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

Supplementary Information

For:

2-formyl phenyl boronic acid (2FPBA)-maleimide crosslinker: a versatile platform for cys-peptide – hydrazine conjugation and interplay

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1. General Remarks

NMR spectra were recorded in a Bruker Fourier 300 using $CDCl_3$, D_2O or $(CD_3)_2SO$ as deuterated solvents. All coupling constants are expressed in Hz and chemical shifts (δ) in ppm. Multiplicities are given as: s (singlet), d (doublet), dd (double doublet), dt (double triplet), t (triplet), td (triple triplet), tt (triple triplet), q (quartet), quint (quintuplet) and m (multiplet). Low Resolution Mass spectra were recorded in LCQ Fleet Ion Trap Mass Spectrometer, Thermo Fisher Scientific, Germany. High Resolution Mass spectra were recorded in a Thermo Scientific Q Exactive hybrid quadrupole-Orbitrap mass spectrometer (Thermo Scientific™ Q Exactive™ Plus).

The Liquid chromatography–mass spectrometry (LC-MS) runs were realized using a Dionex Ultimate 3000 UHPLC+ system equipped with a Multiple-Wavelength detector, using an imChem Surf C18 TriF 100A 3 μm 100x2,1mm column connected to Thermo Scientific Q Exactive hybrid quadrupole-Orbitrap mass spectrometer (Thermo Scientific™ Q Exactive™ Plus). Exctracted ion chromatograms (EIC) were ploted with a 10 ppm tolerance from the expected mass.

Elemental analysis was performed in a Flash 2000 CHNS-O analyzer (ThermoScientific, UK).

Reaction mixtures were analysed by thin layer chromatography using Merck silica gel $60F_{254}$ aluminium plates and visualized by UV light. Column chromatography was performed with silica gel Geduran® Si 60 (0.040-0.063 mm) purchased from Merck.

All solvents were of analytical reagent grade and were purchased from Merck, Fluorochem, Alfa Aesar, TCI, Carlo Erba or Sigma-Aldrich. Cysteine, Maleic anhydride and H_2SO_4 were purchased from Merck. $B_2(Pin)_2$, TBTU, N-Acetyl-L-cysteine (NAC) (**a**), Benzylhydrazine (**d**), tert-Butyl carbazate (**g**) and *p*-Toluenesulfonyl hydrazide (**h**) were purchased from Fluorochem. $PhNTF_2$ and Pd(dppf) were purchased from TCI. Phenylhydrazine (**f**), Dansyl chloride and $MgCl_2$ were purchased from AlfaAesar. Methylhydrazine (**e**) and TCEP were purchased from Aldrich. Formaldehyde was purchased from Panreac and KOAc was purchased from BDH. *C*-Ovalbumin (**10**), Laminin fragment (**9**), Cys-Bombesin (**15**), F3 (**16**), GV1001 (**17**) and AcGKCG (**18**) peptides were purchased from GeneCust.

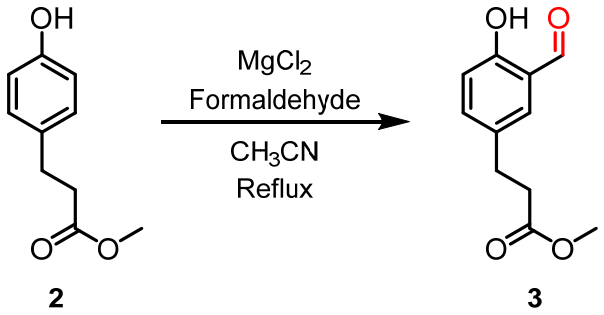
Methyl 3-(4-hydroxyphenyl)propanoate (**2**),[1] Methyl 3-(3-formyl-4-hydroxyphenyl)propanoate (**3**),[2] 2-(2,5-dioxo-2,5-dihydro-1H-pyrrol-1-yl)ethan-1-aminium 2,2,2-trifluoroacetate,[3] 2-((7-nitrobenzo[c][1,2,5]oxadiazol-4-yl)amino)ethane-1-thiol (**b**),[4] 5-(dimethylamino)-N-(2-mercaptoethyl)naphthalene-1-sulfonamide (**c**),[5] carbohydrazine coumarine (**j**)[6] and benzohydrazide (**i**),[7] are known compounds and were prepered according to the reported procedures.

[1] V. Rauniyar, D. G. Hall, *J. Org. Chem.* **2009**, *74*, 4236–4241.
[2] Z.-N. Sun, H.-L. Wang, F.-Q. Liu, Y. Chen, P. K. H. Tam, D. Yang, *Org. Lett.* **2009**, *11*, 1887–1890.
[3] F. Liu, A. Soh Yan Ni, Y. Lim, H. Mohanram, S. Bhattacharjya, B. Xing, *Bioconjug. Chem.* **2012**, *23*, 1639.
[4] L.-Y. Niu, H.-R. Zheng, Y.-Z. Chen, L.-Z. Wu, C.-H. Tung, Q.-Z. Yang, *Analyst* **2014**, *139*, 1389.
[5] C. Blaszykowski, S. Sheikh, P. Benvenuto and M. Thompson, *Langmuir*, 2012, **28**, 2318–2322.
[6] L. Long, Y. Wu, L. Wang, A. Gong, F. Hu, C. Zhang, *Chem. Commun.* **2015**, *51*, 10435.
[7] S. Panda and S. Nayak, *Supramol. Chem.*, 2015, **27**, 679–689.

2. Chemical Synthesis

2.1 Isolation of the reagents and products

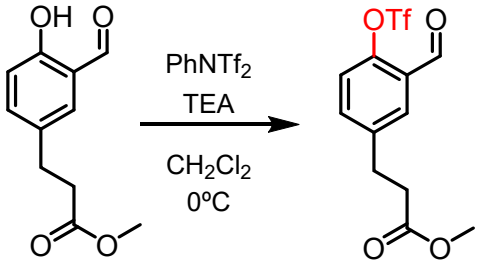
2.1.1 Methyl 3-(3-formyl-4-hydroxyphenyl)propanoate (3)



2 **3**

To a solution of methyl 3-(4-hydroxyphenyl) propanoate (**2**) (5 g, 27.75 mmol, 1.0 eq.) and anhydrous magnesium dichloride (3.96 g, 41.62 mmol, 1.5 eq.) in 240 mL of CH_3CN was added dry paraformaldehyde (7.75 g, 188.68 mmol, 6.8 eq.). The reaction mixture was heated to reflux for 8 h. The reaction mixture was cooled to room temperature and pour into 600 mL of 5% HCl. The mixture was extracted with diethyl ether (3x300 mL) and the organic phase was washed with a saturated NaCl solution and dried using anhydrous Na_2SO_4. The crude product was purified by silica gel column chromatography (hexane/ethyl acetate 7:3) to yield methyl 3-(3-formyl-4-hydroxyphenyl)propanoate (**3**) as a white solid (4.62 g, 22.19 mmol, 80% yield). **^{1}H-NMR:** (300 MHz, Chloroform-d) δ 10.73 (s, 1H), 9.70 (s, 1H), 7.31 – 7.19 (m, 2H), 6.75 (d, J = 8.4 Hz, 1H), 3.52 (s, 3H), 2.79 (t, J = 7.5 Hz, 2H), 2.50 (t, J = 7.5 Hz, 2H). **^{13}C-NMR:** (75 MHz, Chloroform-d) δ 196.4, 172.7, 159.8, 137.0, 132.9, 131.9, 120.3, 117.4, 51.4, 35.2, 29.5.

2.1.2 Methyl 3-(3-formyl-4-(((trifluoromethyl)sulfonyl)oxy)phenyl)propanoate (4)



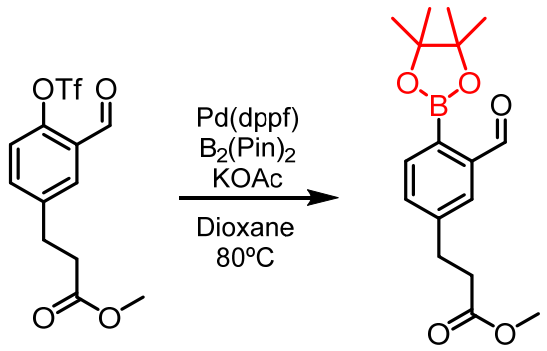
3 **4**

(85%)

To a solution of methyl 3-(3-formyl-4-hydroxyphenyl)propanoate (**3**) (0.677 g, 3.25 mmol) in DMF (7.8 ml), triethylamine (1.36 ml, 9.75 mmol) was added, and the solution stirred for 1 h. Then, *N*-Phenyltrifluoromethanesulfonimide (1.859 g, 5.20 mmol) was added portionwise and the reaction stirred for another 2 h. Water (50 ml) was added, and the mixture extracted with methyl tert-butyl ether (50 mL). The organic layer was washed with water, LiCl (aq), brine, dried over Na_2SO_4, concentrated, and purified by silica gel column chromatography (hexane/ethyl acetate 8:2) to give:

Methyl 3-(3-formyl-4-(((trifluoromethyl)sulfonyl)oxy)phenyl)propanoate (**4**) (0.936 g, 2.75 mmol, 85 % yield). **^{1}H NMR** (300 MHz, CDCl3) δ 10.20 (d, J = 0.6 Hz, 1H), 7.80 (d, J = 2.4 Hz, 1H), 7.55 (dd, J = 8.5, 2.4 Hz, 1H), 7.30 (d, J = 8.5 Hz, 1H), 3.64 (s, 3H), 3.02 (t, J = 7.5 Hz, 2H), 2.66 (t, J = 7.5 Hz, 2H). **^{13}C NMR** (75 MHz, CDCl3) δ 186.7 (CHO), 172.6 (C), 148.4 (C), 142.1 (C), 136.0 (CH), 130.6 (CH), 128.4 (C), 122.6 (CH), 118.7 (q, J = 320.6 Hz, CF_3), 51.9 (CH_3), 34.9 (CH_2), 20.0 (CH_2). **LRMS** (m/z, ESI$^-$): 339 $(M-H)^-$, 325, 311, 297. **Anal. Calc** for $C_{12}H_{11}F_3O_6S$: C, 42.36; H, 3.26; S, 9.42. Found: C, 42.32; H, 3.25; S, 9.23.

2.1.3 Methyl 3-(3-formyl-4-(4,4,5,5-tetramethyl-1,3,2-dioxaborolan-2-yl)phenyl)propanoate (5)

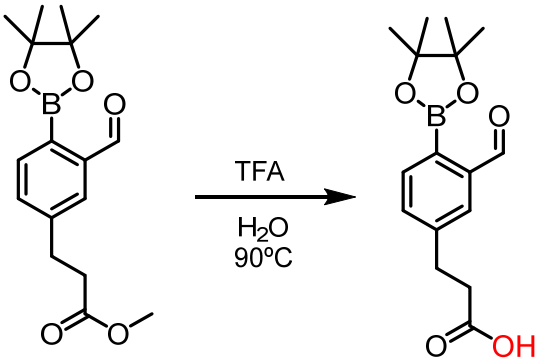


4 **5**

(59%)

To a solution of bis(pinacolato)diborane (1.37 g, 5.39 mmol) in dry dioxane (14 ml) under argon, was added dry potassium acetate (1.22 g, 12.4 mmol). After degassed for 15 min, $Pd(dppf)Cl_2 \cdot CH_2Cl_2$ (0.338 g, 0.414 mmol) and methyl 3-(3-formyl-4-(((trifluoromethyl)sulfonyl)oxy)phenyl)propanoate (**4**) (1.41 g, 4.14 mmol) were added to the reaction mixture. The mixture was stirred at 80 °C for 30 min. The reaction was quenched by adding ice-water (9 ml). The resulting mixture was extracted with 50% ethyl acetate/hexanes (30 ml). The extract was washed with brine, dried, and concentrated to dryness. The residue was purified by chromatography on silica gel (hexane/ethyl acetate 7:3) to give methyl 3-(3-formyl-4-(4,4,5,5-tetramethyl-1,3,2-dioxaborolan-2-yl)phenyl)propanoate (**5**) (0.779 g, 2.45 mmol, 59 % yield). **^{1}H NMR** (300 MHz, Chloroform-d) δ 10.57 (s, 1H), 7.84 – 7.77 (m, 2H), 7.44 (dd, J = 7.7, 1.8 Hz, 1H), 3.66 (s, 3H), 3.02 (t, J = 7.7 Hz, 2H), 2.65 (t, J = 7.7 Hz, 2H), 1.38 (s, 12H). **^{13}C NMR** (75 MHz, CDCl3) δ 194.9 (CHO), 173.0 (C), 143.7 (C), 141.7 (C), 136.2 (CH), 133.2 (CH), 127.3 (CH), 84.4 (C), 51.8 (CH_3), 35.2 (CH_2), 30.7 (CH_2), 24.9 (CH_3). **LRMS** (m/z, ESI): 341 $(M+Na)^+$, 319 $(M+H)^+$, 260, 237, 219.

2.1.4 3-(3-formyl-4-(4,4,5,5-tetramethyl-1,3,2-dioxaborolan-2-yl)phenyl)propanoic acid (6)

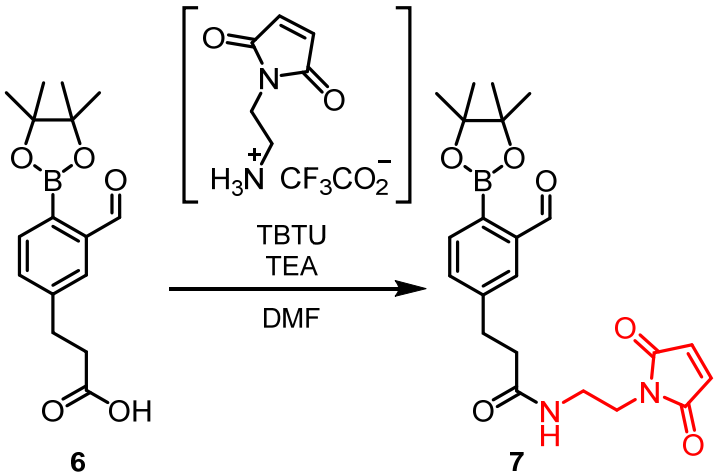


5 **6**

(95%)

In a round bottom flask, under inert atmosphere, methyl 3-(3-formyl-4-(4,4,5,5-tetramethyl-1,3,2-dioxaborolan-2-yl)phenyl)propanoate (**5**) (750 mg, 2.357 mmol) was stirred in a solution of trifluoroacetic acid (3.6 ml, 47.1 mmol) and water (15 ml) at 90 °C for 2h, after which the solvent was concentrated in vacuo. The crude was suspended in toluene (15 mL) and evaporated until dryness 3 times. Then, the residue was suspended in hexane, triturated, and decanted. The precipitate was washed 3 times with hexane, and the filtrate is evaporated until dryness to give 3-(3-formyl-4-(4,4,5,5-tetramethyl-1,3,2-dioxaborolan-2-yl)phenyl)propanoic acid (**6**) (0.688 g, 2.24 mmol, 95 % yield). **^{1}H NMR** (300 MHz, $CDCl_3$) δ 10.82 (ls, 1H), 10.55 (s, 1H), 7.85 – 7.78 (m, 2H), 7.44 (dd, J = 7.7, 1.8 Hz, 1H), 3.01 (t, J = 7.7 Hz, 2H), 2.69 (t, J = 7.6 Hz, 2H), 1.37 (s, 12H). **^{13}C NMR** (75 MHz, $CDCl_3$) δ 194.9 (CHO), 178.5 (C), 143.5 (C), 141.7 (C), 136.2 (CH), 133.2 (CH), 127.4 (CH), 84.5 (C), 35.1 (CH_2), 30.4 (CH_2), 25.0 (CH_3). **LRMS** (m/z, ESI): 305 $(M+H)^+$, 246, 223, 205. **Anal. Calcd** for $C_{16}H_{21}BO_5$: C, 63.18; H, 6.96; Found: C, 63.06; H, 7.01.

2.1.5 *N*-(2-(2,5-dioxo-2,5-dihydro-1H-pyrrol-1-yl)ethyl)-3-(3-formyl-4-(4,4,5,5-tetramethyl-1,3,2-dioxaborolan-2-yl)phenyl)propanamide (7)



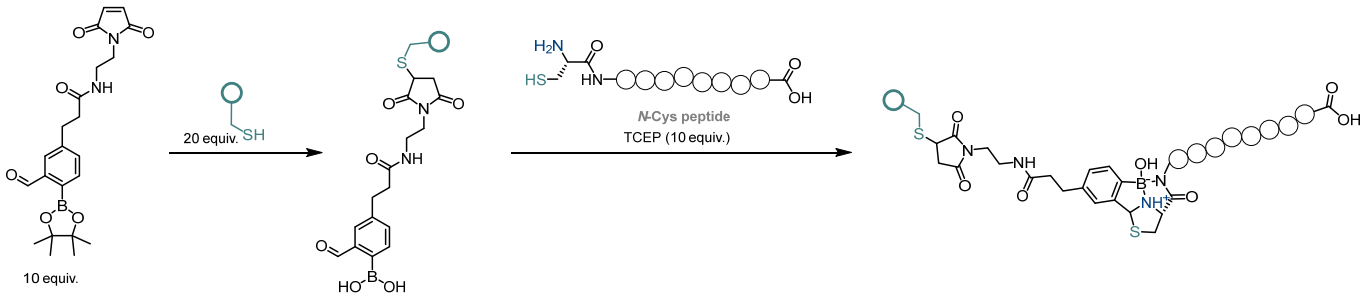
6 **7**

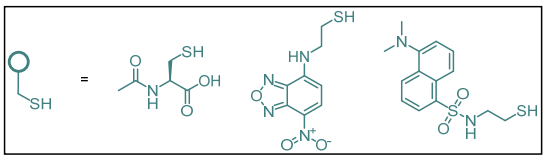
(35%)

To a solution of 3-(3-formyl-4-(4,4,5,5-tetramethyl-1,3,2-dioxaborolan-2-yl)phenyl)propanoic acid (**6**) (483 mg, 1.588 mmol) in CH_2C_{l2} (8 ml) was added TEA (66 µl, 4.7 mmol), and the solution stirred for 10 min, then 2-(1H-benzo[d][1,2,3]triazol-1-yl)-1,1,3,3-tetramethyluronium tetrafluoroborate (TBTU) (612 mg, 1.91 mmol) was added and the solution stirred for 30 min more. Finally 2-(2,5-dioxo-2,5-dihydro-1H-pyrrol-1-yl)ethan-1-aminium 2,2,2-trifluoroacetate (804 mg, 3.18 mmol) was added and the reaction was stirred at rt for 20 h. Solvent was concentrated in vacuo and the residual crude product was purified by flash column chromatography (hexane/EtOAc 3/7) to afford: N-(2-(2,5-dioxo-2,5-dihydro-1H-pyrrol-1-yl)ethyl)-3-(3-formyl-4-(4,4,5,5-tetramethyl-1,3,2-dioxaborolan-2-yl)phenyl)propanamide (**7**) (236 mg, 0.553 mmol, 35 % yield). **^{1}H NMR** (300 MHz, $CDCl_3$) δ 10.51 (s, 1H), 7.80 – 7.71 (m, 2H), 7.40 (dd, J = 7.4, 2.0 Hz, 1H), 6.67 (s, 2H), 5.98 (t, J = 5.4 Hz, 1H), 3.69 – 3.58 (m, 2H), 3.46 – 3.34 (m, 2H), 2.96 (t, J = 7.7 Hz, 2H), 2.43 (t, J = 7.7 Hz, 2H), 1.35 (s, 12H). **^{13}C NMR** (75 MHz, $CDCl_3$) δ 194.9 (CHO), 172.1 (C), 171.0 (C), 144.1 (C), 141.7 (C), 136.1 (CH), 134.3 (CH), 133.3 (CH), 127.4 (CH), 84.4 (C), 39.0 (CH_2), 37.6 (CH_2), 37.5 (CH_2), 31.2 (CH_2), 25.0 (CH_3). **LRMS** (m/z, ESI): 427.2 $(M+H)^+$, 331.3, 225.2, 188.1. **HRMS** Calculated for $[M+H]^+$, $C_{22}H_{28}BN_2O_6$: 427.2035, found 427.2029.

3. Crosslinking thiols with *N*-terminal Cys peptides

3.1 General procedure for the crosslinking of thiols with *N*-terminal Cys peptides





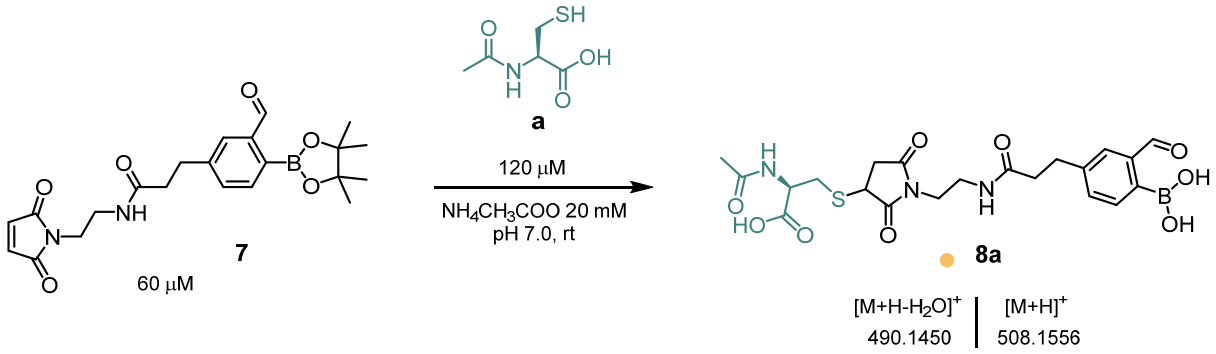
To a solution of *N*-terminal peptide (≈ 1.0 mM (10 µL, 0.010 µmol) in ammonium acetate 20 mM pH = 7.0 (500 µL) was added a Tris(2-carboxyethyl)phosphine hydrochloride (TCEP) (10 mM in water, 10 µL, 0.10 µmol) and the solution mixed for 30 at 25 ºC.

In a separated Eppendorf, crosslinker **7** (10 mM in ACN) (10 µL, 0.10 µmol) was added to a solution of the thiol (10 mM in ACN or water) (20 µL, 0.20 µmol) in ammonium acetate 20 mM pH = 7.0 (500 µL) and mixed for 30 min.

Then both solutions were mixed and allow to react for 1h and the reaction monitored in Positive Mode of ESI-MS.

3.1.1 Crosslink between NAC and c-Ovalbumin/Laminin fragment

3.1.1.1 Reaction of NAC with crosslinker 7 (8a)



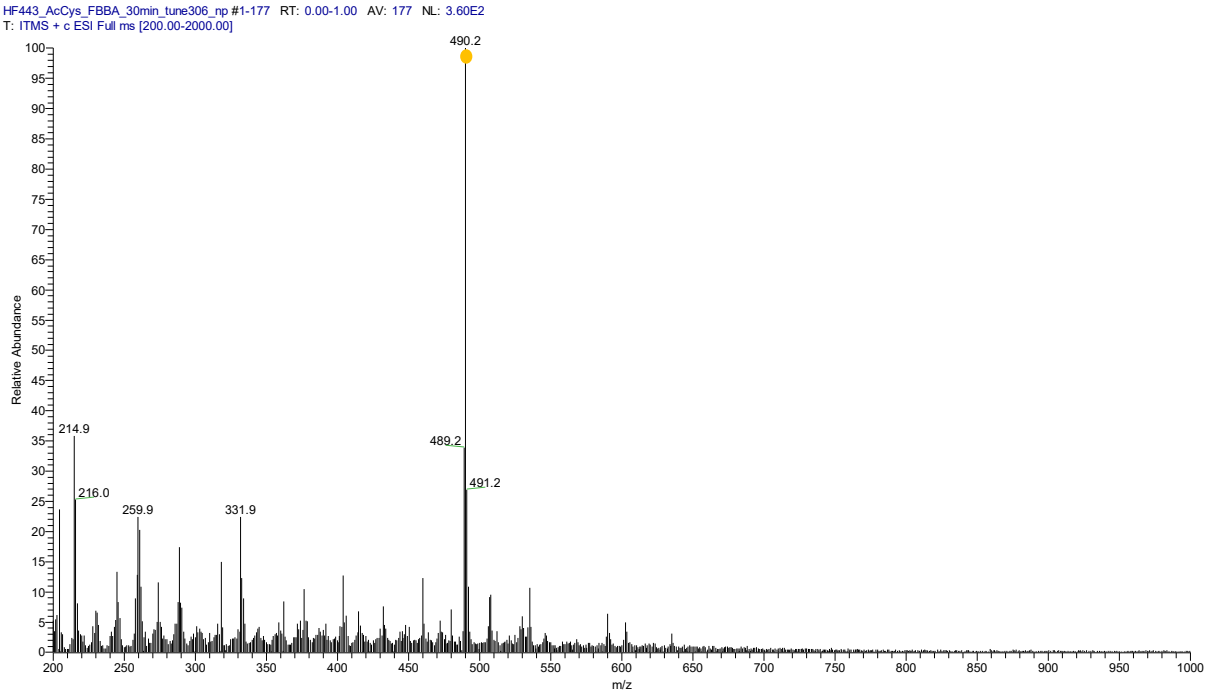


Figure S 1 - ESI$^+$-MS spectrum of reaction of NAC with **7** after 30 min to produce **8a**.

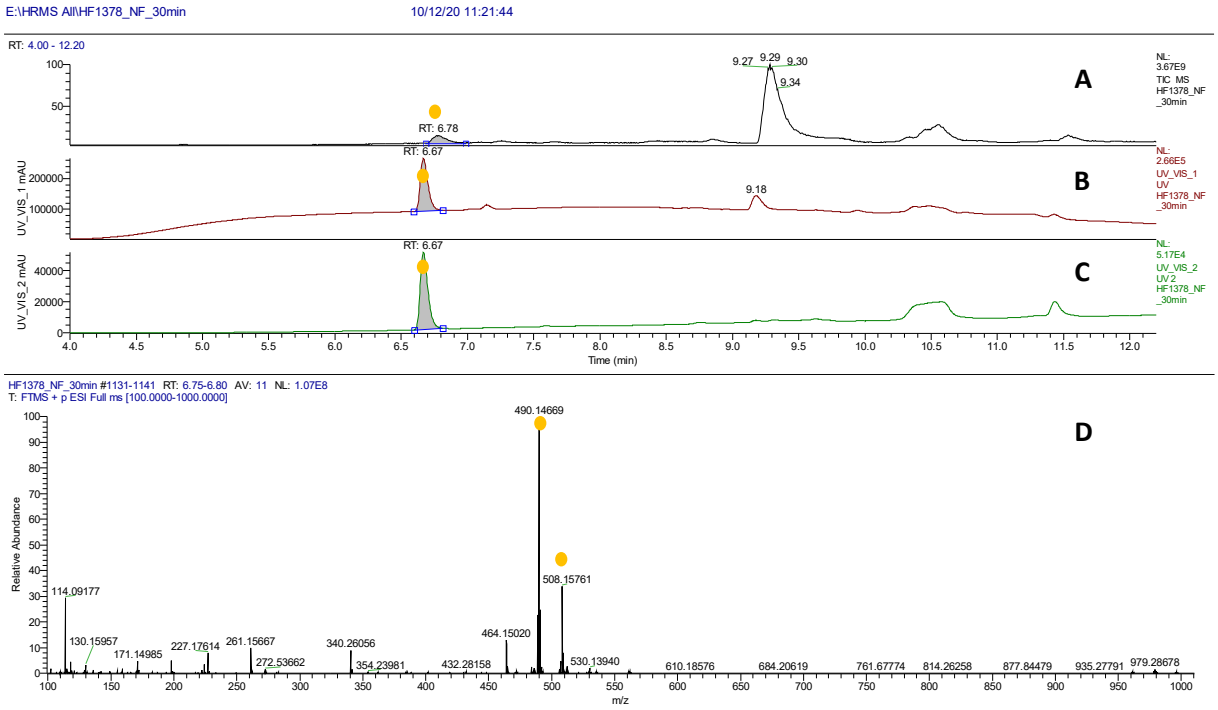
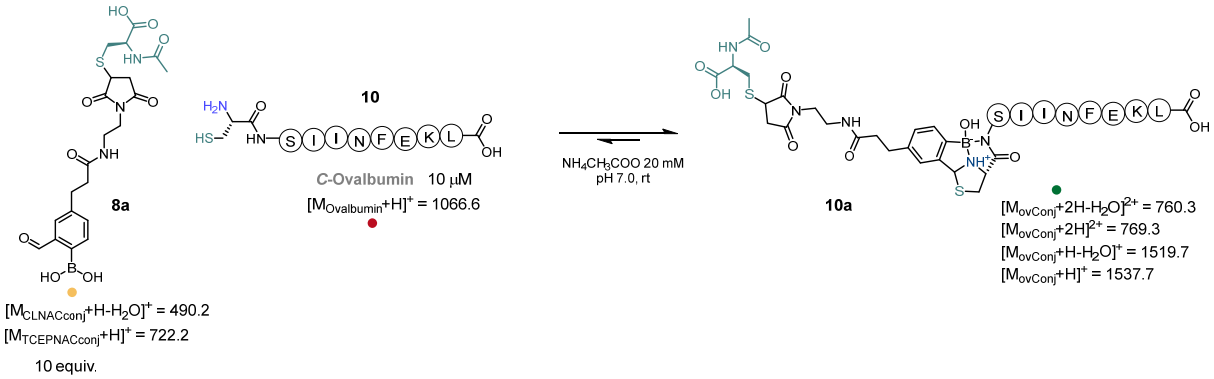


Figure S 2 –[8] Monitoring of the reaction of crosslinker **7** with NAC after 30 min. **A** – Total ion current (TIC); **B** - HPLC UV trace at 210 nm; **C** - HPLC UV trace at 254 nm; **D** – HRMS-ESI$^+$-MS of the peak at 6.7 min corresponding to the thiosuccinimide **8a**.

[8] LC-MS were acquired in positive mode and the HPLC runs were carried out with a gradient of A (Milli Q water containing 0.1% v/v Formic acid (FA)) and B (acetonitrile containing 0.1% v/v FA). The mobile phase was t = 0 min, 5% B; t = 1min, 5% B; t = 10 min, 95% B; t = 11 min, 95% B; t = 12 min, 5% B; t = 15 min, stop, at a flow rate of 0.3 mL/min.

3.1.1.2 Reaction of c-Ovalbumin with 2FBBA-NAC conjugate (10a)



Ovalbumin sequence: CSIINFEKL

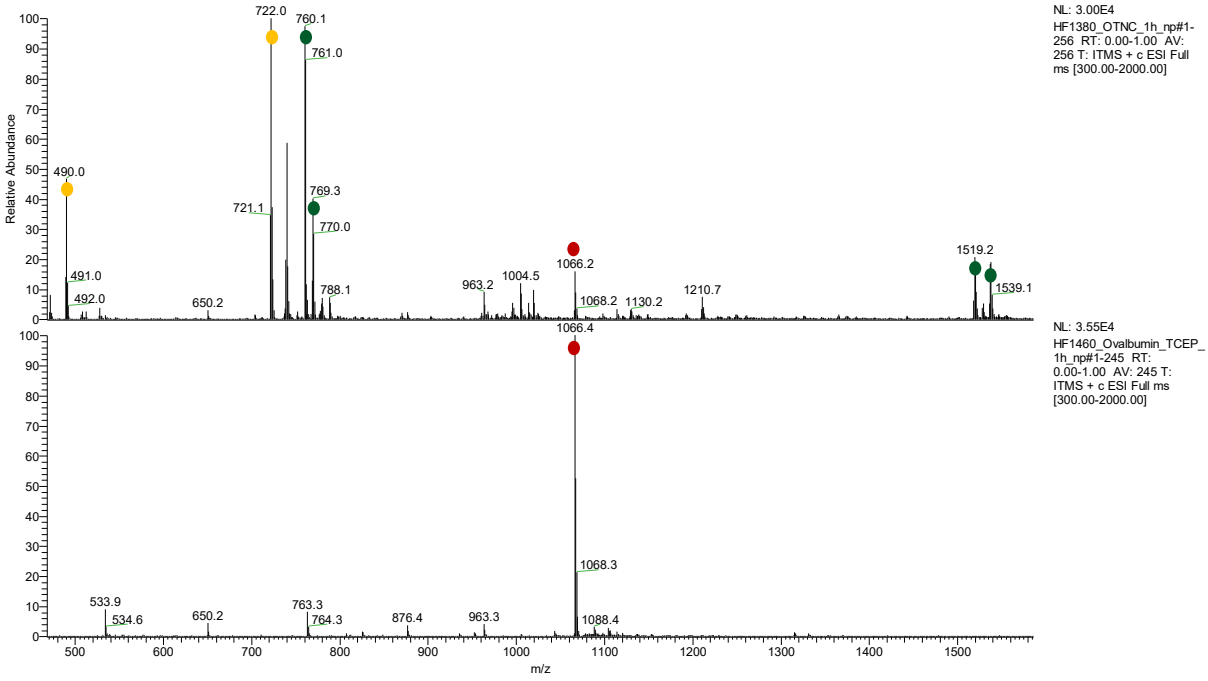


Figure S 3.1 - ESI$^+$-MS spectrum of Ovalbumin reaction with **8a** after 1h (top) and ESI$^+$-MS spectrum of Ovalbumin (**10**) (bottom).

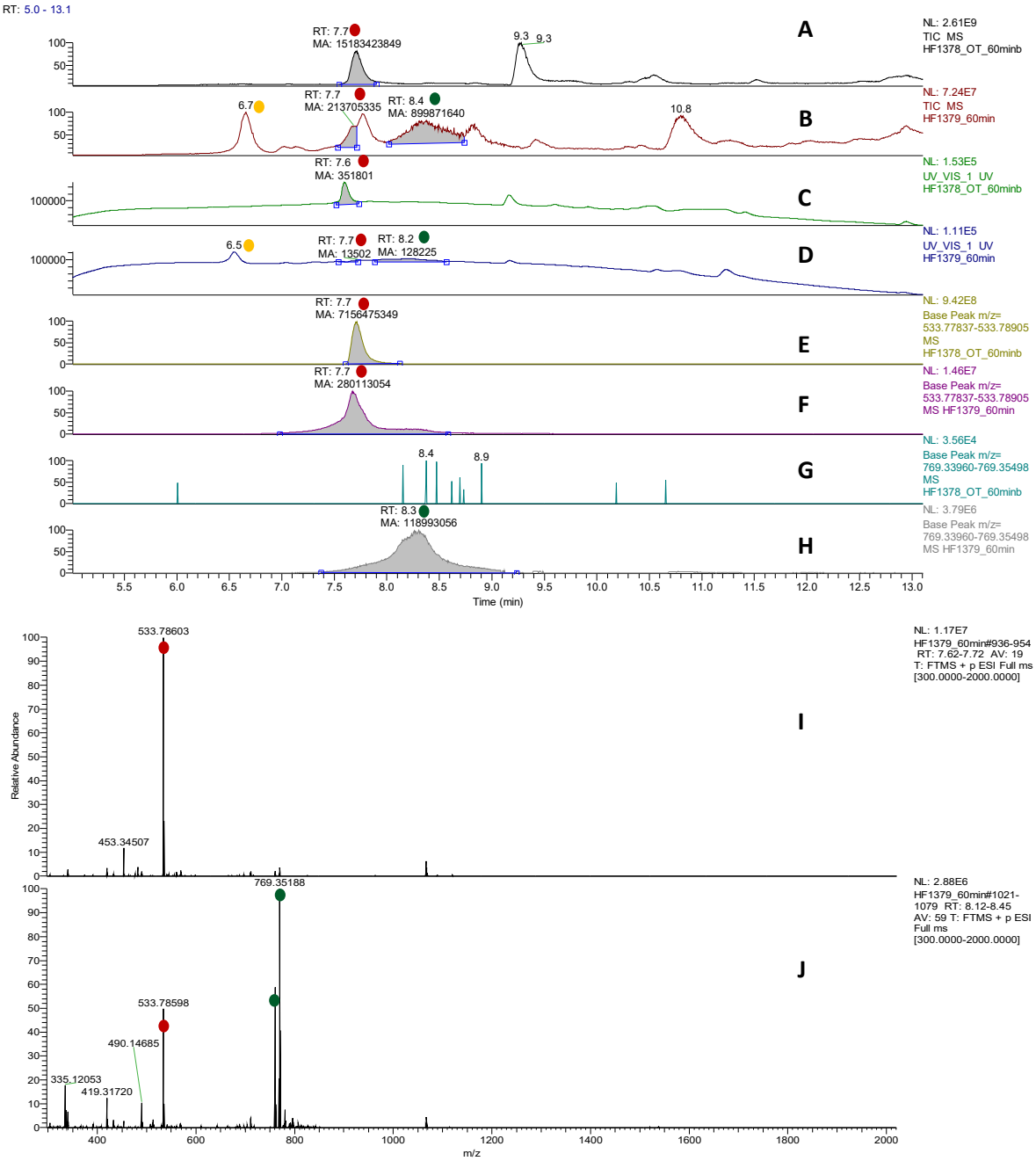
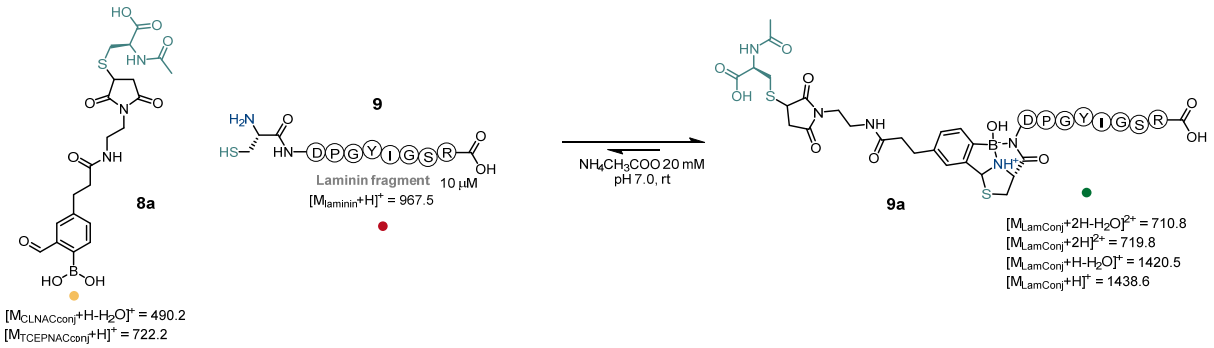


Figure S 3.2 -[9] **A** - TIC chromatogram of ovalbumin (**10**) after 60 min reduction with TCEP. **B** - TIC chromatogram of reaction between ovalbumin (**10**) and **8a** after 60 min to form **10a**; **C** - HPLC UV trace at 210 nm of ovalbumin (**10**) after 60 min reduction with TCEP. **D** - HPLC UV trace at 210 nm of reaction between ovalbumin (**10**) and **8a** after 60 min to form **10a**. **E** – EIC at 533.78371 (m/z for $[\mathbf{10}+2H]^{2+}$) for ovalbumin (**10**) after 60 min reduction with TCEP. **F** - EIC at 533.78371 (m/z for $[\mathbf{10}+2H]^{2+}$) of reaction between ovalbumin (**10**) and **8a** after 60 min to form **10a**. **G** – EIC at 769.34729 (m/z for $[\mathbf{10a}+2H]^{2+}$) for **10a** in the reduction of ovalbumin **10** after 60 min reduction with TCEP. **H** - EIC at 769.34729 (m/z for $[\mathbf{10a}+2H]^{2+}$) of reaction between ovalbumin (**10**) and **8a** after 60 min to form **10a**. **I** - HRMS ESI^{+}-MS spectrum of the peak at 7.6 min (ovalbumin (**10**)); **J** - HRMS ESI^{+}-MS spectrum of the peak at 8.3 min (**10a**). The reversibility of the boronated thiazolidine linkage is probably causing the broadening of the chromatographic peaks of conjugate **10a**. Although the baseline noise in UV-trace chromatograms do not allow accurate conversion calculation, it indicate near quantitative conversion of **10**, also confirmed by EIC area of **10** before (**E**) and after (**F**) the addition of **8a** (96% conversion).

[9] LC-MS were acquired in positive mode and the HPLC runs were carried out with a gradient of A (Milli Q water containing 0.1% v/v Formic acid (FA)) and B (acetonitrile containing 0.1% v/v FA). The mobile phase was t = 0 min, 5% B; t = 1min, 5% B; t = 10 min, 95% B; t = 11 min, 95% B; t = 12 min, 5% B; t = 15 min, stop, at a flow rate of 0.3 mL/min.

