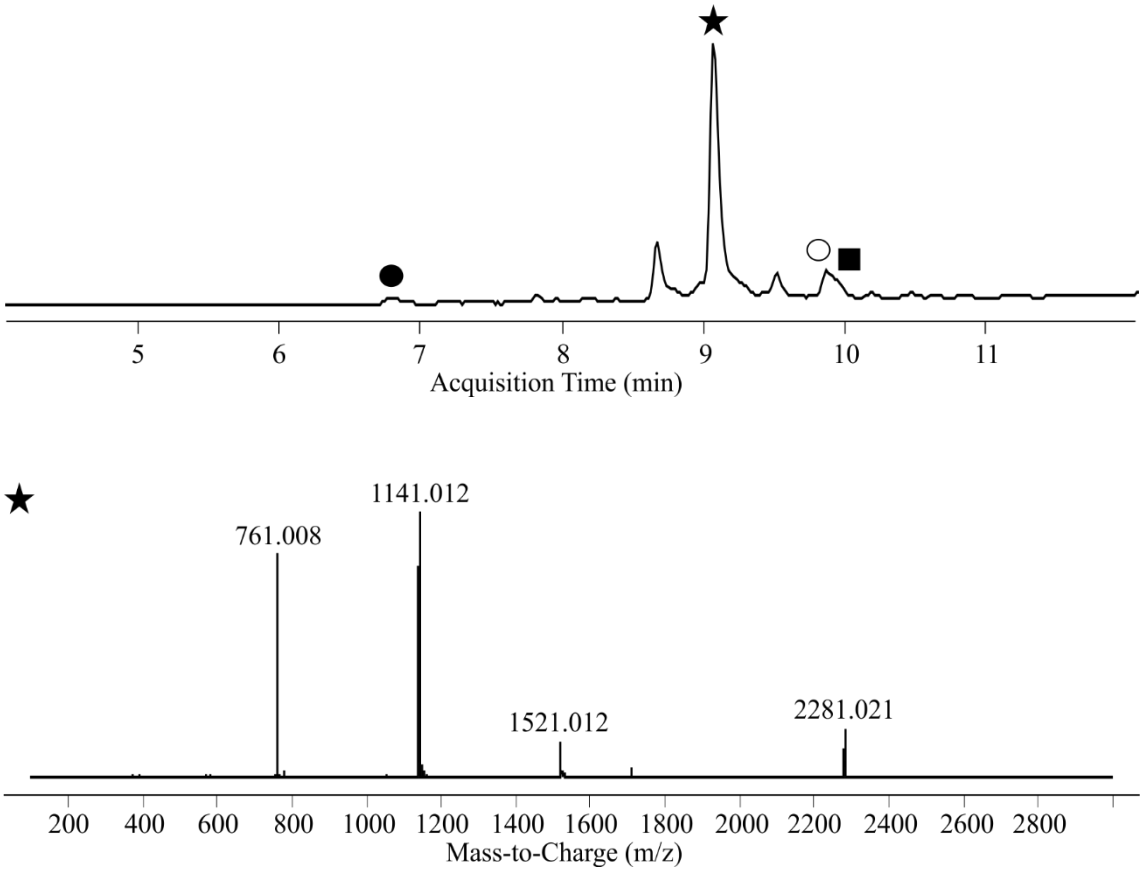




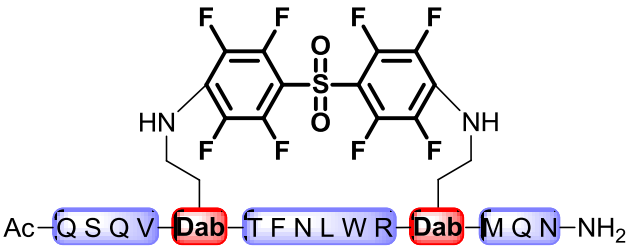
(**31a**) : Prepared according to the representative protocol **(C)** using peptide **31** (0.5 mM), electrophile **6** (0.625 mM), and DIEA (10 mM) at room temperature for 4 h. The diluted reaction mixture was analyzed using LC-MS *Method A*. TIC trace of crude reaction and Mass spectrum of product **31a**. Signal of the starting material **31** (yield = 1 %) is marked with a black circle (●). Signal of the double-arylation product (yield = 3 %) is marked with a black square (■). Signal of the stapling product **31a** (yield = 95 %) is marked with a star (★). Analytical data for **31a** : m/z calcd. for $C_{96}H_{131}F_8N_{27}O_{25}S_2$ $[M+2H]^{2+}$: 1139.96 found 1139.96.



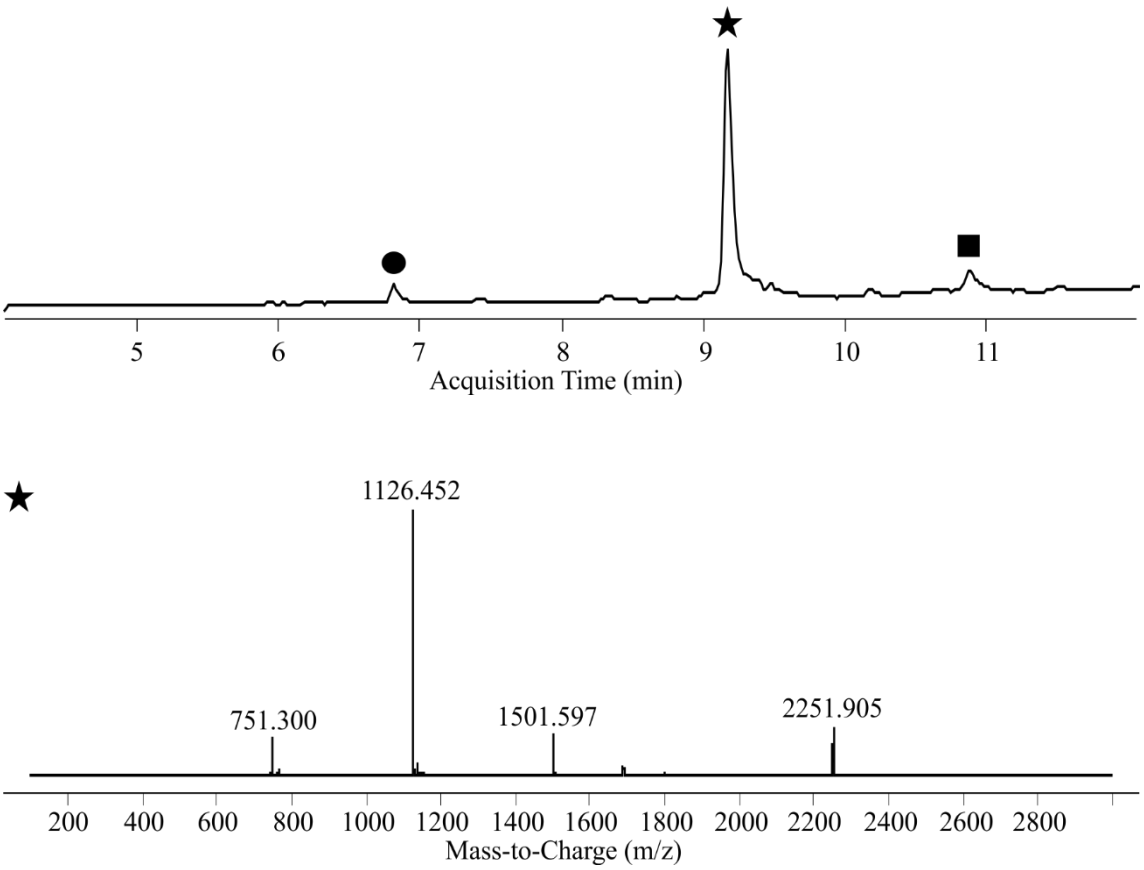
31b



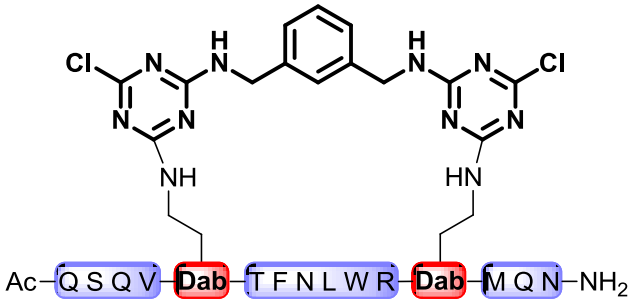
(**31b**) : Prepared according to the representative protocol **(E)** using peptide **31** (1 mM), electrophile **7** (2 mM), and DIEA (20 mM) at room temperature for 4 h. The diluted reaction mixture was analyzed using LC-MS *Method A*. TIC trace of crude reaction and Mass spectrum of product **31b**. Signals of the starting material **31** (yield = 1 %) and **7** are marked with a full black circle (●) and an empty black circle (○), respectively. Signal of the double-arylation product (yield = 5 %) is marked with a black square (■). Signal of the stapling product **31b** (yield = 85 %) is marked with a star (★). Analytical data for **31b** : m/z calcd. for $C_{98}H_{141}Cl_2N_{35}O_{23}S$ $[M+2H]^{2+}$: 1140.01 found 1140.01.



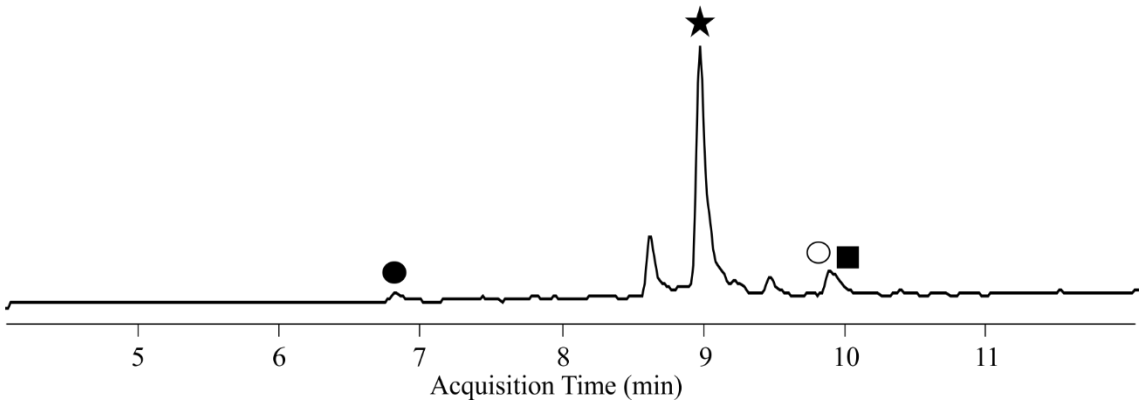
32a

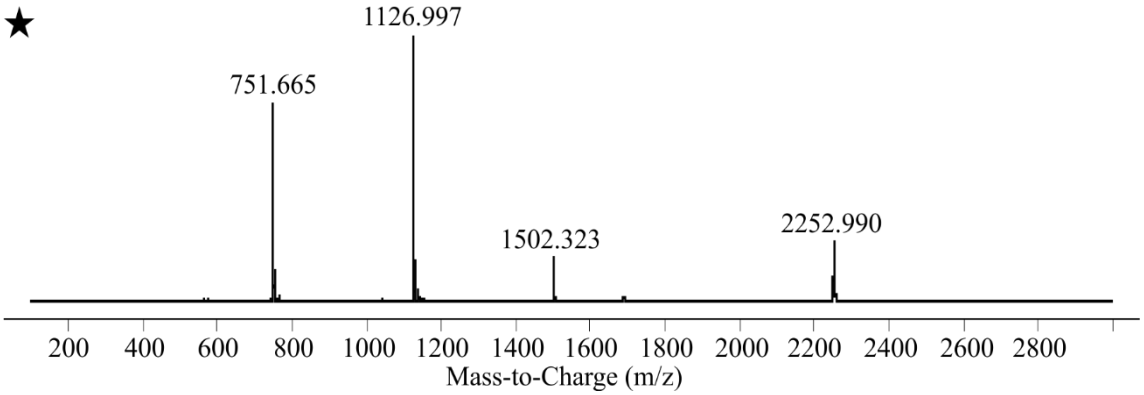


(**32a**) : Prepared according to the representative protocol **(C)** using peptide **32** (0.5 mM), electrophile **6** (0.625 mM), and DIEA (10 mM) at room temperature for 4 h. The diluted reaction mixture was analyzed using LC-MS *Method A*. TIC trace of crude reaction and Mass spectrum of product **32a**. Signal of the starting material **32** (yield = 3 %) is marked with a black circle (●). Signal of the double-arylation product (yield = 5 %) is marked with a black square (■). Signal of the stapling product **32a** (yield = 91 %) is marked with a star (★). Analytical data for **32a** : m/z calcd. for $C_{94}H_{127}F_8N_{27}O_{25}S_2$ $[M+2H]^{2+}$: 1125.95 found 1126.00.

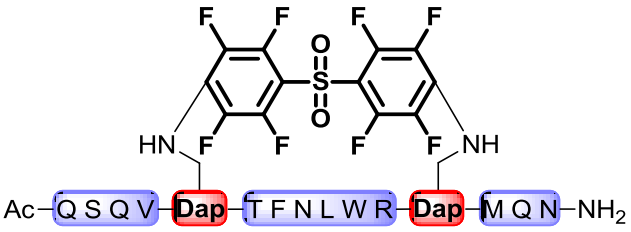


32b

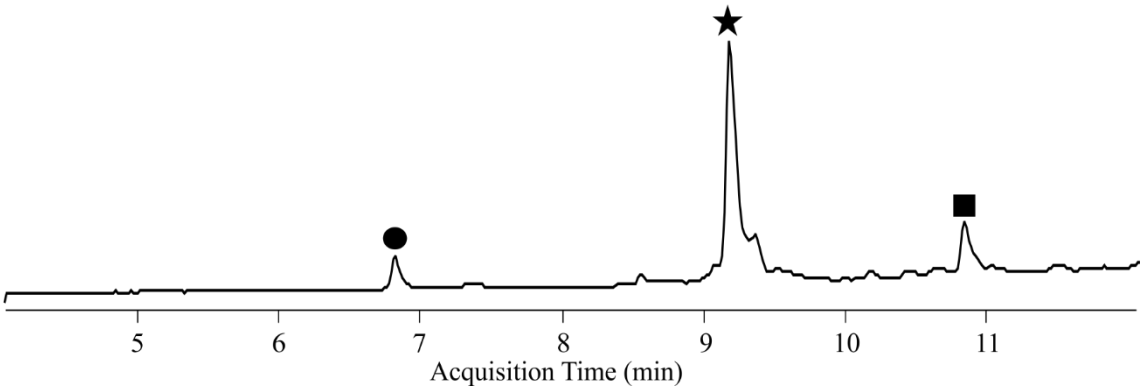


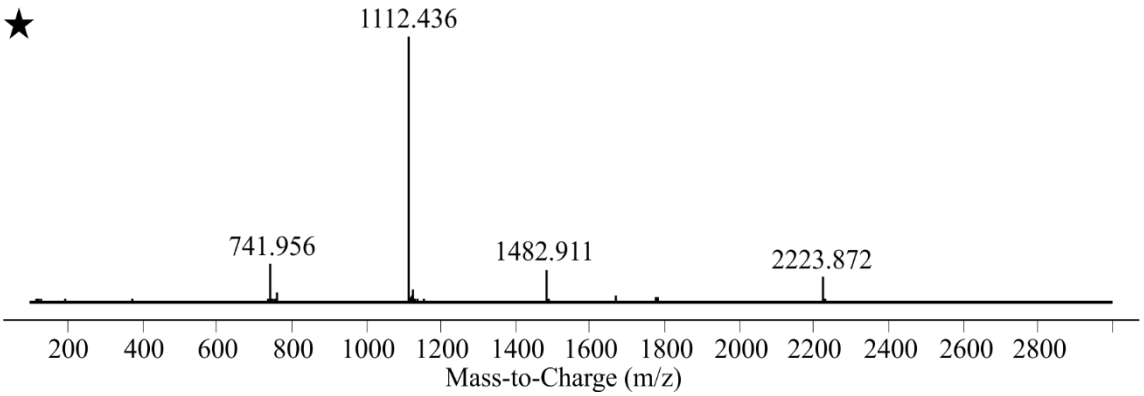


(**32b**) : Prepared according to the representative protocol **(E)** using peptide **32** (1 mM), electrophile **7** (2 mM), and DIEA (20 mM) at room temperature for 4 h. The diluted reaction mixture was analyzed using LC-MS *Method A*. TIC trace of crude reaction and Mass spectrum of product **32b**. Signals of the starting material **32** (yield = 2 %) and **7** are marked with a full black circle (●) and an empty black circle (○), respectively. Signal of the double-arylation product (yield = 4 %) is marked with a black square (■). Signal of the stapling product **32b** (yield = 89 %) is marked with a star (★). Analytical data for **32b** : m/z calcd. for $C_{96}H_{137}Cl_2N_{35}O_{23}S$ $[M+2H]^{2+}$: 1125.99 found 1126.00.

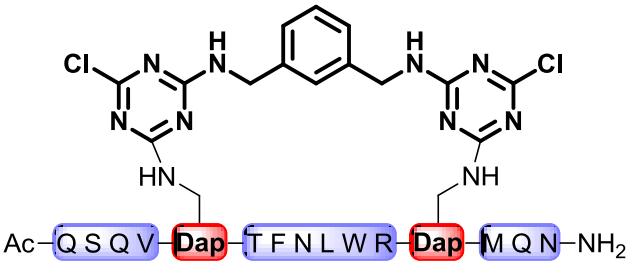


33a

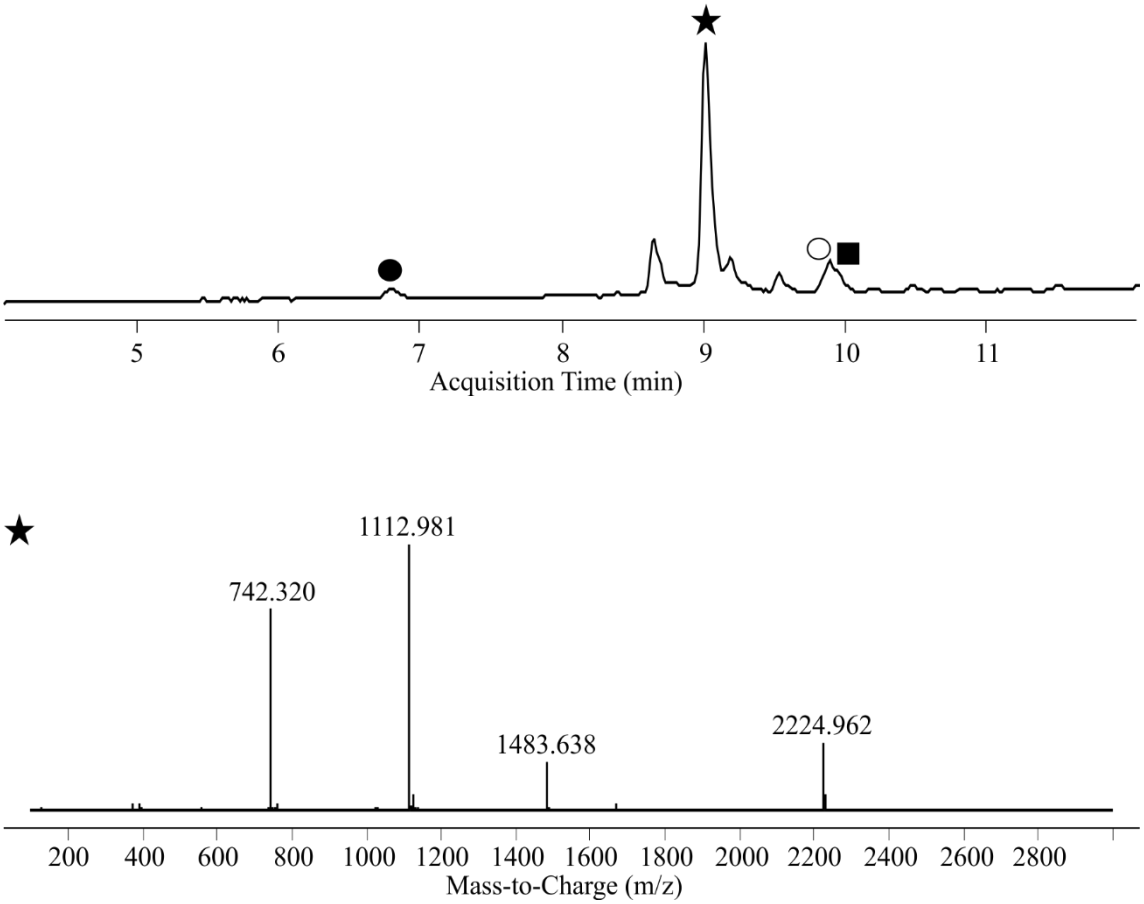




(**33a**) : Prepared according to the representative protocol **(C)** using peptide **33** (0.5 mM), electrophile **6** (0.625 mM), and DIEA (10 mM) at room temperature for 4 h. The diluted reaction mixture was analyzed using LC-MS *Method A*. TIC trace of crude reaction and Mass spectrum of product **33a**. Signal of the starting material **33** (yield = 7 %) is marked with a black circle (●). Signal of the double-arylation product (yield = 15 %) is marked with a black square (■). Signal of the stapling product **33a** (yield = 72 %) is marked with a star (★). Analytical data for **33a** : m/z calcd. for $C_{92}H_{123}F_8N_{27}O_{25}S_2$ $[M+2H]^{2+}$: 1111.93 found 1111.93.



