Design of ultrasensitive probes for human neutrophil elastase through hybrid combinatorial substrate library profiling

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Supplemental Section

Kinetic analysis of individual substrates

To validate the contribution of subsites preferences we synthesized individual substrates and determined specificity constants (k_{cat}/K_m).

In the P2 position we selected the "best" unnatural amino acids Oic, Nle(O-Bzl), Lys(2-Cl-Z), dhPro and hCit, the "best" natural amino acid Pro, and one of the "worst" amino acid Asp. Ac-Ala-Ala-Oic-Ala-ACC was about two and a half times better than the reference substrate. Ac-Ala-Ala-hCit-Ala-ACC catalytic efficiency was almost equal to the reference substrate, while Ac-Ala-Ala-dhPro-Ala-ACC had a k_{cat}/K_m lower by 50% than the reference substrate. Due to a problem with precipitation of Ac-Ala-Ala-Nle(O-Bzl)-Ala-ACC and Ac-Ala-Ala-Lys(2-Cl-Z)-Ala-ACC, we did not determine kinetic parameters for these. Ac-Ala-Ala-Asp-Ala-ACC was not cleaved by HNE in our assay.

Table S1. Kinetic analysis of tetrapeptide substrates of the general formula Ac-Ala-Ala-P2-Ala-ACC, where P2 is a natural or unnatural amino acid selected for validation after HyCoSuL screening. Data represent the mean S.D. of three or more experiments. NH – no hydrolysis.

	$k_{cat}/K_m[M^{-1}s^{-1}]$
Ac-Ala-Ala-**Pro**-Ala-ACC	989 ± 36
Ac-Ala-Ala-**hCit**-Ala-ACC	999 ± 25
Ac-Ala-Ala-**Oic**-Ala-ACC	2408 ± 132
Ac-Ala-Ala-**dhPro**-Ala-ACC	498 ± 99
Ac-Ala-Ala-**Asp**-Ala-ACC	NH

Analysis of the P3 position confirmed that the $Met(O)_2$ derivative (Ac-Ala-$Met(O)_2$-Pro-Ala-ACC) is “best”. Its catalytic efficiency was around 22-fold higher than for the sequence with “best” natural amino acid Ac-Ala-Gln-Pro-Ala-ACC and the reference substrate Ac-Ala-Ala-Pro-Ala-ACC. Ac-Ala-$Phe(F_5)$-Pro-Ala-ACC was around five times better and Ac-Ala-Glu(O-Me)-Pro-Ala-ACC and Ac-Ala-Cha-Pro-Ala-ACC two times better than the reference substrate. Ac-Ala-Gly-Pro-Ala-ACC was not cleaved by HNE confirming library screening data.

Table S2. Kinetic analysis of tetrapeptide substrates of the general formula Ac-Ala-P3-Pro-Ala-ACC, where P3 is a natural or unnatural amino acid selected for validation after HyCoSuL screening. Data represent the mean S.D. of three or more experiments. NH – no hydrolysis.

	k_{cat}/K_m [$M^{-1}s^{-1}$]
Ac-Ala-**Ala**-Pro-Ala-ACC	989 ± 36
Ac-Ala-**Gln**-Pro-Ala-ACC	1044 ± 21.3
Ac-Ala-**Cha**-Pro-Ala-ACC	1789 ± 316.4
Ac-Ala-**Glu(OMe)**-Pro-Ala-ACC	2167 ± 34.2
Ac-Ala-**$Met(O)_2$**-Pro-Ala-ACC	22296 ± 997
Ac-Ala-**$Phe(F_5)$**-Pro-Ala-ACC	4750 ± 316
Ac-Ala-**Gly**-Pro-Ala-ACC	NH

P4 position analysis with individual substrates demonstrated that Ac-Bpa-Ala-Pro-Ala-ACC, Ac-Nle(O-Bzl)-Ala-Pro-Ala-ACC and Ac-Oic-Ala-Pro-Ala-ACC are the most preferred in this position and they are twenty five, nineteen and fourteen times better cleaved by HNE than the reference substrate. Ac-Cha-Ala-Pro-Ala-ACC and Ac-Thr(Bzl)-Ala-Pro-Ala-ACC were around six and three times better than the reference sequence. Arg, which was the

"best" natural amino acid in the P4 position, was 2.7 times better compared to Ac-Ala-Ala-Pro-Ala-ACC, while Ac-Thr-Ala-Pro-Val-ACC was not cleaved by HNE.

Table S3. Kinetic analysis of tetrapeptide substrates of general formula Ac-P4-Ala-Pro-Ala-ACC, where P4 is a natural or unnatural amino acid selected for validation after HyCoSuL screening. Data represent the mean S.D. of three or more experiments. NH – no hydrolysis.

	k_{cat}/K_m $[M^{-1}s^{-1}]$
Ac-**Ala**-Ala-Pro-Ala-ACC	989 ± 36
Ac-**Thr**-Ala-Pro-Ala-ACC	NH
Ac-**Arg**-Ala-Pro-Ala-ACC	2734 ± 120
Ac-**Nle(O-Bzl)**-Ala-Pro-Ala-ACC	19153 ± 661
Ac-**Cha**-Ala-Pro-Ala-ACC	6656 ± 198
Ac-**Thr(Bzl)**-Ala-Pro-Ala-ACC	2905 ± 146
Ac-**Bpa**-Ala-Pro-Ala-ACC	25028 ± 200
Ac-**Oic**-Ala-Pro-Ala-ACC	14287 ± 846

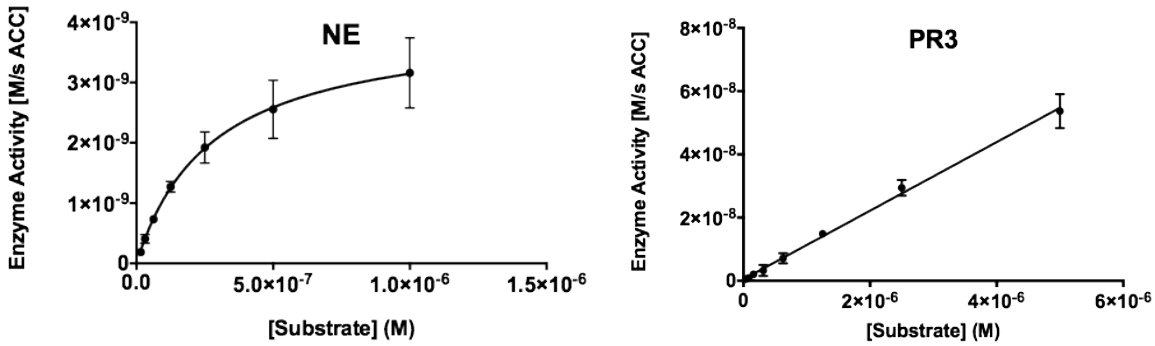
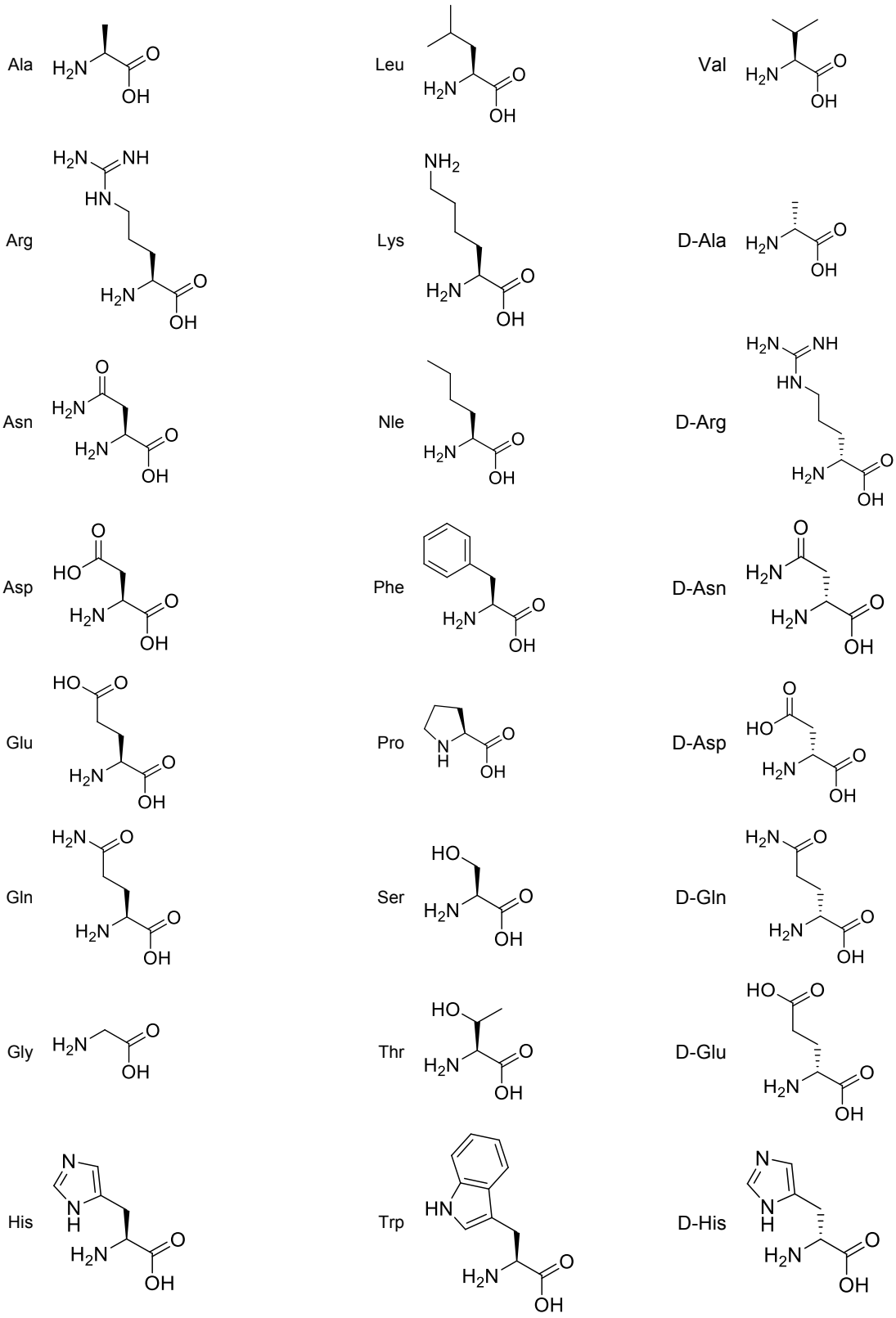
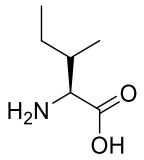
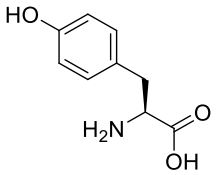


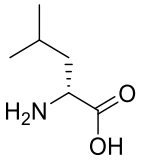
Fig. S1. Substrate/velocity plots of NE and PR3 assayed with the NE optimal substrate Ac-Nle(O-Bzl)-Met(O)$_2$-Oic-Abu-ACC. Final concentrations of 0.3 nM NE or 200 nM PR3 were equilibrated for 10 min in 50 mM Hepes, 0.1 M NaCl, 0.1% (vol/vol) igepal CA-630, pH 7.4 at 37°C, followed by addition of substrate equilibrated in the same buffer at 37°C at the indicated final concentrations. Non-linear fitting was applied to the NE plot, but since no saturation was observed for PR3, a linear fit was applied to this plot. The derived kinetic constants are listed in Table 2 of the main manuscript.

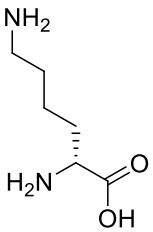
Fig. S2. Amino acids structures (without protecting groups) used in the design of HyCoSuL.

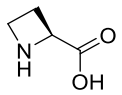


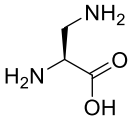
Ile 

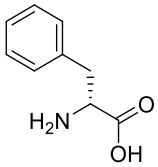
Tyr 

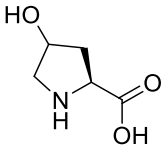
D-Leu 

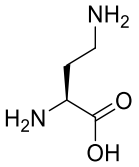
D-Lys 

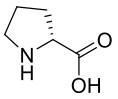
Aze 

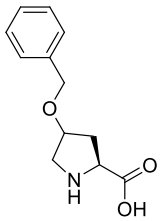
Dap 

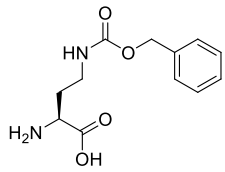
D-Phe 

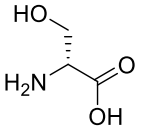
hPro 

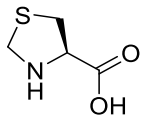
Dab 

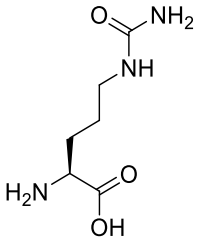
D-Pro 

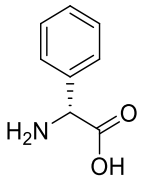
Hyp(O-Bzl) 

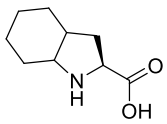
Dab(Z) 

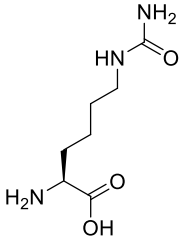
D-Ser 

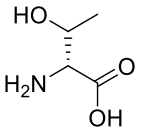
Thz 

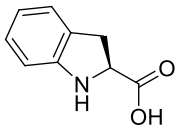
Cit 

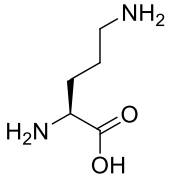
D-Phg 

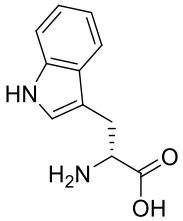
Oic 

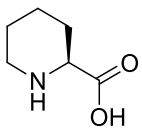
hCit 

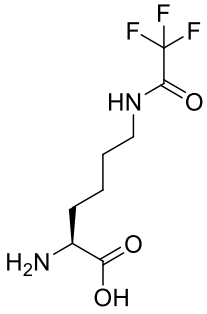
D-Thr 

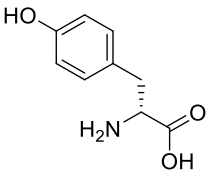
Idc 

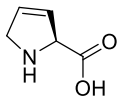
Orn 

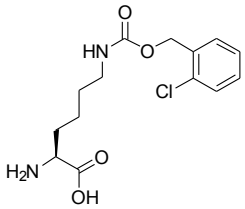
D-Trp 

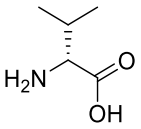
Pip 

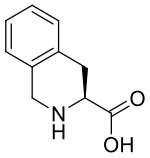
Lys(TFA) 

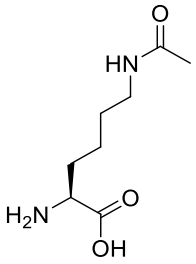
D-Tyr 

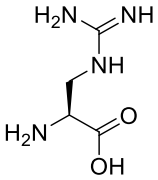
dhPro 

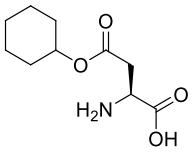
Lys(2-Cl-Z) 

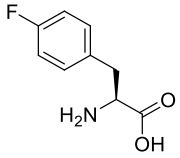
D-Val 

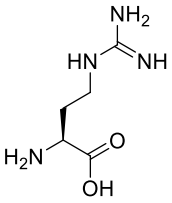
Tic 

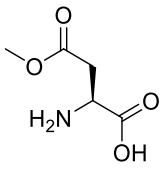
Lys(Ac) 

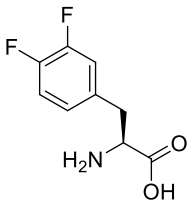
Agp 

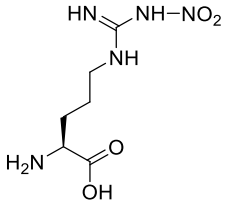
Asp(O-cHx) 

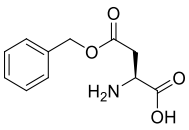
Phe(4-F) 

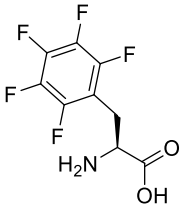
Agb 

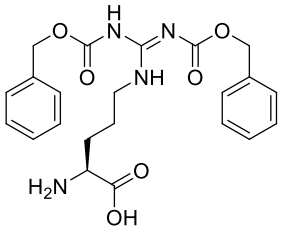
Asp(O-Me) 

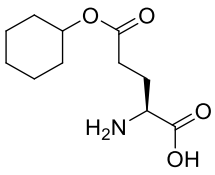
Phe(3,4-F_2) 

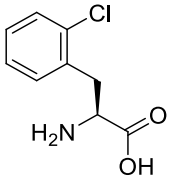
Arg(NO_2) 

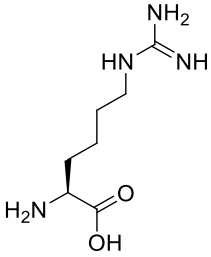
Asp(O-Bzl) 

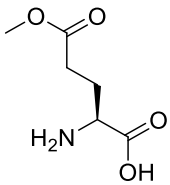
Phe(F_5) 

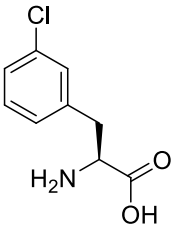
Arg(Z_2) 

Glu(O-cHx) 

Phe(2-Cl) 

hArg 

Glu(O-Me) 

Phe(3-Cl) 

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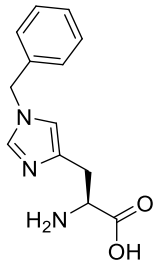

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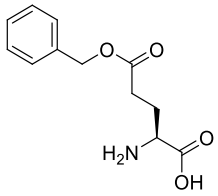

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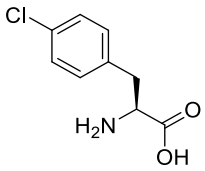

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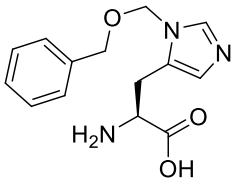

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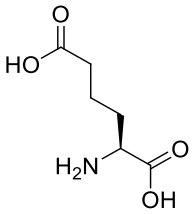

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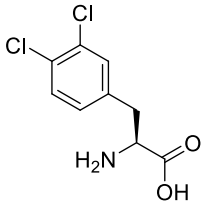

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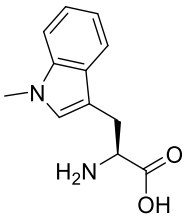

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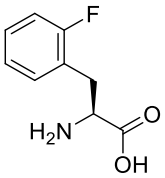

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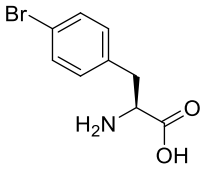

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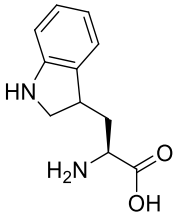

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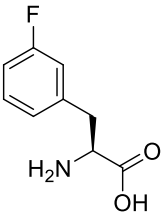

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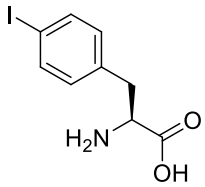

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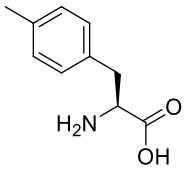

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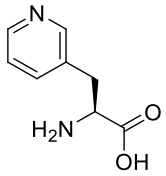

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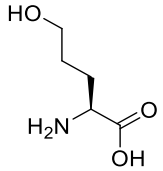

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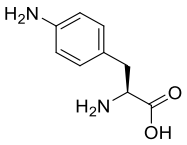

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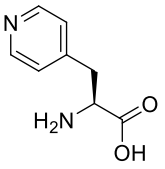
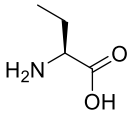


Abu



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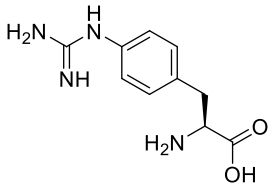

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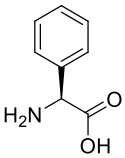

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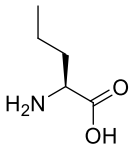

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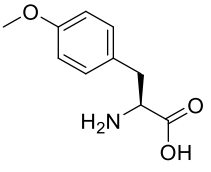