3.1.1.3 Reaction of Laminin with 2FBBA-NAC conjugate (9a)



Laminin sequnce: CDPGYIGSR

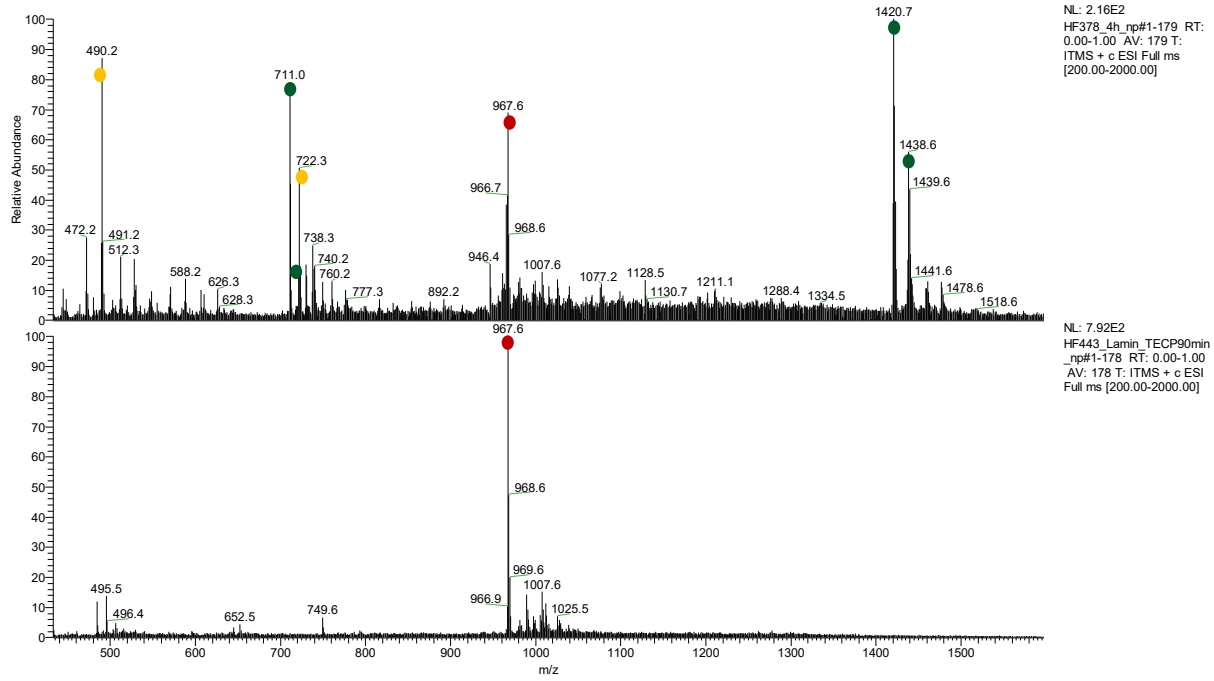
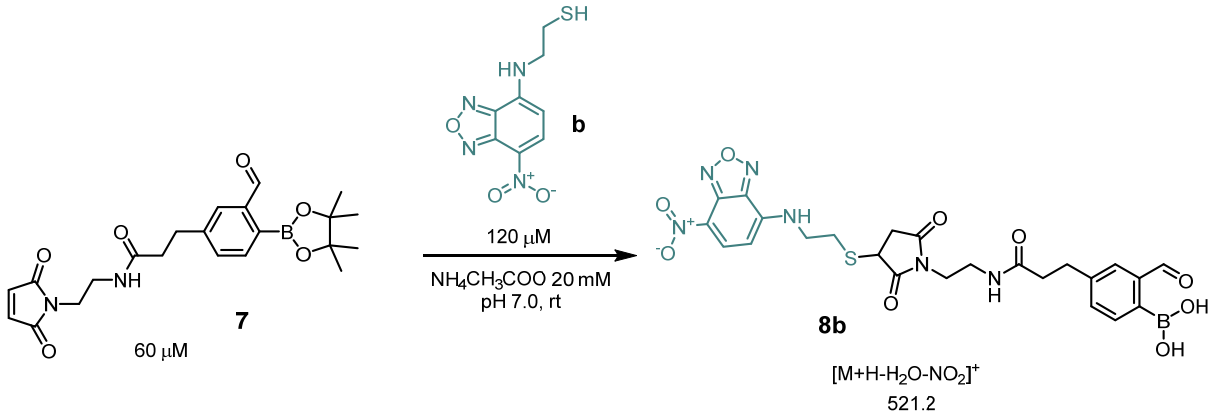


Figure S 4 - ESI$^+$-MS spectrum of Laminin (**9**) reaction with **8a** after 1h (top) and ESI$^+$-MS spectrum of Laminin (**9**) (bottom).

3.1.2 Crosslink betwenn thiol-furanzan and c-Ovalbumin/Laminin

3.1.2.1 Reaction of thiol-furanzan with crosslinker 7 to form 8b.



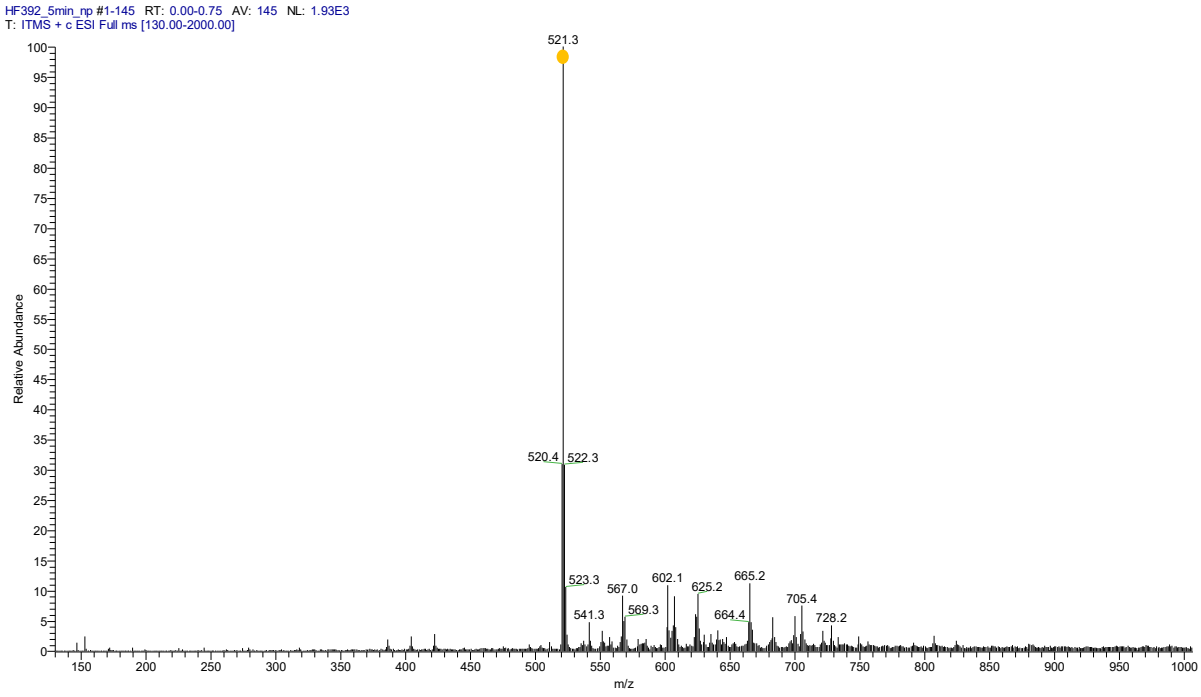


Figure S 5 - ESI$^+$-MS spectrum after reaction of crosslinker **7** with furanzan-thiol (**b**) after 5 min.

3.1.2.2 Reaction of Ovalbumin with 2FBBA-thiol-furanzan conjugate to form 10b

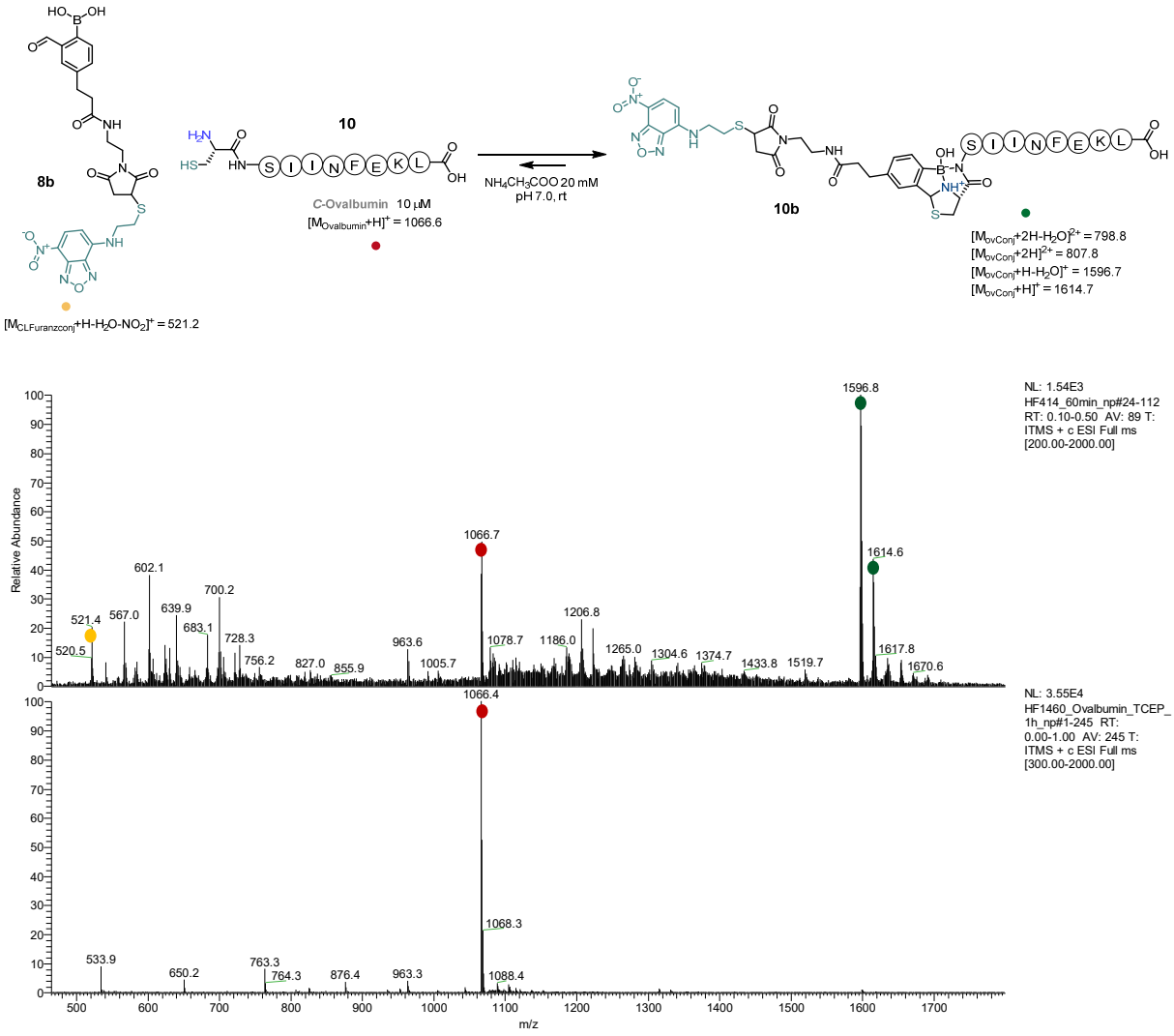


Figure S 6 - ESI⁺-MS spectrum of Ovalbumin (**10**) reaction with **8b** after 1h (top) and ESI⁺-MS spectrum of Ovalbumin (**10**) (bottom).

3.1.2.3 Reaction of Laminin with 2FBBA-thiol-furanzan conjugate to form 9b.

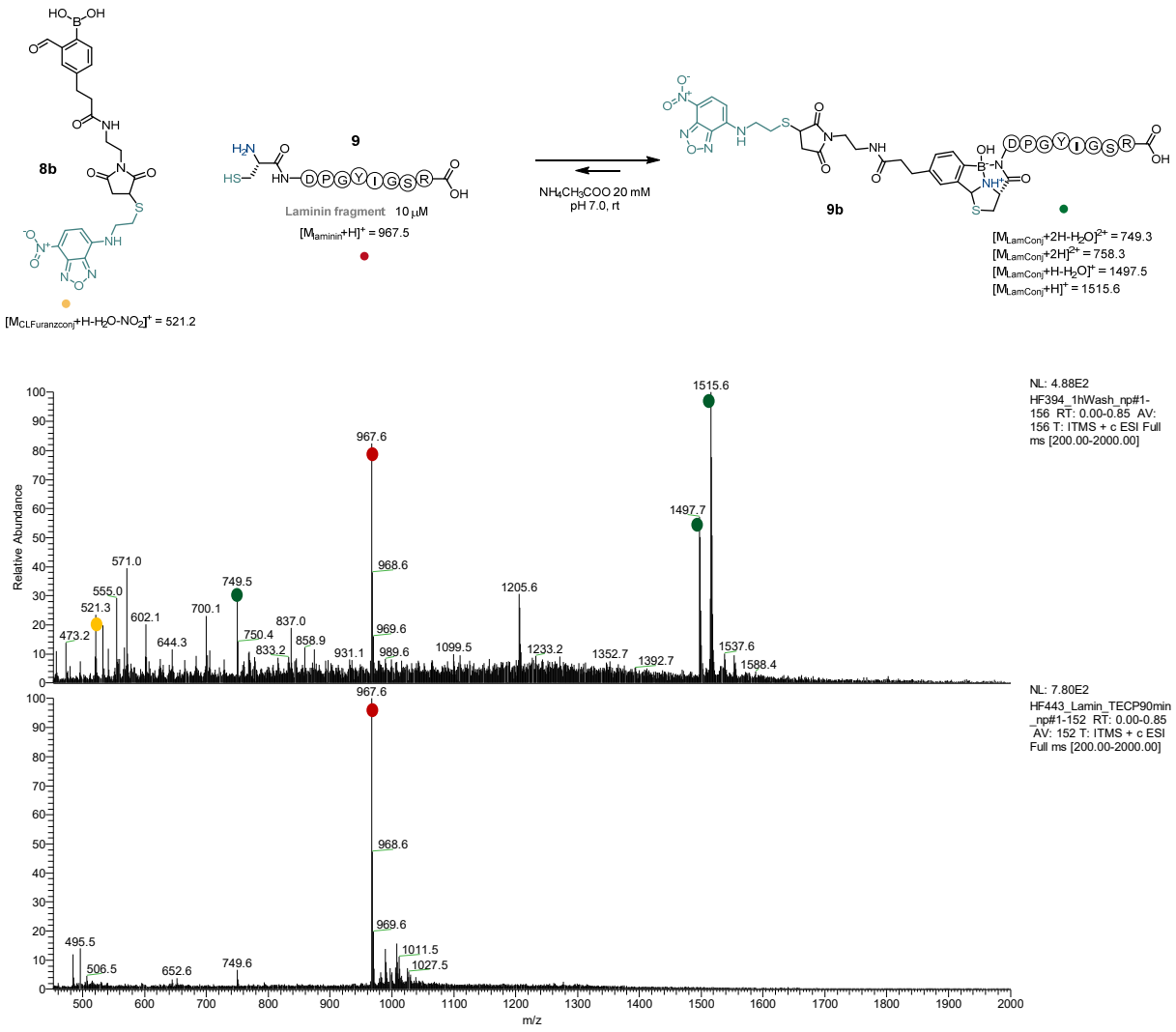
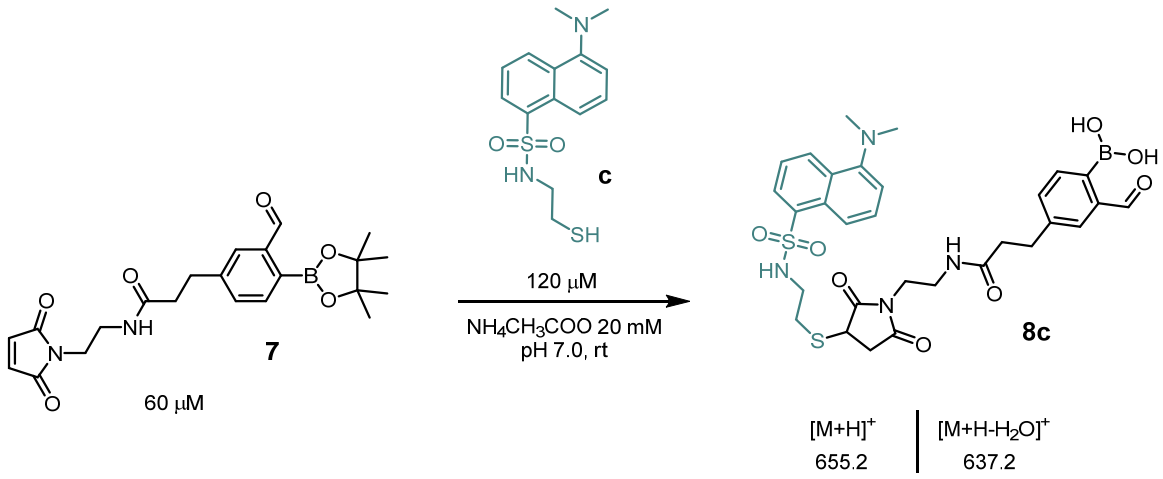


Figure S 7 - ESI$^+$-MS spectrum of Laminin (**9**) reaction with **8b** after 1h (top) and ESI$^+$-MS spectrum of Laminin (**9**) (bottom).

3.1.3 Crosslink between thiol-Dansyl and Laminin

3.1.3.1 Reaction of thiol-Dansyl with crosslinker 7 to form 8c.



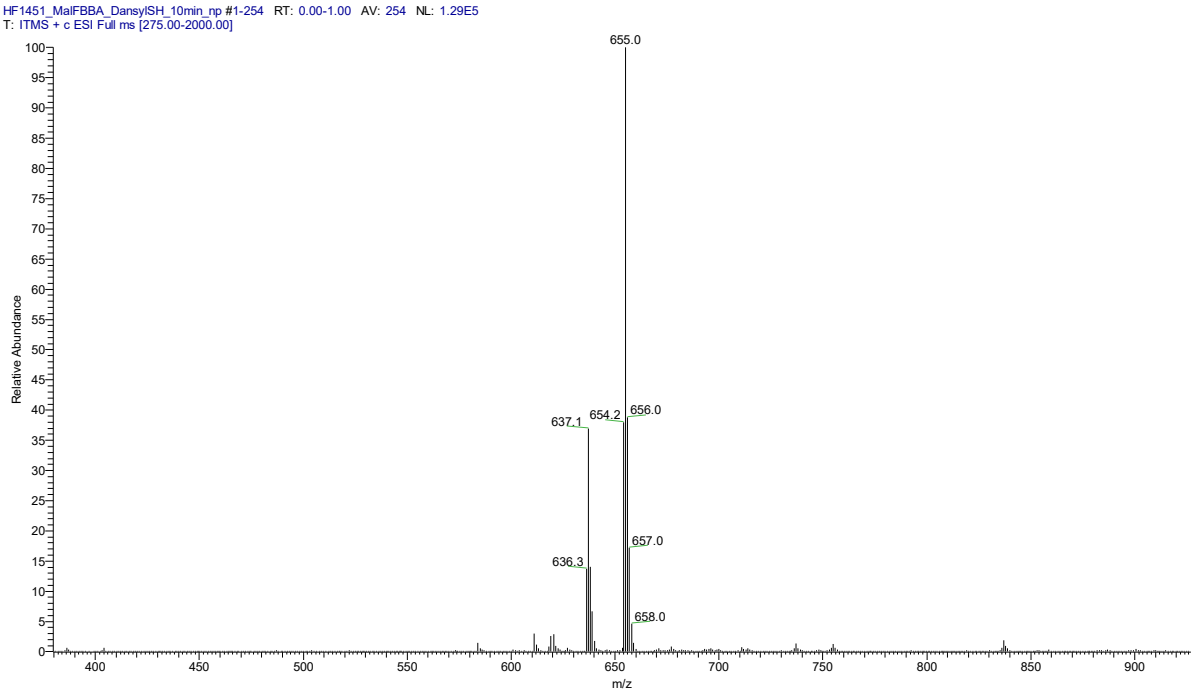


Figure S 8 - ESI$^+$-MS spectrum of the reaction bewteeen crosslinker **7** with Dansyl-thiol **(c)** after 10 min.

3.1.3.2 Reaction of Laminin with FBBA-thiol-Dansyl conjugate 7 to form 9c.

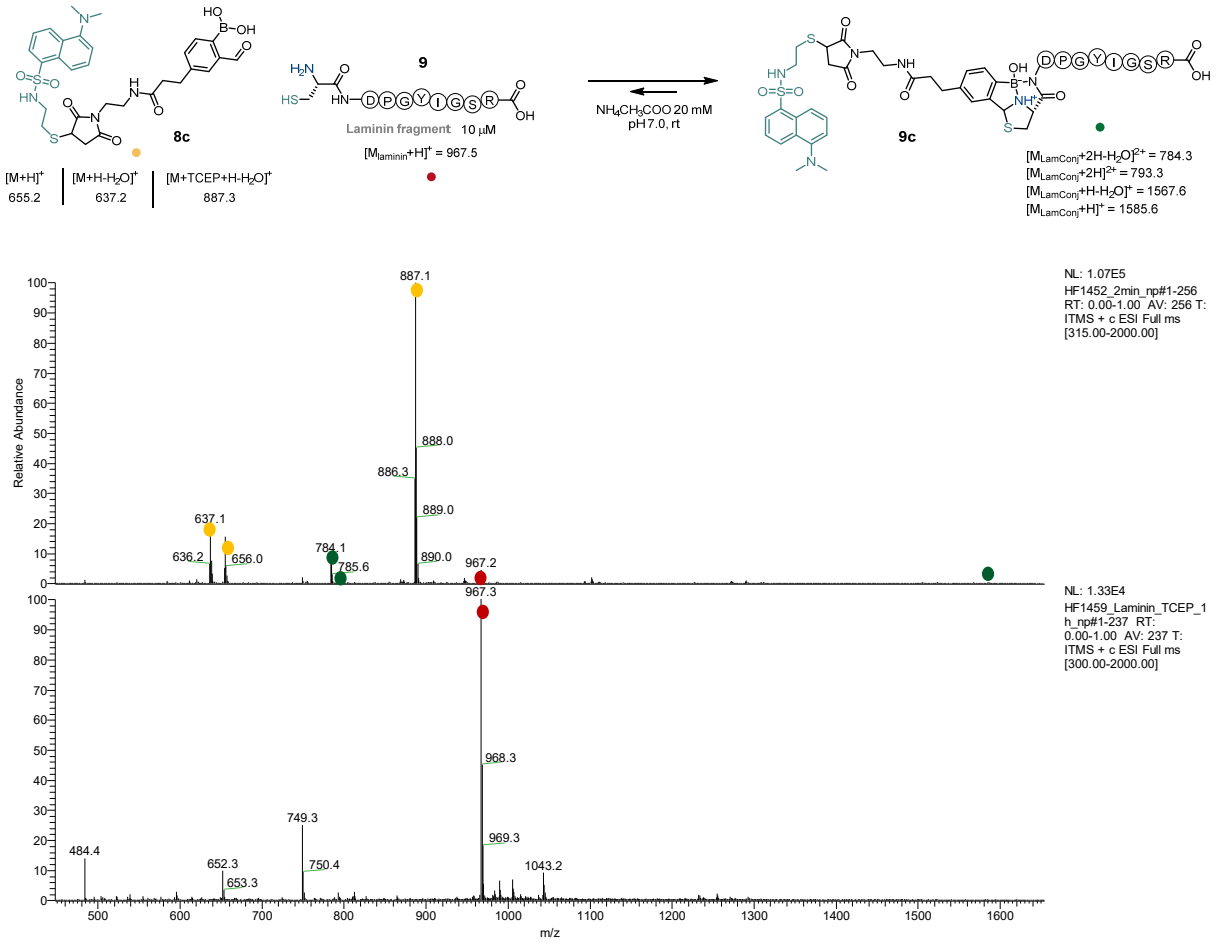
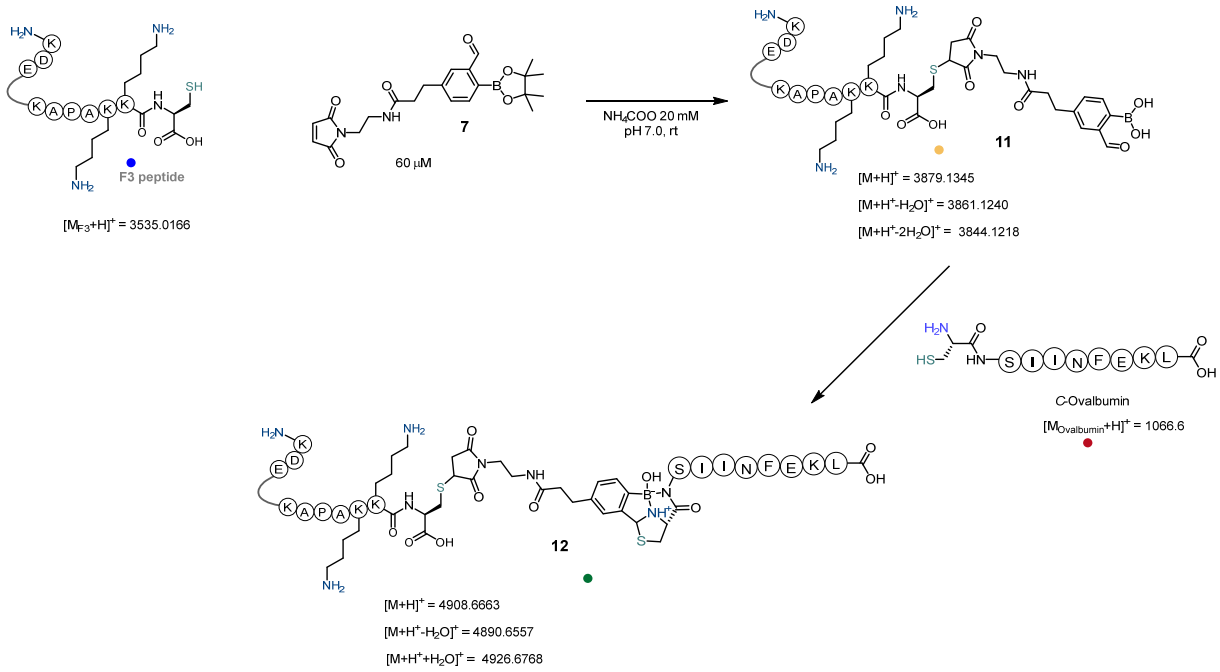


Figure S 9 - ESI$^+$-MS spectrum of the reaction bewteeen Laminin (**9**) and **8c** after 1h (top) and ESI$^+$-MS spectrum of Laminin (**9**) (bottom).

3.1.4 Crosslink between F3 peptide and Ovalbumin

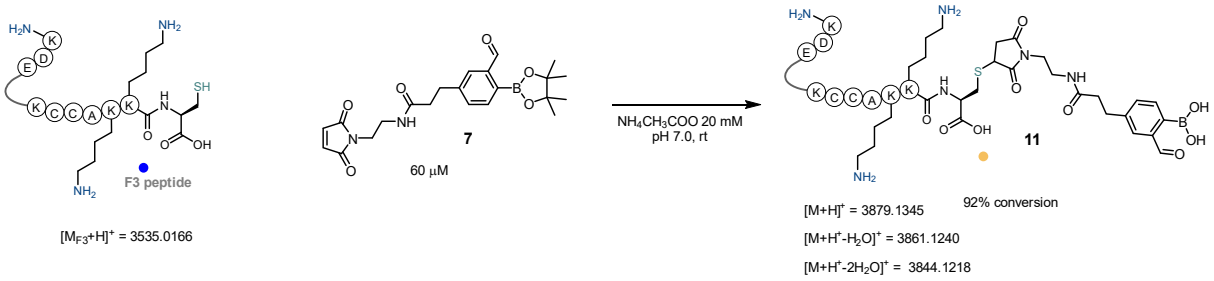


To a solution of F3 (0.283 mM in water, 1mg/mL) (35 µL, 0.010 µmol) in Acetate solution 20 mM pH 7.0 (495 µL) was added Tris(2-carboxyethyl)phosphine hydrochloride (TCEP) (10 mM in water) (10 µL, 0.10 µmol) and allow to reduce for 30 min. Then crosslink **7** (10mM in ACN, 1.1 µL, 0.011 µmol) was added and allow to react for 15 min and the reaction was monitored by LC-MS. A 92% conversion (calculated from TIC peak) is observed for the starting peptide.

In a second eppendorf, to a solution of c-Ovalbumin (1mg/mL, 0.847 mM (35.1 µL, 0.030 µmol)) in Acetate solution 20 mM pH 7.0 (495 µL) was added TCEP (10 mM in water, 2 mg in 0.698 mL) (10 µL, 0.10 µmol) and the solution was mixed for 30 min at 25 ºC.

Then, the solutions of **11** and Ovalbumin were mixed and allowed to react for 150 min and monitored by LC-MS. A 78% conversion is observed from the **11** starting material and the desired F3-Ovalbumin (**12**) peak observed at 6.4 min.

3.1.4.1 Reaction of F3 with crosslinker 7 to form 11.



$[M_{F3}+H]^+ = 3535.0166$

$[M+H]^+ = 3879.1345$

$[M+H^+-H_2O]^+ = 3861.1240$

$[M+H^+-2H_2O]^+ = 3844.1218$

F3 Alone

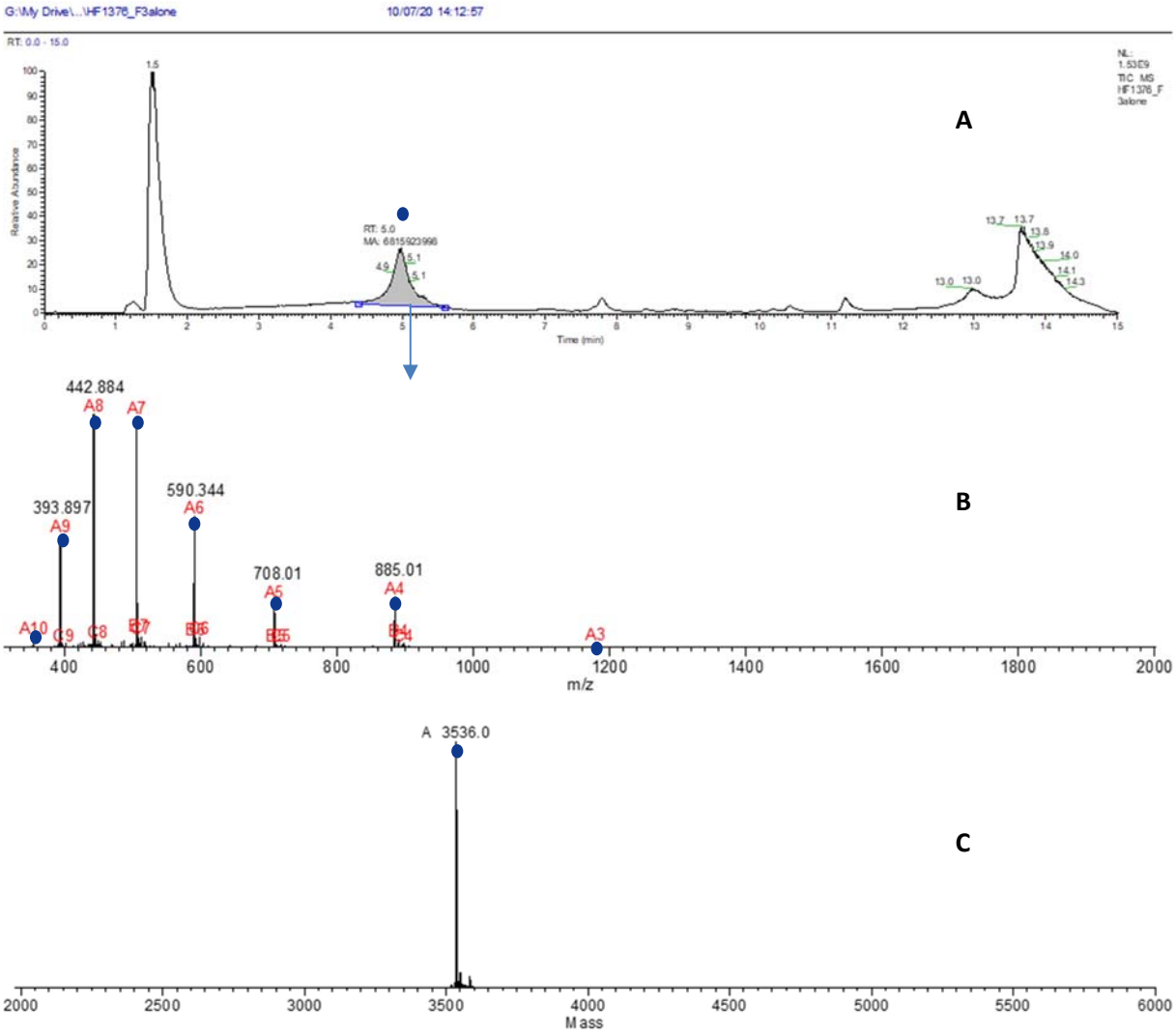


Figure S 10 –[10] **A** - TIC chromatogram of F3 (**11**); **B** – HRMS ESI$^+$-MS spectrum of the peak at 5.0 min (F3 (**11**)); **C** - Deconvoluted spectrum of the peak at 5.0 min, in MagTran software.[11]

[10] LC-MS were acquired in positive mode and the HPLC runs were carried out with a gradient of A (Milli Q water containing 0.1% v/v Formic acid (FA)) and B (acetonitrile containing 0.1% v/v FA). The mobile phase was t = 0 min, 5% B; t = 1min, 5% B; t = 10 min, 95% B; t = 11 min, 95% B; t = 12 min, 5% B; t = 15 min, stop, at a flow rate of 0.3 mL/min.

[11] Z. Zhang and A. G. Marshall, *J. Am. Soc. Mass Spectrom.*, 1998, **9**, 225–233.

F3 + Crosslink (11)

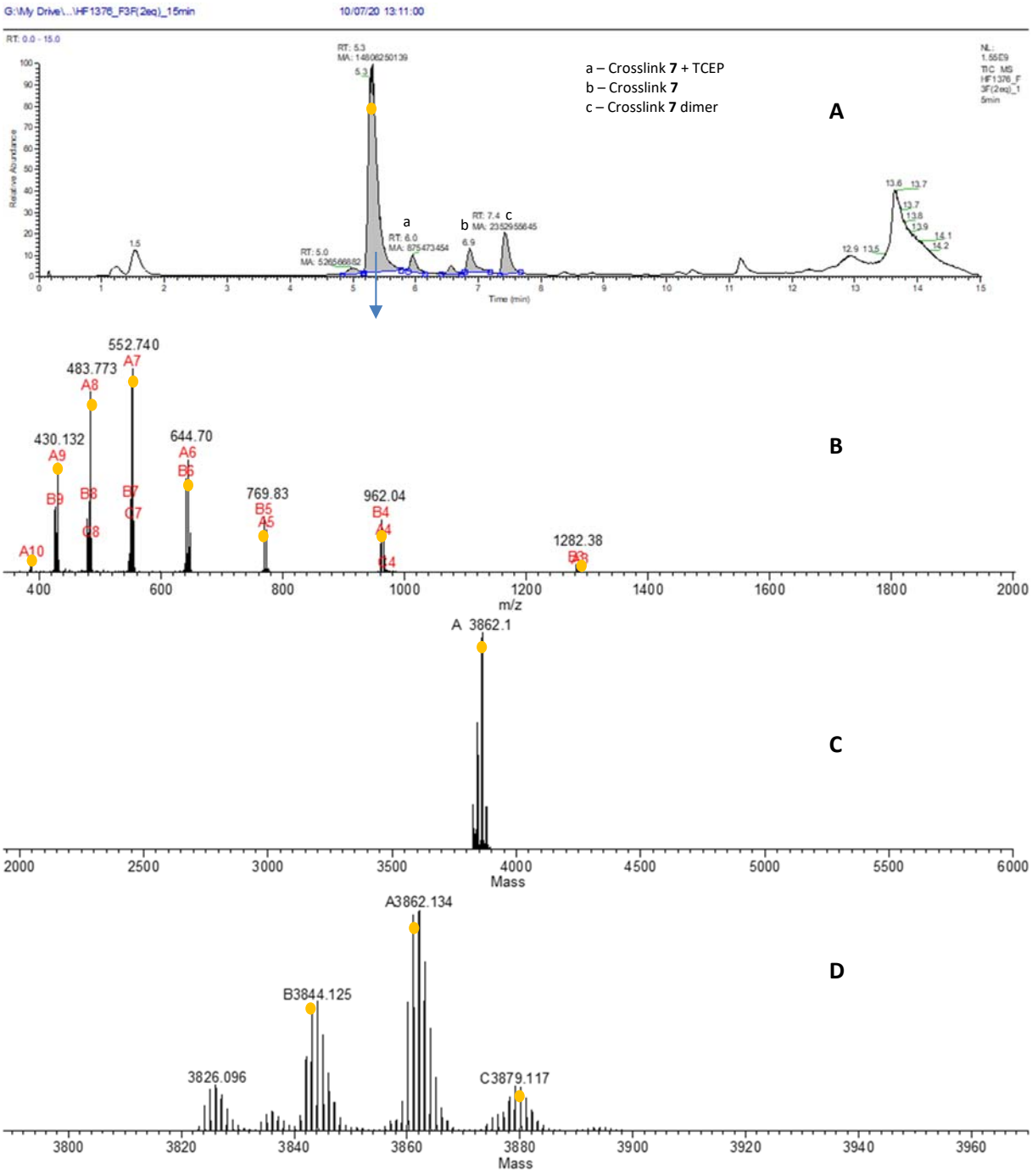


Figure S 11 –[12] **A** - TIC chromatogram of reaction between F3 (**11**) and crosslink **7** after 5 min to form **11**; **B** – HRMS ESI$^+$-MS spectrum of the peak at 5.3 min (**11**); **C** - Deconvoluted spectrum of the peak at 5.3 min (**11**), in MagTran software.[11] **D** – Zoom of the deconvoluted peak at 5.3 min (**11**).

[12] LC-MS were acquired in positive mode and the HPLC runs were carried out with a gradient of A (Milli Q water containing 0.1% v/v Formic acid (FA)) and B (acetonitrile containing 0.1% v/v FA). The mobile phase was t = 0 min, 5% B; t = 1min, 5% B; t = 10 min, 95% B; t = 11 min, 95% B; t = 12 min, 5% B; t = 15 min, stop, at a flow rate of 0.3 mL/min.

F3-ovalbumin crosslink (12)

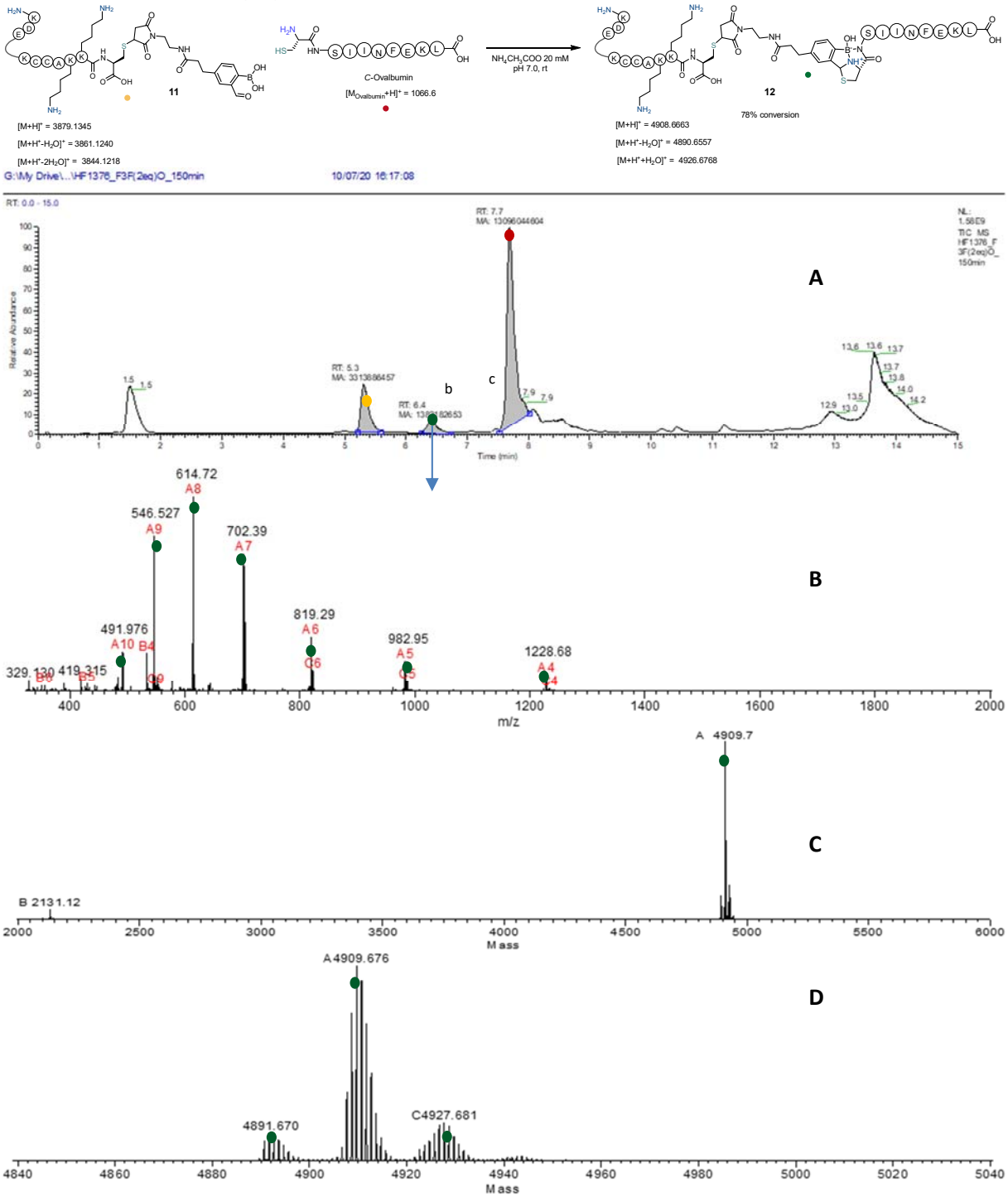
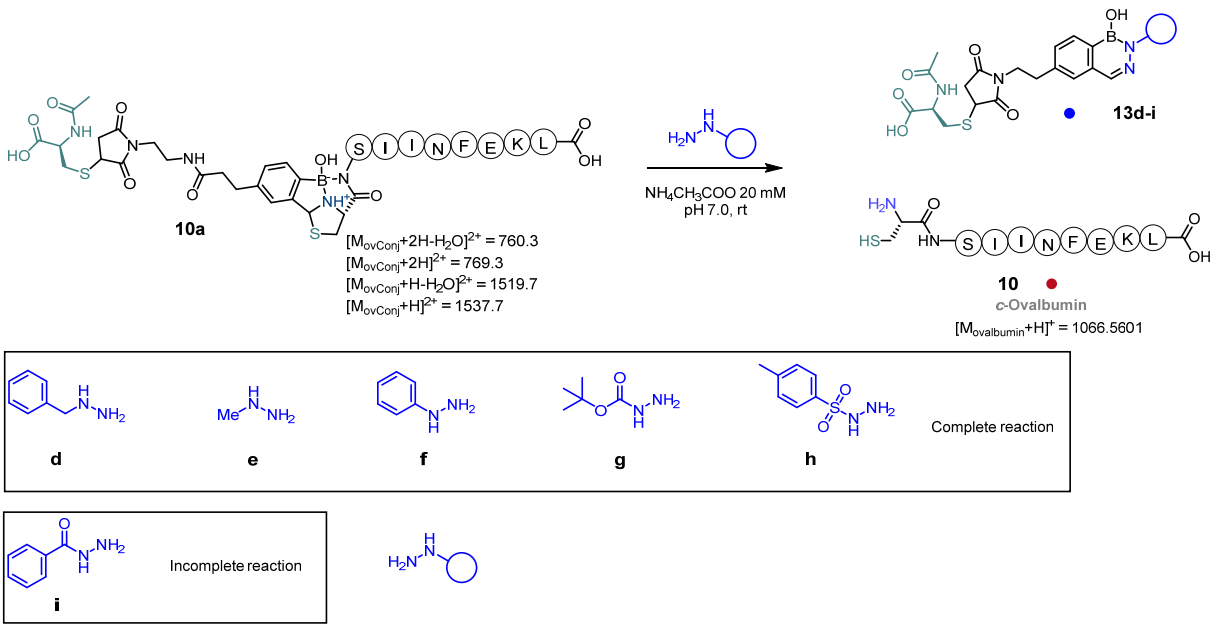


Figure S 12 –[13] **A** - TIC chromatogram of reaction between **11** and c-Ovalbumin **10** after 150 min to form **12**; **B** – HRMS ESI$^+$-MS spectrum of the peak at 6.4 min (**12**); **C** - Deconvoluted spectrum of the peak at 6.4 min (**12**), in MagTran software.[11] **D** – Zoom of the deconvoluted peak at 6.4 min (**12**).