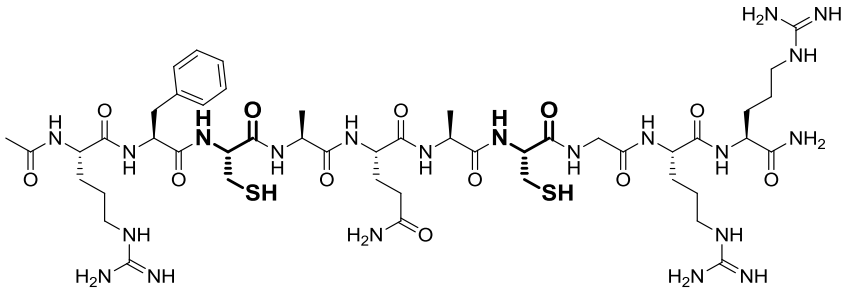
33b



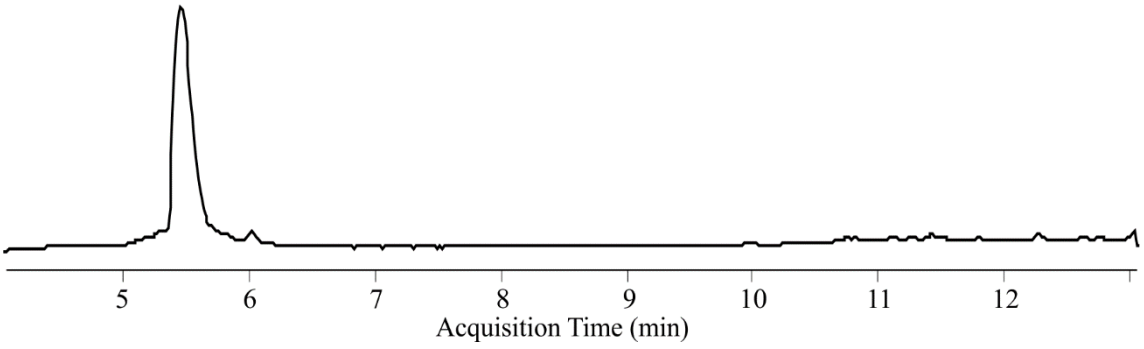
(**33b**) : Prepared according to the representative protocol **(E)** using peptide **33** (1 mM), eletrophile **7** (2 mM), and DIEA (20 mM) at room temperature for 4 h. The diluted reaction mixture was analyzed using LC-MS *Method A*. TIC trace of crude reaction and Mass spectrum of product **33b**. Signals of the starting material **33** (yield = 2 %) and **7** are marked with a full black circle (●) and an empty black circle (○), respectively. Signal of the double-arylation product (yield = 5 %) is marked with a black square (■). Signal of the stapling product **33b** (yield = 84 %) is marked with a star (★). Analytical data for **33b** : m/z calcd. for $C_{94}H_{133}Cl_2N_{35}O_{23}S$ $[M+2H]^{2+}$: 1111.98 found 1111.98.

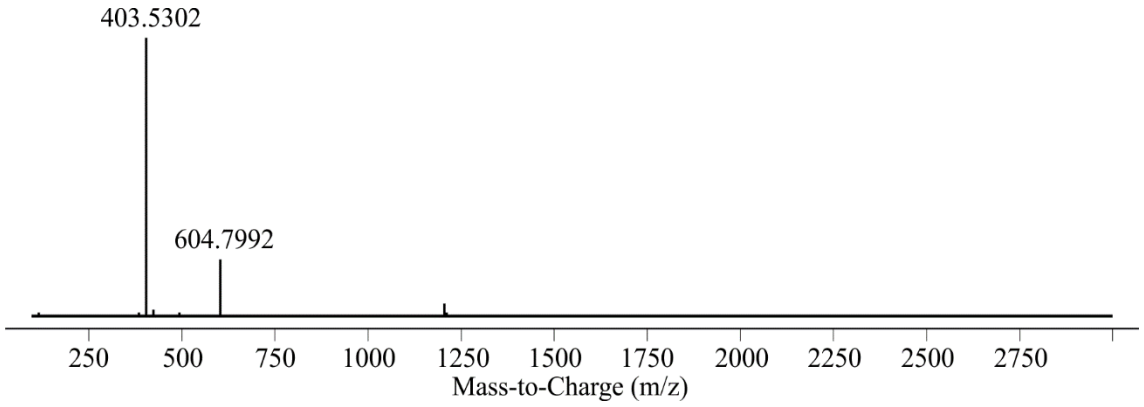
9. Chemical stability assay

LC-MS analytical data of purified peptides **34**, **35**, **36** *and* **37**

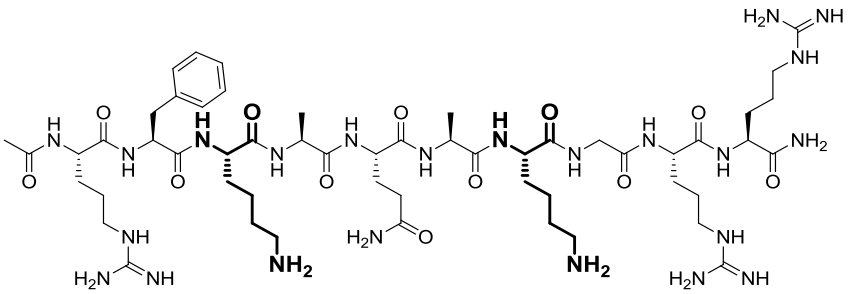


34: AcNH-Arg-Phe-**Cys**-Ala-Asn-Ala-**Cys**-Gly-Arg-Arg-$CONH_2$

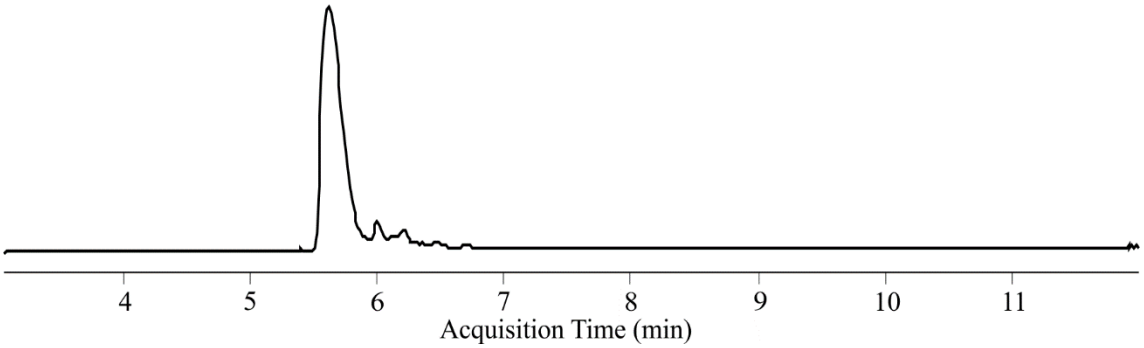


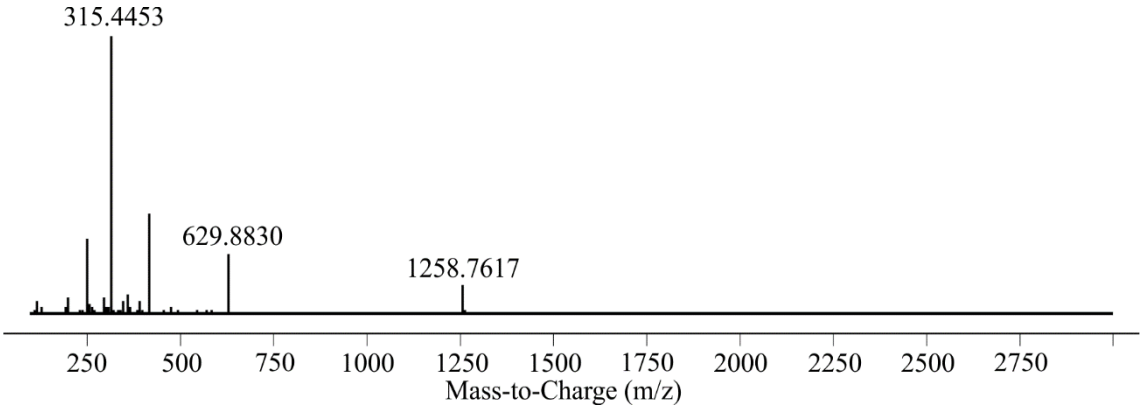


Peptide **34**: LC-MS analysis *Method A*. TIC trace and Mass spectrum of peptide **34**. m/z calcd. for $C_{48}H_{81}N_{21}O_{12}S_2$ $[M+2H]^{2+}$: 604.80 found 604.80.

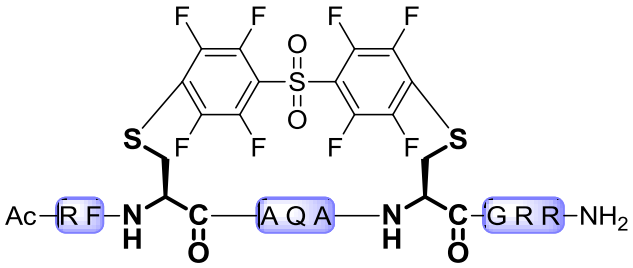


35: AcNH-Arg-Phe-**Lys**-Ala-Asn-Ala-**Lys**-Gly-Arg-Arg-$CONH_2$

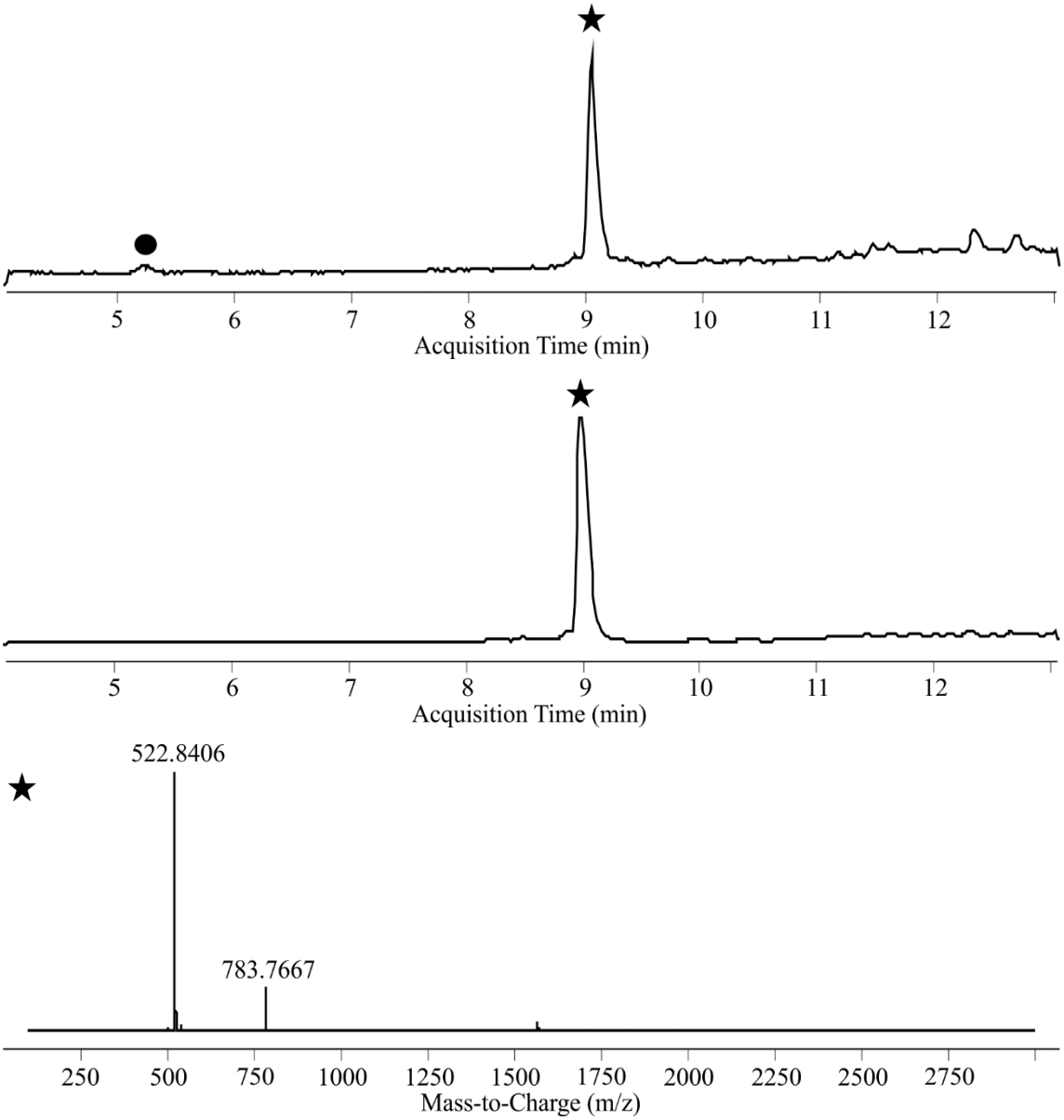




Peptide **35**: LC-MS analysis *Method C*. TIC trace and Mass spectrum of peptide **35**. m/z calcd. for $C_{54}H_{95}N_{23}O_{12}$ $[M+H]^+$: 1258.76 found 1258.76.

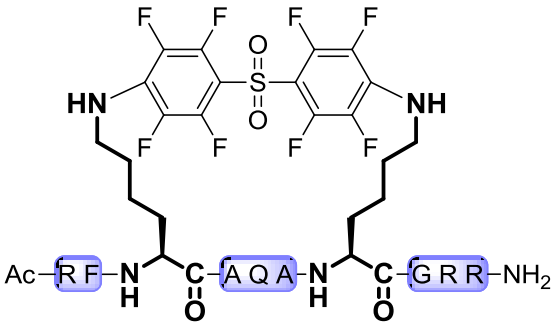


36

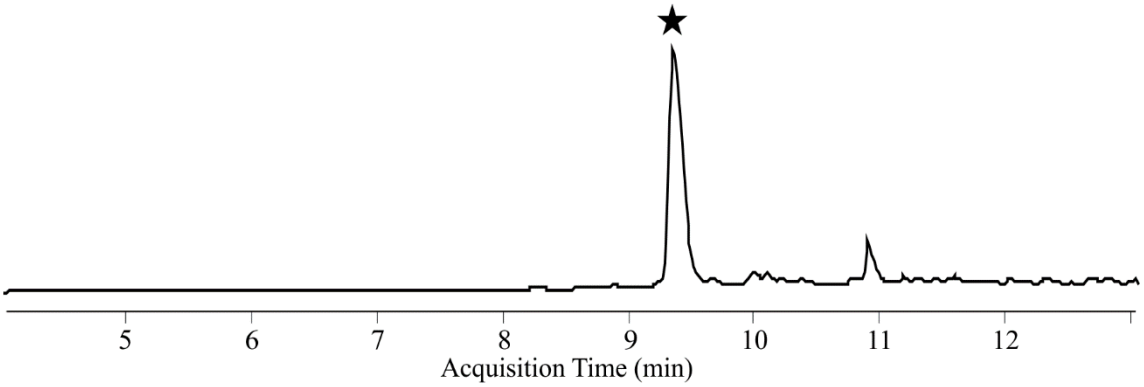


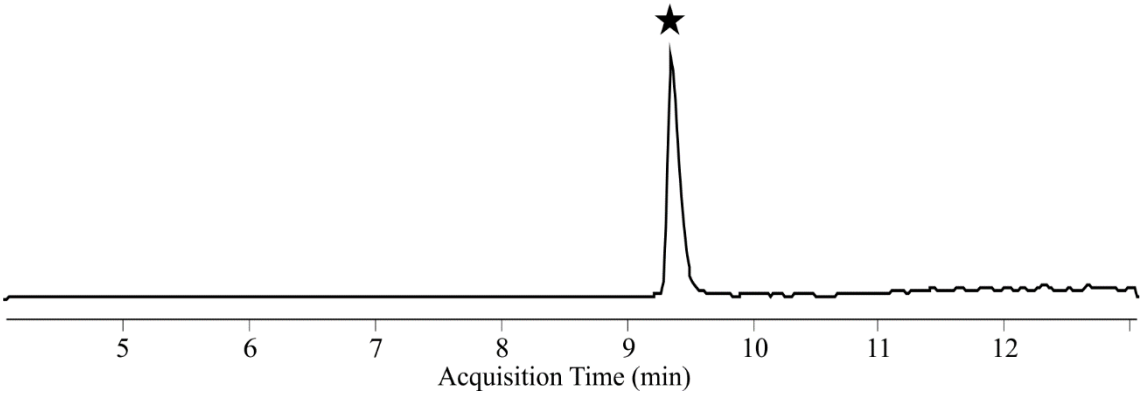
Peptide **36**: Prepared according to the representative protocol **(C)** using peptide **34** (0.5 mM), electrophile **6** (0.625 mM), and DIEA (10 mM) at room temperature for 6 h. The diluted reaction mixture was analyzed using LC-MS *Method A*. TIC trace of crude reaction, pure isolated peptide **36** and Mass spectrum of product **36**. Signals of the starting material **34** and stapling product **36** are marked with a full black circle (●) and a star (★), respectively. Analytical data for **36** : m/z calcd. for

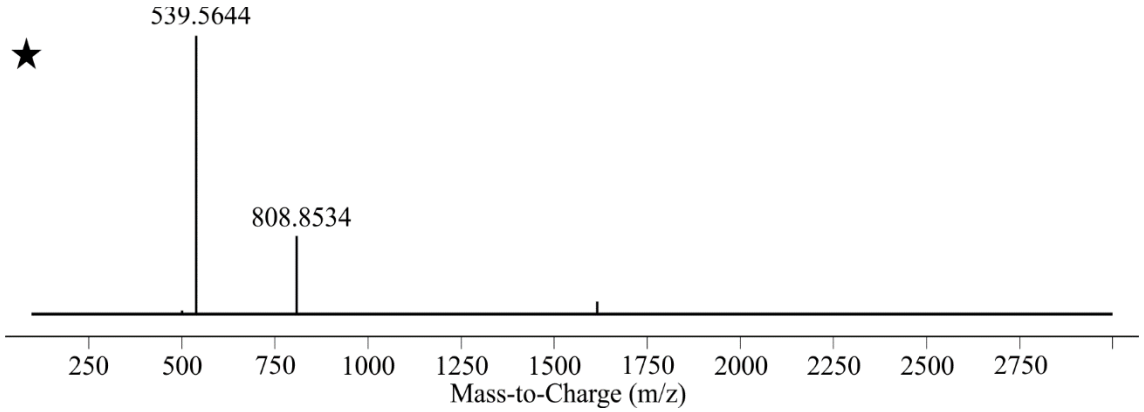
$C_{60}H_{79}F_8N_{21}O_{14}S_3$ $[M+2H]^{2+}$: 783.77 found 783.77. **36** was obtained as a white powder (6.8 mg, 53 %) after concentration, HPLC purification and lyophilization.



37







Peptide **37**: Prepared according to the representative protocol **(C)** using peptide **35** (0.5 mM), electrophile **6** (0.625 mM), and DIEA (10 mM) at room temperature for 6 h. The diluted reaction

mixture was analyzed using LC-MS *Method A*. TIC trace of crude reaction, pure isolated peptide **37** and Mass spectrum of product **37**. Signal of the stapling product **37** is marked with a star (★). Analytical data for **37** : m/z calcd. for $C_{66}H_{93}F_8N_{23}O_{14}S$ $[M+2H]^{2+}$: 808.85 found 808.85. **37** was obtained as a white powder (7.2 mg, 56 %) after concentration, HPLC purification and lyophilization.