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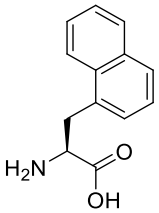

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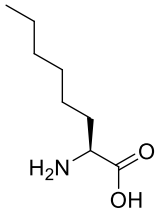

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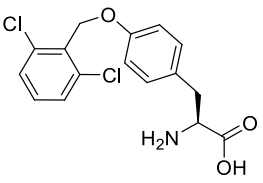

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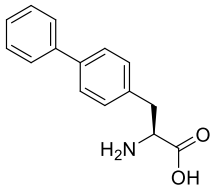
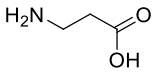


β-Ala



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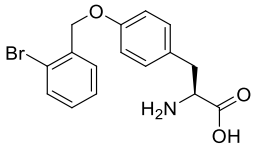

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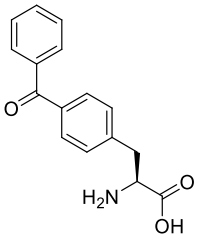

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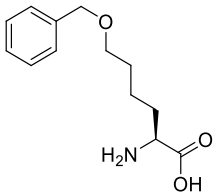

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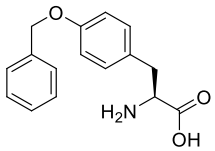

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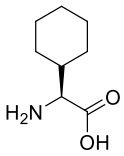

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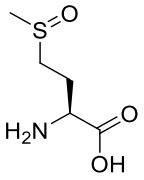

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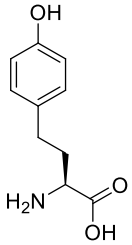

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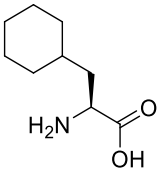

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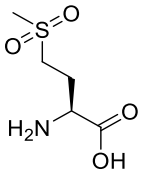

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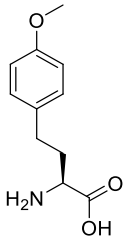

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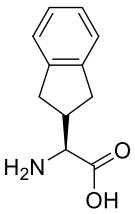

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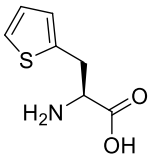

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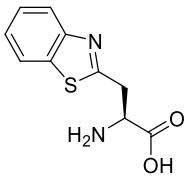

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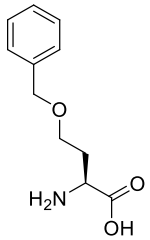

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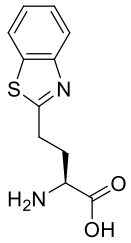

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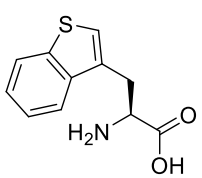
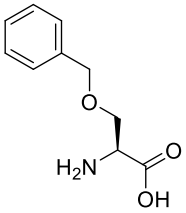
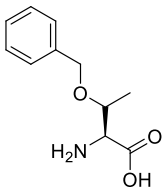
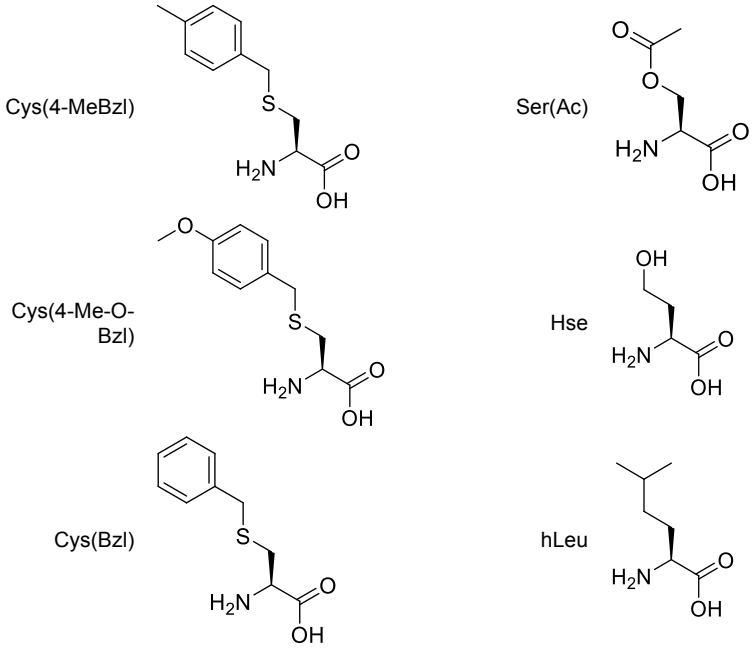


Ser(O-Bzl)



Thr(O-Bzl)





End of Fig. S2.

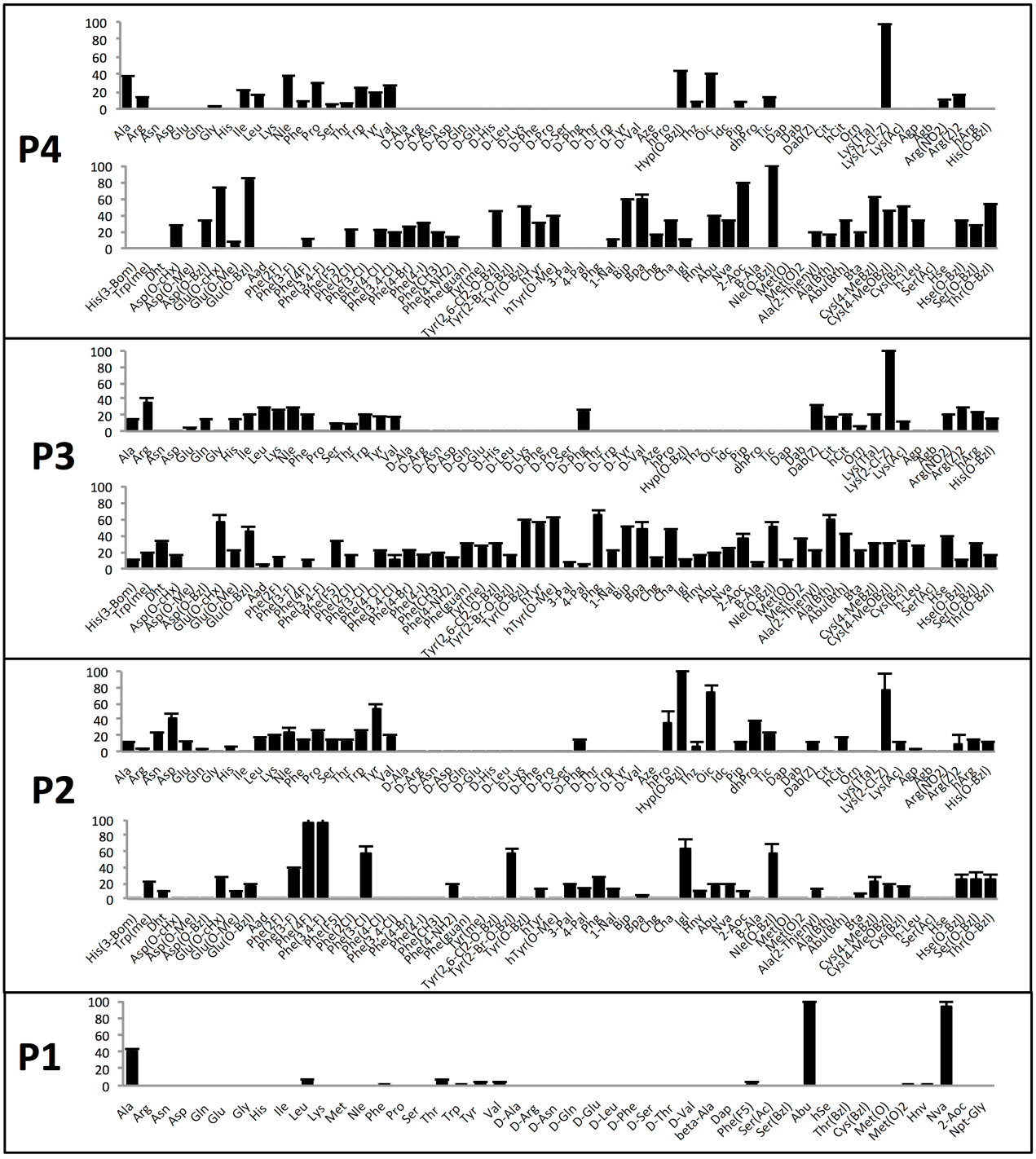


Fig. S3. Preferences at the P4–P1 subsites of proteinase 3. The P4, subsite preference of proteinase 3 was determined using combinatorial substrate libraries of the general structure Ac-P4-X-X-Ala-ACC, where P4 represents a natural or unnatural amino acid and X represents an isokinetic mixture of natural amino acids (Cys and Met were omitted because of a problem with oxidation; Nle was used instead of Met). The P3 and P2 preferences were determined in a similar way. The P1 subsite preference was determined using individual substrate libraries of the general structure Ac-Ala-Ala-Pro-P1-ACC, where P1 is a natural or unnatural amino acid. Abbreviated amino acid names (SI Appendix, Fig. S2) are shown on the x axis. The y axis displays the average relative activity expressed as a percentage of the best amino acid. Error bars represent the S.D. (n = 3).

METHODS

Materials

Chemicals were obtained from commercial suppliers and used without further purification. To libraries and individual substrates synthesis, Rink amide RA resin (particle size 200-300 mesh, loading 0.48 mmol/g, Iris Biotech GmbH), Fmoc-protected amino acids (purity > 99% Iris Biotech GmbH, Bachem, Creosalus), *N*-hydroxybenzotriazole (HOBt purity > 98%, Creosalus), diisopropylcarbodiimide (DICI, peptide grade, Iris Biotech GmbH), *N,N*-diisopropylethylamine (DIPEA, peptide grade, VWR), *O*-benzotriazole-*N,N,N',N'*-tetramethyl-uronium-hexafluoro-phosphate (HBTU, peptide grade, Iris Biotech GmbH), 2-(1-H-7-azabenzotriazol-1-yl)-1,1,3,3-tetramethyl uranium hexafluorophosphate methanaminium (HATU, peptide grade, Iris Biotech GmbH), 2,4,6-trimethylpyridine (collidine, peptide grade, Sigma Aldrich), *N,N'*-dimethylformamide (DMF, peptide grade, J.T. Baker), dichloromethane (DCM, pure for analysis, POCh), methanol (MeOH, pure for analysis, POCh), acetonitrile (ACN, HPLC gradient grade, J.T. Baker), diethyl ether (Et_2O, pure for analysis, POCh), piperidine (PIP, purity > 99%, Sigma Aldrich), trifluoroacetic acid (TFA, purity 99%, Iris Biotech GmbH), triisopropylsilane (TIPS, purity 99%, Sigma Aldrich), acetic acid (AcOH, purity >98%, POCh), phosphorus pentoxide (P_2O_5, purity 98%, POCh) were used. Individual substrates and ABP were purified by HPLC on a Waters M600 solvent delivery module with a Waters M2489 detector system using a semi-preparative Waters Spherisorb S10ODS2 column. The solvent composition was as follows: phase A (water/0.1% TFA, vol/vol) and phase B (ACN/H_2O 80%/20% (vol/vol) with 0.1% of TFA (vol/vol)). The purity of each individual compound was confirmed by analytical HPLC using a Waters Spherisorb S5ODS2 column. Finally, the molecular weight of each substrate was confirmed by high-resolution mass spectrometry on High Resolution Mass Spectrometer WATERS LCT Premier XE

with Electrospray ionization (ESI) and Time of Flight (TOF). Data files for these studies can be found in the Supplementary Data section. NMR spectrum was obtained on Bruker Avance DRX 300. Human neutrophil elastase (NE) used for library screening was purchased from Biocentrum, Kraków, Poland. NE and human neutrophil proteinase 3 (PR3) used for optimal substrate validation and activity based probe characterizations were purchased from Athens Research Technology, Athens, Georgia, USA.

Synthesis of NH_2-ACC-resin

Preparation of ACC was carried out as described previously according to Maly et al. (1) 1eq (5.76 mmol, 12 g) of Rink AM resin was added to glass reaction vessel and stirred gently once per 10 minutes in DCM for 1h, then filtered and washed 3 times with DMF. Fmoc protecting group was removed using 20% piperidine (vol/vol) in DMF (5 min, 5 min, 25 min), filtered each time and washed with DMF (six times). Next, 2.25 eq of Fmoc-ACC-OH (12.96 mmol, 5.7 g) was preactivated with 2.25 eq HOBt (12.96 mmol, 1.9 g) and 2.25 eq DICI (12.96 mmol, 1.7 ml) in DMF and mixture was added to the resin. Reaction was stirred gently for 24 h at room temperature. Resin was washed four times with DMF and reaction was repeated using 1.5 eq of above reagents to improve yield of ACC coupling to the resin. After reaction, resin was washed with DMF and Fmoc group was removed using 20% piperidine (vol/vol) in DMF (5 min, 5 min, 25 min), filtered and washed with DMF (six times).

Synthesis of H_2N-P1-ACC-resin exemplified in detail with H_2N-Ala-ACC-resin

2.5 eq Fmoc-Ala-OH (14.4 mmol, 4.5 g) with 2.5 eq HATU (14.4 mmol, 5.5 g), 2.5 eq collidine (14.4 mmol, 1.9 ml) in DMF were activated for two minutes and added to

filter cannula with 1eq (5.76 mmol) NH_2-ACC-resin and reaction was carried out for 24 h. Next, resin was washed four times with DMF and reaction was repeated using 1.5 eq of above reagents. After washing with DMF, Fmoc protecting group was removed using 20% piperidine (vol/vol) in DMF (5 min, 5 min, 25 min). Resin was additional washed with DCM (3 times) and MeOH (3 times) and dried over P_2O_5.

Synthesis of P1 library and individual optimized substrates

2.5 eq Fmoc-P2-OH was preactivated with 2.5 eq HOBt and 2.5 eq DICI in DMF and added to cartridge with 1 eq NH_2-P1-ACC-resin (P1-natural or unnatural amino acid) and followed by gentle agitation for 3 h. Then filtered and washed with DMF (six times). Fmoc-protecting group was removed using 20% piperidine (vol/vol) in DMF (5 min, 5 min, 25 min). Ninhydrin test was carried out each time after coupling and deprotection. A solution of 2.5eq Fmoc-P3-OH, 2.5 eq HOBt and 2.5 eq DICI in DMF was added to the resin and the slurry was agitated for 3 h. After removal of the solution, the resin was washed with DMF (six times), and coupling and deprotection of Fmoc-P4-OH was carried in identical conditions like P2 position. N-terminus was protected with acetyl group using 5 eq AcOH, 5 eq HBTU and 5 eq DIPEA in DMF as previous described. After solvent removal, the resin was washed with DMF (six times), DCM (three times) and MeOH (three times) dried over P_2O_5 and cleaved from the resin with a mixture of TFA : TIPS : H_2O (v/v/v 95/2.5/2.5). The crude product was purified by HPLC and lyophilized. Its purity was confirmed by analytical HPLC. Each optimized substrate was analyzed using HRMS. P1 library and optimized substrates were dissolved to 20 mM concentration and stored in -80^{o} C until use.

Synthesis of P2, P3 and P4 HyCoSuL sub-library exemplified in detail with the P2 sub-library

General: Each library consisted of 120 sub-libraries in which each of the natural amino acids (minus cysteine and methionine) and pool of unnatural amino acids were used at a defined position and an isokinetic mixture of 19 amino acids (without cysteine and methionine, with norleucine) was coupled. Equivalents ratio of amino acids in the isokinetic mixture was created based on their reported coupling rates. 5-fold excess (over resin load) of mixture was used. For fixed positions, a 2.5 equimolar excess (eq) of single amino acid was used. Reactions were carried out with coupling reagent DIC and HOBt.

P2 coupling: The synthesis of library was performed using MultiChem 48-wells synthesis apparatus (FlexChem). 1 eq of dry NH_2-Ala-ACC-resin (0.04 mmol, 80 mg) was added to wells of reaction apparatus and stirred gently for 1h in DCM and then washed with DMF (four times). In separated vials, 2.5 eq (0.1 mmol) Fmoc-P2-OH were preactivated with 2.5eq HOBt (0.1 mmol, 15 mg) and 2.5 eq DICI (0.1 mmol, 14 µl) in DMF. Next, preactivated amino acids were added to wells of the apparatus containing NH_2-Ala-ACC-resin, followed by 3 h agitation. Then reaction mixture was filtered, washed with DMF (4 times) and ninhydrin test was carried out. Subsequently Fmoc-protecting group was removed using 20% piperidine (vol/vol) in DMF (5 min, 5 min, 25 min).

P3 and P4 positions coupling: An isokinetic mixture for 48 portions was prepared of 18 Fmoc-protected natural amino acids (omitting cysteine) and with norleucine mimicking methionine (19 amino acids in total). 5 eq of isokinetic mixture, 5 eq HOBt (9.22 mmol, 1.4 g), 5 eq DICI (9.22 mmol, 1.2 ml) were diluted in DMF and preactivated for 3 min. Then to each of 48 wells containing 1 eq of H_2N-P2-Ala-ACC-resin, the activated isokinetic mixture was added. After 3h agitation, the slurry was

filtered and washed with DMF (4 times). Ninhydrin test was carried out and Fmoc-protecting group was removed using 20% piperidine (vol/vol) in DMF (5 min, 5 min, 25 min). The same procedure was applied for the remaining substrates. In the same manner the isokinetic mixture was added to sample the P4 position.

N-terminus acetylation: To a vial were added 5eq AcOH (9.22 mmol, 527 µl), 5eq HBTU (9.22 mmol, 3.5 g) and 5eq DIPEA (9.22 mmol, 1.60 ml) in DMF. After stirring for 1 minute, the mixture was added to each well in the reaction apparatus, containing NH_2-X-X-P2-Ala-ACC-resin, and followed by gentle agitation for 30 min. Next, the resin was washed with DMF (5 times), DCM (3 times) and MeOH (3 times) and dried over P_2O_5.

Cleavage from the resin: After completing of the synthesis, peptides were cleaved from resin by treating for 2 h (shaking once per 15 minutes) with a mixture of TFA : TIPS : H_2O (v/v/v 95/2.5/2.5; 2 ml/well). The solution from each well was collected separately and the resin was washed once by a portion of cleavage solution (1 ml) and diethyl ether (Et_2O, 14 ml) was added. After precipitation (0.5 h in -20° C) the mixture was centrifuged and washed again with Et_2O (4 ml). After centrifugation, a white precipitate was dissolved in ACN:H_2O (v/v, 1/1.4 ml) and lyophilized. The final products were dissolved in DMSO to a concentration of 20 mM and used without further purification.

Synthesis of P3 and P4 sub-libraries

In the same manner as described above, P3 and P4 sub-libraries were synthesized by coupling fixed amino acid residues to P3 (isokinetic mixture to P2 and P4) and respectively P4 position (isokinetic mixture to P2 and P3).

Characterization of neutrophil elastase specificity using HyCoSuL

Standard enzyme assay conditions for P2, P3 and P4 positions were as follows: reaction volume, 100 µl; total final substrate mixture concentration, 50 µM; enzyme concentration, 43 nM. Assay conditions for P1 library screening: reaction volume, 100 µl; total final substrate concentration, 5 µM; enzyme concentration, 43 nM. The total assay time was 90 min, and the linear portion of the progress curve (generally 15–30 min) was used to calculate velocity. All experiments were repeated at least three times and the results presented are means. The difference between individual values was in every case less than 15%. Analysis of the results was based on total RFUs (relative fluorescence units) for each sub-library, setting the highest value to 100% and adjusting the other results accordingly.

Activity Based Probe synthesis

Synthesis of HBr × NH_2-$Abu^P(OPh)_2$: In round bottom flask fitted with stirring bar 1 eq of triphenyl phosphate (1.2 mmol, 340 µl), 1.1 eq of propionealdehyde (1.3 mmol, 95 µl), 1.1 eq (1.3 mmol, 200 mg) of benzyl carbamate were dissolved in ACN. Then reaction mixture was cooled in ice bath and HBF_4 (10% in Et_2O, (vol/vol)) was added. After 4h of stirring, solvent was evaporated. Obtained crude oil was dissolved in MeOH and left for crystallization at -20° C. White precipitate was filtered under reduced pressure and dried at room temperature. Deprotection of carboxybenzyl group was carried out in 30% HBr/AcOH (vol/vol) for 1 h. After evaporation using rotary vapour, crude oil was treated with Et_2O, what resulted in formation of white precipitate of HBr × NH_2-$Abu^P(OPh)_2$, which was filtered off, washed using Et_2O and used in next step without purification. 25 % yield. ^{1}H NMR (600 MHz, DMSO-d6) δ 9,097-9,026 (m, 2H), 7,26-7.10 (m, 10H), 4,12-3,89 (m,1H), 1,85-1,63 (m, 2H), 1,08 (t, J=7,98, 3H); ^{31}P NMR (243 MHz, DMSO-d6) δ 13.83 (s, 56%), δ12.18 (s, 44%).

