Singlet oxygen-mediated one-pot chemoselective peptide-peptide ligation

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Part A: General experimental procedures

Materials and Methods

Rink Amide AM (0.69 mmol/g) and 2-chlorotrityl chloride (2-Cl-(Trt)-Cl, 1.60 mmol/g) resins were obtained from Iris Biotech GmbH. All amino acids and coupling reagents such as 2-(1H-Benzotriazol-1-yl)-1,1,3,3-tetramethyluronium hexafluorophosphate (HBTU) and 2-(1H-7-Azabenzotriazol-1-yl)-1,1,3,3-tetramethyluronoium hexafluorphosphate (HATU) were purchased from Iris Biotech GmbH. Fmoc-L-Furylalanine (Fua) was purchased from Chem-Impex International. L-amino acids were used throughout the synthesis. Dimethylformamide (DMF) peptide synthesis grade and *N*-methylpyrrolidone (NMP) were purchased from Biosolve. Phosphate Buffered Saline (PBS) without calcium or magnesium was purchased from Lonza. Dichloromethane (DCM) and *N,N*-diisopropylethylamine (DIPEA) were obtained from Sigma Aldrich. Trifluoroacetic acid (TFA) was obtained from Iris Biotech GmbH. All reagents used for automated peptide synthesis were peptide synthesis grade. Tri-Boc-hydrazinoacetic acid, hydrazine hydrate, 4-(Fmoc-aminomethyl)benzoic acid, Carbonyldiimidazole (CDI) and tert-butyl carbazate were purchased from Sigma Aldrich. All chemicals were used without further purification.

Peptide synthesis

Automated peptide synthesis was performed on a 24-reactor block SYRO Multiple Peptide Synthesizer equipped with a vortexing unit (Multisyntech, Witten, Germany). Peptides were synthesized by standard Fmoc/*t*Bu strategy using HBTU/DIPEA couplings.

Analyses

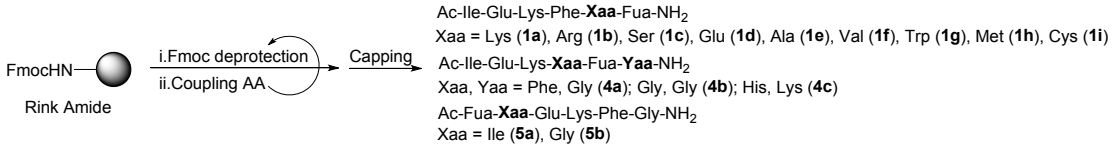
RP-HPLC analyses were performed on an Agilent 1100 Series instrument with a Phenomenex Luna C18 column (250 x 4.6 mm, 5 µm particle size at 35 °C). A flow rate of 1 ml/min was used with the following solvent systems: 0.1% TFA in H_2O (A) and MeCN (B). The column was flushed for 3 min with 100% A, then a gradient from 0 to 100% B over 15 min, followed by 5 min of flushing with 100% B.

Semi-preparative purification was performed on an Agilent Technologies PrepStar instrument using a Phenomenex Luna C18 column (250 × 21.2 mm, 5 µm particle size at 35 °C). The analyses were executed with a flow rate of 3 mL/min and with the following solvent systems: 0.1% TFA in H_2O (A) and MeCN (B). The column was flushed for 2 min with 100% A, then a gradient from 0 to 100% B, followed by 5 min of flushing with 100% B.

ESI-LC/MS data were recorded on an Agilent 1100 Series instrument with a Phenomenex Kinetex C18(2) column (150 x 4.6 mm, 5 µm at 35 °C) connected to an G1956B type SL mass detector with a flow rate of 1 mL/min was used with the following solvent systems: 5 mM NH_4OAc in H_2O (A) and MeCN (B). The column was flushed with 100% A for 2 min, then a gradient from 0 to 100% B over 15 min was used, followed by 5 min of flushing with 100% B.

Part B: Peptide synthesis and modifications

I) General procedure for the preparation of furan-containing peptides 1a-1i, 4a-4c and 5a, 5b:



The resin was preswollen in DMF for 30 min and then filtered off. Peptide synthesis was performed on an automated peptide synthesizer using the following protocols for Fmoc deprotection and coupling.

Fmoc deprotection: A solution of 20% piperidine in DMF was added to the resin. The resin was shaken for 3 min and filtered off. The procedure was repeated and the reaction mixture was shaken for 12 min. The resin was filtered and washed with DMF (6 x 30 s).

Coupling: 5.0 equiv of a 0.5 M solution of amino acid in DMF, 5.0 equiv of a 0.5 M solution of HBTU in DMF and 10.0 equiv of a 2.0 M solution of DIPEA in NMP were added to the resin. The reaction mixture was shaken for 40 min. The resin was filtered off and washed with DMF (4 x 30 s). For difficult couplings, this step is repeated once. Fmoc-L-Furylalanine (Fua) was coupled manually: 3.0 equiv. of the amino acid, HATU as coupling reagent (3.0 equiv.) and DIPEA (6.0 equiv.) in DMF (1.0 mL) were added to the resin (34.5 μmol) and the reaction mixture is shaken for 2 h.

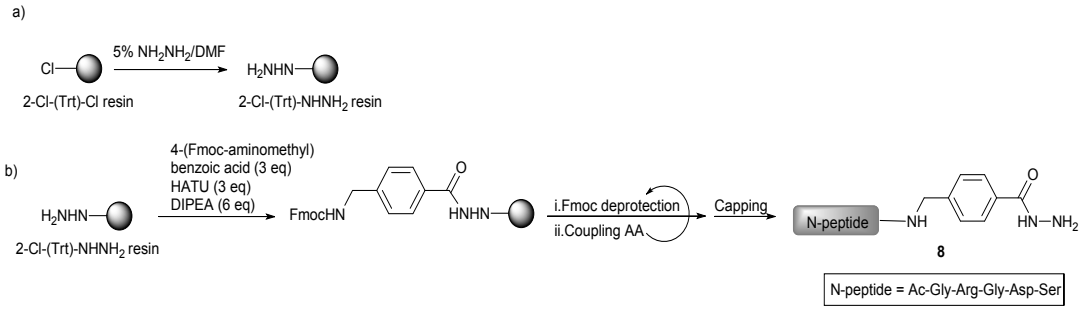
Capping: 6.0 equiv. of acetic anhydride and DIPEA in DMF were added to the resin. The reaction mixture was shaken for 30 min, filtered off and washed with DMF (3 x 30 s). The capping was repeated once to ensure complete conversion.

Cleavage and deprotection of peptides: 1 mg of resin was treated with 300 μl of TFA/Triisopropylsilane (TIS)/H_2O (95:2.5:2.5). After 2 h, the liquid was filtered off and the resin was washed with TFA (3 times). The filtrate was evaporated and the residue was redissolved in H_2O for HPLC and LC-MS analysis.

II) Procedure for the preparation of N-terminal hydrazine peptide 2:

N-terminal hydrazine peptide was synthesized by solid phase peptide synthesis as described above (Section I). The N-terminus of the peptide was capped by tri-Boc hydrazinoacetic acid (3.0 equiv.), using HATU as coupling reagent (3.0 equiv.) and DIPEA (6.0 equiv.) in DMF (1.0 mL). The mixture of tri-Boc hydrazinoacetic acid, HATU and DIPEA was added to the resin (34.5 μmol) and shaken for 120 min. Next, the solution was filtered off and the resin was washed with DMF/DCM/DMF (6 x 10 seconds each). Cleavage and deprotection were performed as described previously.

III) Procedure for the preparation of C-terminal hydrazide peptide 8[1]



Preparation of 2-Cl-(Trt)-$NHNH_2$ resin

The 2-Cl-(Trt)-$NHNH_2$ resin was freshly prepared for stability reasons. 50 mg of 2-Cl-(Trt)-Cl resin was washed with DMF/DCM/DMF (3 x 10 seconds each). Then, the resin was swollen in DMF/DCM (1:1) for 30 minutes and the solution was filtered off. Then 2 mL of 5% (vol/vol) NH_2NH_2/DMF was added to the resin for hydrazination. The mixture was agitated for 30 min under microwave irradiation at 40 °C and then filtered off. The resin was washed three times with DMF/DCM/DMF and the hydrazination procedure was repeated. Then the mixture was filtered off, the resin was washed again and 2 mL of 5% (vol/vol) MeOH/DMF were added to the resin for 10 min. Then the resin was washed and drained. The resin was colored yellow-light green.

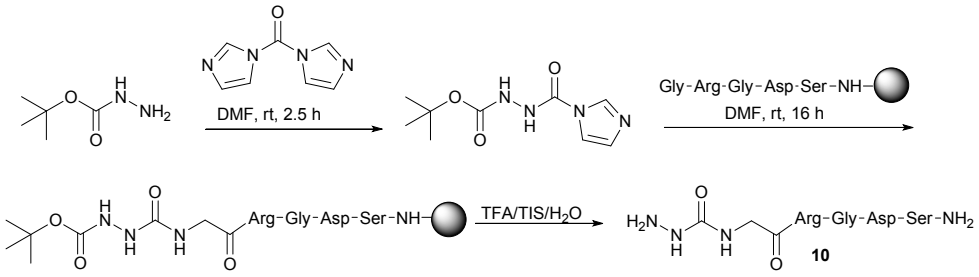
Coupling of 4-(Fmoc-aminomethyl)benzoic acid

After the hydrazination of the resin, 4-(Fmoc-aminomethyl)benzoic acid was coupled to the resin. For the coupling reaction, 3.0 equiv. of 4-(Fmoc-aminomethyl)benzoic acid were used together with HATU (3.0 equiv.) and DIPEA (6.0 equiv.) in DMF (1.5 mL). The resin was shaken for 90 minutes. Then it was washed with DMF/DCM/DMC (6 x 10 seconds each). The peptide synthesis was further performed using an automated peptide synthesizer. Acetylation and cleavage were conducted as described above (Section I).

IV) Procedure for the preparation of N-terminal hydrazide peptide 10[2]

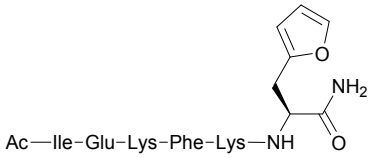
[1] J.-S. Zheng, S. Tang, Y.-K. Qi, Z.-P. Wang and L. Liu, *Nat. Protoc.*, 2013, **8**, 2483–2495.

[2] D. V Siev and J. E. Semple, *Org. Lett.*, 2000, **2**, 19–22.



To a suspension of 1,1′-Carbonyldiimidazole (210 μmol) in dry DMF (300 μL), tert-butyl carbazate (210 μmol) in dry DMF (200 μL) was added portionwise under N_2 for 2.5 h. Then the mixture was added to the resin-bound peptide (35 μmol) and the reaction was left for 16 hours at room temperature to ensure coupling. The resin was then filtered off, washed and cleaved as described above (Section I).

Part C: Spectral data of furan and α-effect nucleophilic peptides



1a

Chemical Formula: $C_{41}H_{63}N_9O_{10}$

Exact Mass: 841,46979

Molecular Weight: 841,99322

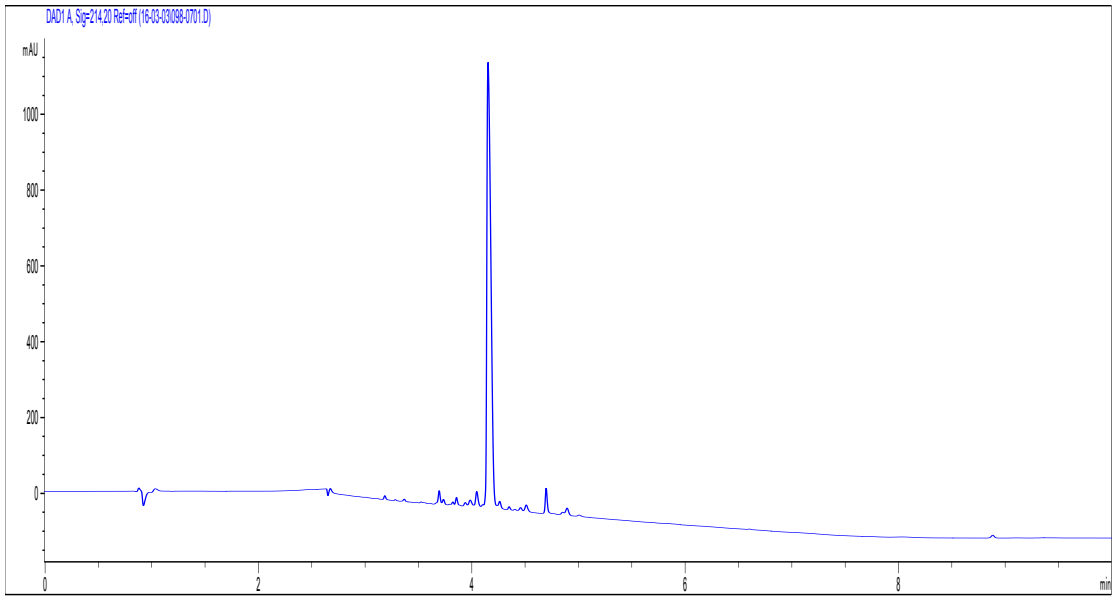


Figure 1: LC-MS chromatogram of the crude peptide **1a** at λ = 214 nm.

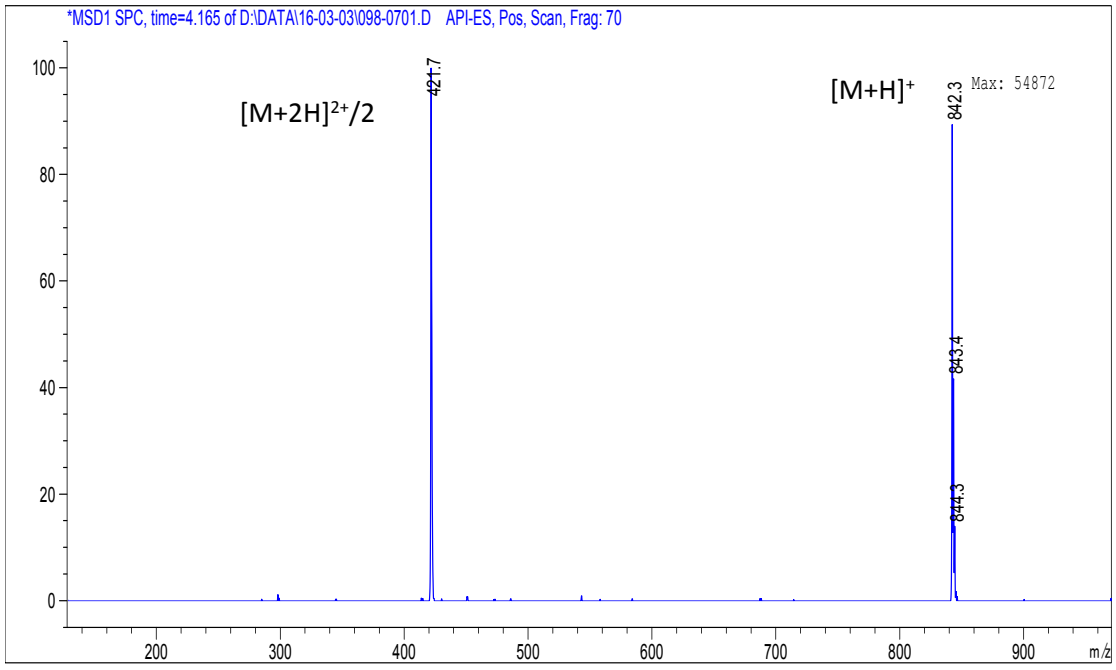
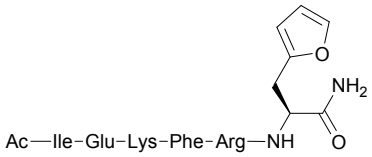


Figure 2: ESI-MS from LC-MS at t_R = 4.165 min (obs: 842.3 $[M+H]^+$; calc: 842.5).



1b

Chemical Formula: $C_{41}H_{63}N_{11}O_{10}$

Exact Mass: 869,47594

Molecular Weight: 870,02200

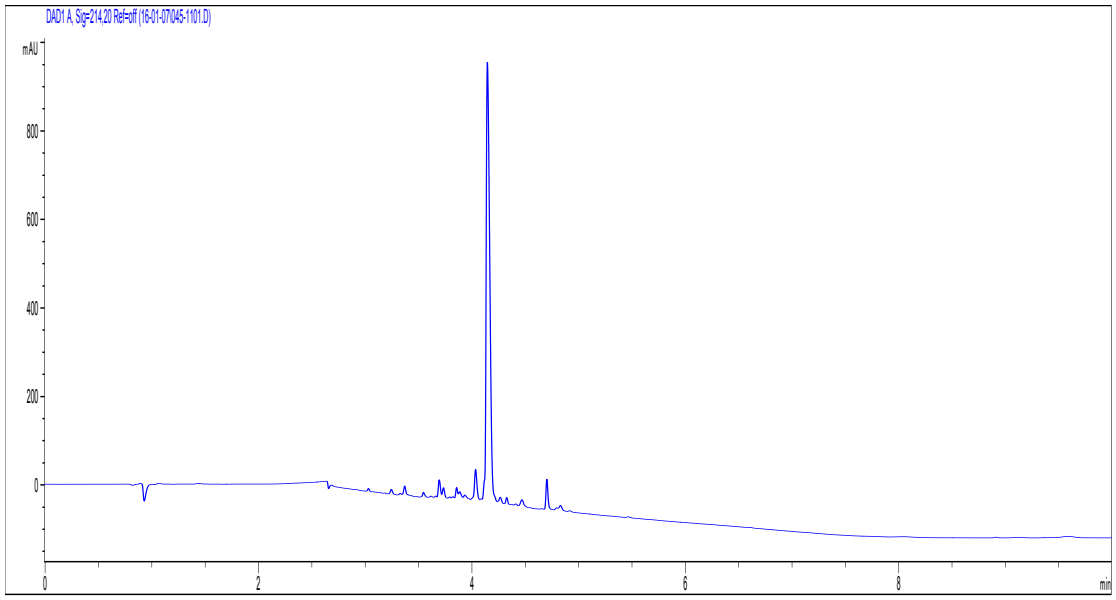


Figure 3: LC-MS chromatogram of the purified peptide **1b** at λ = 214 nm.

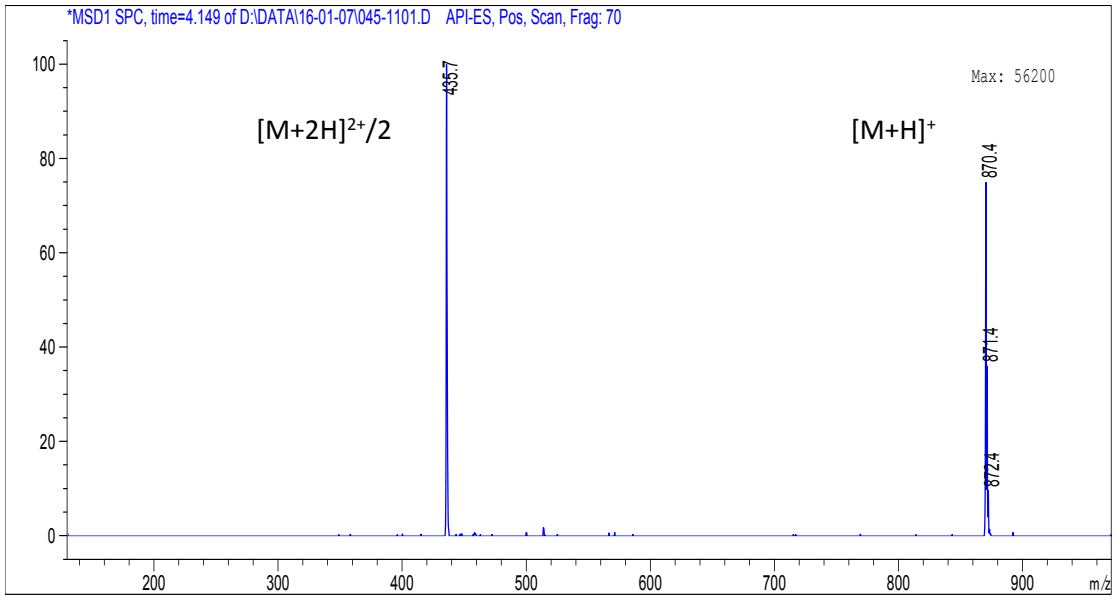
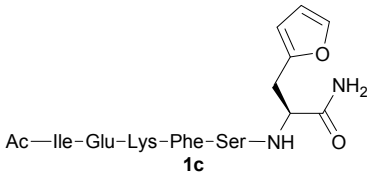


Figure 4: ESI-MS from the LC-MS at t_R = 4.149 min (obs: 870.4 $[M+H]^+$; calc: 870.5).



1c

Chemical Formula: $C_{38}H_{56}N_8O_{11}$

Exact Mass: 800,40685

Molecular Weight: 800,91100

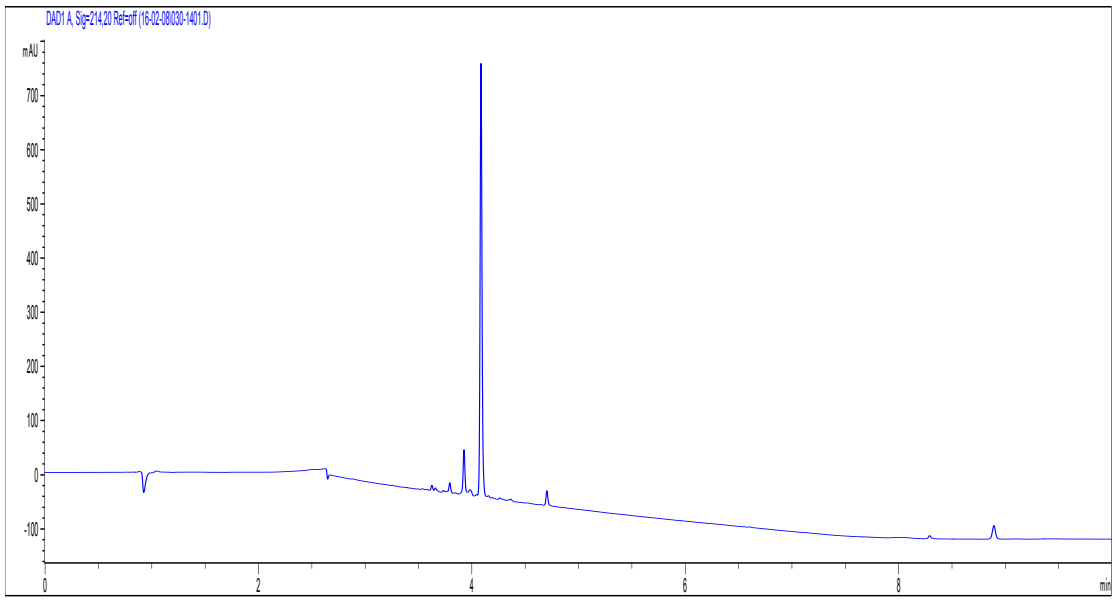


Figure 5: LC-MS chromatogram of the crude peptide **1c** at λ= 214 nm.

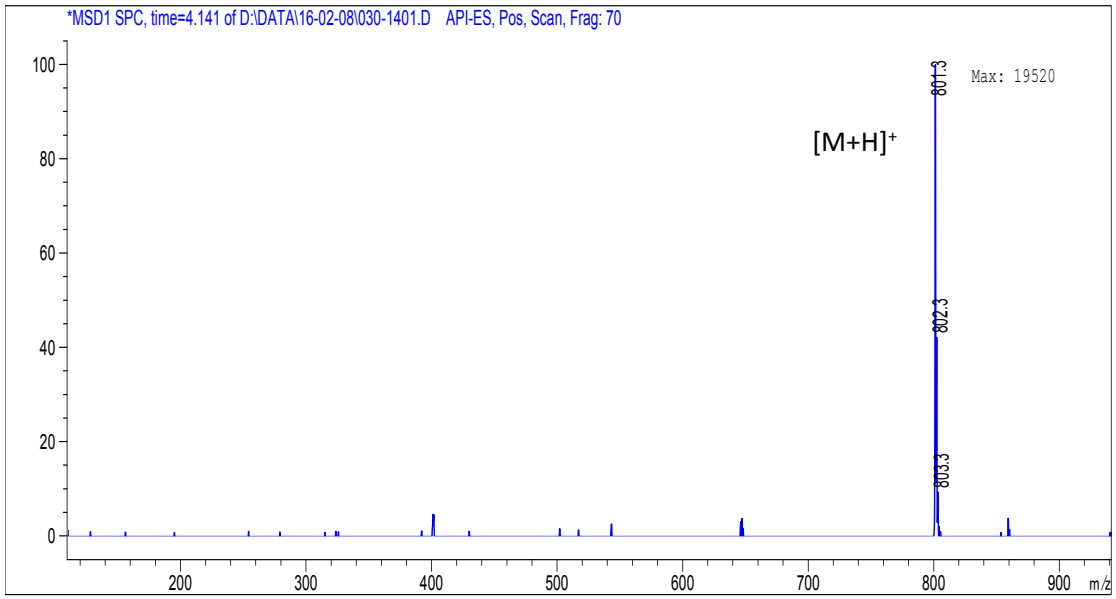
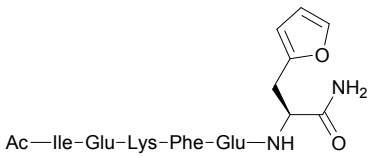


Figure 6: ESI-MS from LC-MS at t_R = 4.141 min (obs: 801.3 $[M+H]^+$; calc: 801.4).



1d

Chemical Formula: $C_{40}H_{58}N_8O_{12}$

Exact Mass: 842,41742

Molecular Weight: 842,93492

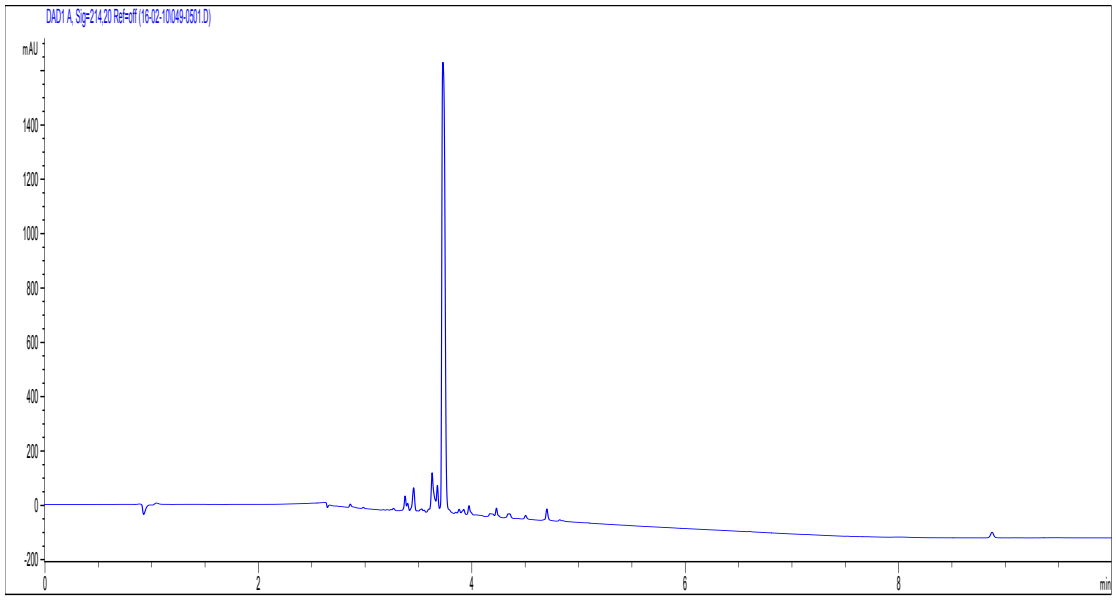


Figure 7: LC-MS chromatogram of the crude peptide **1d** at λ = 214 nm.

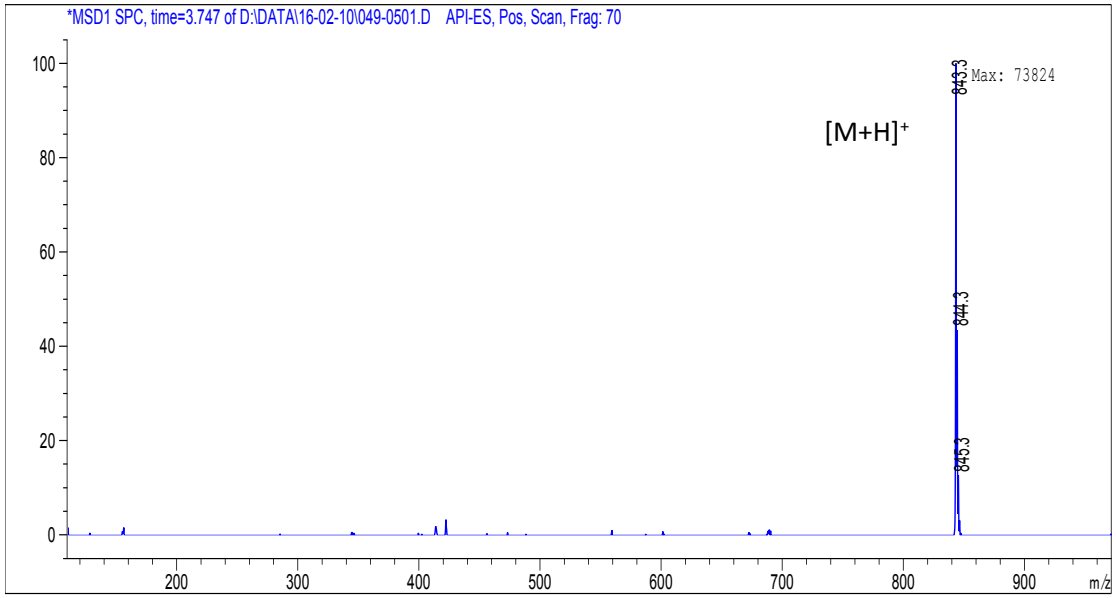
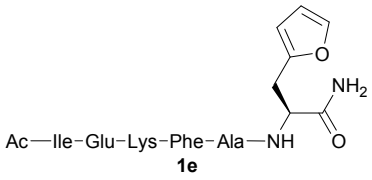


Figure 8: ESI-MS from LC-MS at t_R = 3.747 min (obs: 843.3 $[M+H]^+$; calc: 843.4).