[13] LC-MS were acquired in positive mode and the HPLC runs were carried out with a gradient of A (Milli Q water containing 0.1% v/v Formic acid (FA)) and B (acetonitrile containing 0.1% v/v FA). The mobile phase was t = 0 min, 5% B; t = 1min, 5% B; t = 10 min, 95% B; t = 11 min, 95% B; t = 12 min, 5% B; t = 15 min, stop, at a flow rate of 0.3 mL/min.

3.2 Reversibility of the boronated-thiazolidine ligation upon addition of hydrazines



To a solution of *C*-Ovalbumin (**10**) (0.847 mM (11.8 µL, 0.010 µmol) in ammonium acetate 20 mM pH = 7.0 (500 µL) was added a Tris(2-carboxyethyl)phosphine hydrochloride (TCEP) (10 mM in water, 10 µL, 0.10 µmol) and the solution mixed for 30 at 25 ºC.

In a separated Eppendorf, crosslinker **7** (10 mM in ACN) (10 µL, 0.10 µmol) was added to a solution of the thiol (10 mM in ACN or water) (20 µL, 0.20 µmol) in ammonium acetate 20 mM pH = 7.0 (500 µL) and mixed for 15 min.

Then, both solutions were mixed and allow to react for 1h and the reaction monitored in Positive Mode of ESI-MS (direct infusion).

Then, the hydrazine (**d-i**) (100 mM in ACN or ammonium acetate pH 7 20mM) (10 µL, 1.0 µmol) was added and allow to react for 1h and the reaction monitored in Positive Mode of ESI-MS (direct infusion).

3.2.1 Reversibility of the boronated thiazolidine 10a with benzylhydrazine d

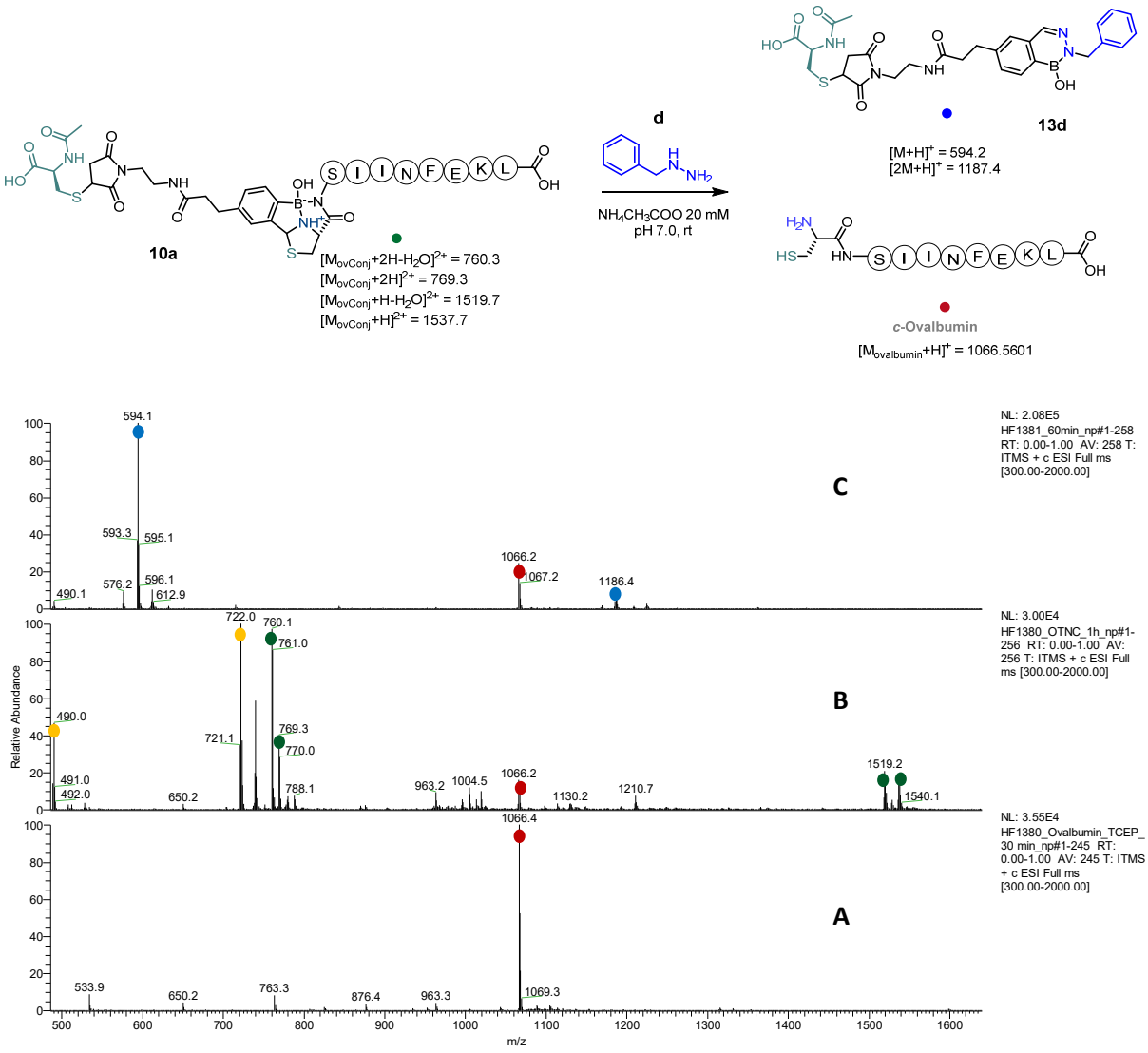


Figure S 13.1 – **A** - ESI$^+$-MS spectrum of Ovalbumin (**10**); **B** - ESI$^+$-MS spectrum of Ovalbumin reaction with **8a** after 1h; **C** - ESI$^+$-MS spectrum of **10a** with benzylhydrazine **d** after 1h.

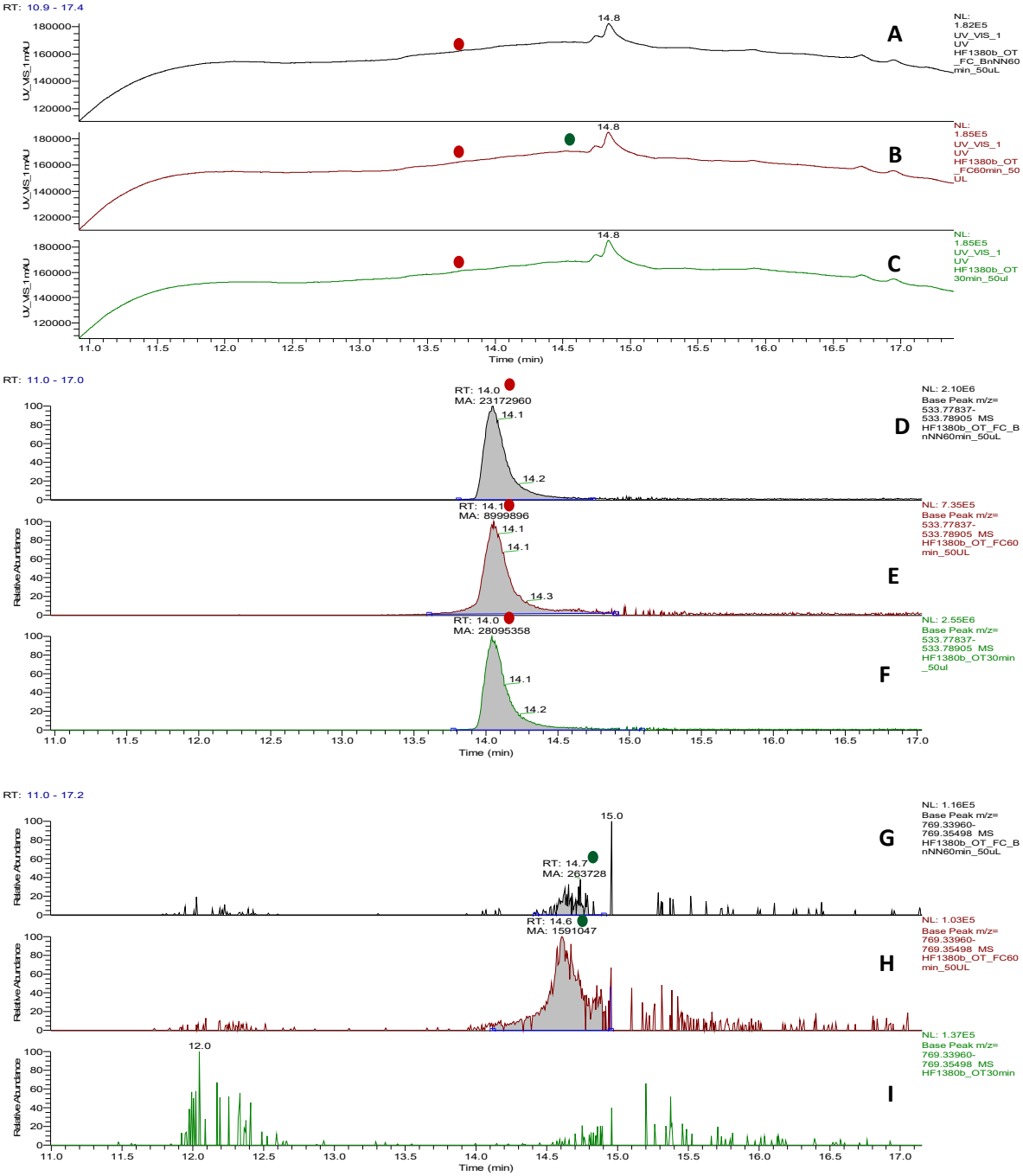


Figure S 13.2 -[14] **A** - HPLC UV trace at 210 nm 60 min after the addition of benzylhydrazine **d**. **B** - HPLC UV trace at 210 nm for the formation of **10a**; **C** - HPLC UV trace at 210 nm of ovalbumin (**10**) after 60 min reduction with TCEP. **D** – EIC at 533.78371 (m/z for $[\mathbf{10}+2H]^{2+}$) for ovalbumin (**10**) 60 min after the addition of benzylhydrazine **d**. **E** - EIC at 533.78371 (m/z for $[\mathbf{10}+2H]^{2+}$) of reaction between ovalbumin (**10**) and **8a** after 60 min to form **10a**. **F** - EIC at 533.78371 (m/z for $[\mathbf{10}+2H]^{2+}$) of ovalbumin (**10**) after 60 min reduction with TCEP. **G** – EIC at 769.34729 (m/z for $[\mathbf{10a}+2H]^{2+}$) for **10a** 60 min after the addition of benzylhydrazine **d. H** – EIC at 769.34729 (m/z for $[\mathbf{10a}+2H]^{2+}$) for the formation of **10a. I** – EIC at 769.34729 (m/z for $[\mathbf{10a}+2H]^{2+}$) for ovalbumin (**10**) after 60 min reduction with TCEP. Baseline noise in UV-traces at 210 nm impede extracting relevant information. Comparing EIC **E** and **F** is observed a 68% conversion of ovalbumin **10**. Comparing **F** and **D**, 60 min after the addition of benzylhydrazine **d**, 83% of ovalbumin **10** is observed in the reaction mixture.

[14] LC-MS were acquired in positive mode and the HPLC runs were carried out with a gradient of A (Milli Q water containing 0.1% v/v Formic acid (FA)) and B (acetonitrile containing 0.1% v/v FA). The mobile phase was t = 0 min, 5% B; t = 5 min, 5% B; t = 15 min, 95% B; t = 17 min, 95% B; t = 18 min, 5% B; t = 20 min, stop, at a flow rate of 0.2 mL/min.

3.2.2 Reversibility of the boronated thiazolidine 10a with methylhydrazine e

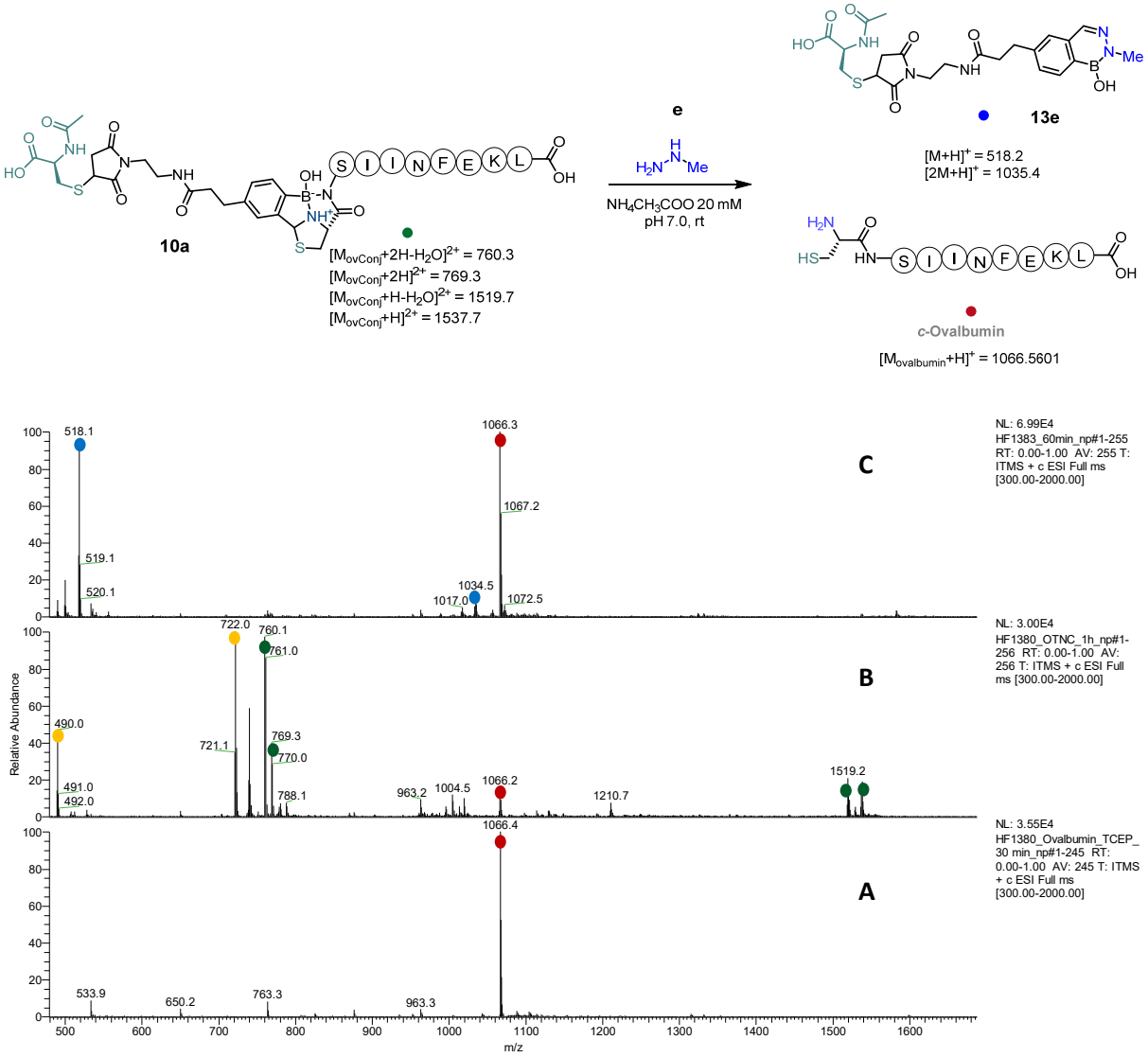


Figure S 14 – **A** - ESI$^+$-MS spectrum of Ovalbumin (**10**); **B** - ESI$^+$-MS spectrum of Ovalbumin reaction with **8a** after 1h; **C**. ESI$^+$-MS spectrum of **10a** with methylhydrazine **e** after 1h.

3.2.3 Reversibility of the boronated thiazolidine 10a with phenylhydrazine f

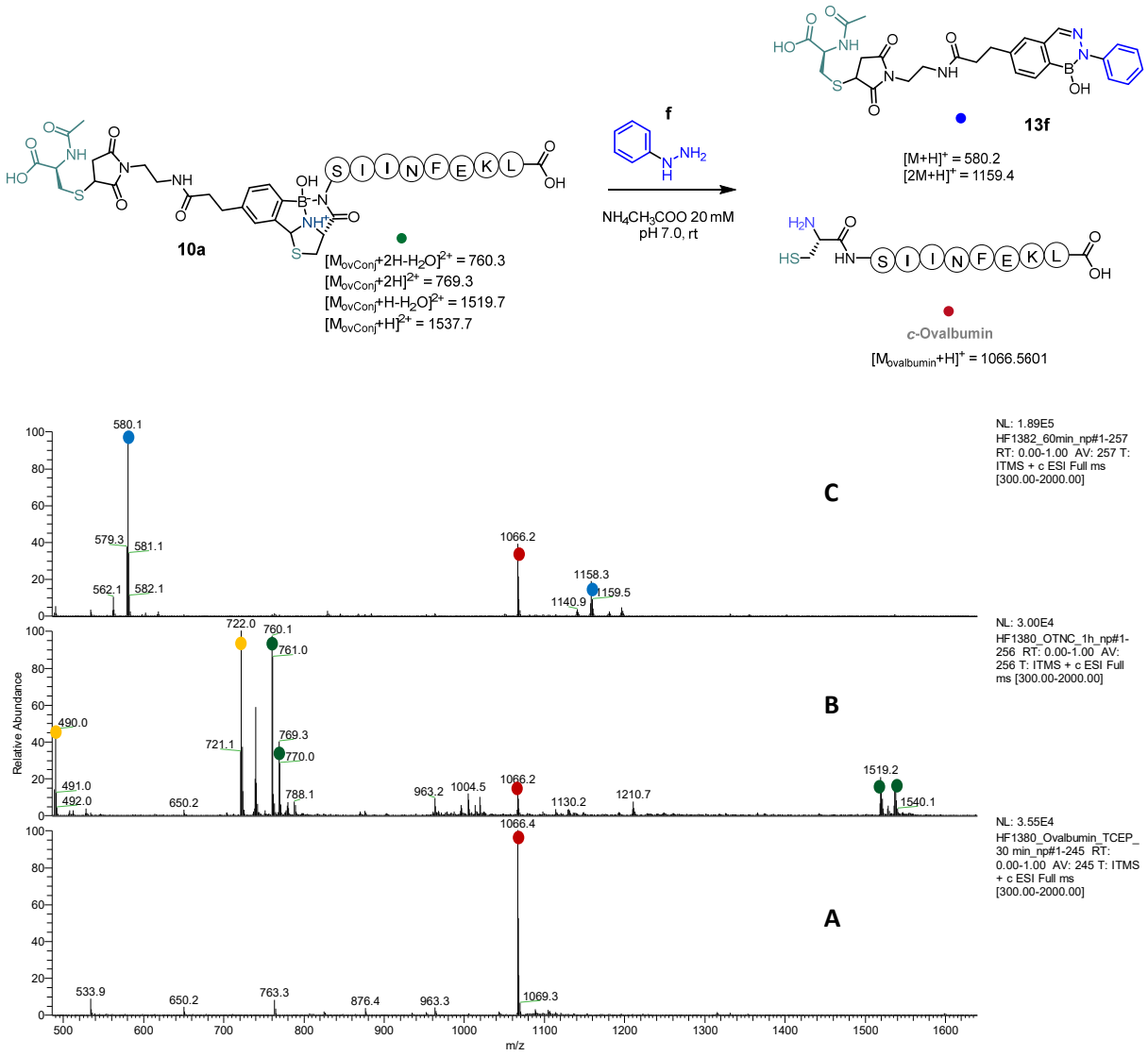


Figure S 15 – A - ESI$^+$-MS spectrum of Ovalbumin (**10**); **B** - ESI$^+$-MS spectrum of Ovalbumin reaction with **8a** after 1h; **C** - ESI$^+$-MS spectrum of **10a** with phenylhydrazine **f** after 1h.

3.2.4 Reversibility of the boronated thiazolidine 10a with BOC-hydrazine g

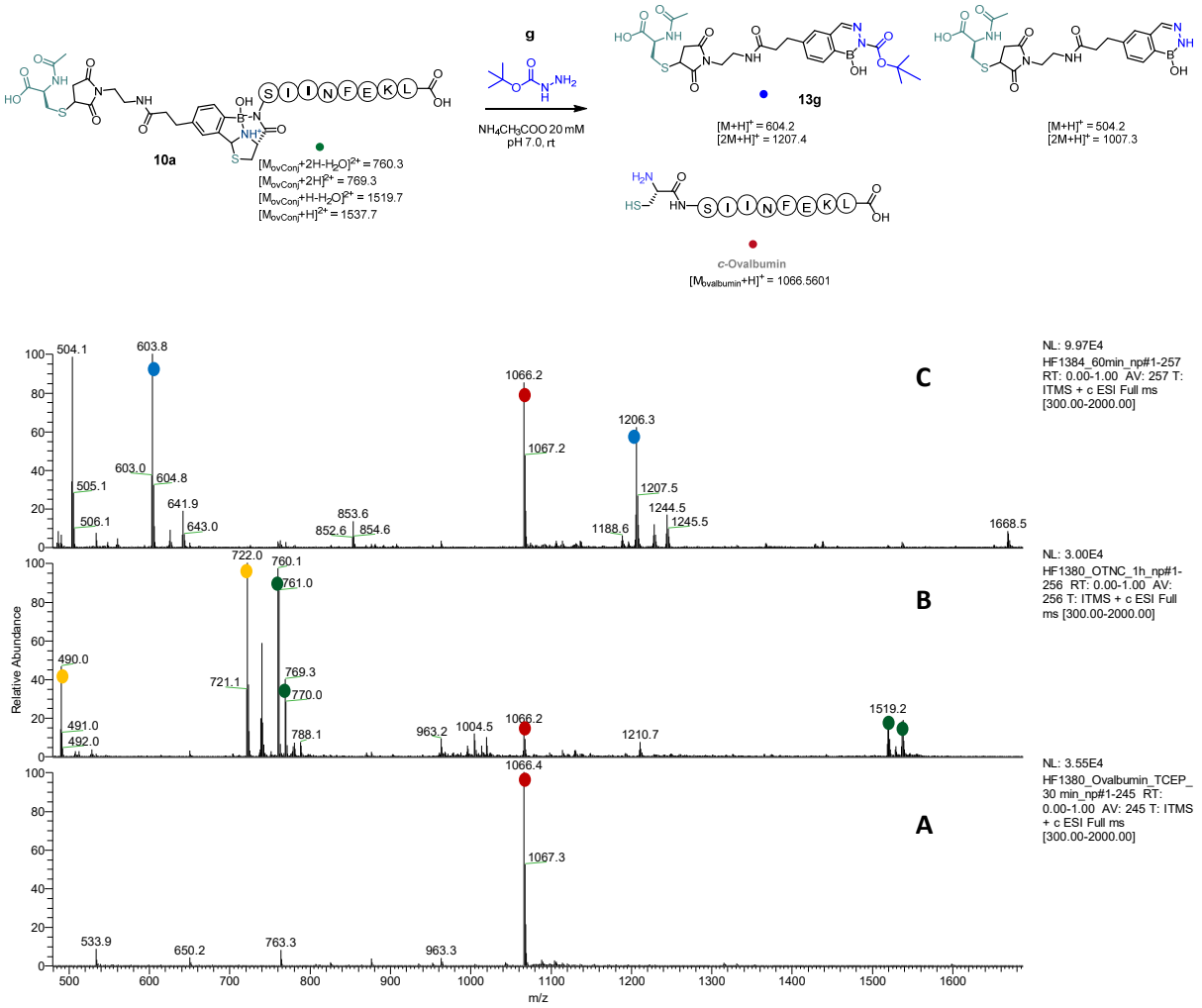


Figure S 16 – **A** - ESI$^+$-MS spectrum of Ovalbumin (**10**); **B** - ESI$^+$-MS spectrum of Ovalbumin reaction with **8a** after 1h; **C** - ESI$^+$-MS spectrum of **10a** with BOC-hydrazine **g** after 1h.

3.2.5 Reversibility of the boronated thiazolidine 10a with tosylhydrazine h

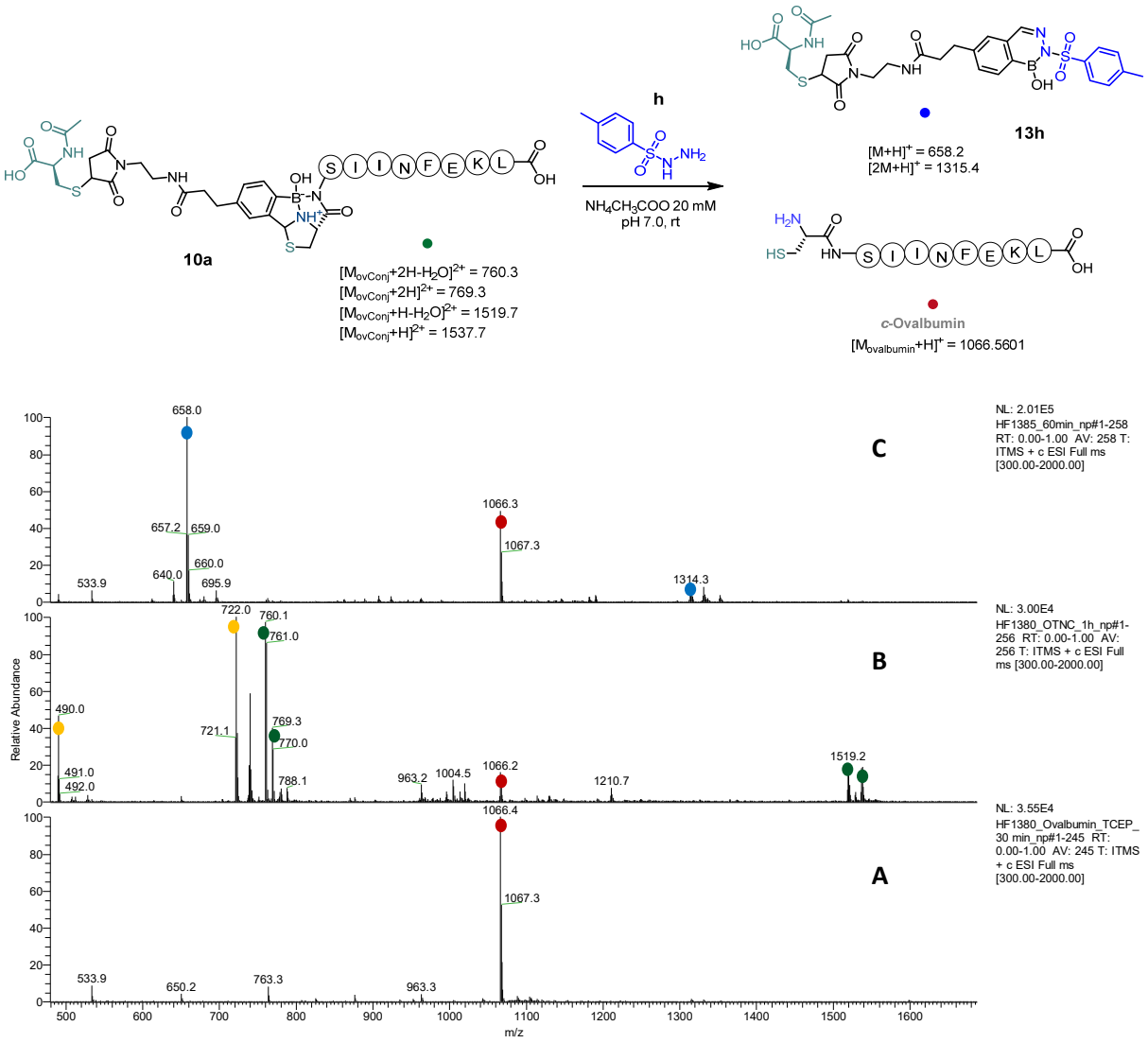


Figure S 17 – **A** - ESI$^+$-MS spectrum of Ovalbumin (**10**); **B** - ESI$^+$-MS spectrum of Ovalbumin reaction with **8a** after 1h; **C** - ESI$^+$-MS spectrum of **10a** with tosylhydrazine **h** after 1h.

3.2.6 Reversibility of the boronated thiazolidine 10a with benzoylhydrazine i

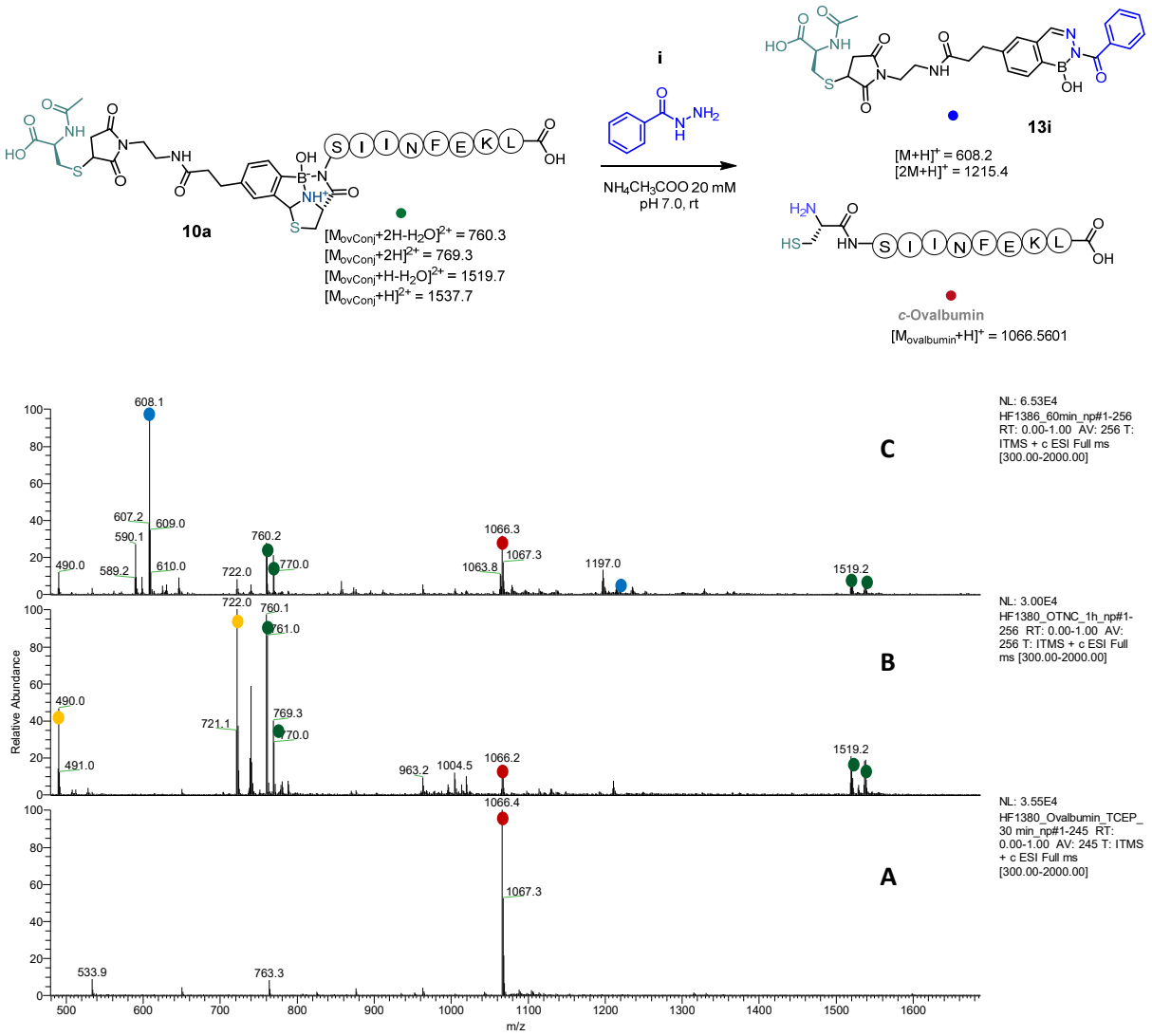
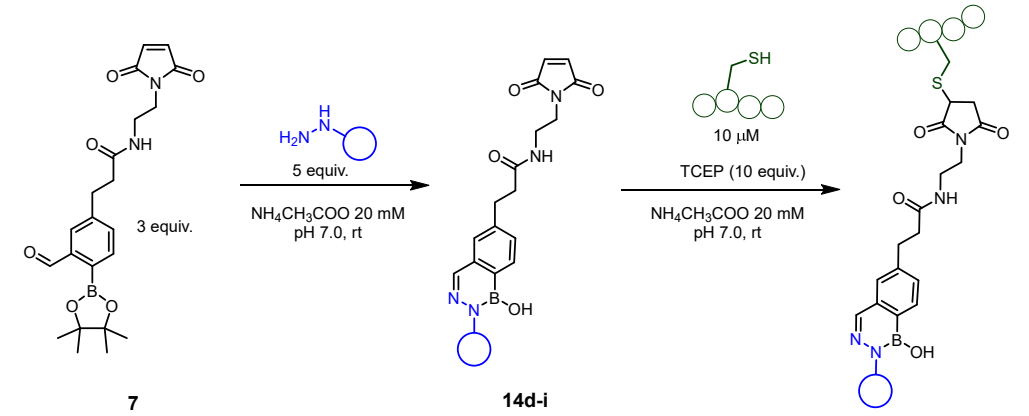


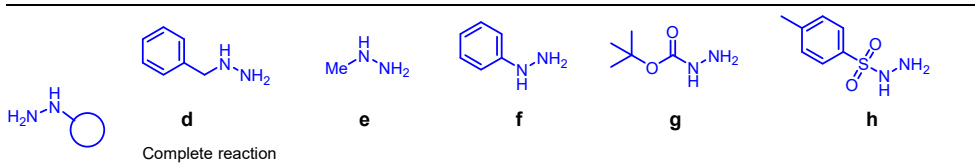
Figure S 18 – **A** - ESI$^+$-MS spectrum of Ovalbumin (**10**); **B** - ESI$^+$-MS spectrum of Ovalbumin reaction with **8a** after 1h; **C** - ESI$^+$-MS spectrum of **10a** with benzoylhydrazine **i** after 1h.

4. Crosslinking hydrazines with Cys peptides

4.1 Diazaborine formation followed by thiol-Michael (A1-A2 pathway)

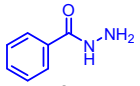


7 **14d-i** **10d-j, 9d and 15d-18d**



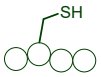
d **e** **f** **g** **h**

Complete reaction



i

Incomplete reaction



9 - Laminin
10 - c-Ovalbumin
15 - Bombesin
16 - F3
17 - GV1001
18 - AcGKCG

General procedure

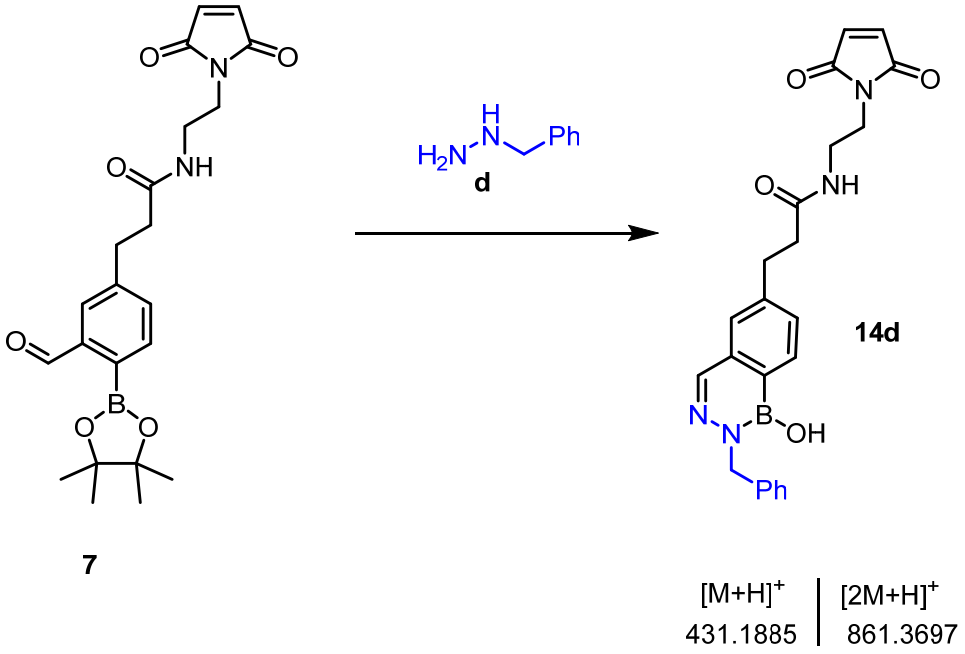
In an eppendorf crosslinker **7** (10mM in ACN, 3.0 µL, 0.03 µmol) was added to ammonium acetate solution 20 mM pH 7.0 (500 µL) and allow for BPin to be hydrolysed over 15 min. Then the hydrazine (5.00 µL, 0.050 µmol) was added and allowed to react for 5 min and the reaction monitored by direct infusion in Positive Mode of ESI-MS.

In a second eppendorf, to a solution of peptide (≈1 mM) (10 µL, 0.010 µmol) in ammonium acetate solution 20 mM pH 7.0 (500 µL) was added a Tris(2-carboxyethyl)phosphine hydrochloride (TCEP) (3.5 mM in water) (28.6 µL, 0.100 µmol) and the solution mixed for 30 min at 25 ºC.

Then both solutions were mixed and allow to react for 1h and the reaction monitored by direct infusion in Positive Mode of ESI-MS.