Biot-Peg(4)-Nle(O-Bzl)-Met(O)$_2$-Oic-OH: In filter cannula to chlorotrityl resin (100 mg, substitution 1.6 mmol/g), dry DCM was added. After 1h resin was filtered and washed twice with DCM. 3eq Fmoc-Oic-OH (0.48 mmol, 187.92 mg), 6 eq DIPEA (0.96 mmol) in dry DCM were added to the resin and gently stirred for 3h. Then reaction mixture was filtered and washed with DMF (four times). Fmoc-protecting group was removed using 20% piperidine (vol/vol) in DMF (5 min, 5 min, 25 min) and ninhydrin test was carried out. Next, 2.5 eq Fmoc-Met(O)$_2$-OH (0.4 mmol, 161 mg) was preactivated in separated vial with 2.5 eq HOBt (0.4 mmol, 60 mg) and 2.5 eq DICI (0.4 mmol, 51.99 ul) in DMF and added to the resin. Using same procedure Fmoc-Nle(O-Bzl)-OH and Fmoc-PEG(4)-OH residues were coupled. Finally, to filter cannula with NH$_2$-PEG(4)-Nle(O-Bzl)-Met(O)$_2$-Oic-resin, preactivated 5 eq biotin (0.8 mmol, 195 mg) with 5eq HOBt (0.8 mmol, 120 mg), 5eq DICI (0.8 mmol, 104 µl) in DMSO:DMF solution was added. The mixture was gently stirred 48 h and then filtered and washed with DMSO (3 times), DMF (3 times), DCM (3times) and MeOH (3 times) and dried over P_2O_5. Product was cleaved from resin by treating with solution of TFE : AcOH : DCM (v:v:v, 0.5:1:3.5). Filtrate was collected and concentrated by evaporation. Then Et_2O was added and product was left in -20^o C for 0.5 h for precipitation. After centrifugation white precipitate was washed with Et_2O and dry at room temperature. Crude product was used in further synthesis without purification.

Biot-Peg(4)-Nle(O-Bzl)-Met(O)$_2$-Oic-Abu^P(OPh)$_2$ synthesis (PK101): To a round bottom flask fitted with a stirring bar, 1 eq Biot-Peg(4)-Nle(O-Bzl)-Met(O)$_2$-Oic-OH, 1 eq HBr × AbuP(OPh)$_2$, 1 eq HBTU, 2 eq DIPEA in DMSO:DMF (1:9) were added.

After 48h solvents were removed under reduced pressure. Crude material was dissolved in DMSO and purified by HPLC. Purity was confirmed by analytical HPLC and HRMS. 15.8 % yield. HRMS *(m/z):* [MNa^+] calcd for $C_{63}H_{92}N_7NaO_{16}PS_2$, 1320.5677; found, 1320.5699.

Enzymatic kinetic studies

These were performed using a fMax fluorimeter (Molecular Devices) operating in the kinetic mode in 96-well Corning® plates. The excitation wavelength was 355 nm, and the emission wavelength was 460 nm with a cutoff of 455 nm. For HyCoSuL screening the assay buffer (0.1M Hepes, 0.5M NaCl, pH 7.5) was made at 23° C, and assays were performed at 37°C. NE was preincubated for 10 min at 37°C before adding to the wells containing substrate.

Comparison of Kinetic Parameters (k_{cat}, K_m, and k_{cat}/K_m) for individual substrates

Substrates were screened against NE at 37°C in the above assay buffer. Buffer was prepared at 23°C. NE solution in buffer was preincubated for 30 min at 37 °C before adding to the substrate in the wells of a 96-well plate reader operating in the kinetic mode. Total assay time 10-20 min. Releasable ACC concentrations were calculated by the hydrolysis of eight independent ACC-coupled substrate solutions at known concentration, and an average value was determined. Enzyme kinetic assay conditions were as follows: 100 µl total reaction volume, eight different substrate concentrations, and enzymes at 0.5-40 nM. Release of ACC fluorophore was monitored as above. Each experiment was repeated at least three times, and the results are presented as an average with the error bars describing the S.D. Final

substrate concentrations for k_{cat}/K_m determination ranged from 0.05 to 500 μM. Concentration of DMSO in the assay was less than 2% (vol/vol). Calculation of kinetic parameters was carried out using GraphPadPrism software.

Kinetic Parameters (k_{cat}, K_m, and k_{cat}/K_m) for the optimal substrate for NE and PR3

At this point we switched from the buffer used in the library screening (0.1M Hepes, 0.5M NaCl, pH 7.5) to 50 mM Hepes, 0.1 M NaCl, 0.1% (vol/vol) igepal CA-630, pH 7.4, in which PR3 and NE are both optimally stable at 37°C. Kinetic parameters were determined as above, using a range of 0.05-5 μM Ac-Nle(O-Bzl)-Met$(O)_2$-Oic-Abu-ACC, 0.01-1 nM NE or 10-100 nM PR3. Each experiment was repeated three times, and the results are presented as an average with the error bars describing the S.D.

Determination of Inhibition Kinetics (k_{obs}/I) of NE and PR3 by the activity based probe PK101.

Under pseudo-first order conditions, a constant amount of NE (0.3 nM) or PR3 (200 nM) was mixed with different concentrations of PK101 (at least 5-fold excess over enzyme) and 10 mM substrate. Substrate and inhibitor were equilibrated together at 37°C in 50 mM Hepes, 0.1 M NaCl, 0.1% igepal (vol/vol) CA-630, pH 7.4, and the reaction was started by adding NE or PR3 pre-equilibrated in the same buffer at 37°C. Fluorescent product time courses were captured as described above. The amount of product formation (P) proceeds at an initial velocity (V) and is inhibited over time (t) at a rate of (k_a): $P=V/k_a*(1-e^{-k_a*t})+C$. The value of k_a – the apparent rate of inhibition in the presence of substrate - at each inhibitor concentration was determined by curve fitting using non-linear regression analysis and GraphPadPrism

software, and this was plotted against the inhibitor concentration I to yield the apparent second order rate constant for inhibition, k_a/I. The absolute value of the second order rate constant k_{obs}/I was then calculated as k_{obs}/I =k_a/I *(1+[S]/K_m), where the K_m for NE in the assays had been determined to be 0.28 µM (see Results). Since we could not saturate PR3 with substrate k_{obs}/I =k_a/I for this protease : inhibitor pair.

NE and PR3 reactivity with PK101 revealed by dotblotting and Western *analysis.*

PK101 binding to NE and PR3 was analyzed by dotblotting, and SDS-PAGE followed by Western blotting. The indicated concentrations of PK101 were allowed to react with 100 nM NE or PR3 for 20 min at 37°C in 50 mM Hepes, 0.1 M NaCl, 0.1% igepal (vol/vol) CA-630, pH 7.4. Samples were boiled for 10 min in SDS-PAGE sample buffer, and divided for analysis by dotblot, or separation in 8-18% SDS-PAGE gels followed by Western blot transfer to Amersham Hybond nitrocellulose membrane (GE Healthcare). Membranes were blocked with 2% BSA in TBST (vol/vol), probed Streptavidin-IRDye800CW at a 1:5000 dilution for 30 min at room temperature, and analyzed using an Odyssey fluorescence imaging system (Li-Cor Biosciences).

Preparation and analysis of granule extracts

To stimulate the release of granule proteins into the extracellular medium, neutrophils (2 x 10^7/mL) were pre-treated with 10 µM DPI and 5 µg/mL cytochalsin B for 10 min at 37°C then stimulated with 100 nM fMLP for 20 min at 37°C. Cells were pelleted (500 *g*, 5 min) and the supernatant collected and stored at -80°C until required. For SDS-PAGE followed by Western blotting, granule extracts (5 µL) were incubated with

PK101 (10 nM, 20 nM & 50 nM) for 20 min at 37°C in a total reaction mix of 50 µL diluted in NE buffer (0.05 M Hepes; 0.1 M NaCl; 0.05% (vol/vol) NP40; pH 7.4). Where appropriate granule extracts were pre-treated with MeOSuc-AAPV-CH_2Cl (10 µM) for 30 min before addition of PK101. After incubation with PK101, samples were boiled in SDS sample reducing buffer for 5 min to stop the reaction then run on a 12% SDS-PAGE gel followed by transfer to PVDF. Membranes were blocked with 3% (vol/vol) BSA overnight at 4°C, washed then incubated with streptavidin-HRP (1/1000) for 2 h at room temperature. After further washing, PK101 binding was detected by ECL and images taken using a chemidoc (UVITEC Cambridge).

Neutrophil isolation, NET formation and immunofluorescence.

Heparinized peripheral blood was taken from healthy volunteers with informed consent. Neutrophils were isolated by dextran sedimentation followed by Ficoll-Paque PLUS centrifugation. Contaminating red blood cells were removed by H_2O lysis and the remaining granulocytes were suspended in RPMI (without phenol red) containing 2% (vol/vol) fetal calf serum. To induce NETs, neutrophils (5×10^5) were treated with phorbol myristate acetate (20 nM) then seeded onto sterile glass coverslips in 24-well cell culture plates. Cells were incubated at 37°C in 5% (vol/vol) CO_2 for 2.5 h then treated at 37°C in 5% (vol/vol) CO_2 for 20 min with 40 nM PK101. After washing briefly in PBS, cells and NETs were carefully fixed with 4% (vol/vol) paraformaldehyde (20 min at room temperature), washed twice with PBS, blocked for 1 hour with 10% (w/v) BSA in PBS, then incubated with 1/1000 dilution of Alexa Fluor® 488 streptavidin (Life Technologies) for 1 hour at room temperature in 3% (w/v) PBS. NETs were stained with propidium iodide (20 µg/mL, 10 min, room temperature). For immuno=detection of total NE, after blocking with BSA, NETs were incubated with a 1/300 dilution of rabbit anti-NE (Abcam) for 1 h at 37°C then washed

and incubated with goat anti-rabbit DyLight® 594 (1/500) (Abcam) and Alexa Fluor® 488 Steptavidin (1/1000) for 1 h at 37°C. Coverslips were mounted in Fluoromount G and viewed on a Zeiss Axio Imager fluorescence microscope (Germany). Images were taken using a Photometrics KAF1400 charge-coupled device camera and AxioVision Rel. 4.6 software (Zeiss) and formatted using Adobe Photoshop.

Compounds Analysis

All individual compounds used for kinetic and biological studies were purified by HPLC on a Waters M600 solvent delivery module with a Waters M2489 detector system using Waters Spherisorb S5ODS2 column and characterized by high-resolution mass spectrometry (HRMS) using on High Resolution Mass Spectrometer WATERS LCT Premier XE with Electrospray ionization (ESI). Overall yields for the complete synthesis and HRMS data are listed below.

Ac-Ala-Ala-Pro-Ala-ACC 31.9% yield. HRMS (m/z): $[MNa]^+$ calcd for $C_{27}H_{34}N_6NaO_8$, 593.2336; found, 593.2333

Ac-Ala-Ala-hCit-Ala-ACC 39.5% yield. HRMS (m/z): $[MNa^+]$ calcd for $C_{29}H_{40}N_8NaO_9$, 667.2816; found, 667.2814

Ac-Ala-Ala-Lys(2-Cl-Z)-Ala-ACC 27.5% yield. HRMS (m/z): $[MNa^+]$ calcd for $C_{36}H_{44}ClN_7NaO_{10}$, 792.2736; found, 792.2737

Ac-Ala-Ala-Nle(O-Bzl)-Ala-ACC 31.3% yield. HRMS (m/z): $[MNa^+]$ calcd for $C_{35}H_{44}N_6NaO_9$, 715.3068; found, 715.3058

Ac-Ala-Ala-Oic-Ala-ACC 26.9% yield. HRMS (m/z): $[MNa^+]$ calcd for $C_{31}H_{40}N_6NaO_8$, 647.2806; found, 647.2805

Ac-Ala-Ala-dhPro-Ala-ACC 41.7% yield. HRMS (m/z): $[MNa^+]$ calcd for $C_{27}H_{32}N_6NaO_8$, 591.2180; found, 591.2178

Ac-Ala-Ala-Asp-Ala-ACC 28.0% yield. HRMS (m/z): $[MNa^+]$ calcd for $C_{26}H_{32}N_6NaO_{10}$, 611.2078; found, 611.2088

Ac-Ala-Gln-Pro-Ala-ACC 34.9% yield. HRMS (m/z): $[MNa^+]$ calcd for $C_{29}H_{37}N_7NaO_9$, 650.2551; found, 650.2554

Ac-Ala-Cha-Pro-Ala-ACC 39.0% yield. HRMS (m/z): $[MNa^+]$ calcd for $C_{33}H_{44}N_6NaO_8$, 675.3119; found, 675.3124

Ac-Ala-Glu(O-Me)-Pro-Ala-ACC 29.5% yield. HRMS (m/z): [MNa^+] calcd for $C_{30}H_{38}N_6NaO_{10}$, 665.2548; found, 665.2532

Ac-Ala-Met(O)$_2$-Pro-Ala-ACC 37.8% yield. HRMS (m/z): [MNa^+] calcd for $C_{29}H_{38}N_6NaO_{10}S$, 685.2268; found, 685.2260

Ac-Ala-Phe(F$_5$)-Pro-Ala-ACC 36.8% yield. HRMS (m/z): [MNa^+] calcd for $C_{33}H_{33}F_5N_6NaO_8$, 759.2178; found, 759.2166

Ac-Ala-Gly-Pro-Ala-ACC 48.5% yield. HRMS (m/z): [MNa^+] calcd for $C_{26}H_{32}N_6NaO_8$, 579.2180; found, 579.2181

Ac-Thr-Ala-Pro-Ala-ACC 39.7% yield. HRMS (m/z): [MNa^+] calcd for $C_{28}H_{36}N_6NaO_9$, 623.2441; found, 623.2431.

Ac-Arg-Ala-Pro-Ala-ACC 34.9% yield. HRMS (m/z): [MH^+] calcd for $C_{30}H_{42}N_9O_8$, 656.3157; found, 656.3157

Ac-Nle(O-Bzl)-Ala-Pro-Ala-ACC 32.2% yield. HRMS (m/z): [MNa^+] calcd for $C_{37}H_{46}N_6NaO_9$, 741.3224; found, 741.3226

Ac-Cha-Ala-Pro-Ala-ACC 26.0% yield. HRMS (m/z): [MNa^+] calcd for $C_{33}H_{44}N_6NaO_8$, 675.3119; found, 675.3103

Ac-Thr(Bzl)-Ala-Pro-Ala-ACC 39.0% yield. HRMS (m/z): [MNa^+] calcd for $C_{35}H_{42}N_6NaO_9$, 713.2911; found, 713.2918

Ac-Bpa-Ala-Pro-Ala-ACC 26.8% yield. HRMS *(m/z):* [MNa^+] calcd for $C_{40}H_{42}N_6NaO_9$, 773.2911; found, 773.2919

Ac-Nle-Ala-Pro-Ala-ACC 46.4% yield. HRMS *(m/z):* [MNa^+] calcd for $C_{30}H_{40}N_6NaO_8$, 635.2806; found, 635.2811

Ac-Oic-Ala-Pro-Ala-ACC 27.9% yield. HRMS *(m/z):* [MNa^+] calcd for $C_{33}H_{42}N_6NaO_8$, 673.2962; found, 673.2980

Ac-Ala-Ala-Pro-Val-ACC 19.1% yield. HRMS *(m/z):* [MNa^+] calcd for $C_{29}H_{38}N_6NaO_8$, 621.2649; found, 621.2549

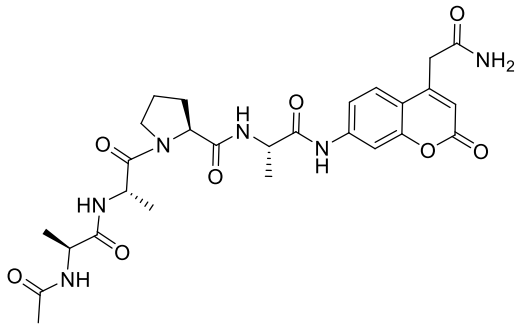
Ac-Ala-Ala-Pro-Abu-ACC 32% yield. HRMS *(m/z):* [MNa^+] calcd for $C_{28}H_{36}N_6NaO_8$, 607.2493; found, 607.2473

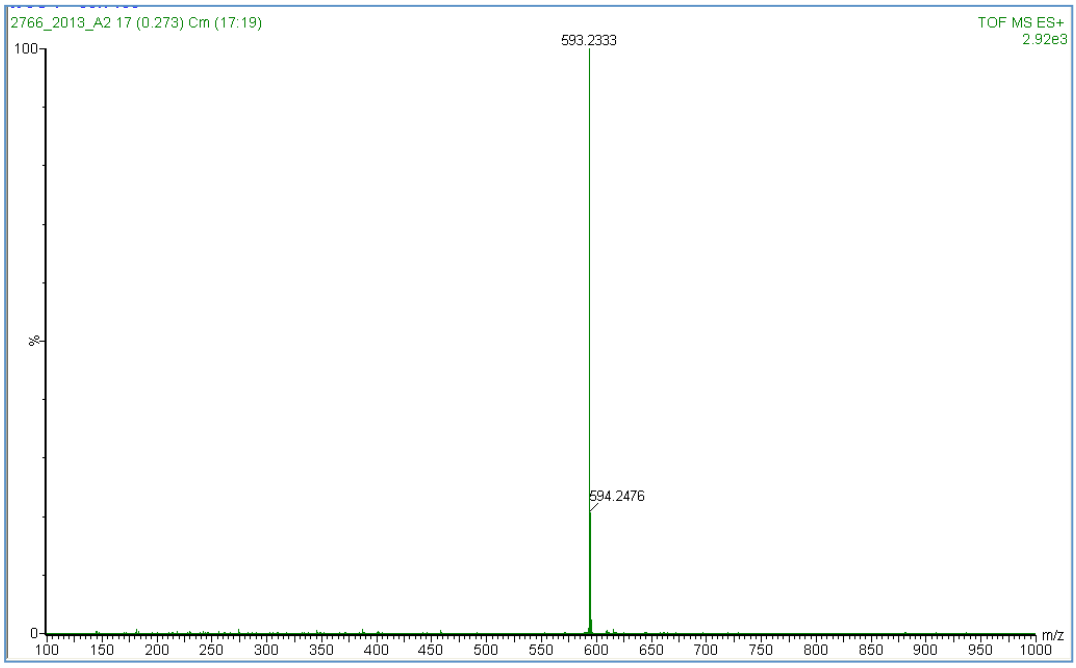
Ac-Nle(O-Bzl)-Met(O)$_2$-Oic-Abu-ACC 50.6% yield. *(m/z):* [MNa^+] calcd for $C_{44}H_{58}N_6NaO_{11}S$, 901.3783; found, 901.3768

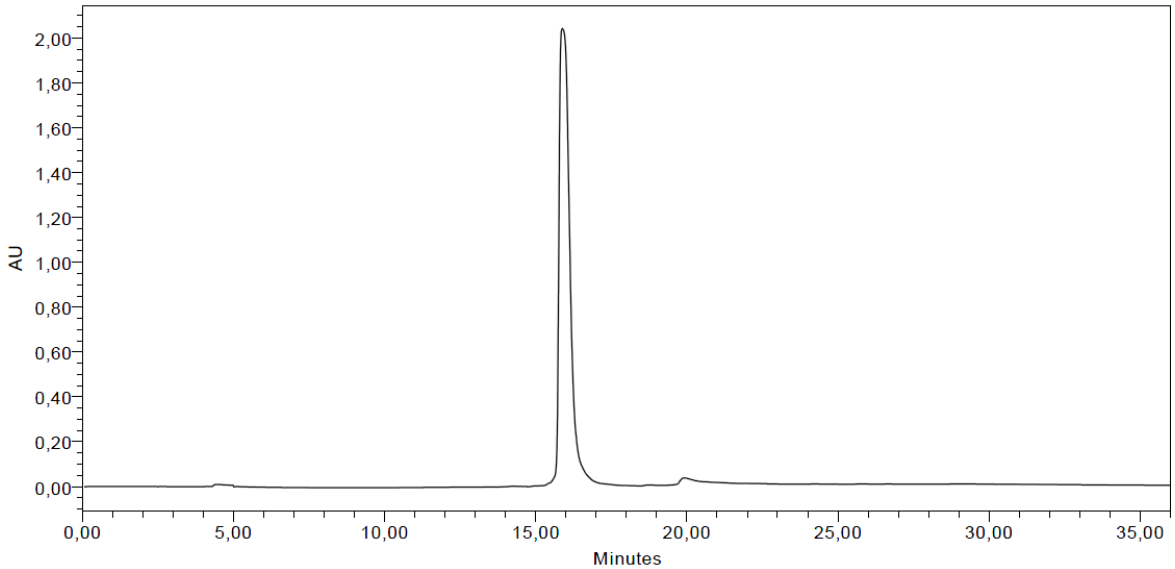
PK101 15.8 % yield. HRMS *(m/z):* [MNa^+] calcd for $C_{63}H_{92}N_7NaO_{16}PS_2$, 1320.5677; found, 1320.5699