1e

Chemical Formula: $C_{38}H_{56}N_8O_{10}$

Exact Mass: 784,41194

Molecular Weight: 784,91200

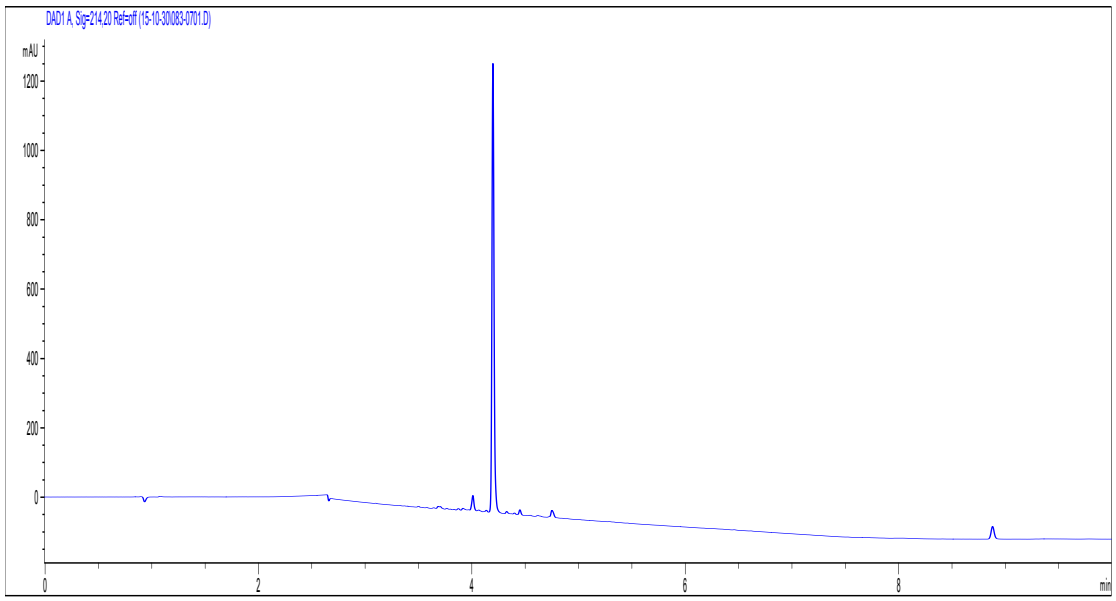


Figure 9: LC-MS chromatogram of the crude peptide **1e** at λ = 214 nm.

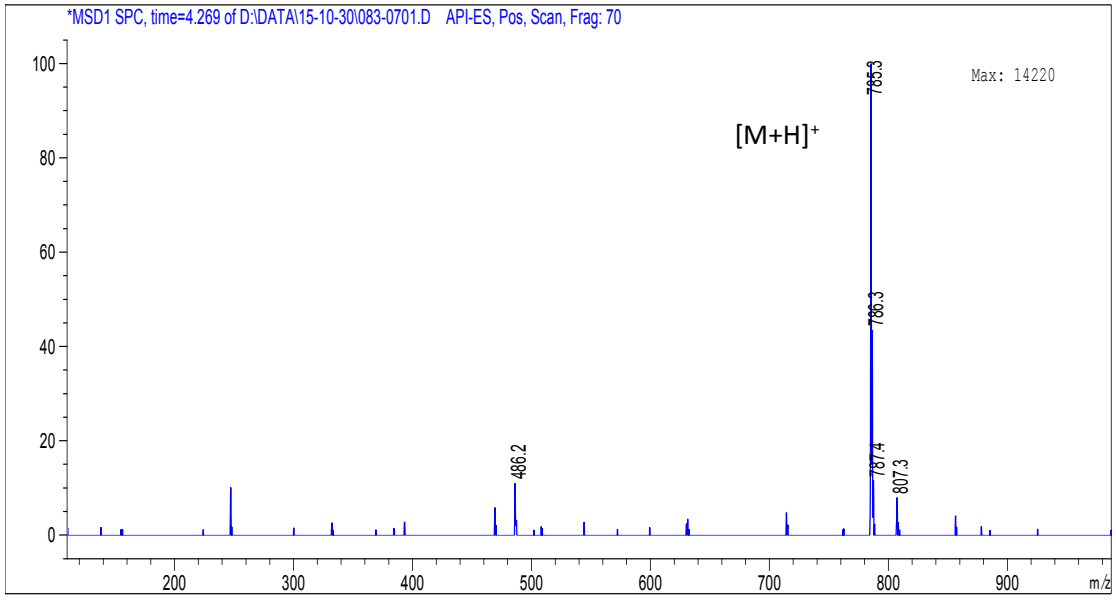
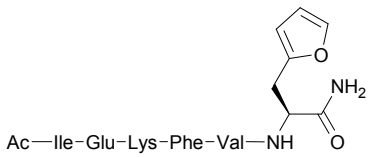


Figure 10: ESI-MS from LC-MS at t_R = 4.269 min (obs: 785.3 $[M+H]^+$; calc: 785.4).



1f

Chemical Formula: $C_{40}H_{60}N_8O_{10}$

Exact Mass: 812,44324

Molecular Weight: 812,95200

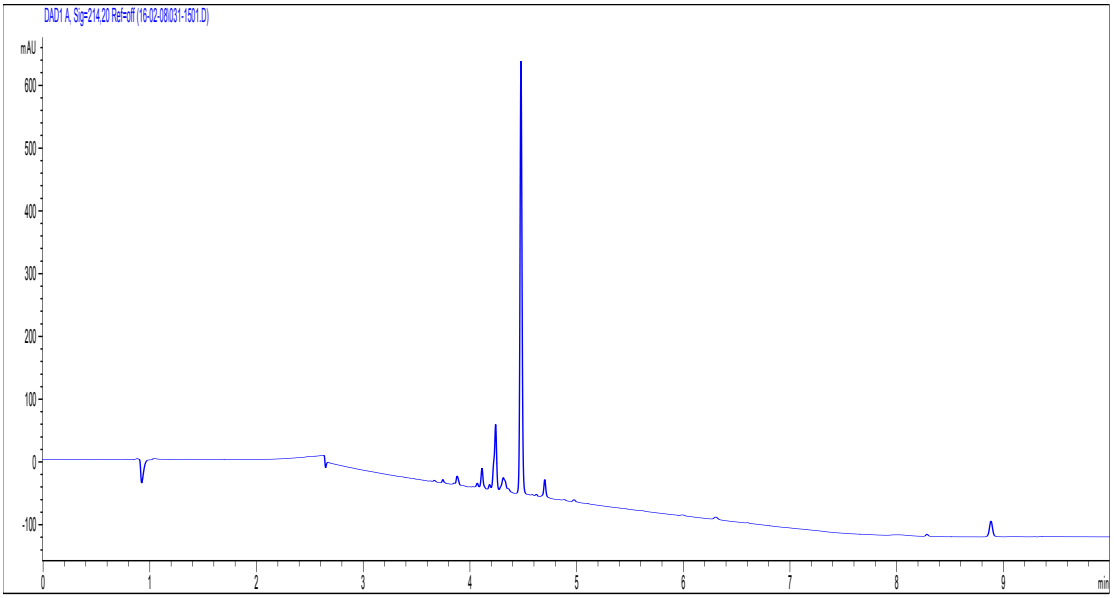


Figure 11: LC-MS chromatogram of the crude peptide **1f** at λ = 214 nm.

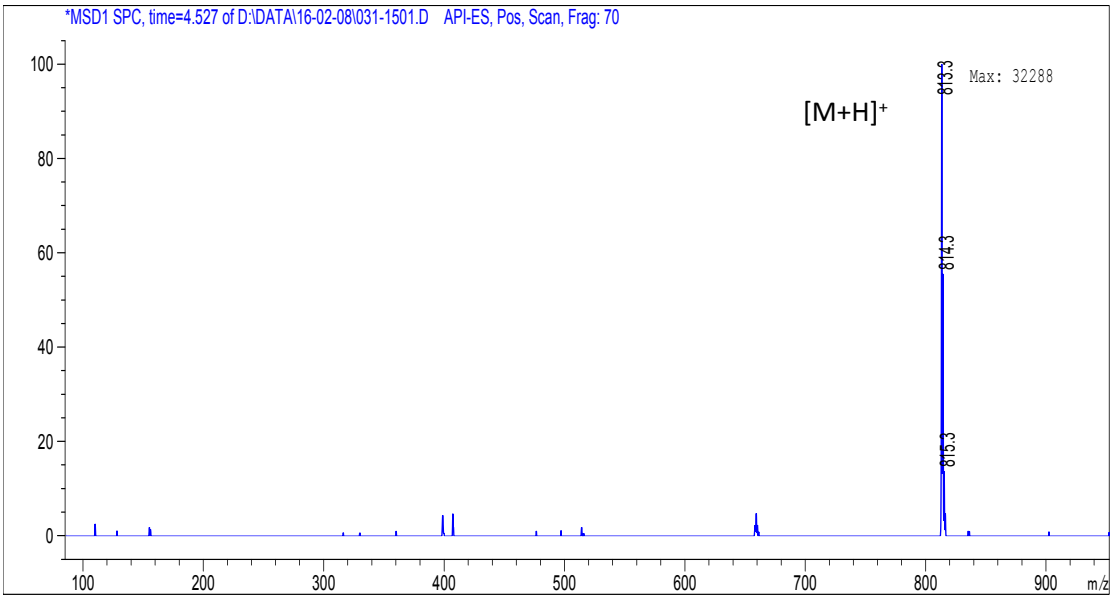
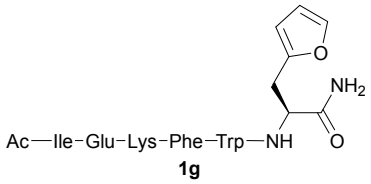


Figure 12: ESI-MS from LC-MS at t_R = 4.527 min (obs: 813.3 $[M+H]^+$; calc: 813.5).



1g

Chemical Formula: $C_{46}H_{61}N_9O_{10}$
Exact Mass: 899,45414
Molecular Weight: 900,04700

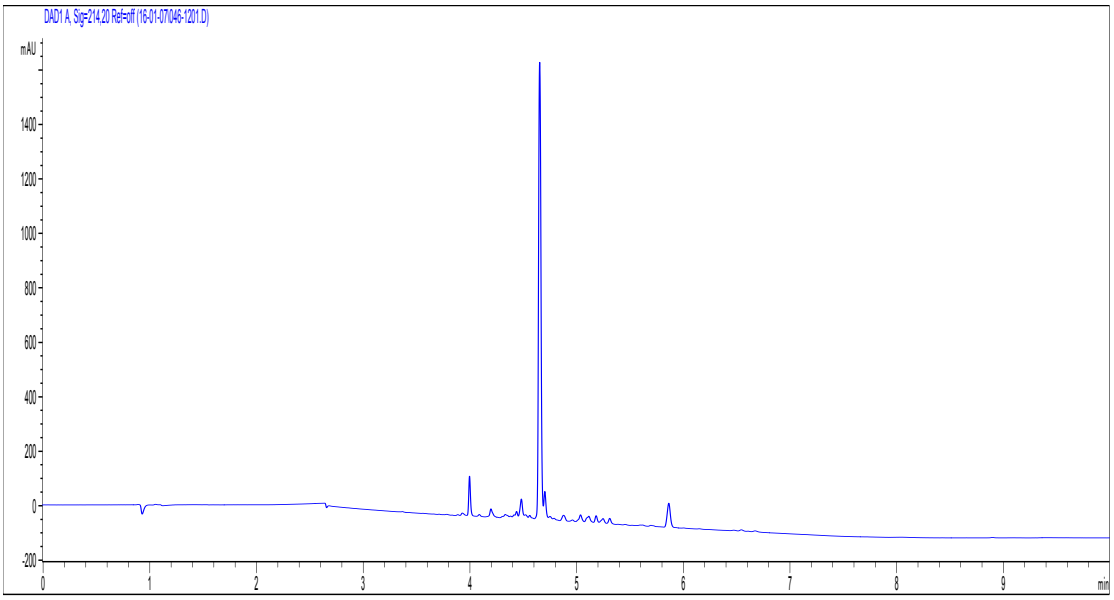


Figure 13: LC-MS chromatogram of the purified peptide **1g** at λ = 214 nm.

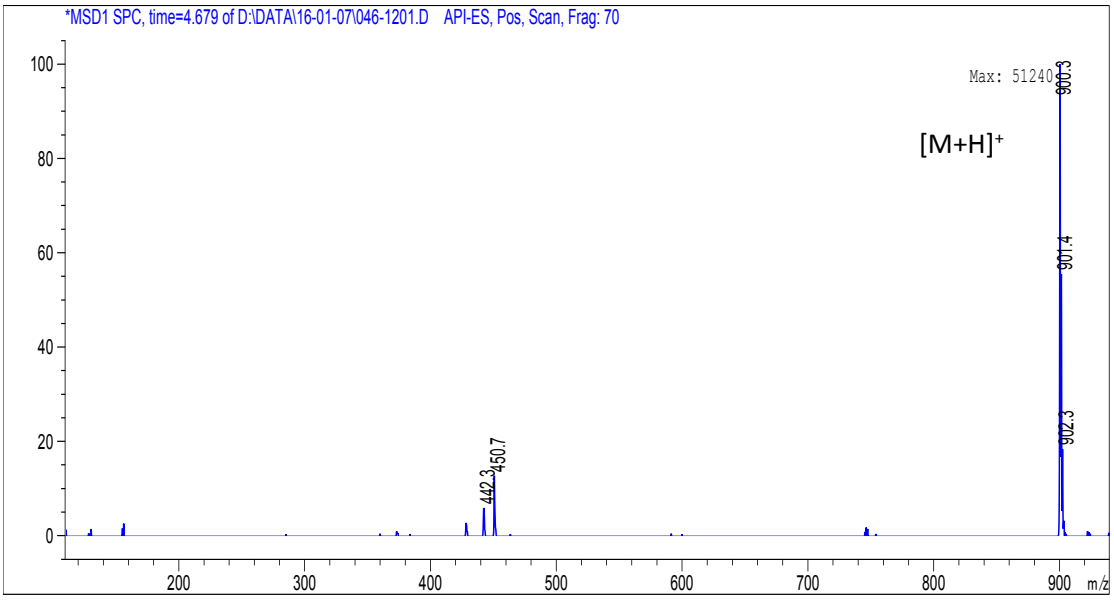
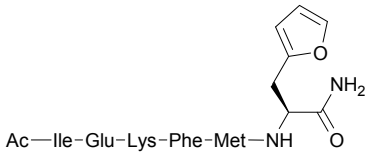


Figure 14: ESI-MS from the LC-MS at t_R = 4.679 min (obs: 900.3 $[M+H]^+$; calc: 900.5).



1h

Chemical Formula: $C_{40}H_{60}N_8O_{10}S$

Exact Mass: 844,41531

Molecular Weight: 845,01700

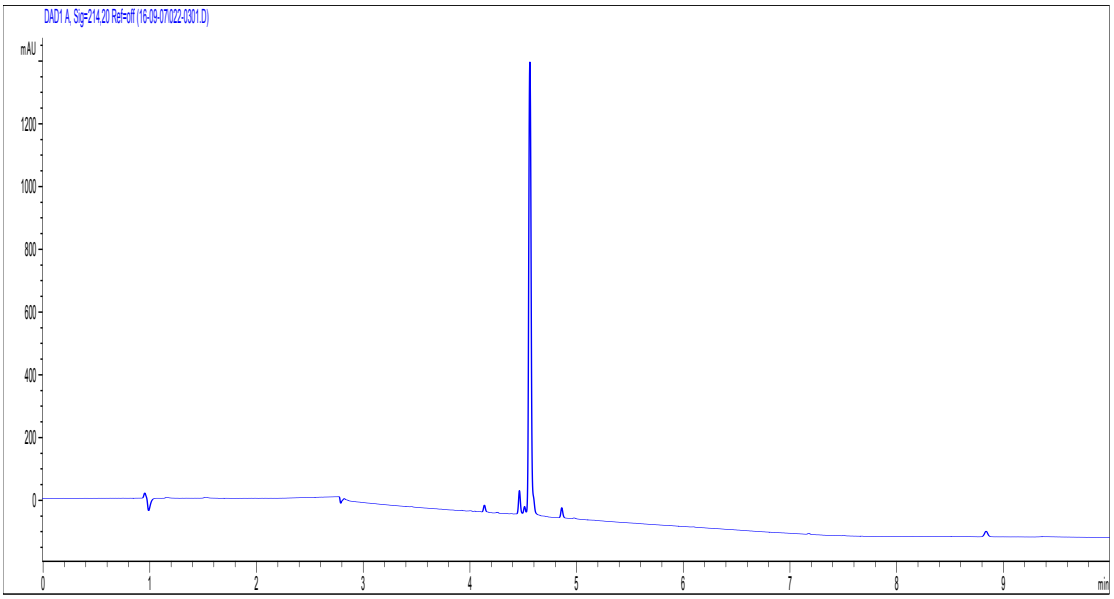


Figure 15: LC-MS chromatogram of the purified peptide **1h** at λ= 214 nm.

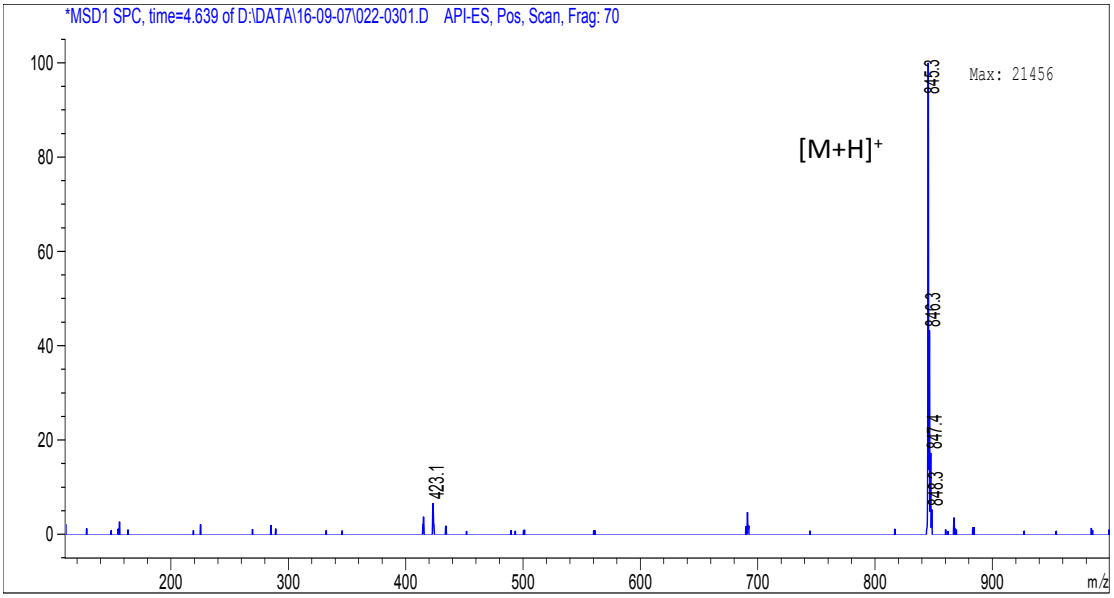
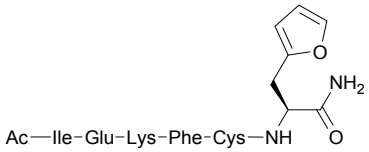


Figure 16: ESI-MS from LC-MS at t_R = 4.639 min (obs: 845.3 $[M+H]^+$; calc: 845.4).



1i

Chemical Formula: $C_{38}H_{56}N_8O_{10}S$
Exact Mass: 816,38401
Molecular Weight: 816,96384

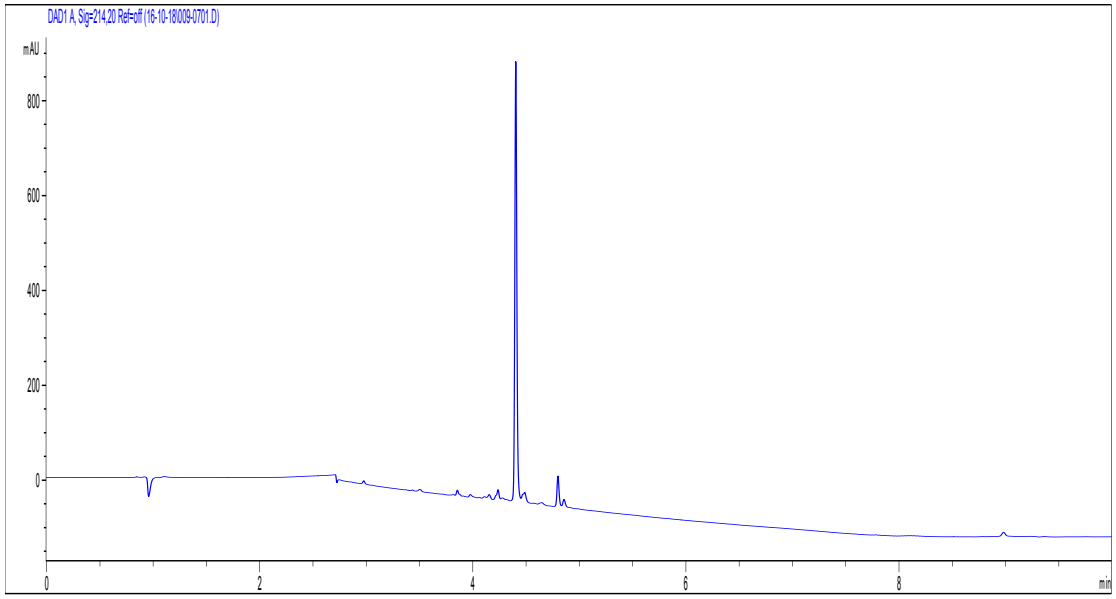


Figure 17: LC-MS chromatogram of the purified peptide **1i** at λ = 214 nm.

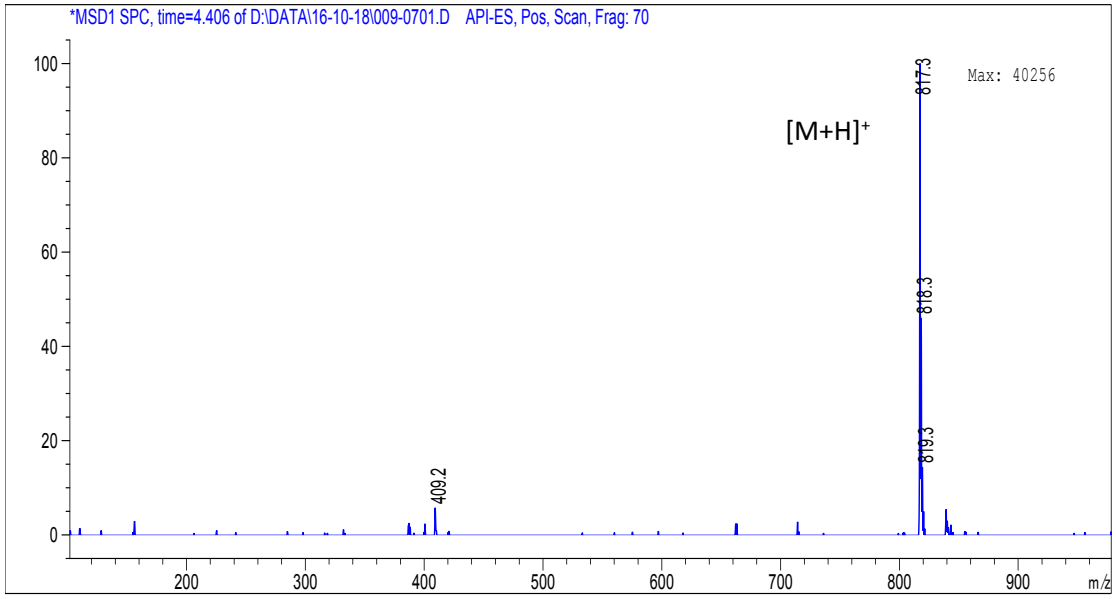
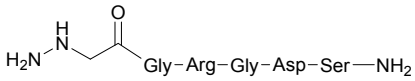


Figure 18: ESI-MS from the LC-MS at t_R = 4.406 min (obs: 817.3 $[M+H]^+$; calc: 817.4).



2

Chemical Formula: $C_{19}H_{35}N_{11}O_9$

Exact Mass: 561,26192

Molecular Weight: 561,54950

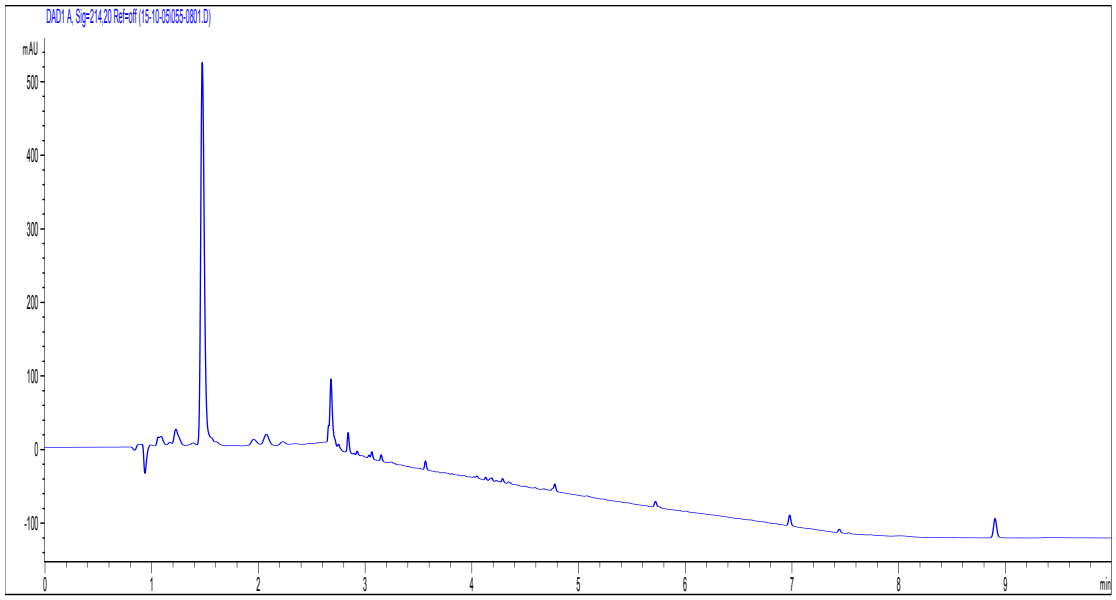


Figure 19: LC-MS chromatogram of the crude peptide **2** at λ = 214 nm.

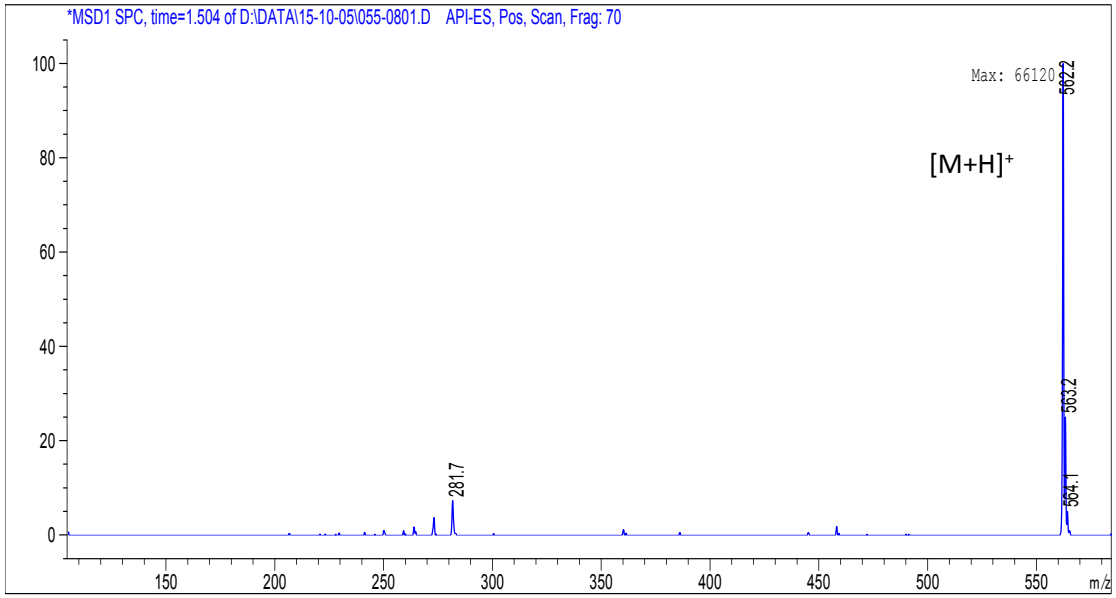
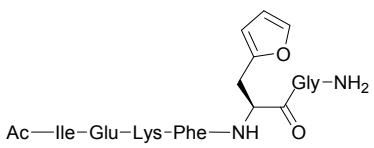


Figure 20: ESI-MS from LC-MS at t_R = 1.504 min (obs: 562.2 $[M+H]^+$; calc: 562.3).