4.1.1 Peptide screening in the Crosslink with $BnNHNH_2$

4.1.1.1 Reaction of benzylhydrazine (d) with crosslinker 7



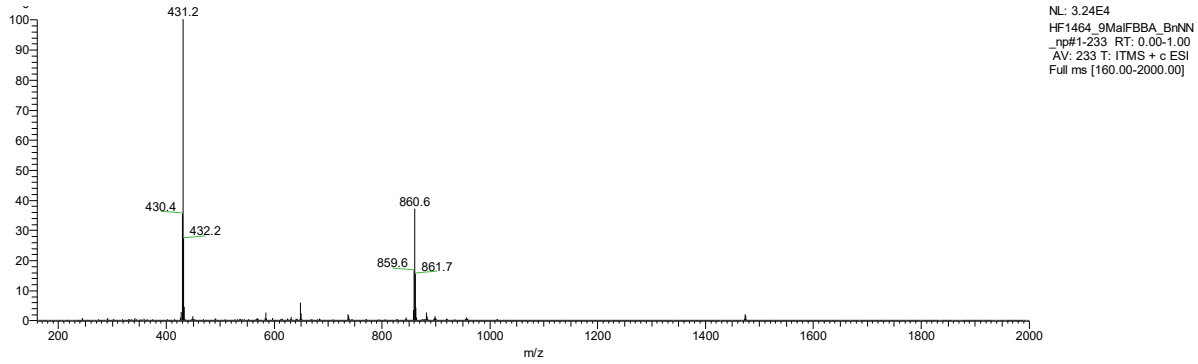


Figure S 19 - ESI$^+$-MS spectrum of reaction of benzylhydrazine **(d)** with **7** after 5 min to produce **14d**.

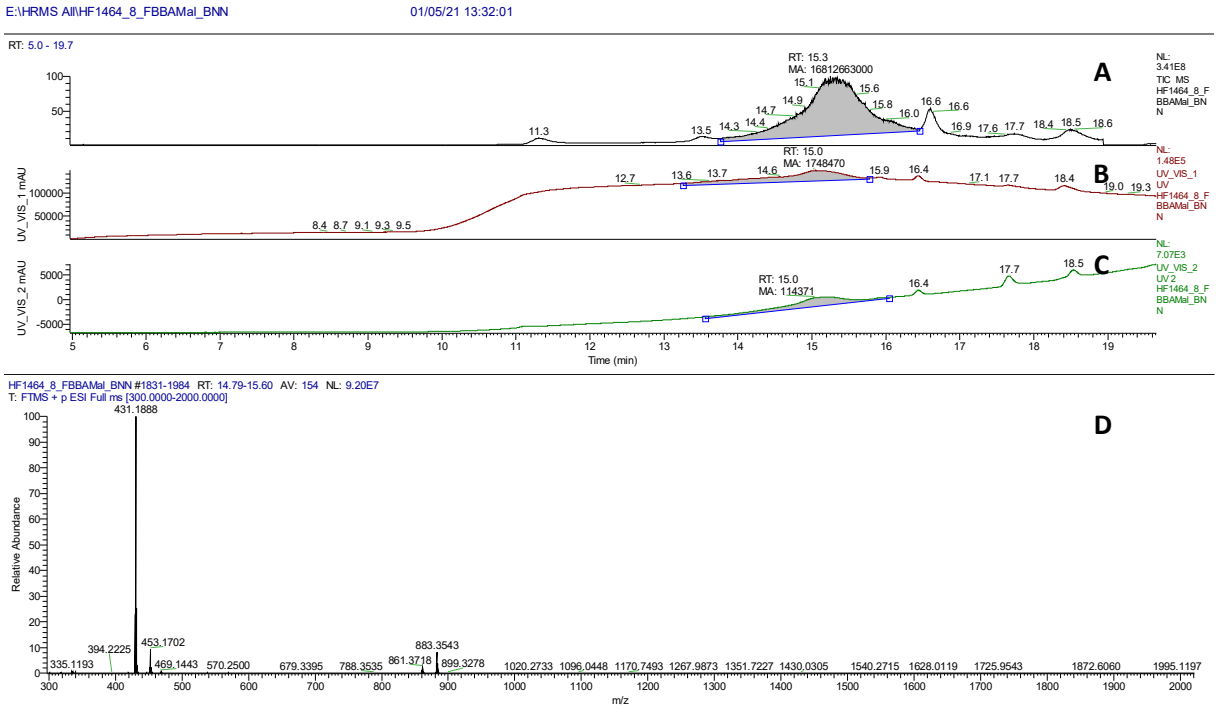


Figure S 20 –[15] Monitoring of the reaction of crosslinker **7** with benzylhydrazine **(d)** after 5 min. **A** – Total ion current (TIC); **B** - HPLC UV trace at 210 nm; **C** - HPLC UV trace at 254 nm; – HRMS-ESI^{+}-MS of the peak at 15.3 min corresponding to the DAB **14d**.

[15] LC-MS were acquired in positive mode and the HPLC runs were carried out with a gradient of A (Milli Q water containing 0.1% v/v Formic acid (FA)) and B (acetonitrile containing 0.1% v/v FA). The mobile phase was t = 0 min, 5% B; t = 5 min, 5% B; t = 15 min, 95% B; t = 17 min, 95% B; t = 18 min, 5% B; t = 20 min, stop, at a flow rate of 0.2 mL/min.

4.1.1.2 Reaction of c-Ovalbumin with benzylhydrazine-crosslink conjugate 14d.

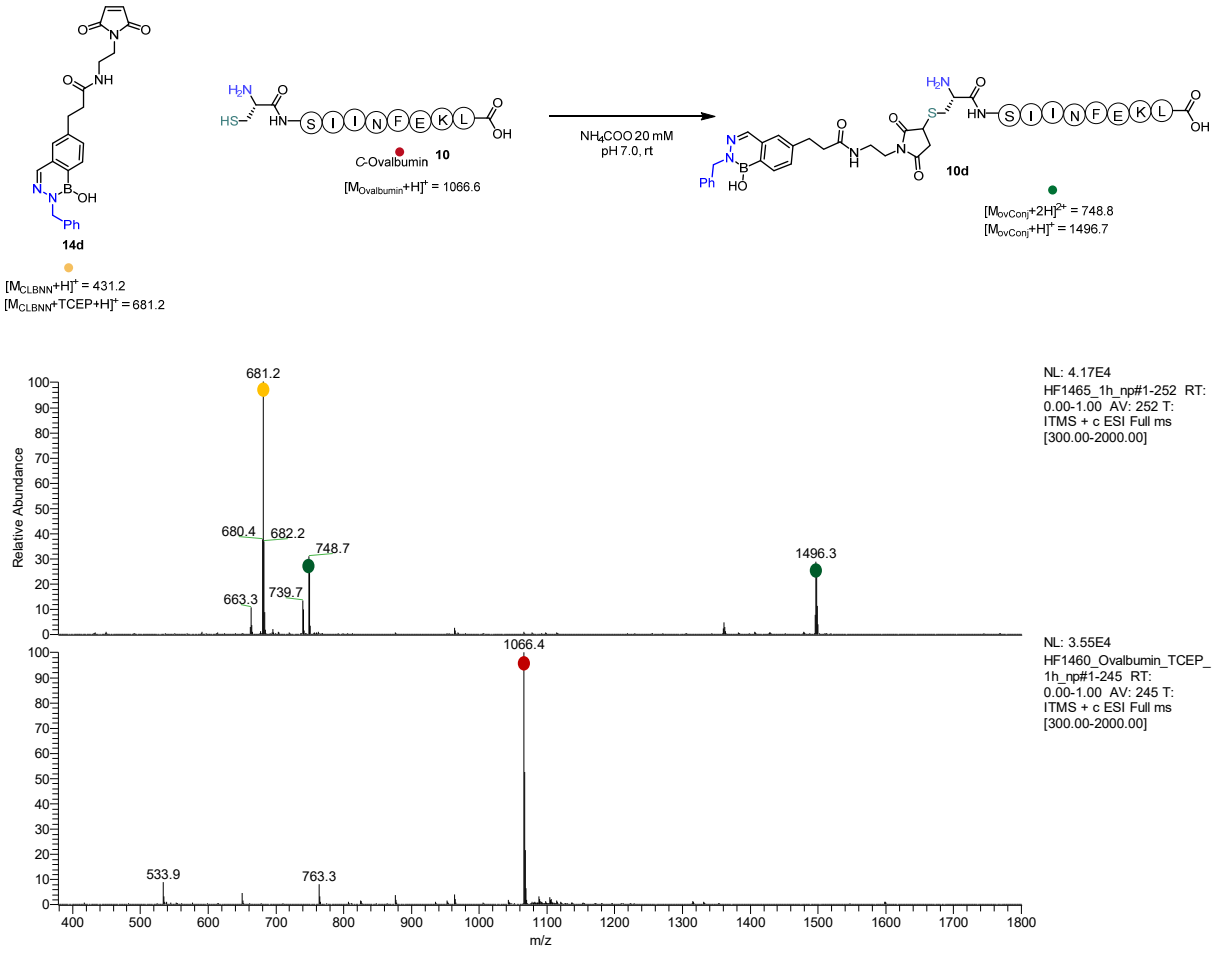


Figure S 21.1 - ESI$^+$-MS spectrum of Ovalbumin reaction with **14d** after 1h (top) and ESI$^+$-MS spectrum of Ovalbumin (**10**) (bottom).

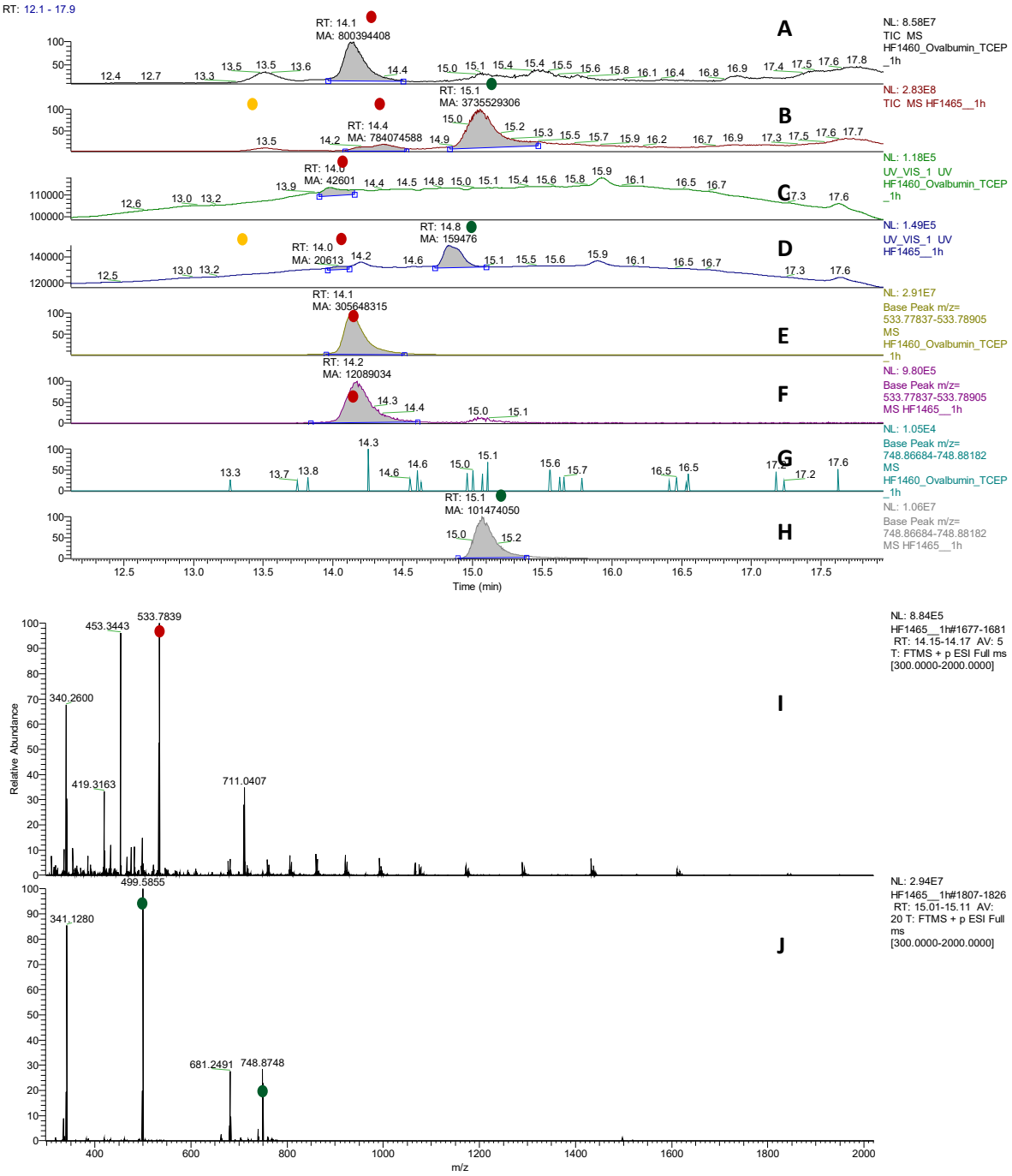


Figure S 21.2 -[16] **A** - TIC chromatogram of ovalbumin (**10**) after 60 min reduction with TCEP. **B** - TIC chromatogram of reaction between ovalbumin (**10**) and **14d** after 60 min to form **10d**; **C** - HPLC UV trace at 210 nm of ovalbumin (**10**) after 60 min reduction with TCEP. **D** - HPLC UV trace at 210 nm of reaction between ovalbumin (**10**) and **14d** after 60 min to form **10d**. **E** – EIC at 533.78371 (m/z for [**10**+2H]$^{2+}$) for ovalbumin (**10**) after 60 min reduction with TCEP. **F** - EIC at 533.78371 (m/z for [**10**+2H]$^{2+}$) of reaction between ovalbumin (**10**) and **14d** after 60 min to form **10d**. **G** – EIC at 748.87433 (m/z for [**10d**+2H]$^{2+}$) for **10d** in the reduction of ovalbumin **10** after 60 min reduction with TCEP. **H** - EIC at 748.87433 (m/z for [**10d**+2H]$^{2+}$) of reaction between ovalbumin (**10**) and **14d** after 60 min to form **10d**. **I** - HRMS ESI^{+}-MS spectrum of the peak at 14.1 min (ovalbumin (**10**)); **J** - HRMS ESI^{+}-MS spectrum of the peak at 15.1 min (**10d**). Although the baseline noise in UV-trace chromatograms do not allow accurate conversion calculation, it indicate near quantitative conversion of **10**, also confirmed by EIC area of **10** before (**E**) and after (**F**) the addition of **14d** (96% conversion).

[16] LC-MS were acquired in positive mode and the HPLC runs were carried out with a gradient of A (Milli Q water containing 0.1% v/v Formic acid (FA)) and B (acetonitrile containing 0.1% v/v FA). The mobile phase was t = 0 min, 5% B; t = 5 min, 5% B; t = 15 min, 95% B; t = 17 min, 95% B; t = 18 min, 5% B; t = 20 min, stop, at a flow rate of 0.2 mL/min.

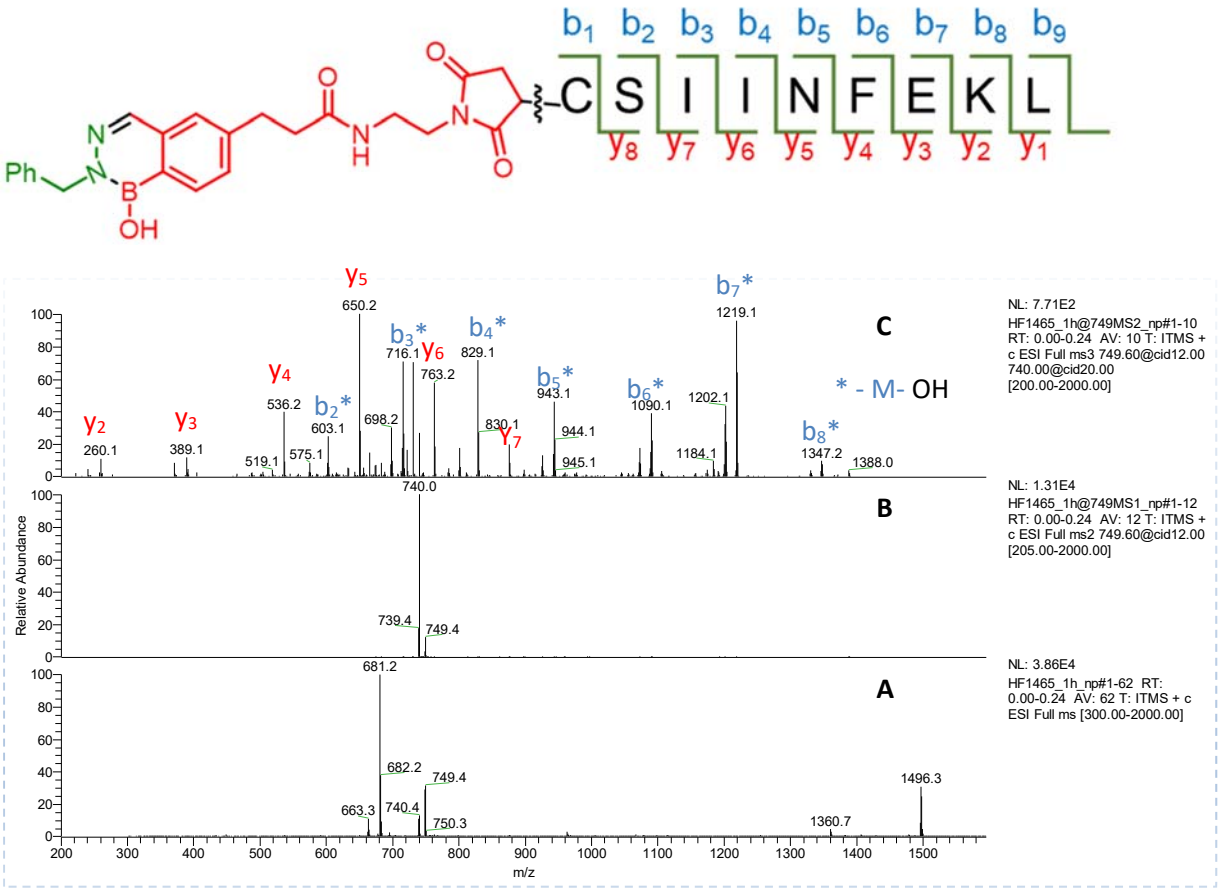


Figure S 22 – A - ESI+-MS spectrum of direct injection of the reaction between Ovalbumin (**10**) and **14d** to form **10d**. **B** – MS^2 fragmentation of conjugate **10d**. **C** – MS^3 fragmentation of conjugate **10d**

4.1.1.3 Reaction of Laminin with benzylhydrazine-crosslink conjugate 14d.

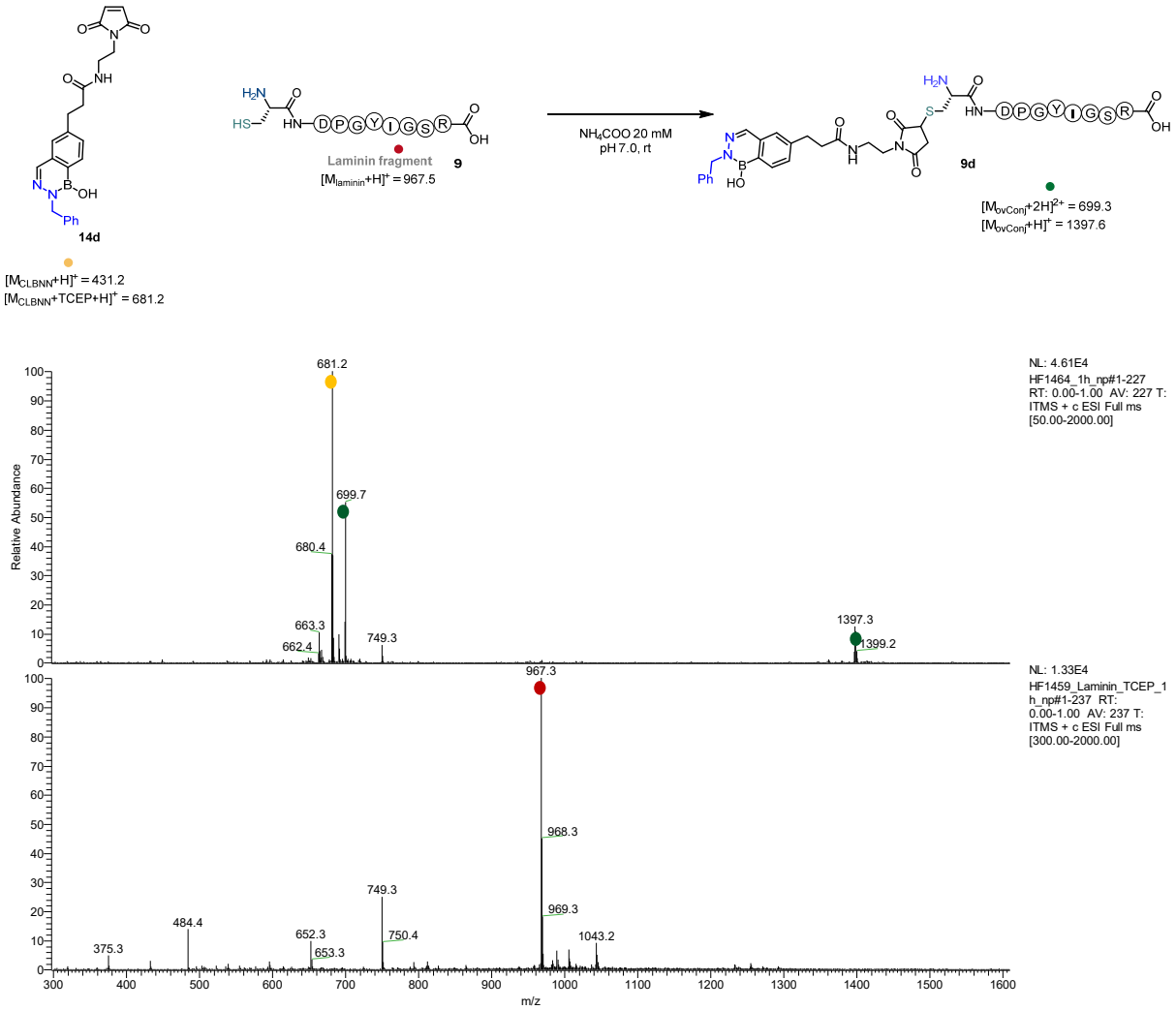


Figure S 23 - ESI$^+$-MS spectrum of Laminin (**9**) reaction with **14d** after 1h (top) and ESI$^+$-MS spectrum of Laminin (**9**) (bottom).

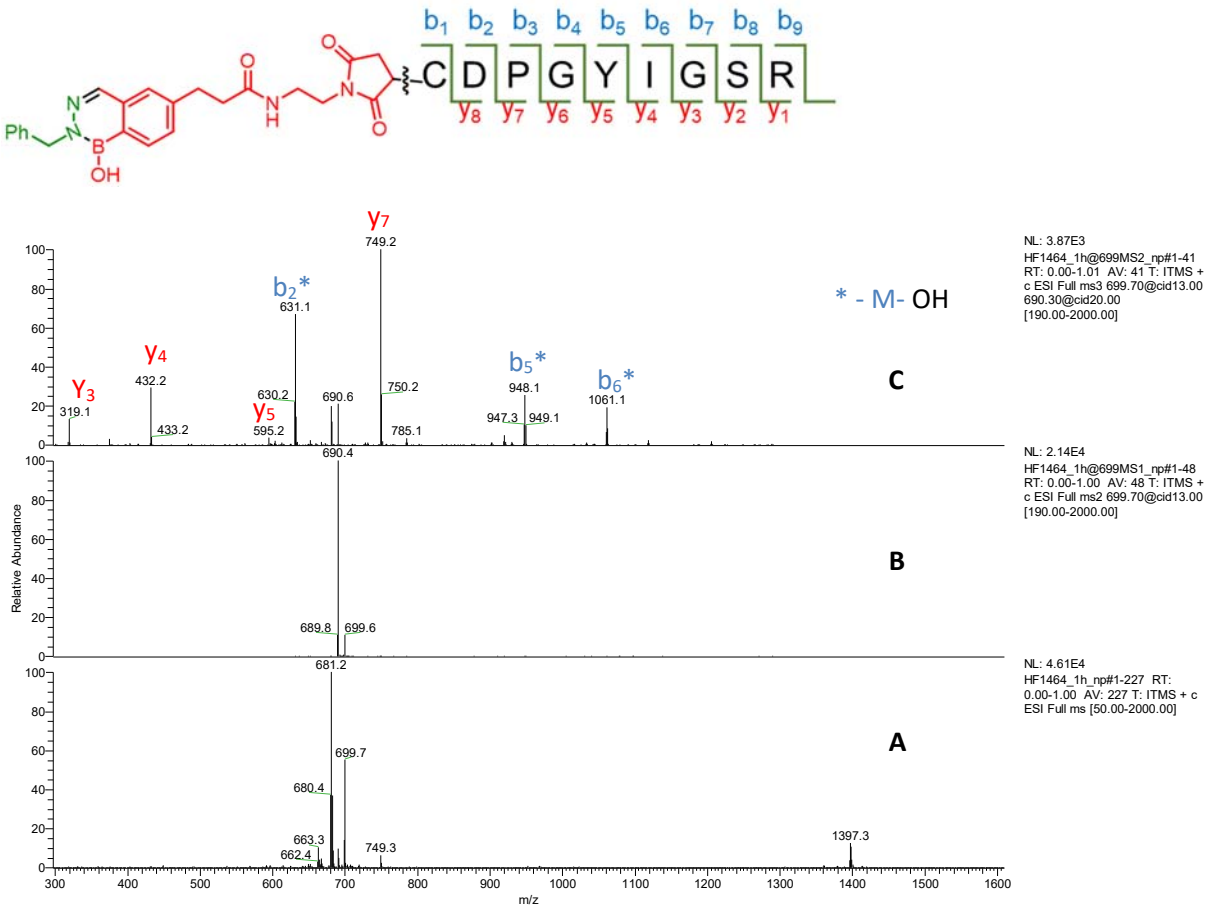


Figure S 24 – A - ESI+-MS spectrum of direct injection of the reaction between Laminin (**9**) and **14d** to form **9d**. **B** – MS^2 fragmentation of conjugate **9d**. **C** – MS^3 fragmentation of conjugate **9d**

4.1.1.4 Reaction of Cys-Bombesin with benzylhydrazine-crosslink conjugate 14d.

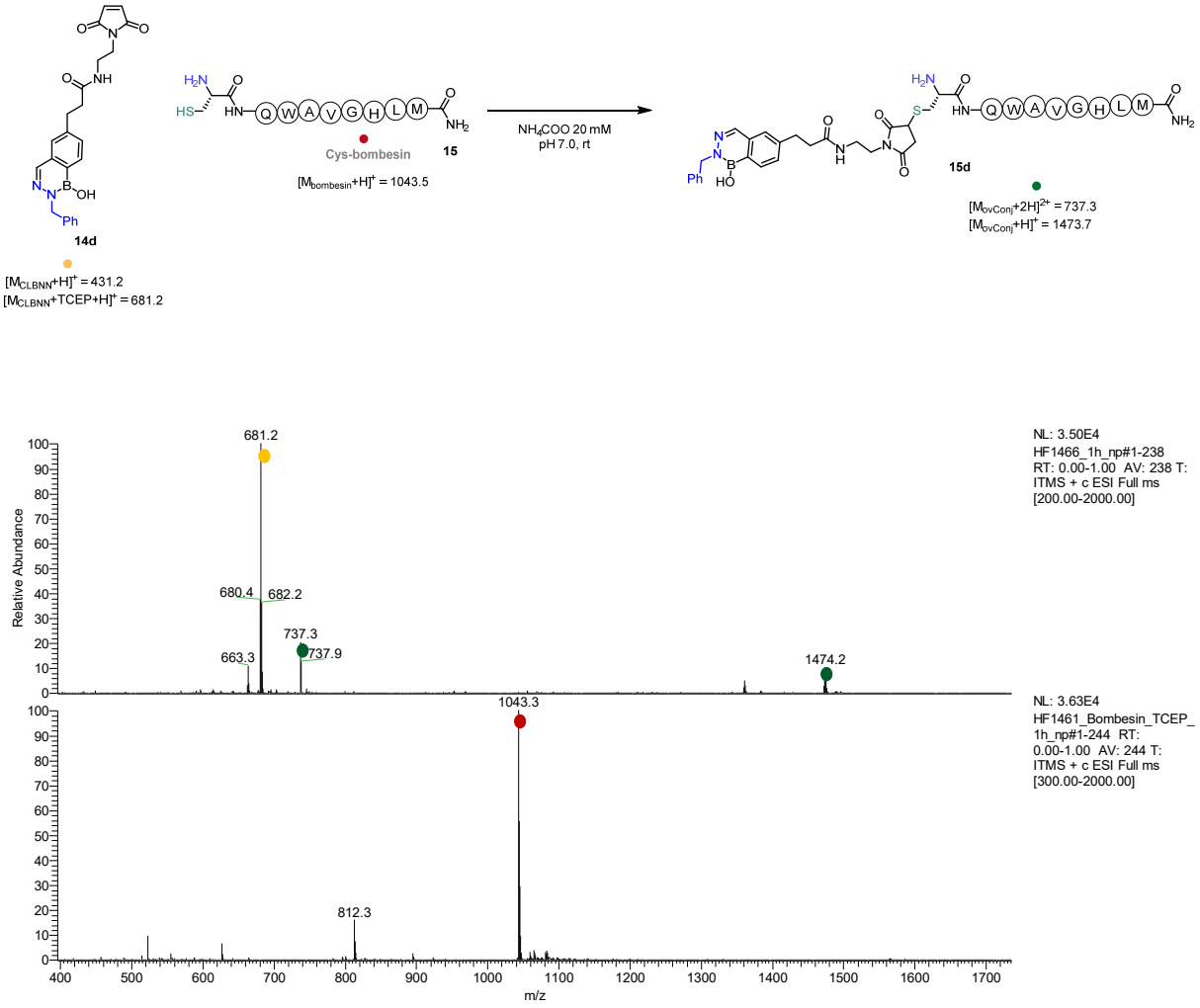


Figure S 25 - ESI$^+$-MS spectrum of Cys-Bombesin (**15**) reaction with **14d** after 1h (top) and ESI$^+$-MS spectrum of Cys-Bombesin (**15**) (bottom).

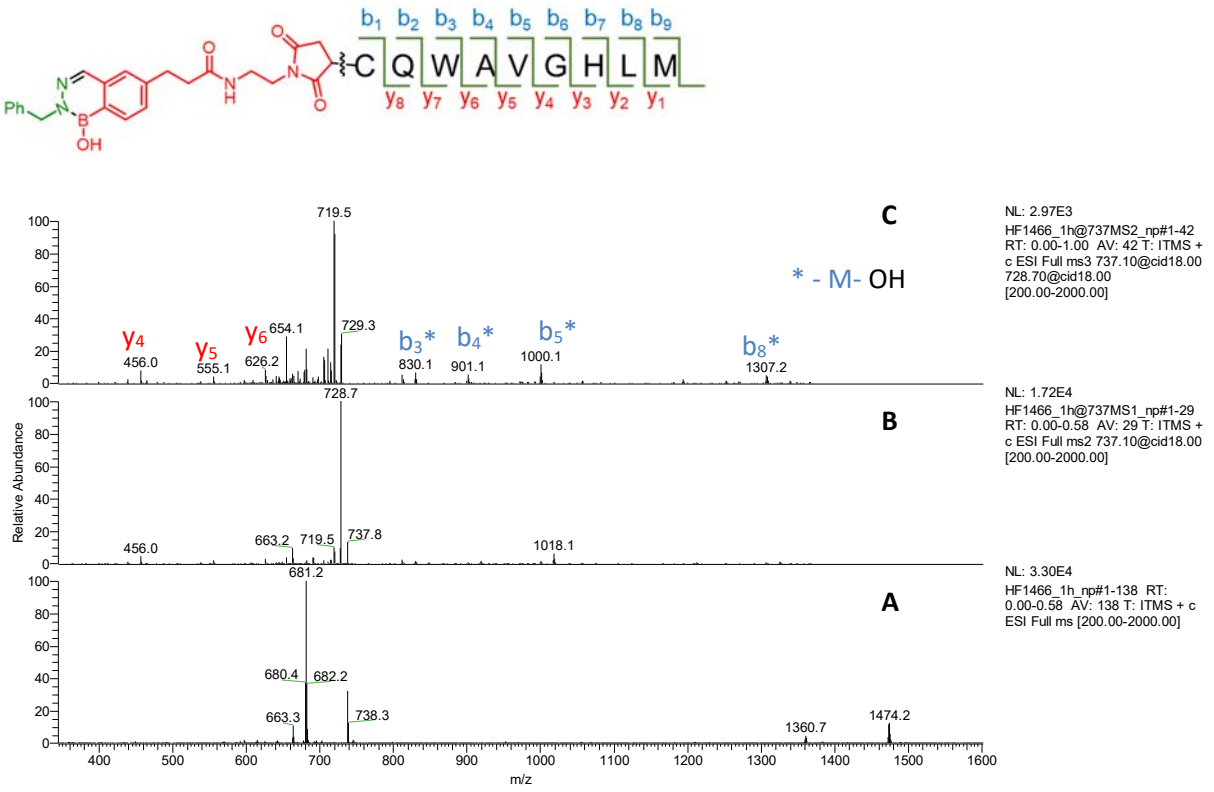
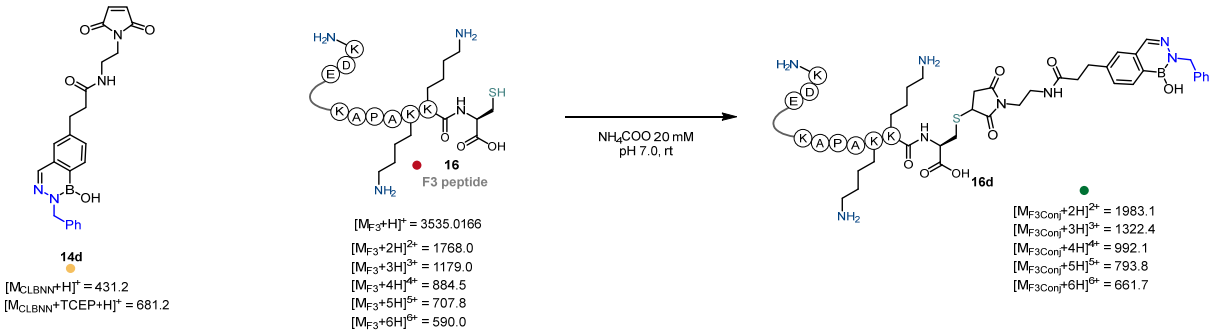


Figure S 26 – A - ESI+-MS spectrum of direct injection of the reaction between Cys-Bombesin (**15**) reaction with **14d** to form **15d**. **B** – MS^2 fragmentation of conjugate **15d**. **C** – MS^3 fragmentation of conjugate **15d**

4.1.1.5 Reaction of F3 with benzylhydrazine -crosslink conjugate 14d.



F3 sequence: KDEPQRRSARLSAKPAPPKPEPKPKKAPAKKC

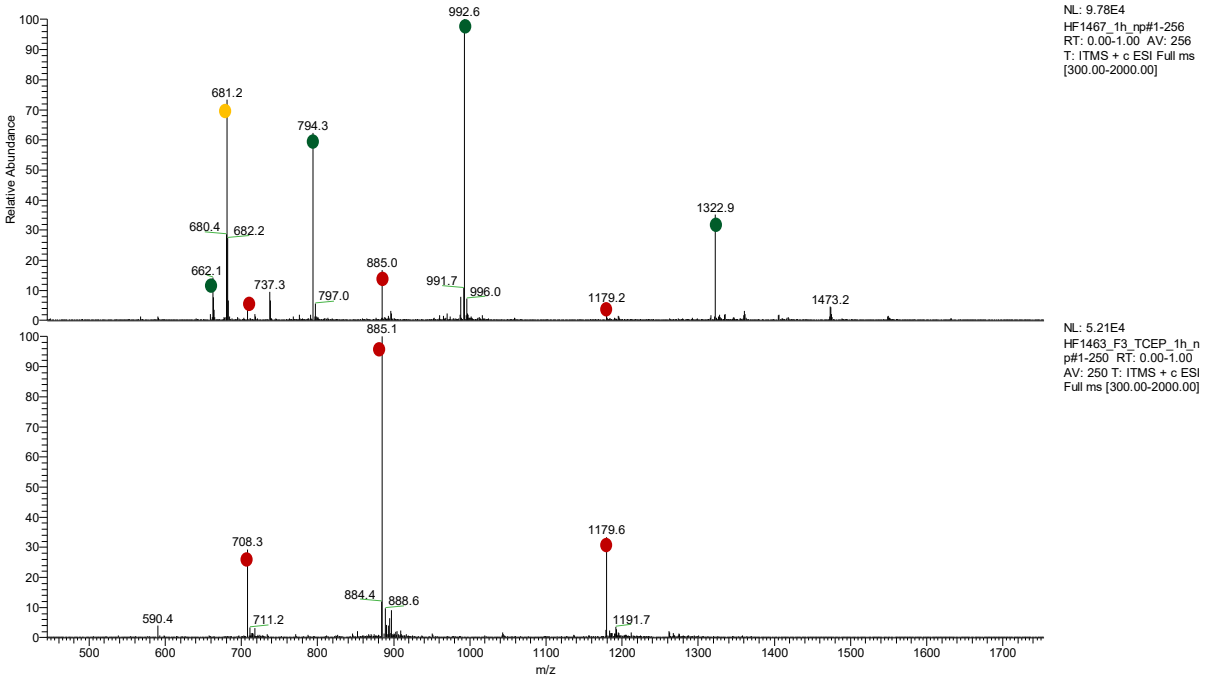


Figure S 27 - ESI$^+$-MS spectrum of F3 (**16**) reaction with **14d** after 1h (top) and ESI$^+$-MS spectrum of F3 (**16**) (bottom).

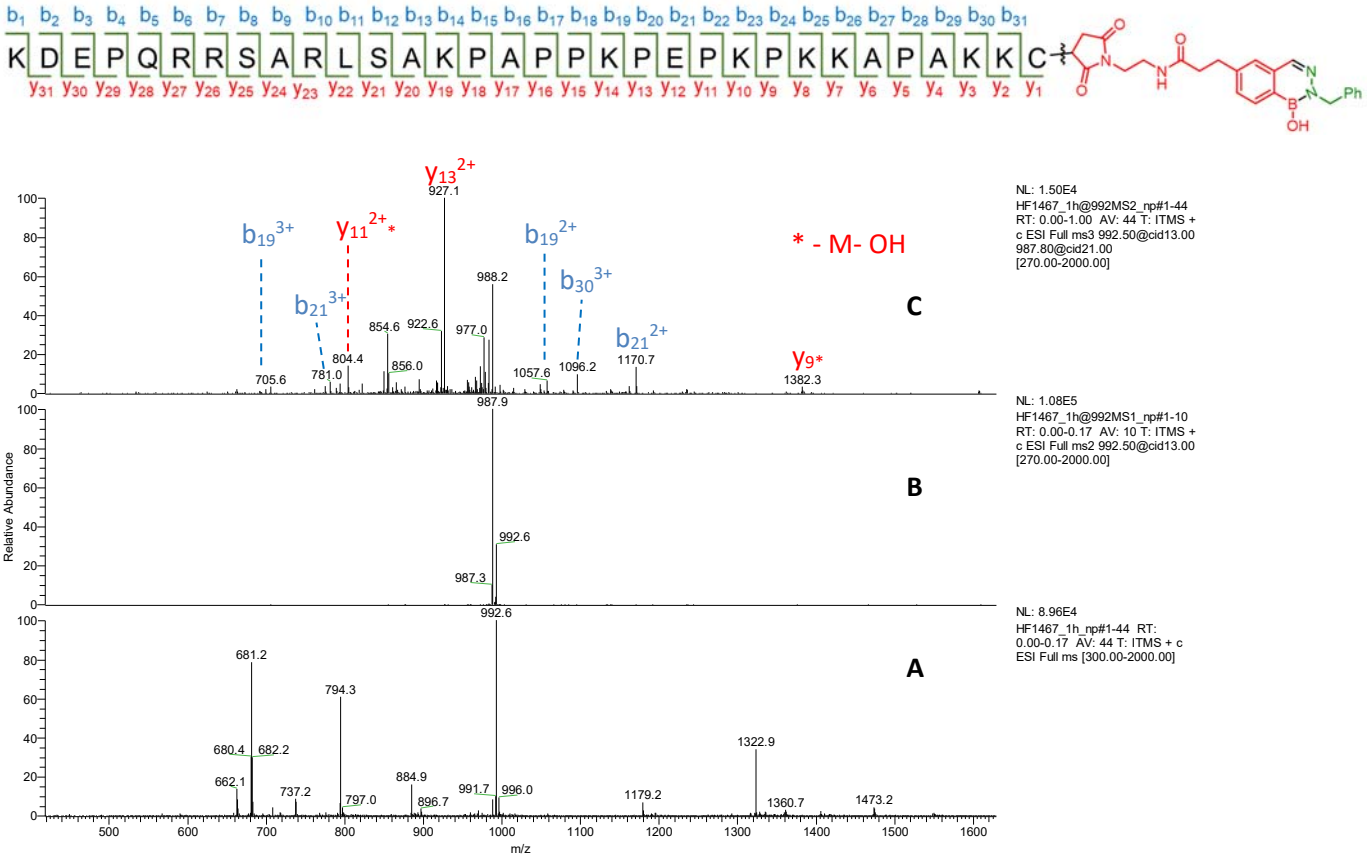
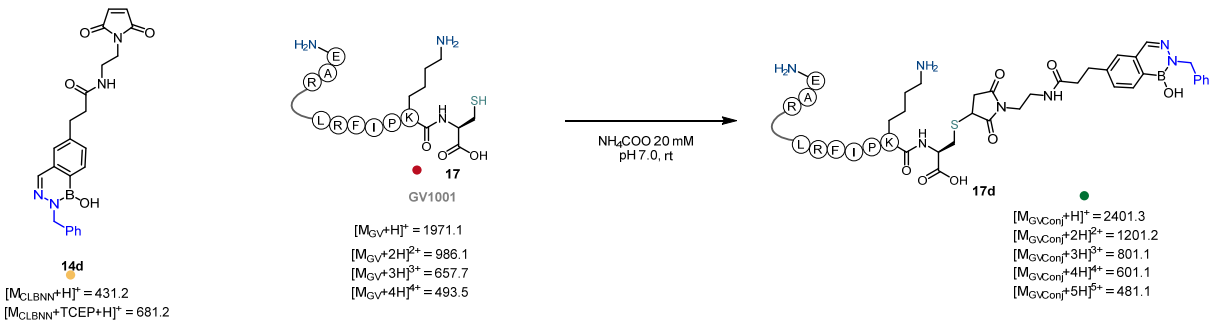


Figure S 28 – A - ESI+-MS spectrum of direct injection of the reaction between F3 (**16**) and **14d** to form **16d**. **B** – MS^2 fragmentation of conjugate **16d**. **C** – MS^3 fragmentation of conjugate **16d**.