HRMS and HPLC:

Ac-Ala-Ala-P-Ala-ACC







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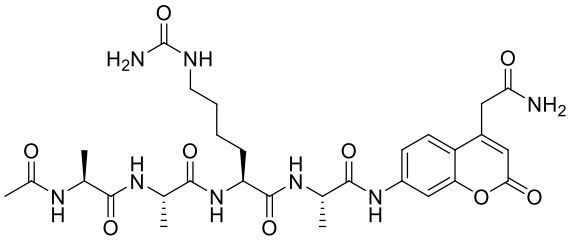

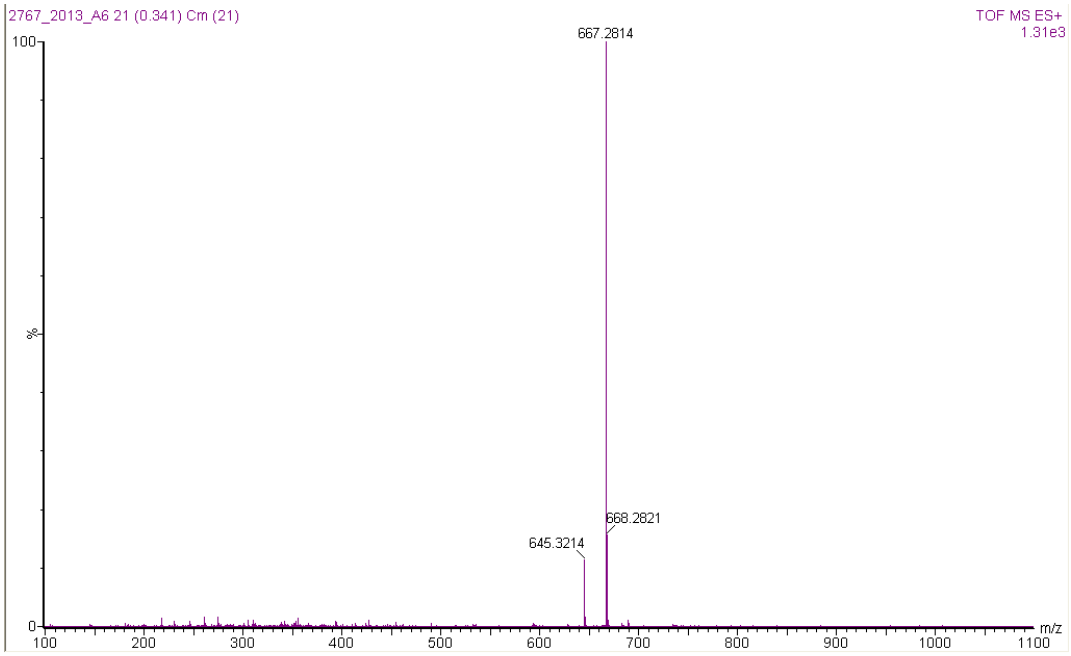

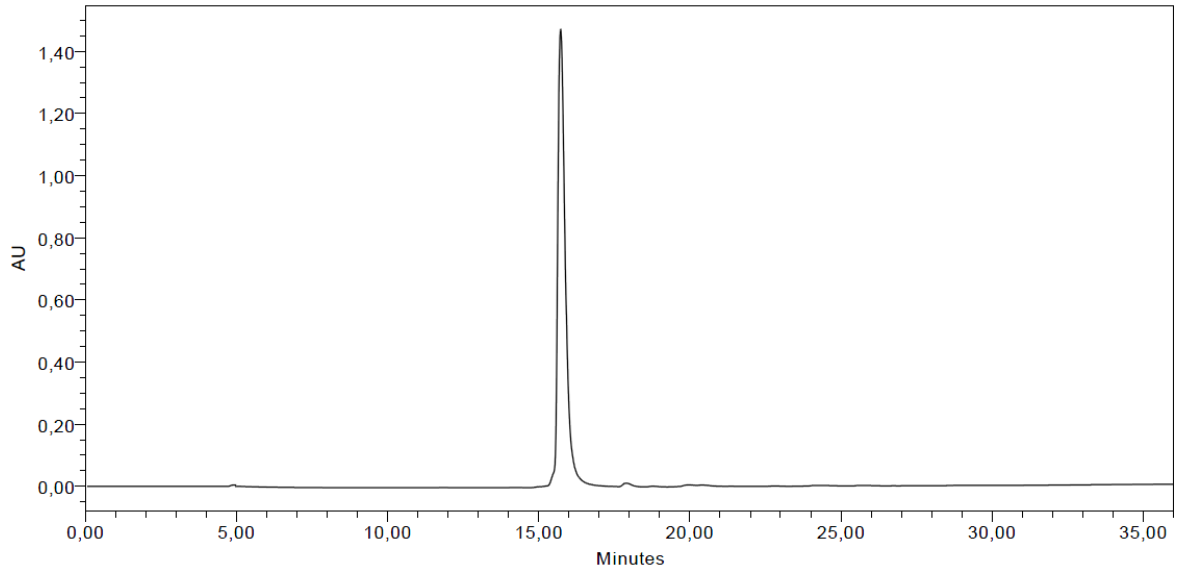

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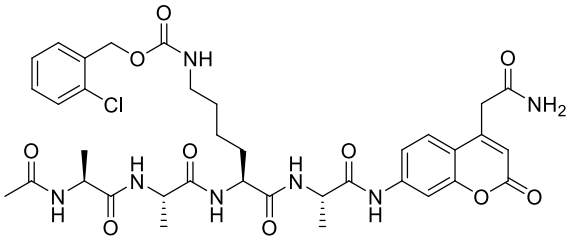

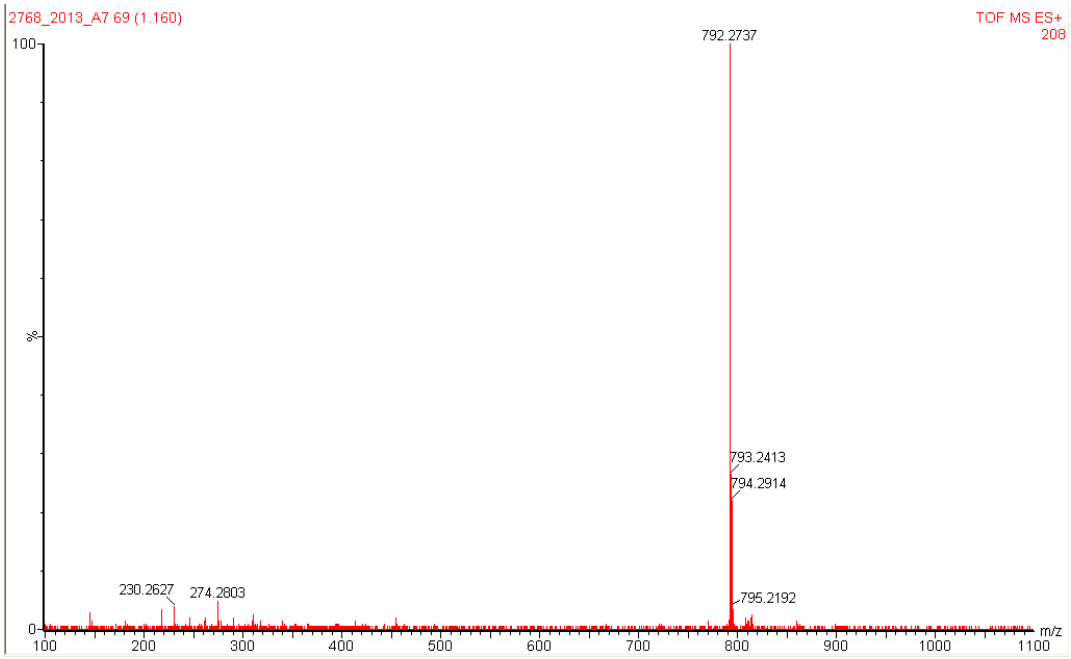

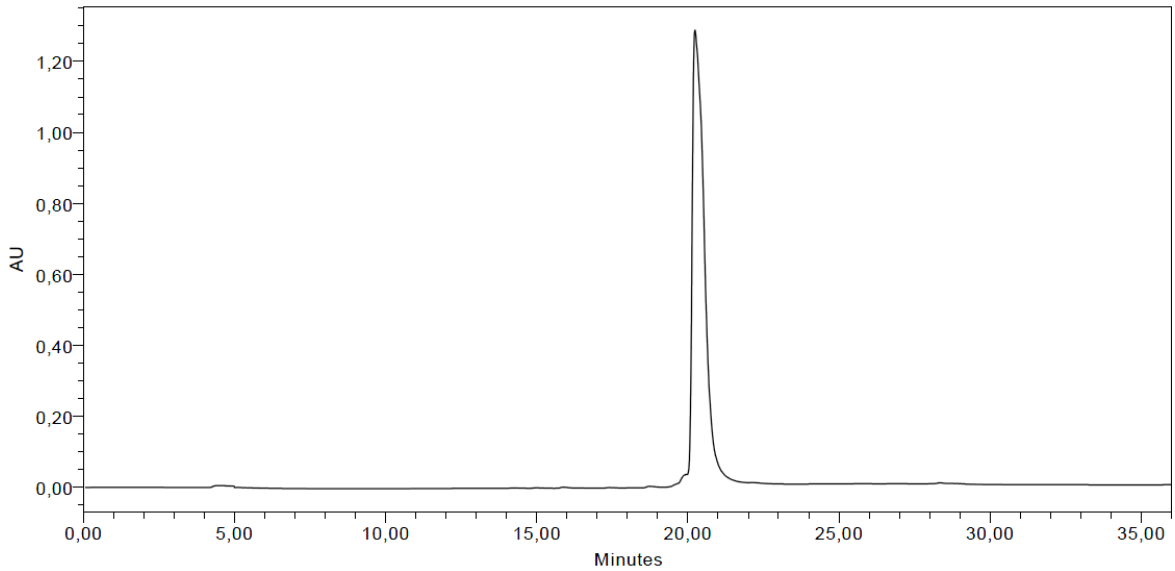

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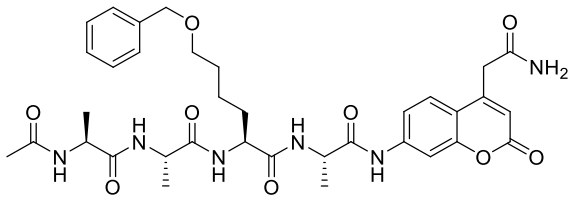
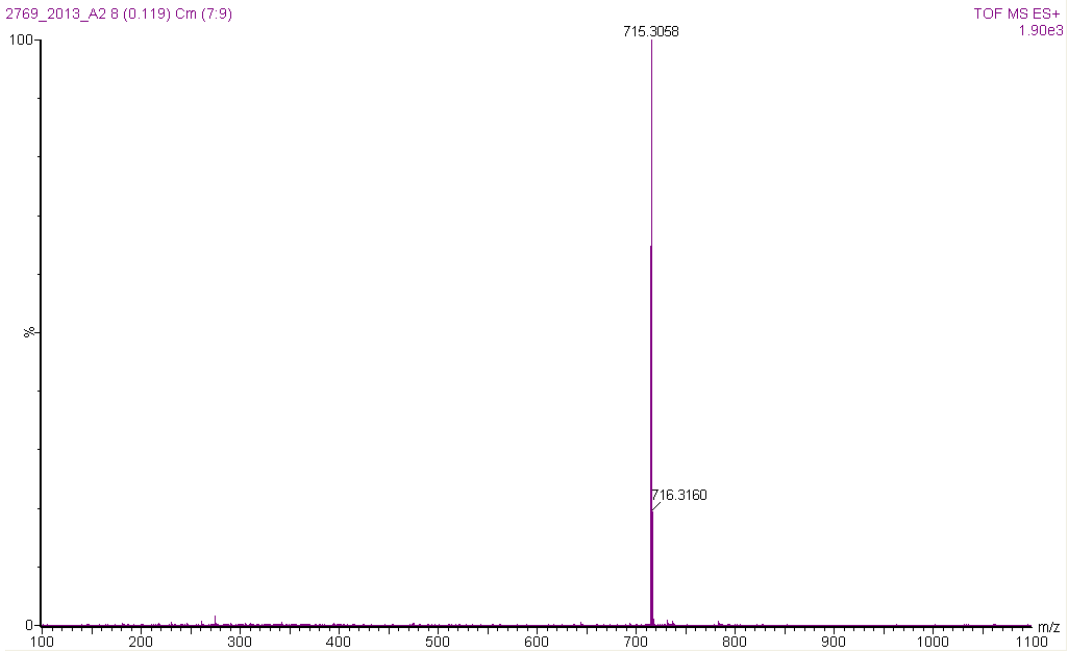
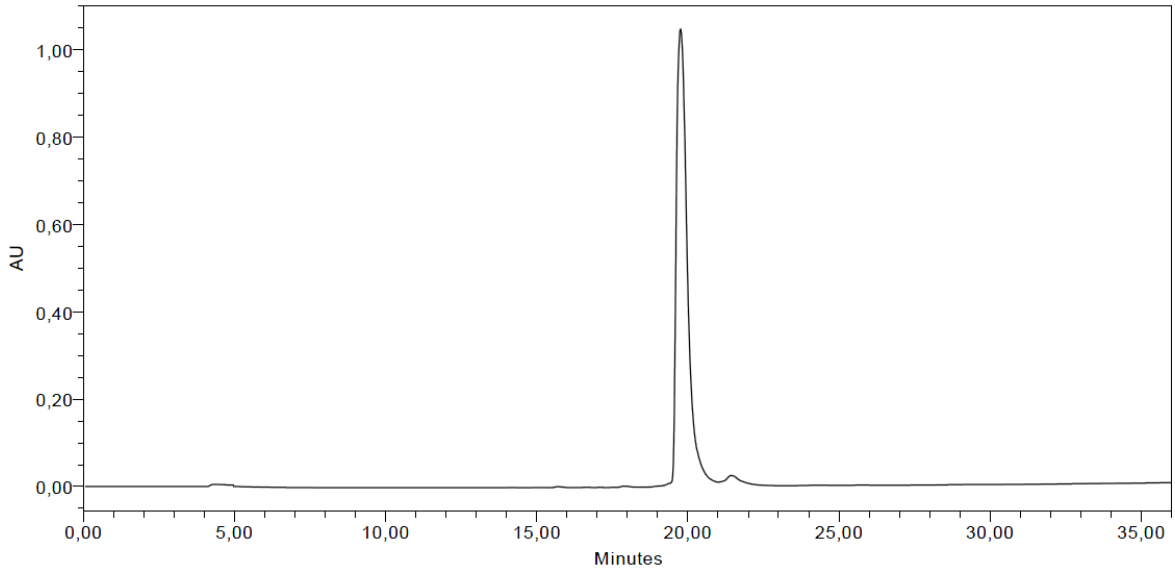
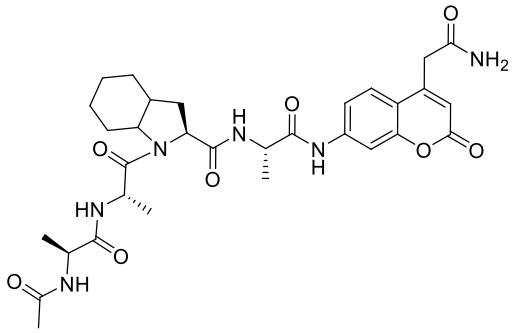
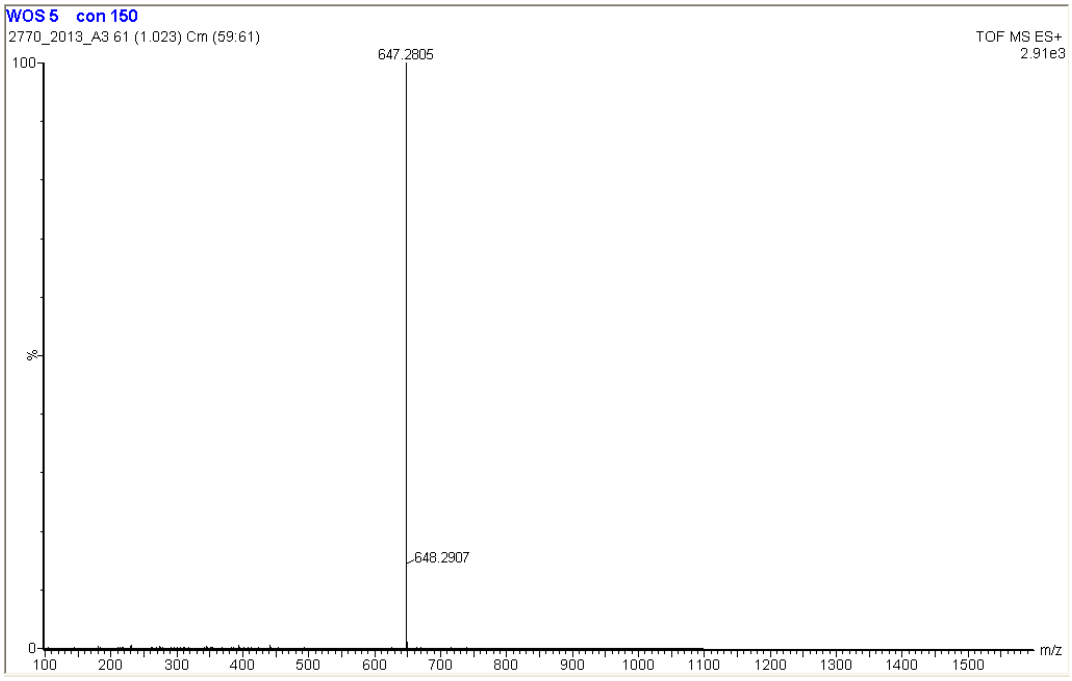


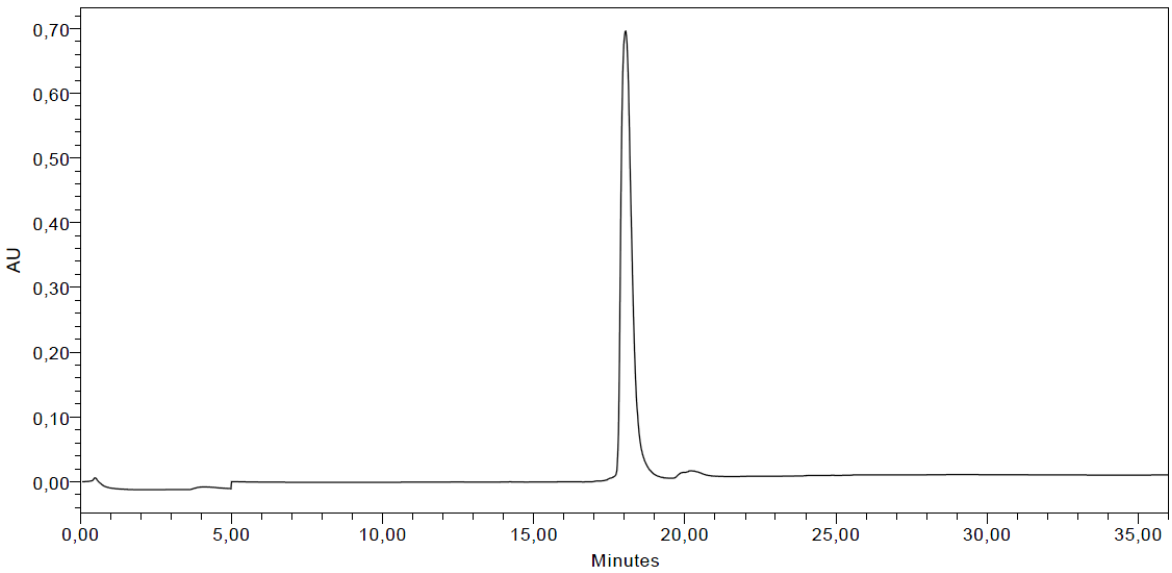




Ac-Ala-Ala-Oic-Ala-ACC







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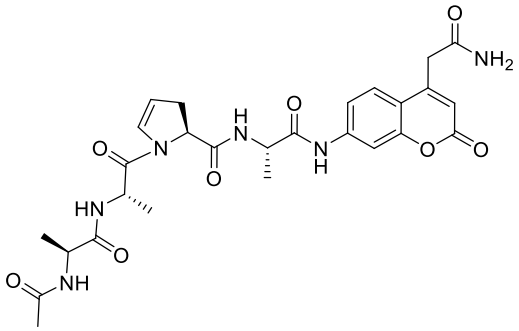