4a

Chemical Formula: $C_{37}H_{54}N_8O_{10}$

Exact Mass: 770,39629

Molecular Weight: 770,88500

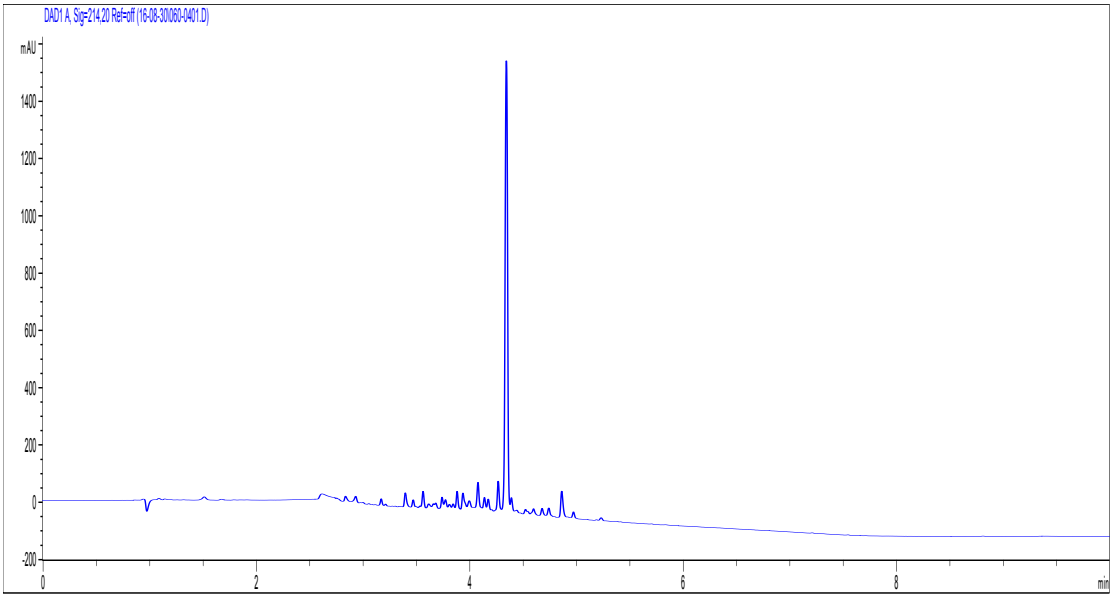


Figure 21: LC-MS chromatogram of the crude peptide **4a** at λ = 214 nm.

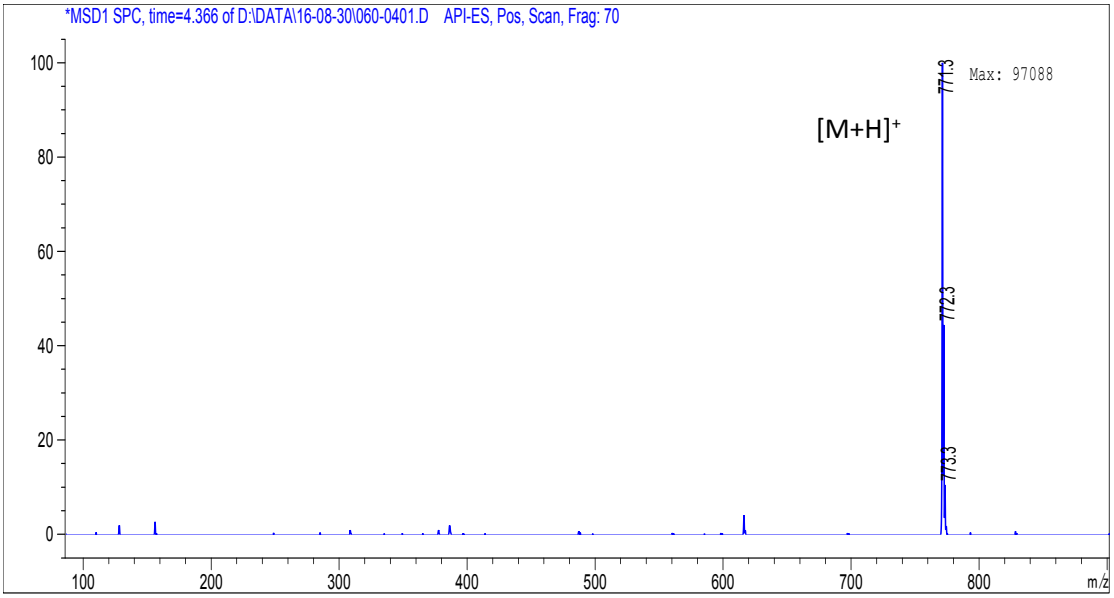
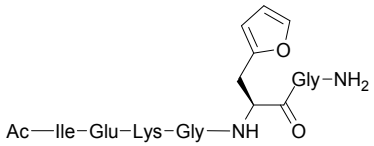


Figure 22: ESI-MS from LC-MS at t_R = 4.366 min (obs: 771.3 $[M+H]^+$; calc: 771.4).



4b

Chemical Formula: $C_{30}H_{48}N_8O_{10}$
Exact Mass: 680,34934
Molecular Weight: 680,76000

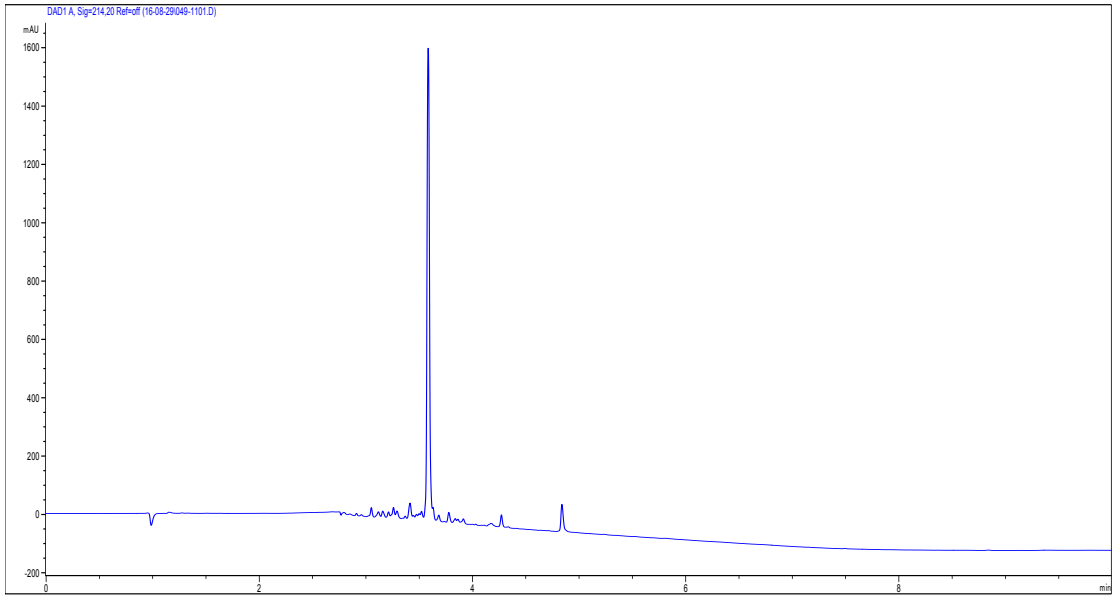


Figure 23: LC-MS chromatogram of the purified peptide **4b** at λ = 214 nm.

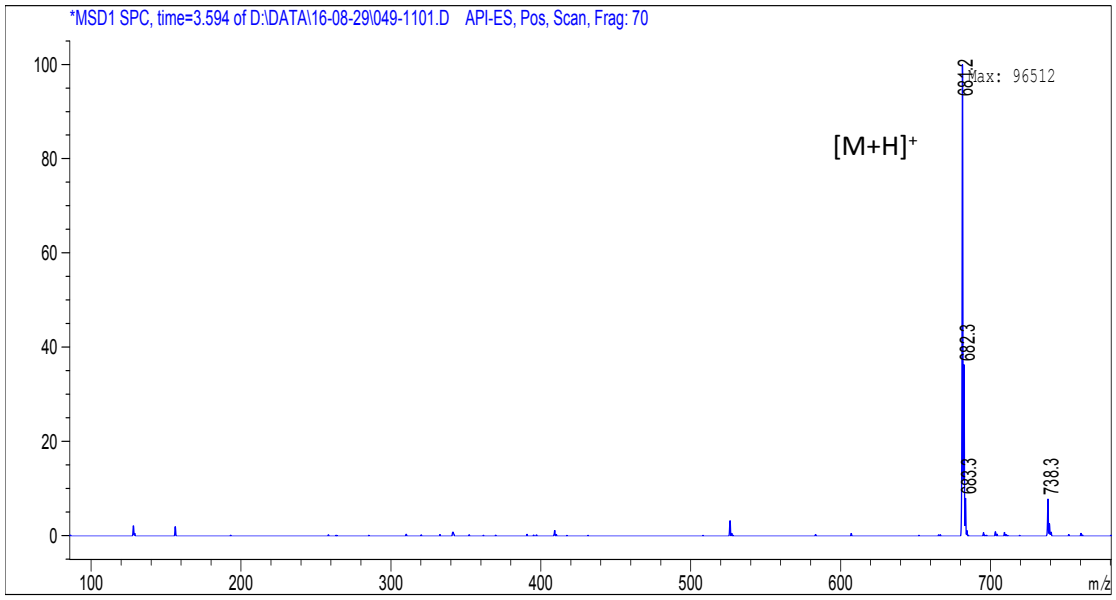
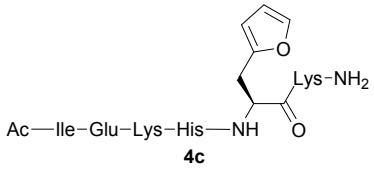


Figure 24: ESI-MS from the LC-MS at t_R = 3.594 min (obs: 681.2 $[M+H]^+$; calc: 681.4).



4c

Chemical Formula: $C_{38}H_{61}N_{11}O_{10}$
Exact Mass: 831,46029
Molecular Weight: 831,95864

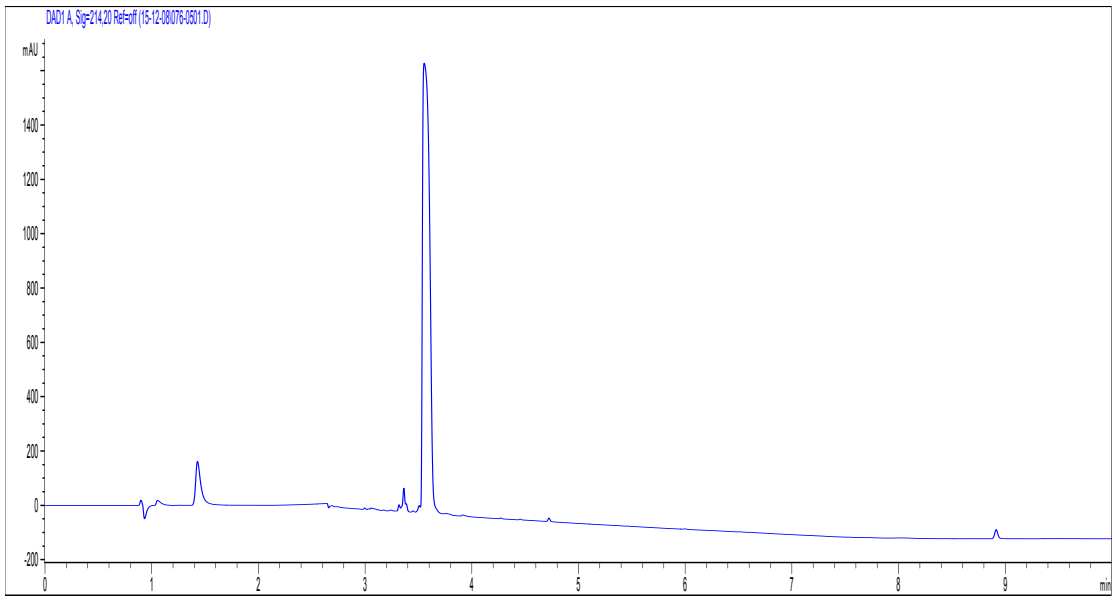


Figure 25: LC-MS chromatogram of the crude peptide **4c** at λ = 214 nm.

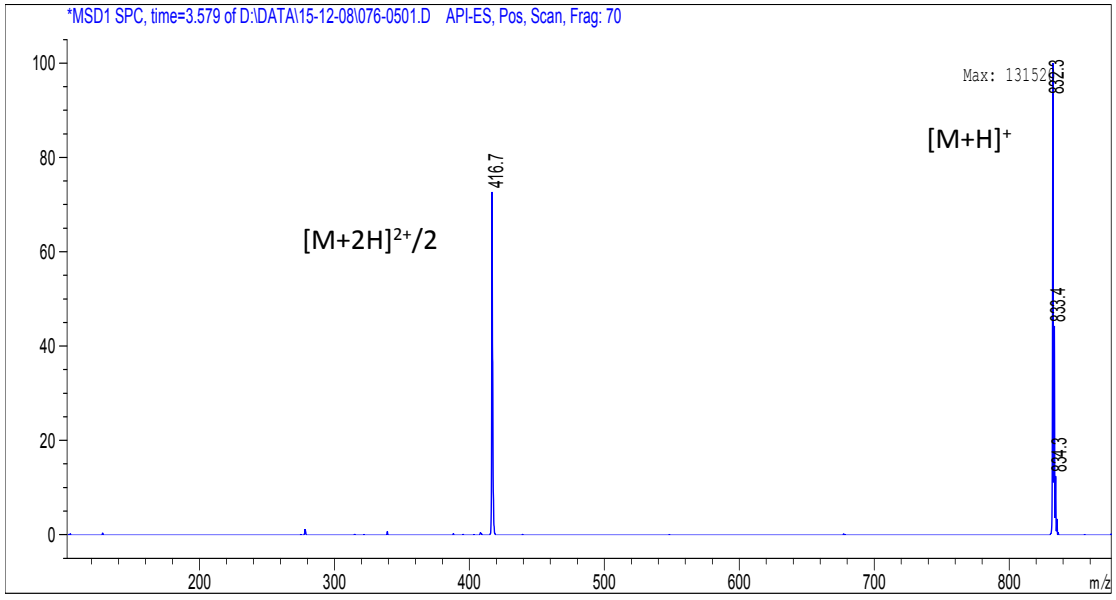
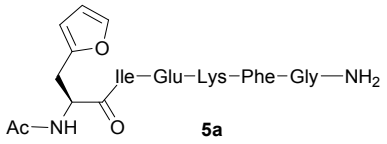


Figure 26: ESI-MS from LC-MS at t_R = 3.579 min (obs: 832.3 $[M+H]^+$; calc: 832.5).



5a

Chemical Formula: $C_{37}H_{54}N_8O_{10}$
Exact Mass: 770,39629
Molecular Weight: 770,88500

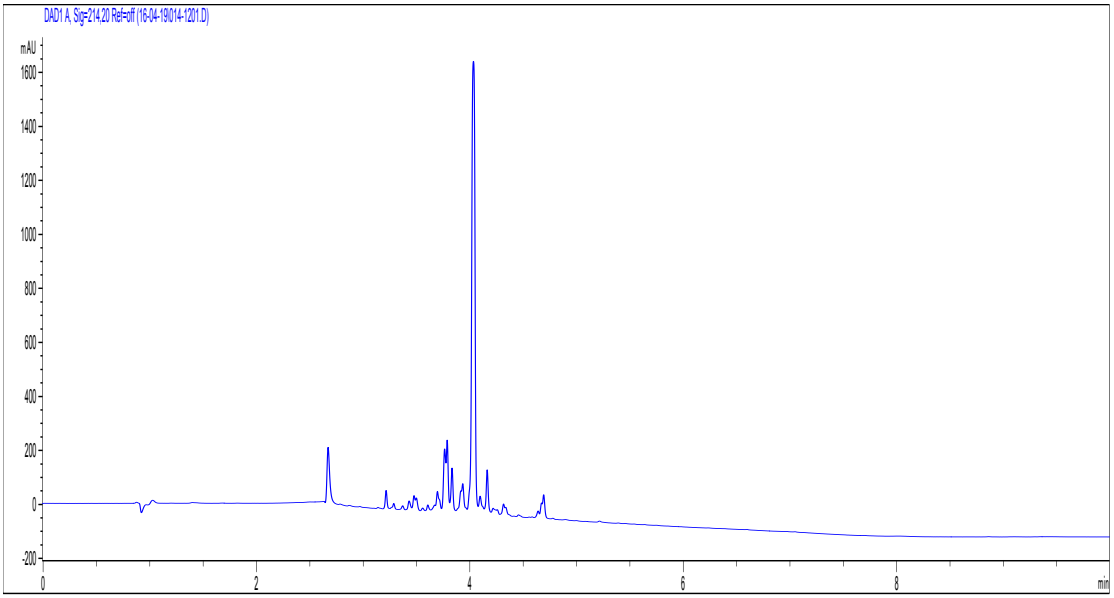


Figure 27: LC-MS chromatogram of the crude peptide **5a** at λ = 214 nm.

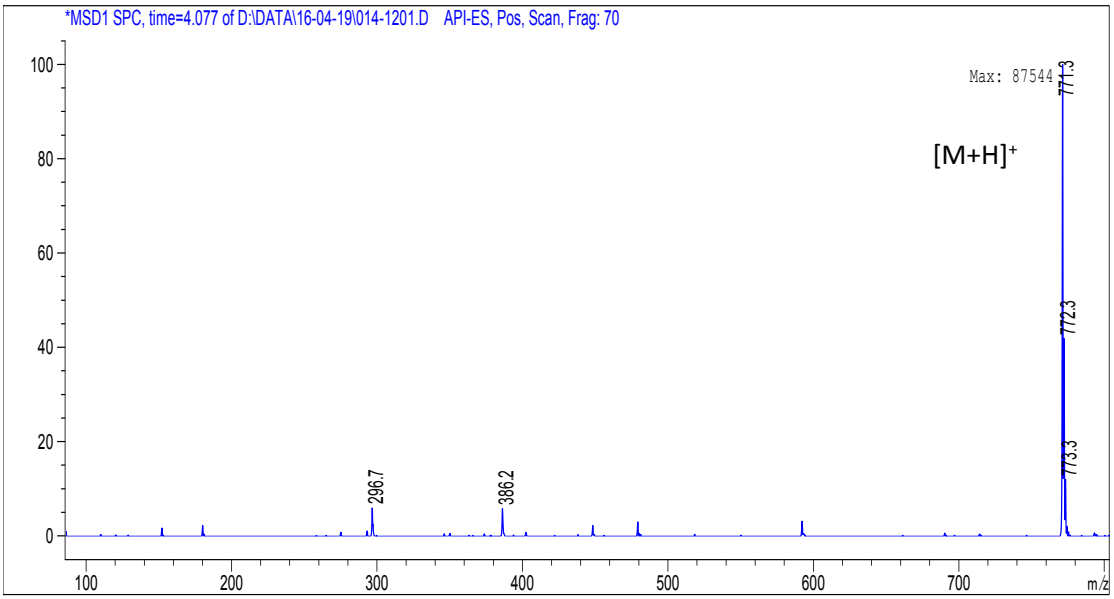
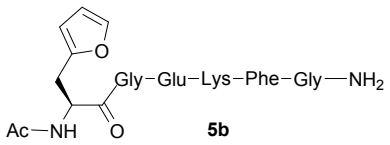


Figure 28: ESI-MS from LC-MS at t_R = 4.077 min (obs: 771.3 $[M+H]^+$; calc: 771.4).



5b

Chemical Formula: $C_{33}H_{46}N_8O_{10}$

Exact Mass: 714,33369

Molecular Weight: 714,77700

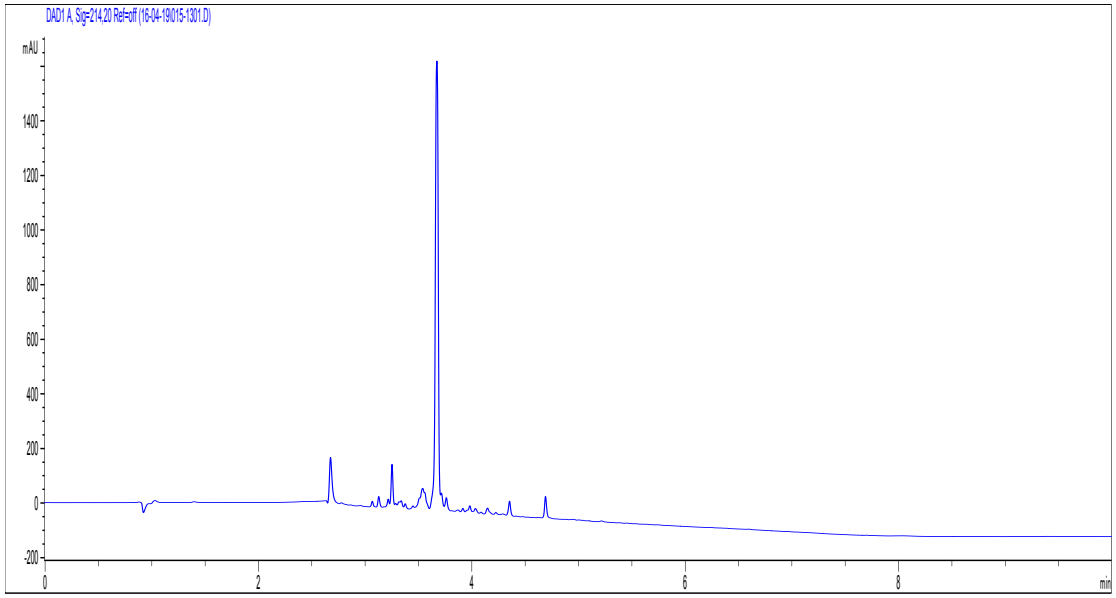


Figure 29: LC-MS chromatogram of the crude peptide **5b** at λ= 214 nm.

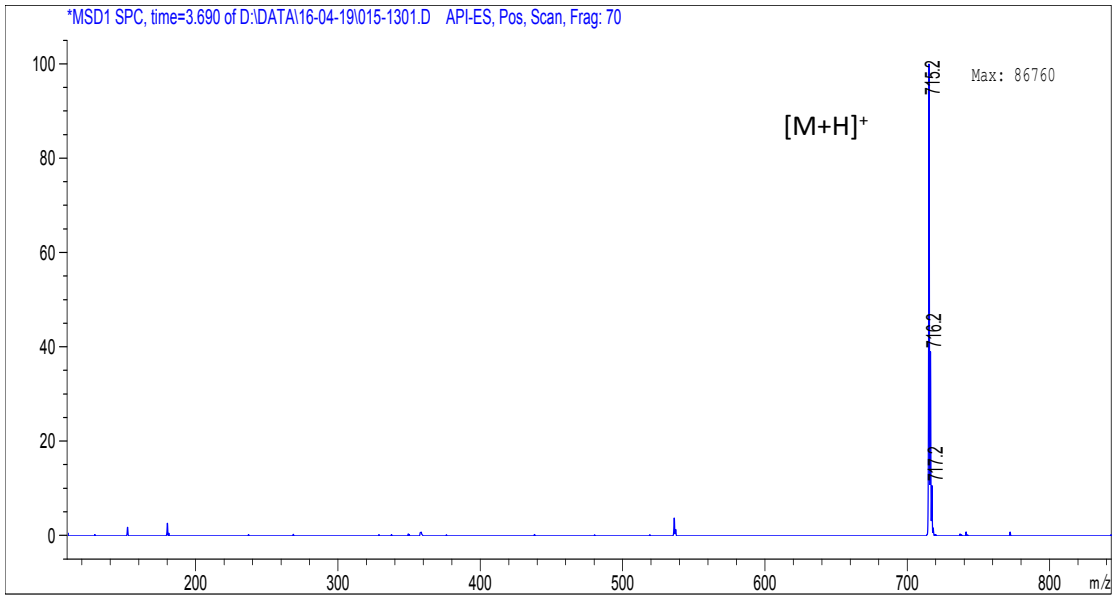
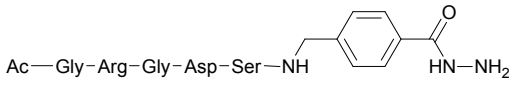


Figure 30: ESI-MS from LC-MS at t_R = 3.690 min (obs: 715.2 $[M+H]^+$; calc: 715.3).



8

Chemical Formula: $C_{27}H_{41}N_{11}O_{10}$
Exact Mass: 679,30379
Molecular Weight: 679,69200

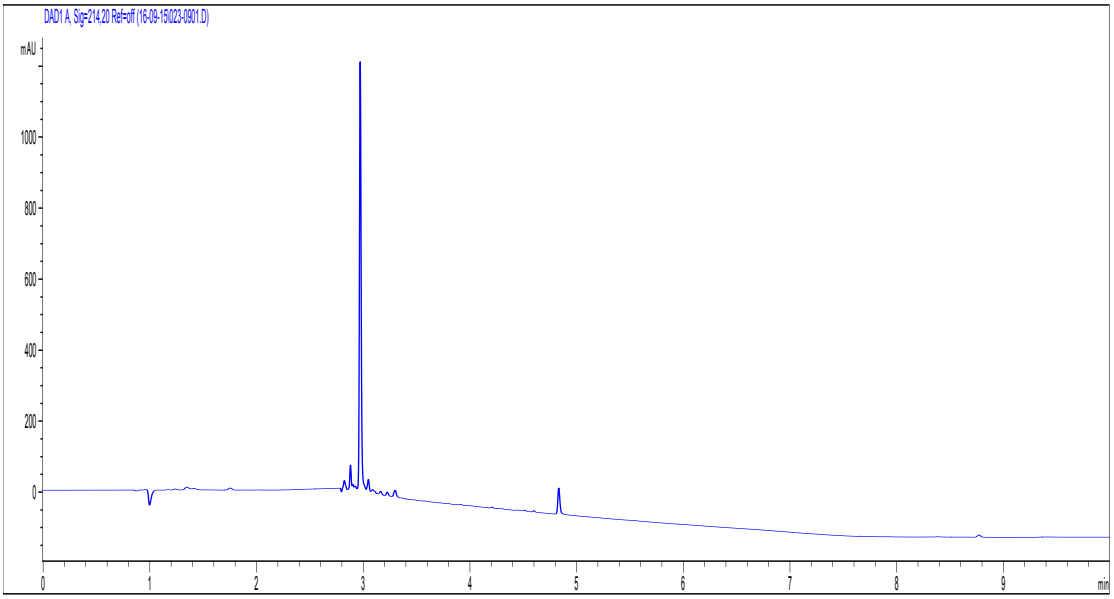


Figure 31: LC-MS chromatogram of the crude peptide **8** at λ = 214 nm.

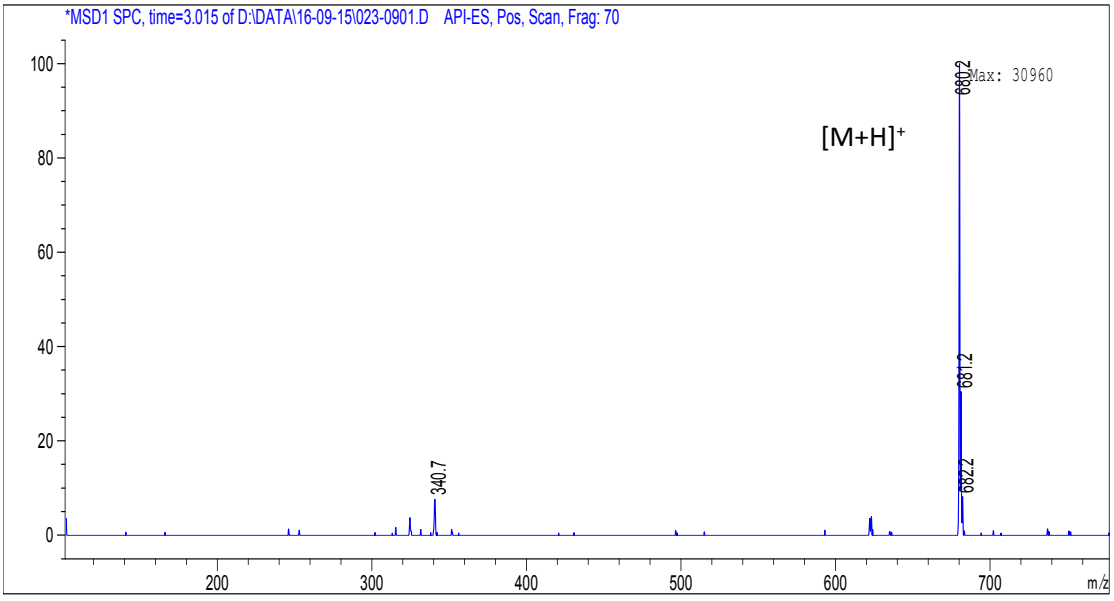
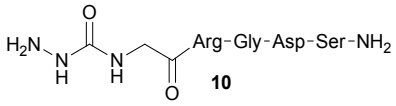


Figure 32: ESI-MS from LC-MS at t_R = 3.015 min (obs: 680.2 $[M+H]^+$; calc: 680.3).