Stability under oxidative conditions

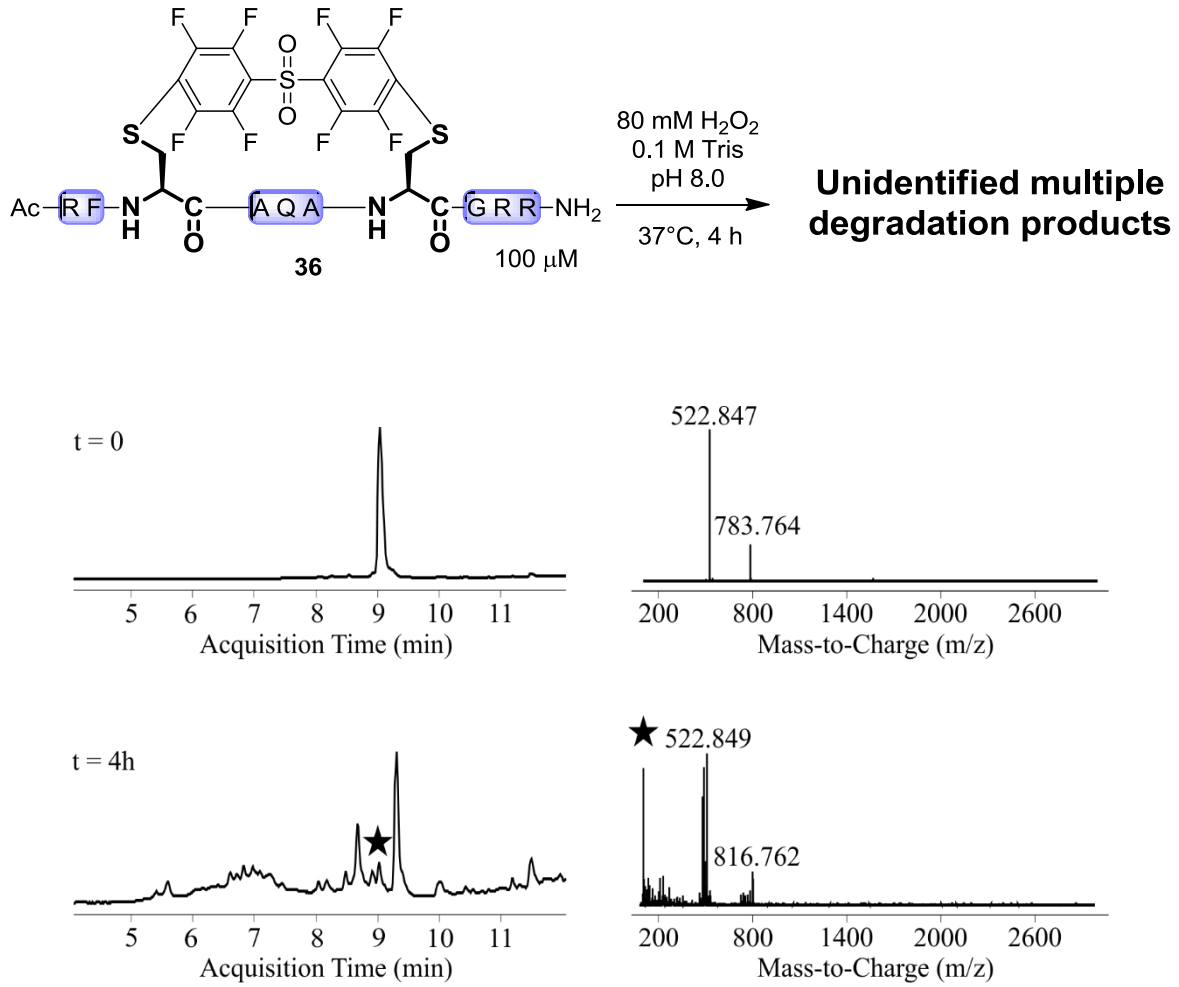


Figure S5. A 0.6 mL tube was charged with 160 µL H_2O_2 (100 mM stock solution in water), 20 µL of 1.0 M Tris Buffer (pH 8.0), 20 µL of peptide (1 mM stock solution). The resulting reaction mixture was capped and incubated in 37 °C water bath for 4 hours. 5 µL of the crude reaction was quenched by addition of 195 µL of 50% water: 50% acetonitrile and was subjected to LC-MS analysis *Method A*. TIC trace of reaction at time 0 (starting material peptide **36**) and time 4 hours. Signal of starting material peptide **36** is marked with a star (★). Unidentified multiple degradation products was observed for the cysteine based stapled peptide.

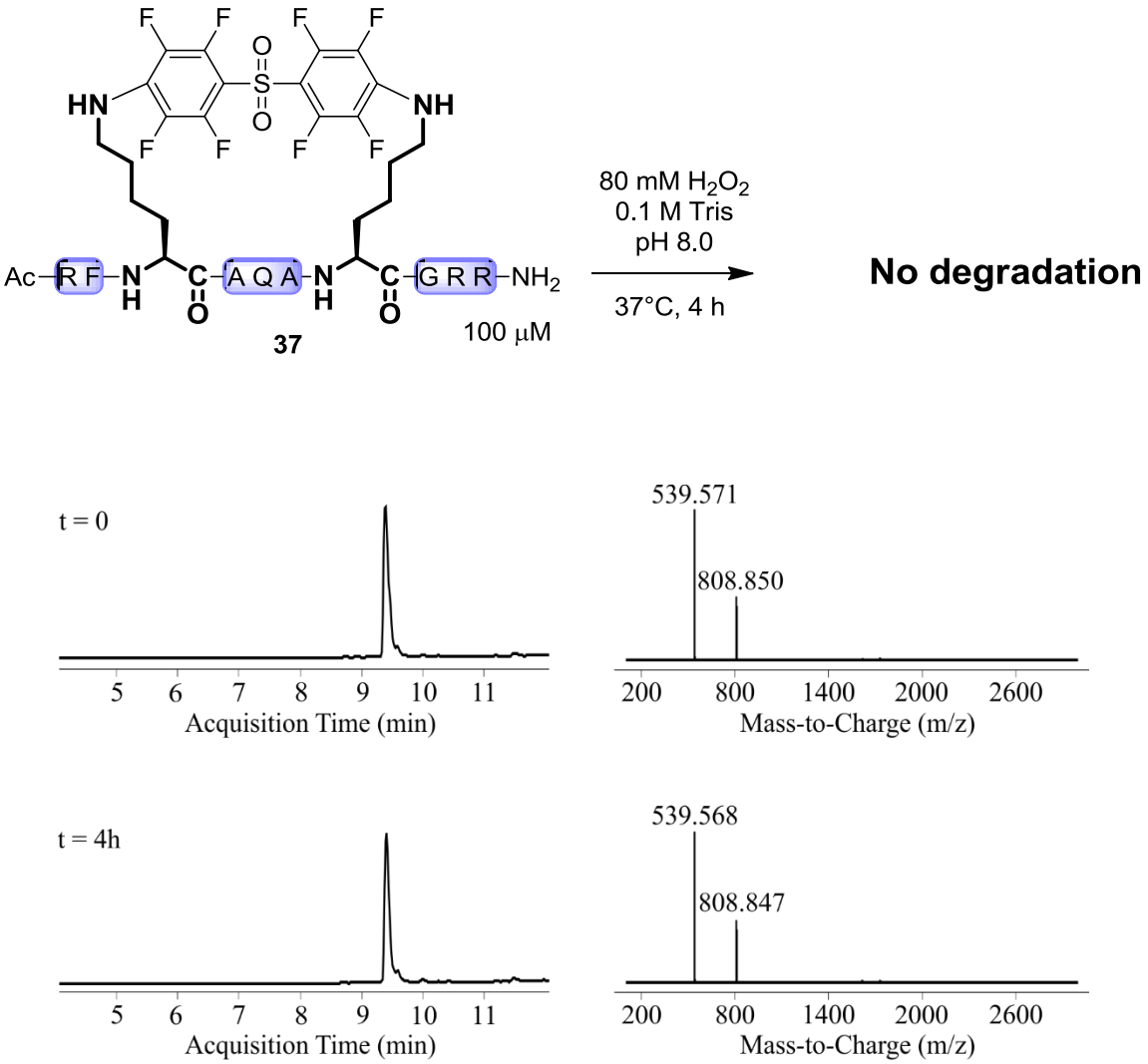


Figure S6. A 0.6 mL tube was charged with 160 µL H_2O_2 (100 mM stock solution in water), 20 µL of 1.0 M Tris Buffer (pH 8.0), 20 µL of peptide (1 mM stock solution). The resulting reaction mixture was capped and incubated in 37 °C water bath for 4 hours. 5 µL of the crude reaction was quenched by addition of 195 µL of 50% water: 50% acetonitrile and was subjected to LC-MS analysis *Method A*. TIC trace of crude reaction at time 0 and time 4 hours. Mass spectrum of starting material peptide **37**. No degradation was observed for the lysine based stapled peptide.

Stability in pH = 10.0 buffer

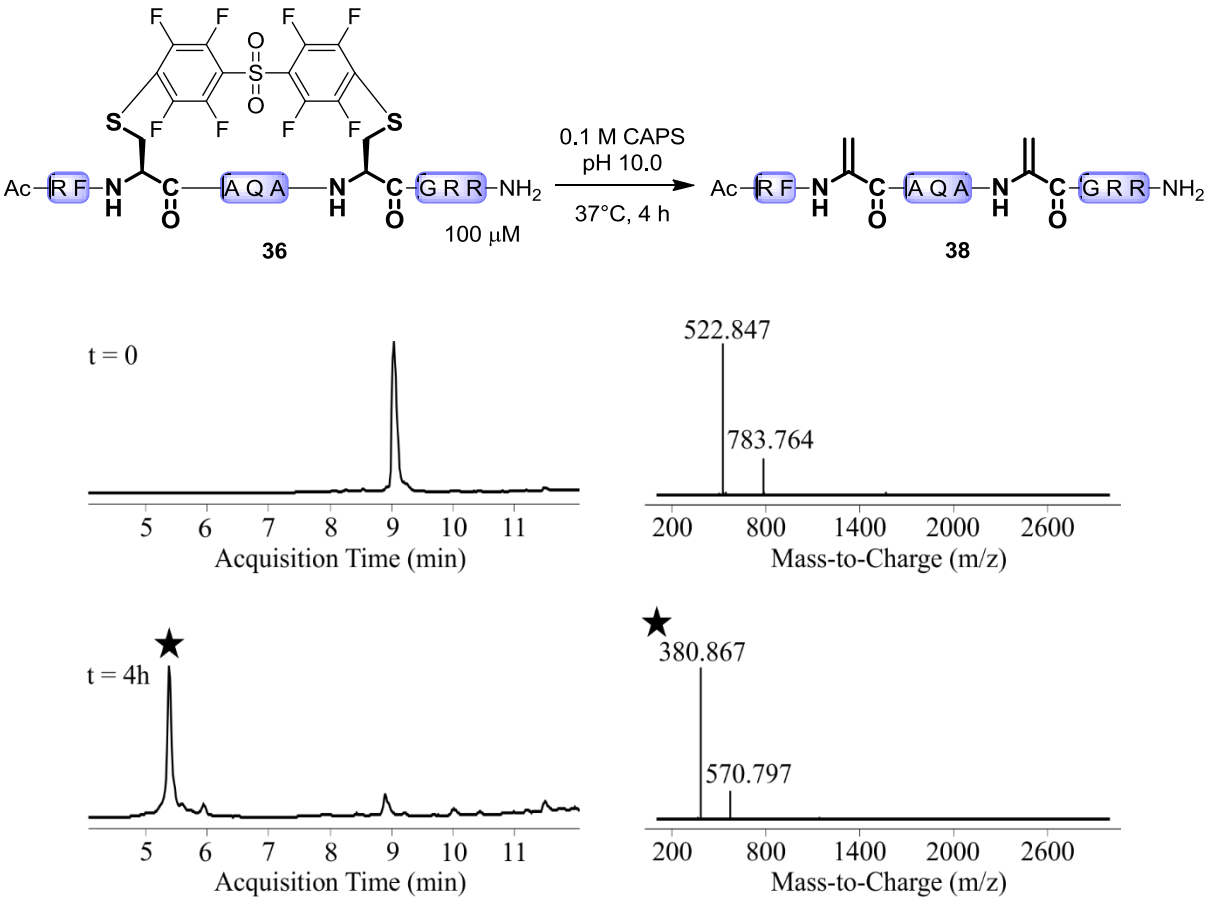


Figure S7. A 0.6 mL tube was charged with 160 µL of deionized H_2O, 20 µL of 1.0 M CAPS Buffer (pH 10.0), 20 µL of peptide (1 mM stock solution). The resulting reaction mixture was capped and incubated in 37 °C water bath for 4 hours. 5 µL of the crude reaction was quenched by addition of 195 µL of 50% water: 50% acetonitrile and was subjected to LC-MS analysis *Method A*. TIC trace of reaction at time 0 (starting material peptide **36**) and time 4 hours. Signal double dehydroalanine product **38** is marked with a star (★). Analytical data for **38** : m/z calcd. for $C_{48}H_{77}N_{21}O_{12}$ $[M+2H]^{2+}$: 570.81 found 570.80.

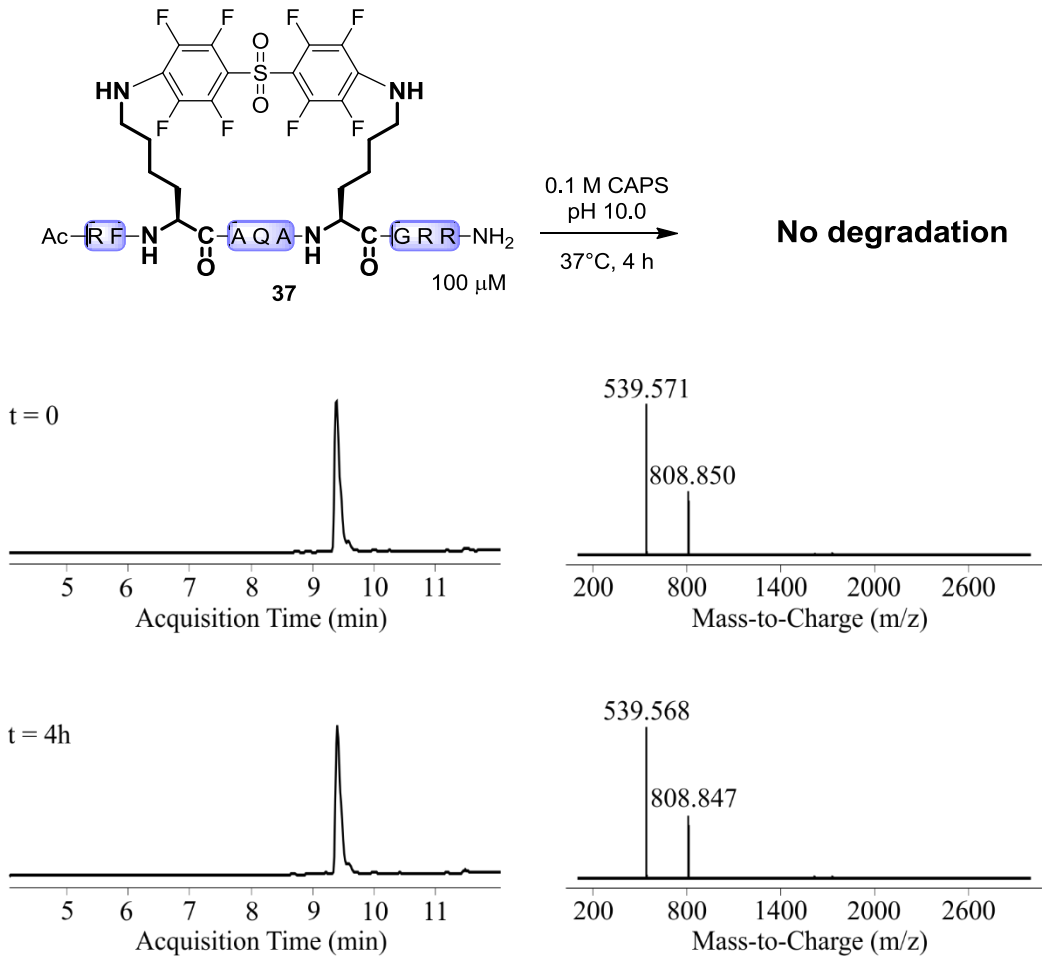
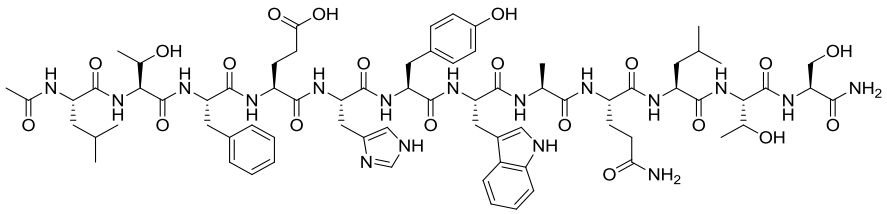


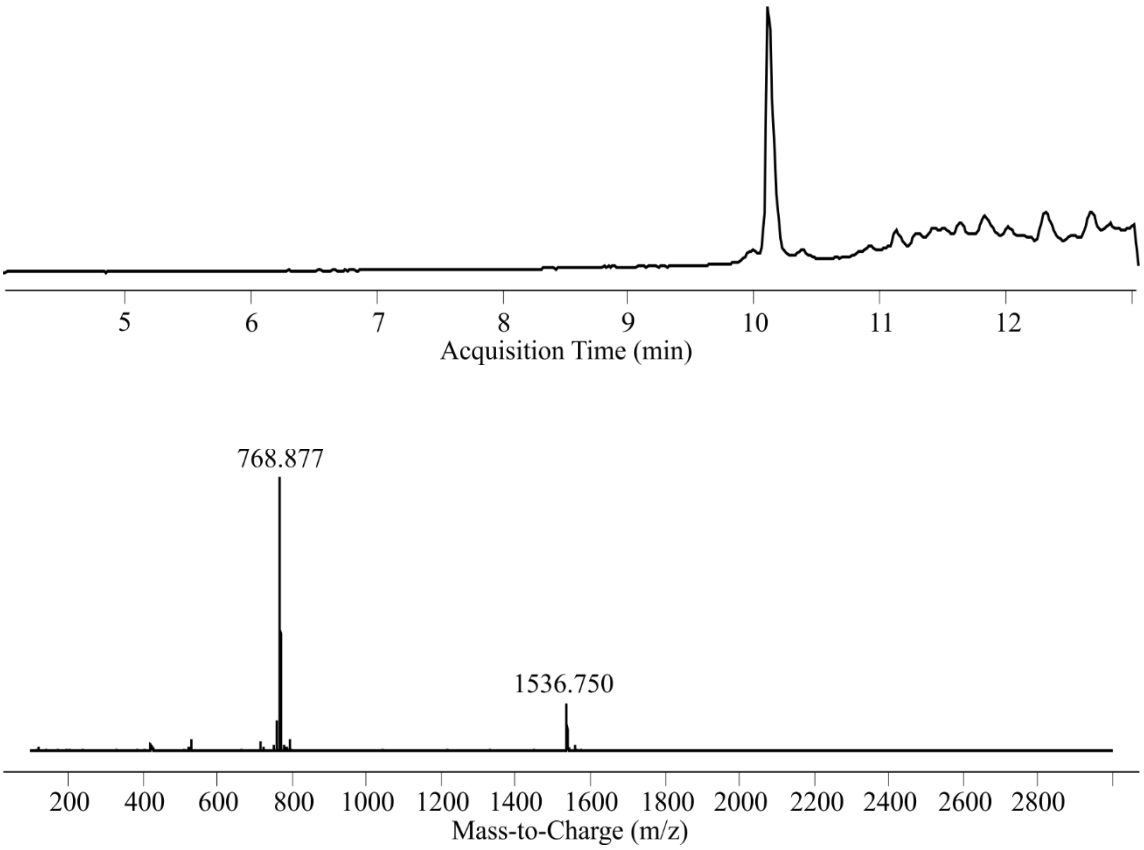
Figure S8. A 0.6 mL tube was charged with 160 µL of deionized H_2O, 20 µL of 1.0 M CAPS Buffer (pH 10.0), 20 µL of peptide (1 mM stock solution). The resulting reaction mixture was capped and incubated in 37 °C water bath for 4 hours. 5 µL of the crude reaction was quenched by addition of 195 µL of 50% water: 50% acetonitrile and was subjected to LC-MS analysis *Method A*. TIC trace of crude reaction at time 0 and time 4 hours. Mass spectra of starting material peptide **37**. No degradation was observed for the lysine based stapled peptide.