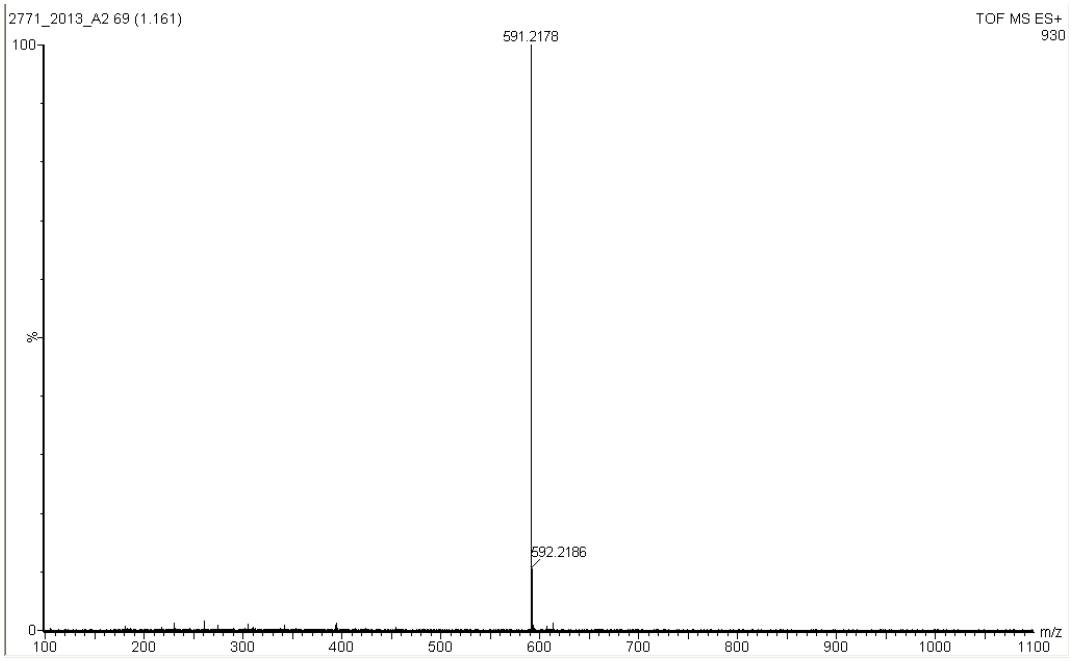

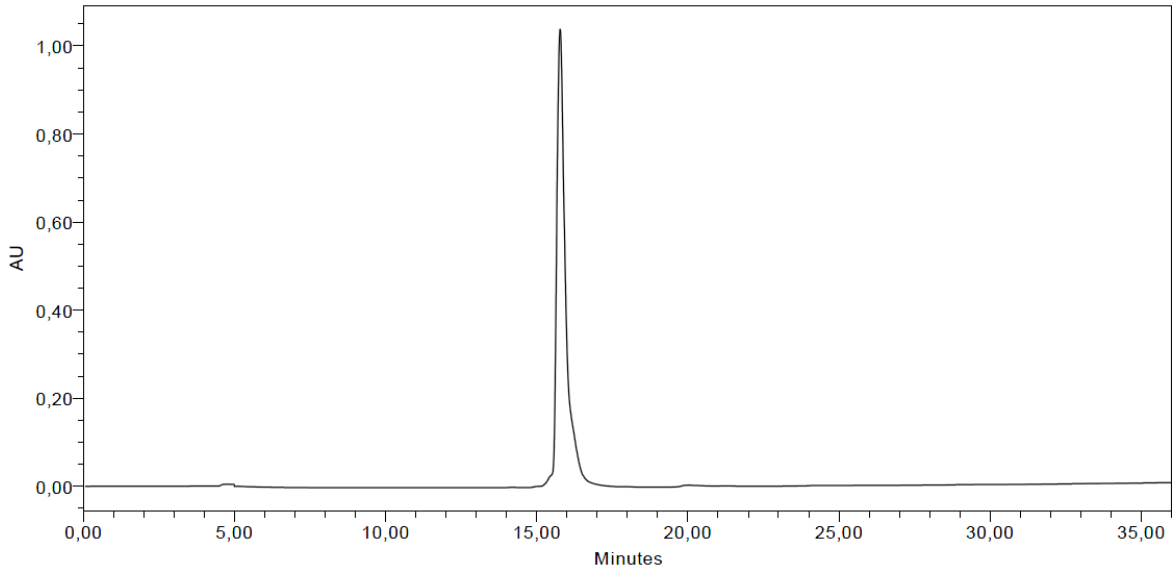

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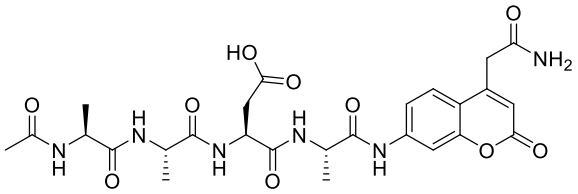
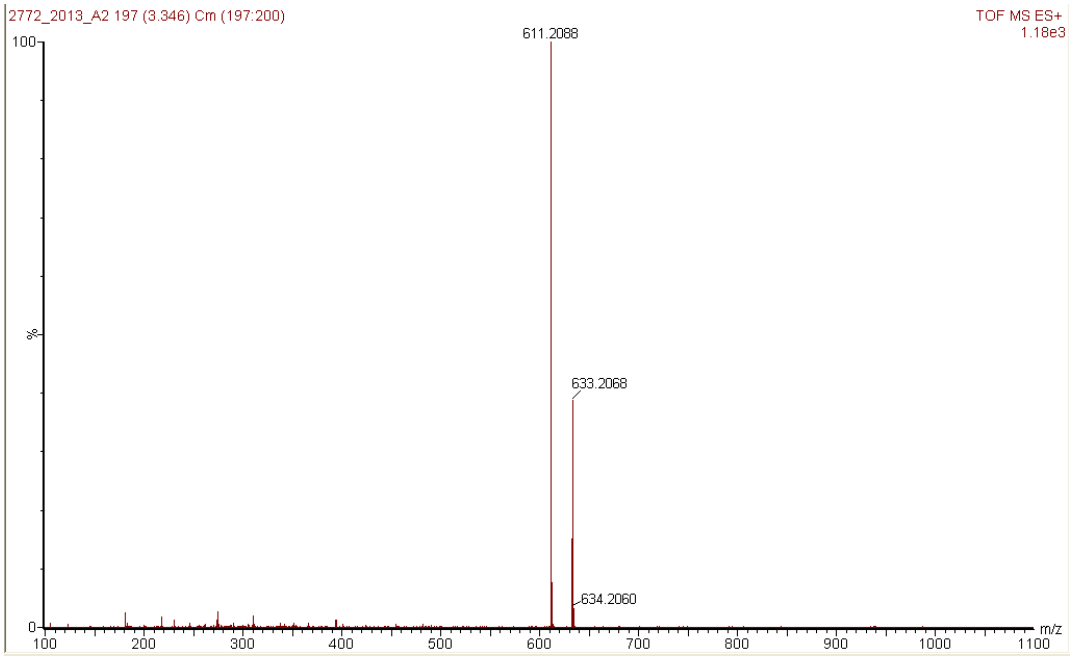
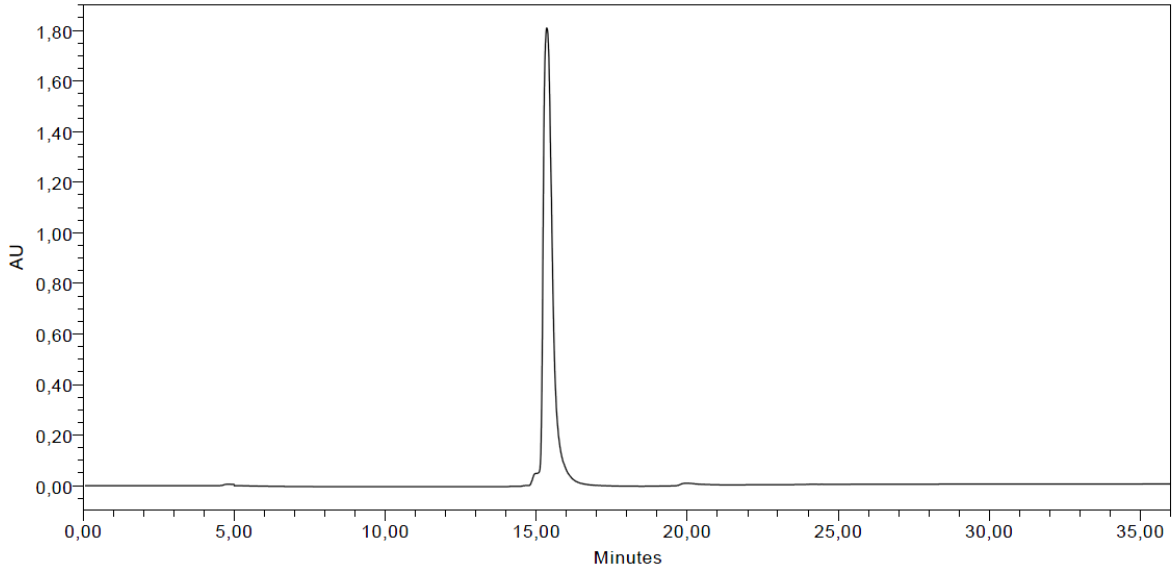
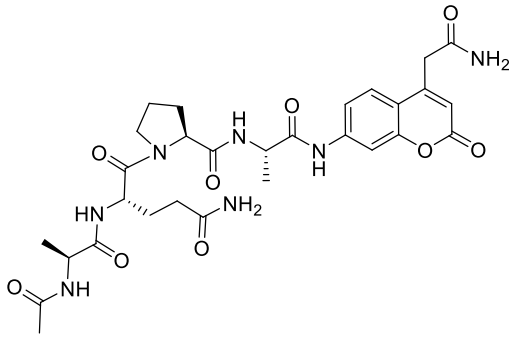
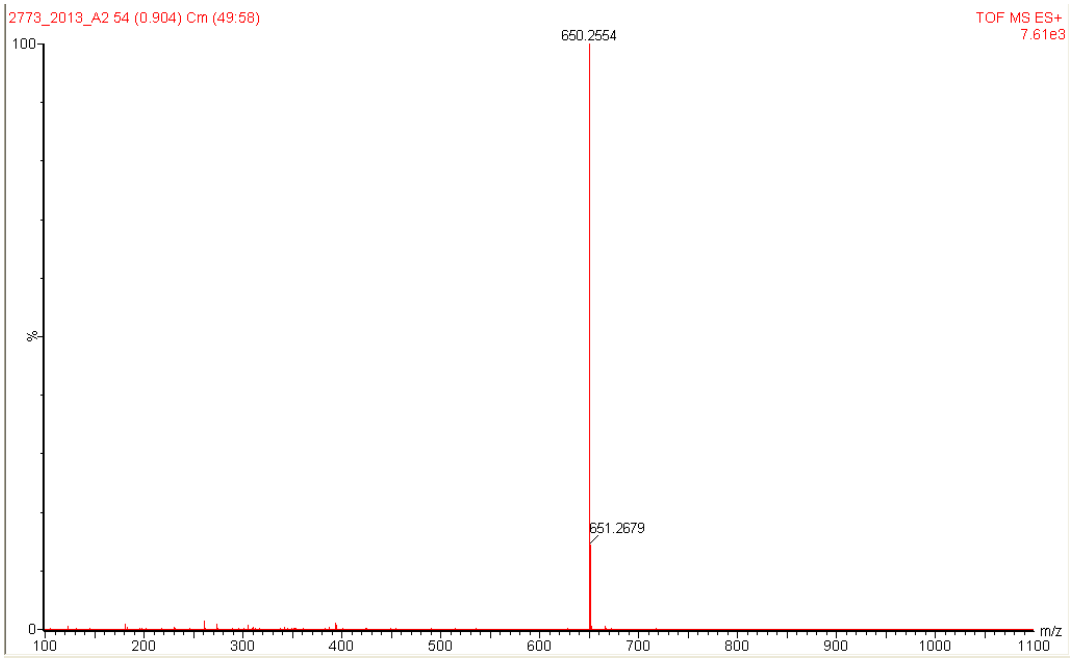


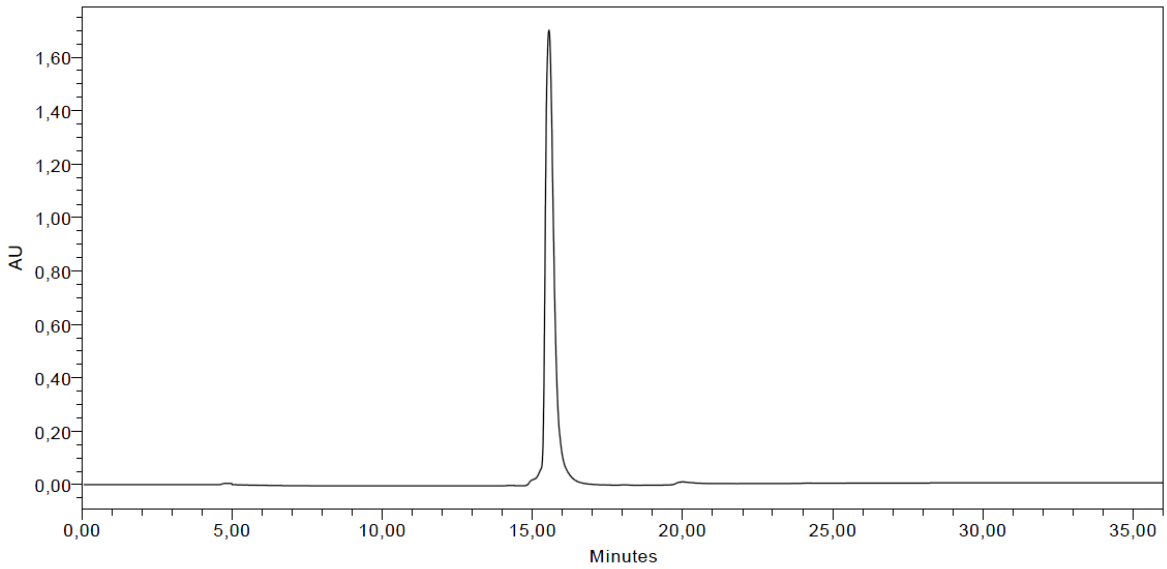




Ac-Ala-Gln-Pro-Ala-ACC







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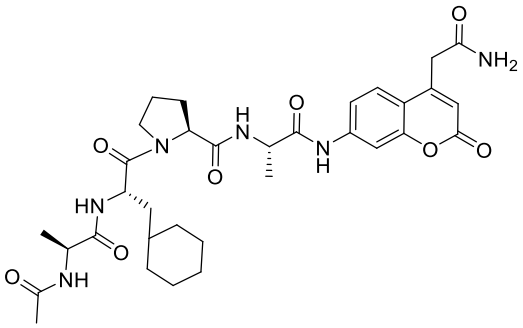
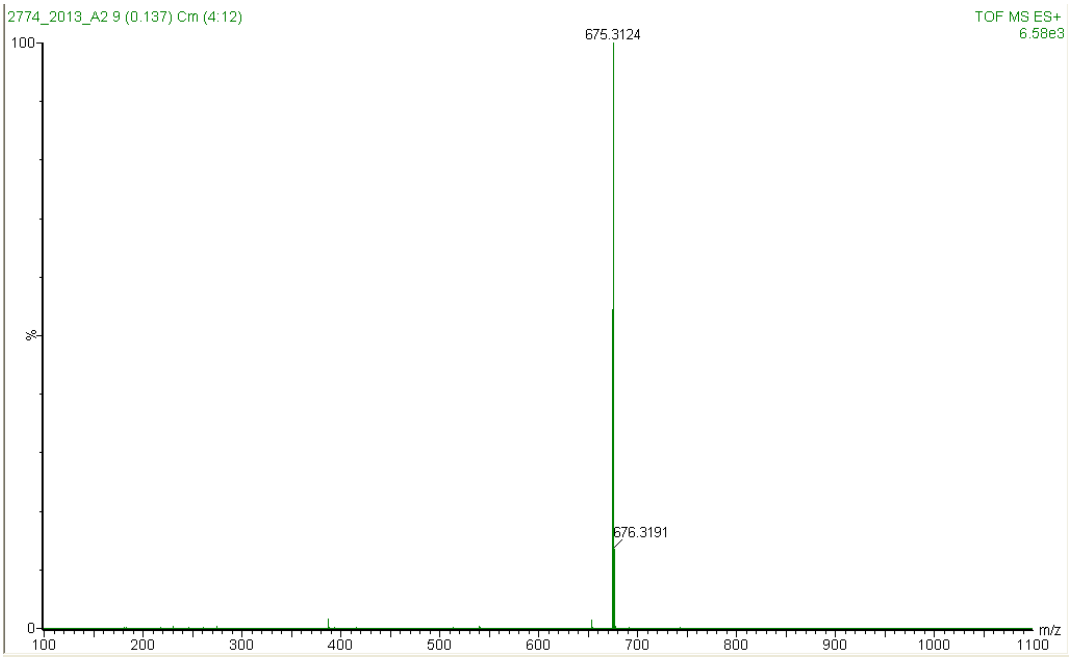
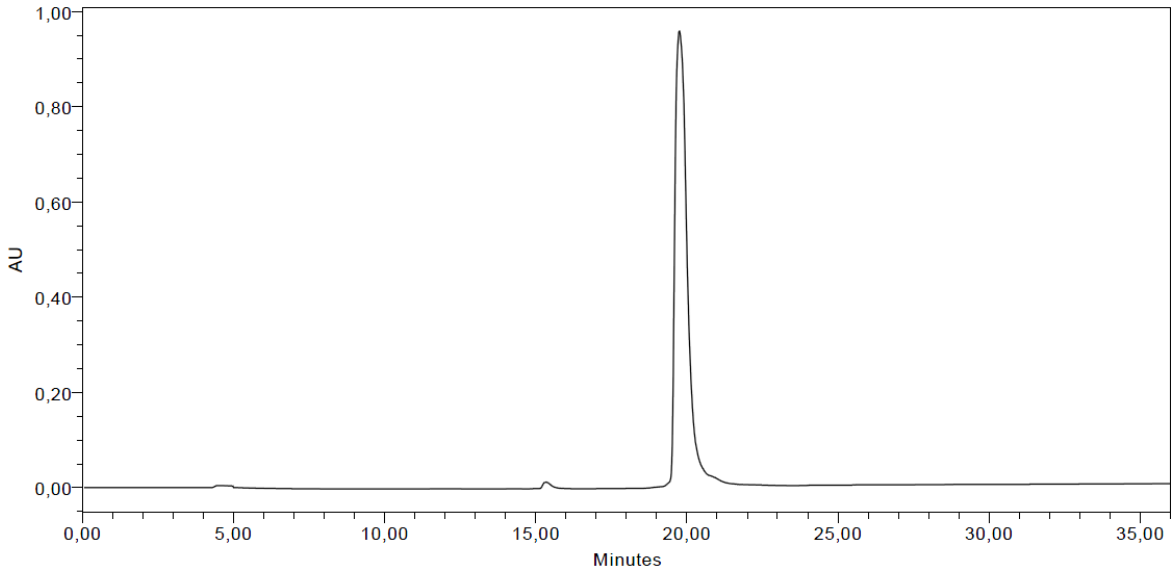
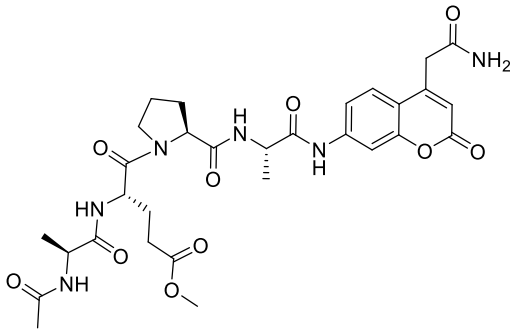
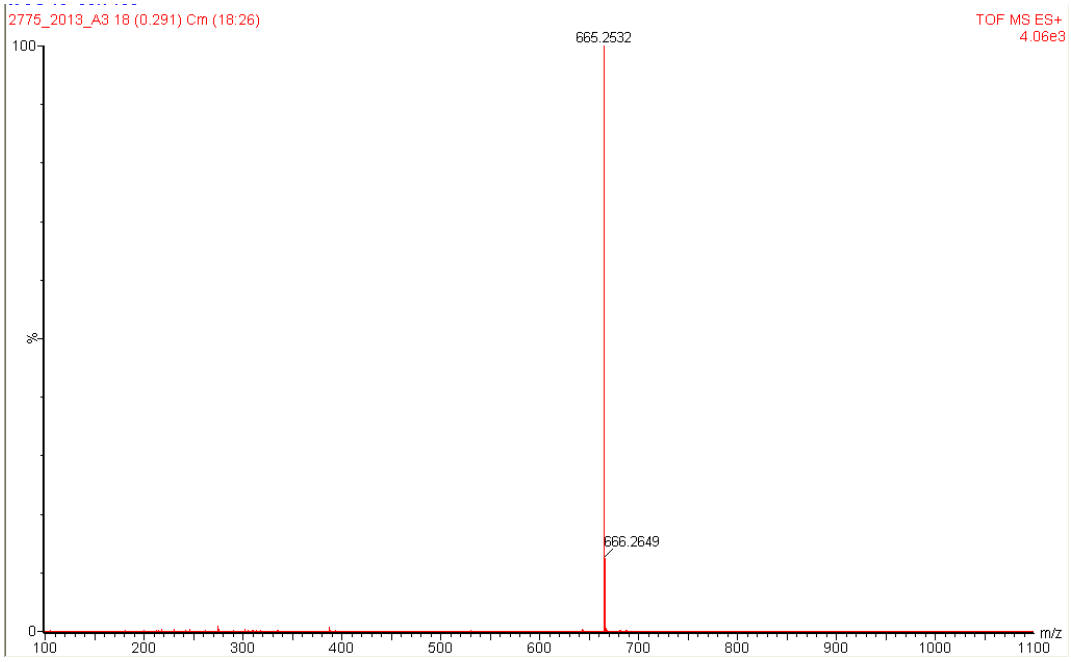