10

Chemical Formula: $C_{18}H_{33}N_{11}O_9$

Exact Mass: 547,24627

Molecular Weight: 547,52292

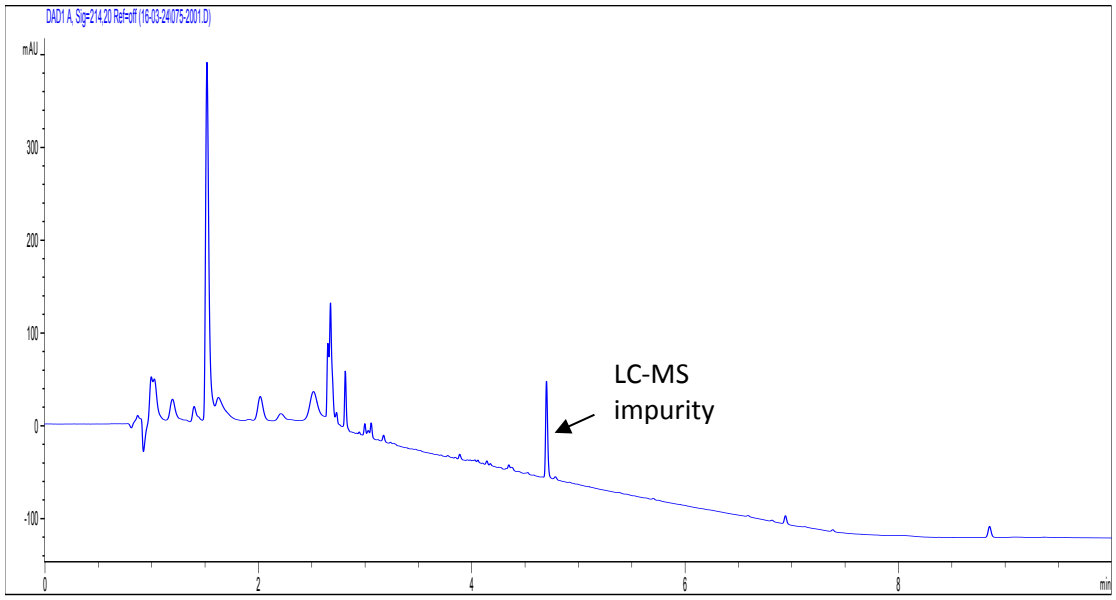


Figure 33: LC-MS chromatogram of the crude peptide **10** at λ = 214 nm.

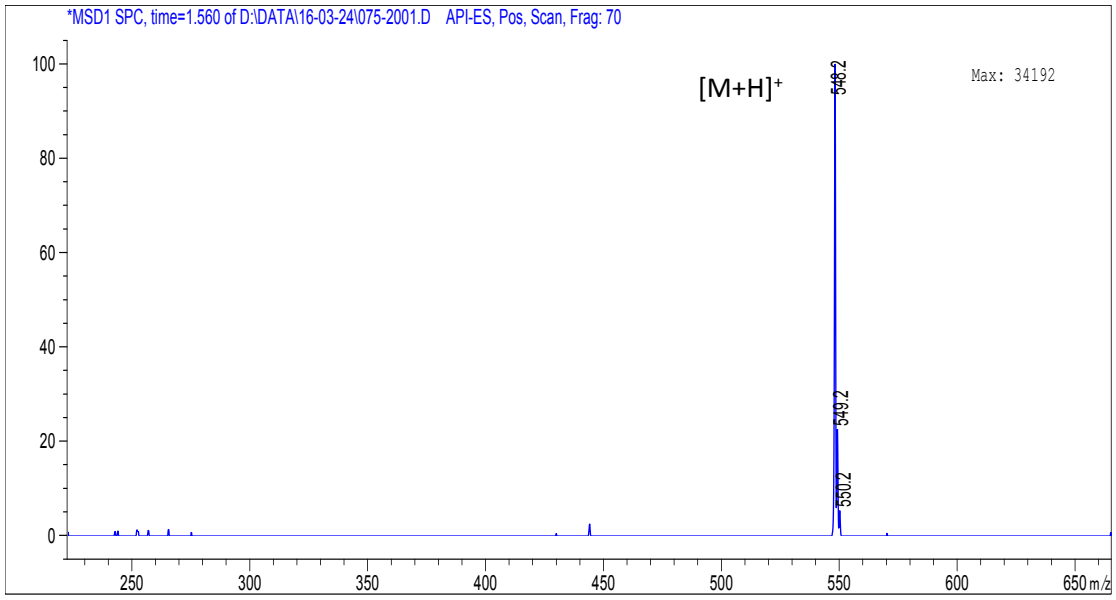
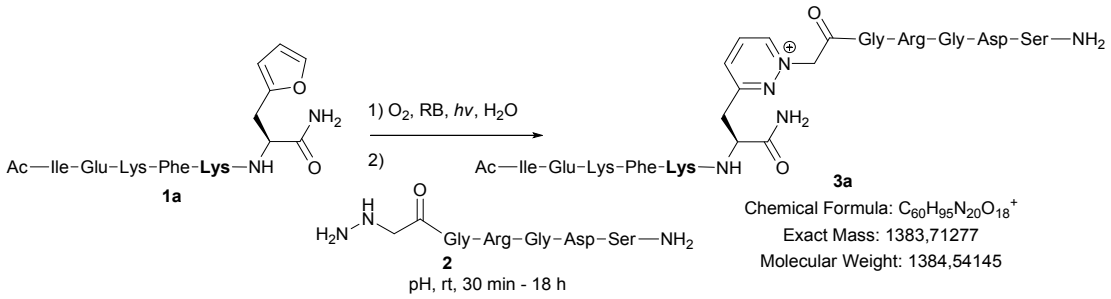


Figure 34: ESI-MS from LC-MS at t_R = 1.560 min (obs: 548.2 $[M+H]^+$; calc: 548.3).

Part D: Pyridazinium-based ligation reactions



Preparation of pyridazinium-based conjugates of C-terminal furan-peptide 1a with N-terminal hydrazine peptide 2

Furan-containing peptide **1a** was dissolved in MilliQ water containing catalytic amounts of rose Bengal, as photosensitizer (10 µM). The solution was cooled with an ice bath. Air was gently bubbled through the solution while it was irradiated with a Euromex 100 W cold light microscope lamp. After completion of the photooxidation reaction (40 min), hydrazine peptide **2** (1.0 equiv.) was added from aqueous stock solution of different pH values (2.9, 4.7, 5.8 and 7.2) and each reaction was shaken for 30 min to 18 h. The reactions were monitored by RP-HPLC and the products were analyzed by MALDI-TOF analysis.

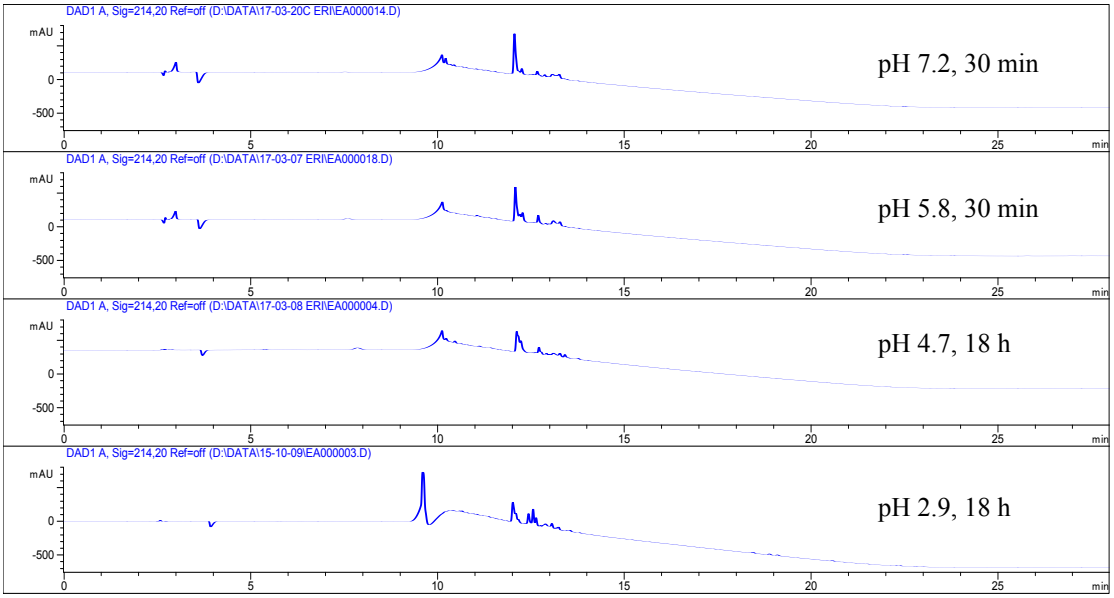
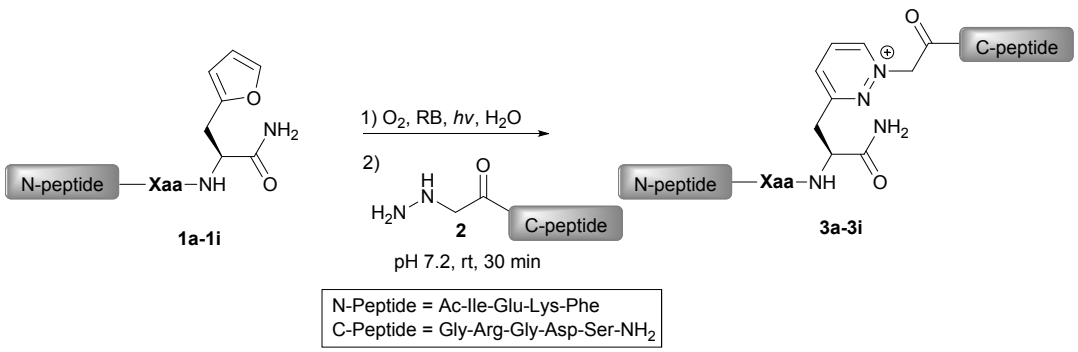
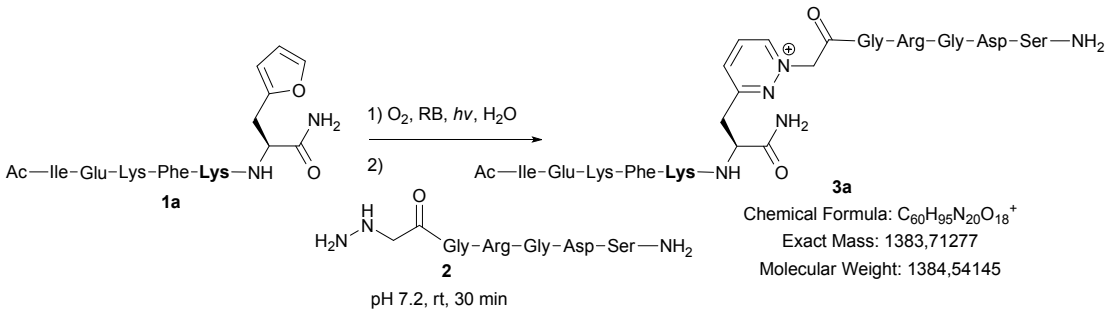


Figure 35: Crude RP-HPLC chromatograms demonstrating the formation of the conjugate. Furan-peptide **1a** was photooxidised for 40 min and then hydrazine peptide was added from stock solutions of different pH values. Peptide **2** in H_2O: pH 2.9. Buffers used for the preparation of hydrazine peptide stock solutions: 0.1 M acetate buffer pH 4.7 and 1.0 M phosphate buffer pH 5.8 and 7.2.



General procedure for the preparation of pyridazinium-based conjugates of C-terminal furan-peptides with N-terminal hydrazine peptide 2

Furan-containing peptides (**1a-1i**) were dissolved in MilliQ water containing catalytic amounts of rose Bengal, as photosensitizer (10 μM). The solutions were cooled with an ice bath. Air was gently bubbled through the solution while it was irradiated with a Euromex 100 W cold light microscope lamp. After completion of the photooxidation reaction (15-40 min), hydrazine peptide **2** (1.0 equiv.) was added from 1.0 M phosphate buffer stock solution of pH 7.2 and the reaction solution was shaken for 30 min. The reactions were monitored by RP-HPLC and the products were analyzed by MALDI-TOF analysis.



Preparation of pyridazinium-based conjugate 3a

The reaction was accomplished according to the general experimental procedure described above, utilizing peptide **1a** (0.6 μmol, added from aqueous stock solution of 18.37 mM) in water (1.2 mL), which was photooxidized for 40 min followed by addition of peptide **2** (0.6 μmol, added from a 1.0 M phosphate buffer stock solution of 13.76 mM, pH 7.2). The reaction progress was monitored by RP-HPLC and the product was analyzed by MALDI-TOF analysis. The ligated product was formed after 30 min. MALDI-TOF spectrum of the collected peak at t_R = 12.054 min demonstrated the formation of the desired conjugate.

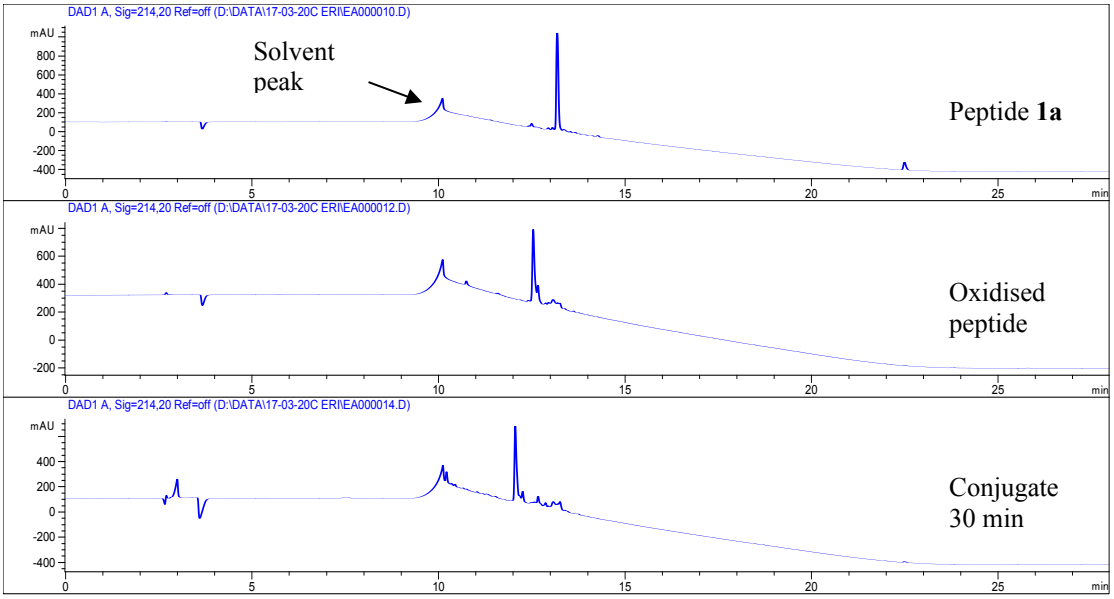


Figure 36: Crude RP-HPLC chromatograms demonstrating the progress of the reaction sequence (**1a**→**3a**) at λ = 214 nm.

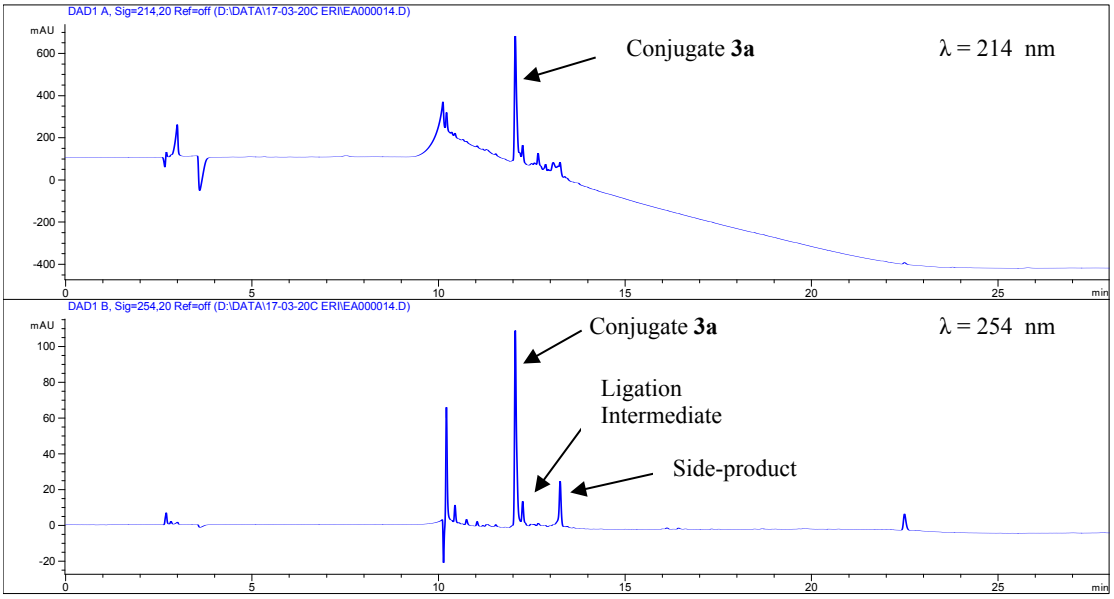


Figure 37: Crude RP-HPLC chromatograms demonstrating the crude reaction mixture (**2**, 30 min) at λ = 214 nm and λ = 254 nm.

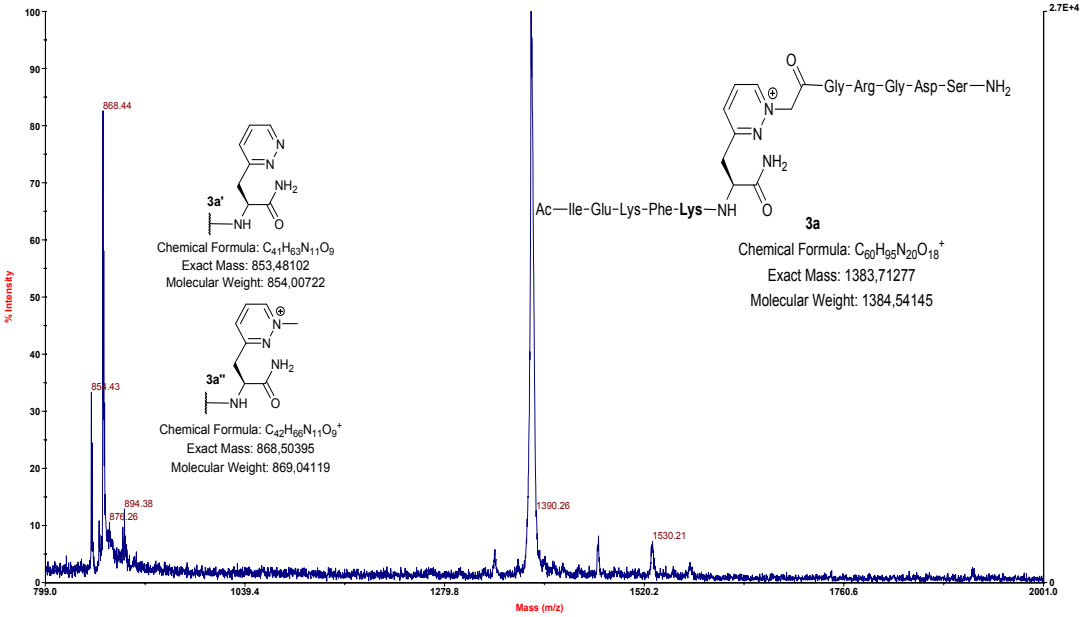


Figure 38: MALDI-TOF spectrum of the collected peak at t_R = 12.054 min (**2**, 30 min) demonstrating the formation of conjugate **3a** (obs: 1383.98 M^+; calc: 1383.71). Fragmentation products occurred during MALDI-TOF ionization: peptide **3a'** (obs: 854.43 $[M+H]^+$; calc: 854.49), peptide **3a''** (obs: 868.44 M^+; calc: 868.50).

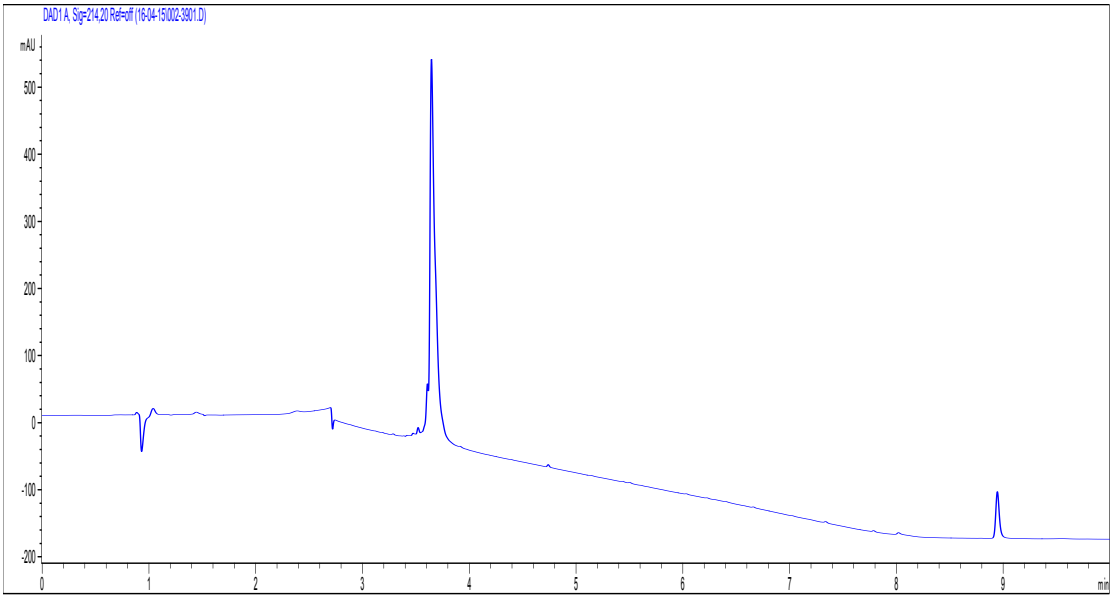
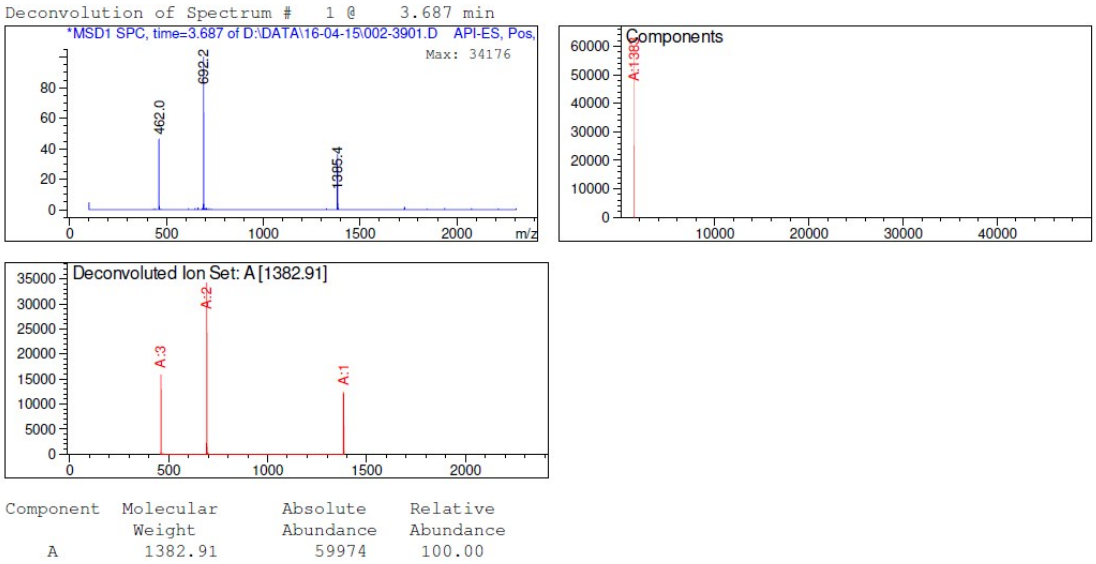


Figure 39: LC-MS chromatogram of the collected peptide **3a** (at t_R = 12.054 min through HPLC analysis) at λ =214 nm.



Component	Molecular Weight	Absolute Abundance	Relative Abundance
A	1382.91	59974	100.00

Figure 40: ESI-MS from LC-MS (obs: 1382.91 M^+; calc: 1383.71).

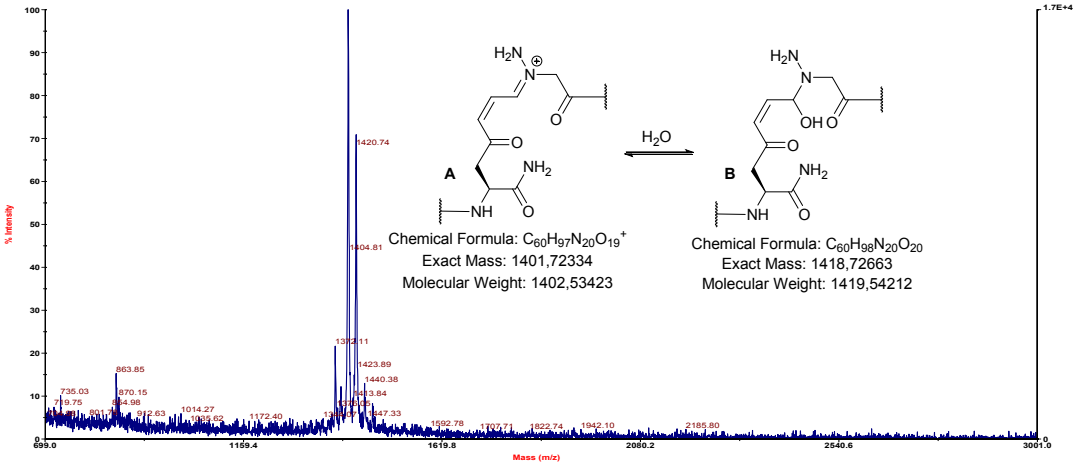


Figure 41: MALDI-TOF spectrum of the collected peak at t_R = 12.251 min (**2**, 30 min) demonstrating the formation of the proposed ligation intermediate structures of type **A** (obs: 1402.04 M^+; calc: 1401.72) and type **B** (obs: 1420.74 $[M+H]^+$; calc: 1419.73).

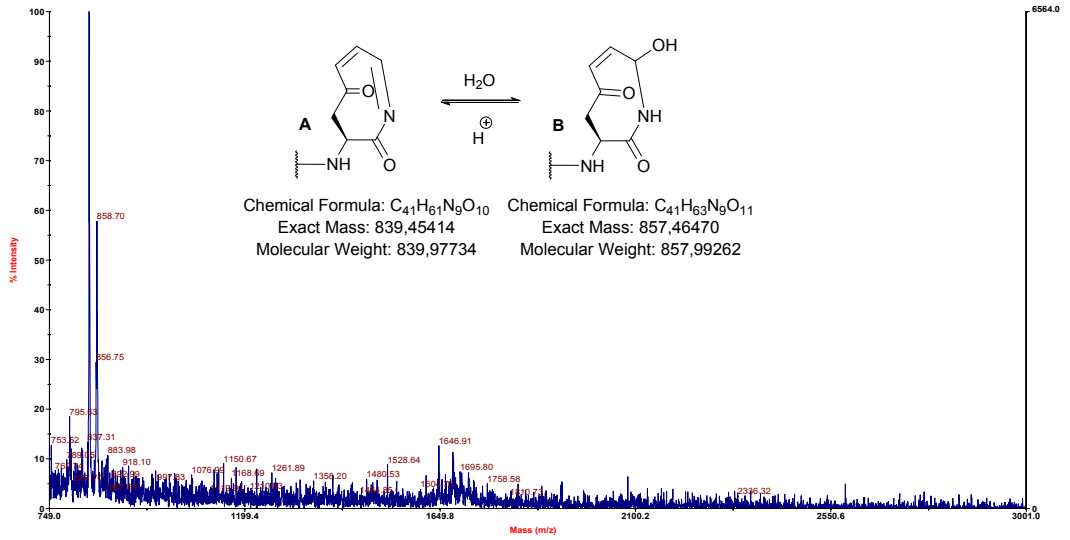


Figure 42: MALDI-TOF spectrum of the collected peak at t_R = 13.257 (peptide **A**, obs: 840.73 $[M+H]^+$; calc: 840.46, peptide **B**, obs: 858.70 $[M+H]^+$; calc: 858.47).[3]

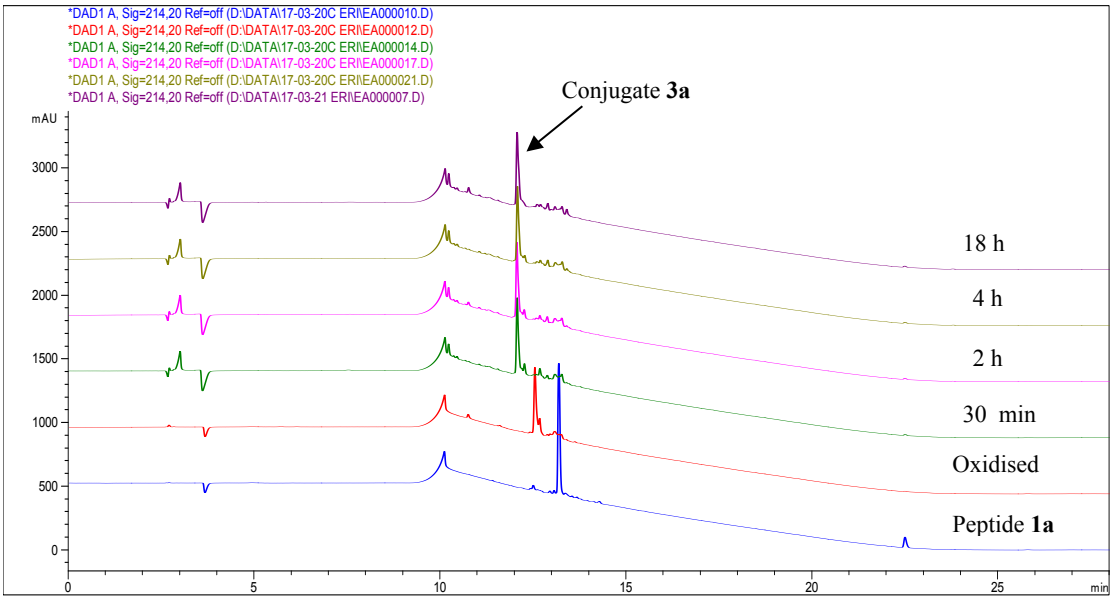
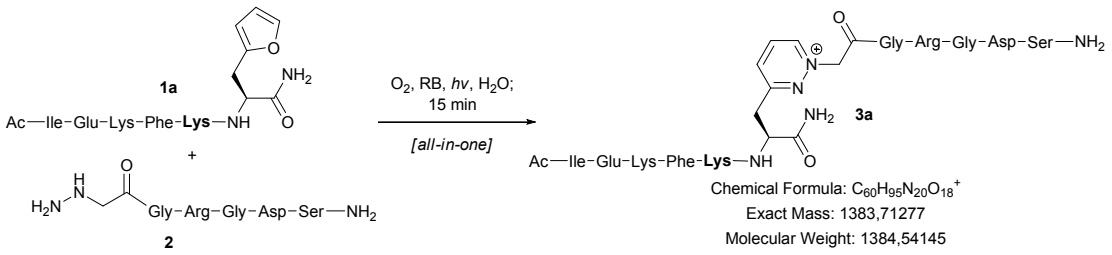


Figure 43: Crude RP-HPLC chromatograms demonstrating the stability of the pyridazinium-based conjugate **3a** over time at rt.