4.1.1.6 Reaction of GV1001 with benzylhydrazine-crosslink conjugate 14d.



GV1001 sequence: EARPALLTSRLRFIPKC

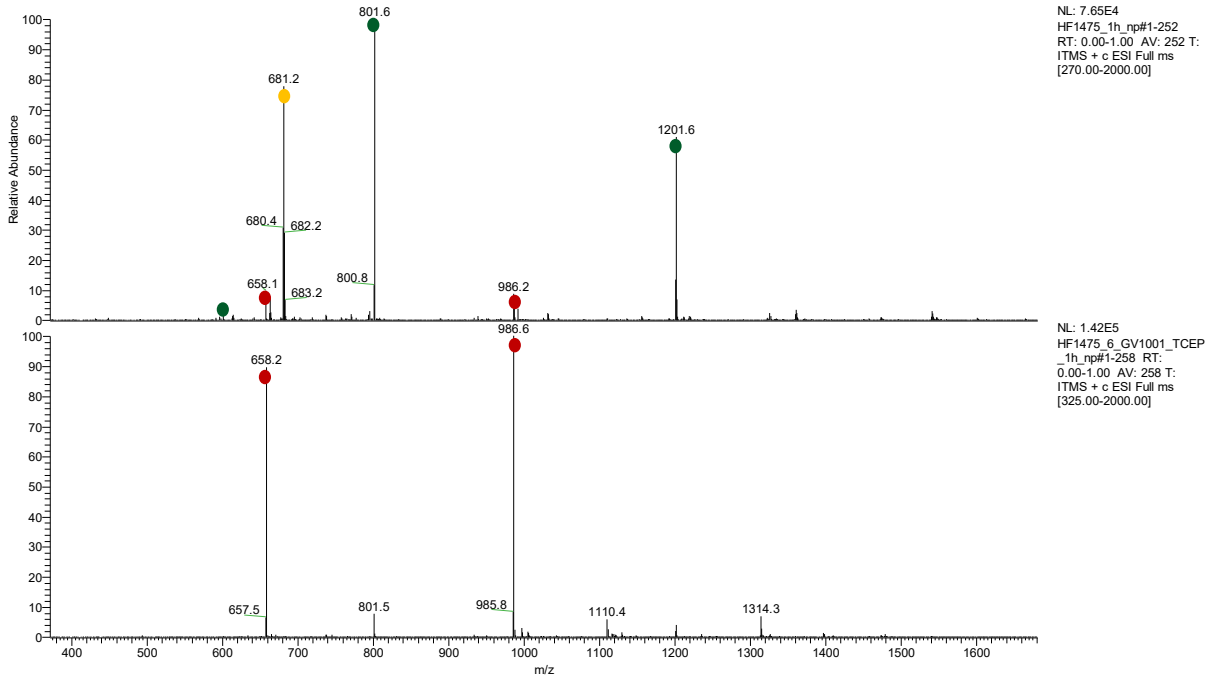


Figure S 29 - ESI$^+$-MS spectrum of GV1001 (**17**) reaction with **14d** after 1h (top) and ESI$^+$-MS spectrum of GV1001 (**17**) (bottom).

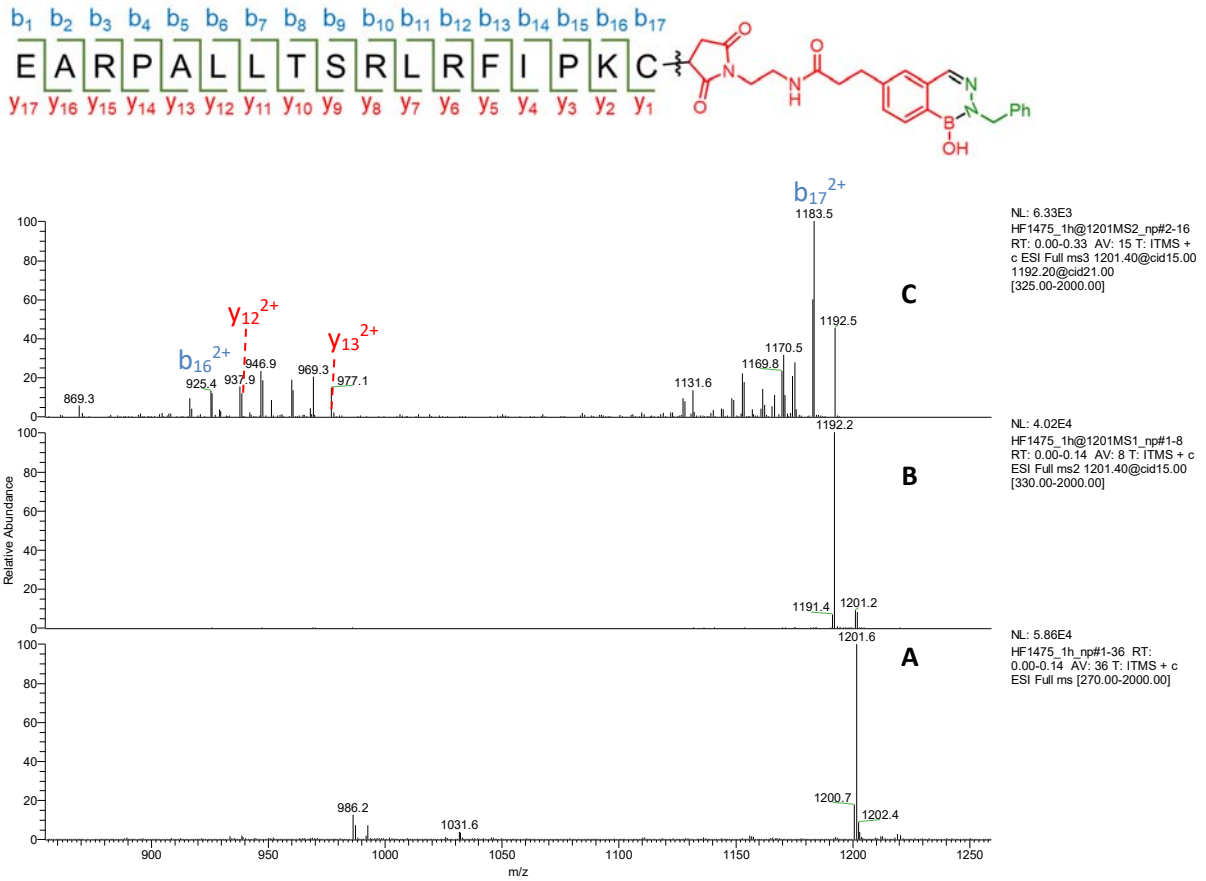
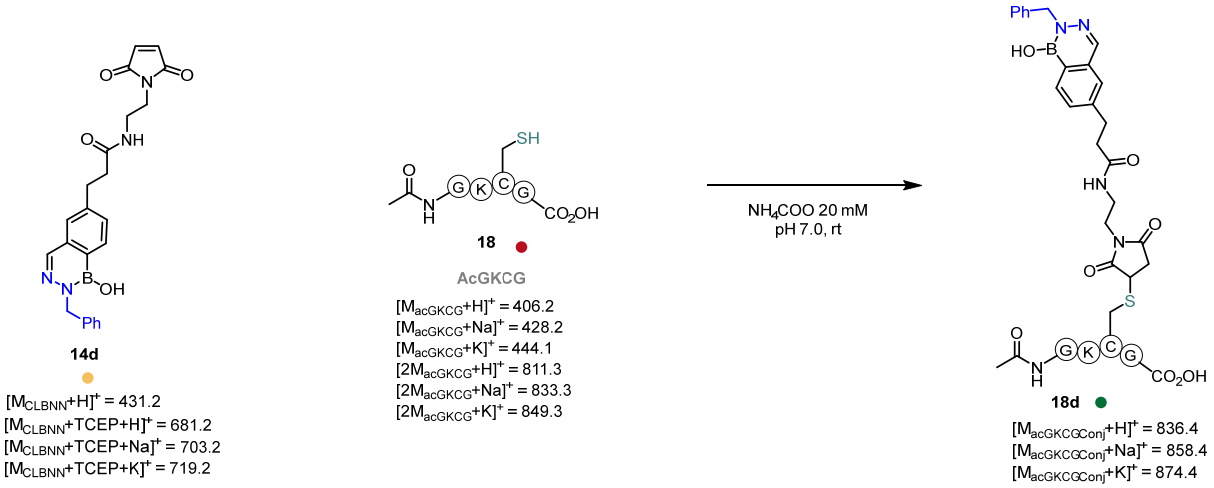


Figure S 30 – A - ESI+-MS spectrum of direct injection of the reaction between GV1001 (**17**) and **14d** to form **17d**. **B** – MS^2 fragmentation of conjugate **17d**. **C** – MS^3 fragmentation of conjugate **17d**.

4.1.1.7 Reaction of AcGKCG with benzylhydrazine-crosslink conjugate 14d.



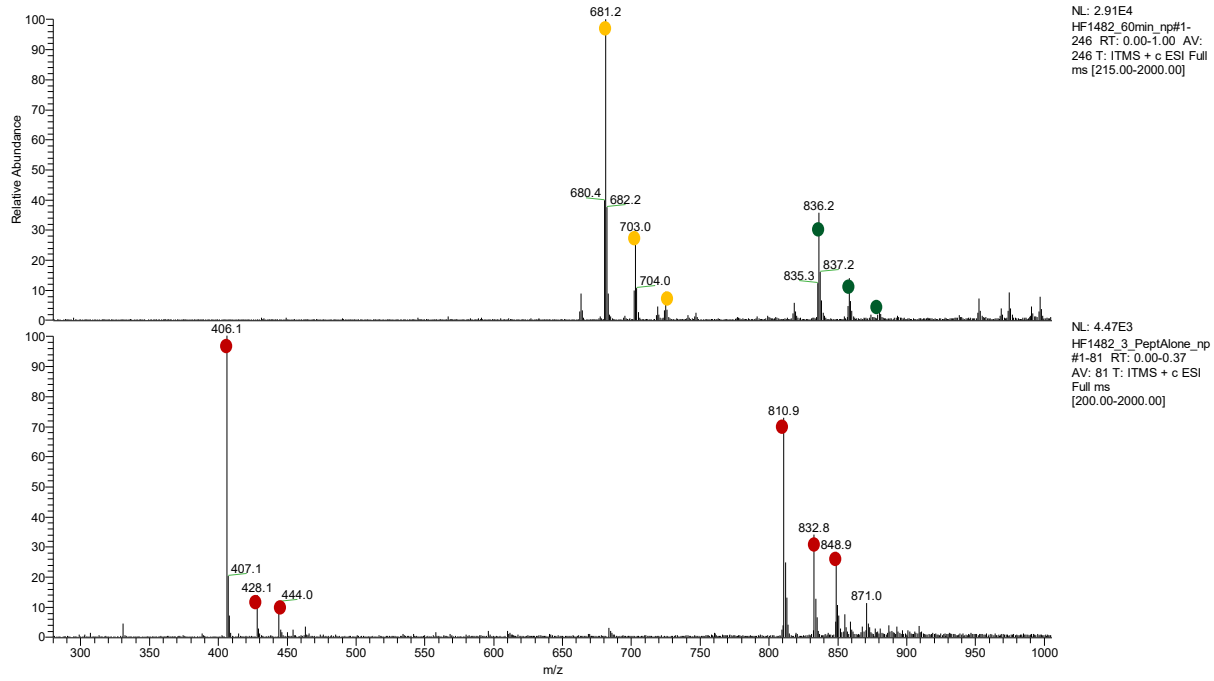


Figure S 31 - ESI$^+$-MS spectrum of AcGKCG (**18**) reaction with **14d** after 1h (top) and ESI$^+$-MS spectrum of AcGKCG (**18**) (bottom).

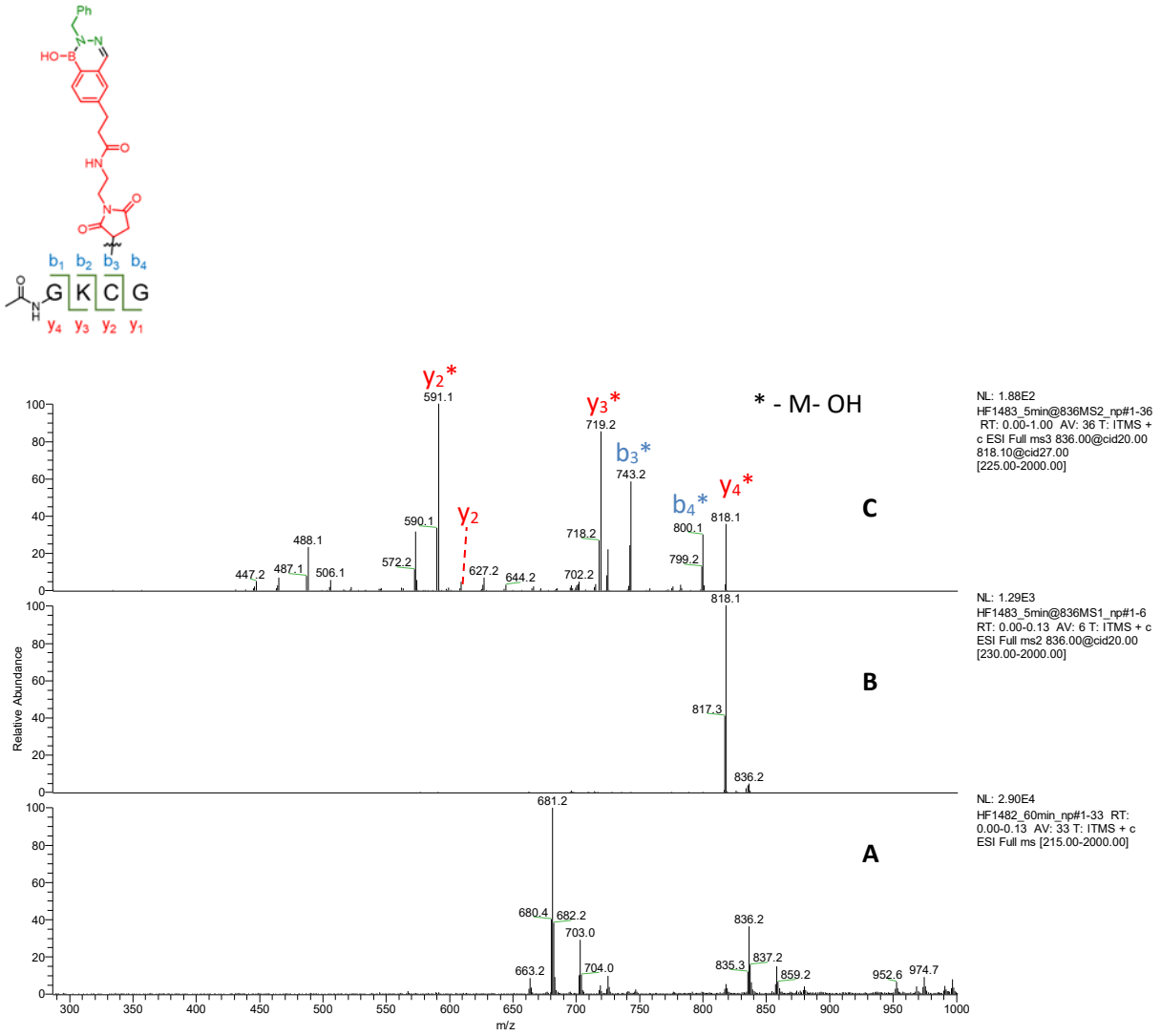


Figure S 32 – A - ESI+-MS spectrum of direct injection of the reaction between AcGKCG (**18**) and **14d** to form **18d**. **B** – MS^2 fragmentation of conjugate **18d**. **C** – MS^3 fragmentation of conjugate **18d**

4.1.2 Hydrazine screening in the Crosslink with *c*-Ovalbumin

4.1.2.1 Reaction of c-Ovalbumin with phenylhydrazine-crosslink conjugate 14f.

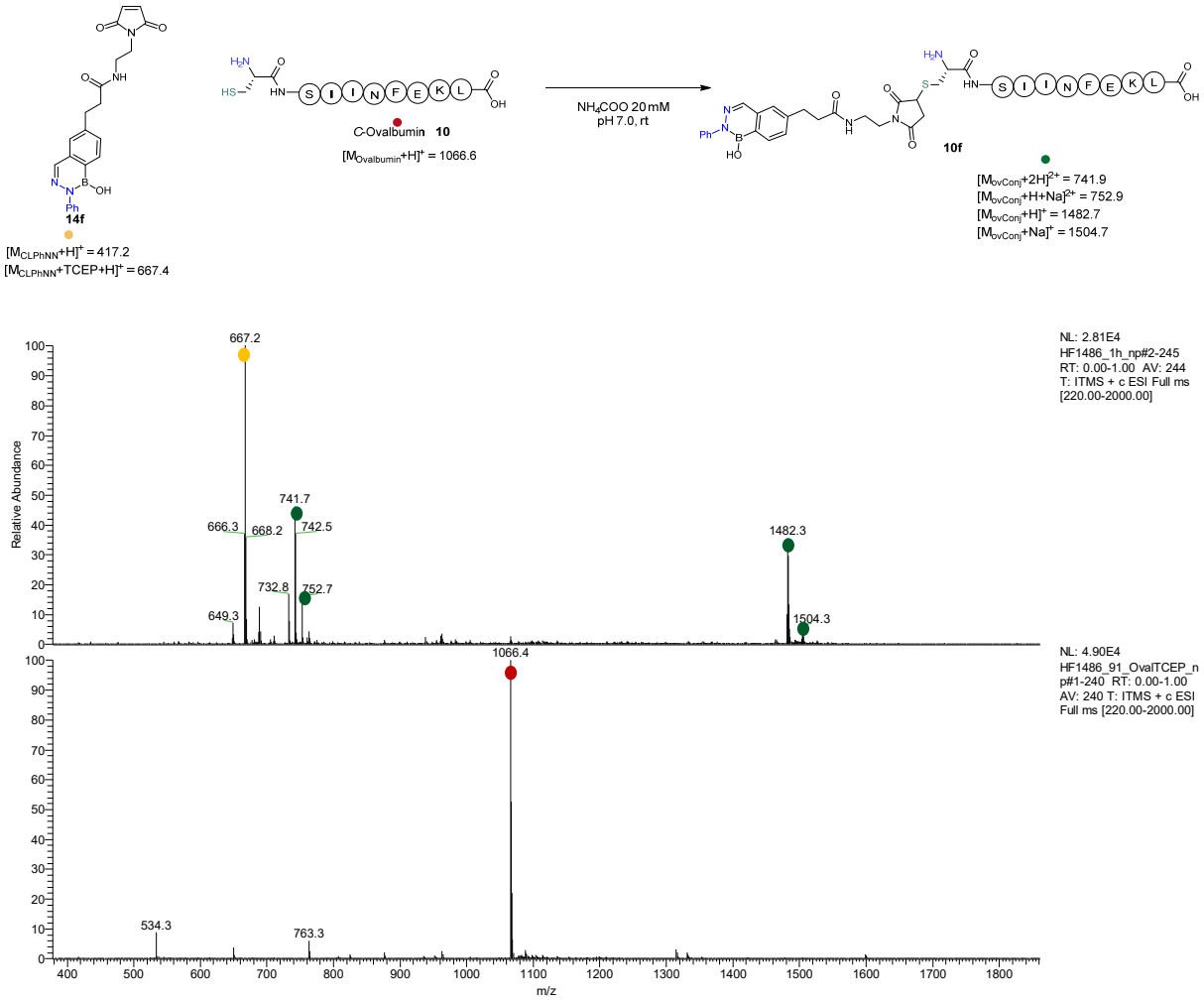


Figure S 33 - ESI$^+$-MS spectrum of Ovalbumin reaction with **14f** after 1h (top) and ESI$^+$-MS spectrum of Ovalbumin (**10**) (bottom).

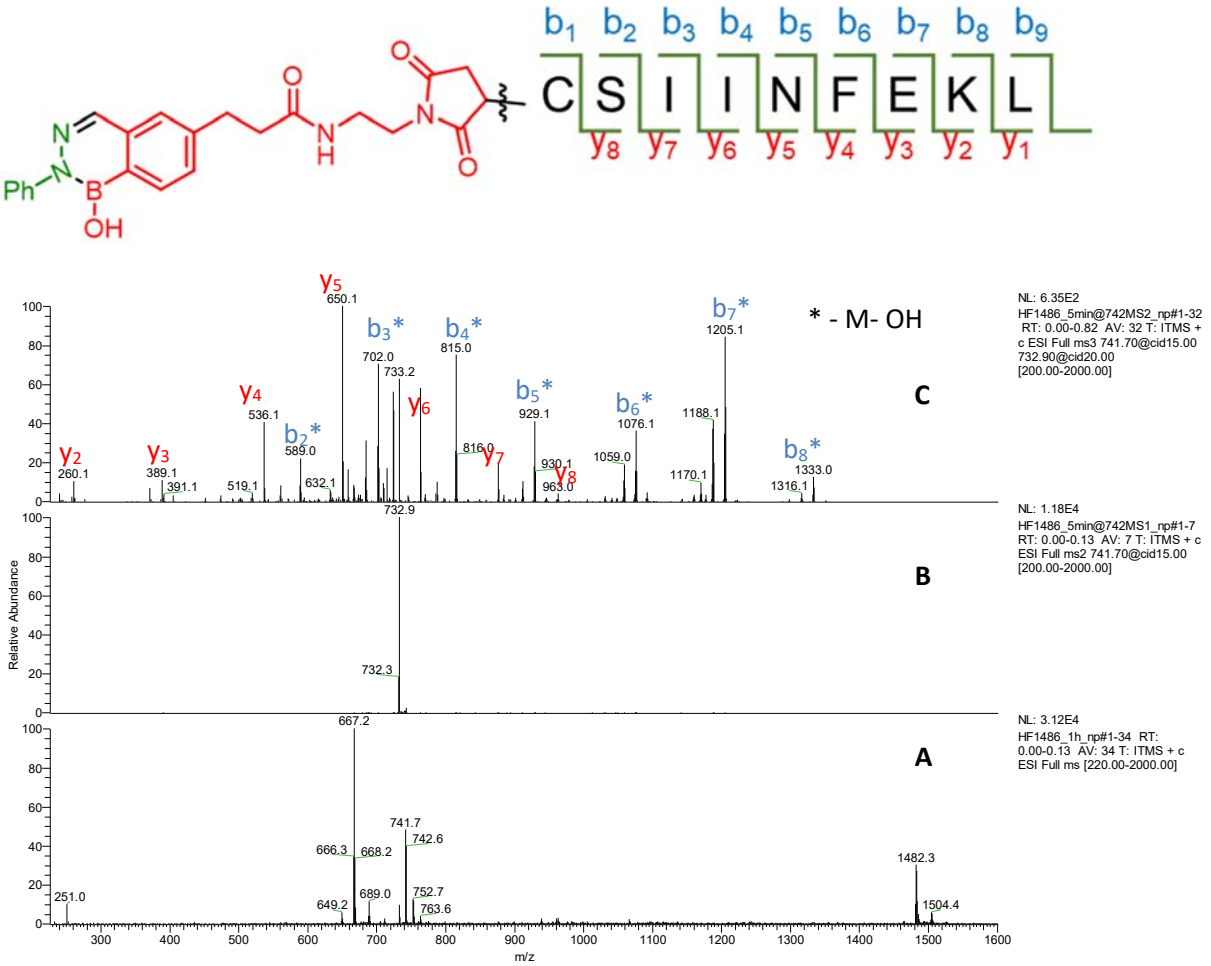


Figure S 34 – A - ESI+-MS spectrum of direct injection of the reaction between Ovalbumin (**10**) and **14f** to form **10f**. **B** – MS^2 fragmentation of conjugate **10f**. **C** – MS^3 fragmentation of conjugate **10f**.

4.1.2.2 Reaction of c-Ovalbumin with methylhydrazine-crosslink conjugate 14e.

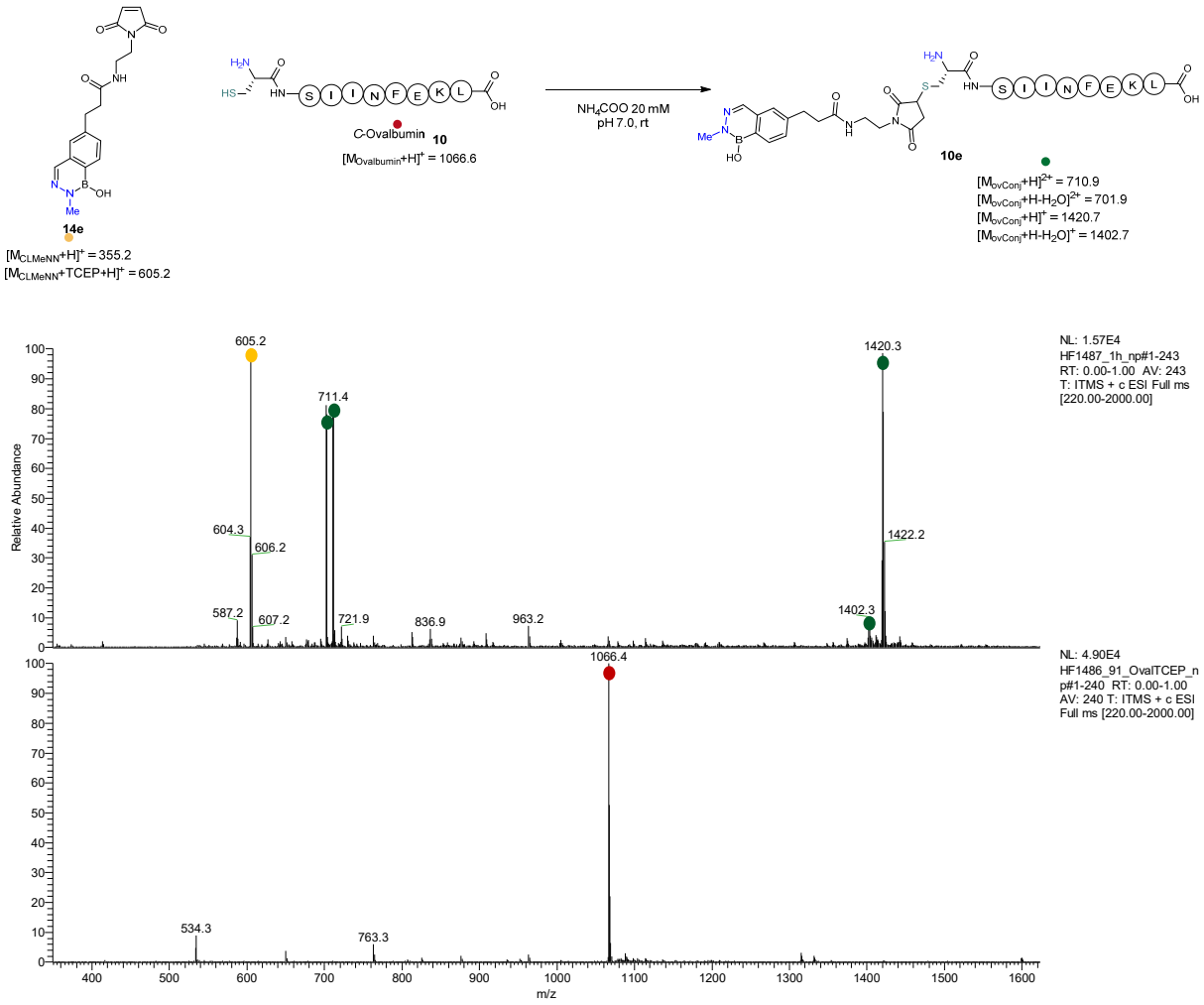


Figure S 35 - ESI$^+$-MS spectrum of Ovalbumin reaction with **14e** after 1h (top) and ESI$^+$-MS spectrum of Ovalbumin (**10**) (bottom).

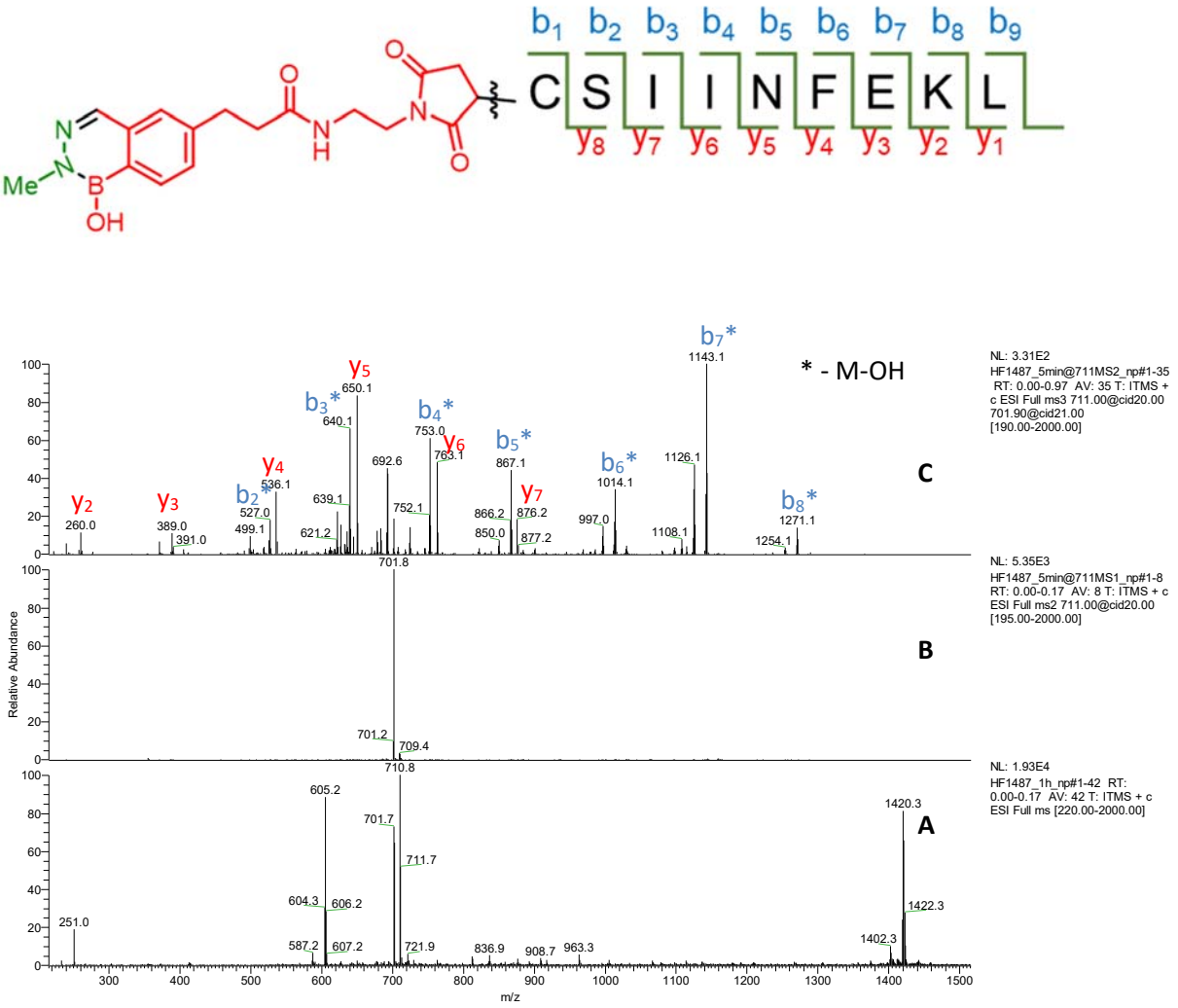


Figure S 36 – A - ESI+-MS spectrum of direct injection of the reaction between Ovalbumin (**10**) and **14e** to form **10e**. **B** – MS^2 fragmentation of conjugate **10e**. **C** – MS^3 fragmentation of conjugate **10e**.

4.1.2.3 Reaction of c-Ovalbumin with BOC-hydrazine-crosslink conjugate 14g.

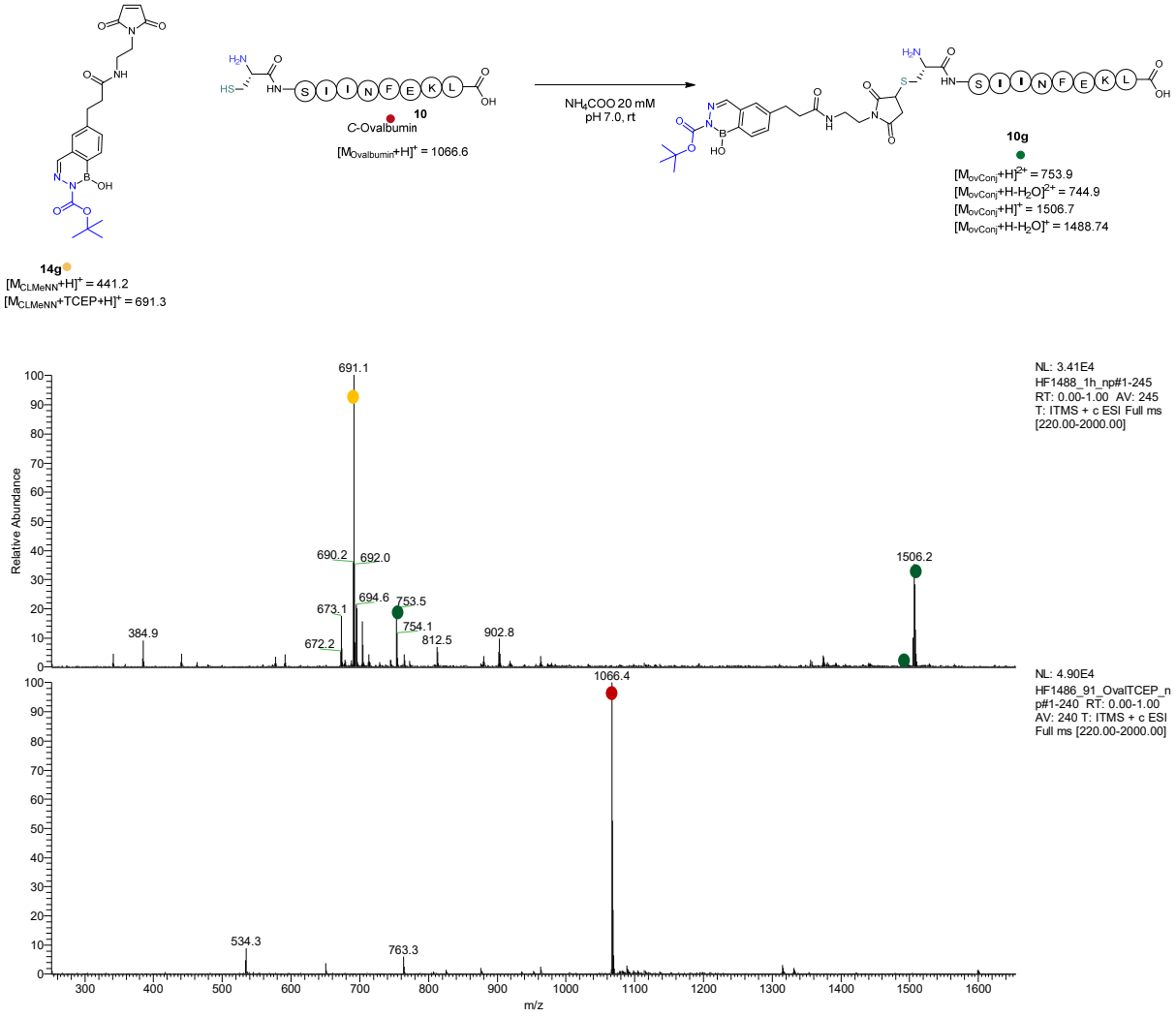


Figure S 37 - ESI$^+$-MS spectrum of Ovalbumin reaction with **14g** after 1h (top) and ESI$^+$-MS spectrum of Ovalbumin (**10**) (bottom).

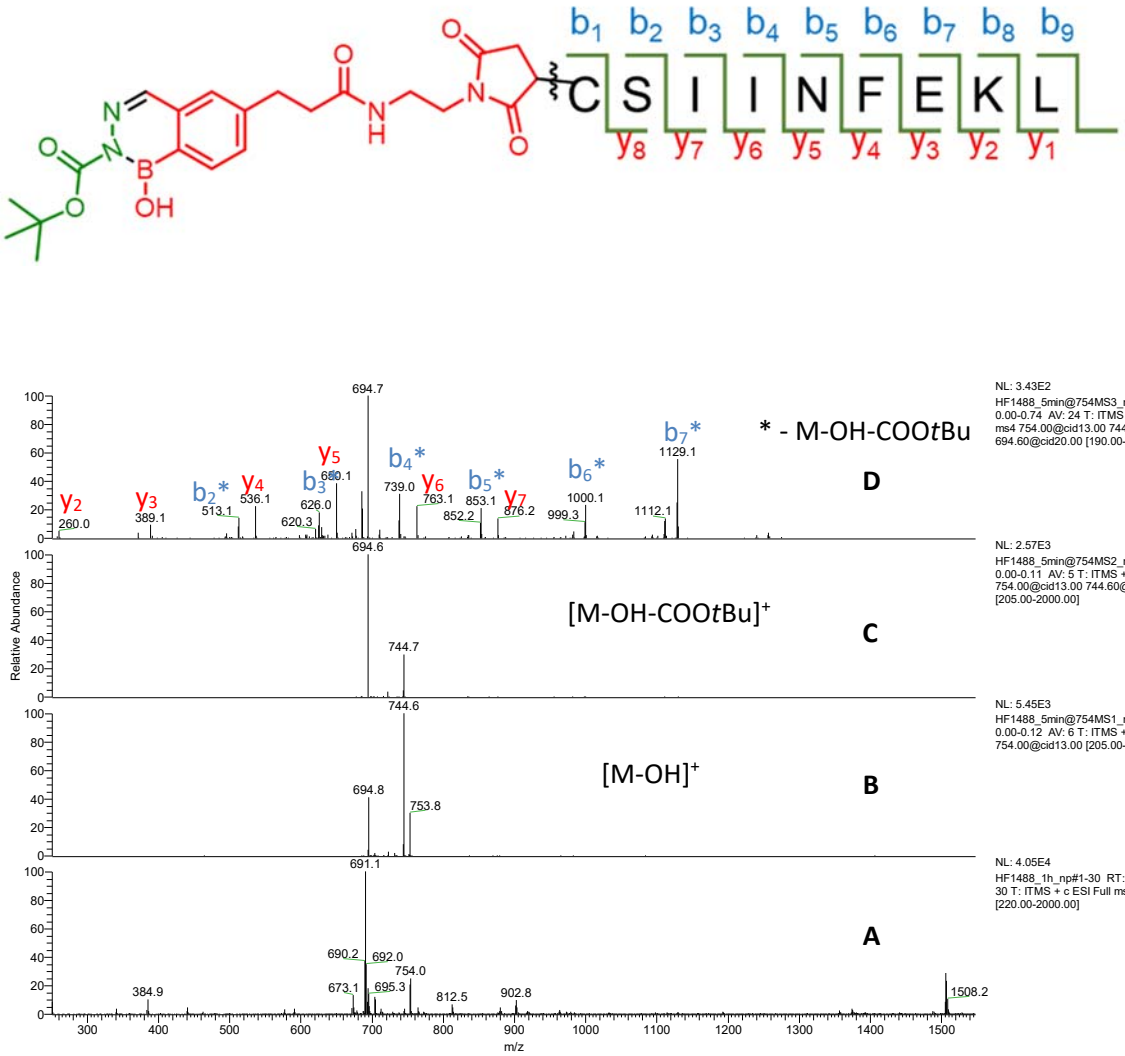


Figure S 38 – A - ESI+-MS spectrum of direct injection of the reaction between Ovalbumin (**10**) and **14g** to form **10g**. **B** – MS^2 fragmentation of conjugate **10g**. **C** – MS^3 fragmentation of conjugate **10g**. **D** – MS^4 fragmentation of conjugate **10g**.