10. Proteolysis assay

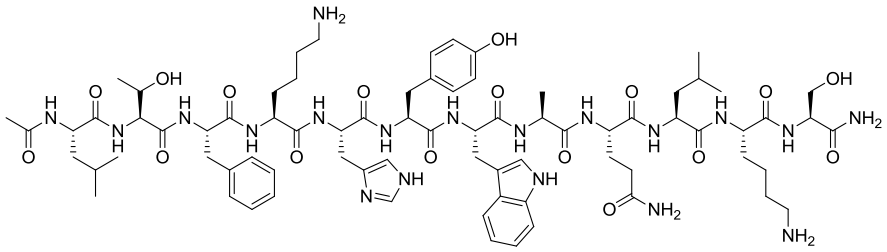
LC-MS analytical data of purified peptides **12a**, **13a** *and* **14a**



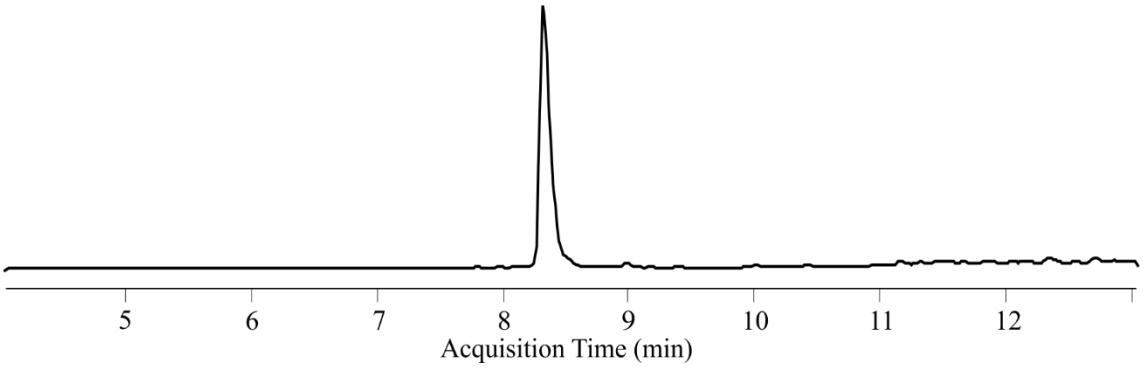
12a: Ac-Leu-Thr-Phe-Glu-His-Tyr-Trp-Ala-Gln-Leu-Thr-Ser-$CONH_2$

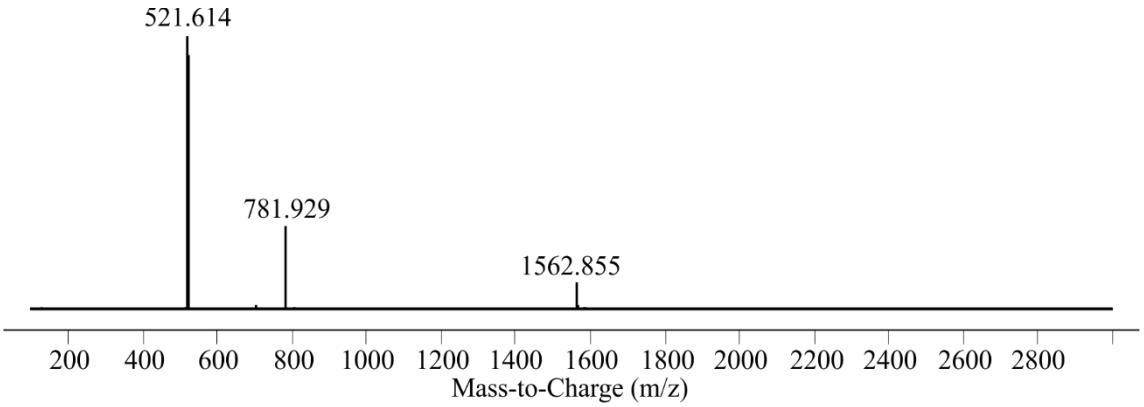


Peptide **12a**: LC-MS analysis *Method A*. TIC trace and Mass spectrum of peptide **12a**. m/z calcd. for $C_{73}H_{101}N_{17}O_{20}$ $[M+H]^+$: 1536.75 found 1536.75.

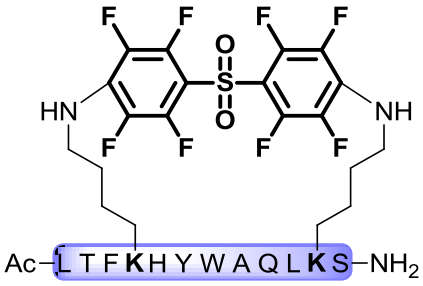


13a: Ac-Leu-Thr-Phe-Lys-His-Tyr-Trp-Ala-Gln-Leu-Lys-Ser-$CONH_2$

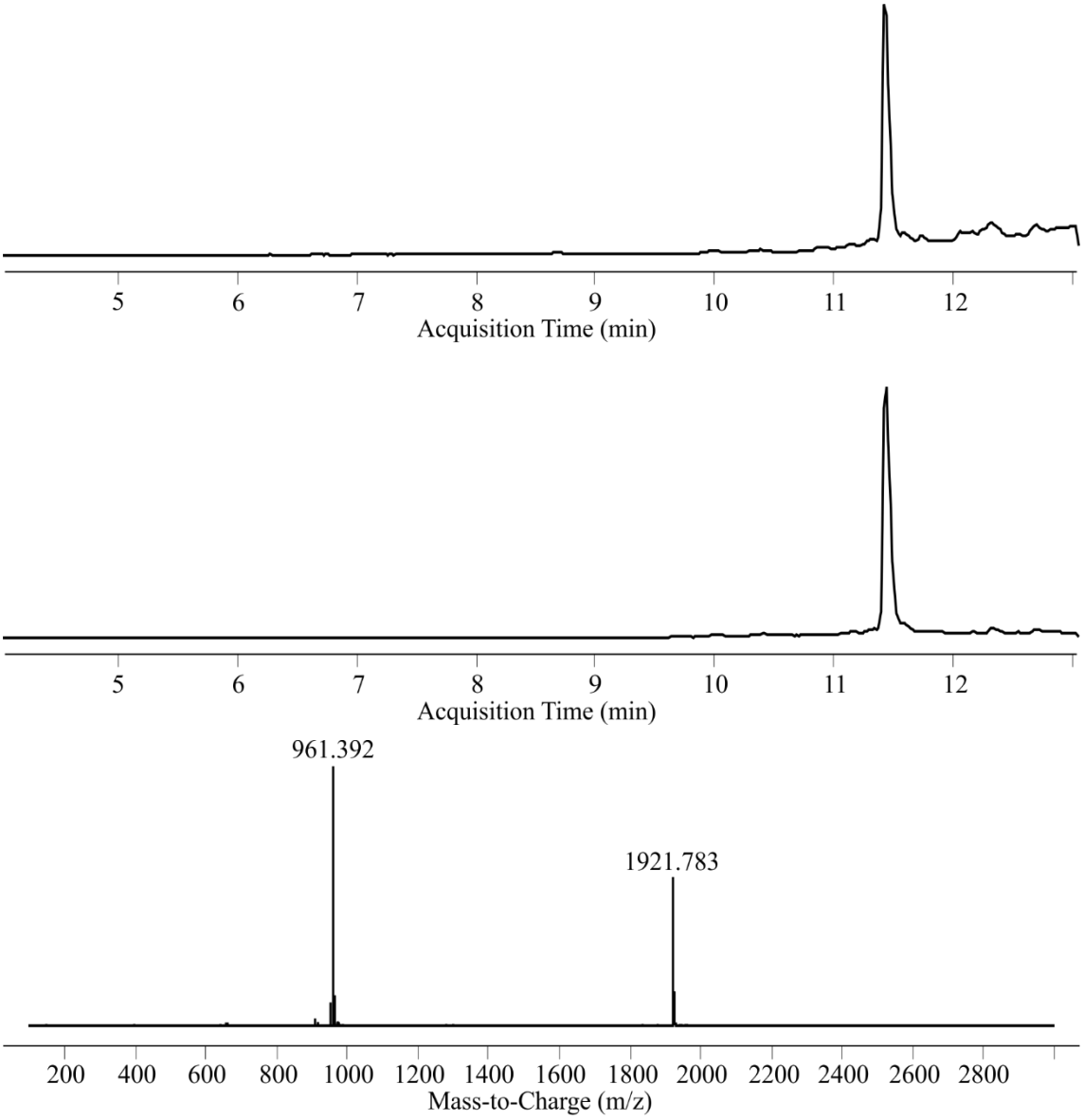




Peptide **13a**: LC-MS analysis *Method A*. TIC trace and Mass spectrum of peptide **13a**. m/z calcd. for $C_{76}H_{111}N_{19}O_{17}$ $[M+H]^+$: 1562.85 found 1562.85.



14a



Peptide **14a**: Prepared according to the representative protocol **(C)** using peptide **13a** (0.5 mM), electrophile **6** (0.625 mM), and DIEA (10 mM) at room temperature for 6 h. The diluted reaction mixture was analyzed using LC-MS *Method A*. TIC trace of crude reaction, pure isolated peptide **14a** and Mass spectrum of product **14a**. Analytical data for **14a** : m/z calcd. for $C_{88}H_{109}F_8N_{19}O_{19}S$ $[M+2H]^{2+}$: 960.89 found 960.89.

Stability under chymotrypsin incubation with peptide **12a** *and* **14a**

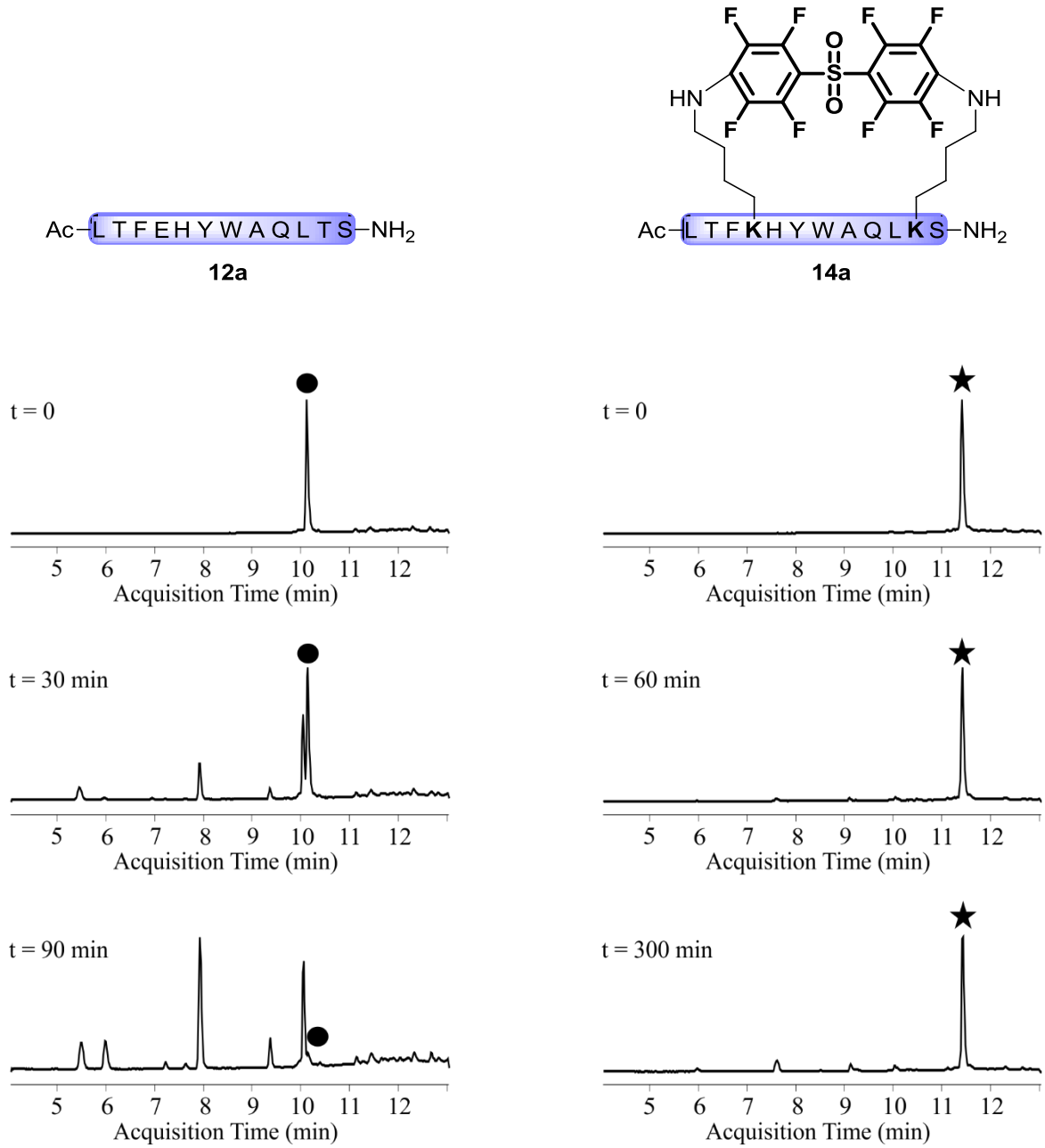


Figure S9. A 0.6 mL tube was charged with 196 µL of phosphate buffer pH 7.4, 2 µL of Chymotrypsin (0.05 mg/mL stock solution in phosphate buffer pH 7.4), 2 µL of peptide (10 mM stock solution). The resulting reaction mixture was capped and incubated at room temperature for 5 hours. 5 µL of the crude reaction was quenched by addition of 195 µL of 50% water: 50% acetonitrile and was subjected to LC-MS analysis *Method A*. TIC trace of crude reaction at time 0, 30 min and 90 min is shown for unstapled peptide **12a** (full back circle ●) whereas TIC trace of crude reaction at time 0, 60 min and 300 min is shown for stapled peptide **14a** (black star ★).

Stability under Proteinase K incubation with peptide **12a** *and* **14a**

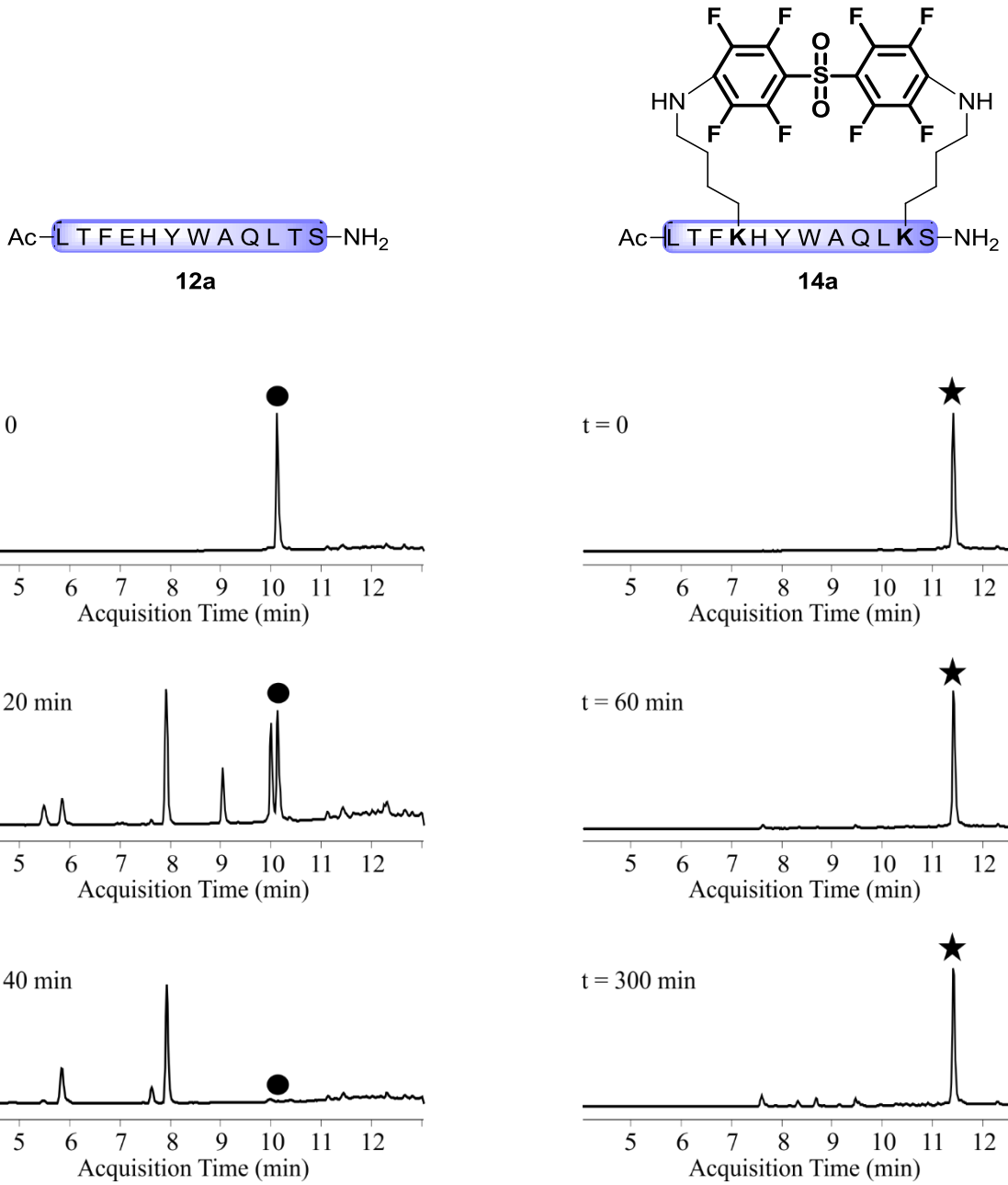
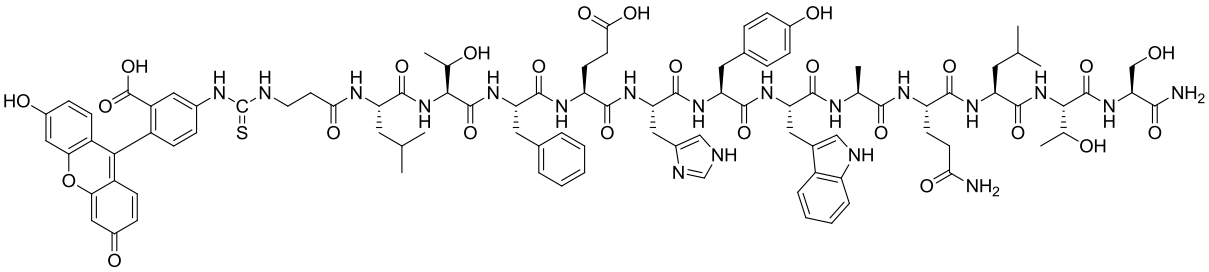


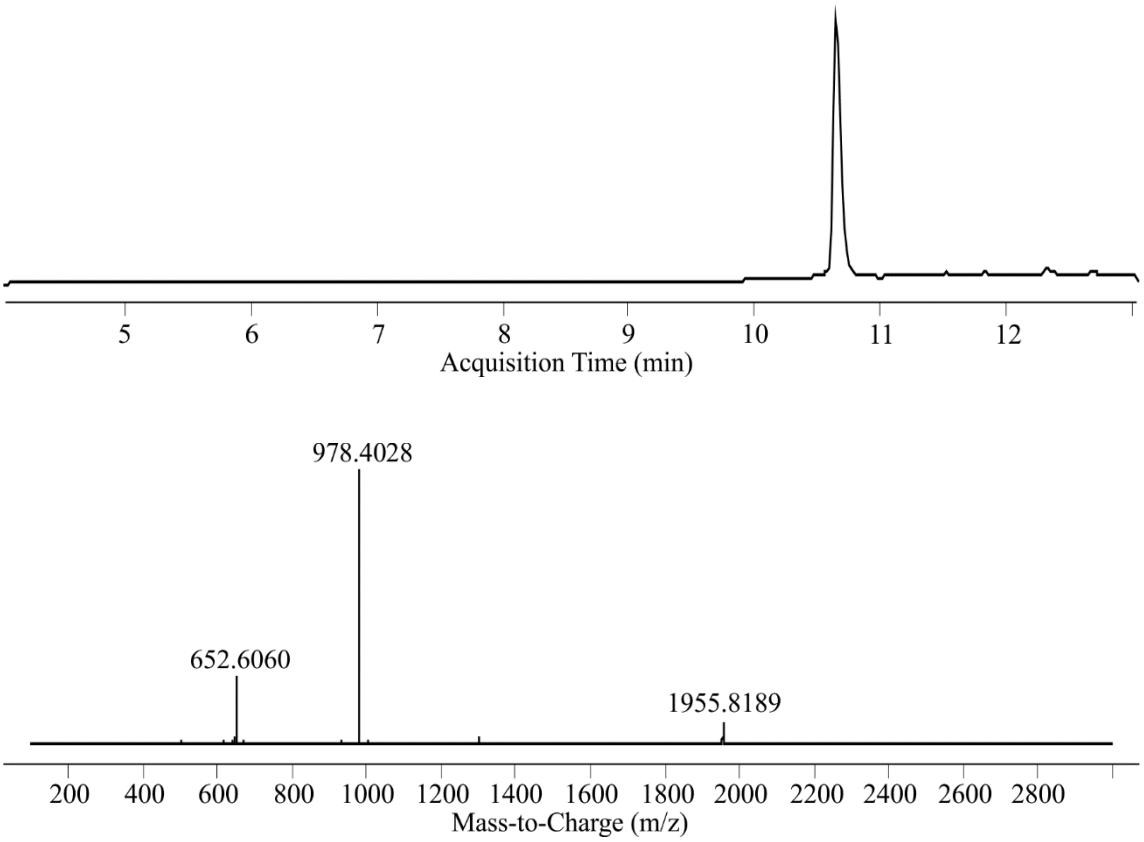
Figure S10. A 0.6 mL tube was charged with 196 µL of phosphate buffer pH 7.4, 2 µL of Proteinase K (0.05 mg/mL stock solution in phosphate buffer pH 7.4), 2 µL of peptide (10 mM stock solution). The resulting reaction mixture was capped and incubated at room temperature for 5 hours. 5 µL of the crude reaction was quenched by addition of 195 µL of 50% water: 50% acetonitrile and was subjected to LC-MS analysis *Method A*. TIC trace of crude reaction at time 0, 20 min and 40 min is shown for unstapled peptide **12a** (full back circle ●) whereas TIC trace of crude reaction at time 0, 60 min and 300 min is shown for stapled peptide **14a** (black star ★).