[3] (a) O. El-Mahdi and O. Melnyk, *Bioconjug. Chem.*, 2013, **24**, 735–765. (b) C. E. Murar, F. Thuaud and J. W. Bode, *J. Am. Chem. Soc.*, 2014, **136**, 18140–18148.



A mixture of peptide **1a** (0.6 µmol, added from aqueous stock solution of 22.8 mM) with hydrazine peptide **2** (3.0 µmol, added from 1.0 M phosphate buffer stock solution of 18.0 mM, pH 7.2) in water (1.2 mL) containing 10 µM of Rose Bengal, was photooxidized for 15 min with the instant formation of ligated product **3a**. The reaction was left to proceed further at rt and reached almost full conversion after 6 h.

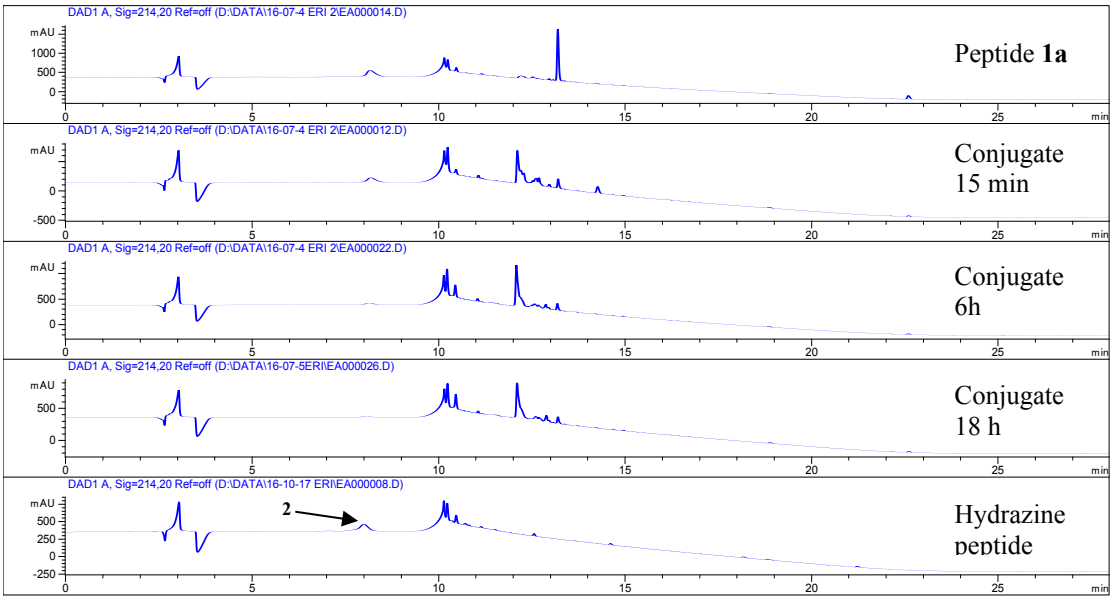
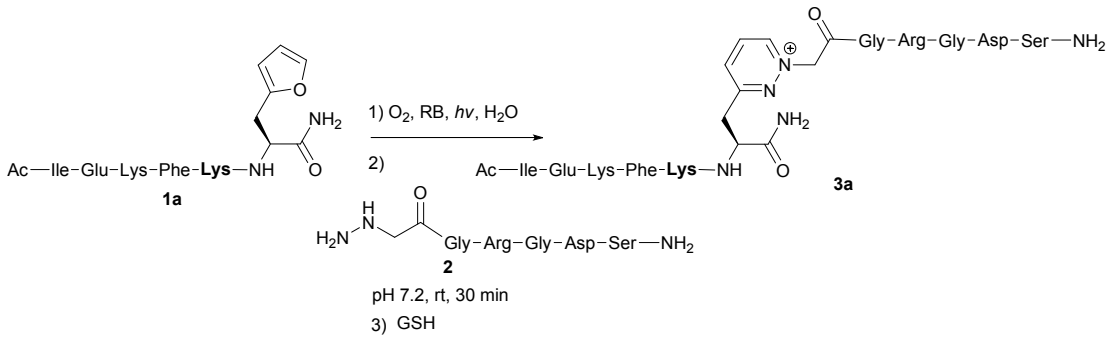


Figure 44: Crude RP-HPLC chromatograms demonstrating the progress of the reaction sequence (**1a→3a**) at λ = 214 nm.



The reaction was accomplished according to the general experimental procedure described above, utilizing the peptide **1a** (0.6 µmol, added from aqueous stock solution of 22.8 mM) in water (1.2 mL), which was photooxidized for 40 min followed by addition of peptide **2** (0.6 µmol, added from 1.0 M phosphate buffer stock solution of

18.0 mM, pH 7.2). After 30 min of the reaction, GSH (6.0 µmol) was added to the crude reaction and possible degradation of the conjugate was monitored over time at rt. The reaction progress was monitored by RP-HPLC and the product was analyzed by MALDI-TOF analysis. No obvious degradation was observed after 72 hours.

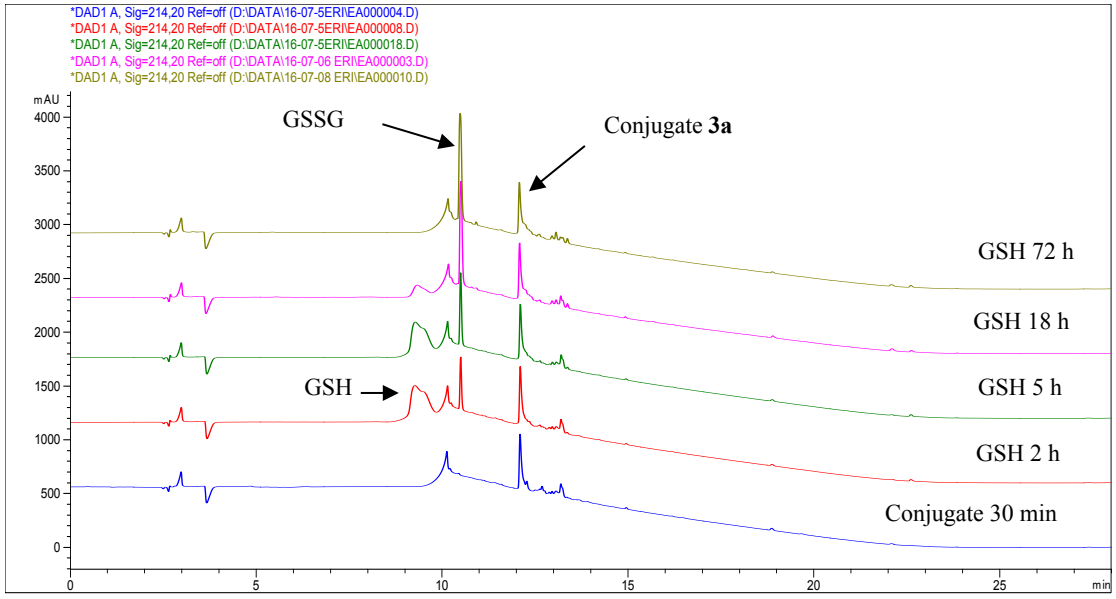


Figure 45: Crude RP-HPLC chromatograms demonstrating the stability of the pyridazinium-based conjugate **3a** over time at rt after the addition of GSH.

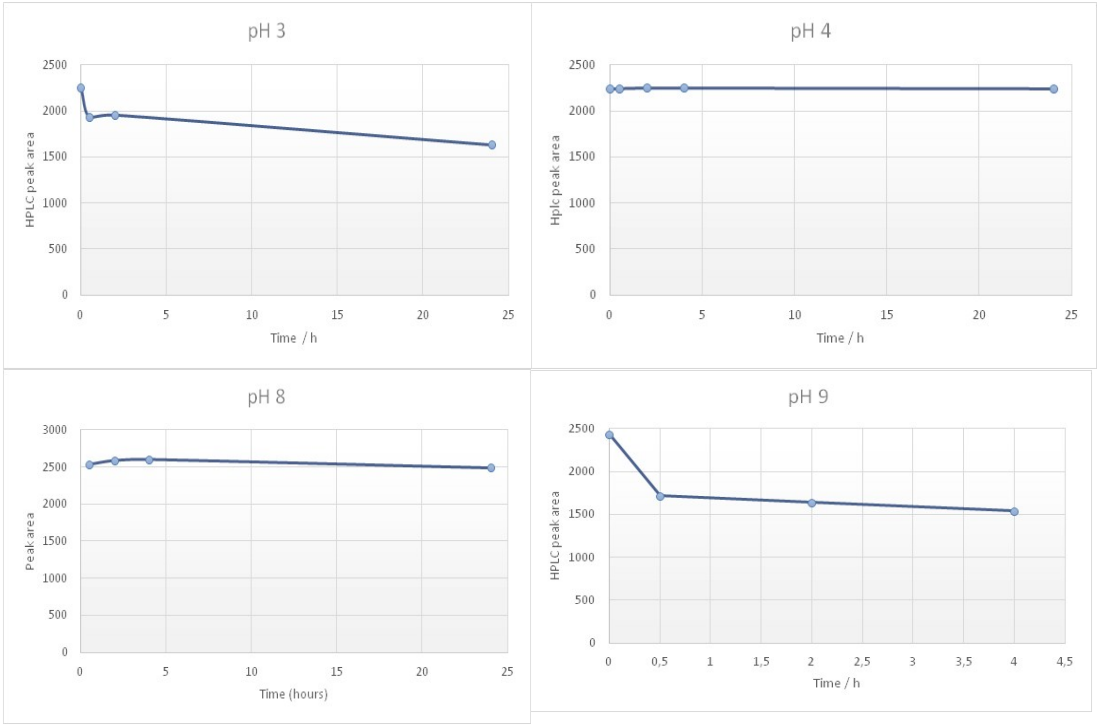
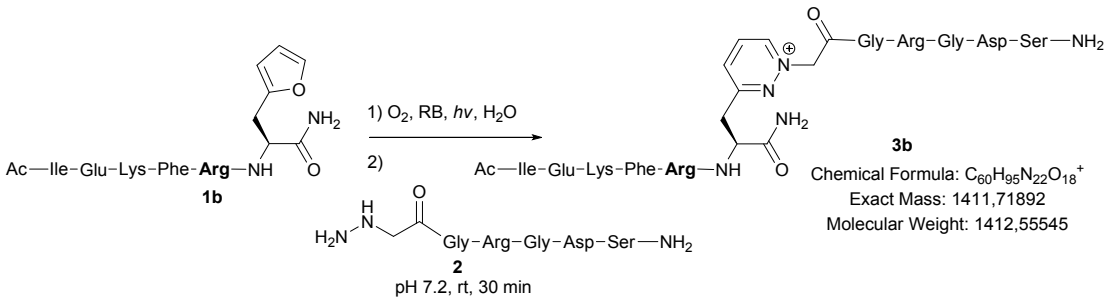


Figure 46: Stability test of ligated product **3a** in different pH values over time.



Preparation of pyridazinium-based conjugate 3b

The reaction was accomplished according to the general experimental procedure described above, utilizing the peptide **1b** (0.6 µmol, added from aqueous stock solution of 25 mM) in water (1.2 mL), which was photooxidized for 40 min followed by addition of peptide **2** (0.6 µmol, added from 1.0 M phosphate buffer stock solution of 13.76 mM, pH 7.2). The reaction progress was monitored by RP-HPLC and the product was analyzed by MALDI-TOF analysis. The desired conjugate was formed in 30 min. MALDI-TOF spectrum of the collected peak at t_R = 12.177 min demonstrated the formation of the desired conjugate.

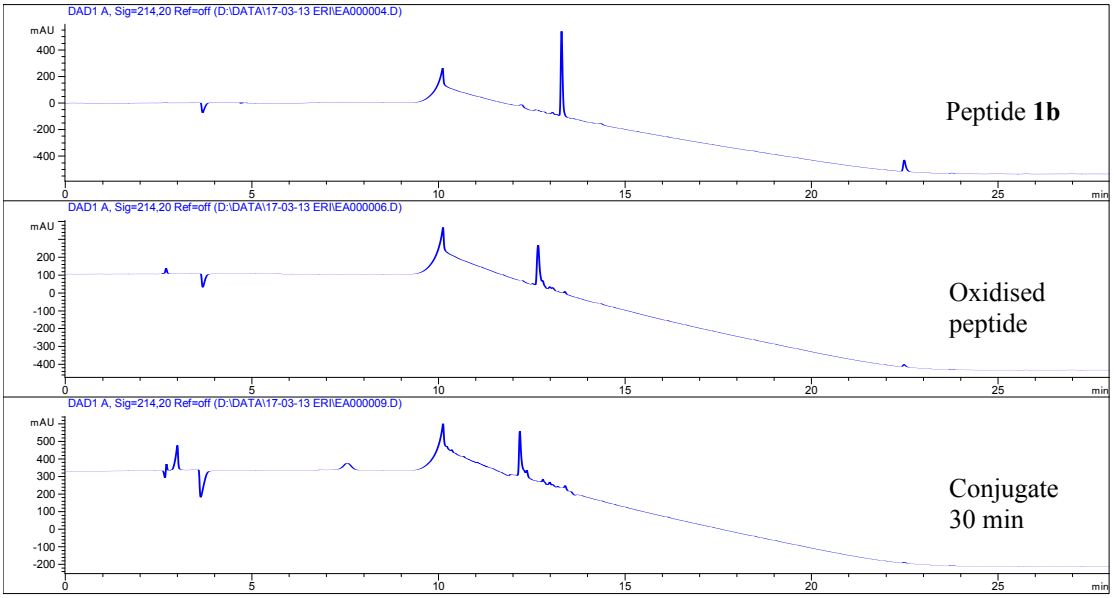


Figure 47: Crude RP-HPLC chromatograms demonstrating the progress of the reaction sequence (**1b**→**3b**) at λ = 214 nm.

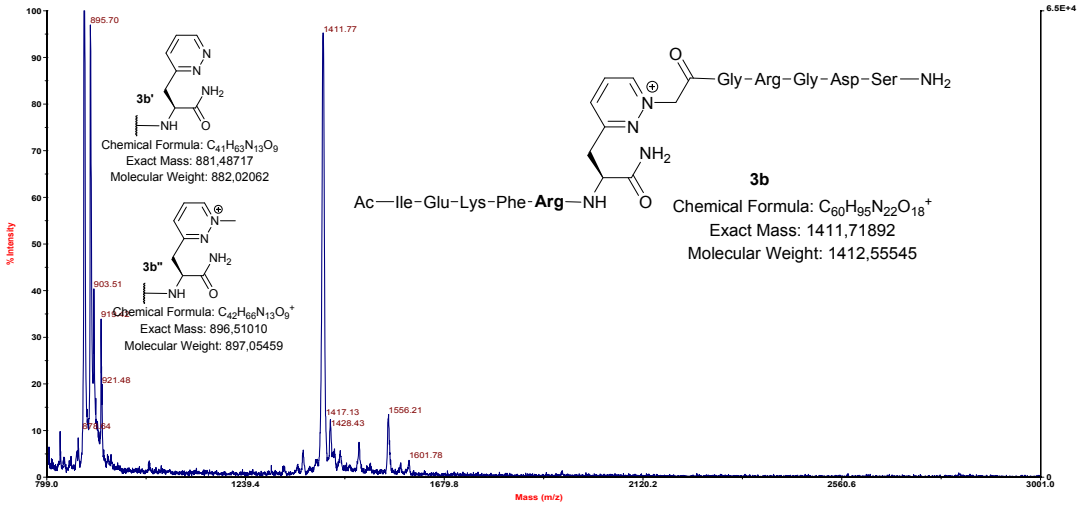
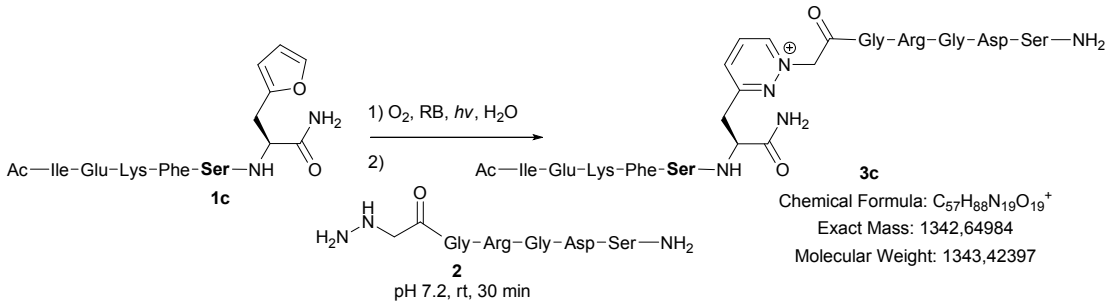


Figure 48: MALDI-TOF spectrum of the collected peak at t_R = 12.177 min (**2**, 30 min) demonstrating the formation of peptide **3b** (obs: 1411.77 M^+; calc: 1411.72). Fragmentation products occurred during MALDI-TOF ionization: peptide **3b'** (obs: 881.77 $[M+H]^+$; calc: 882.49), peptide **3b''** (obs: 895.70 M^+; calc: 896.51).



Preparation of pyridazinium-based conjugate 3c

The reaction was accomplished according to the general experimental procedure described above, utilizing the peptide **1c** (0.6 µmol, added from aqueous stock solution of 23.1 mM) in water (1.2 mL), which was photooxidized for 40 min followed by addition of peptide **2** (0.6 µmol, added from 1.0 M phosphate buffer stock solution of 39.12 mM, pH 7.2). The reaction progress was monitored by RP-HPLC and the product was analyzed by MALDI-TOF analysis. The desired conjugate was formed in 30 min. MALDI-TOF spectrum of the collected peak at t_R = 12.021 min demonstrated the formation of the desired conjugate.

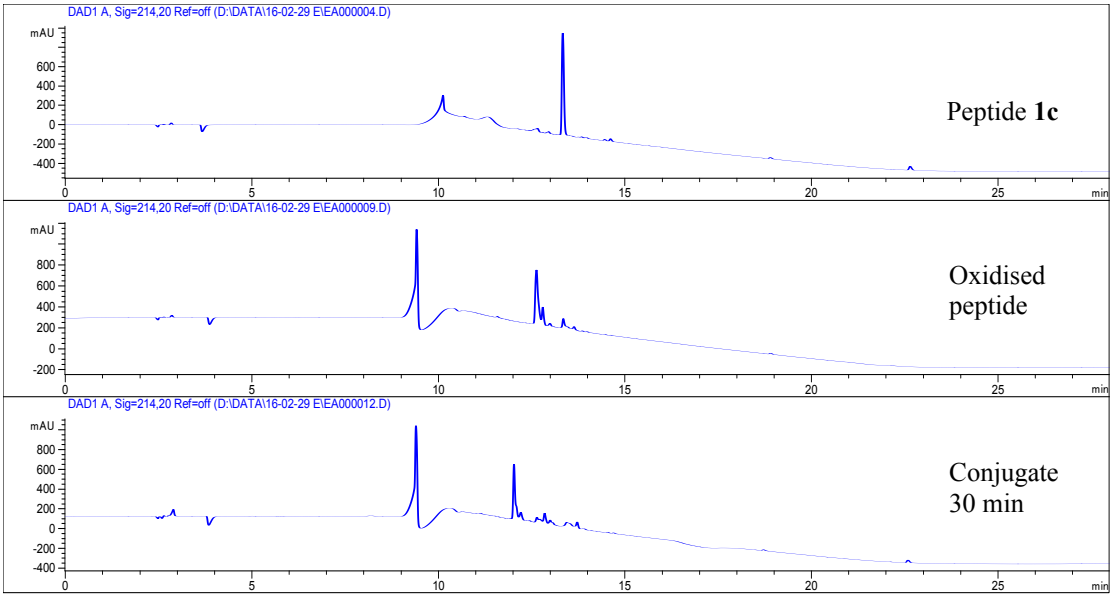


Figure 49: Crude RP-HPLC chromatograms demonstrating the progress of the reaction sequence (**1c→3c**) at λ = 214 nm.

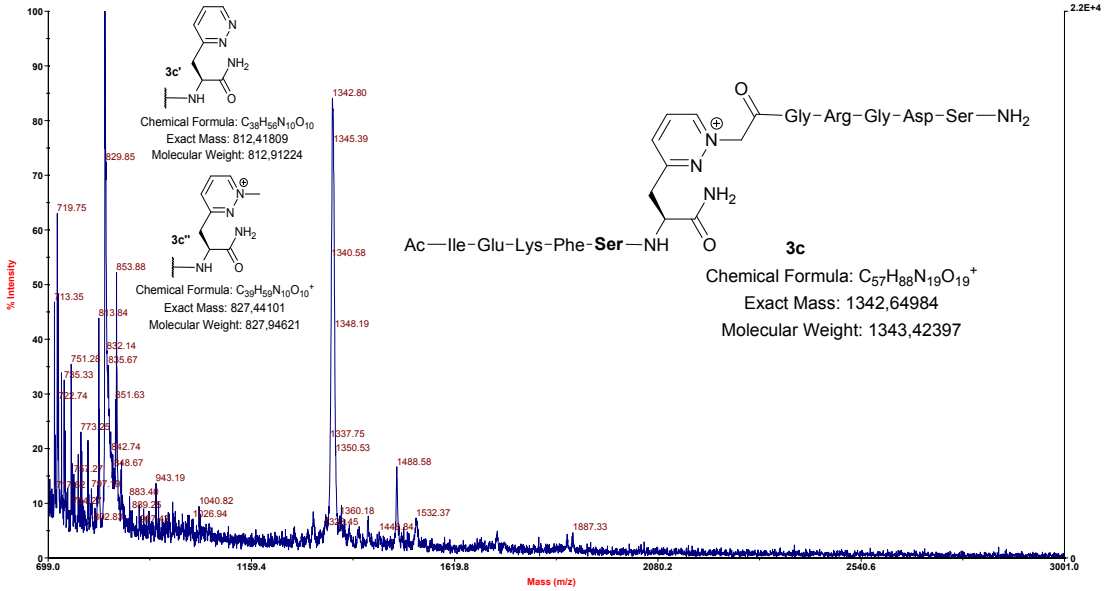
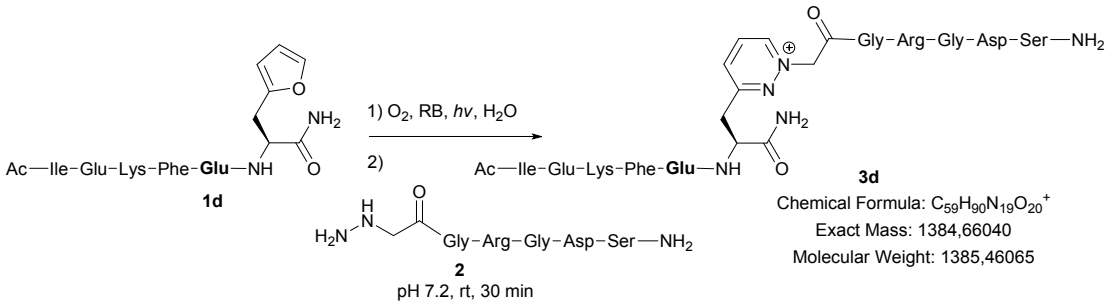


Figure 50: MALDI-TOF spectrum of the collected peak at t_R = 12.021 min (**2**, 30 min) demonstrating the formation of peptide **3c** (obs: 1342.80 M^+; calc: 1342.65). Fragmentation products occurred during MALDI-TOF ionization: peptide **3c'** (obs: 813.84 $[M+H]^+$; calc: 813.43), peptide **3c''** (obs: 827.79 M^+; calc: 827.44).



Preparation of pyridazinium-based conjugate 3d

The reaction was accomplished according to the general experimental procedure described above, utilizing the peptide **1d** (0.6 µmol, added from aqueous stock solution of 14.65 mM containing 9% DMSO) in water (1.2 mL), which was photooxidized for 40 min followed by addition of peptide **2** (0.6 µmol, added from 1.0 M phosphate buffer stock solution of 39.12 mM, pH 7.2). The reaction progress was monitored by RP-HPLC and the product was analyzed by MALDI-TOF analysis. The desired conjugate was formed in 30 min. MALDI-TOF spectrum of the collected peak at t_R = 12.268 min demonstrated the formation of the desired conjugate.

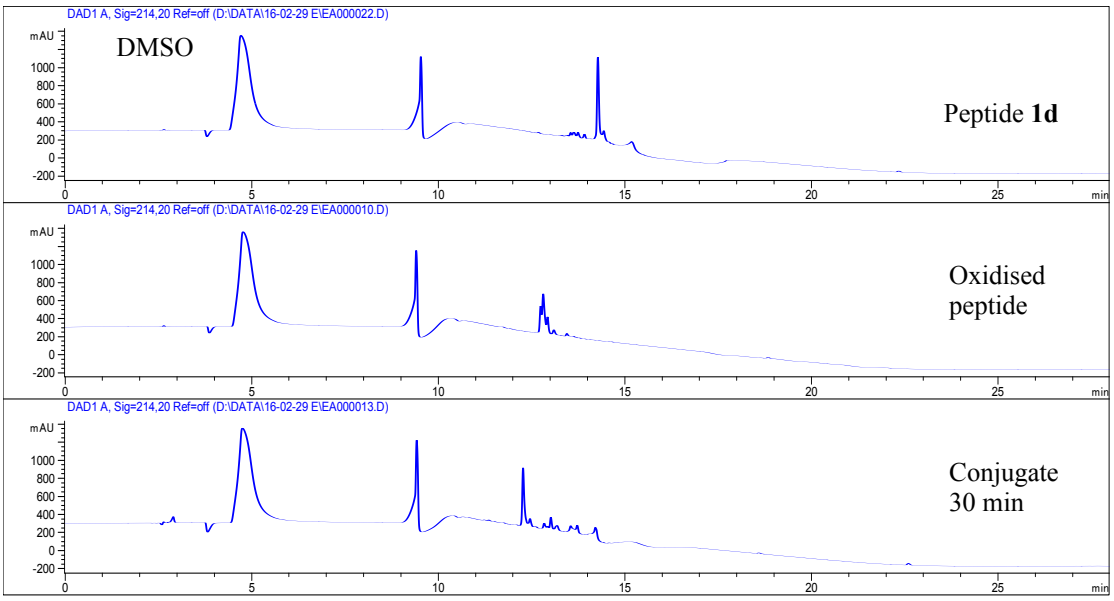


Figure 51: Crude RP-HPLC chromatograms demonstrating the progress of the reaction sequence (**1d**→**3d**) at λ = 214 nm.