4.1.2.4 Reaction of c-Ovalbumin with tosylhydrazine-crosslink conjugate 14h.

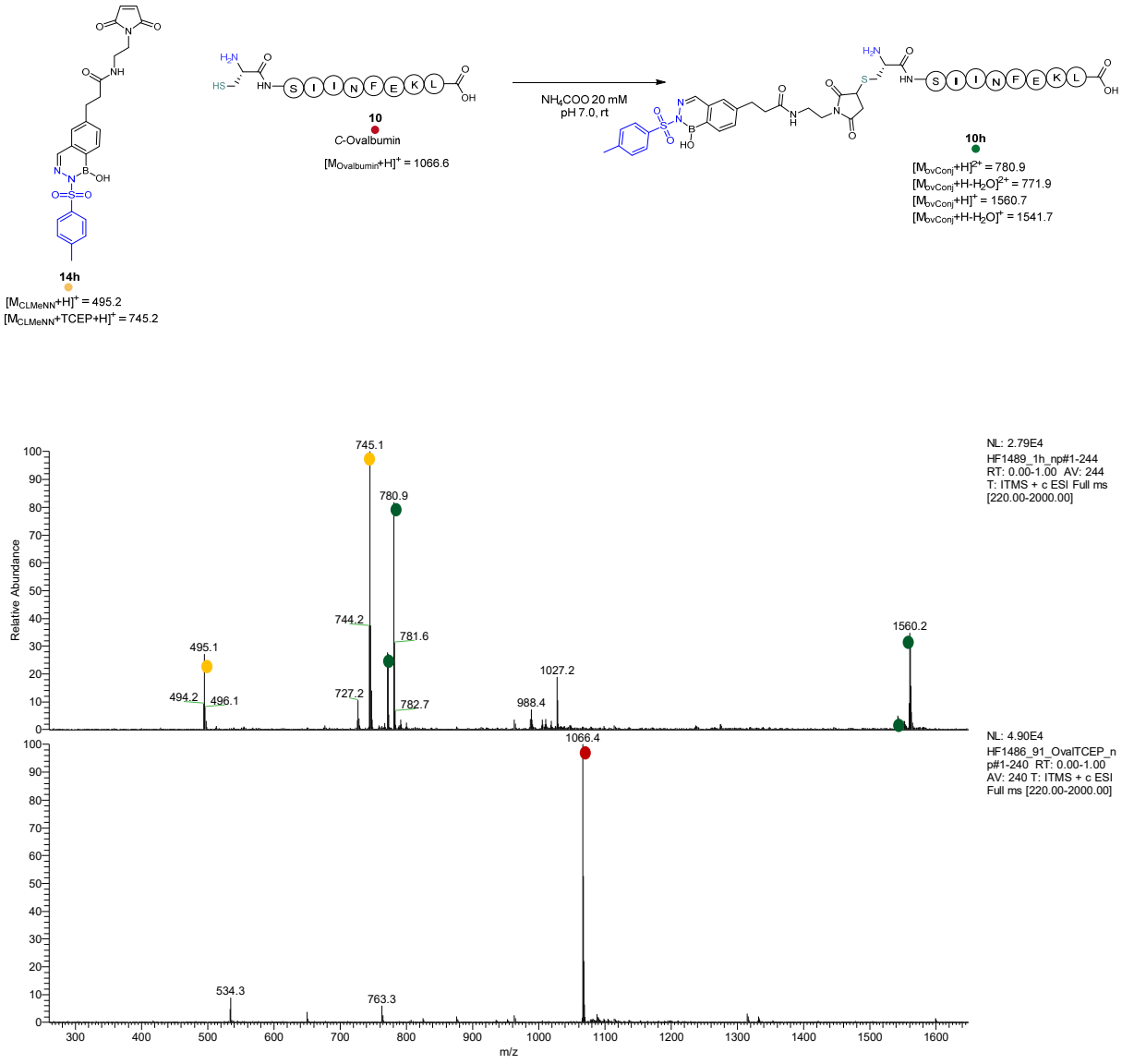


Figure S 39 - ESI$^+$-MS spectrum of Ovalbumin reaction with **14h** after 1h (top) and ESI$^+$-MS spectrum of Ovalbumin (**10**) (bottom).

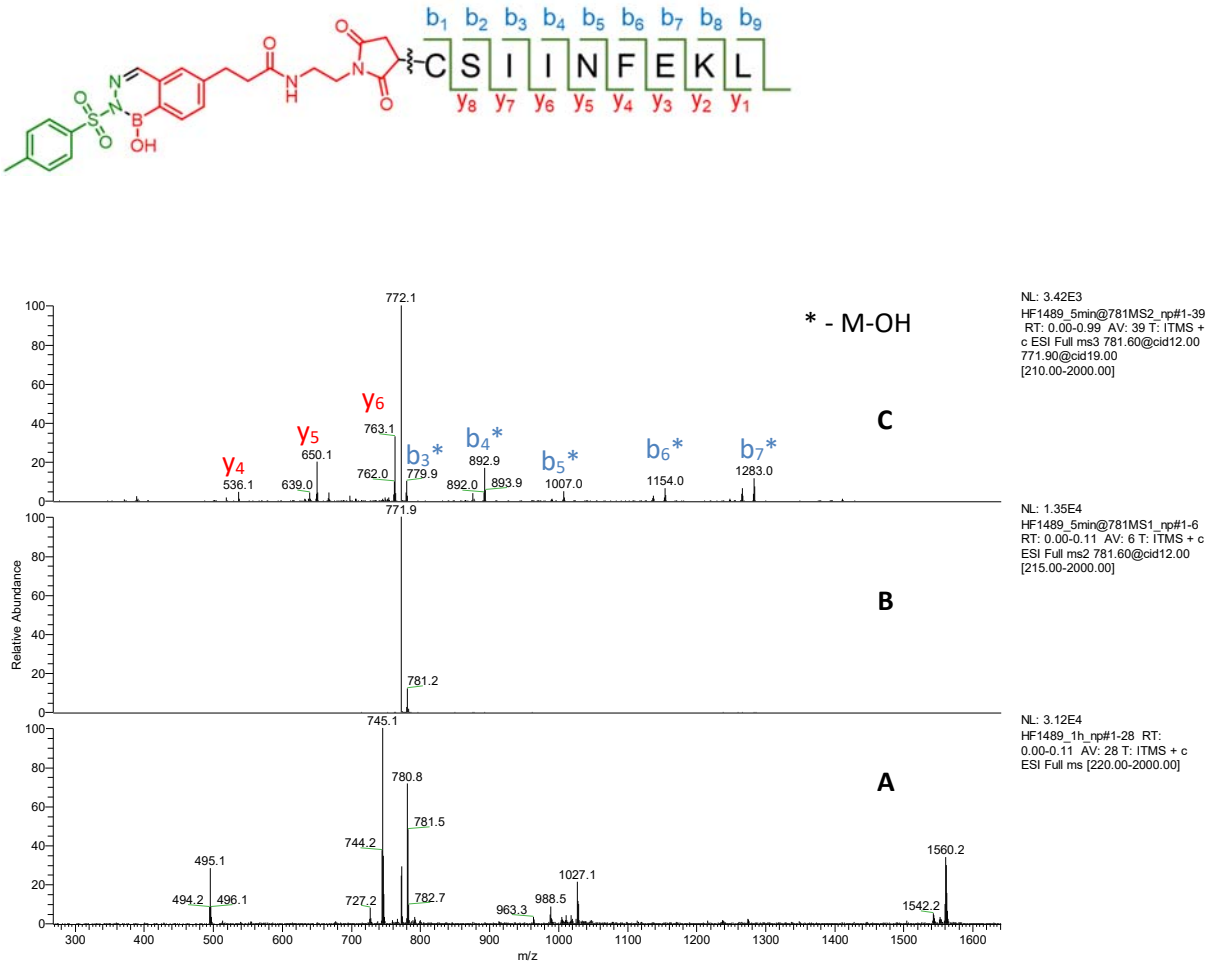


Figure S 40 – A - ESI+-MS spectrum of direct injection of the reaction between Ovalbumin (**10**) and **14h** to form **10h**. **B** – MS^2 fragmentation of conjugate **10h**. **C** – MS^3 fragmentation of conjugate **10h**.

4.1.2.5 Reaction of c-Ovalbumin with Coumarin-NHNH$_2$-crosslink conjugate 14j.

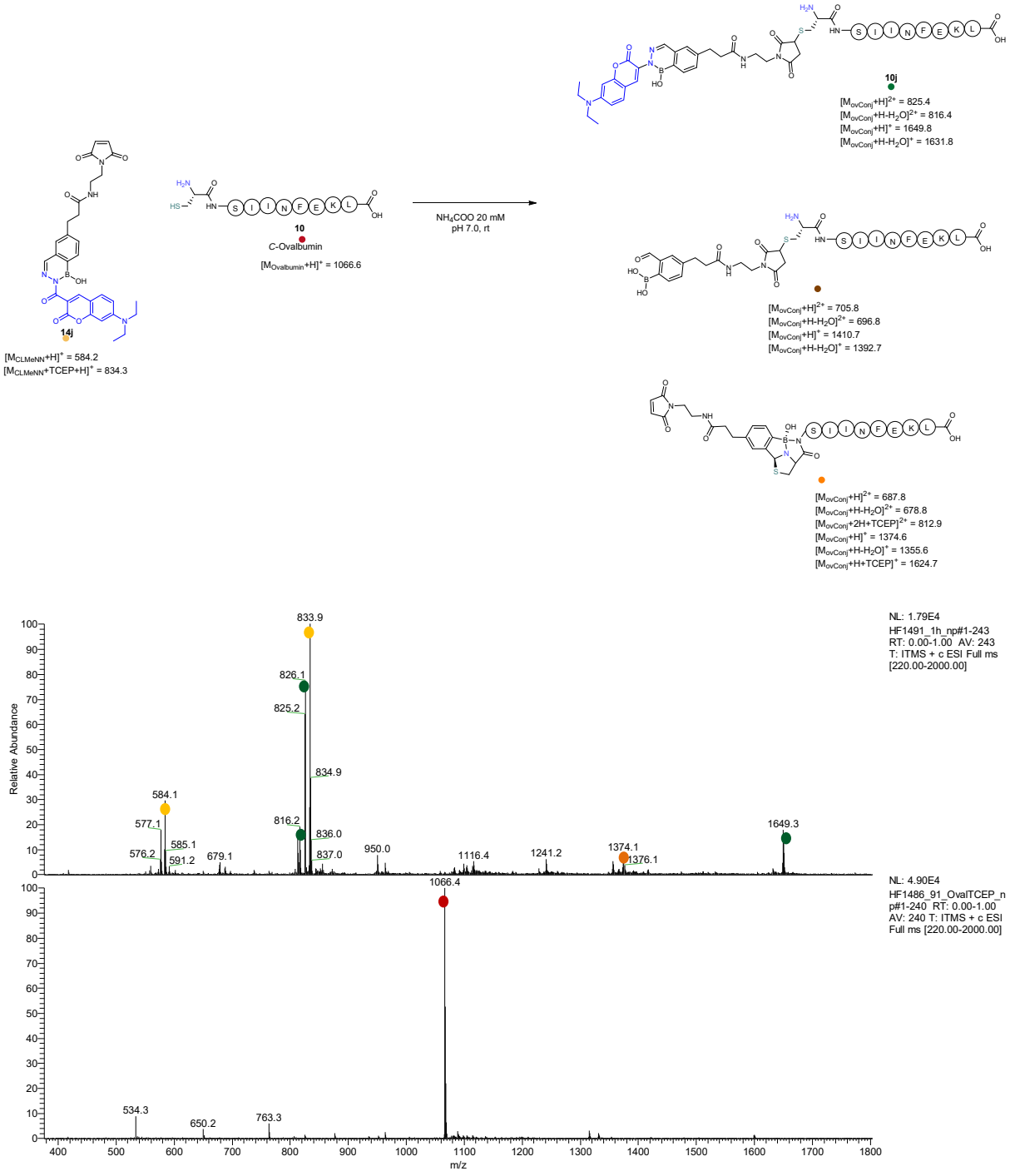


Figure S 41 - ESI$^+$-MS spectrum of Ovalbumin reaction with **14j** after 1h (top) and ESI$^+$-MS spectrum of Ovalbumin (**10**) (bottom).

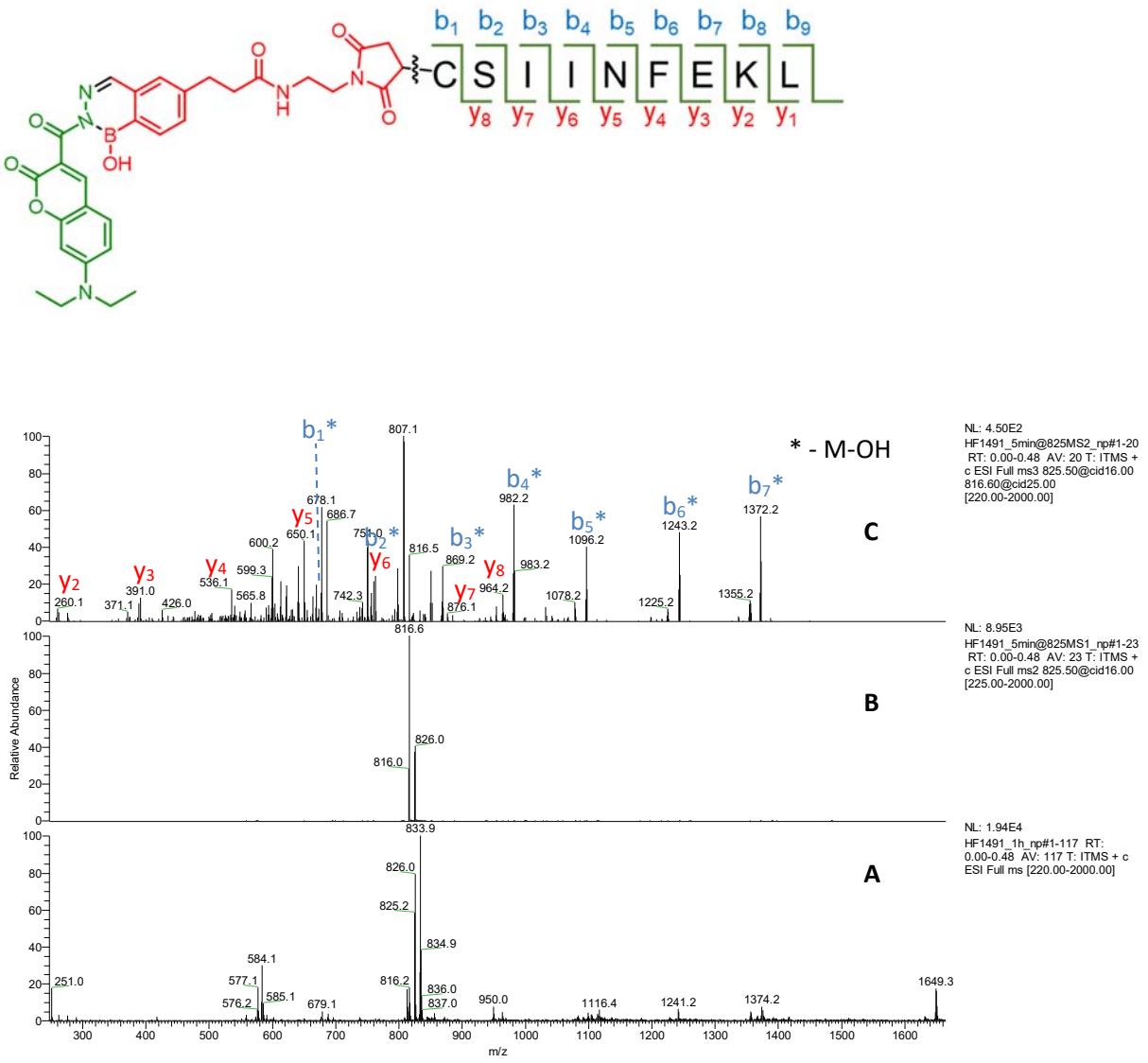


Figure S 42 – A - ESI+-MS spectrum of direct injection of the reaction between Ovalbumin (**10**) and **14j** to form **10j**. **B** – MS^2 fragmentation of conjugate **10j**. **C** – MS^3 fragmentation of conjugate **10j**.

4.1.2.6 Reaction of c-Ovalbumin with benzoylhydrazine-crosslink conjugate 14i.

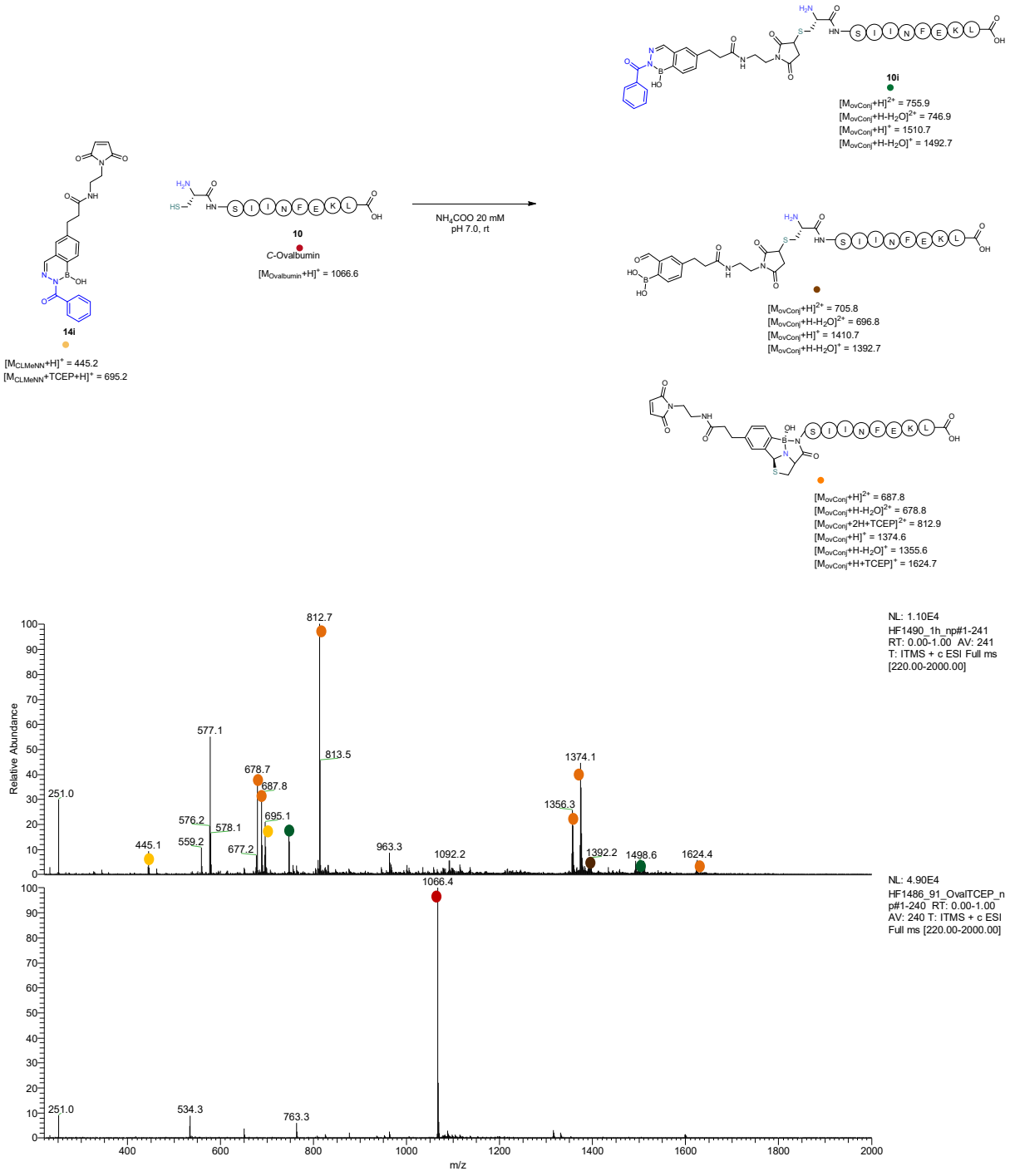


Figure S 43 - ESI$^+$-MS spectrum of Ovalbumin reaction with **14i** after 1h (top) and ESI$^+$-MS spectrum of Ovalbumin (**10**) (bottom).

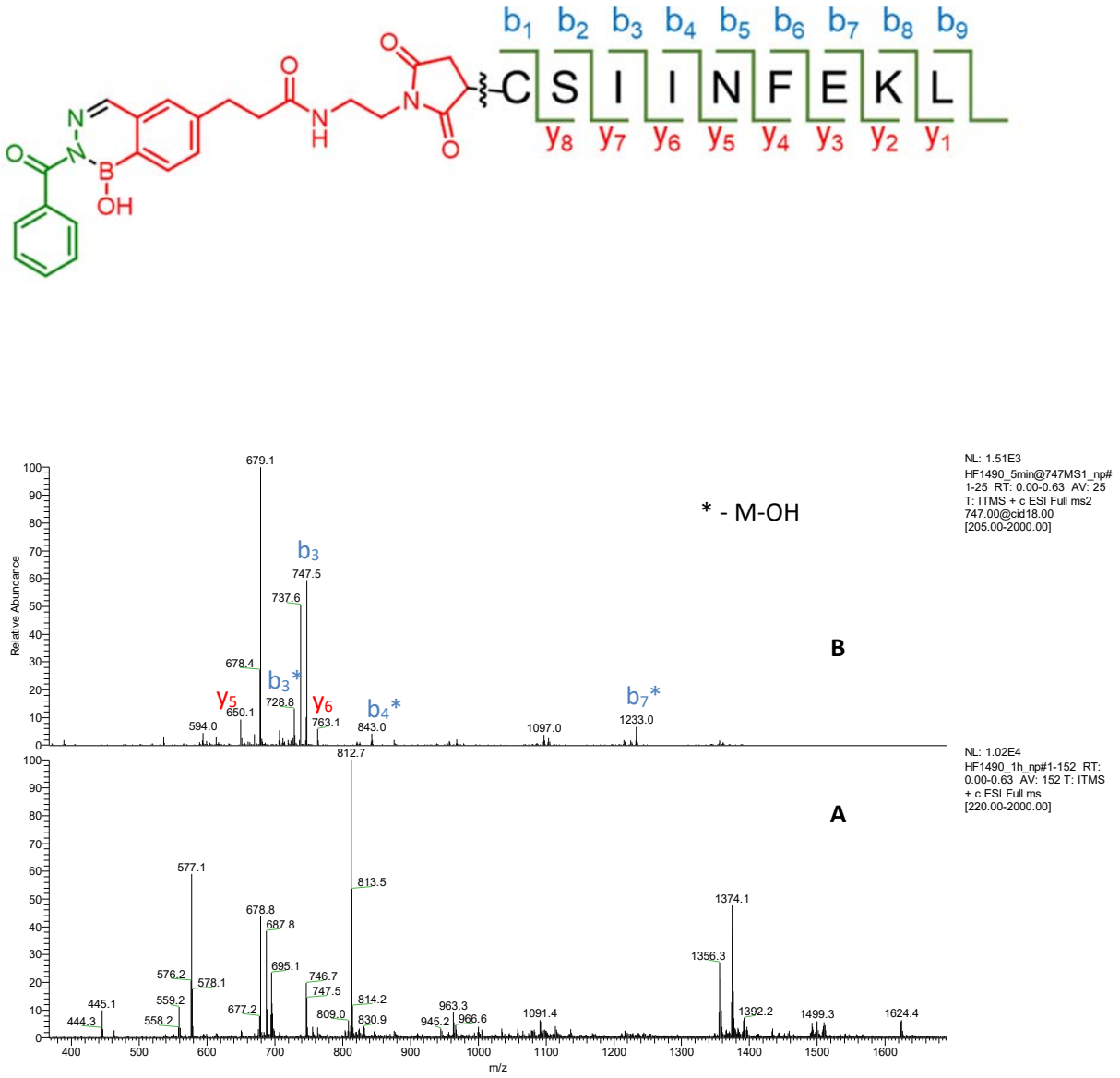
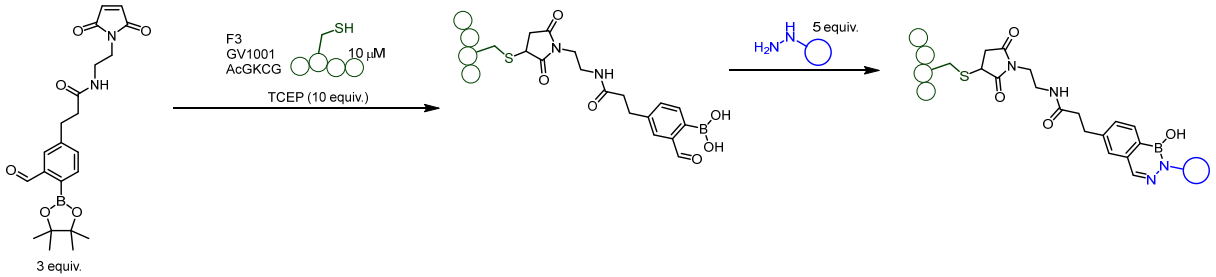


Figure S 44 – A - ESI+-MS spectrum of direct injection of the reaction between Ovalbumin (**10**) and **14i** to form **10i**. **B** – MS^2 fragmentation of conjugate **10i**.

4.2 Thiol-Michael followed by Diazaborine formation (B1-B2 pathway).



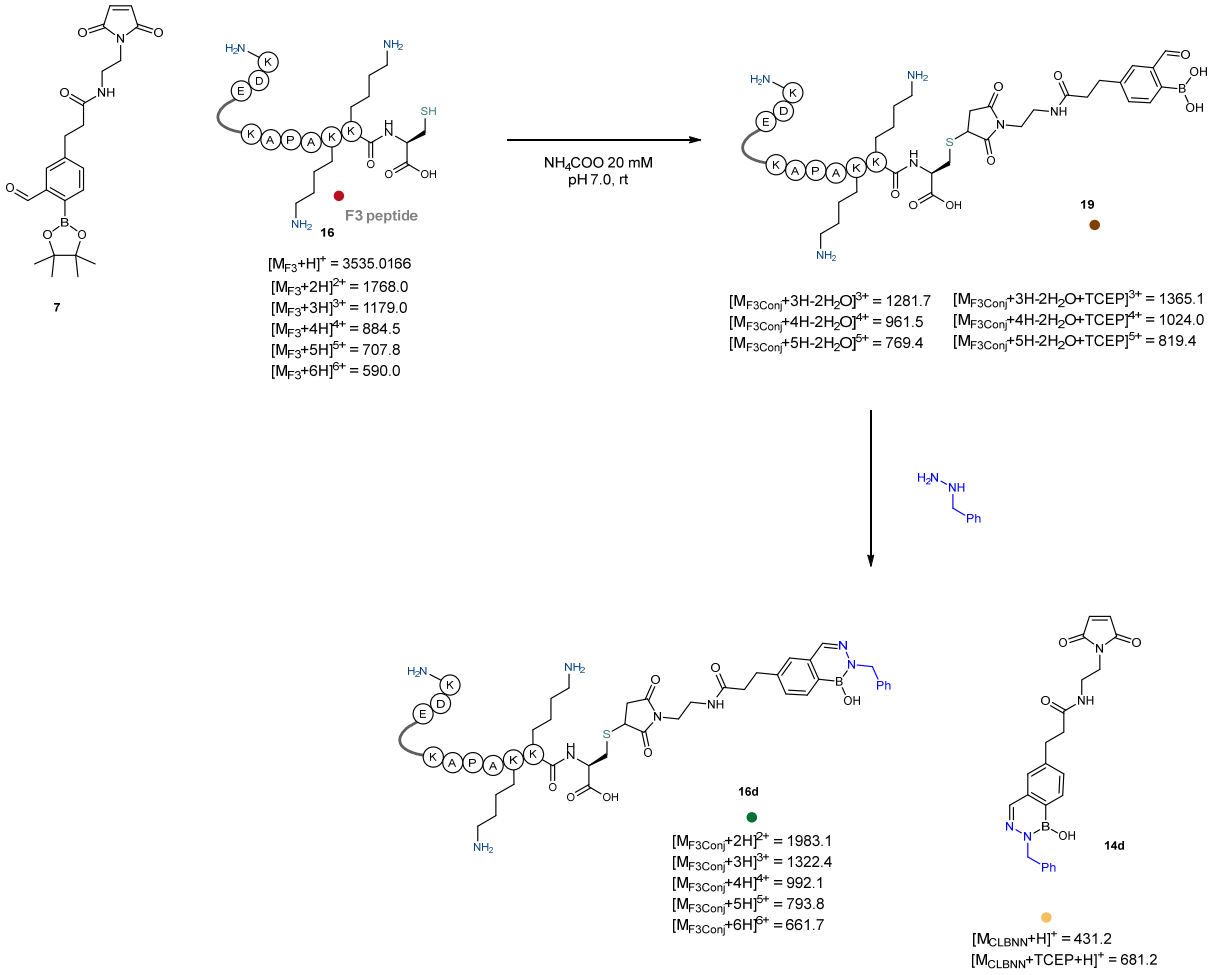
General procedure

To a solution of peptide (≈1 mM) (10 µL, 0.010 µmol) in ammonium acetate solution 20 mM pH 7.0 (1.0 mL) was added a Tris(2-carboxyethyl)phosphine hydrochloride (TCEP) (3.5 mM in water) (28.6 µL, 0.100 µmol) and the solution mixed for 30 min at 25 ºC.

Then crosslinker **7** (10mM in ACN, 3 µL, 0.03 µmol) was added, allowed to react for 1 min and finally the hydrazine (5.0 µL, 0.050 µmol) was added and allowed to react for 60 min and the reaction monitored by direct infusion in Positive Mode of ESI-MS.

4.2.1 Peptide screening in the Crosslink with $BnNHNH_2$

4.2.1.1 Reaction of F3 with crosslinker followed by benzylhydrazine addition.



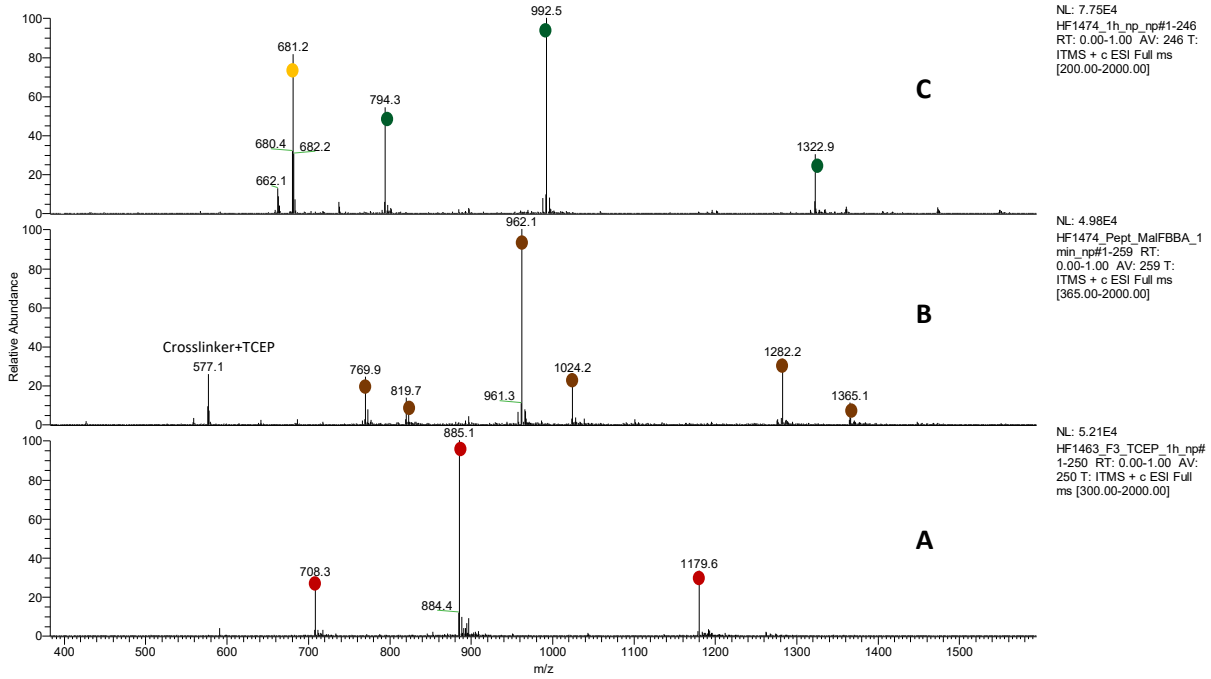


Figure S 45.1 – **A** - ESI$^+$-MS spectrum of F3 (**16**).; **B** - ESI$^+$-MS spectrum of F3 (**16**) reaction with **7** after 1min; **C** ESI$^+$-MS spectrum of the reaction of **19** with benzylhydrazine (**d**) after 1h.

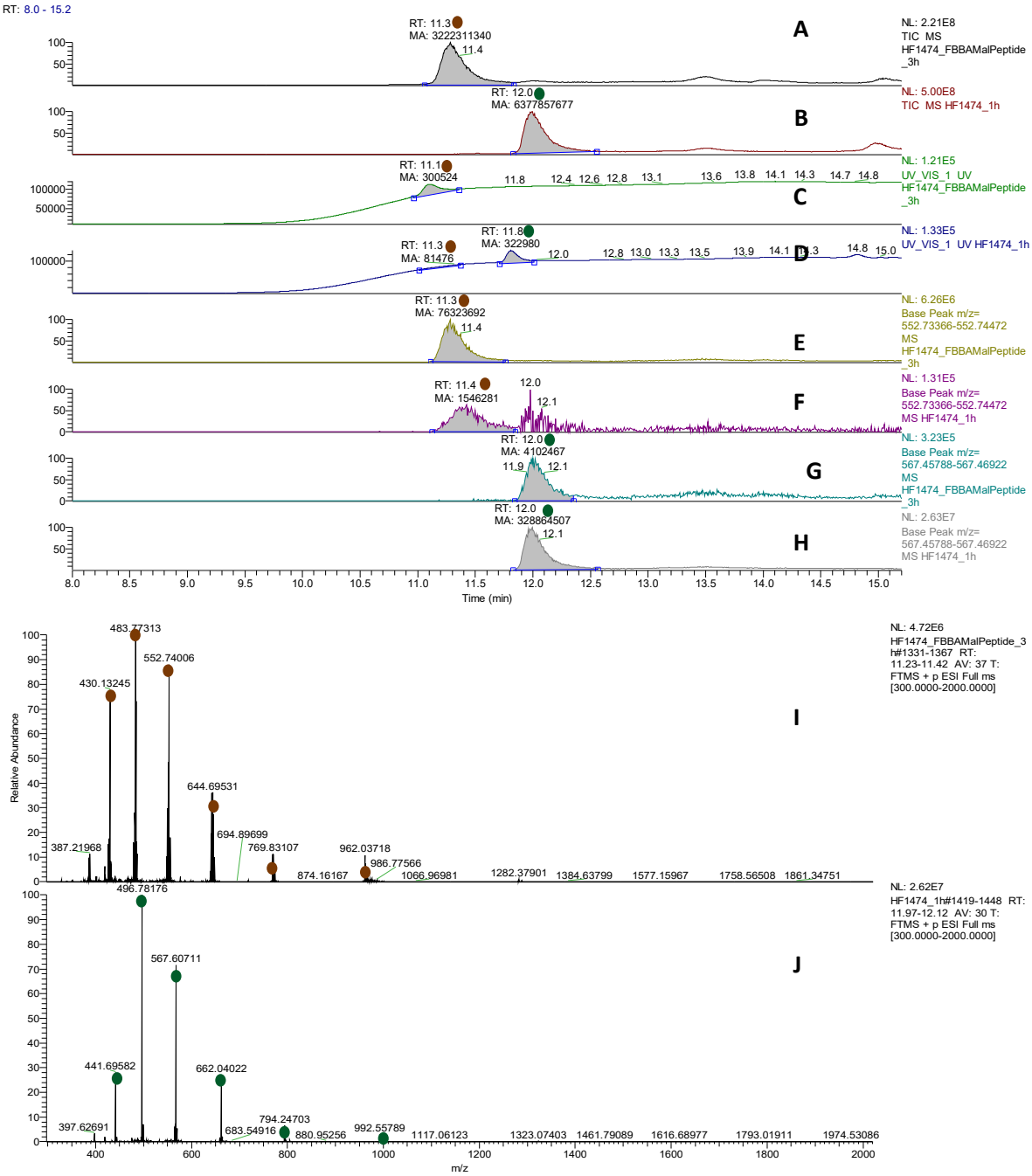


Figure S 45.2 -[17] **A** - TIC chromatogram of reaction of F3 (**16**) with crosslinker **7** to give **19**. **B** - TIC chromatogram of reaction between **19** and benzyl hydrazine **d** after 60 min to form **16d**; **C** - HPLC UV trace at 210 nm of reaction of F3 (**16**) with crosslinker **7** to give **19**. **D** - HPLC UV trace at 210 nm of reaction between **19** and benzyl hydrazine **d** after 60 min to form **16d**. **E** – EIC at 552.73919 (m/z for [**19**+7H]$^{7+}$) for reaction of F3 (**16**) with crosslinker **7** to give **19**. **F** - EIC at 552.73919 (m/z for [**19**+7H]$^{7+}$) of reaction between **19** and benzyl hydrazine **d** after 60 min to form **16d**. **G** – EIC at 567.46355 (m/z for [**16d**+7H]$^{7+}$) for reaction of F3 (**16**) with crosslinker **7** to give **19**. **H** - EIC at 567.46355 (m/z for [**16d**+7H]$^{7+}$) of reaction between **19** and benzyl hydrazine **d** after 60 min to form **16d**. **I** - HRMS ESI$^+$-MS spectrum of the peak at 11.4 min (**19**); **J** - HRMS ESI$^+$-MS spectrum of the peak at 12.0 min (**16d**). Although the baseline noise in UV-trace chromatograms do not allow accurate conversion calculation, it indicate near quantitative conversion of **10**, also confirmed by EIC area of **10** before (**E**) and after (**F**) the addition of benzyl hydrazine **d** (98% conversion).

[17] LC-MS were acquired in positive mode and the HPLC runs were carried out with a gradient of A (Milli Q water containing 0.1% v/v Formic acid (FA)) and B (acetonitrile containing 0.1% v/v FA). The mobile phase was t = 0 min, 5% B; t = 5 min, 5% B; t = 15 min, 95% B; t = 17 min, 95% B; t = 18 min, 5% B; t = 20 min, stop, at a flow rate of 0.2 mL/min.