11. Cell imaging and Flow cytometry assays

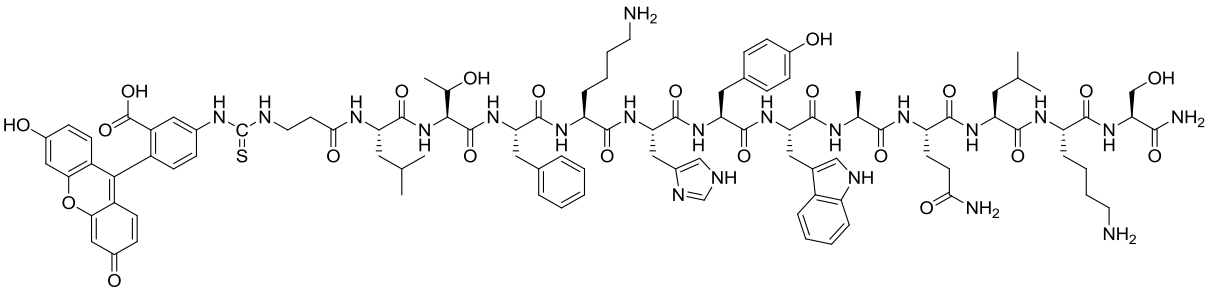
LC-MS analytical data of purified peptides **12b**, **13b**, **14b** *and* **15**



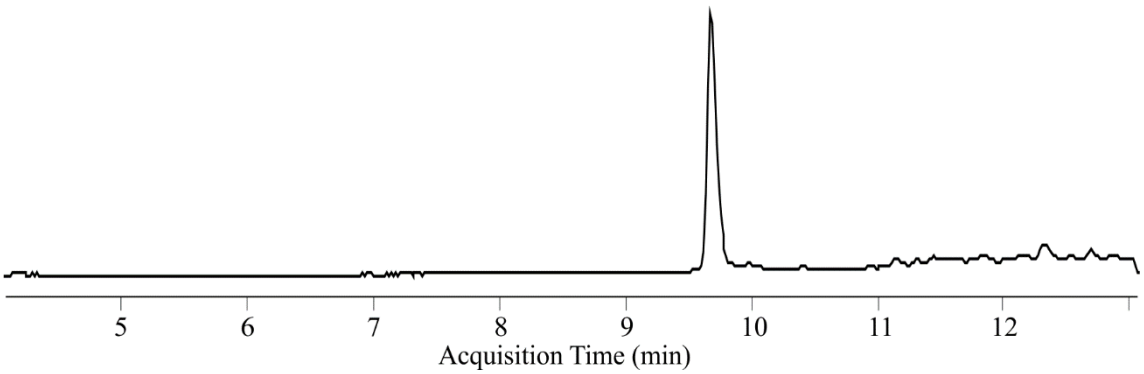
12b: FITC-β-Ala-Leu-Thr-Phe-Glu-His-Tyr-Trp-Ala-Gln-Leu-Thr-Ser-$CONH_2$

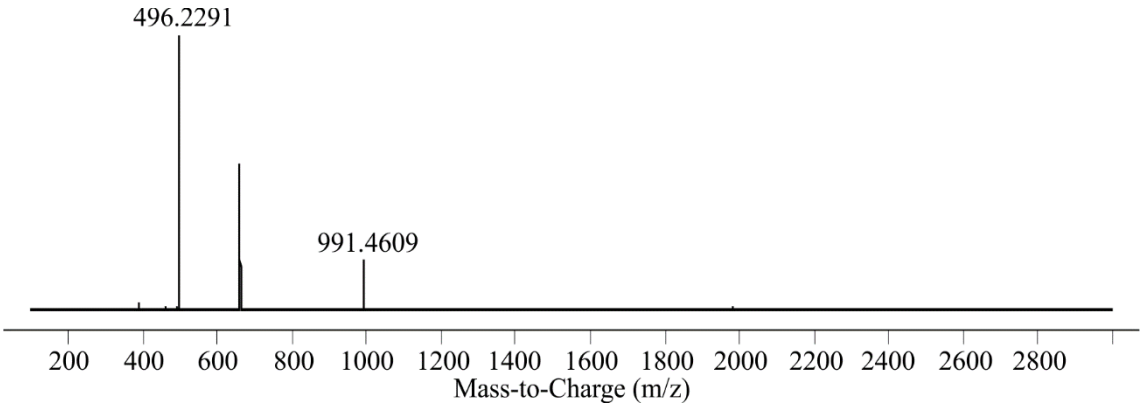


Peptide **12b**: LC-MS analysis *Method A*. TIC trace and Mass spectrum of peptide **12b** prepared according to the representative protocol **(H)**. m/z calcd. for $C_{95}H_{115}N_{19}O_{25}S$ $[M+2H]^{2+}$: 977.91 found 977.90.

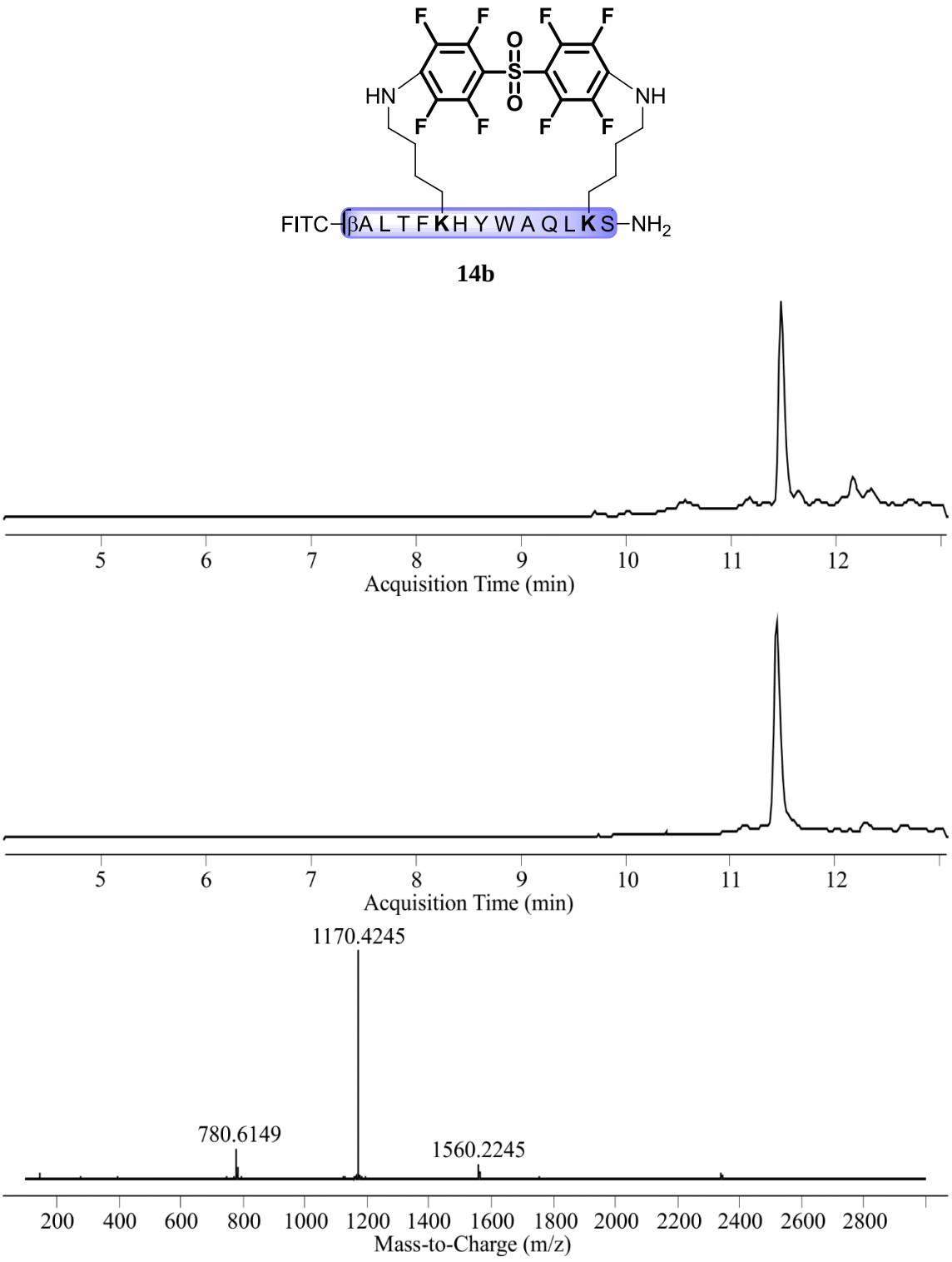


13b: FITC-β-Ala-Leu-Thr-Phe-Lys-His-Tyr-Trp-Ala-Gln-Leu-Lys-Ser-$CONH_2$



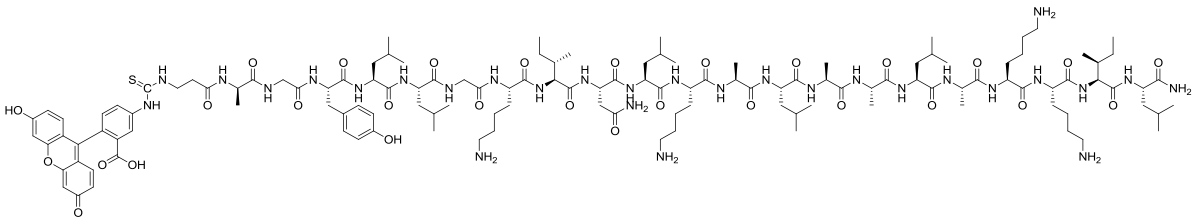


Peptide **13b**: LC-MS analysis *Method A*. TIC trace and Mass spectrum of peptide **13b** prepared according to the representative protocol **(H)**. m/z calcd. for $C_{98}H_{125}N_{21}O_{22}S$ $[M+2H]^{2+}$: 990.96 found 990.96.

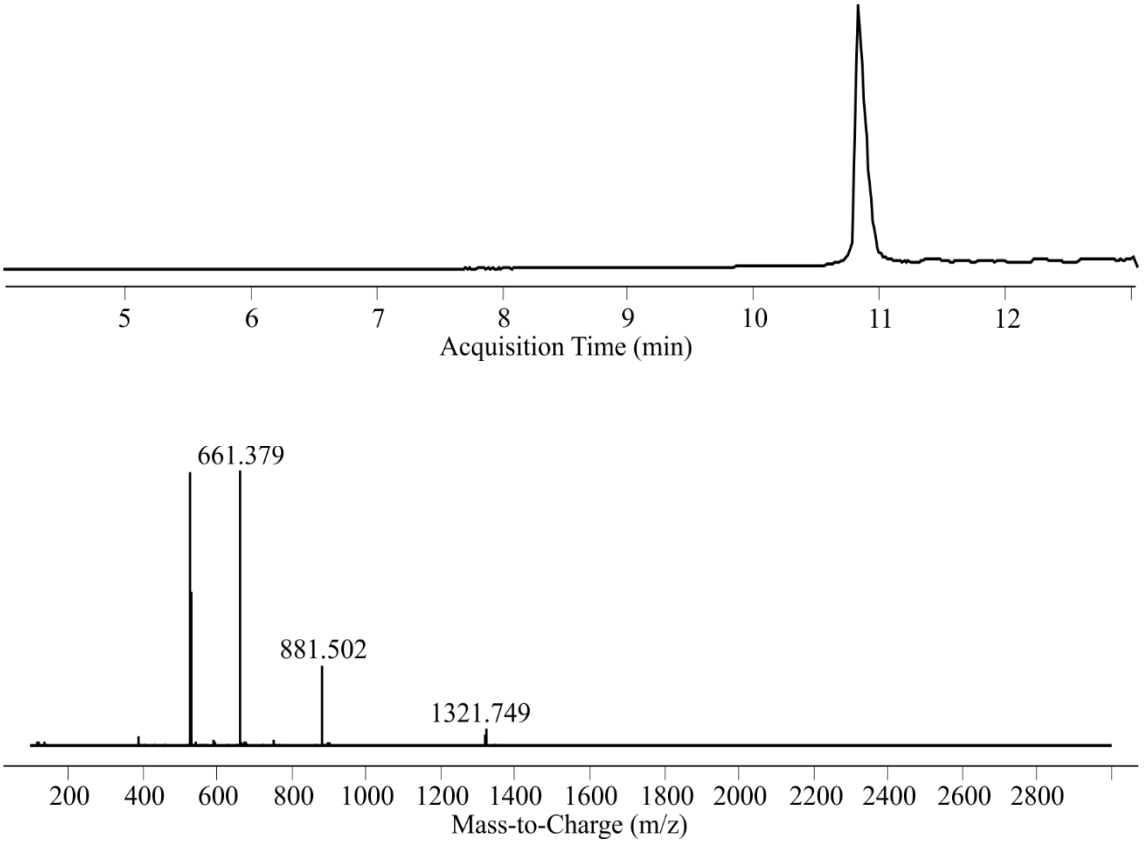


14b

Peptide **14b**: Prepared according to the representative protocol **(C)** using peptide **13b** (0.5 mM), electrophile **6** (0.625 mM), and DIEA (10 mM) at room temperature for 6 h. The diluted reaction mixture was analyzed using LC-MS *Method A*. TIC trace of crude reaction, pure isolated peptide **14b** and Mass spectrum of product **14b**. Analytical data for **14b** : m/z calcd. for $C_{110}H_{123}F_8N_{21}O_{24}S_2$ $[M+2H]^{2+}$: 1169.93 found 1169.92. **14b** was obtained as a white powder (4.9 mg, 42 %) after concentration, HPLC purification and lyophilization.



15: FITC-β-Ala-Ala-Gly-Tyr-Leu-Leu-Gly-Lys-Ile-Asn-Leu-Lys-Ala-Leu-Ala-Ala-Leu-Ala-Lys-Lys-Ile-Leu



Peptide **15**: LC-MS analysis *Method A*. TIC trace and Mass spectrum of peptide **15** prepared according to the representative protocol **(H)**. m/z calcd. for $C_{128}H_{201}N_{29}O_{29}S$ $[M+3H]^{3+}$: 881.17 found 881.17.

Cell imaging

293T HEK cells were cultured in 24 well plates until they reached 80 % confluency. Appropriate amounts of peptides **12b**, **13b**, **14b**, and **15** dissolved in MEM (0.1 % DMSO) were added to the cells to final concentrations of 1 μM, 5 μM, 10 μM and 20 μM. The cells were incubated with the samples for 4 hours at 37°C and 5% CO2. After incubation, cells were washed 2 times with HBSS and a third time with PBS then fixed with 4% formaldehyde (Alfa Aesar, MA) in DPBS for 10 minutes. They are then washed 2 times with PBS and stained with 5 μg/ml wheat germ agglutinin tetramethyl-647 conjugate (Thermo Fisher Scientific, CA) in PBS for 20 minutes. Finally, they were washed 2 more times with PBS and the cover slips were transferred to microscope slides and imaged using scan confocal Leica DMRXE.

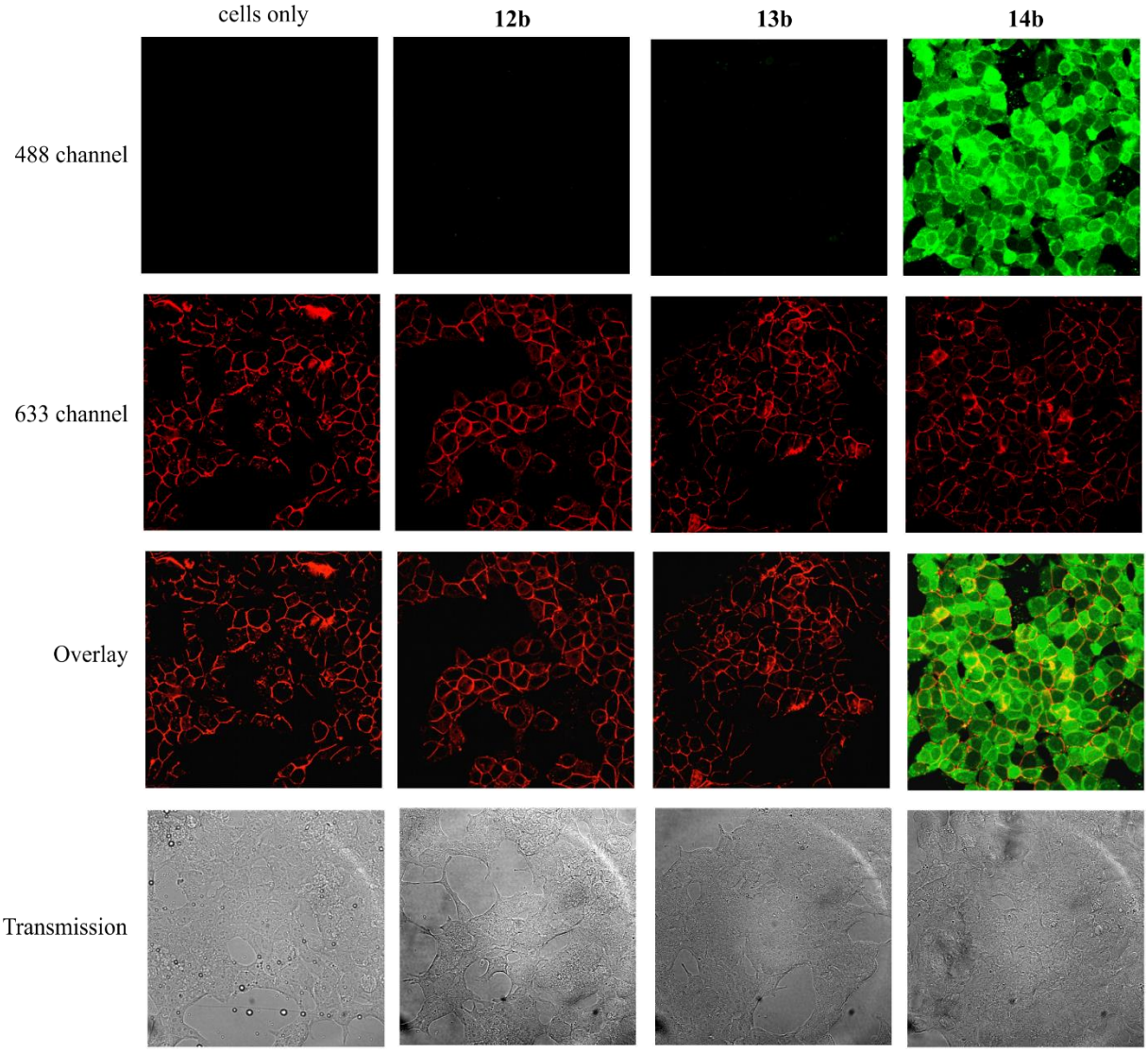


Figure S11. Confocal microscopy images of HEK293T cells treated with 1 μM FITC-labeled peptides. Red colour: membrane; green colour: FITC labeled peptides. Images were normalized using PMT = 550 V in channel 488 corresponding to black image for **12b** 1 μM condition.