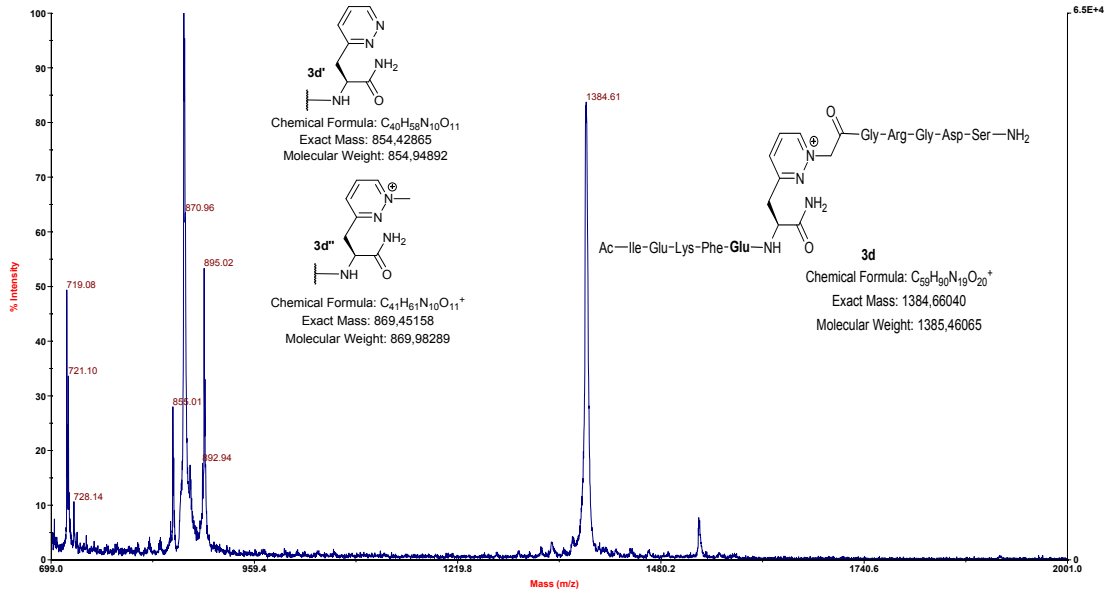
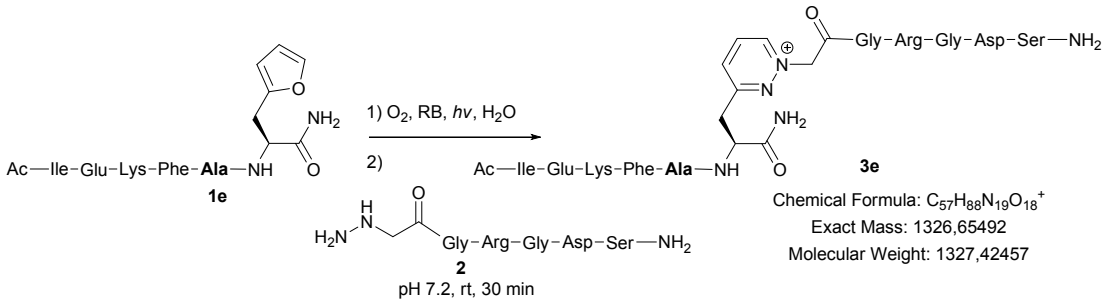


Figure 52: MALDI-TOF spectrum of the collected peak at t_R = 12.268 min (**2**, 30 min) demonstrating the formation of peptide **3d** (obs: 1384.61 M^+; calc: 1384.66). Fragmentation products occurred during MALDI-TOF ionization: peptide **3d'** (obs: 855.01 $[M+H]^+$; calc: 855.44), peptide **3d''** (obs: 869.06 M^+; calc: 869.45).



Preparation of pyridazinium-based conjugate 3e

The reaction was accomplished according to the general experimental procedure described above, utilizing the peptide **1e** (0.6 µmol, added from aqueous stock solution of 22.43 mM containing 2% DMSO) in water (1.2 mL), which was photooxidized for 40 min followed by addition of peptide **2** (0.6 µmol, added from 1.0 M phosphate buffer stock solution of 13.76 mM, pH 7.2). The reaction progress was monitored by RP-HPLC and the product was analyzed by MALDI-TOF analysis. The desired conjugate was formed in 30 min. MALDI-TOF spectrum of the collected peak at t_R = 12.508 demonstrated the formation of the desired conjugate.

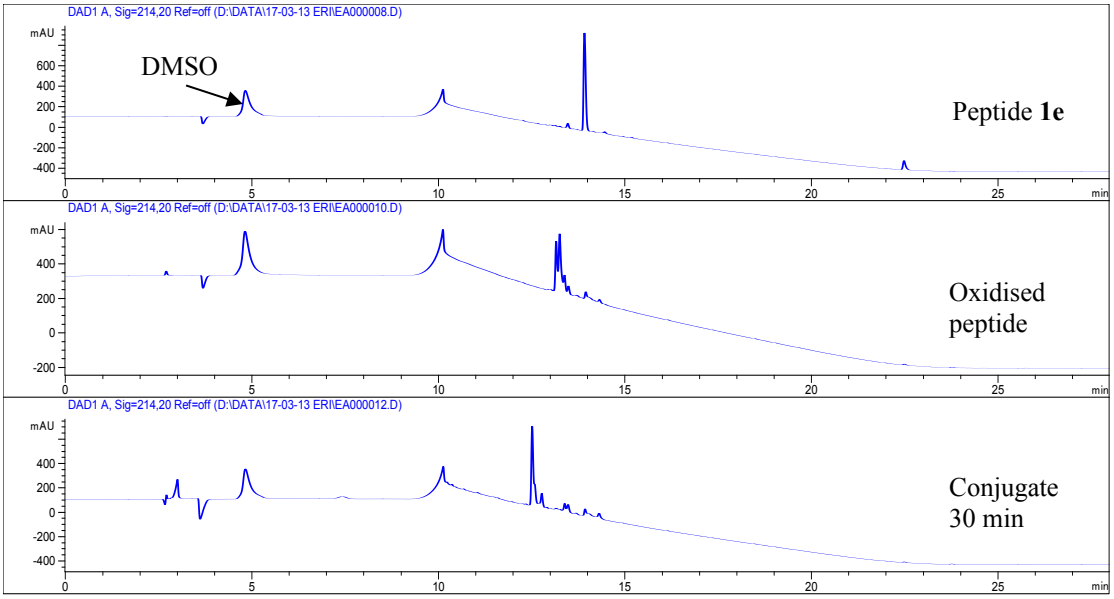


Figure 53: Crude RP-HPLC chromatograms demonstrating the progress of the reaction sequence (**1e**→**3e**) at λ = 214 nm.

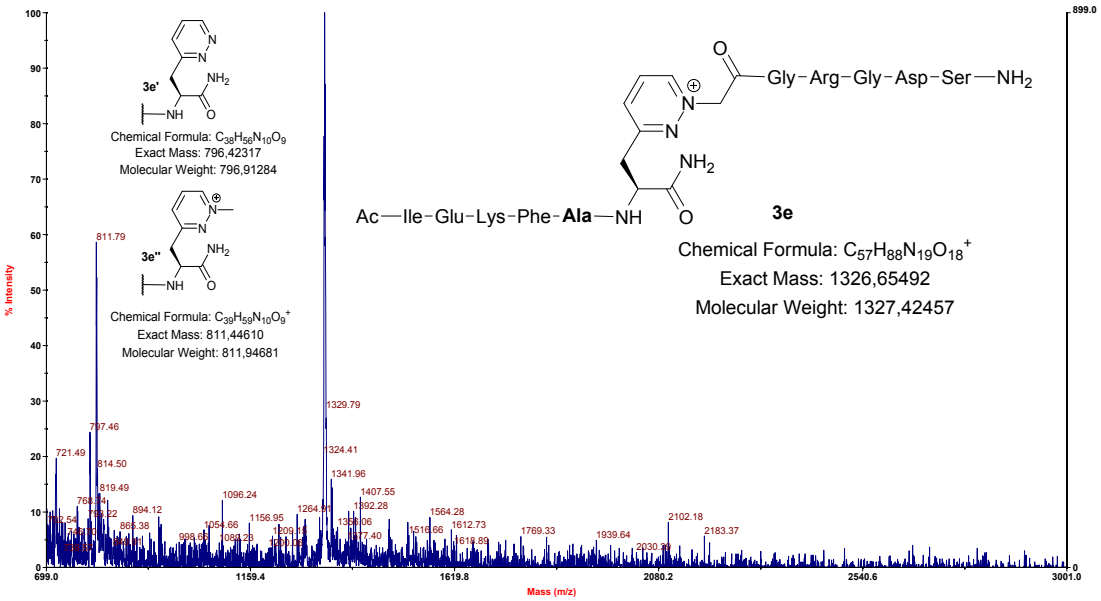
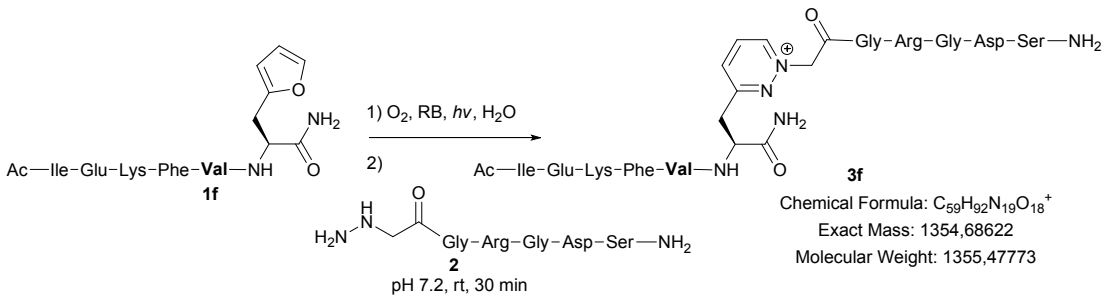


Figure 54: MALDI-TOF spectrum of the collected peak at t_R = 12.508 min (**2**, 30 min) demonstrating the formation of peptide **3e** (obs: 1326.74 M^+; calc: 1326.65). Fragmentation products occurred during MALDI-TOF ionization: peptide **3e'** (obs: 797.46 $[M+H]^+$; calc: 797.43), peptide **3e''** (obs: 811.79 M^+; calc: 811.45).



Preparation of pyridazinium-based conjugate 3f

The reaction was accomplished according to the general experimental procedure described above, utilizing the peptide **1f** (0.6 µmol, added from aqueous stock solution of 12.68 mM containing 30% DMSO) in water (1.2 mL), which was photooxidized for 40 min followed by addition of peptide **2** (0.6 µmol, added from 1.0 M phosphate buffer stock solution of 39.12 mM, pH 7.2). The reaction progress was monitored by RP-HPLC and the product was analyzed by MALDI-TOF analysis. The desired conjugate was already formed in 30 min. MALDI-TOF spectrum of the collected peak at t_R = 12.672 min demonstrated the formation of the desired conjugate.

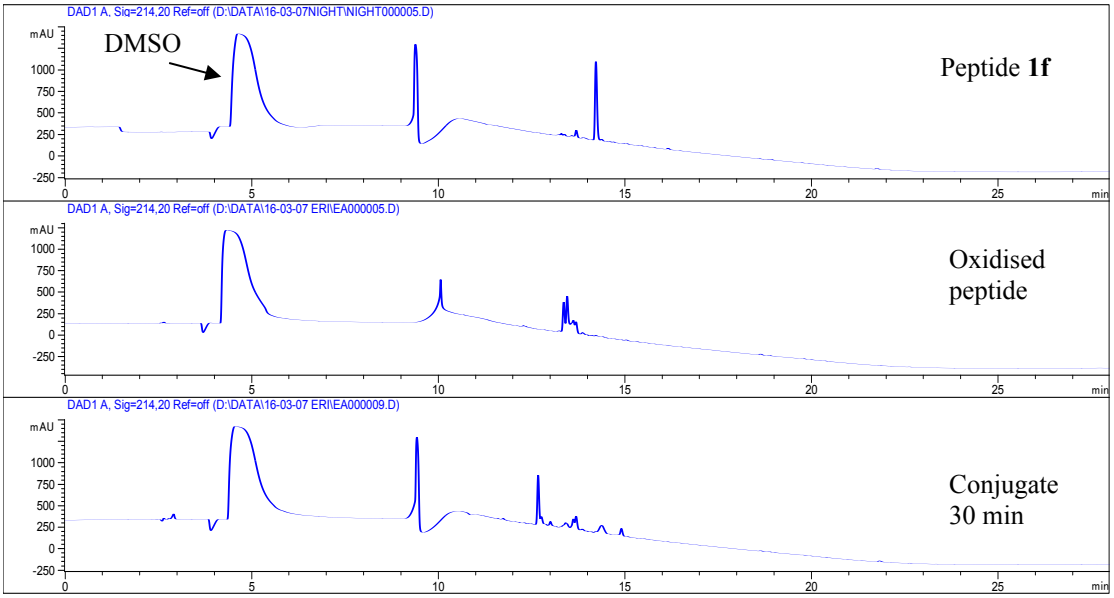


Figure 55: Crude RP-HPLC chromatograms demonstrating the progress of the reaction sequence (**1f→3f**) at λ = 214 nm.

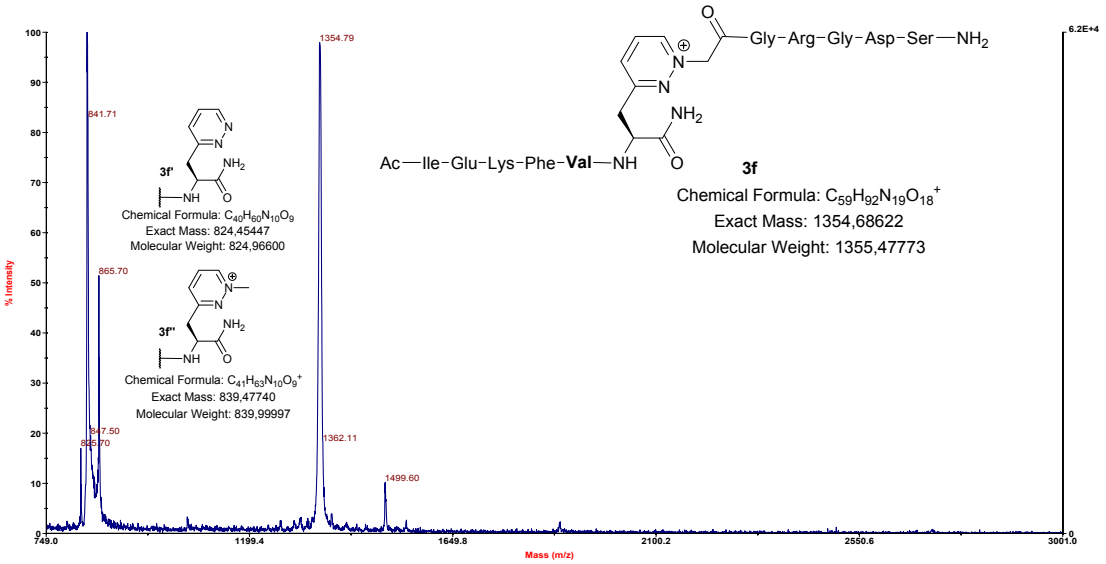
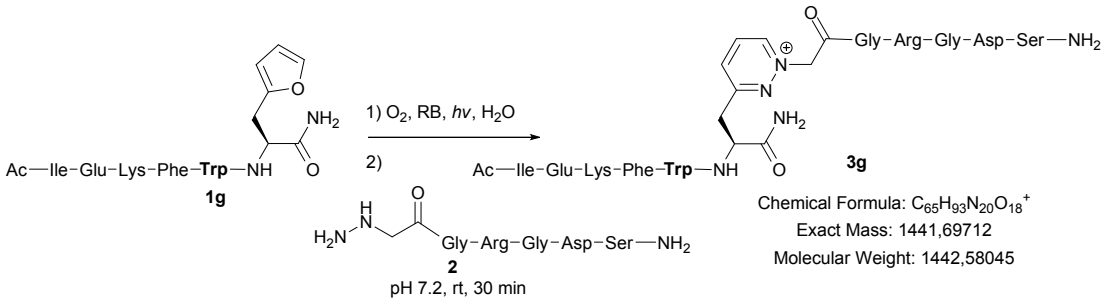


Figure 56: MALDI-TOF spectrum of the collected peak at t_R = 12.672 min (**2**, 30 min) demonstrating the formation of peptide **3f** (obs: 1354.79 M^+; calc: 1354.69). Fragmentation products occurred during MALDI-TOF ionization: peptide **3f'** (obs: 825.70 $[M+H]^+$; calc: 825.46), peptide **3f''** (obs: 839.81 M^+; calc: 839.48).



Preparation of pyridazinium-based conjugate 3g

The reaction was accomplished according to the general experimental procedure described above, utilizing the peptide **1g** (0.6 μmol, added from aqueous stock solution of 10.93 mM containing 4% DMSO) in water (1.2 mL), which was photooxidized for 30 min followed by addition of peptide **2** (0.6 μmol, added from 1.0 M phosphate buffer stock solution of 17.38 mM, pH 7.2). The reaction progress was monitored by RP-HPLC and the product was analyzed MALDI-TOF analysis. The desired conjugate was already formed in 30 min. MALDI-TOF spectrum of the collected peak at t_R = 13.170 min demonstrated the formation of the desired conjugate.

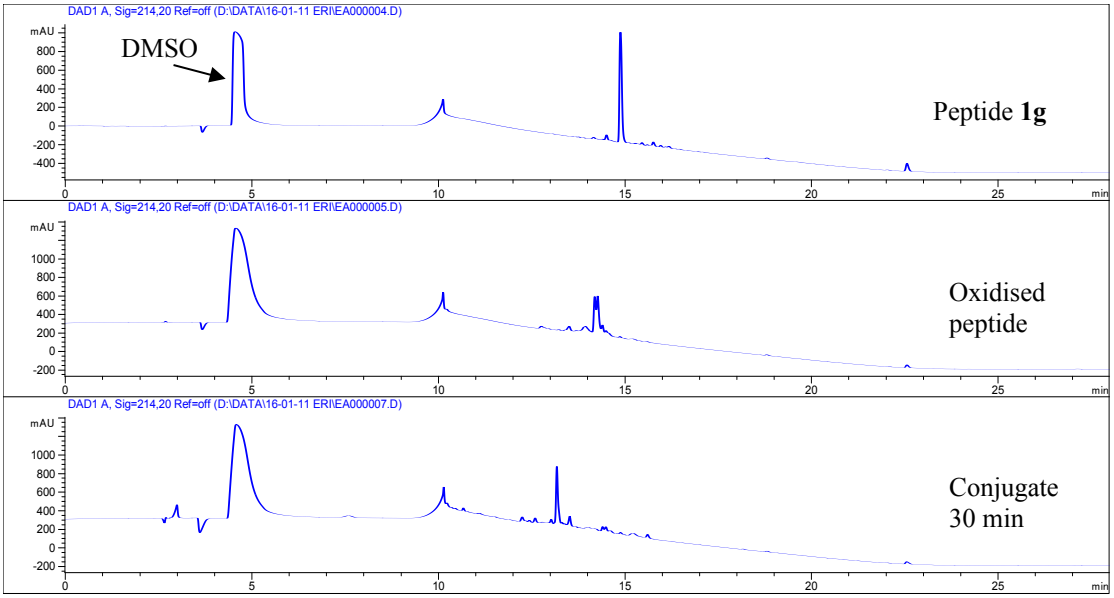


Figure 57: Crude RP-HPLC chromatograms demonstrating the progress of the reaction sequence (**1g**→**3g**) at λ = 214 nm.

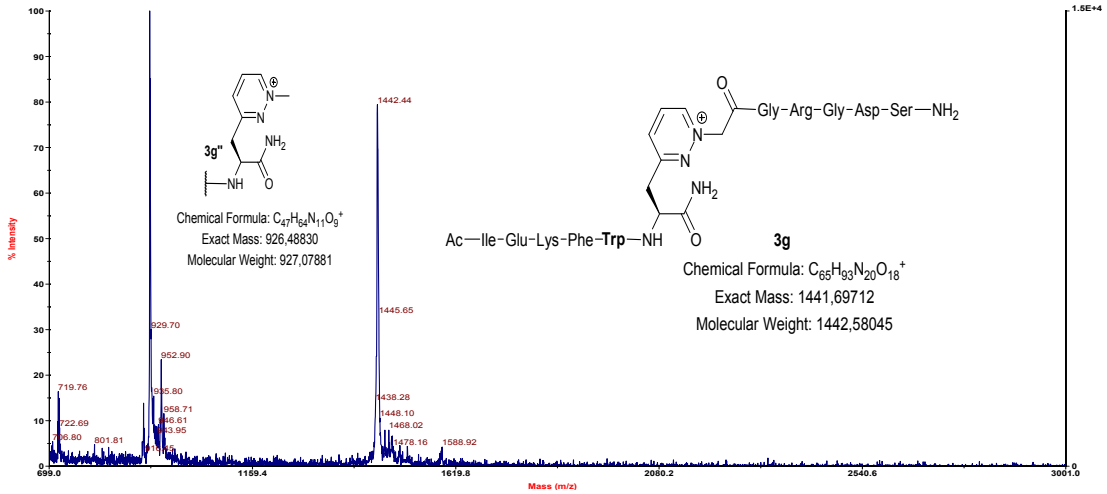
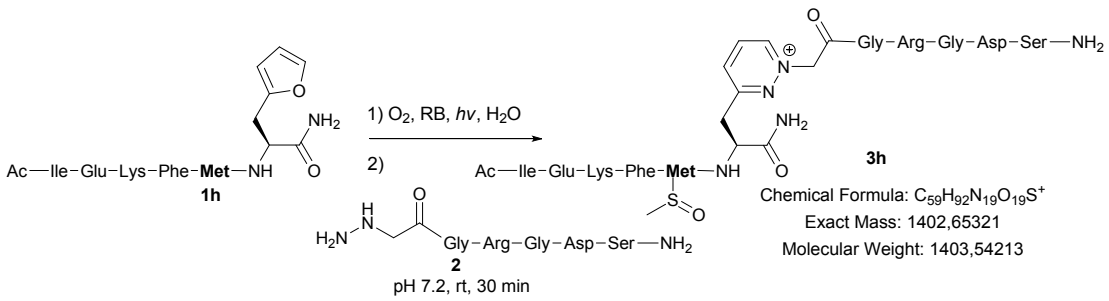


Figure 58: MALDI-TOF spectrum of the collected peak at t_R = 13.170 min (**2**, 30 min) demonstrating the formation of peptide **3g** (obs: 1442.44 M^+; calc: 1441.70). Fragmentation product occurred during MALDI-TOF ionization: peptide **3g''** (obs: 926.81 M^+; calc: 926.49).



Preparation of pyridazinium-based conjugate 3h

The reaction was accomplished according to the general experimental procedure described above, utilizing the peptide **1h** (0.6 µmol, added from aqueous stock solution of 15.58 mM containing 20% DMSO) in water (1.2 mL), which was photooxidized for 30 min followed by addition of peptide **2** (0.6 µmol, added from 1.0 M phosphate buffer stock solution of 17.38 mM, pH 7.2). The reaction progress was monitored by RP-HPLC and the product was analyzed MALDI-TOF analysis. The desired conjugate was already formed in 30 min. MALDI-TOF spectrum of the collected peak at t_R = 12.514 min demonstrated the formation of the desired conjugate.

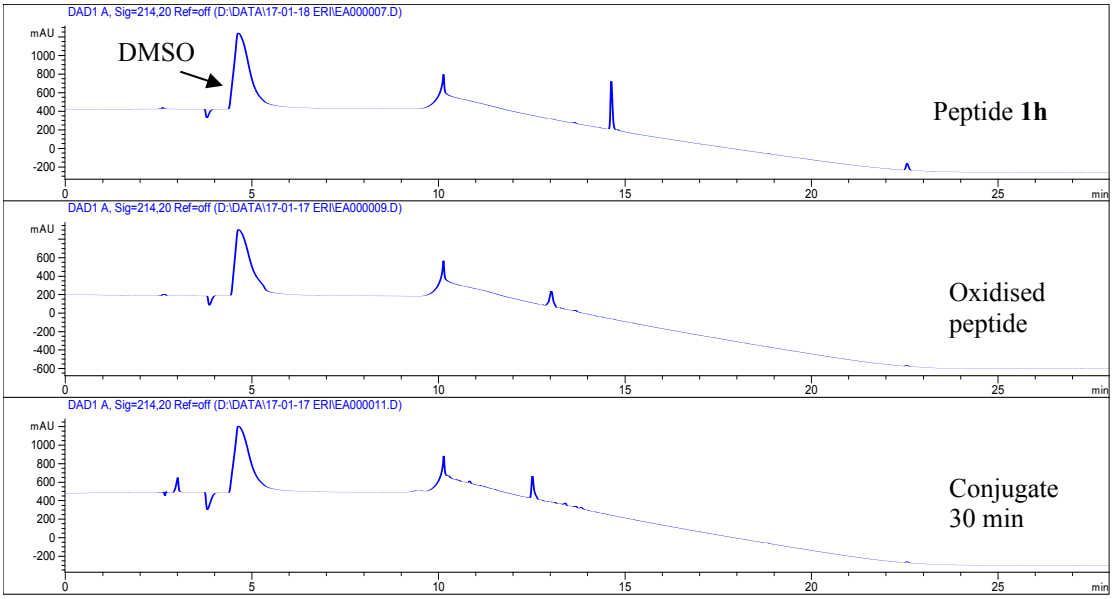


Figure 59: Crude RP-HPLC chromatograms demonstrating the progress of the reaction sequence (**1h**→**3h**) at λ = 214 nm.

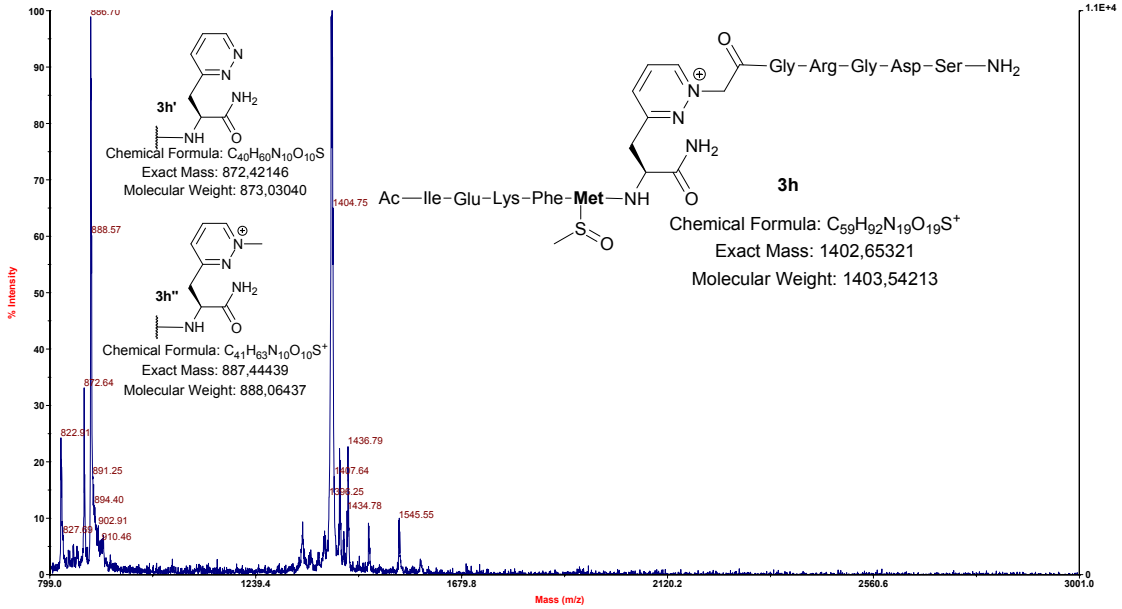
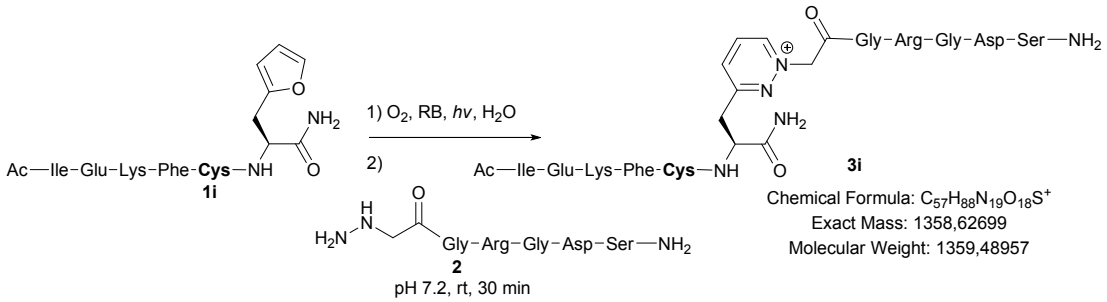


Figure 60: MALDI-TOF spectrum of the collected peak at t_R = 12.514 min (**2**, 30 min) demonstrating the formation of peptide **3h** (obs: 1402.79 M^+; calc: 1402.65). Fragmentation

products occurred during MALDI-TOF ionization: peptide **3h'** (obs: 872.64 $[M+H]^+$; calc: 873.43), peptide **3h''** (obs: 886.70 M^+; calc: 887.44).



Preparation of pyridazinium-based conjugate 3i

The reaction was accomplished according to the general experimental procedure described above, utilizing the peptide **1i** (0.6 µmol, added from aqueous stock solution of 7.8 mM) in water (1.2 mL), which was photooxidized for 30 min followed by addition of peptide **2** (0.6 µmol, added from 1.0 M phosphate buffer stock solution of 17.38 mM, pH 7.2). The reaction progress was monitored by RP-HPLC and the product was analyzed MALDI-TOF analysis. A complicated mixture of products was formed in 30 min, among them the desired conjugate **3i** (t_R = 13.170 min) as well as disulfide-ligated adducts could be identified through MALDI-TOF analysis.