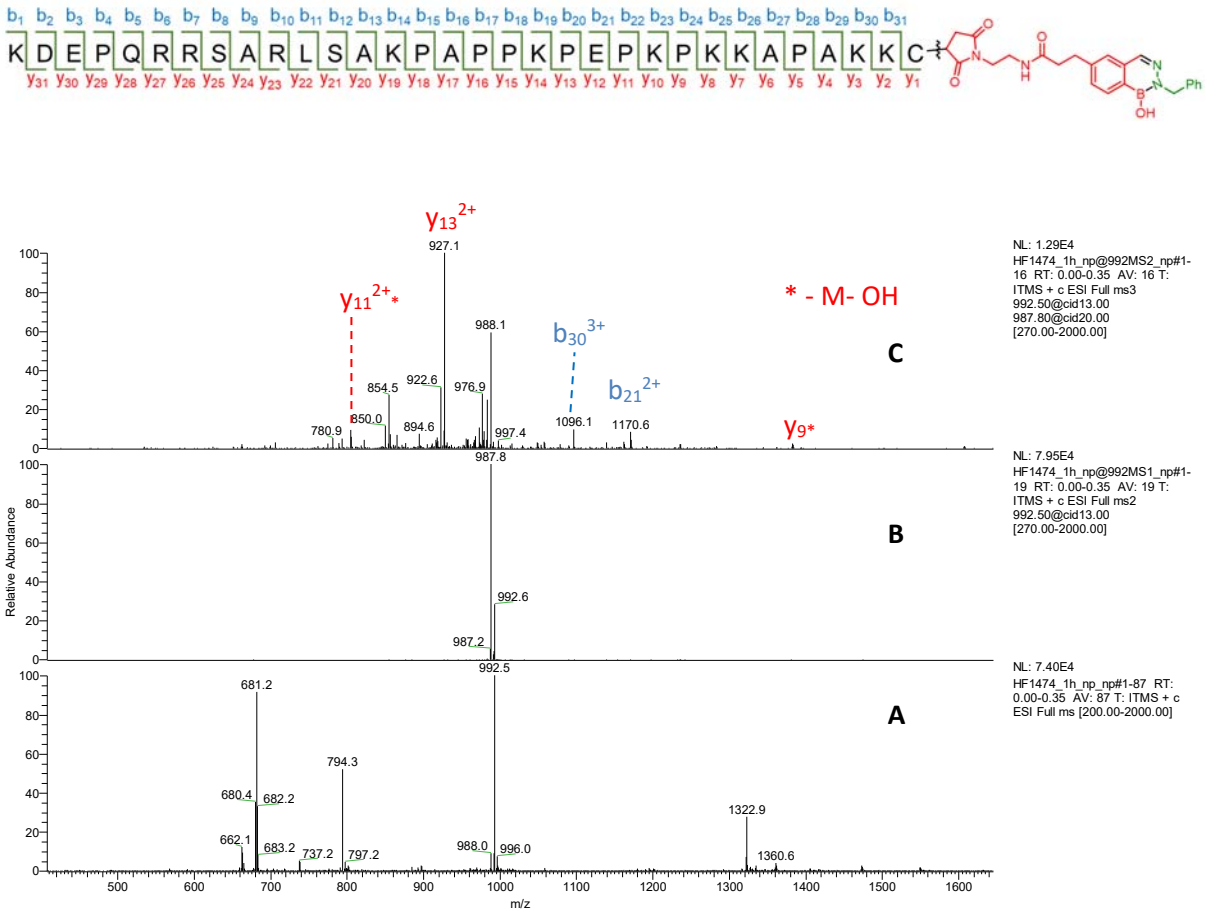
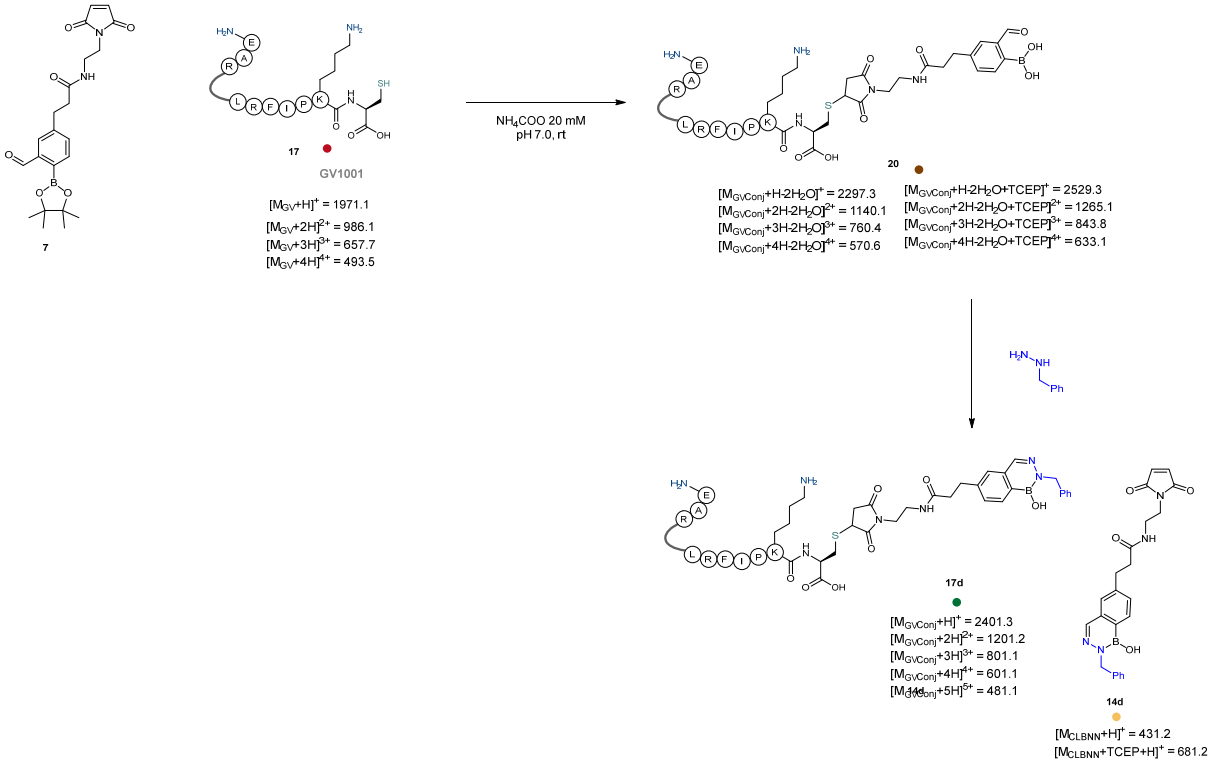


Figure S 46 – A - ESI+-MS spectrum of direct injection of the reaction between F3 (**16**) and **14d** to form **16d**. **B** – MS^2 fragmentation of conjugate **16d**. **C** – MS^3 fragmentation of conjugate **16d**.

4.2.1.2 Reaction of GV1001 with crosslinker followed by benzylhydrazine addition.



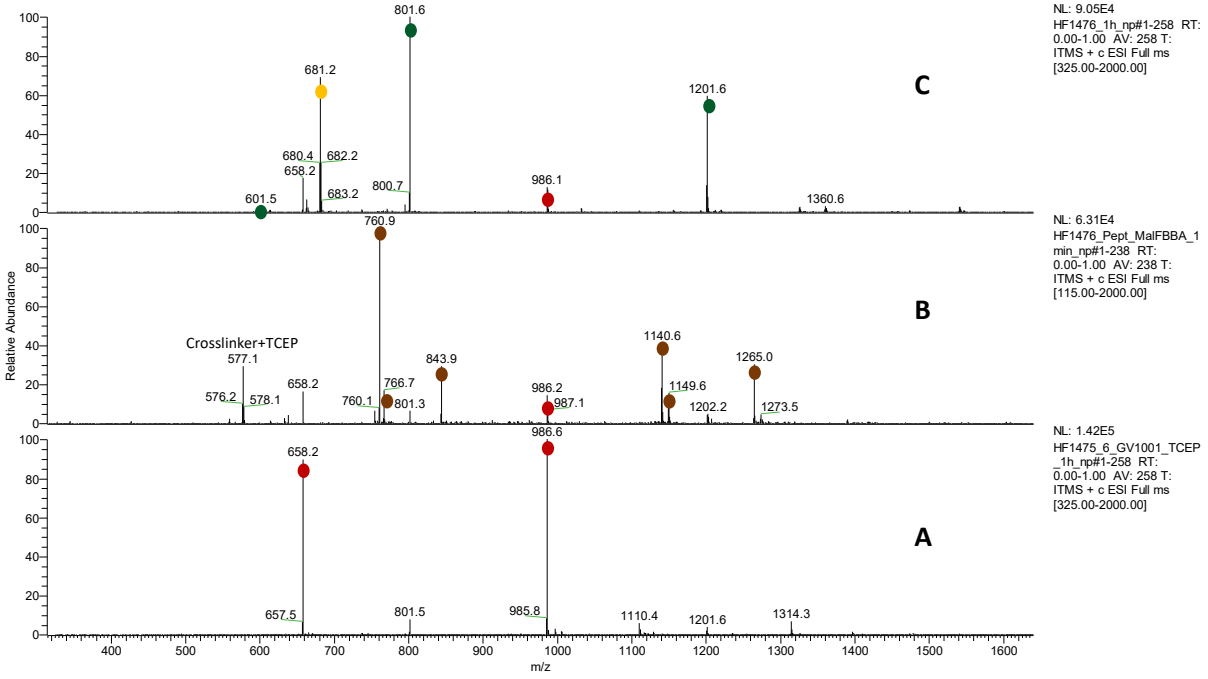
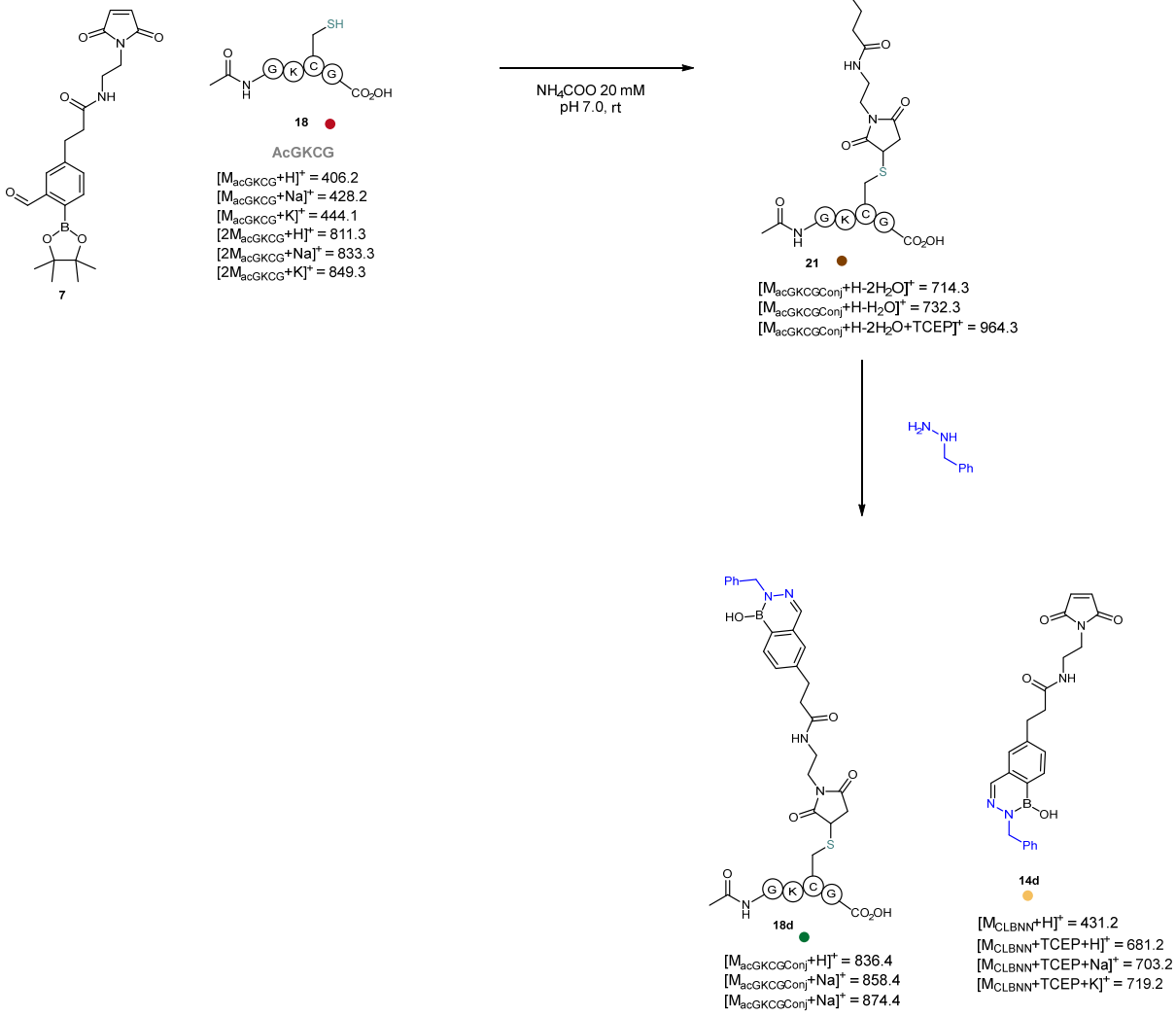


Figure S 47 – **A** - ESI$^+$-MS spectrum of GV1001 (**17**).; **B** - ESI$^+$-MS spectrum of GV1001 (**17**) reaction with **7** after 1min; **C** ESI$^+$-MS spectrum of the reaction of **20** with benzylhydrazine (**d**) after 1h.

4.2.1.3 Reaction of AcGKCG with crosslinker followed by benzylhydrazine addition.



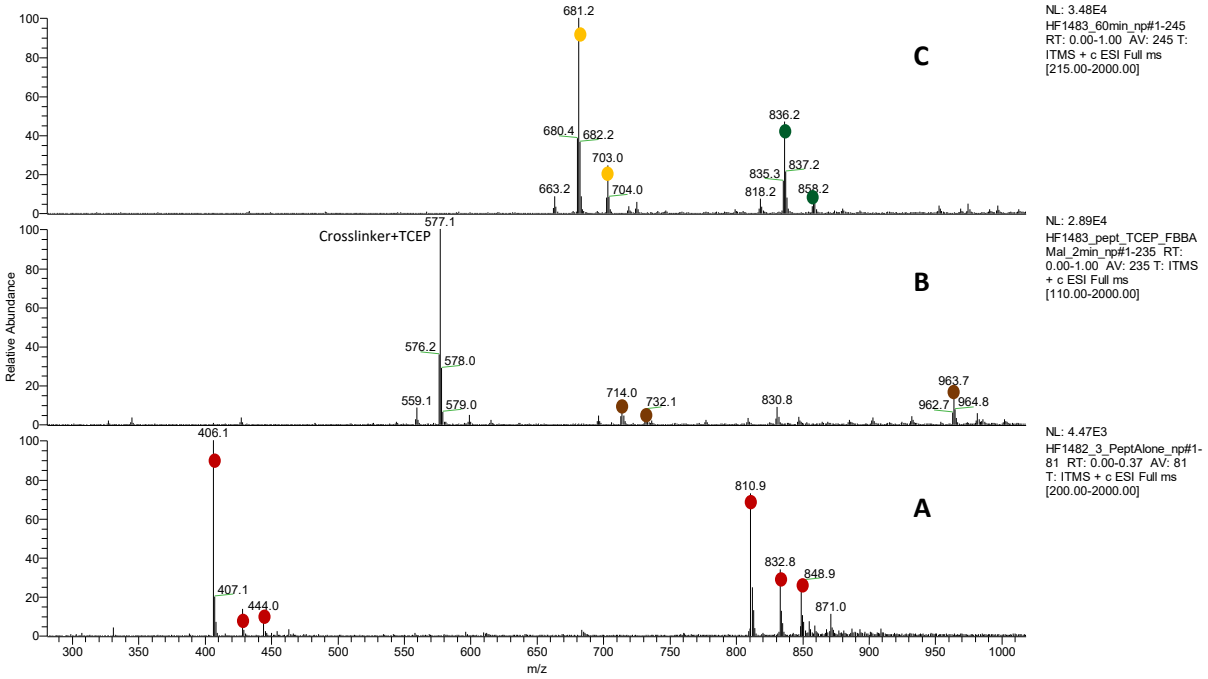
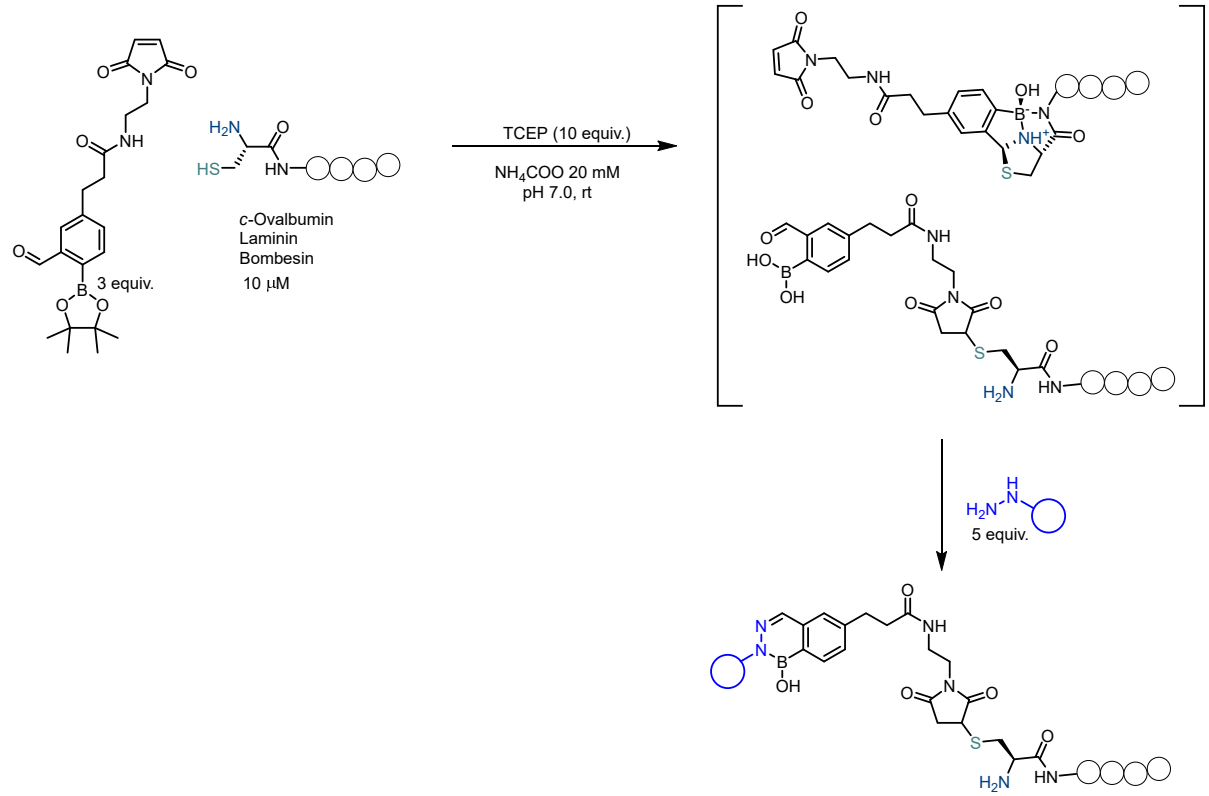
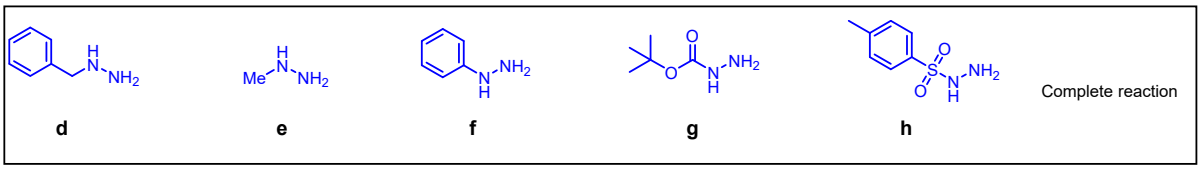


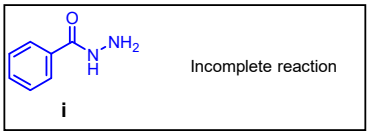
Figure S 48 – **A** - ESI$^+$-MS spectrum of AcGKCG (**18**); **B** - ESI$^+$-MS spectrum of AcGKCG (**18**) reaction with **7** after 2min; **C** ESI$^+$-MS spectrum of the reaction of **21** with benzylhydrazine (**d**) after 1h.

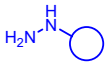
4.3 Thiazolidine followed by Diazaborine formation.

General procedure









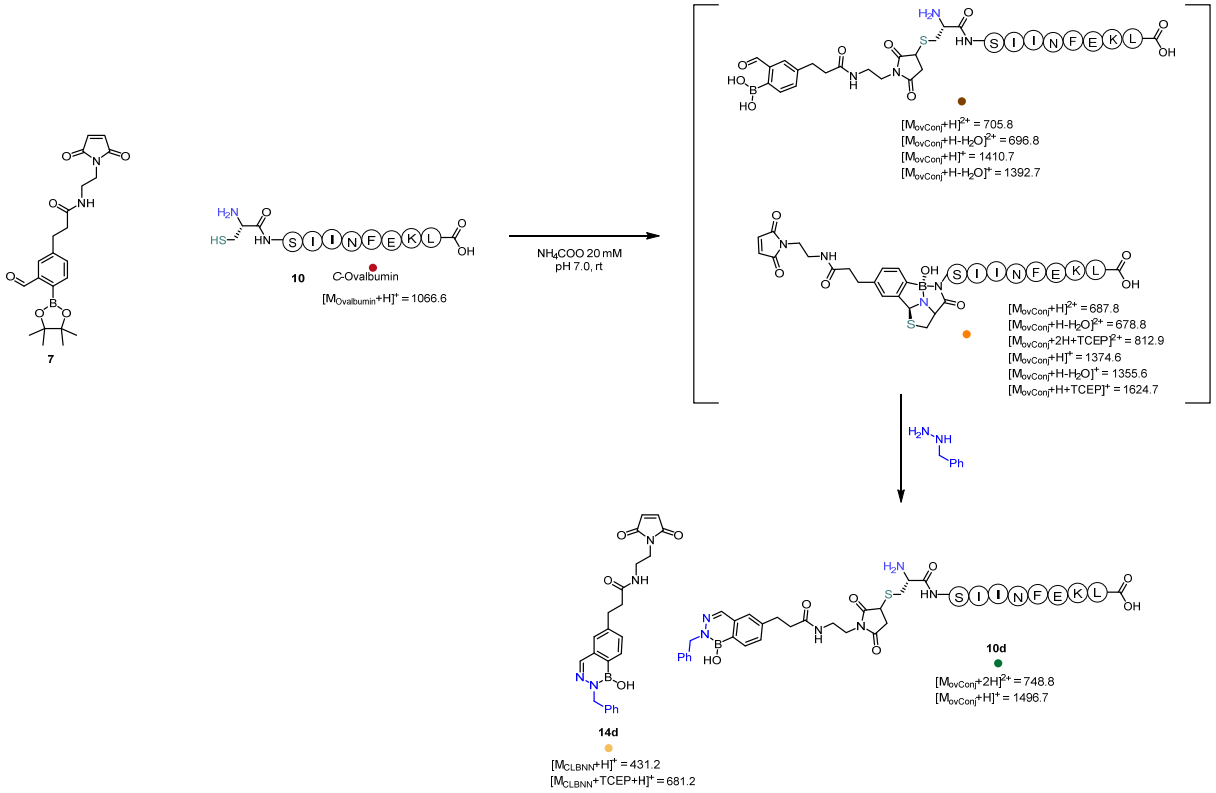
Screening of peptides with $BnNHNH_2$
Screening of hydrazines with c-Ovalbumin

To a solution of peptide (≈1 mM) (10 µL, 0.010 µmol) in Acetate solution 20 mM pH 7.0 (1.0 mL) was added a Tris(2-carboxyethyl)phosphine hydrochloride (TCEP) (3.5 mM in water) (28.6 µL, 0.100 µmol) and the solution mixed for 30 min at 25 ºC.

Then, crosslinker **7** (10mM in ACN, 3 µL, 0.03 µmol) was added, allowed to react for 1 min followed by addition of the hydrazine (5.0 µL, 0.050 µmol). The mixture was allowed to react for 60 min and the reaction was monitored by direct infusion in Positive Mode of ESI-MS.

4.3.1 Peptide screening in the Crosslink with benzylhydrazine

4.3.1.1 Reaction of *c*-Ovalbumin with crosslinker followed by benzylhydrazine addition.



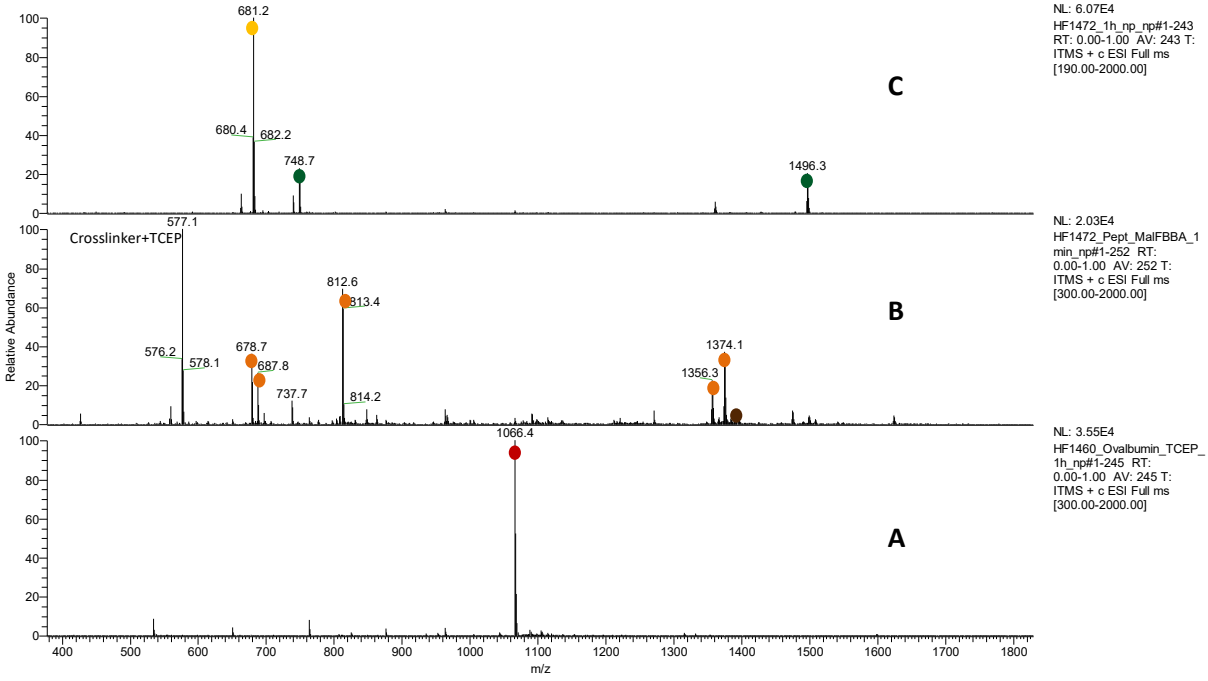


Figure S 49.1 – **A** - ESI$^+$-MS spectrum of Ovalbumin (**10**).; **B** - ESI$^+$-MS spectrum of Ovalbumin (**10**) reaction with **7** after 1min; **C** - ESI$^+$-MS spectrum of the reaction 1h after addition of benzylhydrazine ESI$^+$-MS spectrum of the reaction 1h after addition of benzylhydrazine (**d**) .

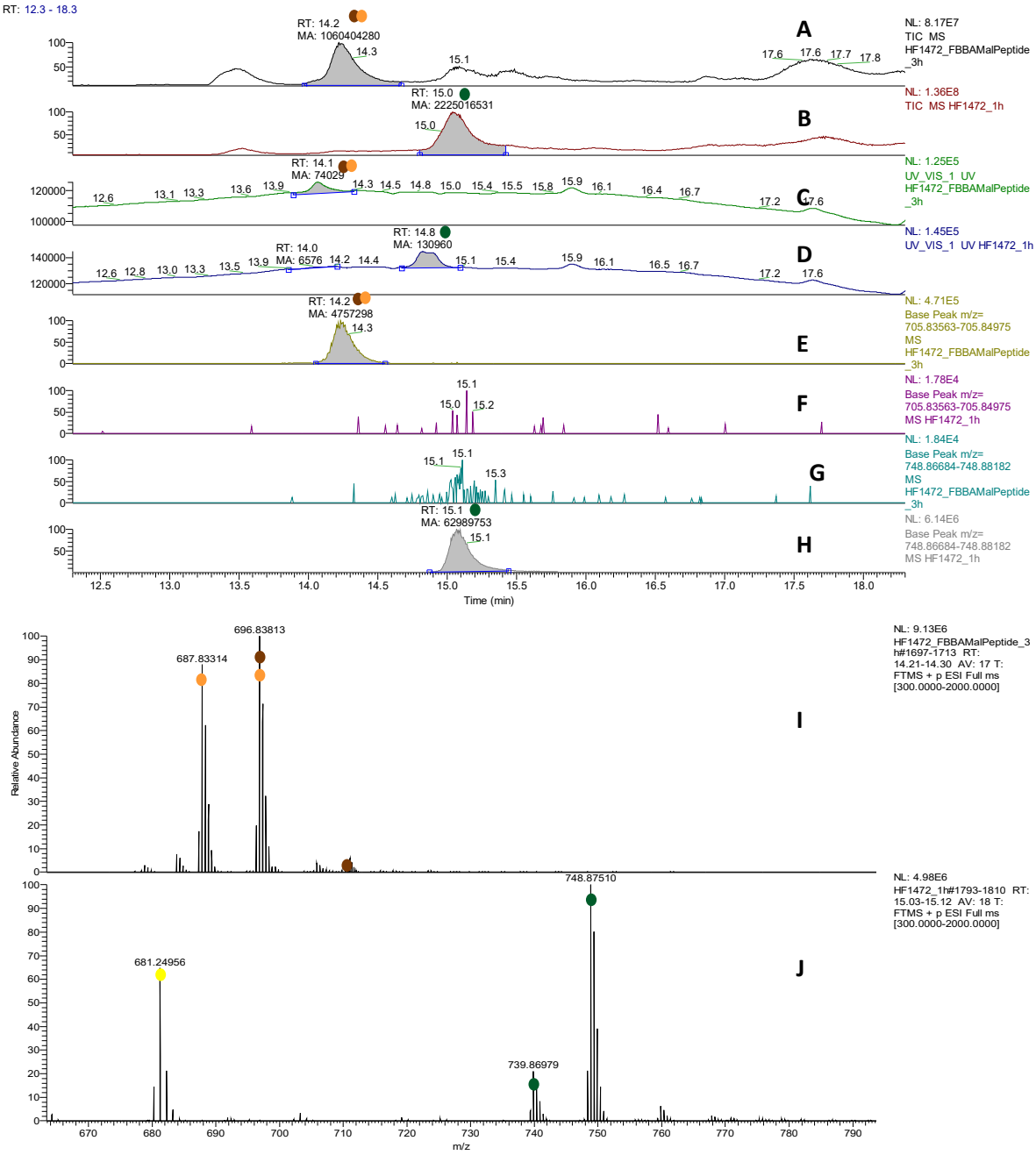


Figure S 49.2 -[18] **A** - TIC chromatogram of reaction of Ovalbumin (**10**) with crosslinker **7** to give a mixture of thiazolidine (●) and thiosuccinimide (●). **B** - TIC chromatogram of reaction between the mixture of thiazolidine (●) and thiosuccinimide (●) and benzyl hydrazine **d** after 60 min to form **10d**; **C** - HPLC UV trace at 210 nm of the mixture of thiazolidine (●) and thiosuccinimide (●). **D** - HPLC UV trace at 210 nm of reaction between the mixture of thiazolidine (●) and thiosuccinimide (●) and benzyl hydrazine **d** after 60 min to form **10d**. **E** – EIC at 705.84269 (m/z for $[(●)+2H]^{2+}$) for mixture of thiazolidine (●) and thiosuccinimide (●). **F** - EIC at 705.84269 (m/z for $[(●)+2H]^{2+}$) of reaction between the mixture of thiazolidine (●) and thiosuccinimide (●) and benzyl hydrazine **d** after 60 min to form **10d**. **G** – EIC at 748.87433 (m/z for $[\mathbf{10d}+2H]^{2+}$) for mixture of thiazolidine (●) and thiosuccinimide (●). **H** - EIC at 748.87433 (m/z for $[\mathbf{10d}+2H]^{2+}$) of reaction between the mixture of thiazolidine (●) and thiosuccinimide (●) and benzyl hydrazine **d** after 60 min to form **10d**. **I** - HRMS ESI$^+$-MS spectrum of the peak at 14.2 min (● and ●); **J** - HRMS ESI$^+$-MS spectrum of the peak at 15.1 min (**10d**). Although the baseline noise in UV-trace chromatograms do not allow accurate conversion calculation, it indicate near quantitative conversion of thiazolidine (●) and thiosuccinimide (●), also confirmed by EIC areas before (**E**) and after (**F**) the addition of benzyl hydrazine **d**.

[18] LC-MS were acquired in positive mode and the HPLC runs were carried out with a gradient of A (Milli Q water containing 0.1% v/v Formic acid (FA)) and B (acetonitrile containing 0.1% v/v FA). The mobile phase was t = 0 min, 5% B; t = 5 min, 5% B; t = 15 min, 95% B; t = 17 min, 95% B; t = 18 min, 5% B; t = 20 min, stop, at a flow rate of 0.2 mL/min.

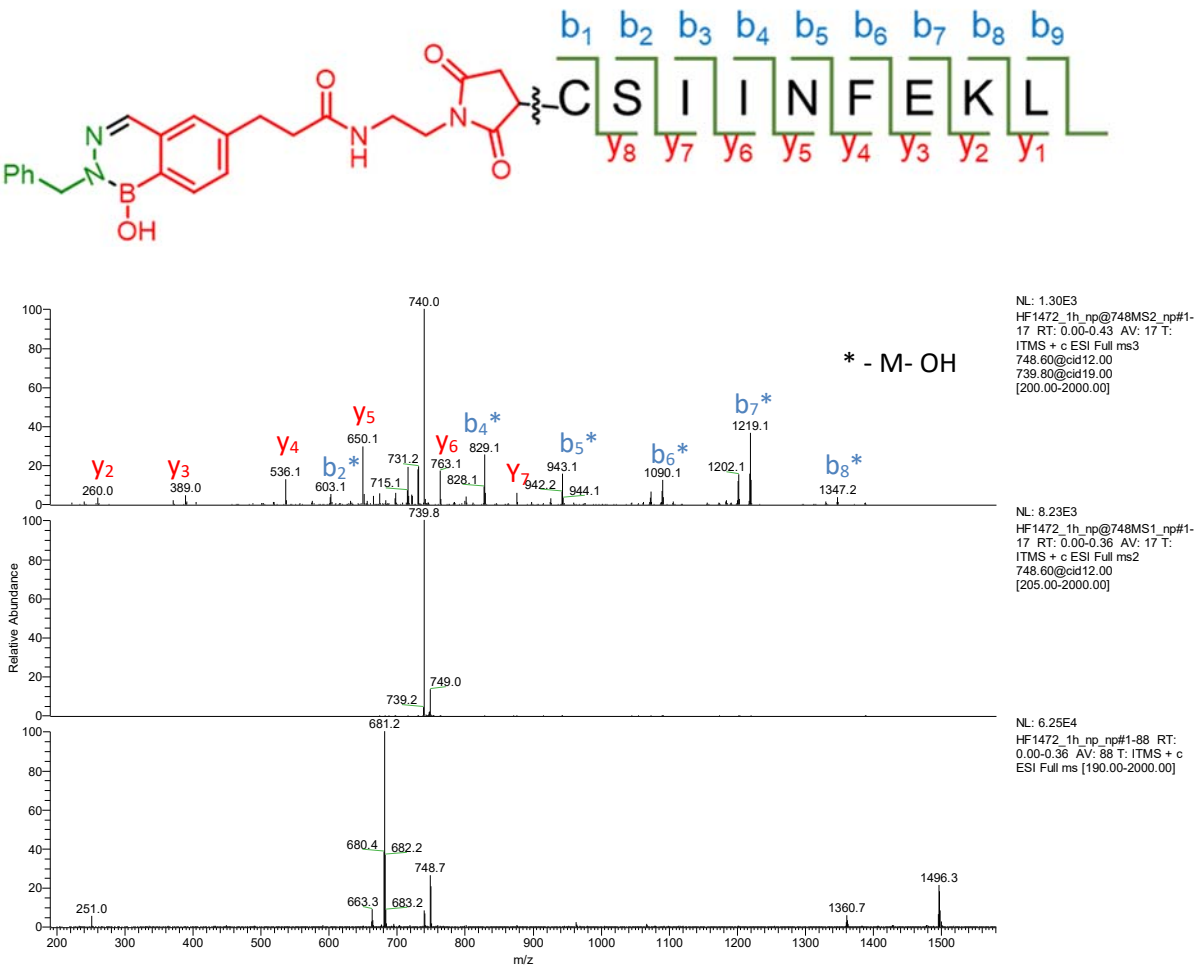


Figure S 50 – A - ESI$^+$-MS spectrum of the reaction 1h after addition of benzylhydrazine (**d**). **B** – MS^2 fragmentation of conjugate **10d**. **C** – MS^3 fragmentation of conjugate **10d**.

4.3.1.2 Reaction of Laminin with crosslinker followed by benzylhydrazine addition.

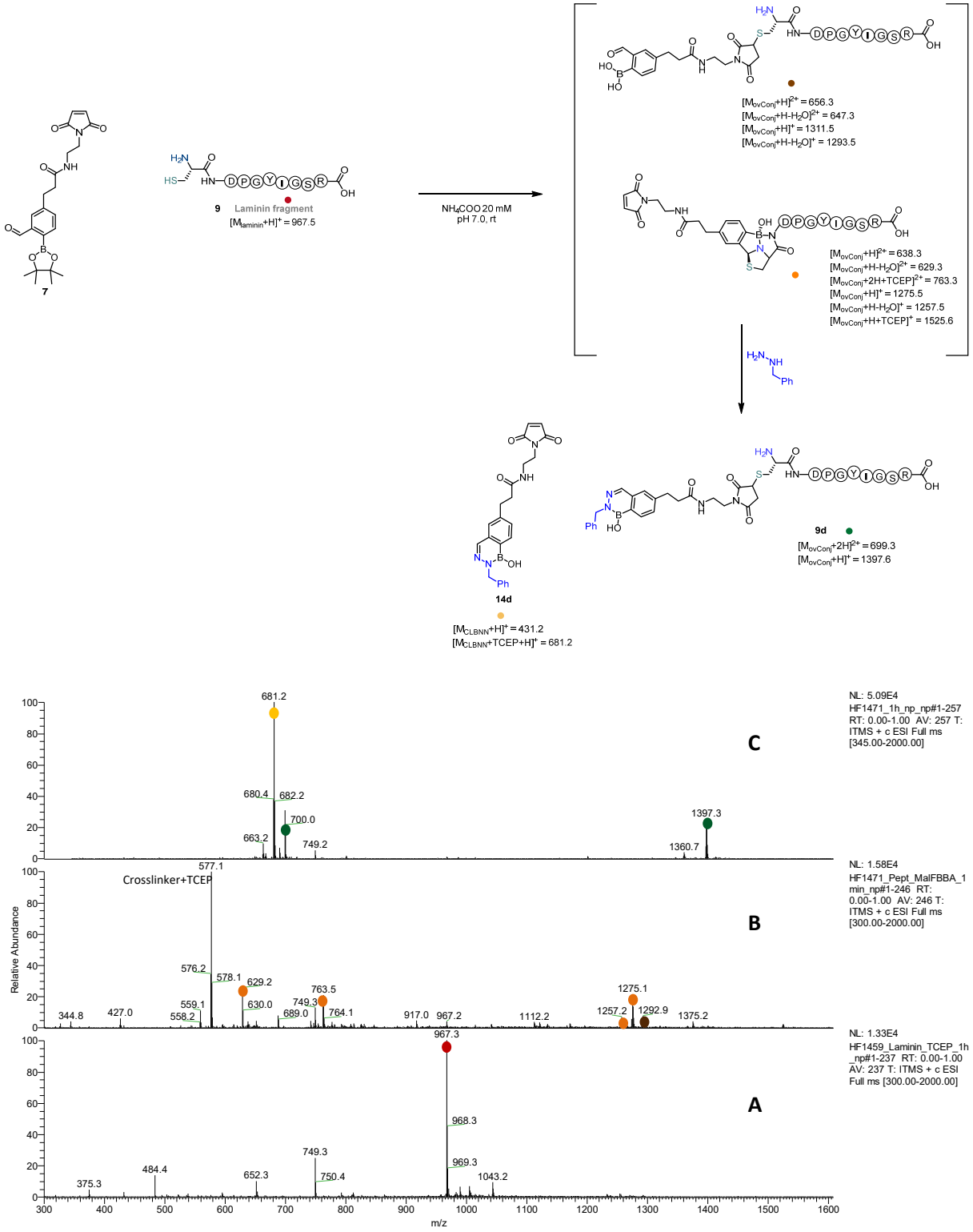


Figure S 51 – **A** - ESI$^+$-MS spectrum of Laminin (**9**).; **B** - ESI$^+$-MS spectrum of Laminin (**9**) reaction with **7** after 1min; **C** - ESI$^+$-MS spectrum of the reaction 1h after addition of benzylhydrazine (**d**) .