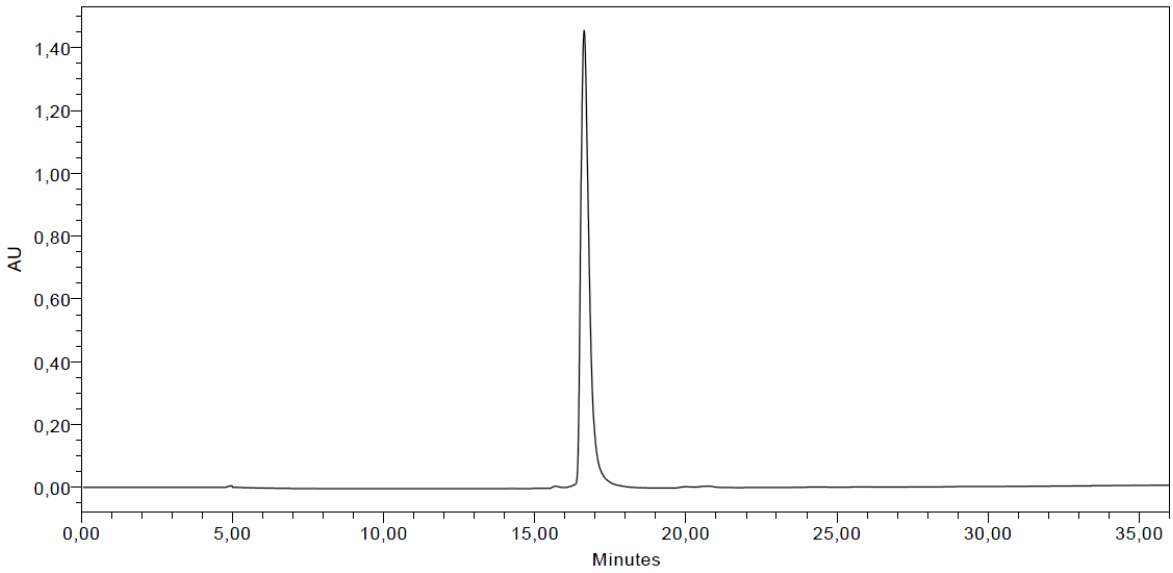




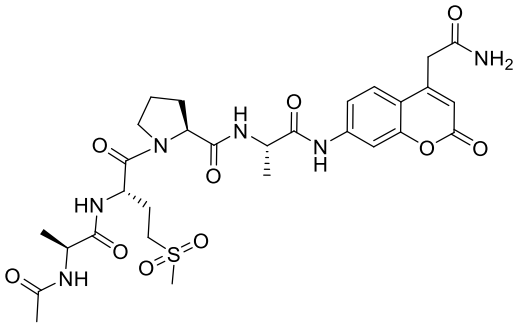
Ac-Ala-Glu(O-Me)-Pro-Ala-ACC

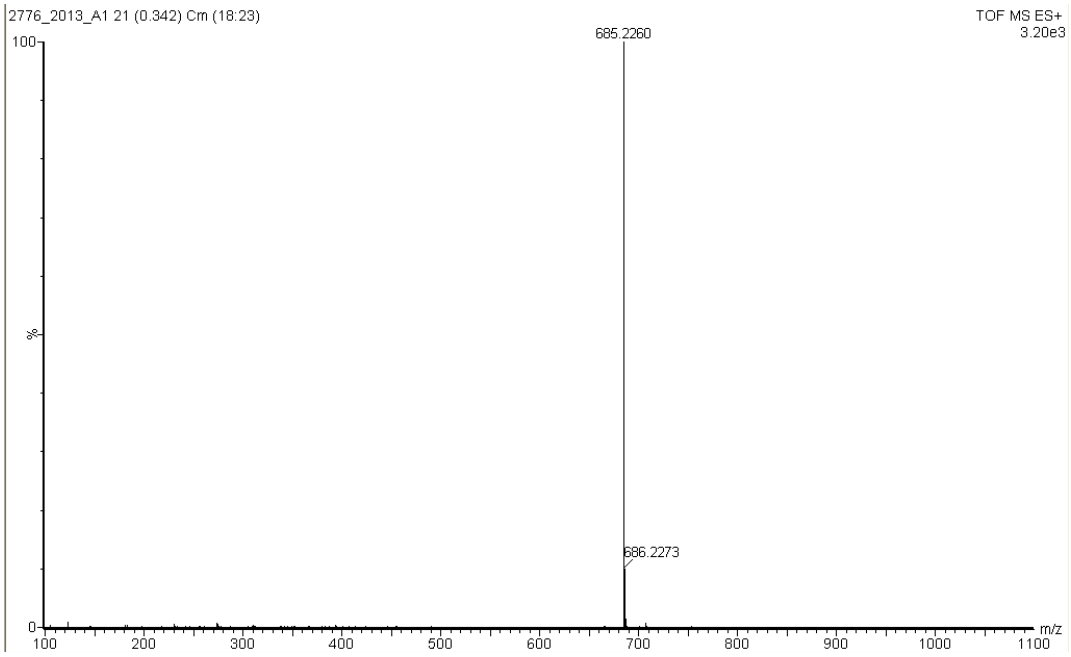


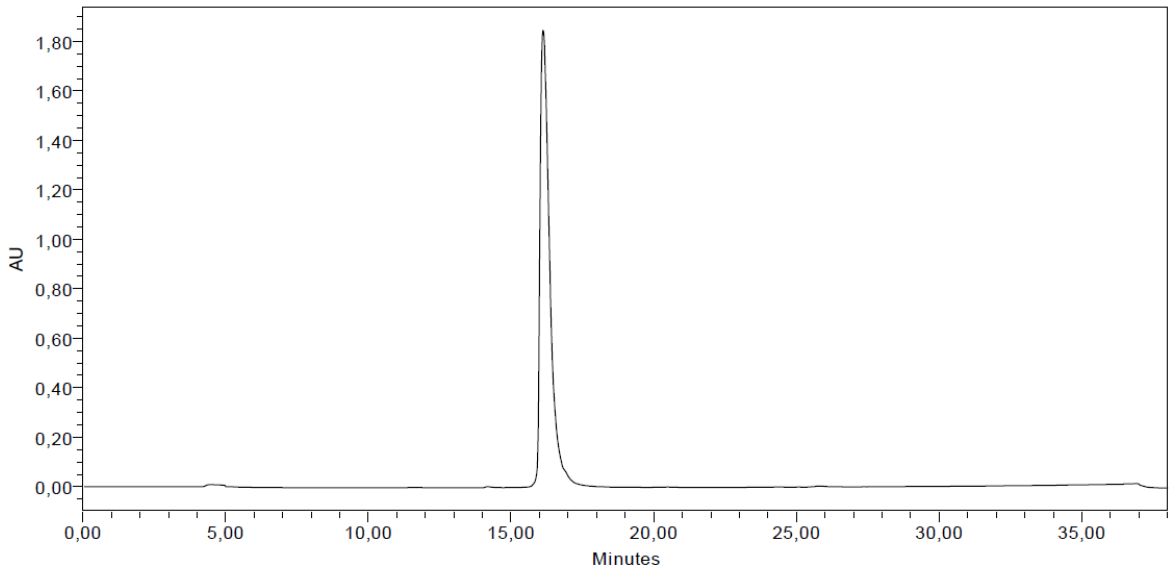




Ac-Ala-Met(O)$_2$-Pro-Ala-ACC







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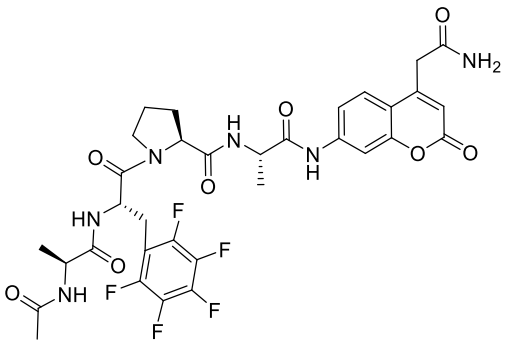

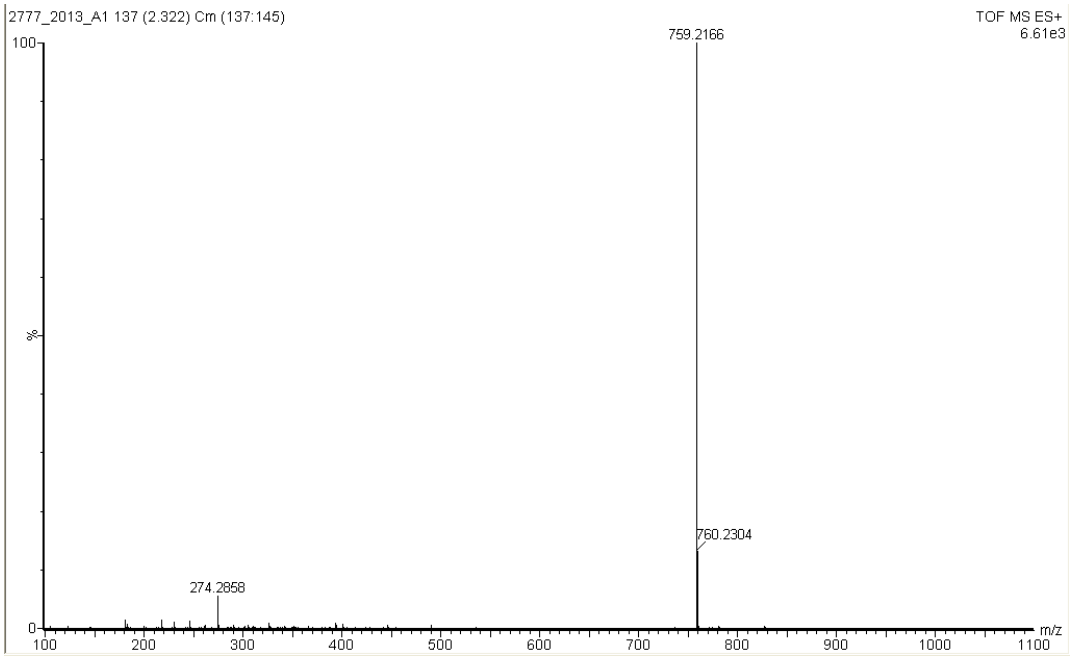

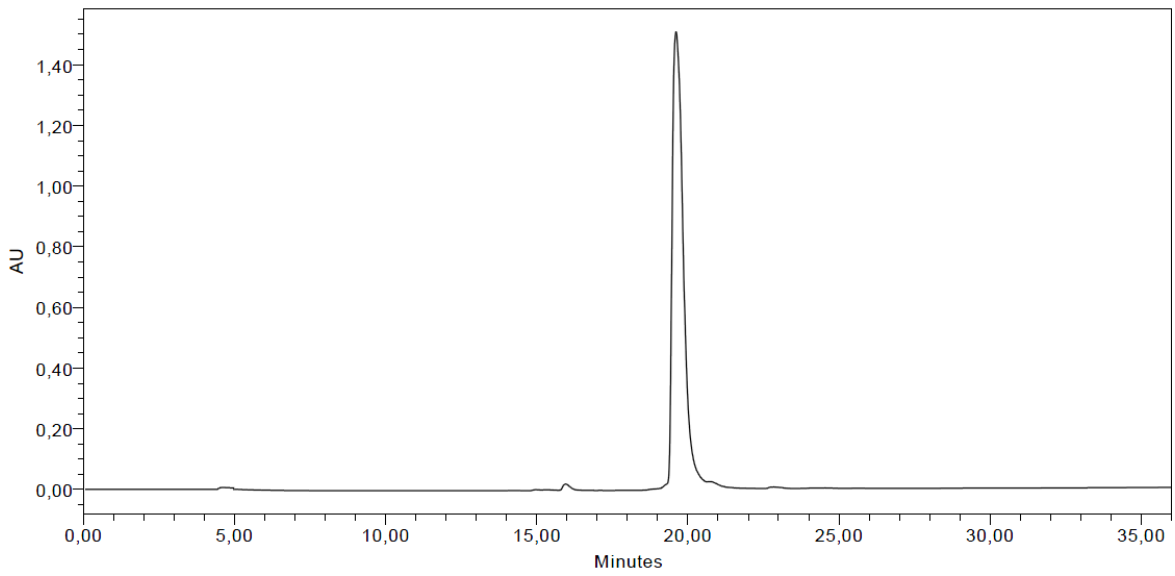

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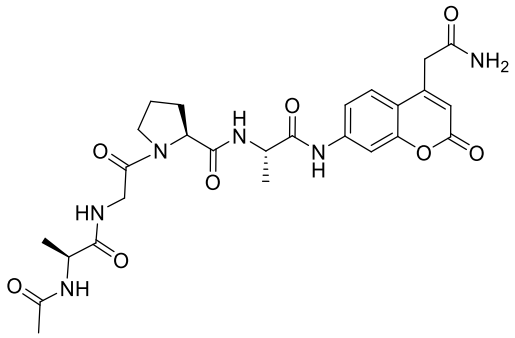

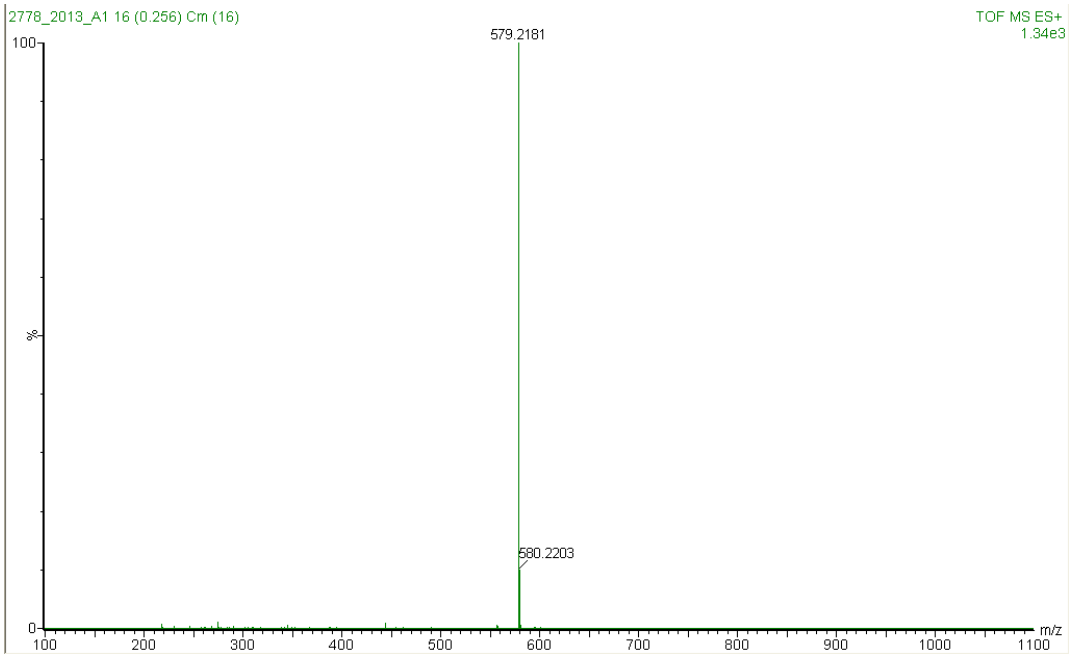

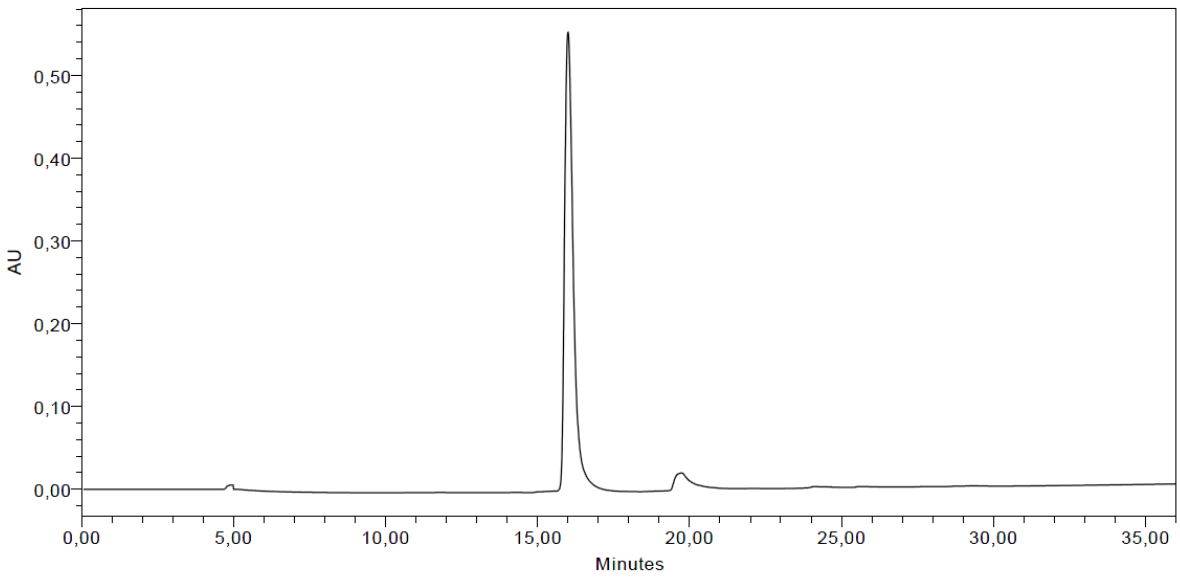

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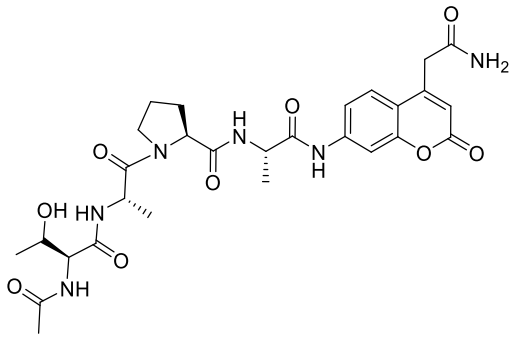