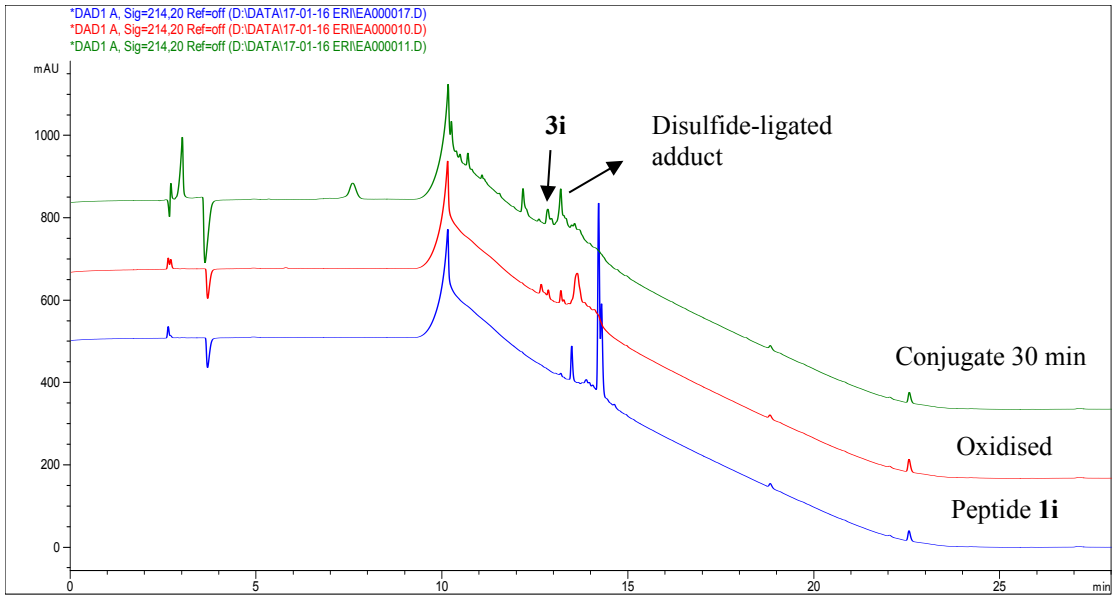


Figure 61: Crude RP-HPLC chromatograms demonstrating the progress of the reaction sequence (**1i→3i**) at λ = 214 nm.

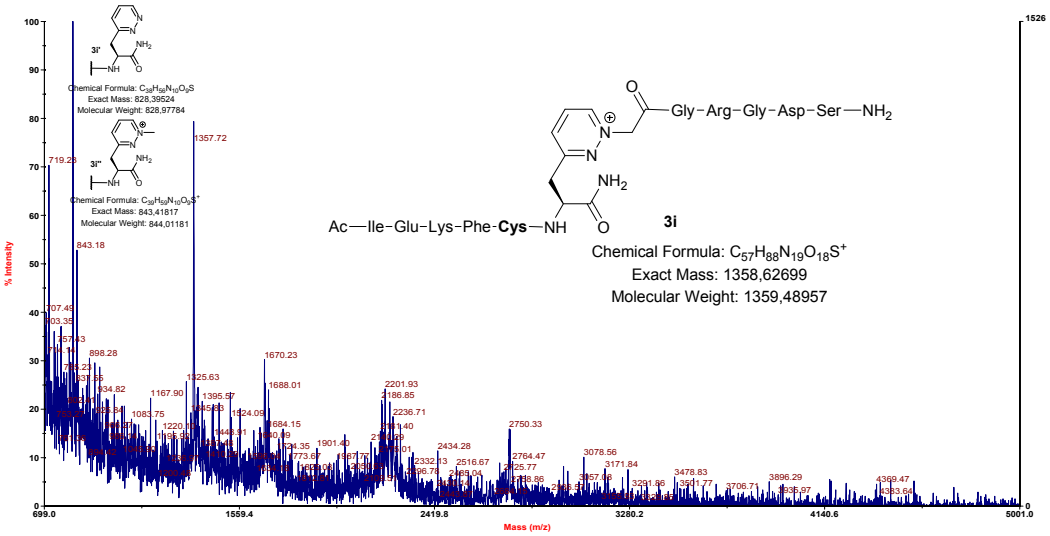


Figure 62: MALDI-TOF spectrum of the collected peak at t_R = 12.824 min (**2**, 30 min) demonstrating the formation of peptide **3i** (obs: 1357.72 M^+; calc: 1358.63). Fragmentation products occurred during MALDI-TOF ionization: peptide **3i'** (obs: 825.27 $[M+H]^+$; calc: 829.40), peptide **3i''** (obs: 843.18 M^+; calc: 843.42).

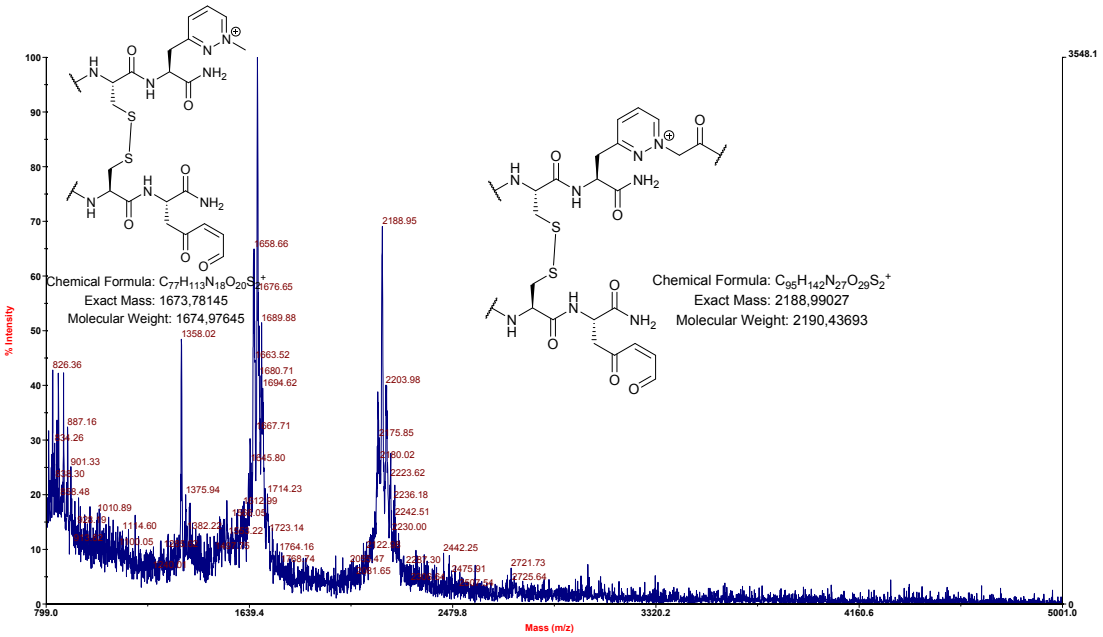
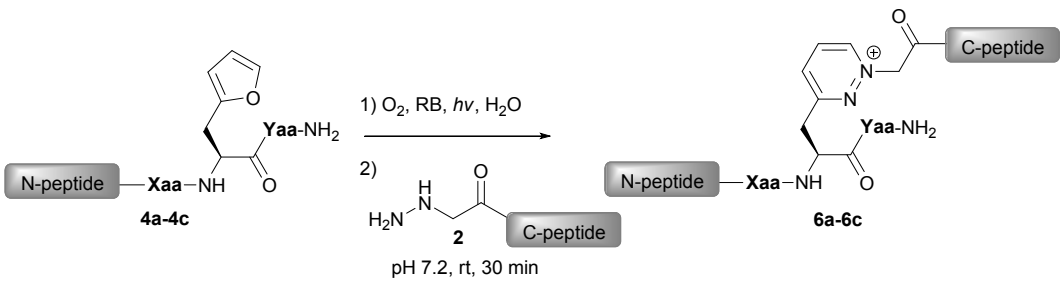
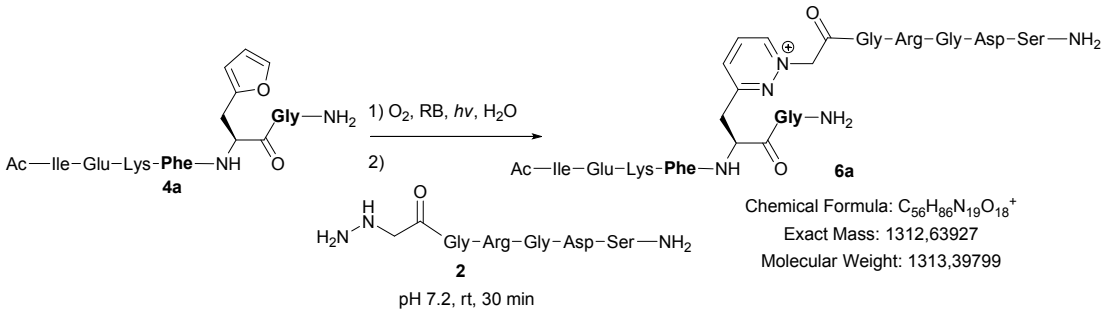


Figure 63: MALDI-TOF spectrum of the collected peak at t_R = 13.172 min (**2**, 30 min) demonstrating the formation of disulfide-ligated peptide (obs: 2188.95 M^+; calc: 2188.99). Fragmentation product occurred during MALDI-TOF ionization: (obs: 1372.75 M^+; calc: 1373.78)



General procedure for the preparation of pyridazinium-based conjugates of internal furan peptides with N-terminal hydrazine peptide

Furan-containing peptides (**4a-4c**) were dissolved in MilliQ water containing catalytic amounts of rose Bengal, as photosensitizer (10 μM). The solutions were cooled with an ice bath. Air was gently bubbled through the solution while it was irradiated with a Euromex 100 W cold light microscope lamp. After completion of the photooxidation reaction (40 min), hydrazine peptide **2** (5.0 equiv.) was added from 1.0 M phosphate buffer stock solution of pH 7.2 and the reaction solution was shaken for 30 min. The reactions were monitored by RP-HPLC and the products were analyzed by MALDI-TOF analysis.



Preparation of pyridazinium-based conjugate 6a

The reaction was accomplished according to the general experimental procedure described above, utilizing the peptide **4a** (0.6 μmol, added from aqueous stock solution of 13.29 mM) in water (1.2 mL), which was photooxidized for 40 min followed by addition of peptide **2** (3.0 μmol, added from 1.0 M phosphate buffer stock solution of 20 mM, pH 7.2). The reaction progress was monitored by RP-HPLC and the product was analyzed MALDI-TOF analysis. The desired conjugate was already formed in 30 min. MALDI-TOF spectrum of the collected peak at t_R = 12.166 min demonstrated the formation of the desired conjugate.

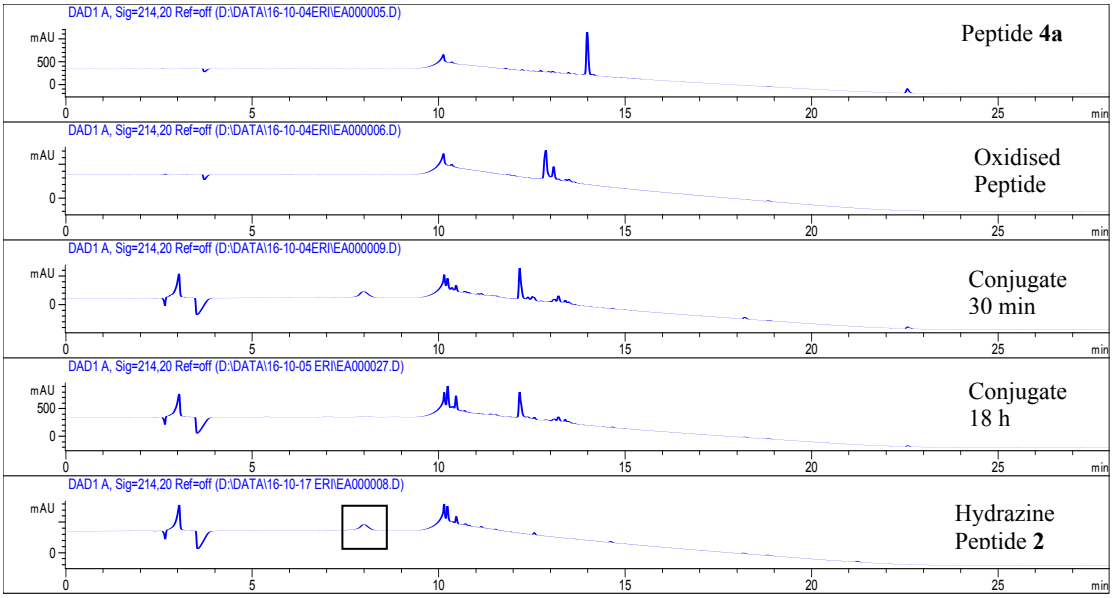


Figure 64: Crude RP-HPLC chromatograms demonstrating the progress of the reaction sequence (**4a**→**6a**) at λ = 214 nm.

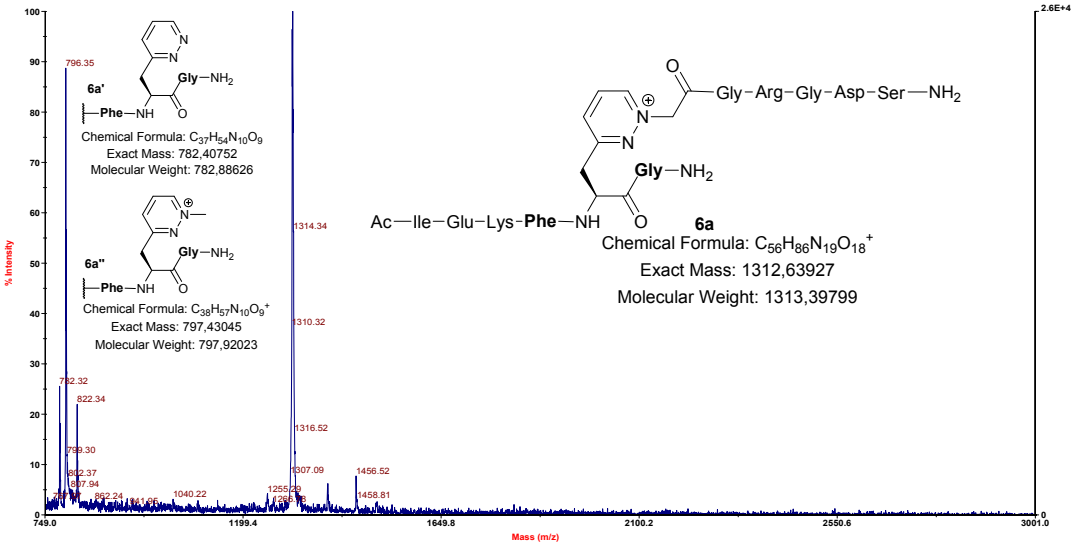
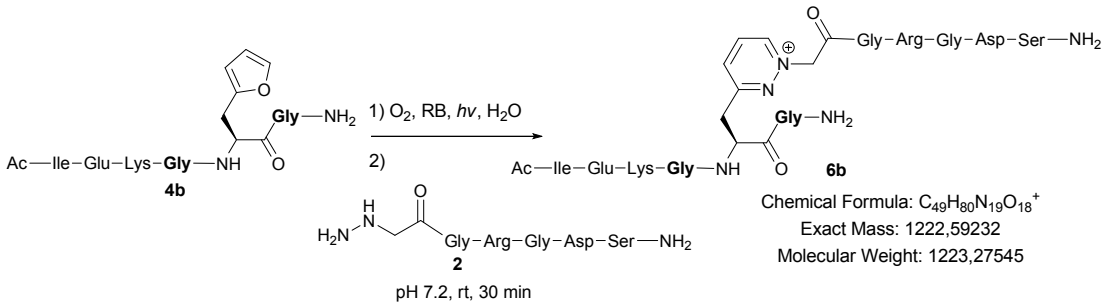


Figure 65: MALDI-TOF spectrum of the collected peak at t_R = 12.166 min (**2**, 30 min) demonstrating the formation of peptide **6a** (obs: 1312.28 M^+; calc: 1312.64). Fragmentation products occurred during MALDI-TOF ionization: peptide **6a'** (obs: 782.32 $[M+H]^+$; calc: 783.41), peptide **6a''** (obs: 796.35 M^+; calc: 797.43).



Preparation of pyridazinium-based conjugate 6b

The reaction was accomplished according to the general experimental procedure described above, utilizing the peptide **4b** (0.6 µmol, added from aqueous stock solution of 22.61 mM) in water (1.2 mL), which was photooxidized for 40 min followed by addition of peptide **2** (3.0 µmol, added from 1.0 M phosphate buffer stock solution of 20 mM, pH 7.2). The reaction progress was monitored by RP-HPLC and the product was analyzed MALDI-TOF analysis. The desired conjugate was already formed in 30 min. MALDI-TOF spectrum of the collected peak at t_R = 11.402 min demonstrated the formation of the desired conjugate.

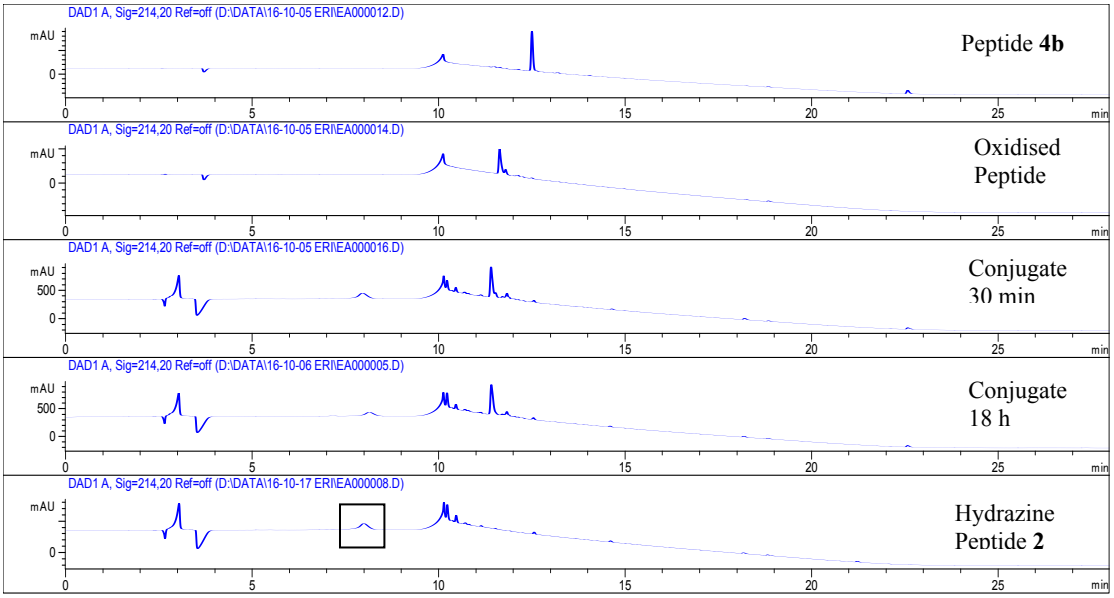


Figure 66: Crude RP-HPLC chromatograms demonstrating the progress of the reaction sequence (**4b→6b**) at λ = 214 nm.

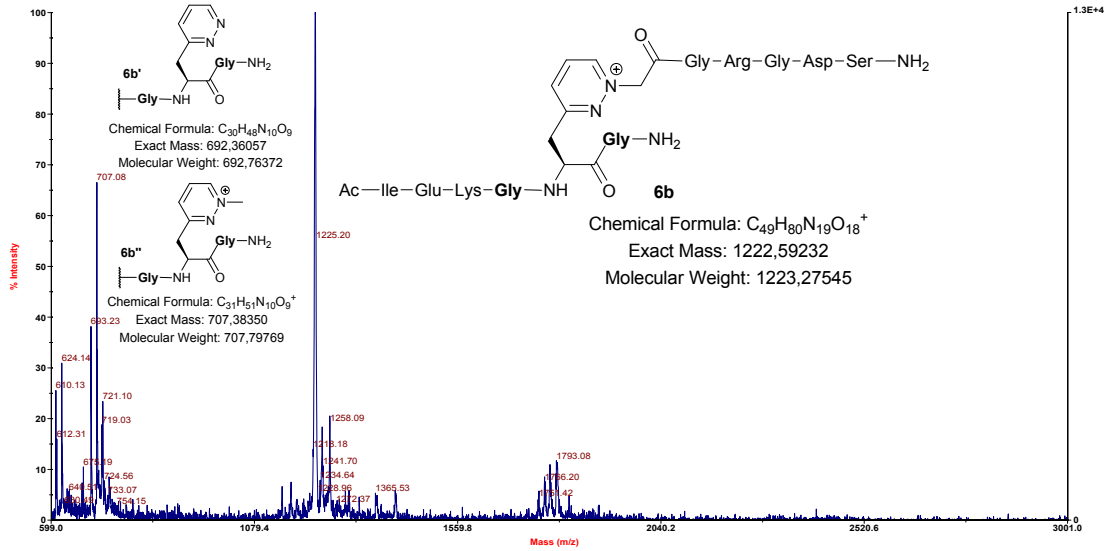
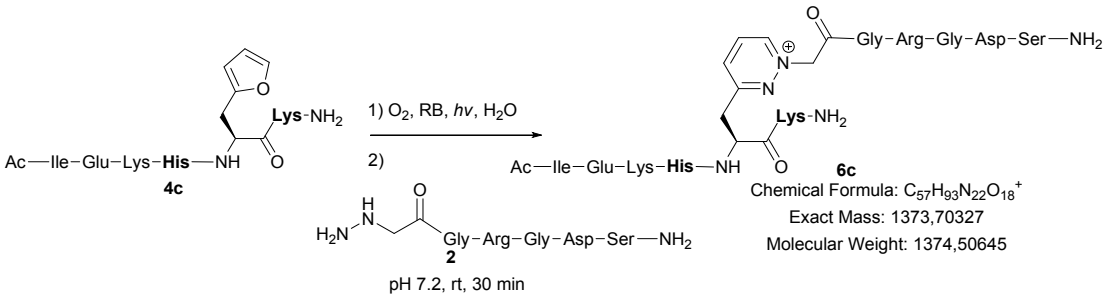


Figure 67: MALDI-TOF spectrum of the collected peak at t_R = 11.402 min (**2**, 30 min) demonstrating the formation of peptide **2** (obs: 1223.07 M^+; calc: 1222.59). Fragmentation products occurred during MALDI-TOF ionization: peptide **6b'** (obs: 693.23 $[M+H]^+$; calc: 693.37), peptide **6b''** (obs: 707.08 M^+; calc: 707.38).



Preparation of pyridazinium-based conjugate 6c

The reaction was accomplished according to the general experimental procedure described above, utilizing the peptide **4c** (0.6 µmol, added from aqueous stock solution of 22 mM) in water (1.2 mL), which was photooxidized for 40 min followed by addition of peptide **2** (3.0 µmol, added from 1.0 M phosphate buffer stock solution of 17.83 mM, pH 7.2). The reaction progress was monitored by RP-HPLC and the product was analyzed MALDI-TOF analysis. The desired conjugate was already formed in 30 min. MALDI-TOF spectrum of the collected peak at t_R = 11.361 min demonstrated the formation of the desired conjugate.

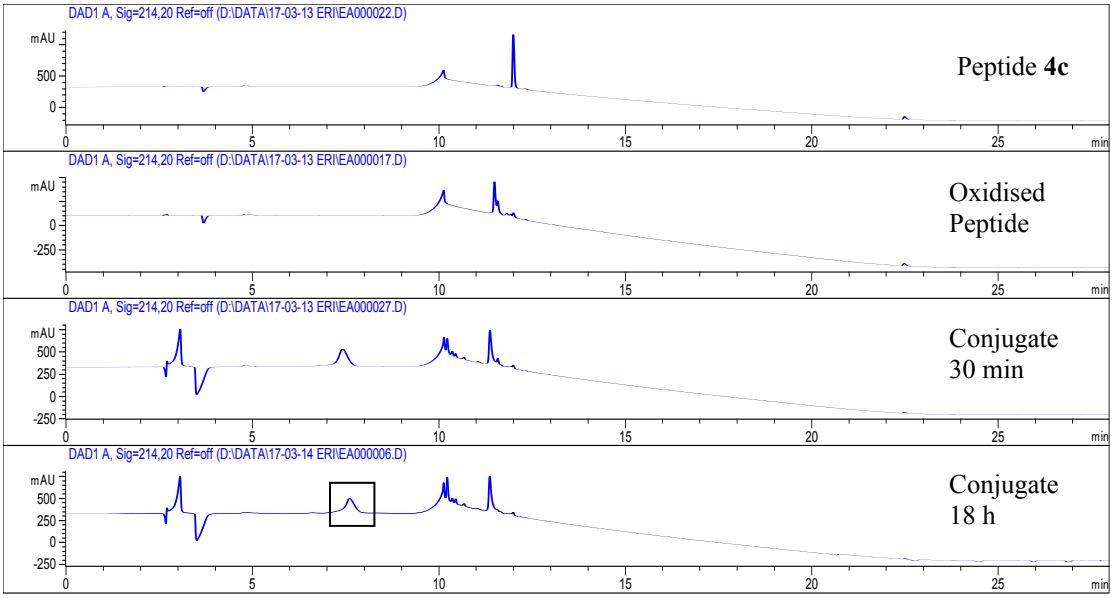


Figure 68: Crude RP-HPLC chromatograms demonstrating the progress of the reaction sequence (**4c**→**6c**) at λ = 214 nm.

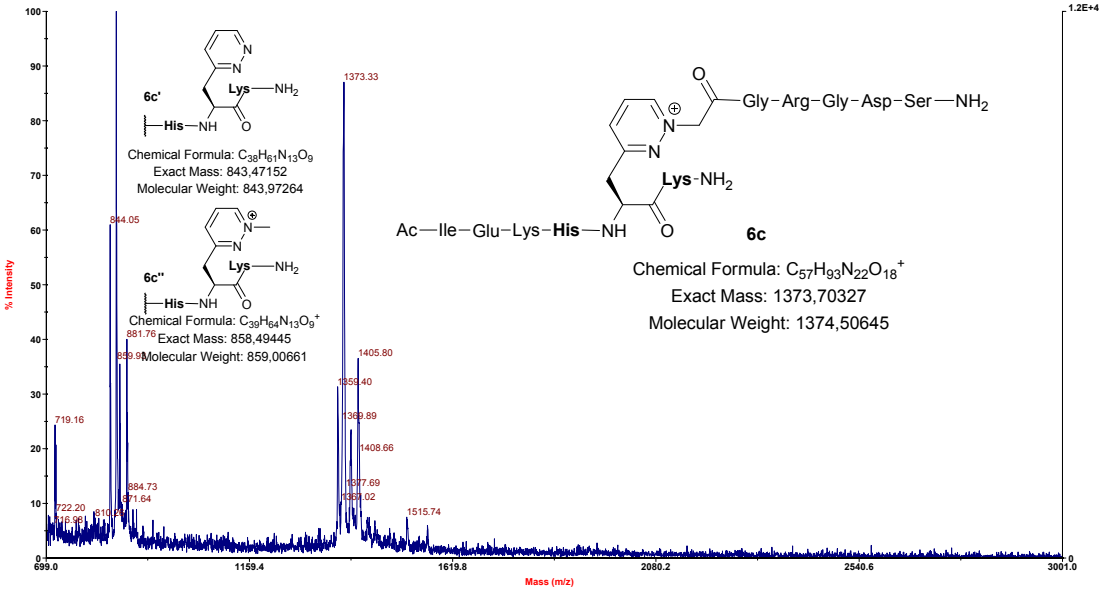
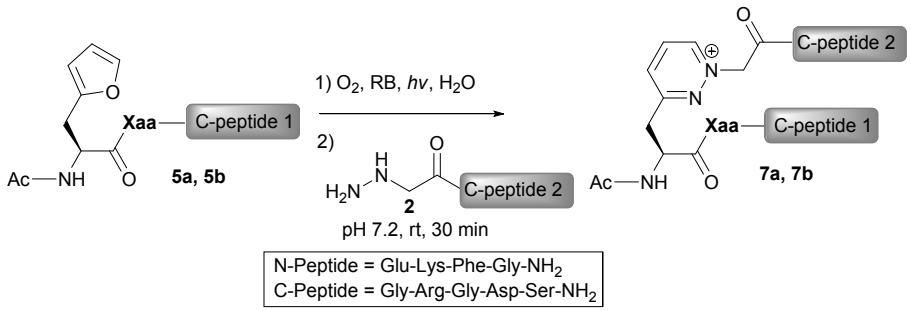
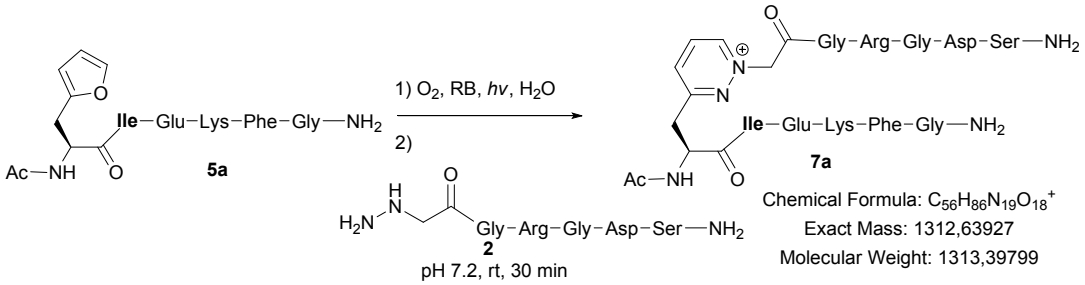


Figure 69: MALDI-TOF spectrum of the collected peak at t_R = 11.361 min (**2**, 30 min) demonstrating the formation of peptide **6c** (obs: 1373.33 M^+; calc: 1373.70). Fragmentation products occurred during MALDI-TOF ionization: peptide **6c'** (obs: 844.05 $[M+H]^+$; calc: 844.48), peptide **6c''** (obs: 857.95 M^+; calc: 858.49).