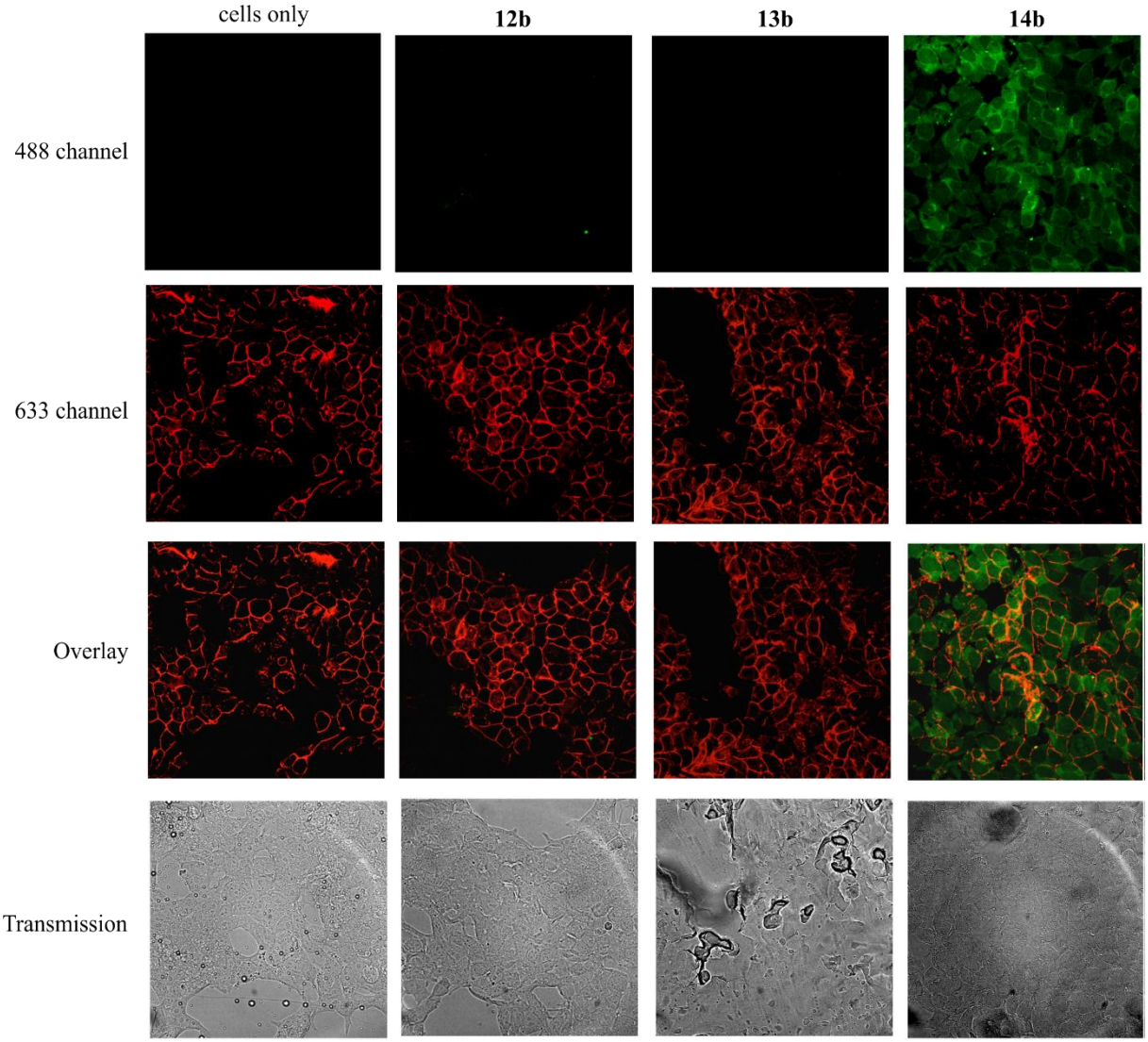


Figure S12. Confocal microscopy images of HEK293T cells treated with 1 μM FITC-labeled peptides. Red colour: membrane; green colour: FITC labeled peptides. Images were normalized using PMT = 470 V in channel 488 corresponding to black image for **12b** 10 μM condition.

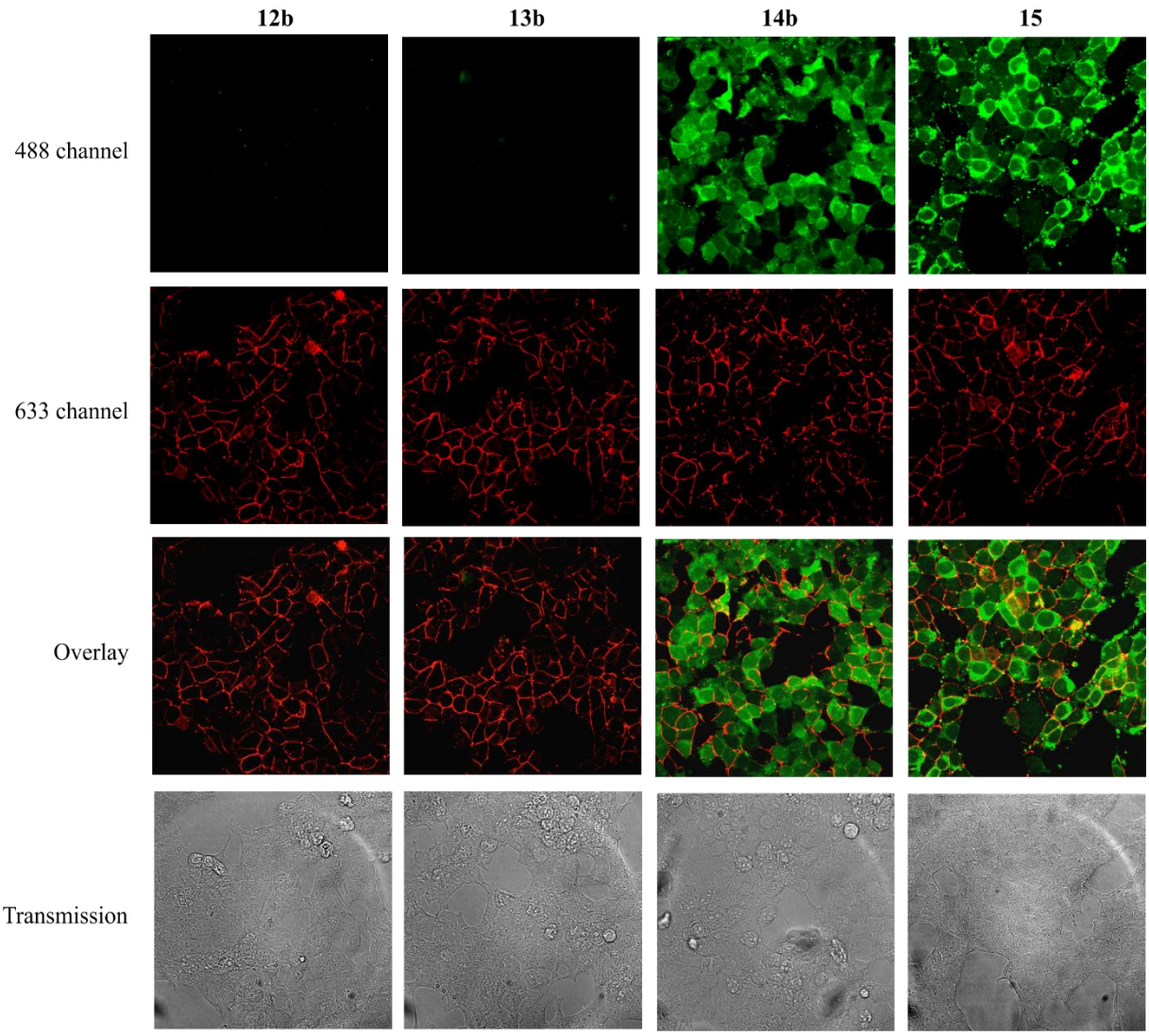


Figure S13. Confocal microscopy images of HEK293T cells treated with 5 μM FITC-labeled peptides. Red colour: membrane; green colour: FITC labeled peptides. Images were normalized using PMT = 470 V in channel 488 corresponding to black image for **12b** 10 μM condition.

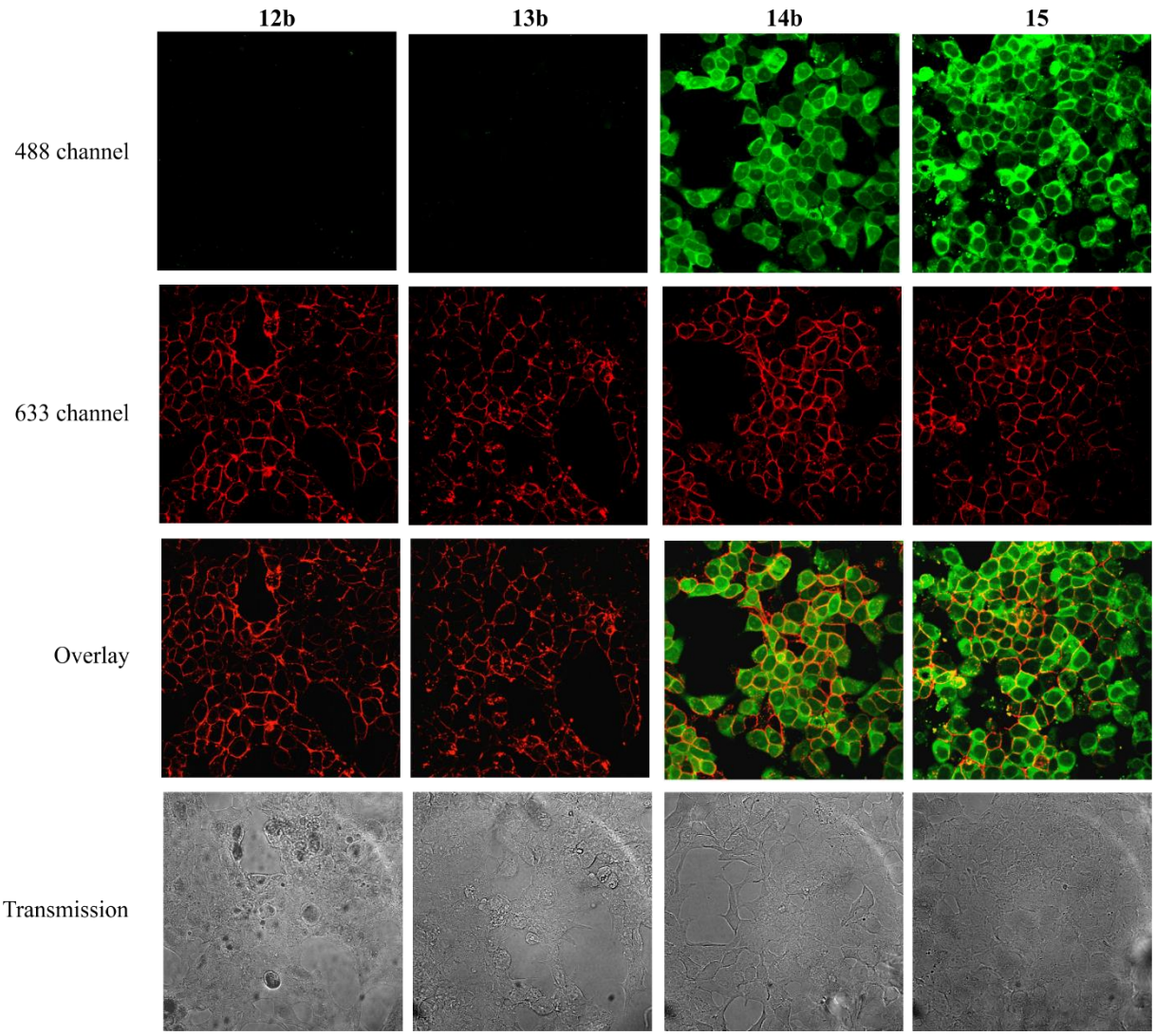


Figure S14. Confocal microscopy images of HEK293T cells treated with 10 μM FITC-labeled peptides. Red colour: membrane; green colour: FITC labeled peptides. Images were normalized using PMT = 470 V in channel 488 corresponding to black image for **12b** 10 μM condition.

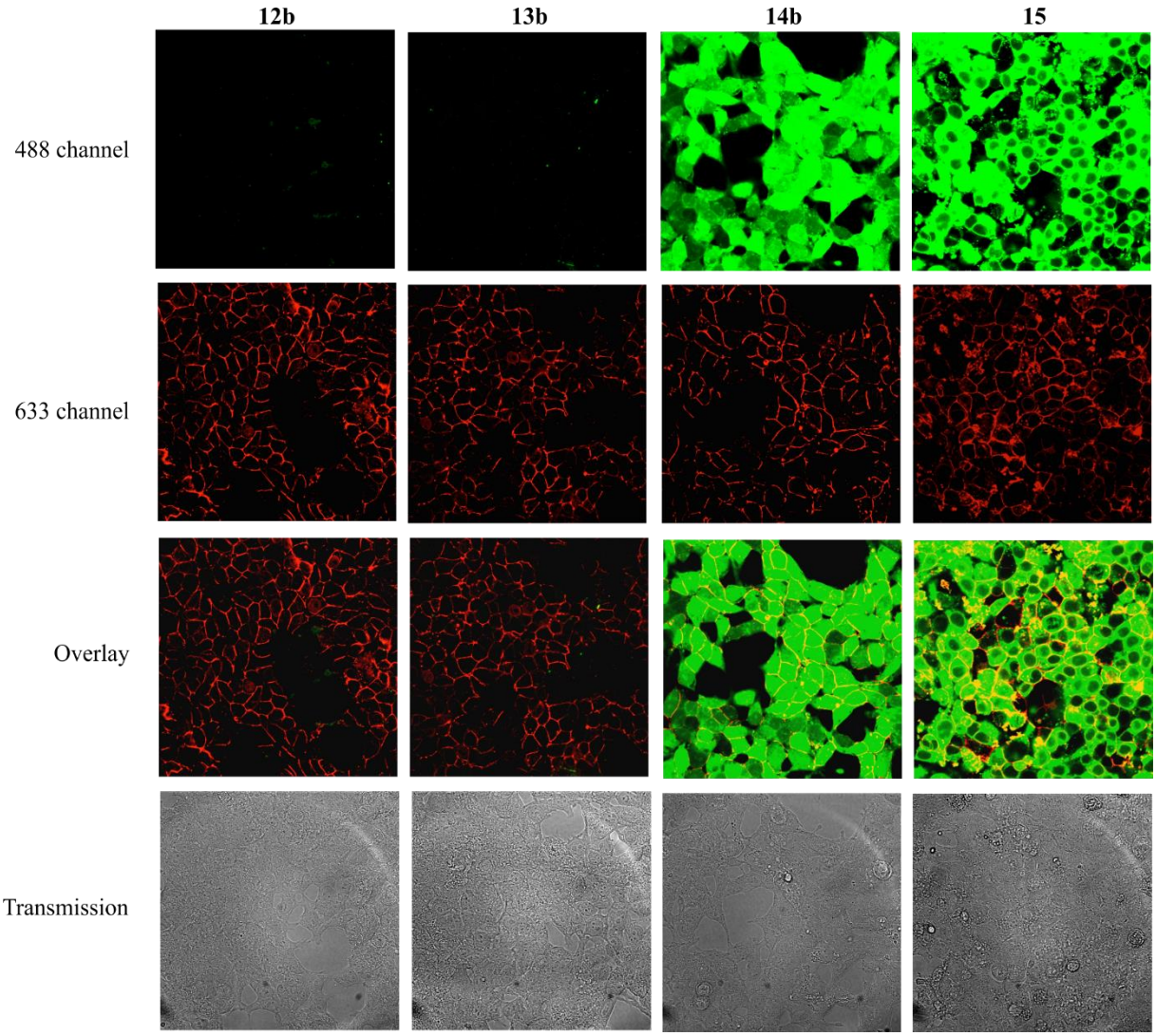


Figure S15. Confocal microscopy images of HEK293T cells treated with 20 μM FITC-labeled peptides. Red colour: membrane; green colour: FITC labeled peptides. Images were normalized using PMT = 470 V in channel 488 corresponding to black image for **12b** 10 μM condition.

Flow cytometry

293T HEK cells were cultured in triplicate in 24 well plates until they reached 90 % confluency. Appropriate amounts of peptides **12b**, **13b**, **14b**, and **15** dissolved in MEM (0.1 % DMSO) were added to the cells to final concentrations of 1 μM, 5 μM, 10 μM and 20 μM. Supernatant was removed and Trypsin-EDTA 0.25 % (0.5 mL) was added to the cells and incubated for 10 minutes at 37°C and 5% CO2. After incubation, cells were recovered by pipetting then transferred to Eppendorf tubes and spun at 2200 rpm for 2 minutes. The pellets were washed 3 times with PBS then re-suspended in PBS with 2% FBS (v/v) before filtration using Cell strainer cap. FACS analysis was carried on BD LSRII Flow Cytometer.

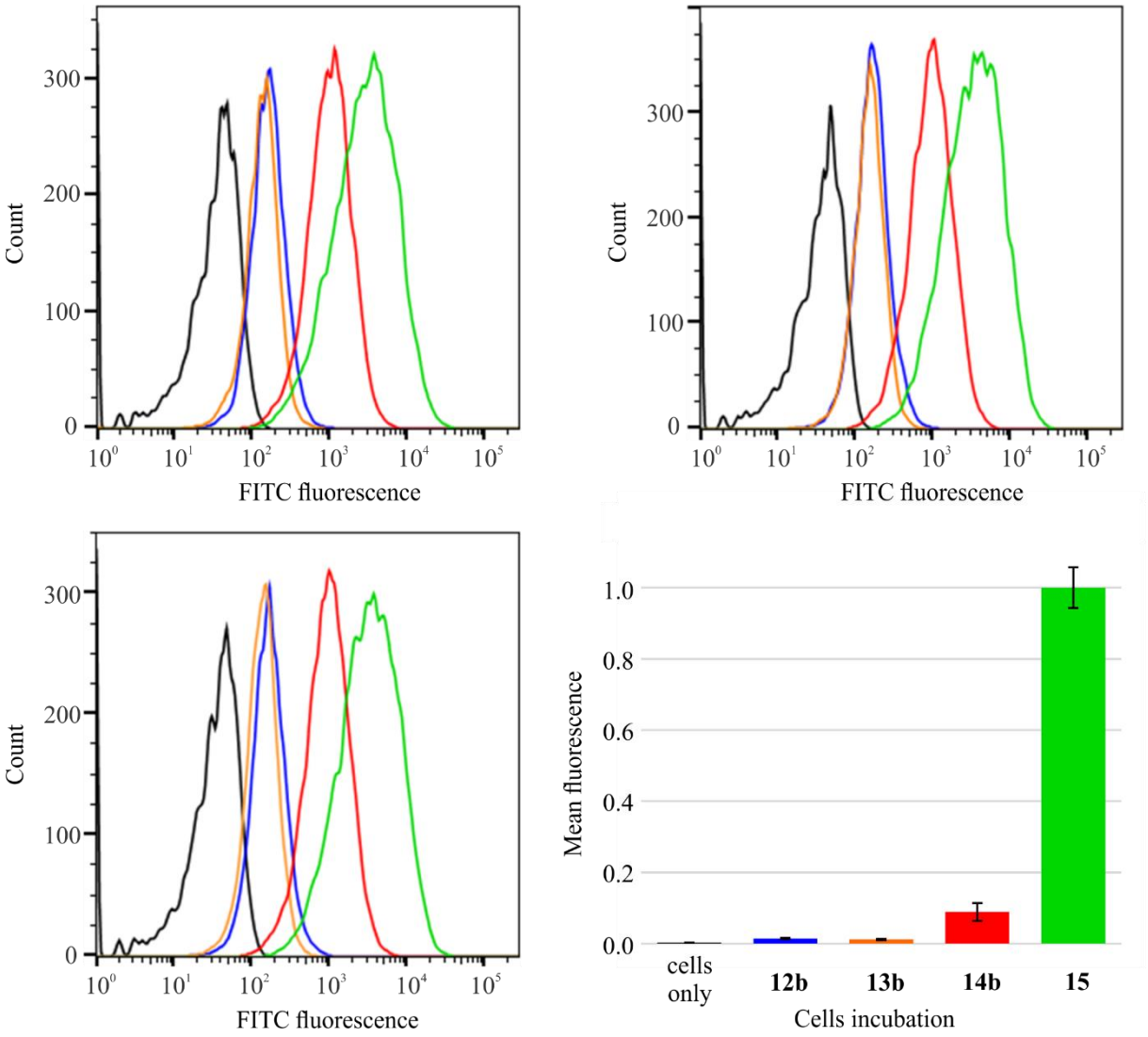


Figure S16. FACS data (in triplicate) obtained from the experiments with 5 μM peptide solutions (blank in black; **12b** in blue; **13b** in orange; **14b** in red; **15** in green).

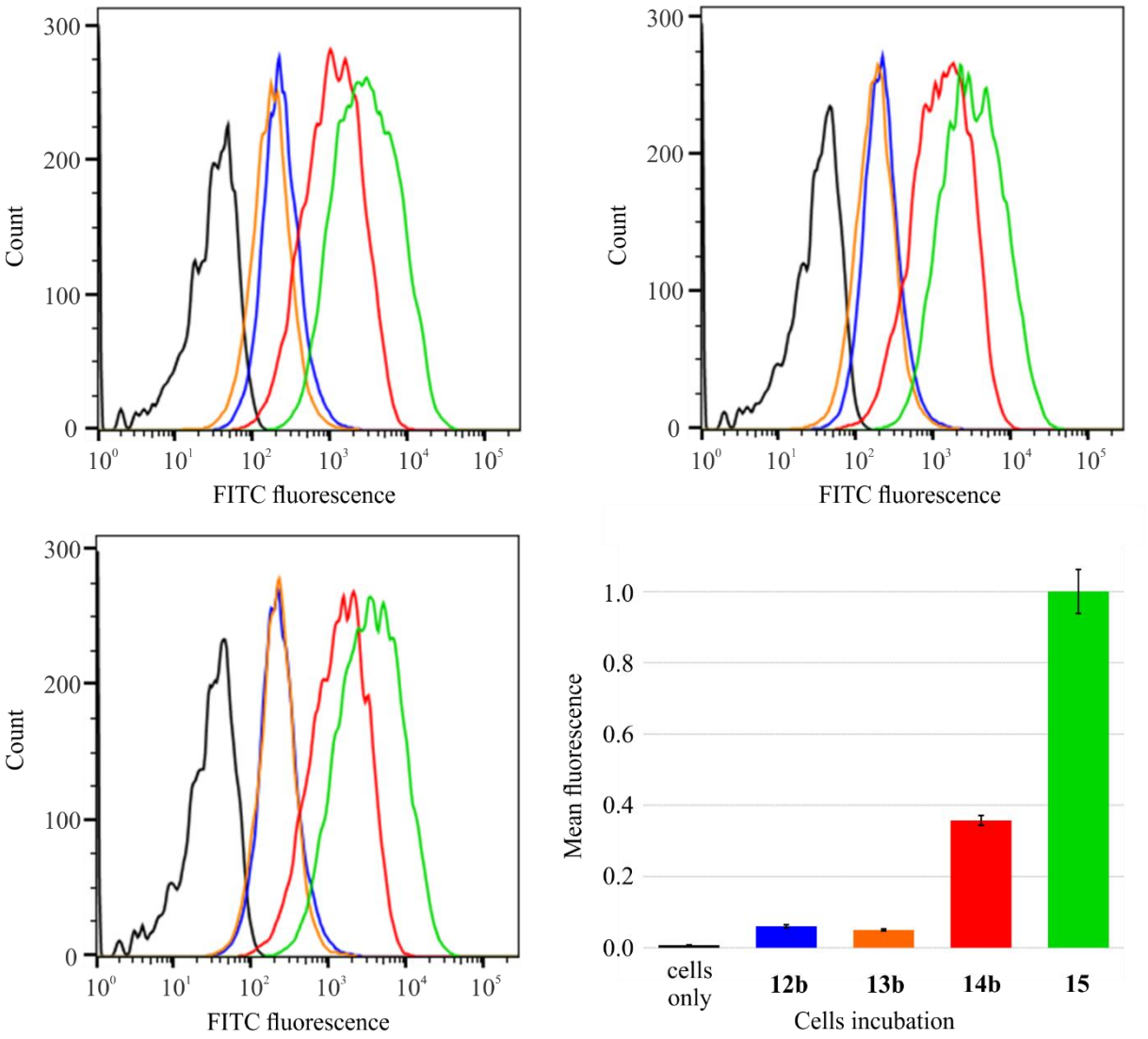


Figure S17. FACS data (in triplicate) obtained from the experiments with 10 μM peptide solutions (blank in black; **12b** in blue; **13b** in orange; **14b** in red; **15** in green).