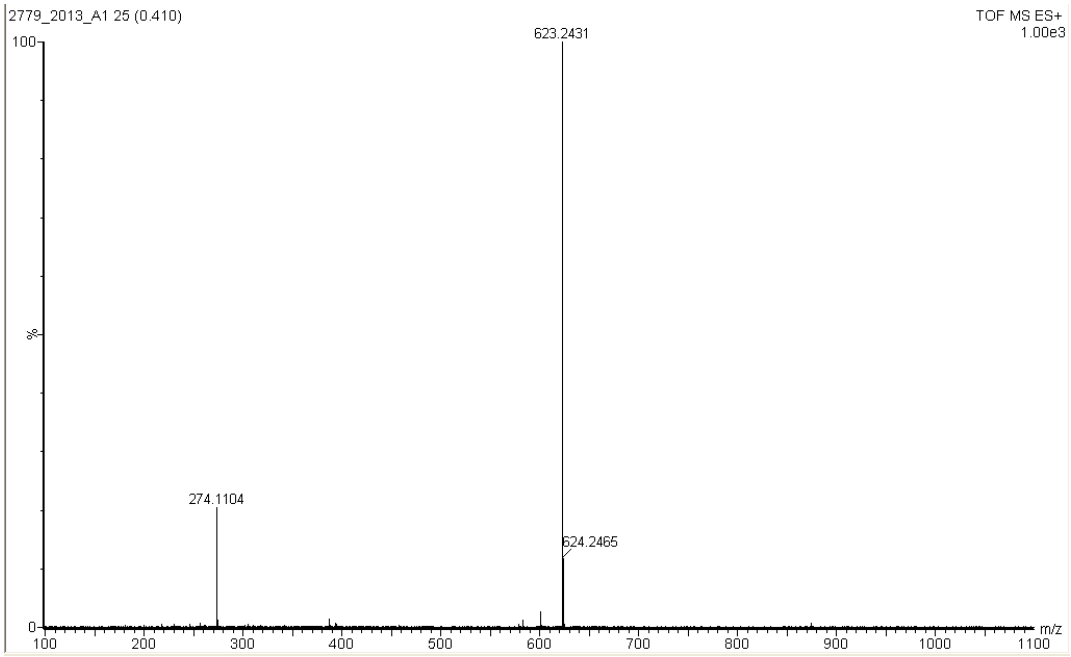

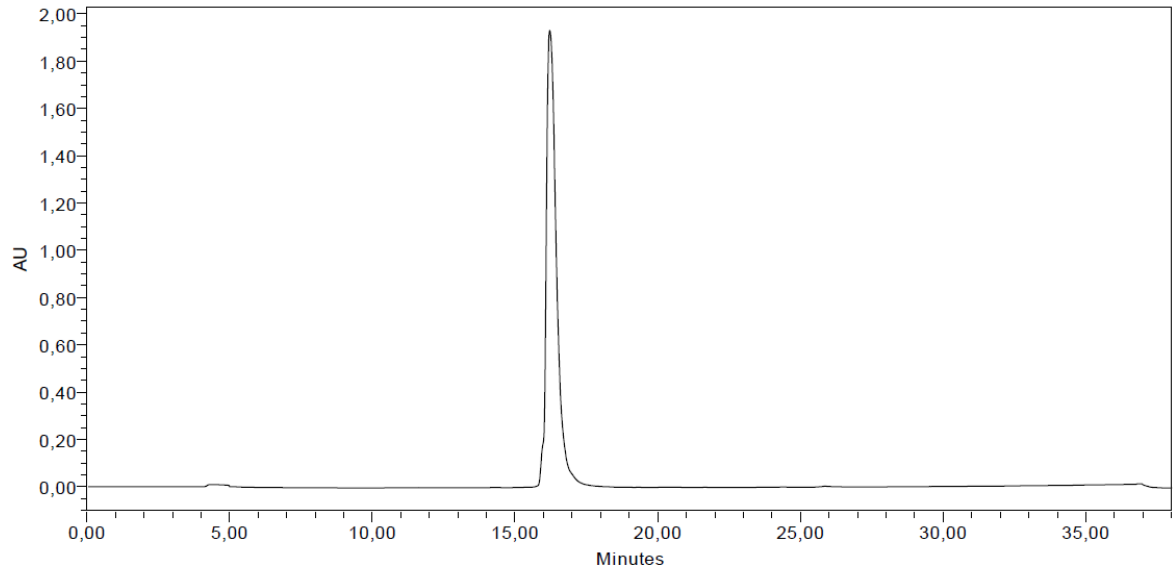

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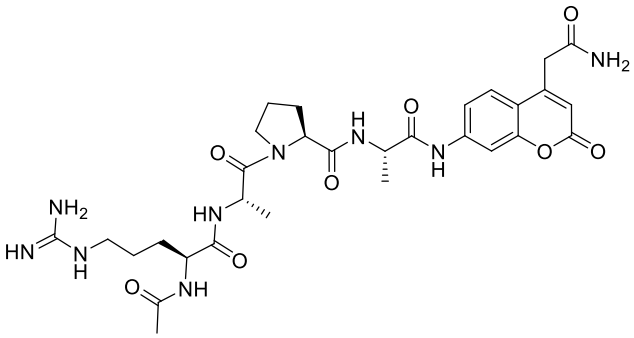
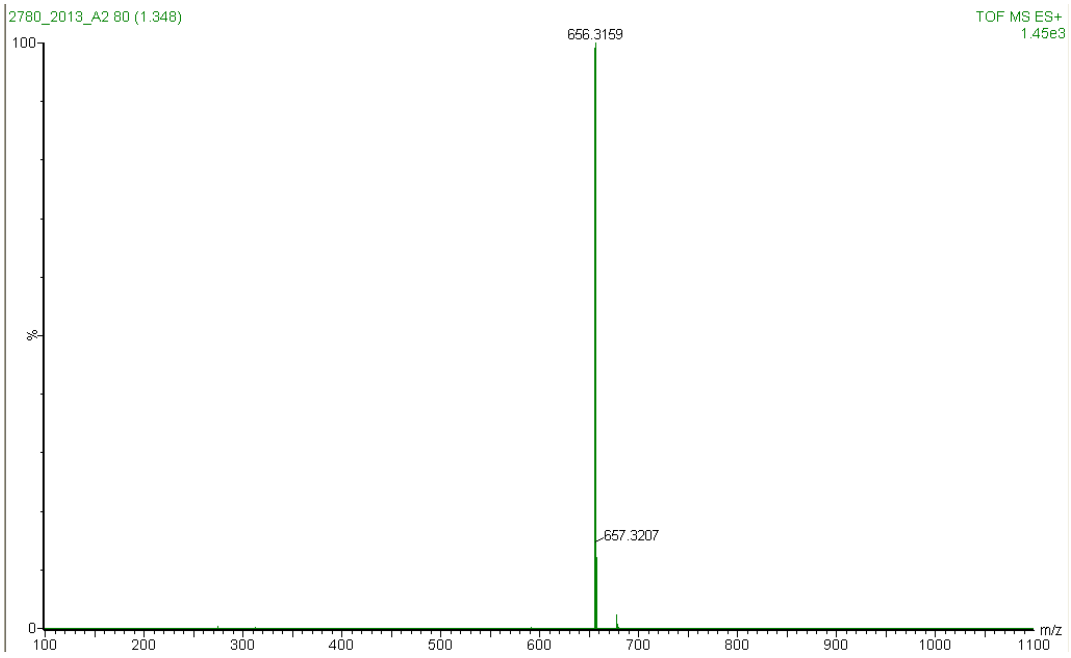
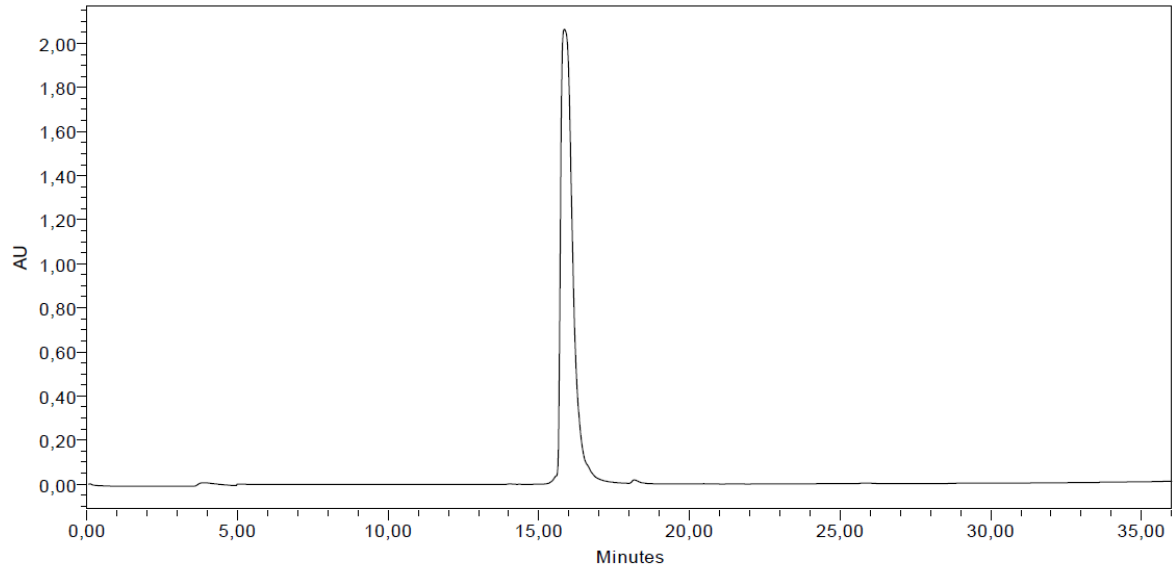
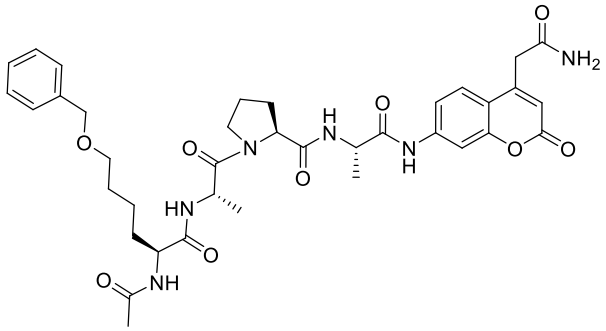
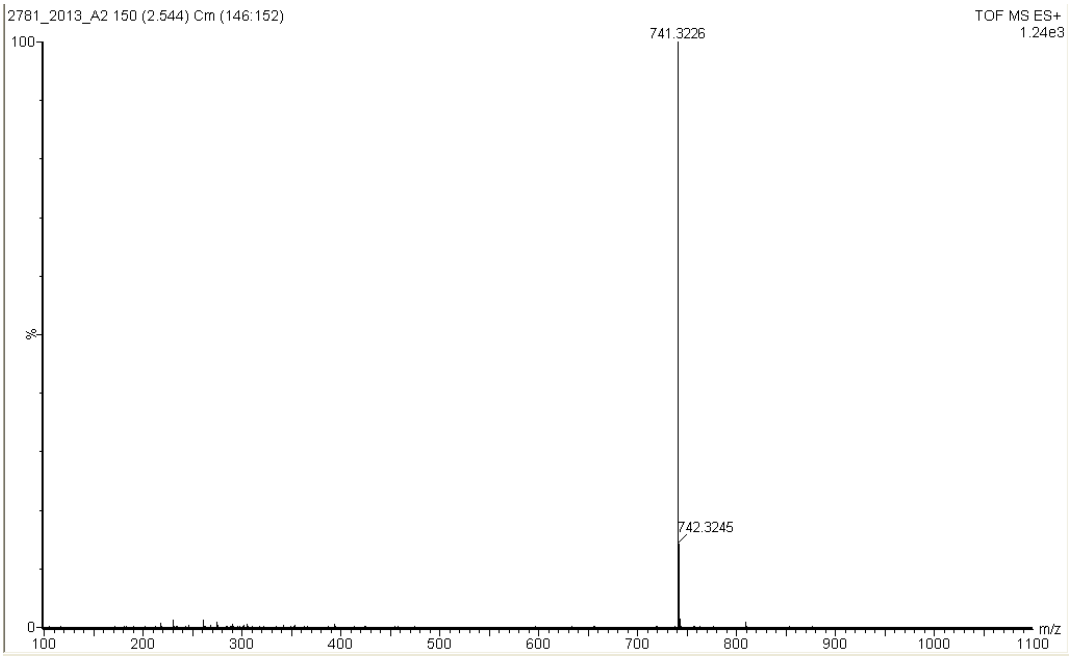


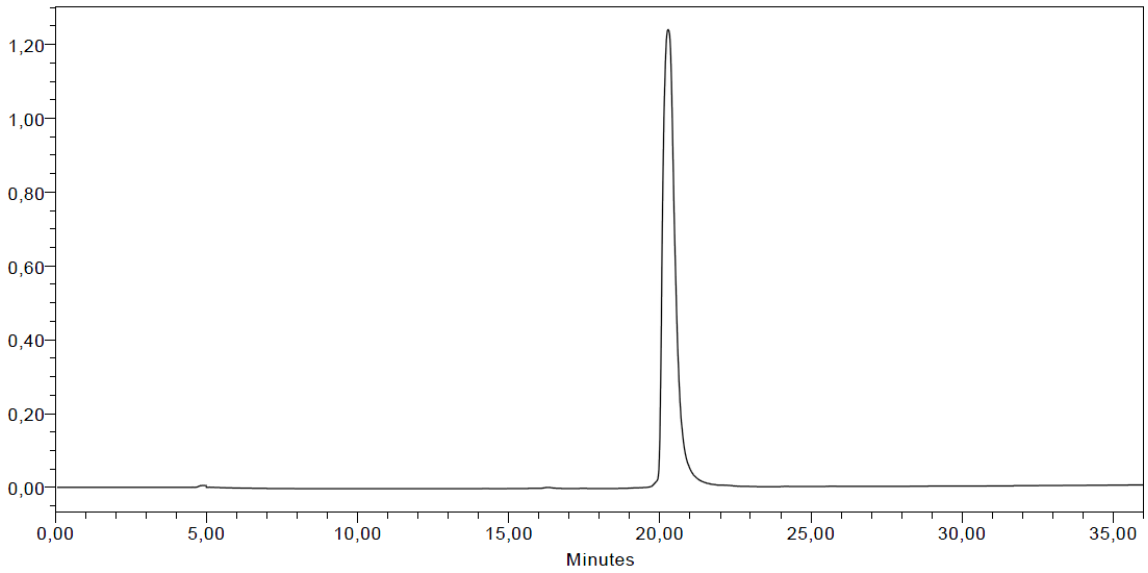




Ac-Nle(O-Bzl)-Ala-Pro-Ala-ACC







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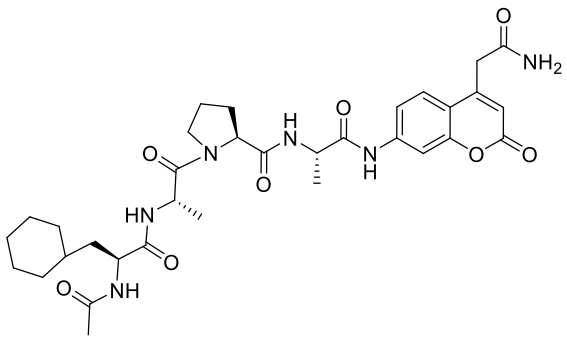

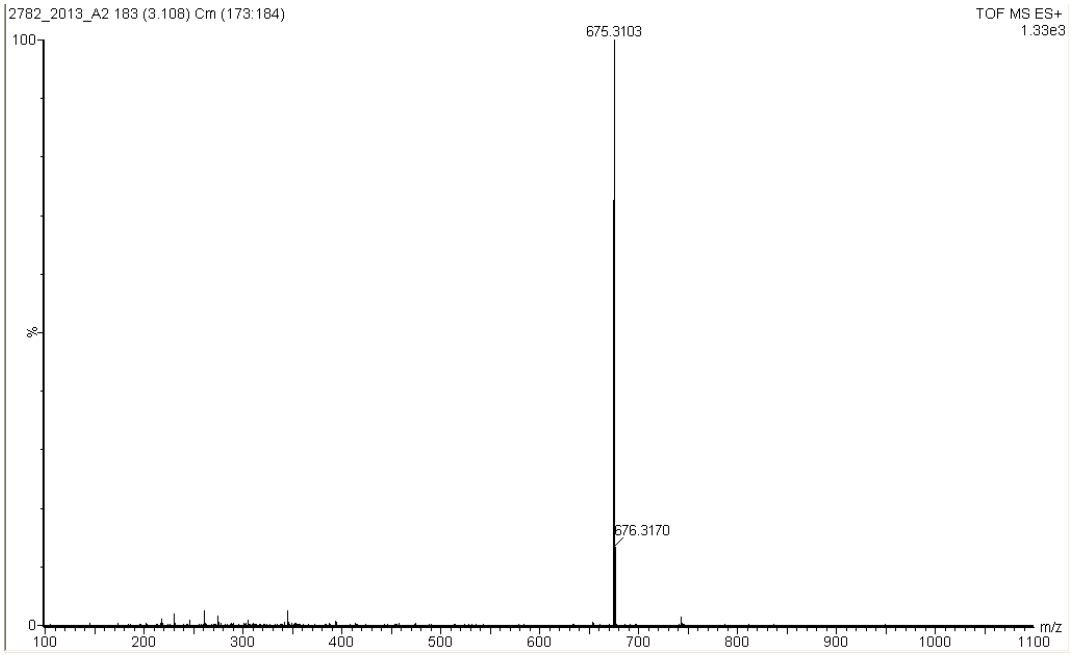

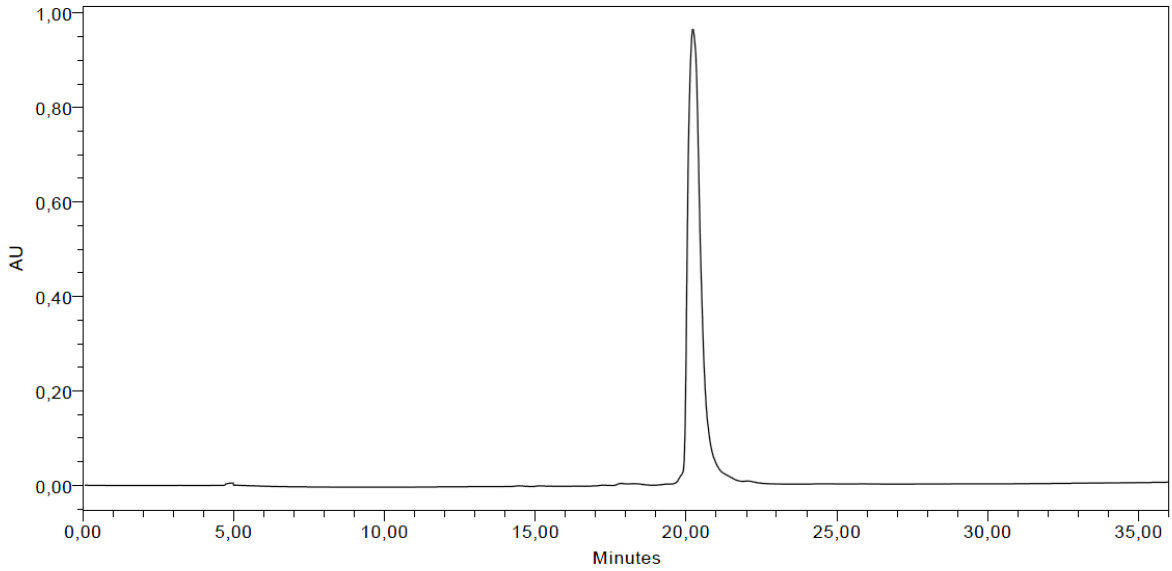

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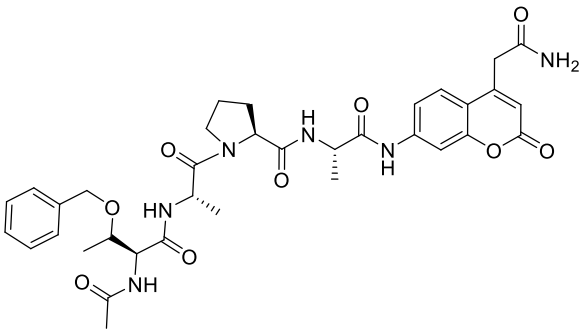