General procedure for the preparation of pyridazinium-based conjugates of N-terminal furan peptides with N-terminal hydrazine peptide

Furan-containing peptides (**5a**, **5b**) were dissolved in MilliQ water containing catalytic amounts of rose Bengal, as photosensitizer (10 μM). The solutions were cooled with an ice bath. Air was gently bubbled through the solution while it was irradiated with a Euromex 100 W cold light microscope lamp. After completion of the photooxidation reaction (40 min), hydrazine peptide **2** was added and the reaction solution was shaken for 30 min. The reactions were monitored by RP-HPLC and the products were analyzed by MALDI-TOF analysis.



Preparation of pyridazinium-based conjugate 7a

The reaction was accomplished according to the general experimental procedure described above, utilizing the peptide **5a** (0.6 μmol, added from aqueous stock solution of 17.5 mM) in water (1.2 mL), which was photooxidized for 40 min followed by addition of peptide **2** (3.0 μmol, added from 1.0 M phosphate buffer stock solution of 21.57 mM, pH 7.2). The reaction progress was monitored by RP-HPLC and the product was analyzed MALDI-TOF analysis. The desired conjugate was already formed in 30 min. MALDI-TOF spectrum of the collected peak at t_R = 12.028 min demonstrated the formation of the desired conjugate.

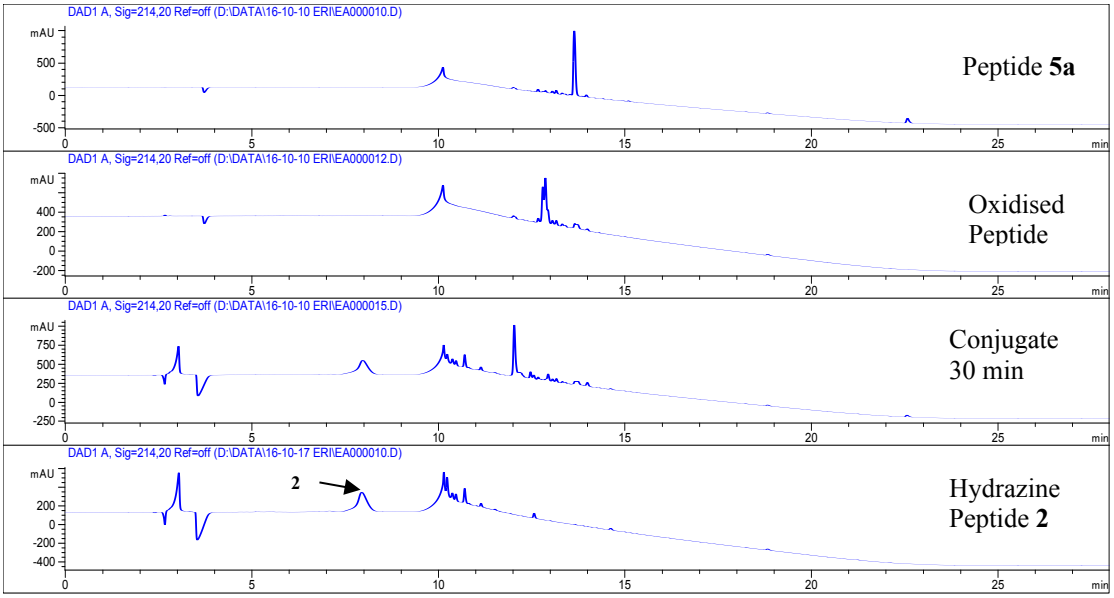


Figure 70: Crude RP-HPLC chromatograms demonstrating the progress of the reaction sequence (**5a**→**7a**) at λ = 214 nm

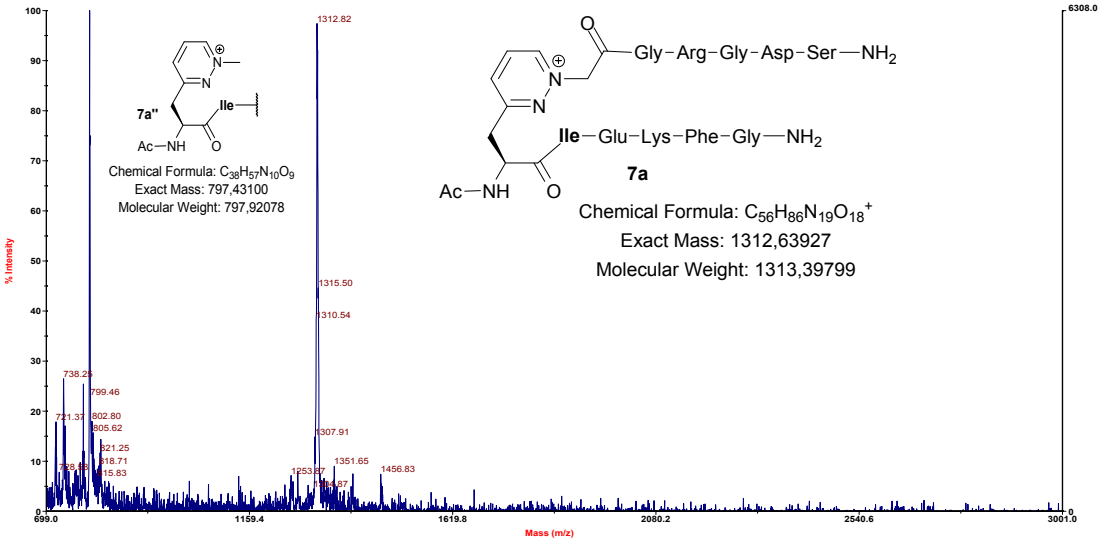
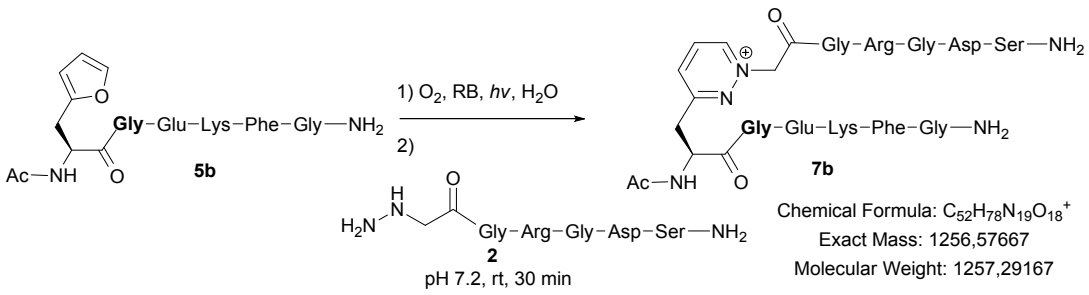


Figure 71: MALDI-TOF spectrum of the collected peak at t_R = 12.028 min (**2**, 30 min) demonstrating the formation of peptide **7a** (obs: 1312.82 M^+; calc: 1312.64). Fragmentation products occurred during MALDI-TOF ionization: peptide **7a"** (obs: 797.34 M^+; calc: 797.43).



Preparation of pyridazinium-based conjugate 7b

The reaction was accomplished according to the general experimental procedure described above, utilizing the peptide **5b** (0.6 µmol, added from aqueous stock solution of 27 mM) in water (1.2 mL), which was photooxidized for 40 min followed by addition of peptide **2** (3.0 µmol, added from 1 M phosphate buffer stock solution of 21.57 mM, pH 7.2). The reaction progress was monitored by RP-HPLC and the product was analyzed MALDI-TOF analysis. The desired conjugate was already formed in 30 min. MALDI-TOF spectrum of the collected peak at t_R = 11.524 min demonstrated the formation of the desired conjugate.

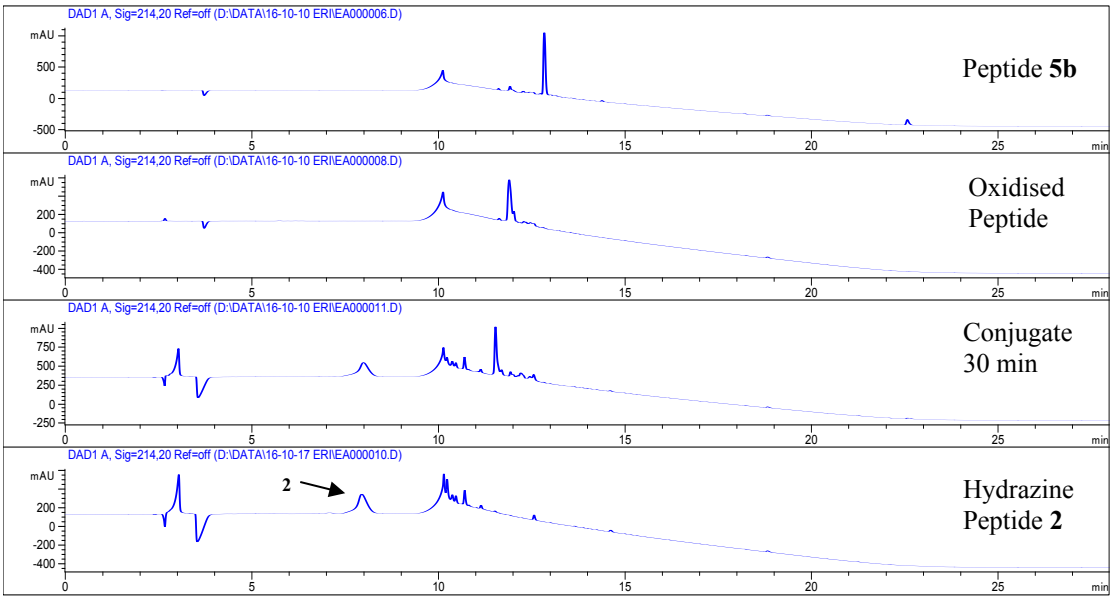


Figure 72: Crude RP-HPLC chromatograms demonstrating the progress of the reaction sequence (**5b**→**7b**) at λ = 214 nm.

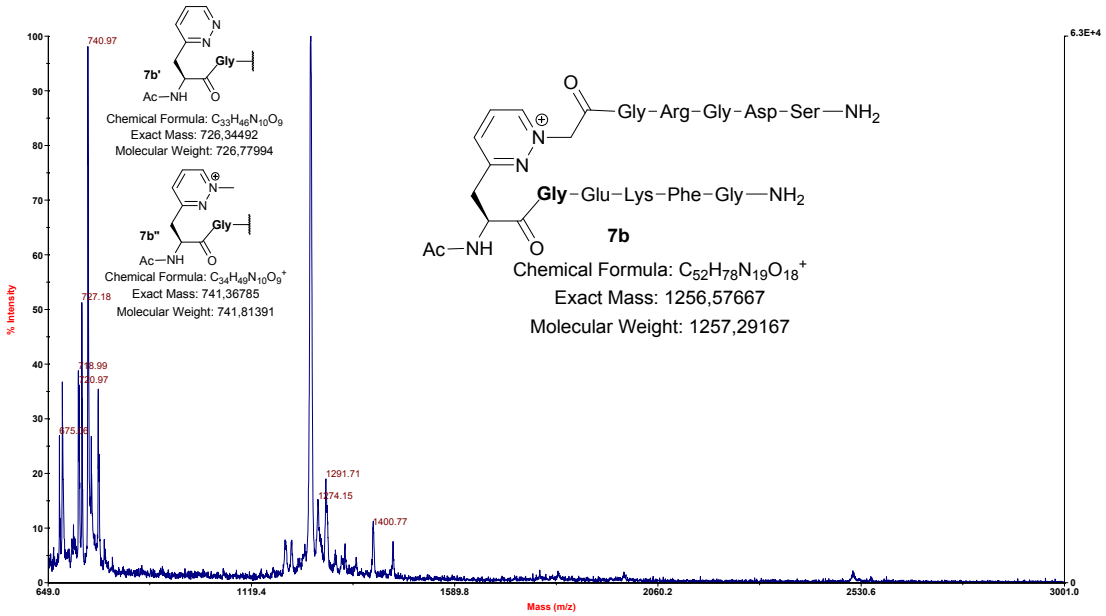
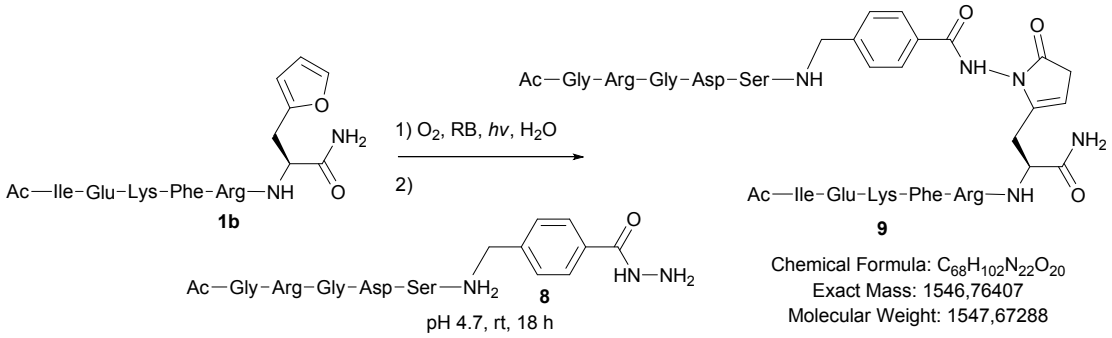


Figure 73: MALDI-TOF spectrum of the collected peak at t_R = 11.524 min (**2**, 30 min) demonstrating the formation of peptide **7b** (obs: 1256.90 M^+; calc: 1256.58). Fragmentation products occurred during MALDI-TOF ionization: peptide **7b'** (obs: 727.18 $[M+H]^+$; calc: 727.35), peptide **7b''** (obs: 740.97 M^+; calc: 741.37).

Part E: Pyrrolidinone-based ligation reactions

General procedure for the preparation of pyrrolidinone-based conjugation of C-terminal furan peptide to C- and N-terminal hydrazide peptides

Furan-containing peptide **1b** was dissolved in MilliQ water containing catalytic amounts of rose Bengal, as photosensitizer (10 μM). The solutions were cooled with an ice bath. Air was gently bubbled through the solution while it was irradiated with a Euromex 100 W cold light microscope lamp. After completion of the photooxidation reaction (40 min), hydrazide peptide **8** or **10** (2.0 equiv.) was added and the reaction solution was shaken for 18 h. The reactions were monitored by RP-HPLC and the products were analyzed by MALDI-TOF analysis.



Preparation of pyrrolidinone-based conjugate 9

The reaction was accomplished according to the general experimental procedure described above, utilizing the peptide **1b** (0.6 μmol, added from aqueous stock solution of 25 mM) in water (1.2 mL), which was photooxidized for 40 min followed by addition of peptide **8** (1.2 μmol, added from 0.1 M acetate buffer of 9.86 mM, pH = 4.7). The reaction progress was monitored by RP-HPLC and the product was analyzed MALDI-TOF analysis. The desired conjugate was already formed in 2 h, however reaction was left for 18 h to ensure the transformation of ligation intermediates to the desired conjugate (see Fig. 75). MALDI-TOF spectrum of the collected peak at t_R = 12.452 min demonstrated the formation of the desired conjugate.

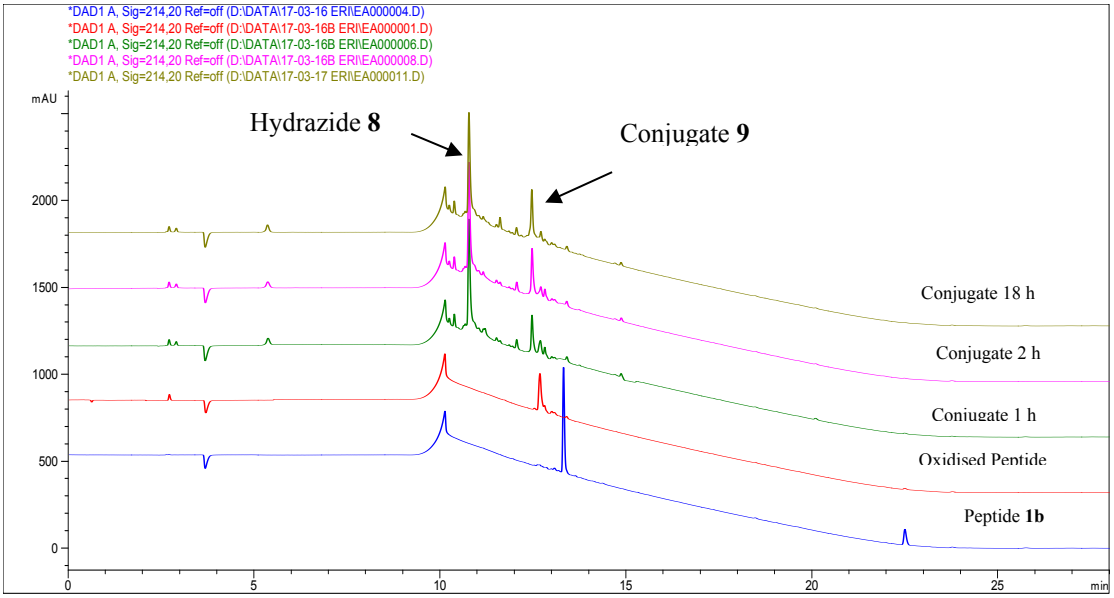


Figure 74: Crude RP-HPLC chromatograms demonstrating the progress of the reaction sequence (**1b**→**9**) at λ = 214 nm.

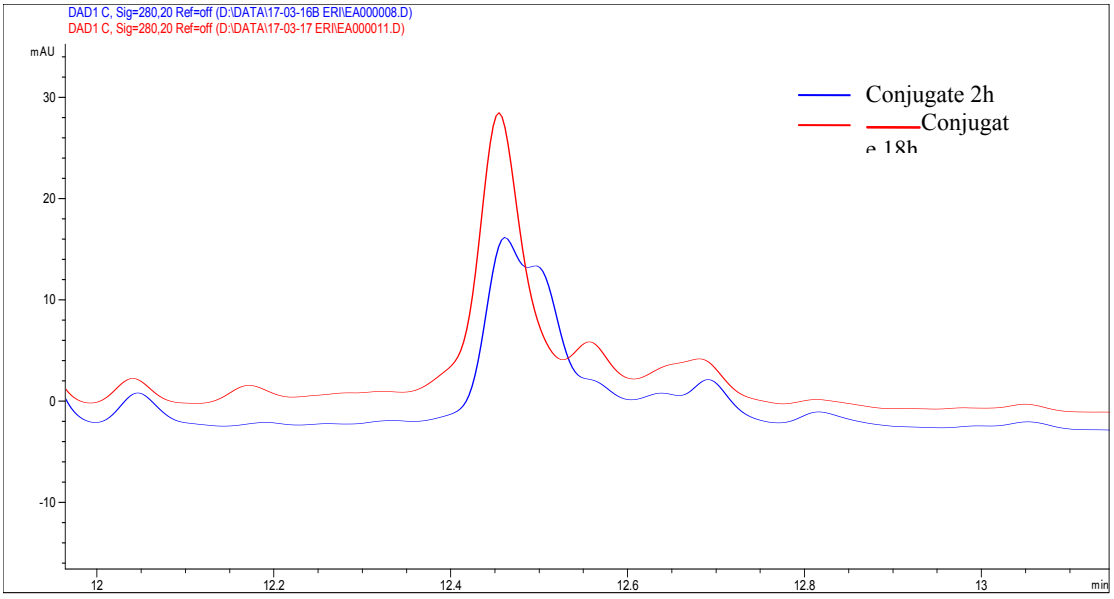


Figure 75: Crude RP-HPLC chromatograms demonstrating the crude reaction mixture at λ = 280 nm.

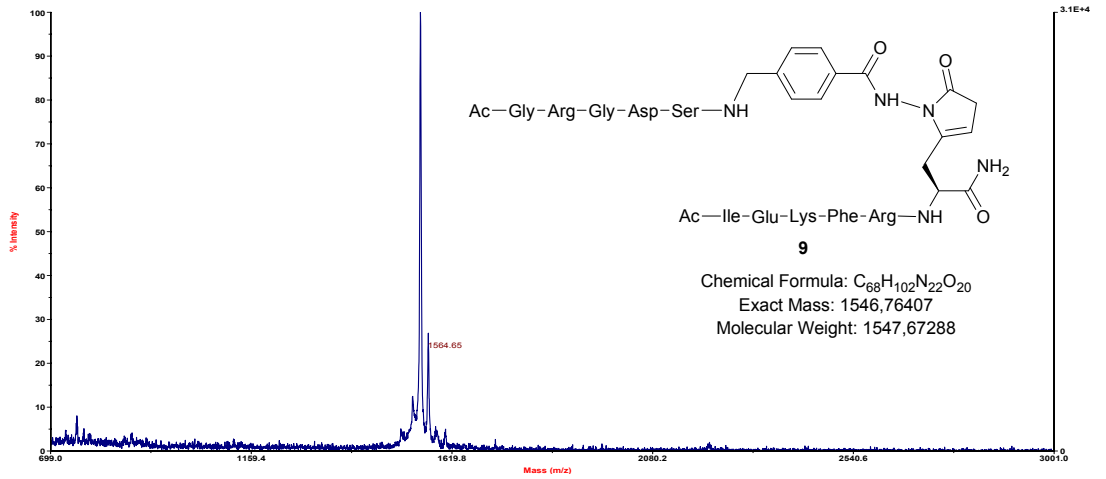
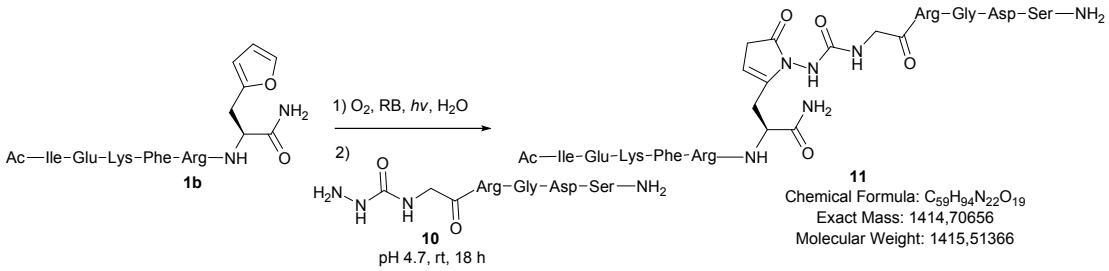


Figure 76: MALDI-TOF spectrum of the collected peak at t_R = 12.452 min (**8**, 18 h) demonstrating the formation of peptide **9** (obs: 1547.60 $[M+H]^+$; calc: 1547.77).



Preparation of pyrrolidinone-based conjugate 11

The reaction was accomplished according to the general experimental procedure described above, utilizing the peptide **1b** (0.6 µmol, added from aqueous stock solution of 25 mM) in water (1.2 mL), which was photooxidized for 40 min followed by addition of peptide **10** (1.2 µmol, added from 0.1 M acetate buffer of 13.7 mM, pH = 4.7). The reaction progress was monitored by RP-HPLC and the product was analyzed MALDI-TOF analysis. The desired conjugate was already already formed in 2 h, however reaction was left for 18 h to ensure the transformation of ligation intermediates to the desired conjugate (see Fig. 78). MALDI-TOF spectrum of the collected peak at t_R = 12.327 min demonstrated the formation of the desired conjugate.

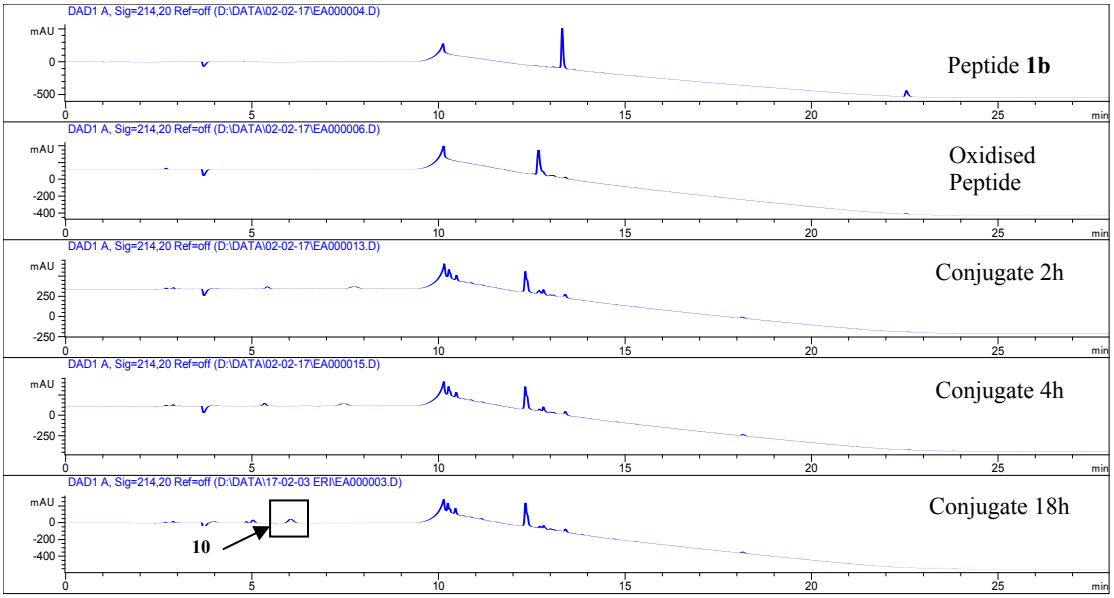


Figure 77: Crude RP-HPLC chromatograms demonstrating the progress of the reaction sequence (**1b→11**) at λ = 214 nm.

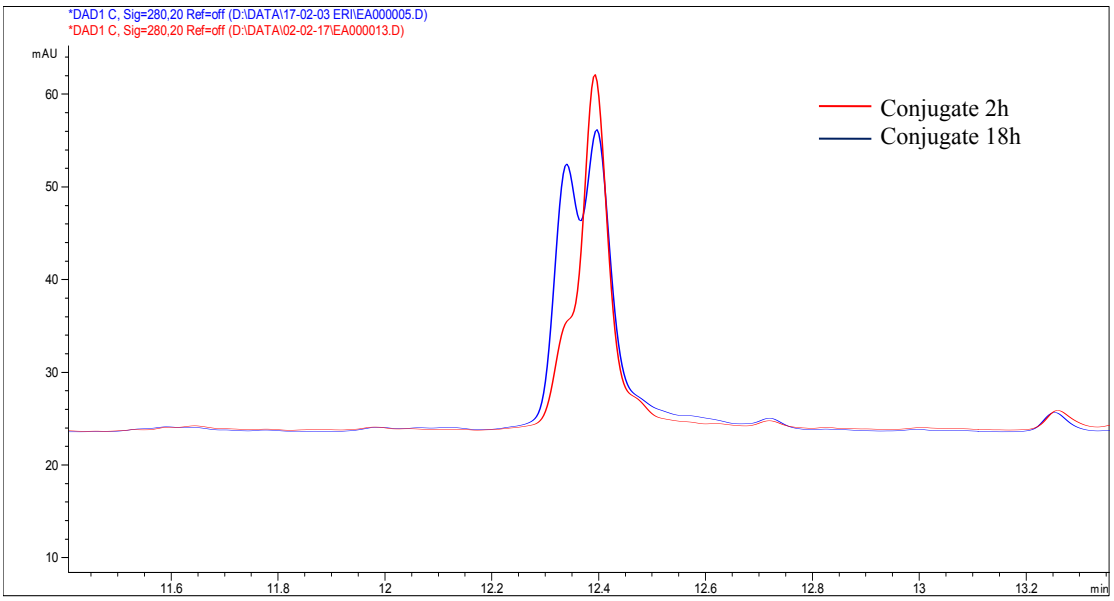


Figure 78: Crude RP-HPLC chromatograms demonstrating the crude reaction mixture at λ = 280 nm.

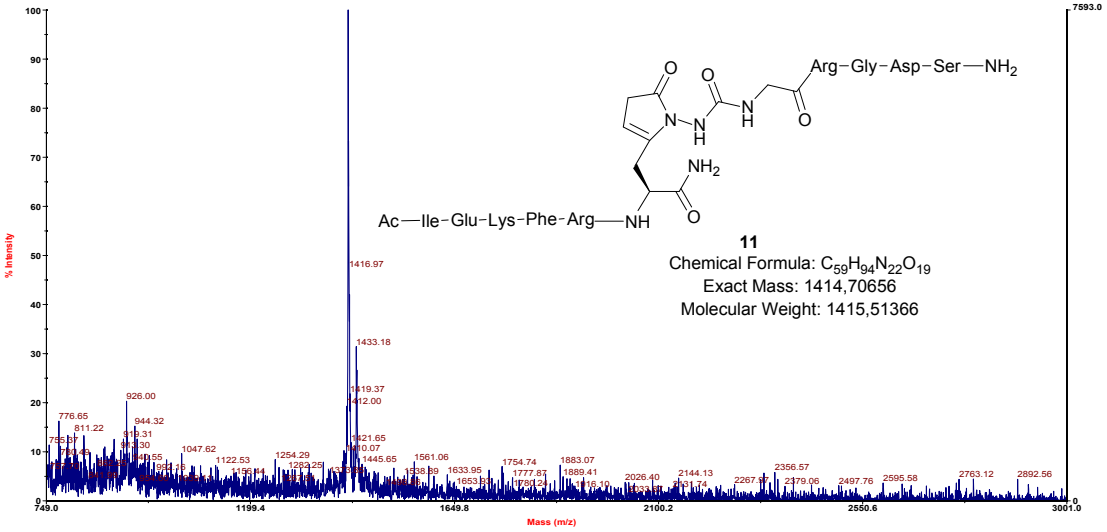


Figure 79: MALDI-TOF spectrum of the collected peak at t_R = 12.327 min (**10**, 18 h) demonstrating the formation of peptide **11** (obs: 1415.07 $[M+H]^+$; calc: 1415.71).