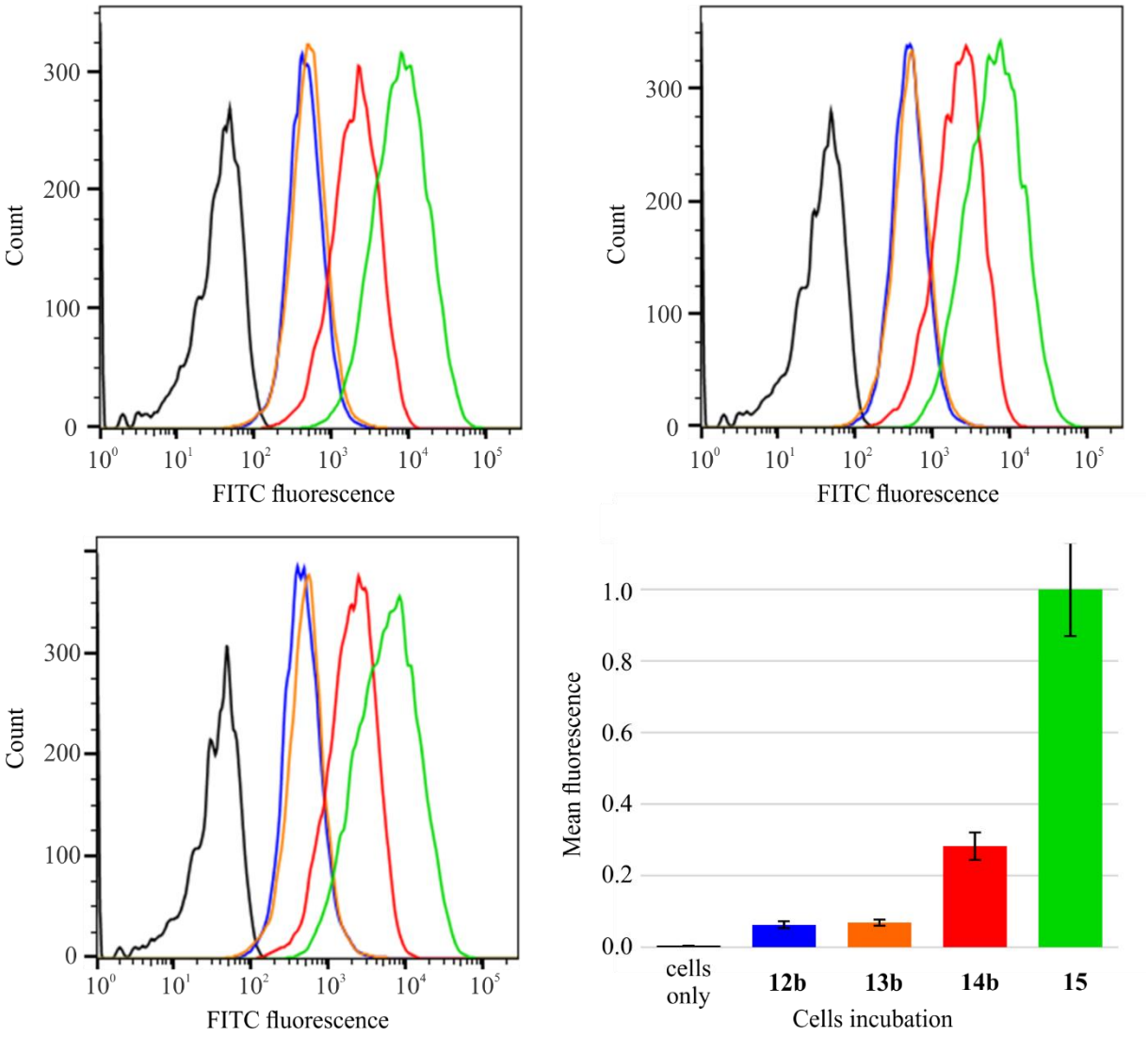
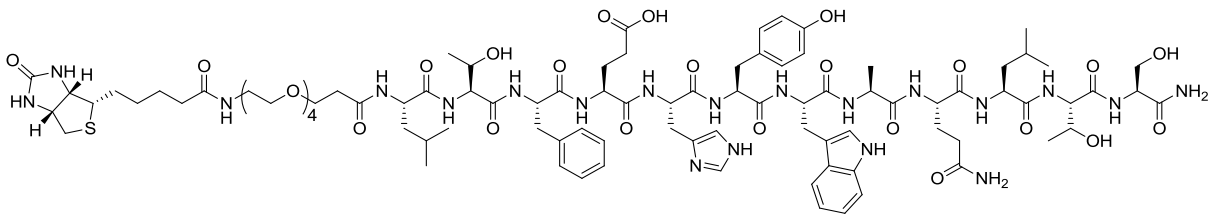


Figure S18. FACS data (in triplicate) obtained from the experiments with 20 μM peptide solutions (blank in black; **12b** in blue; **13b** in orange; **14b** in red; **15** in green).

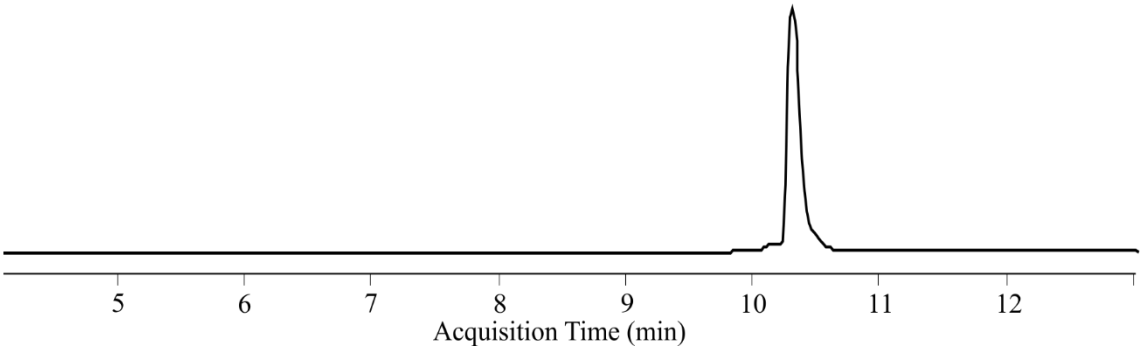
12. Octet BioLayer Interferometry Binding Assay

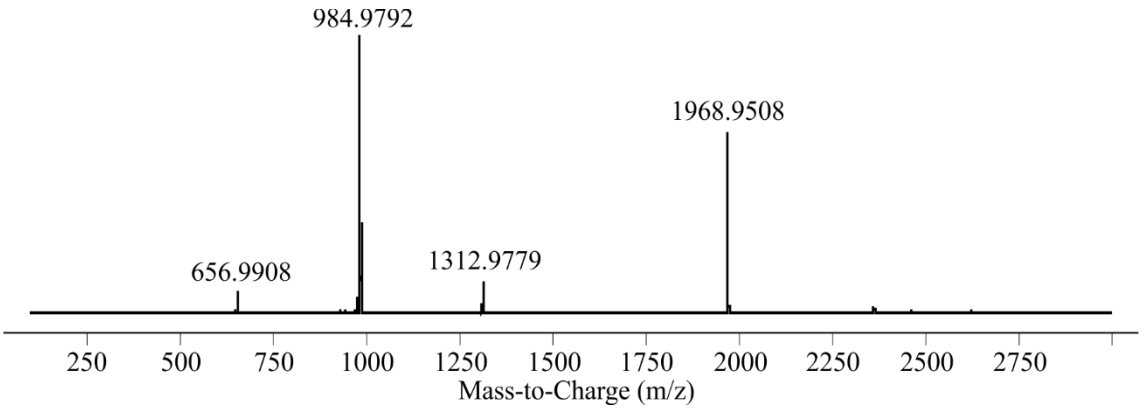
In vitro binding assays were performed using Fortebio Octet® RED96 Bio-Layer Interferometry system at 30 °C. Briefly, super streptavidin tips were dipped into 200 μL of biotin-linked peptide solution (5 μM of **12c** or **13c** or **14c** in PBS with 0.05% tween) for the loading. The tips loaded with peptide were sampled with SUMO-$^{25\text{-}109}$MDM2[8] at various concentrations in PBS with 0.05% tween to obtain the association curve, buffer only serves as the reference. After association, the tips were dipped into PBS with 0.1% BSA and 0.02% tween to obtain the dissociation curve. The association and dissociation curves are fitted with Fortebio Biosystems (global fitting algorism) to obtain the dissociation constant (K_D).

LC-MS analytical data of purified peptides **12c**, **13c** *and* **14c**

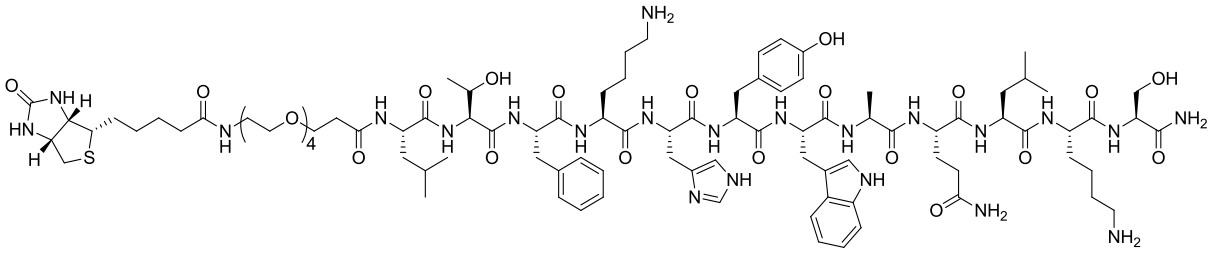


12c: Biotin-$(PEG)_4$-Leu-Thr-Phe-Glu-His-Tyr-Trp-Ala-Gln-Leu-Thr-Ser-$CONH_2$

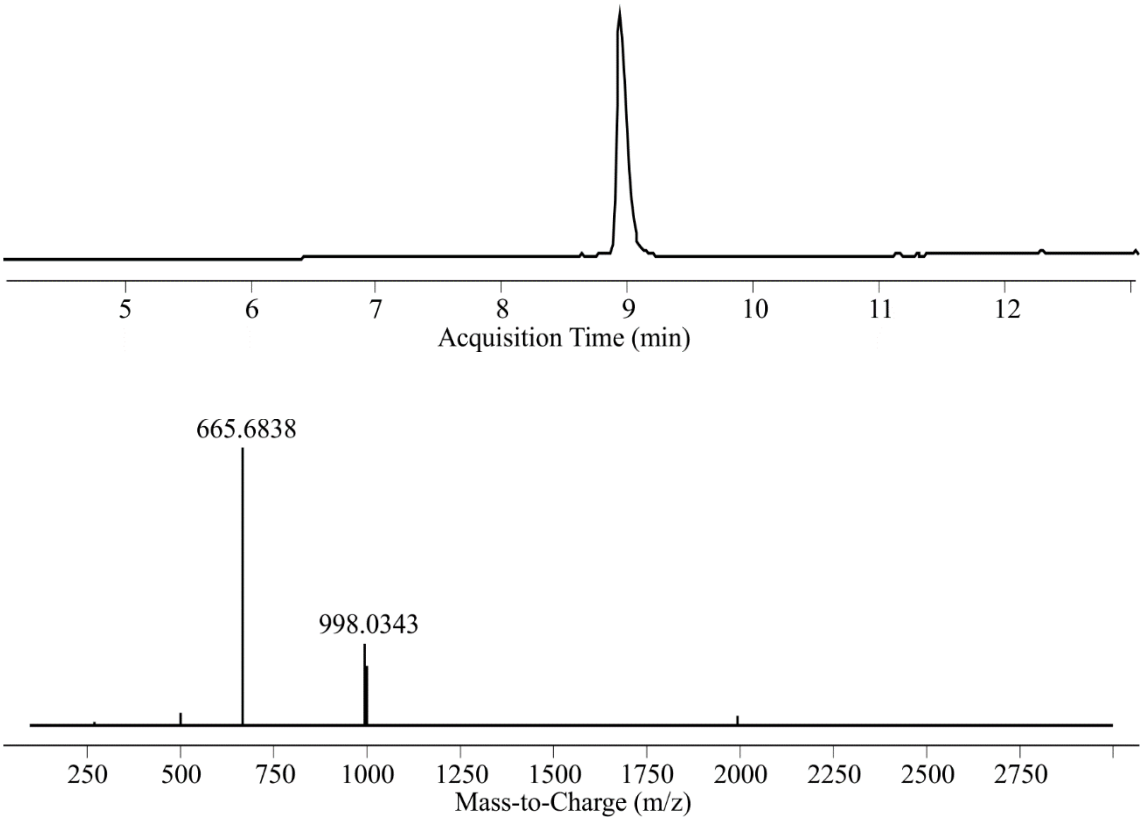




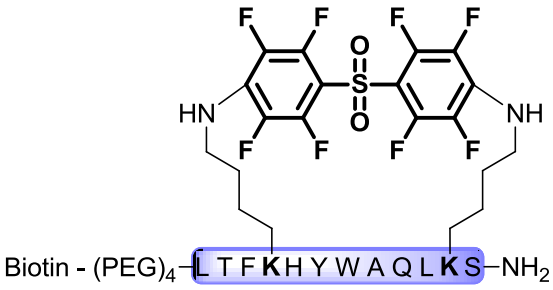
Peptide **12c**: LC-MS analysis *Method A*. TIC trace and Mass spectrum of peptide **12c** prepared according to the representative protocol **(G)**. m/z calcd. for $C_{92}H_{134}N_{20}O_{26}S$ $[M+2H]^{2+}$: 984.48 found 984.48.



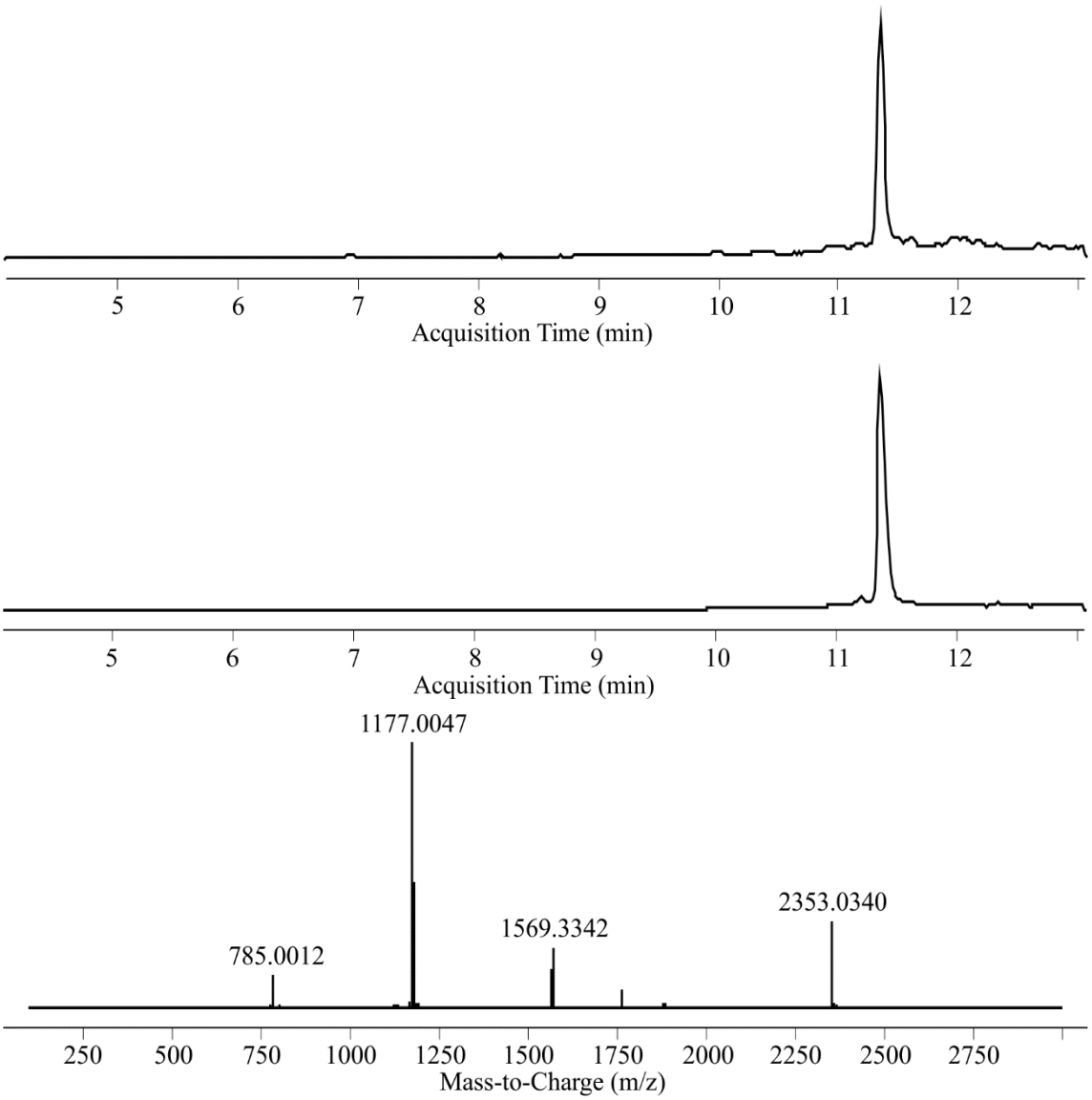
13c: Biotin-$(PEG)_4$-Leu-Thr-Phe-Lys-His-Tyr-Trp-Ala-Gln-Leu-Lys-Ser-$CONH_2$



Peptide **13c**: LC-MS analysis *Method A*. TIC trace and Mass spectrum of peptide **13c** prepared according to the representative protocol **(G)**. m/z calcd. for $C_{95}H_{144}N_{22}O_{23}S$ $[M+2H]^{2+}$: 997.53 found 997.53.



14c



Peptide **14c**: Prepared according to the representative protocol **(C)** using peptide **13c** (0.5 mM), electrophile **6** (0.625 mM), and DIEA (10 mM) at room temperature for 6 h. The diluted reaction mixture was analyzed using LC-MS *Method A*. TIC trace of crude reaction, pure isolated peptide **14c** and Mass spectrum of product **14c**. Analytical data for **14c** : m/z calcd. for $C_{107}H_{142}F_8N_{22}O_{25}S_2$ $[M+2H]^{2+}$: 1176.50 found 1176.50. **14c** was obtained as a white powder (6.1 mg, 52 %) after concentration, HPLC purification and lyophilization.