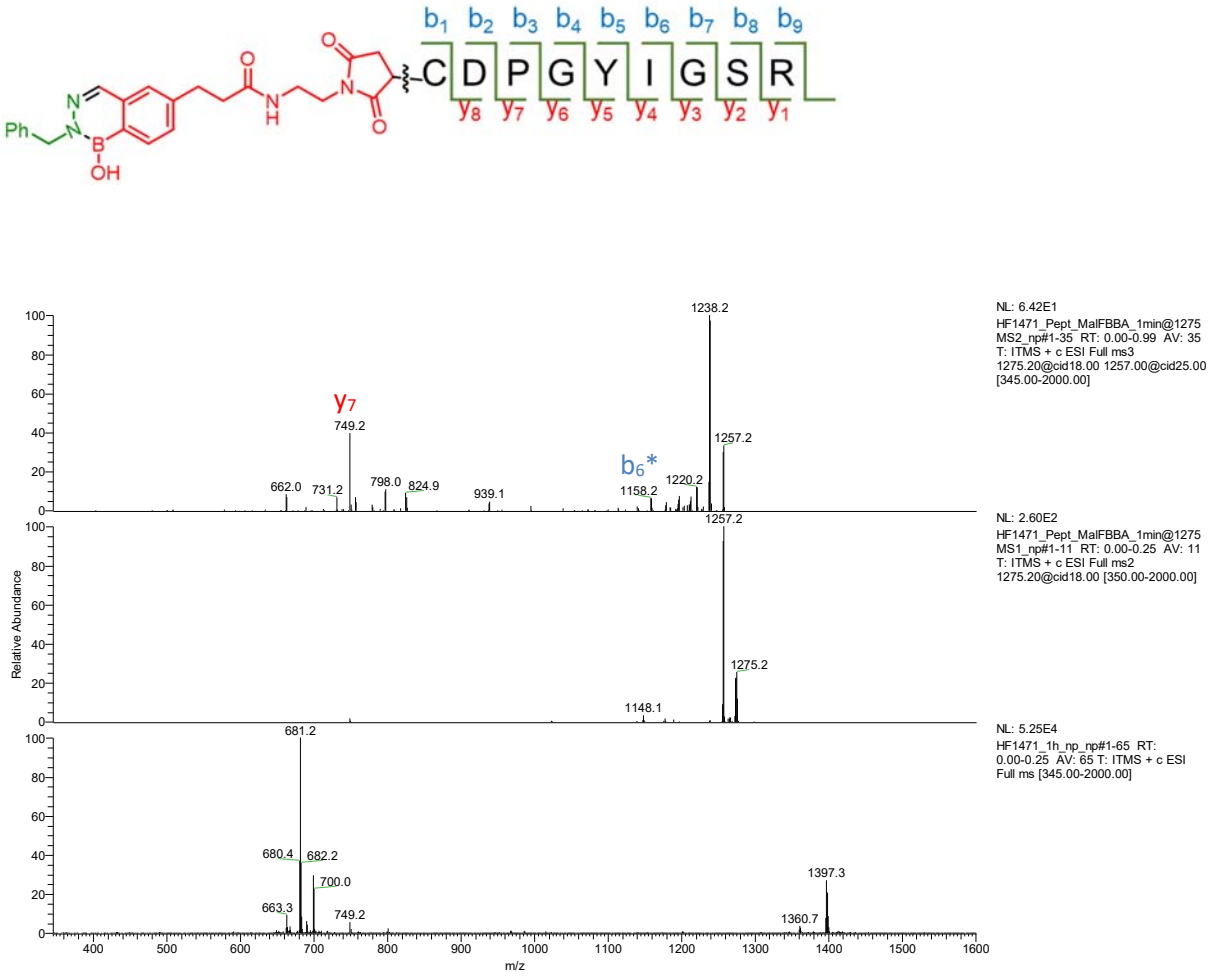
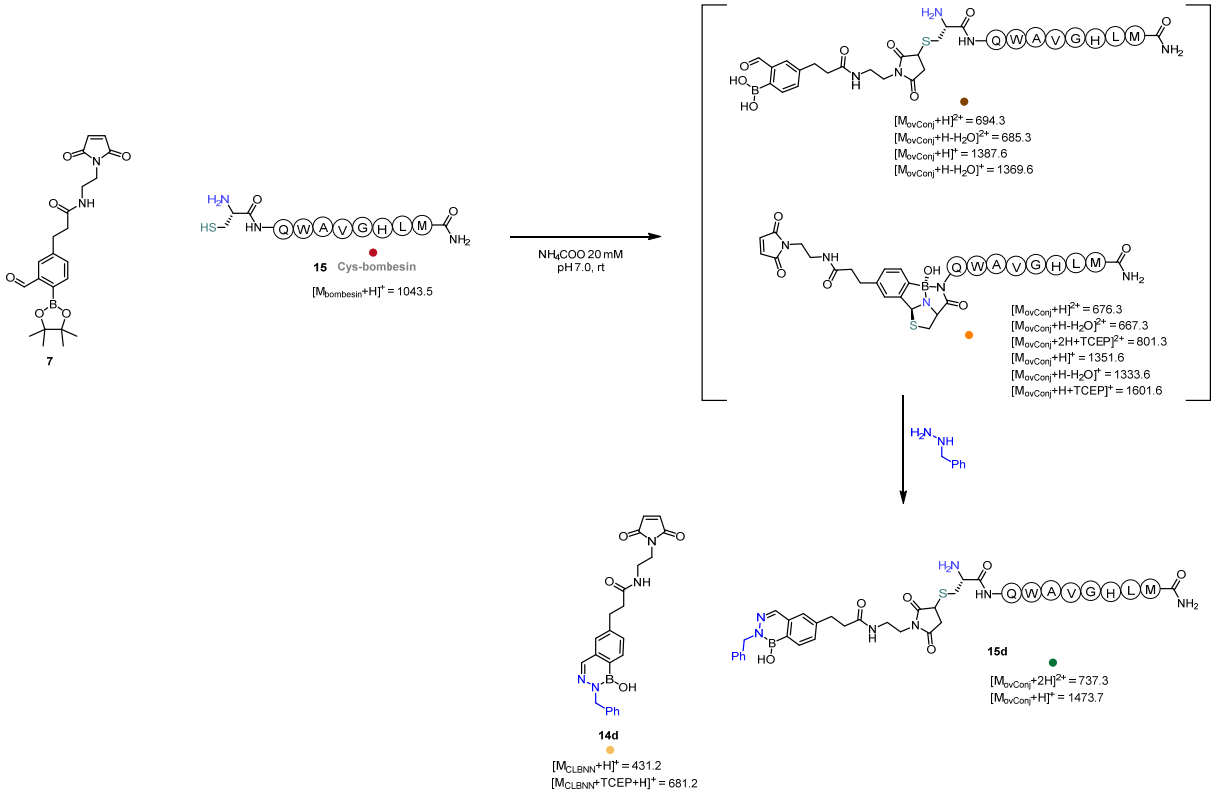


Figure S 52 – A - ESI$^+$-MS spectrum of the reaction 1h after addition of benzylhydrazine (**d**). **B** – MS^2 fragmentation of conjugate **9d**. **C** – MS^3 fragmentation of conjugate **9d**.

4.3.1.3 Reaction of Cys-Bombesin with crosslinker followed by benzylhydrazine addition.



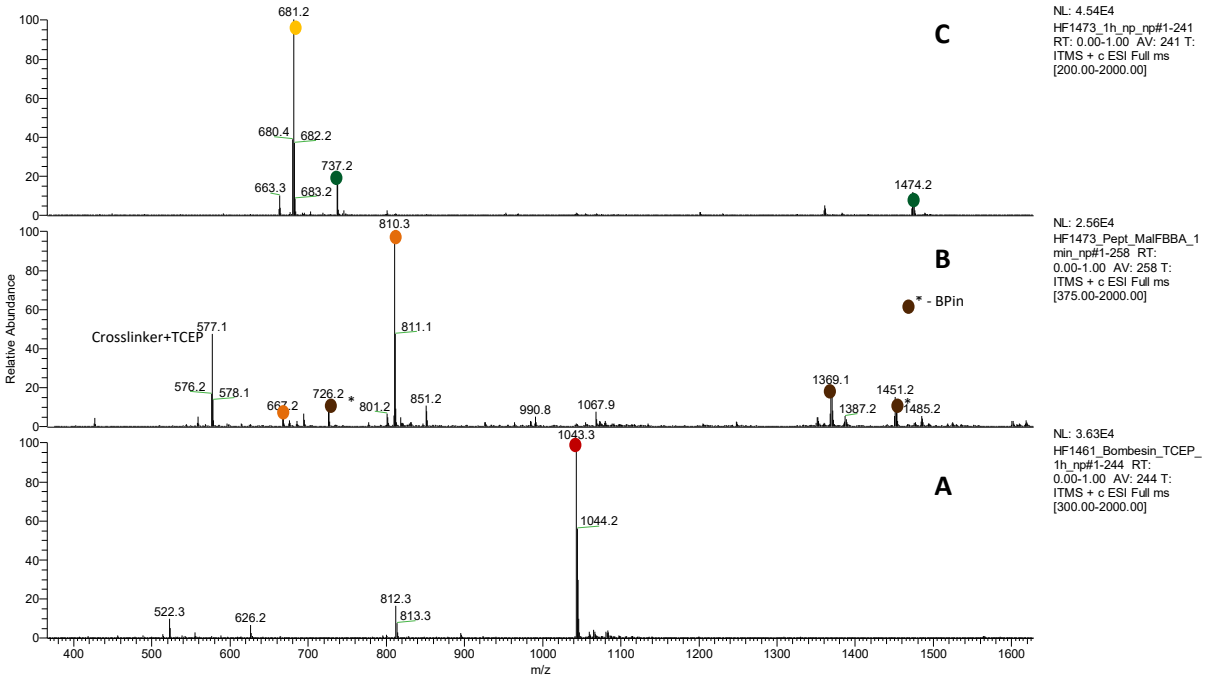


Figure S 53 – **A** - ESI$^+$-MS spectrum of Cys-Bombesin (**15**).; **B** - ESI$^+$-MS spectrum of Cys-Bombesin (**15**) reaction with **7** after 1min; **C** - ESI$^+$-MS spectrum of the reaction 1h after the addition of benzylhydrazine (**d**) .

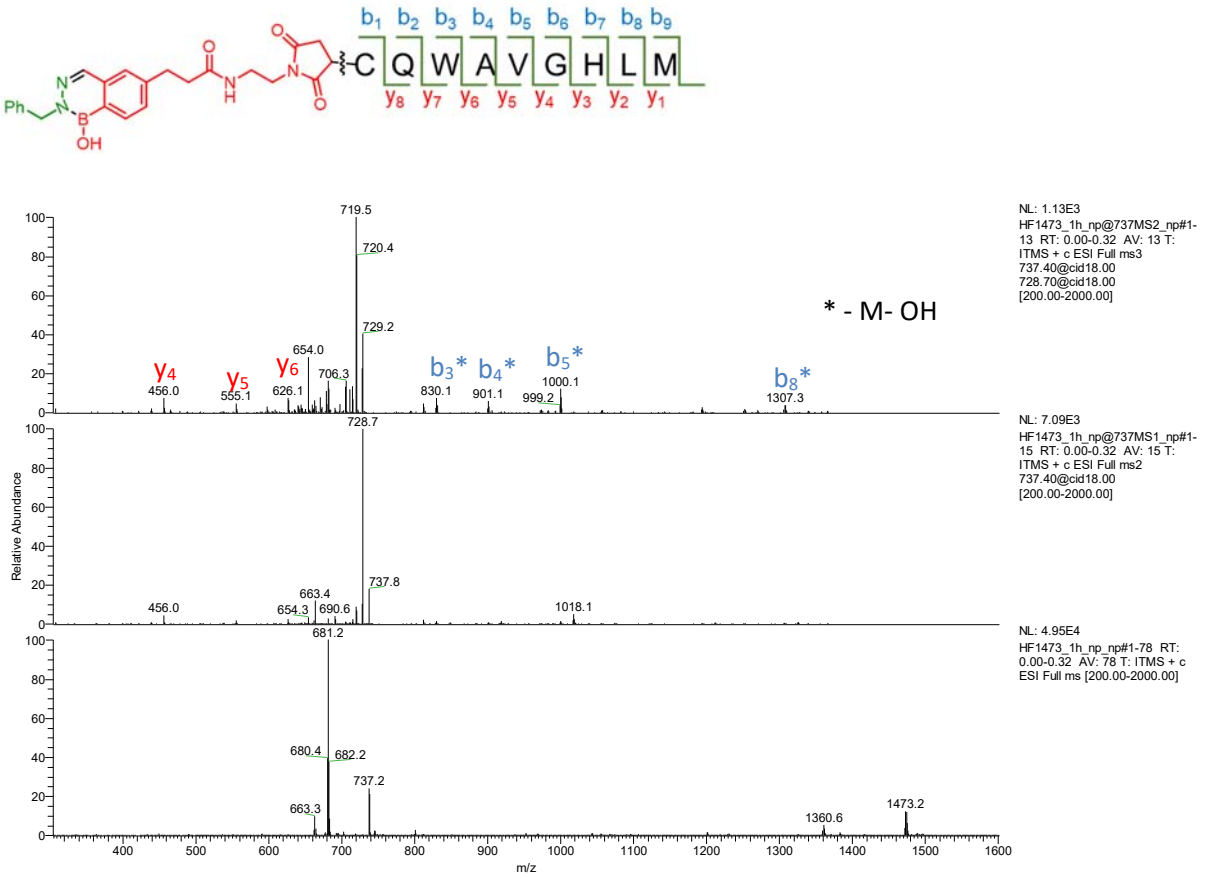
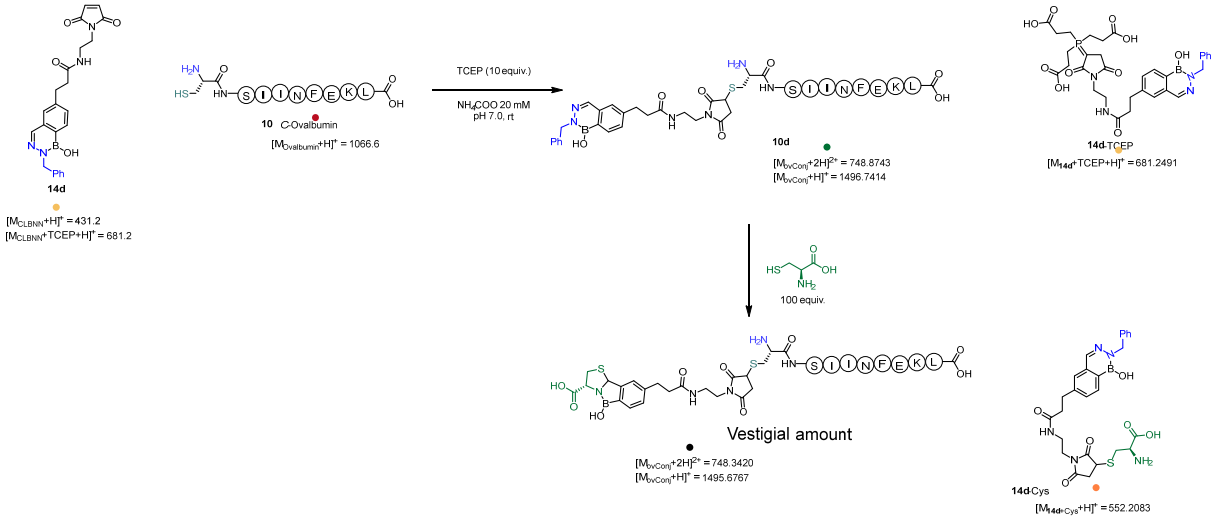


Figure S 54 – A - ESI$^+$-MS spectrum of the reaction 1h after addition of benzylhydrazine (**d**). **B** – MS^2 fragmentation of conjugate **15d**. **C** – MS^3 fragmentation of conjugate **15d**.

4.4 Stability of the DAB-peptide conjugate after addition of excess Cys.

Procedure



In an eppendorf Crosslinker **7** (10mM in ACN, 3.0 µL, 0.03 µmol) was added to ammonium acetate solution 20 mM pH 7.0 (500 µL) and allow for BPin to be hydrolyzed over 15 min. Then the hydrazine (5.0 µL, 0.050 µmol) was added and allowed to react for 5 min and the reaction monitored by direct infusion in Positive Mode of ESI-MS.

In a second eppendorf, to a solution of c-Ovalbumin (1.0 mM) (10 µL, 0.010 µmol) in ammonium acetate solution 20 mM pH 7.0 (500 µL) was added a Tris(2-carboxyethyl)phosphine hydrochloride (TCEP) (3.5 mM in water) (28.6 µL, 0.100 µmol) and the solution mixed for 30 min at 25 ºC.

Then both solutions were mixed and allow to react for 1h and the reaction monitored by direct infusion in Positive Mode of ESI-MS.

Finally L-cysteine (100 mM in water) (10.0 µL, 1.0 µmol) was added and the stability of DAB was monitored by ESI-MS at 1h and 24h and at 24h by LC-HRMS.

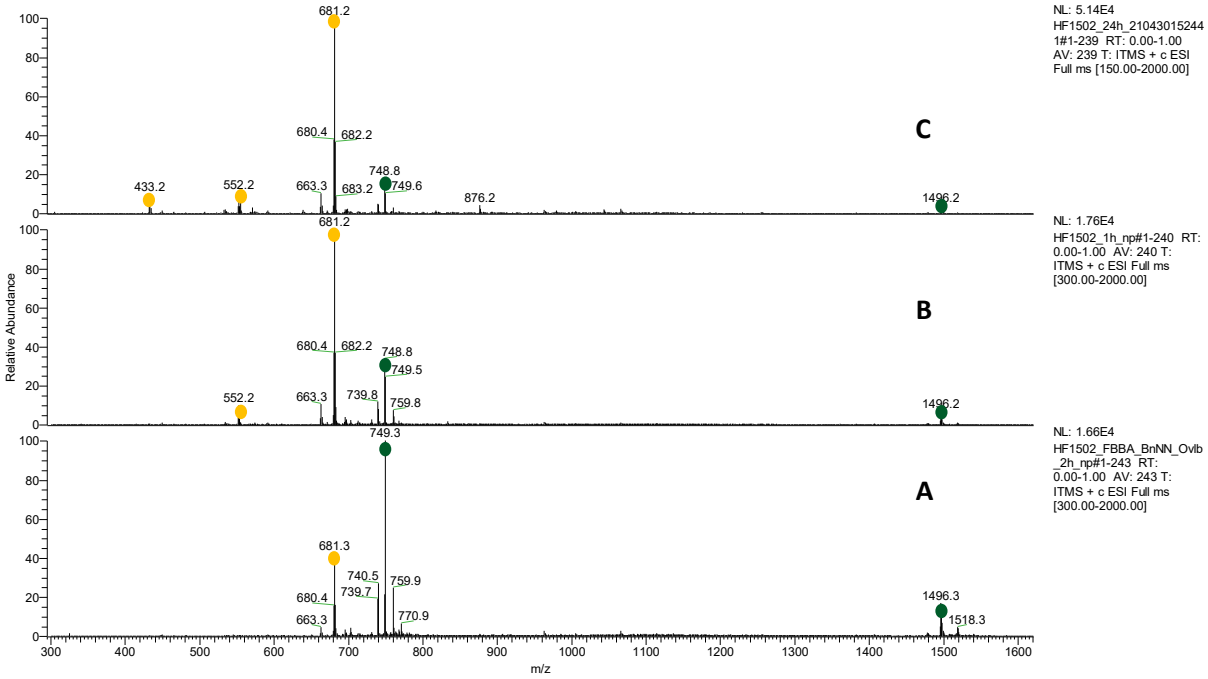


Figure S 55 – A - ESI+-MS spectrum of direct injection of the reaction between Ovalbumin (**10**) and **14d** to form **10d** after 2h without Cys. **B** – ESI+-MS spectrum of direct injection of the reaction between Ovalbumin (**10**) and **14d** to form **10d** after 1h in the presence of 100 equiv. of Cys. **C** – ESI+-MS spectrum of direct injection of the reaction between Ovalbumin (**10**) and **14d** to form **10d** after 24h in the presence of 100 equiv. of Cys. The presence of the product of exchange between hydrazine and cysteine to form a thiazolidine was not detected.

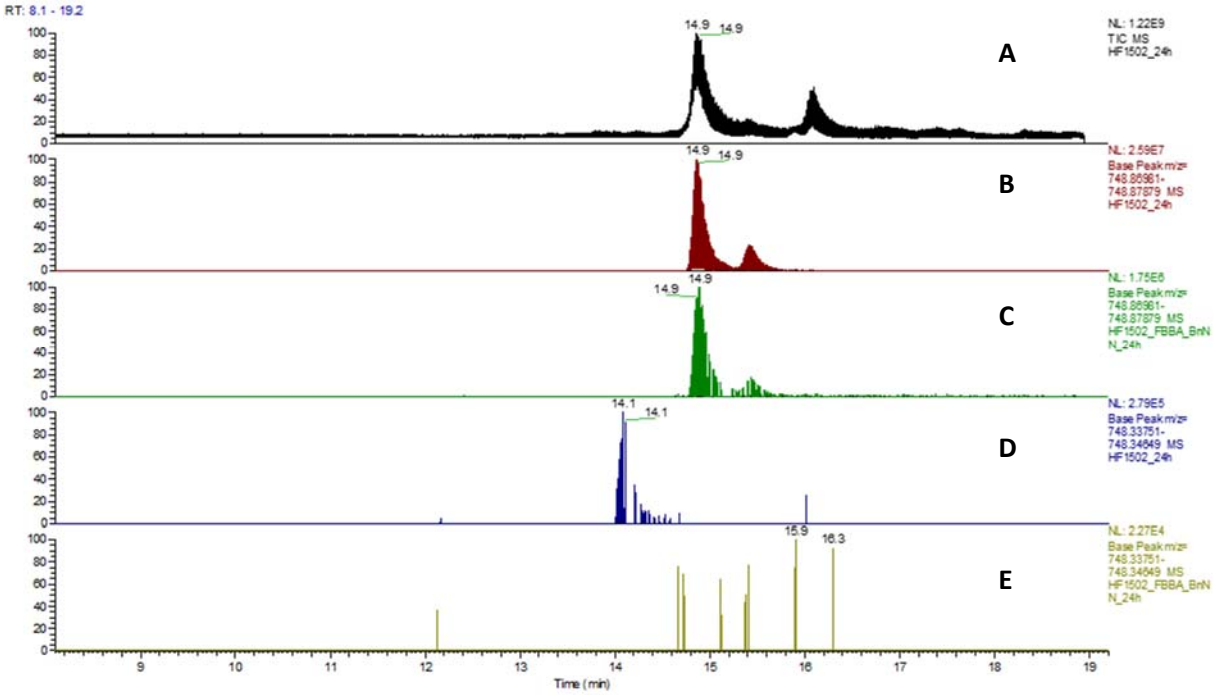


Figure S 56 –.**A** – TIC chromatogram of reaction 24 h after cys addition; **B** – EIC chromatogram of 748.8743 (**10d**$^{2+}$) peak with 6 ppm tolerance for reaction with 100 equiv. Cys; **C** – EIC chromatogram of 748.8743 (DAB-peptide conjugate) peak with 6 ppm tolerance for control reaction **without** 100 equiv. Cys. **D** – EIC chromatogram of 748.3420 ([Cys-peptide conjugate]$^{+2}$) peak with 6 ppm tolerance for reaction with 100 equiv. Cys; **E** – EIC chromatogram of 748.3420 ([Cys-peptide conjugate]$^{+2}$) peak with 6 ppm tolerance for control reaction **without** 100 equiv. Cys. The data demonstrate only vestigial exchange between hydrazine and cysteine.

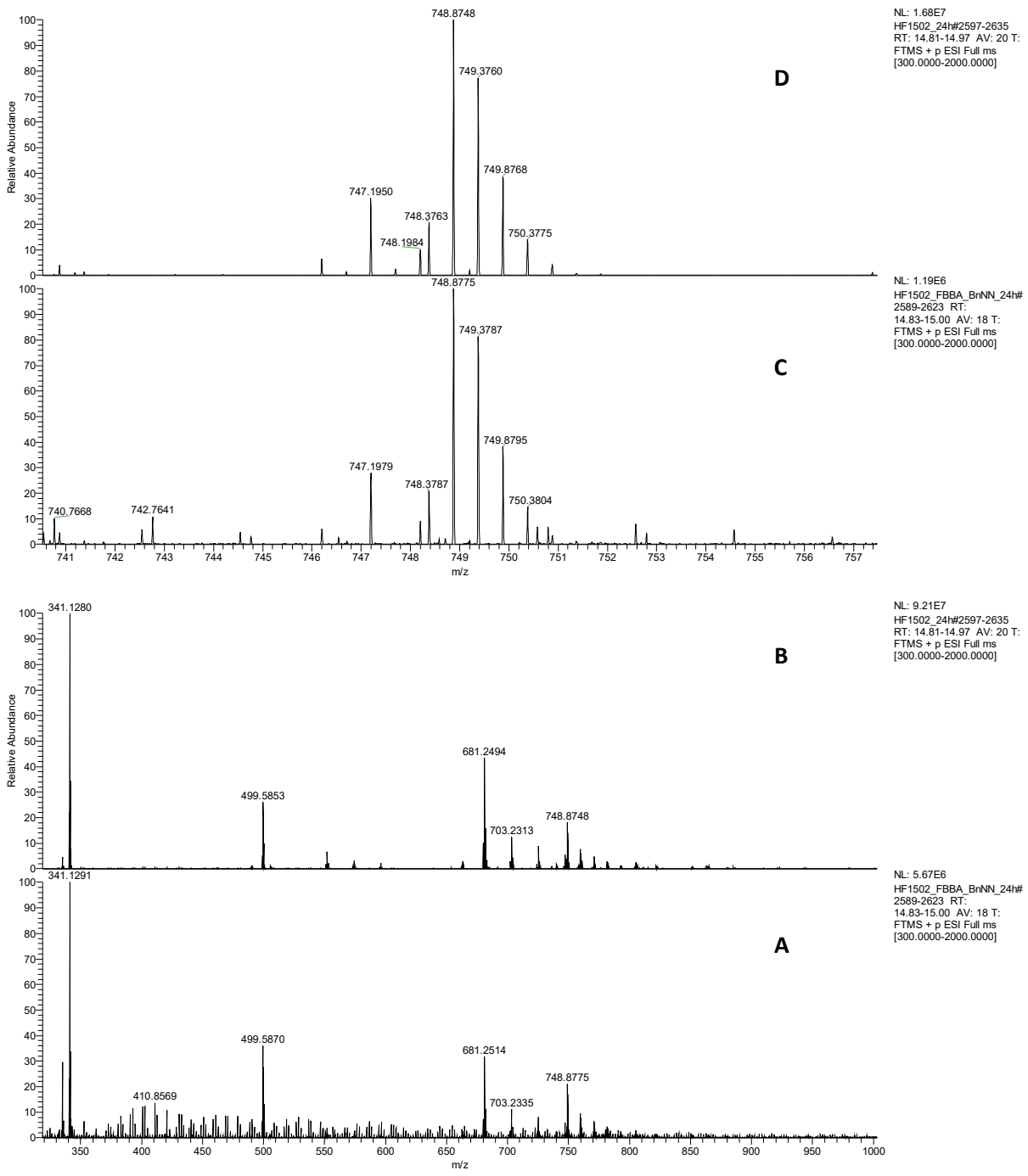
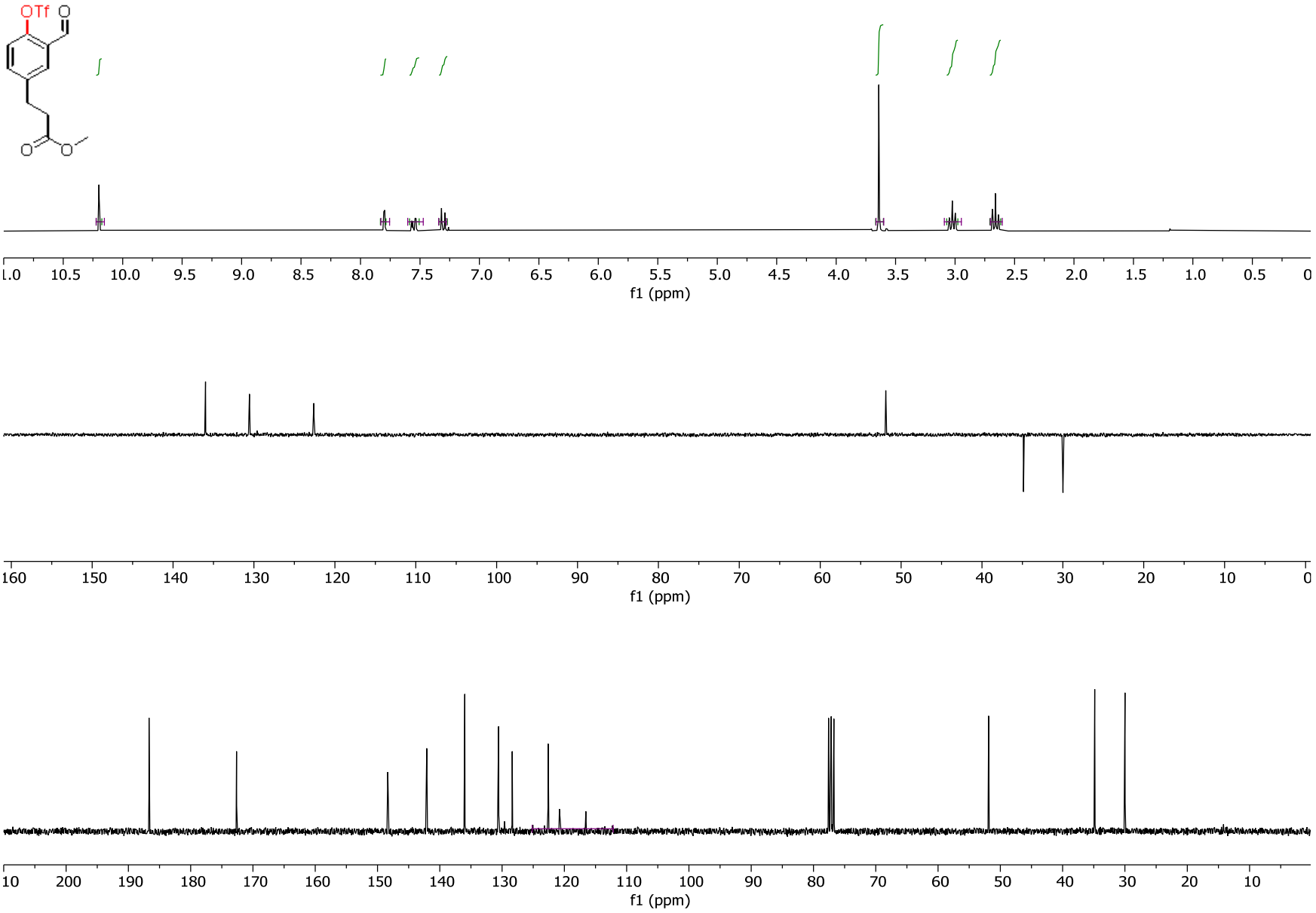
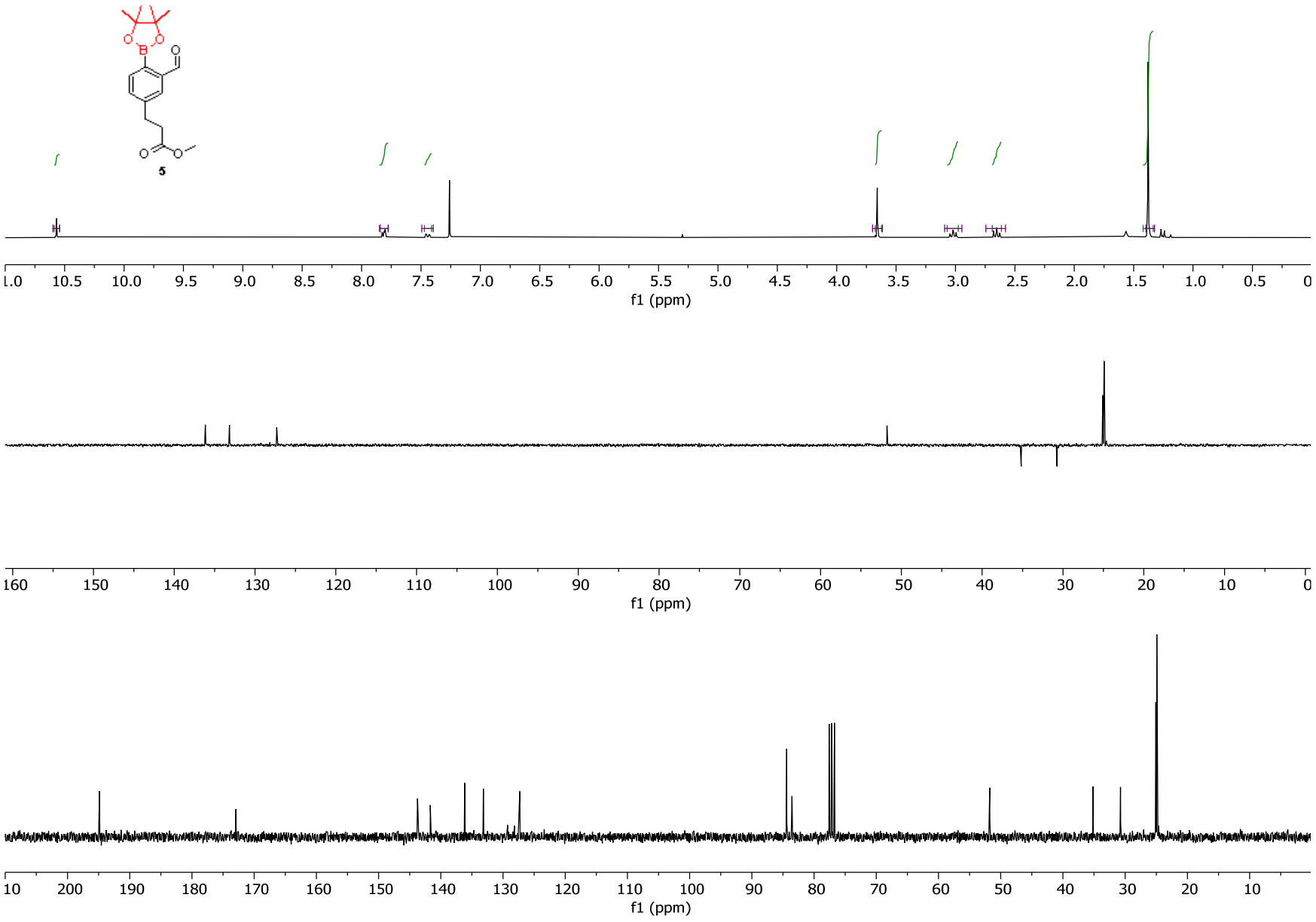
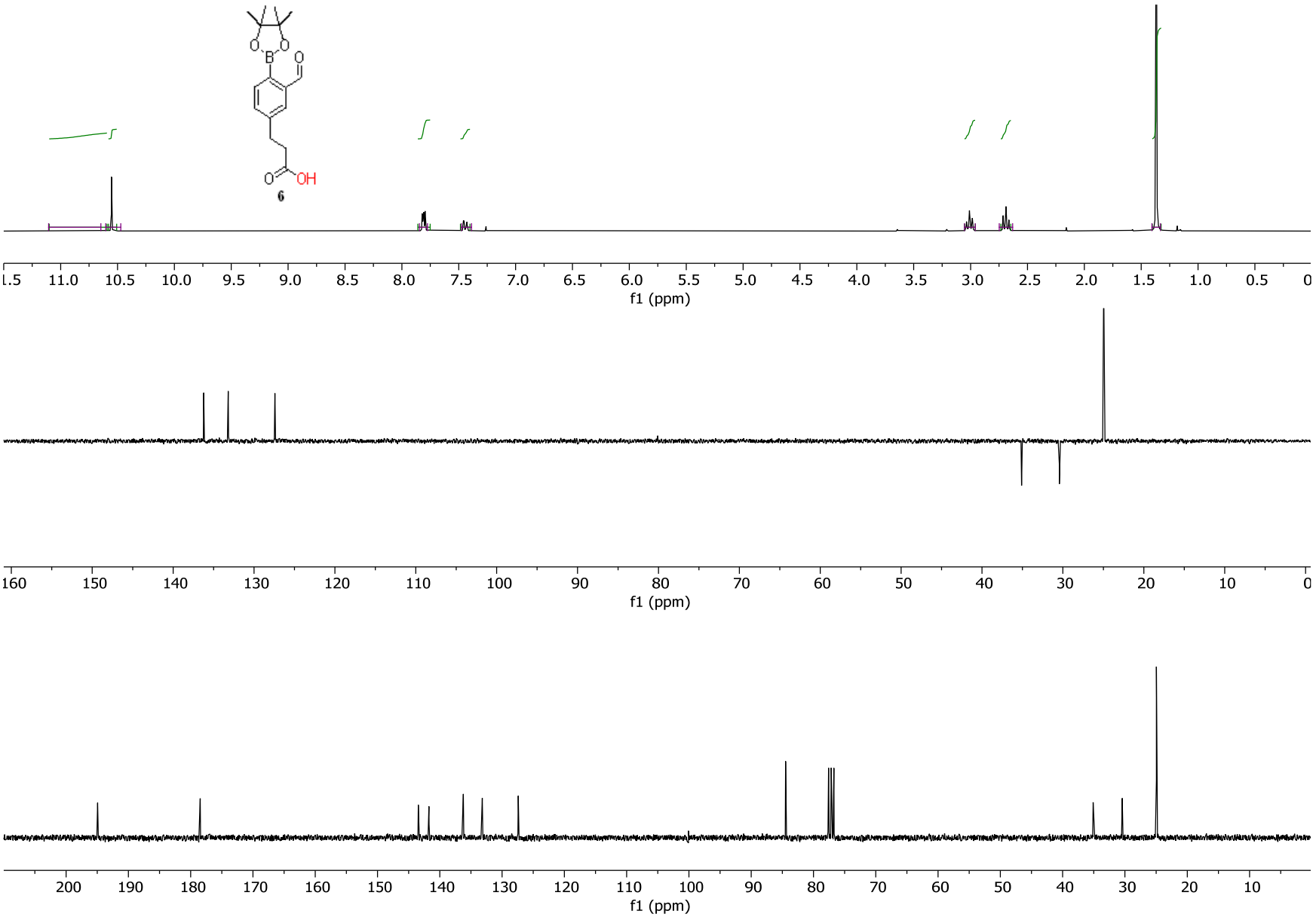


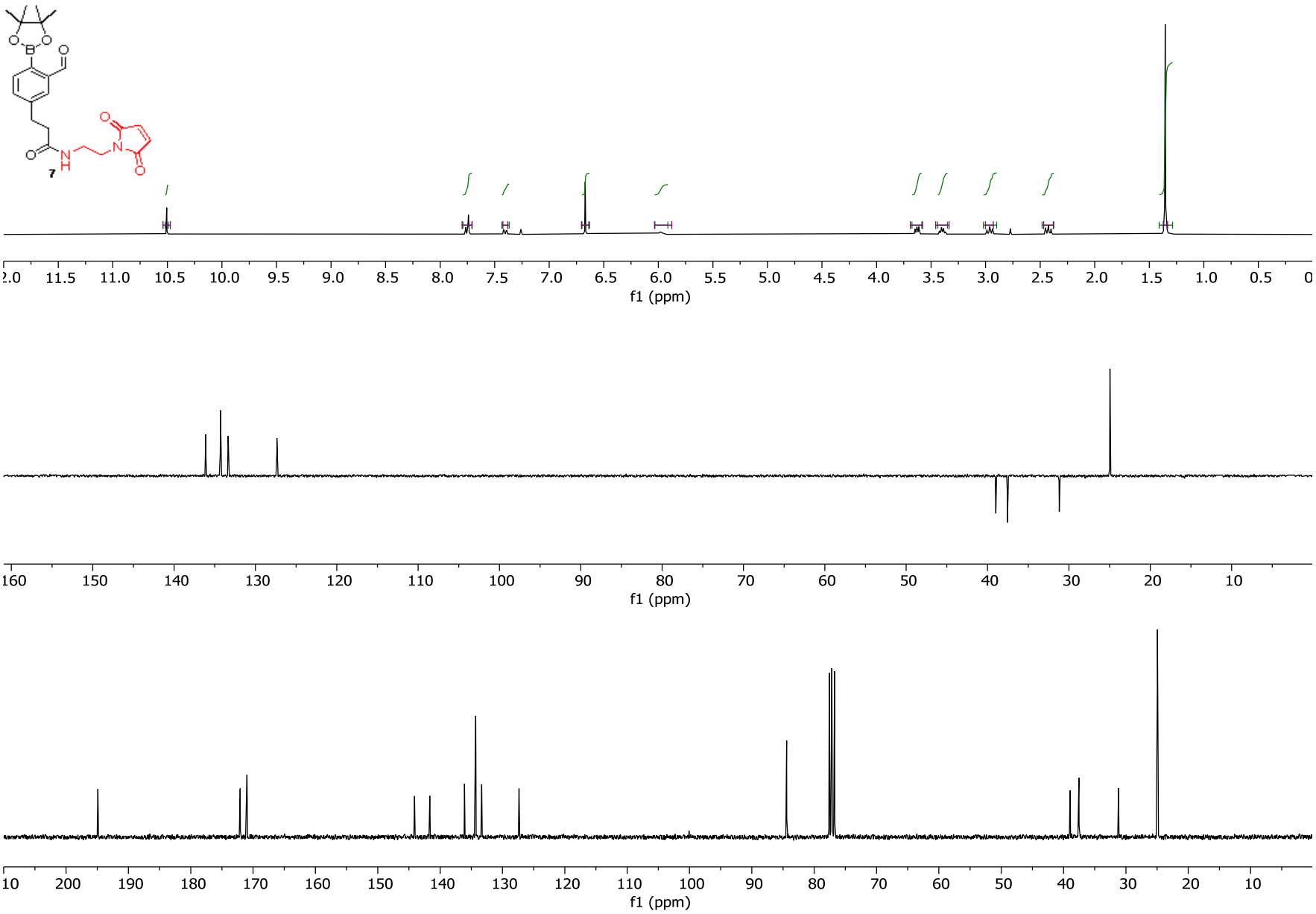
Figure S 57 –.**A** – HRMS ESI$^+$-MS spectrum of the peak at 14.9 min (**10d**) in the for reaction **with** 100 equiv. of Cys; **B** – HRMS ESI$^+$-MS spectrum of the peak at 14.9 min (**10d**) in the for reaction **without** 100 equiv. of Cys; **C** – Zoom of the HRMS-ESI$^+$ peak for **10d^{2+}** in the for reaction **with** 100 equiv. of Cys. **D** – Zoom of the HRMS-ESI$^+$ peak for **10d^{2+}** in the for reaction **without** 100 equiv. of Cys. The data demonstrate that the peak could not correspond to the exchange product between hydrazine and cysteine to form a thiazolidine.

5. NMR spectra


OTf
O
O
O
f1 (ppm)
f1 (ppm)
f1 (ppm)


5
10.5
10.0
9.5
9.0
8.5
8.0
7.5
7.0
6.5
6.0
5.5
5.0
4.5
4.0
3.5
3.0
2.5
2.0
1.5
1.0
0.5
0
f1 (ppm)
160
150
140
130
120
110
100
90
80
70
60
50
40
30
20
10
0
f1 (ppm)
200
190
180
170
160
150
140
130
120
110
100
90
80
70
60
50
40
30
20
10
f1 (ppm)


O
B
O
O
O
OH
6
f1 (ppm)
f1 (ppm)
f1 (ppm)


7
f1 (ppm)
f1 (ppm)
f1 (ppm)