Fitting of association and dissociation curves

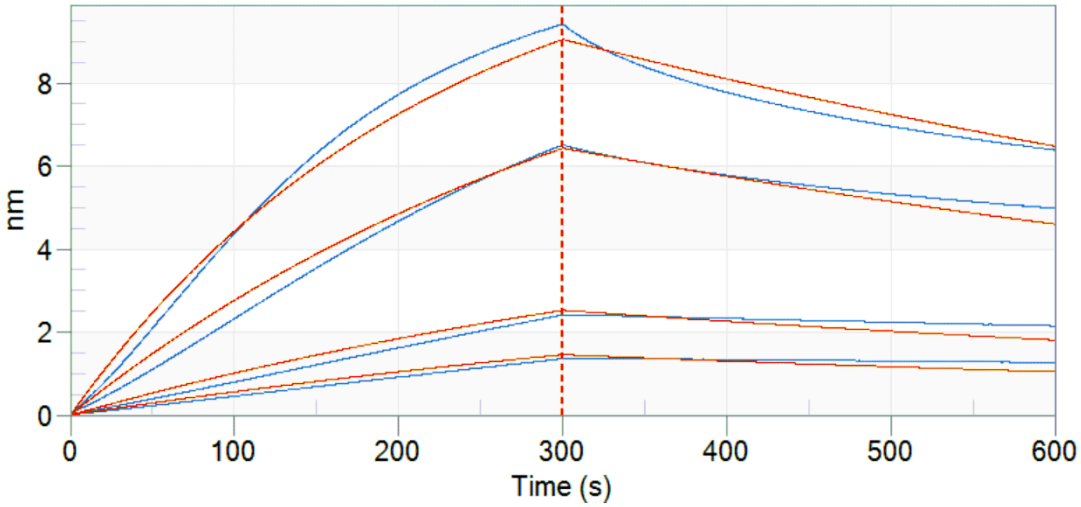


Figure S19. Global fitting of association and dissociation curves of various concentrations of SUMO-$^{25\text{-}109}$MDM2 (200 nM, 100 nM, 50 nM and 25 nM) with biotin-linked peptide **12c** immobilized to super streptavidin sensors. The K_D was found to be 66 ± 2 nM. Coefficient of determination R^2 = 0.9931.

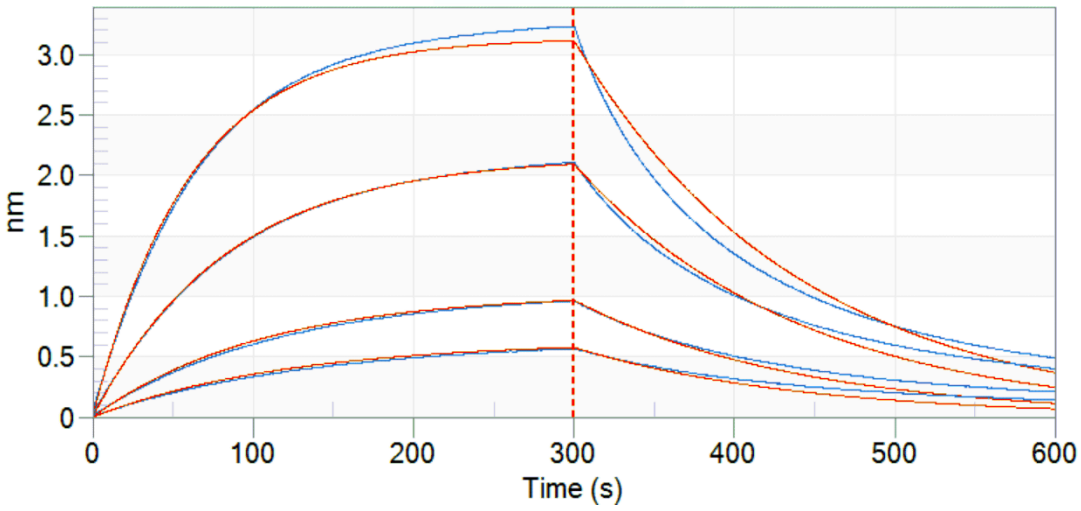


Figure S20. Global fitting of association and dissociation curves of various concentrations of SUMO-$^{25\text{-}109}$MDM2 (200 nM, 100 nM, 50 nM and 25 nM) with biotin-linked peptide **13c** immobilized to super streptavidin sensors. The K_D was found to be 151 ± 2 nM. Coefficient of determination R^2 = 0.9943.

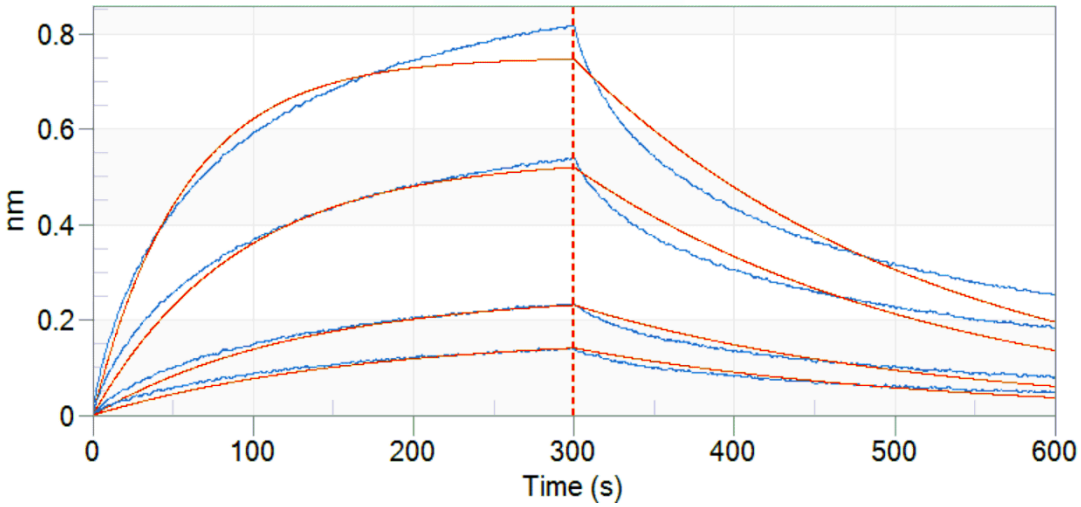


Figure S21. Global fitting of association and dissociation curves of various concentrations of SUMO-$^{25\text{-}109}$MDM2 (200 nM, 100 nM, 50 nM and 25 nM) with biotin-linked peptide **14c** immobilized to super streptavidin sensors. The K_D was found to be 68 ± 2 nM. Coefficient of determination R^2 = 0.9885.

13. References

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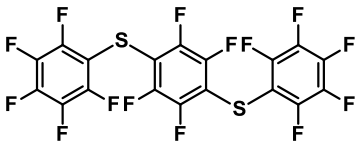
14. ^{1}H NMR, ^{13}C NMR and ^{19}F NMR spectra



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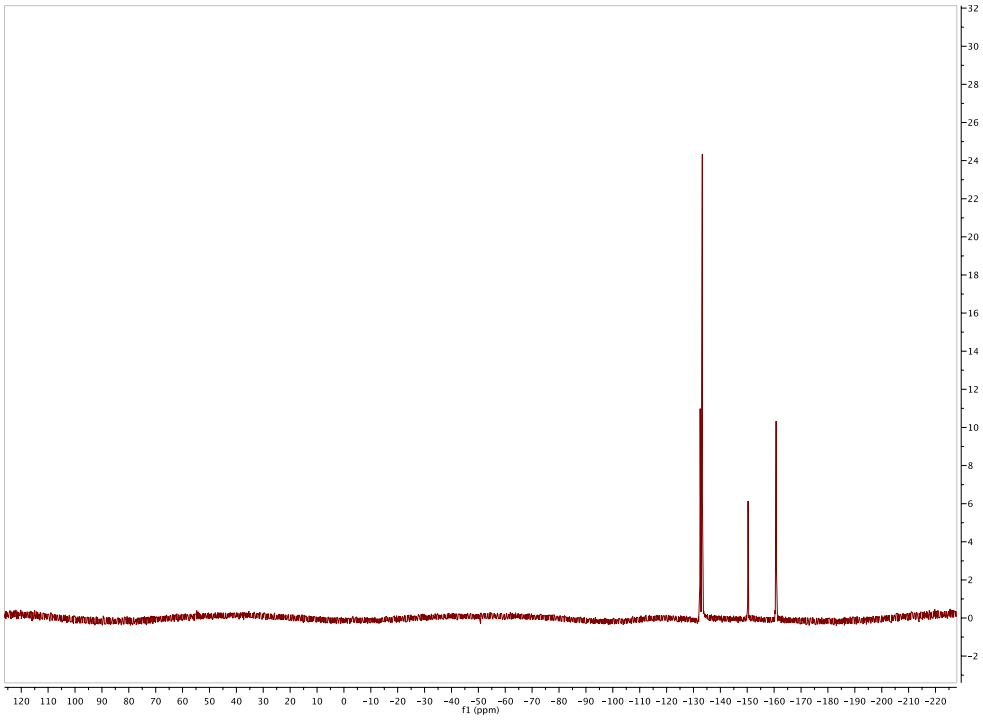
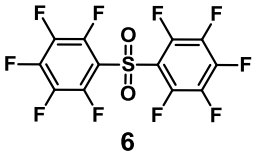


^{19}F NMR spectrum of electrophile **5**.



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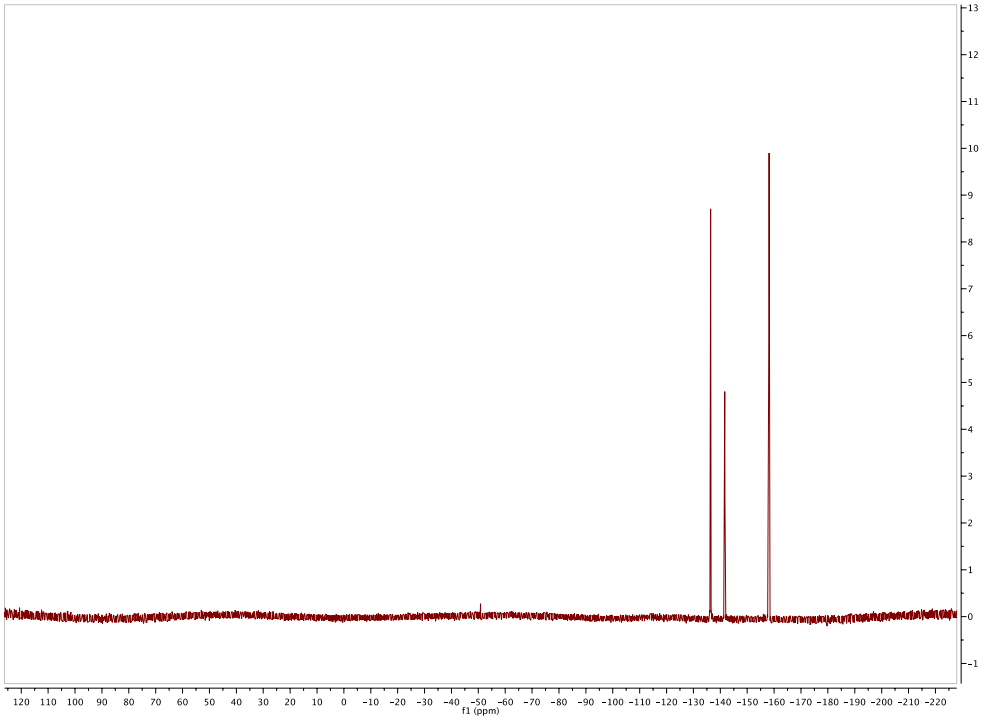
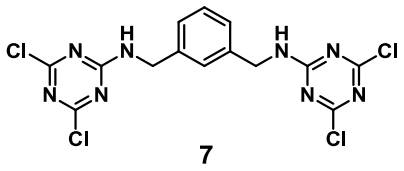


^{19}F NMR spectrum of electrophile **6**.



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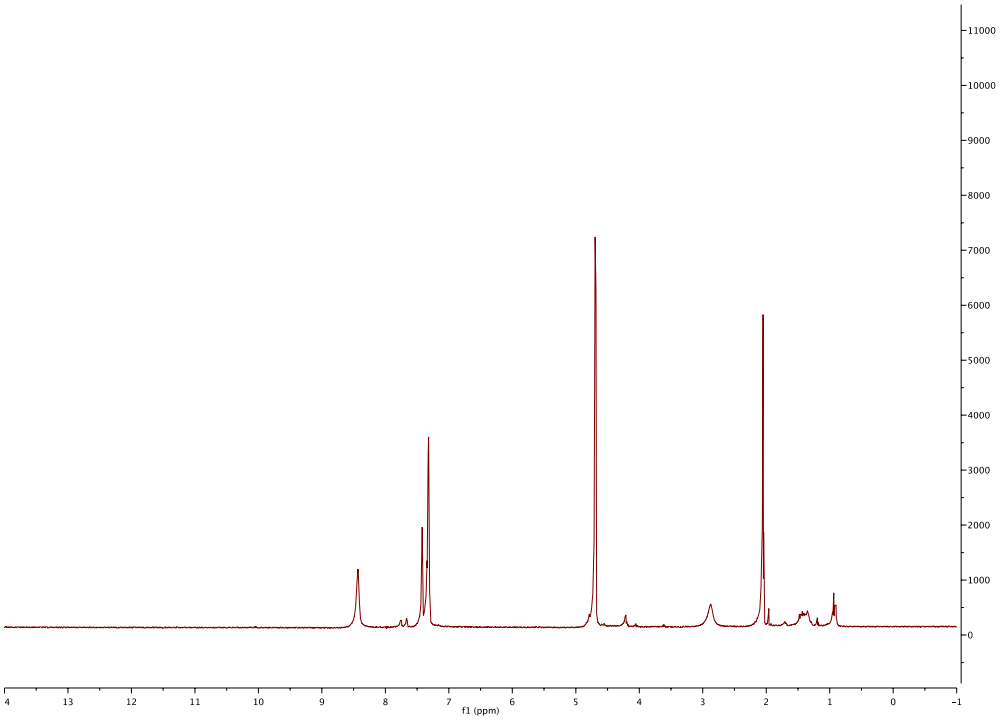
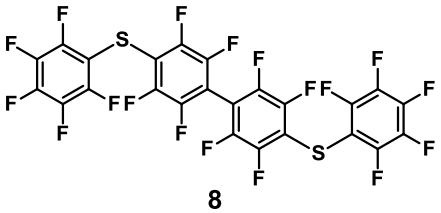


^{1}H NMR spectrum of electrophile **7**.



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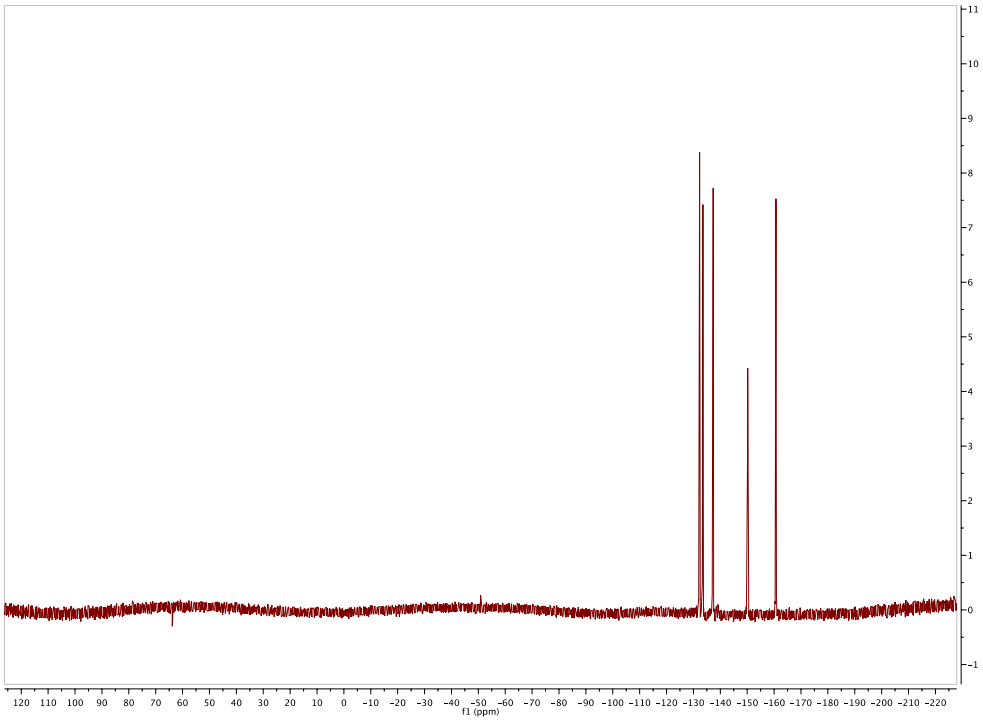
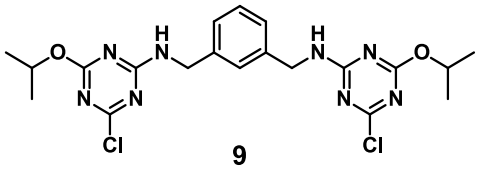
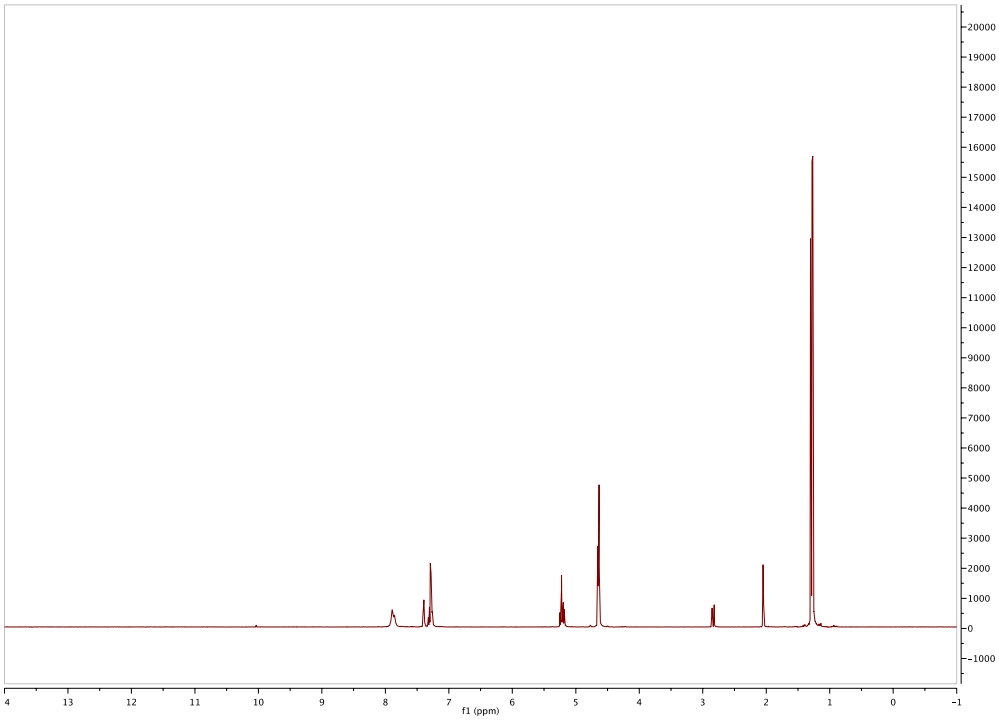


^{19}F NMR spectrum of electrophile **8**.



9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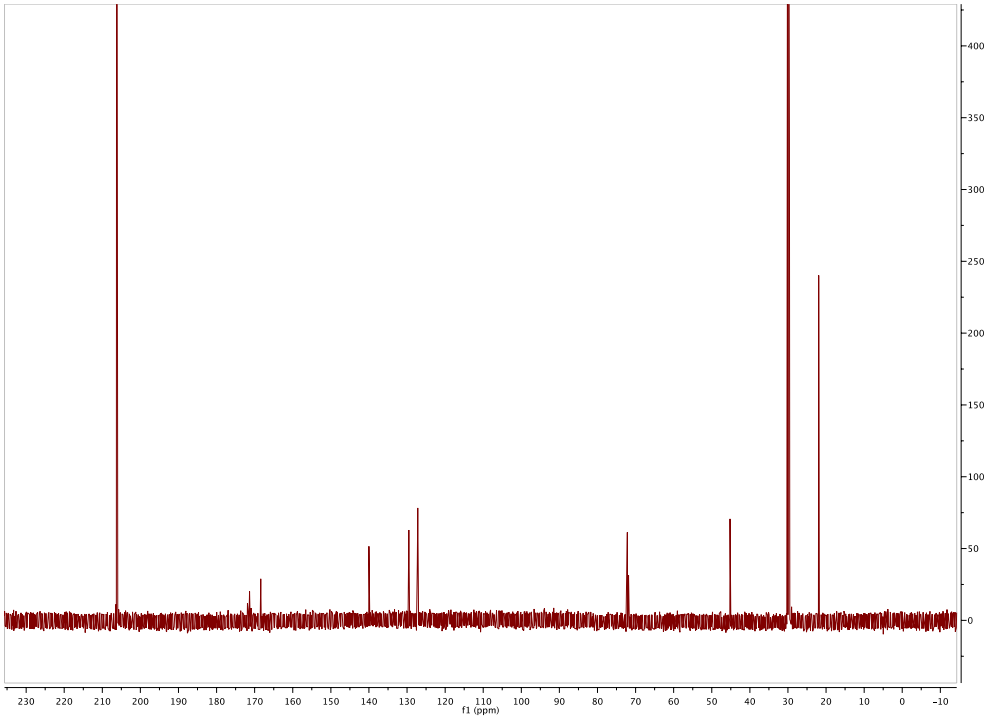

^{1}H NMR and ^{13}C NMR spectra of electrophile **9**.