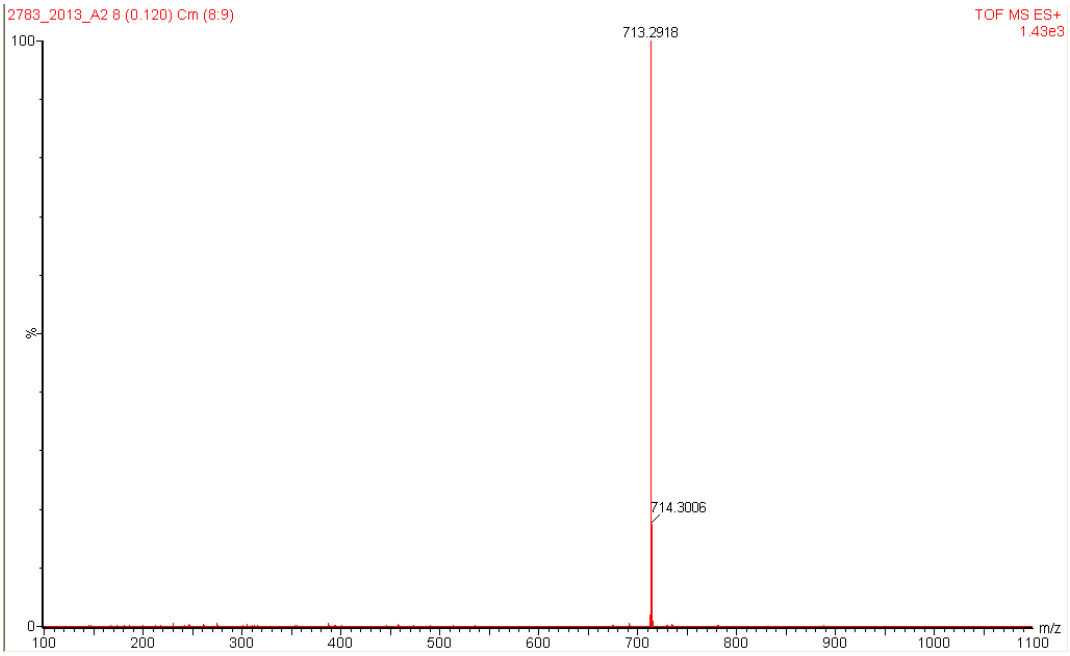

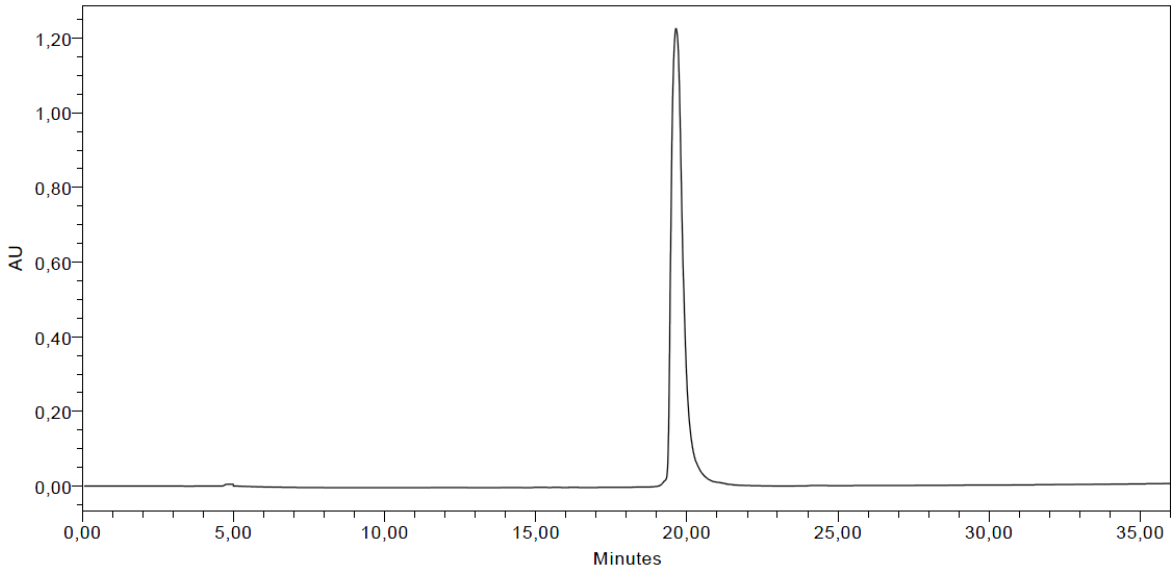

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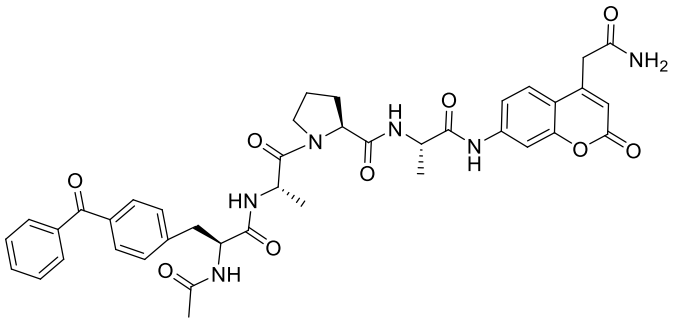

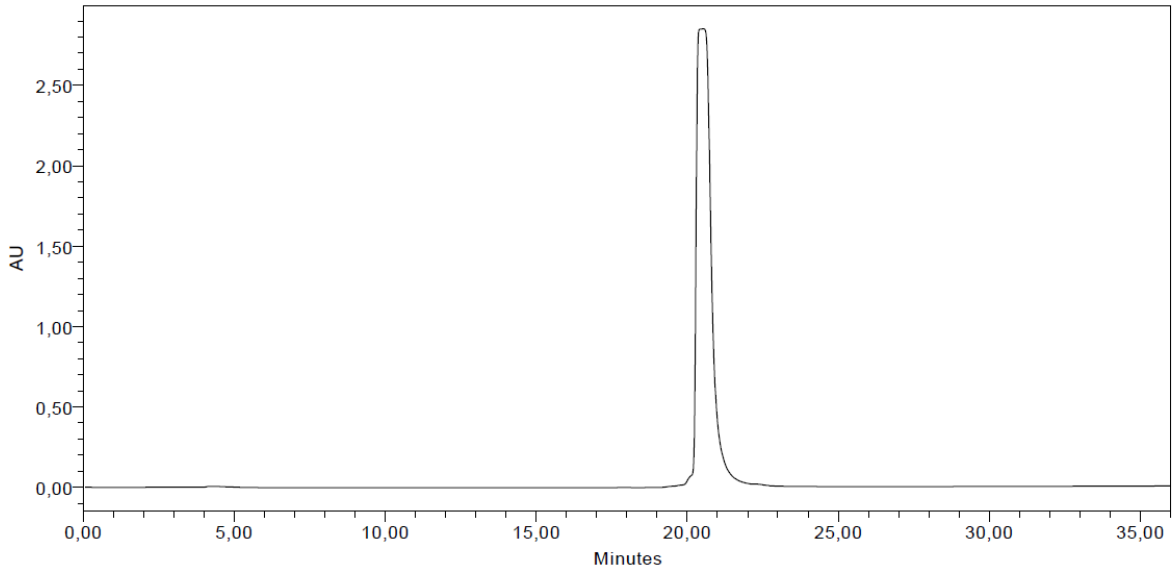

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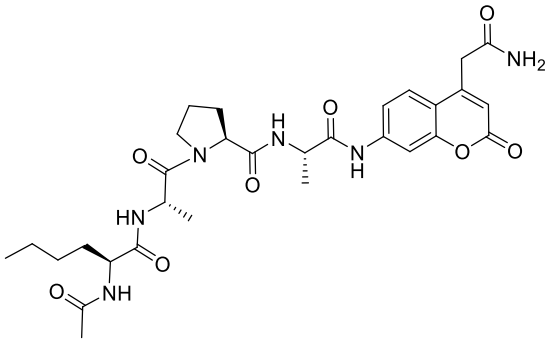

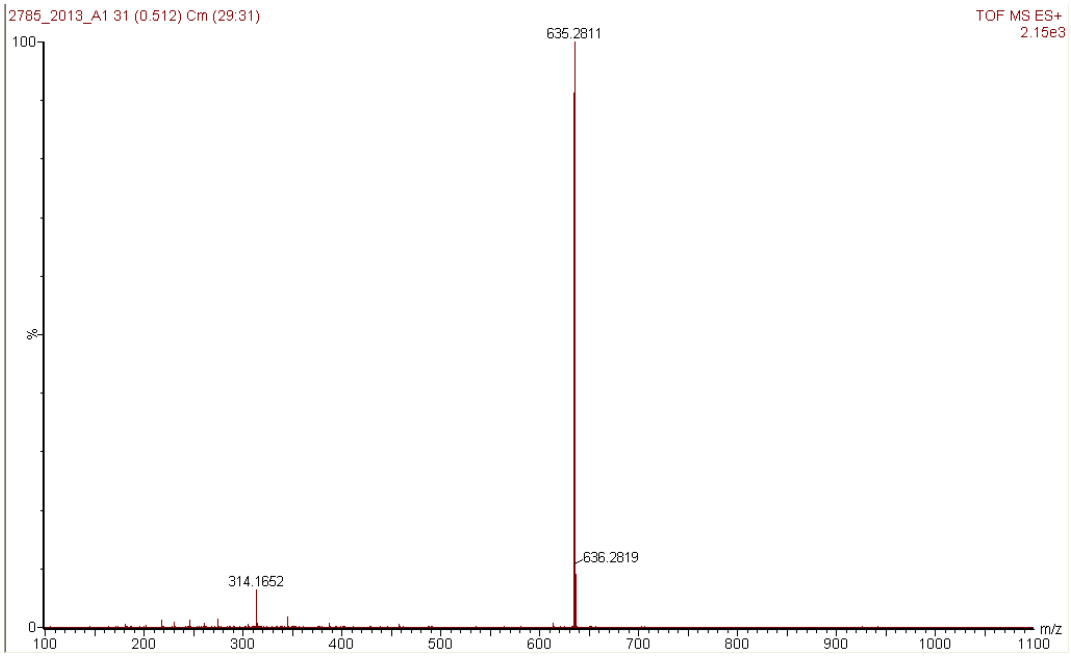

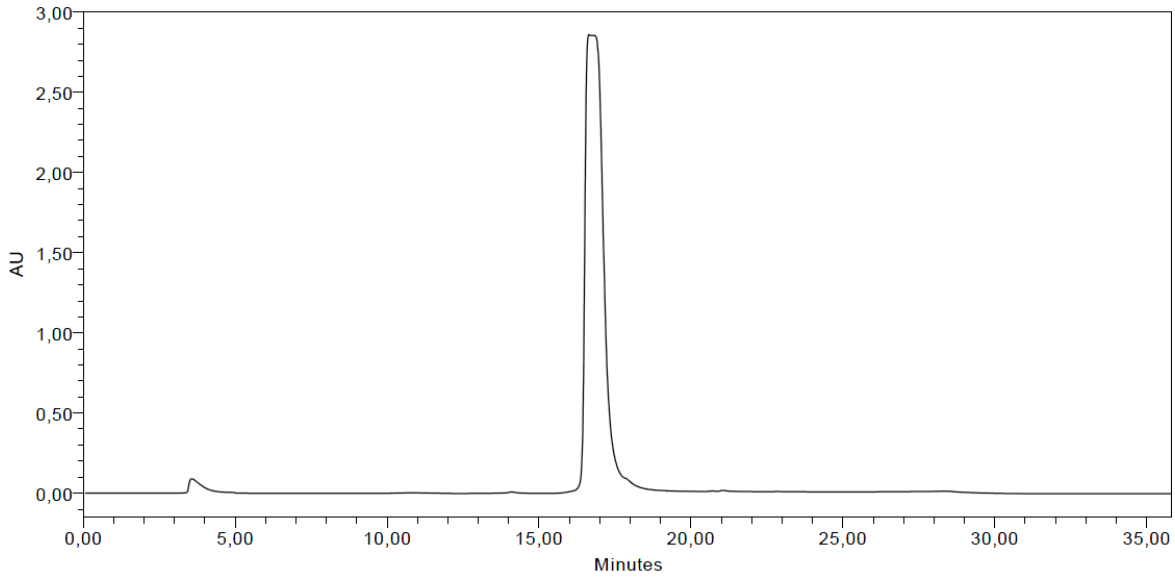

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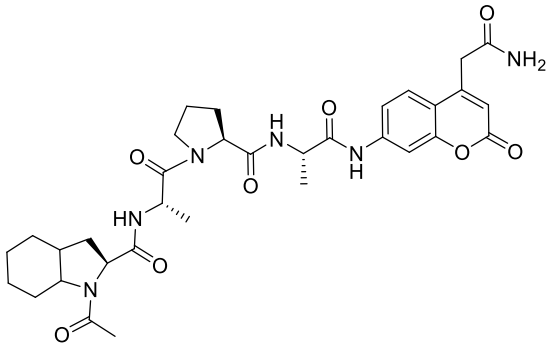
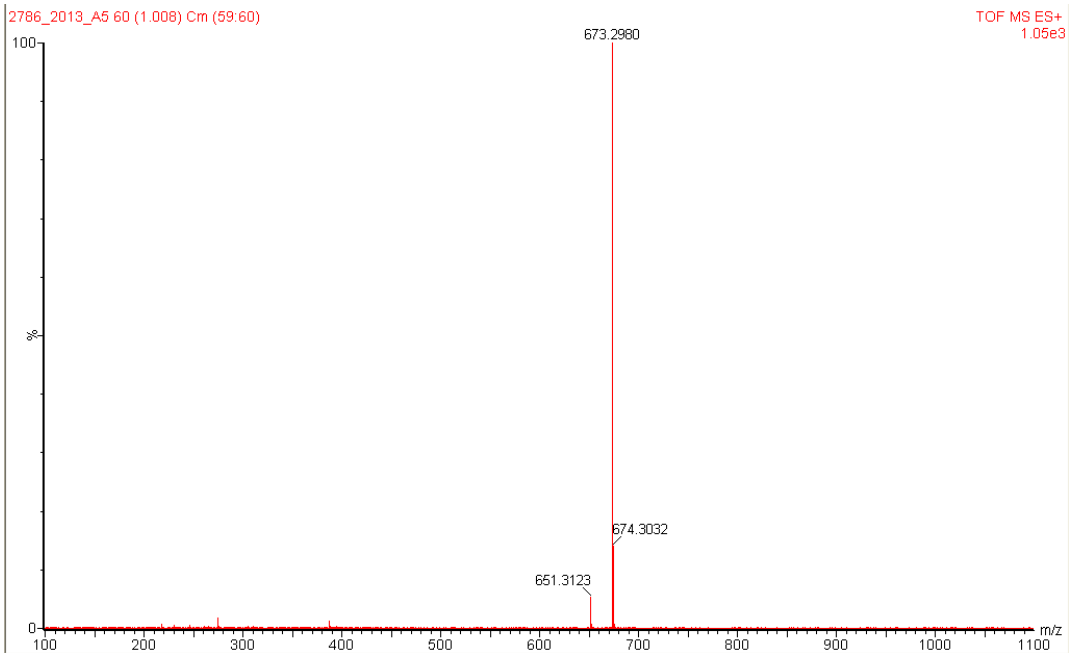
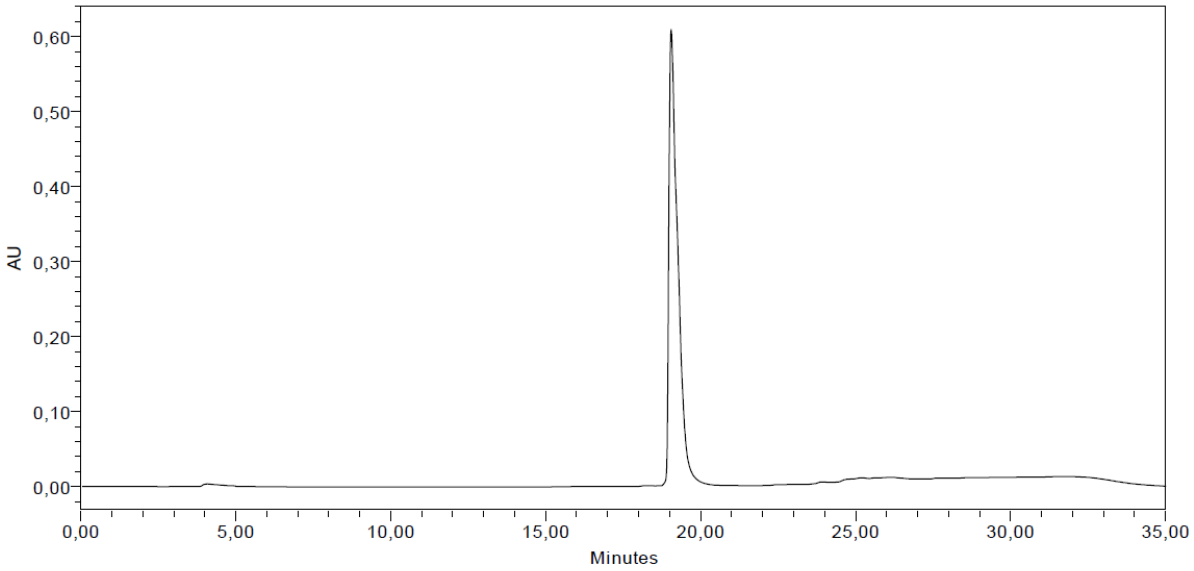
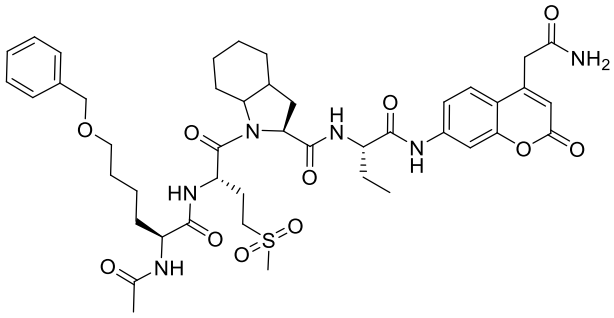
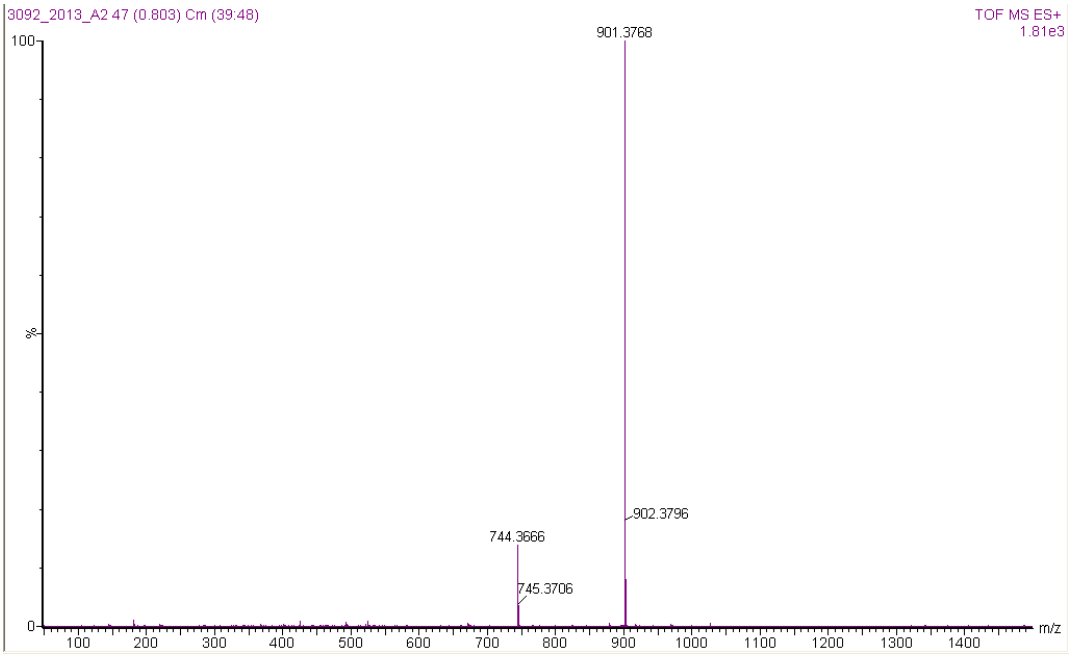


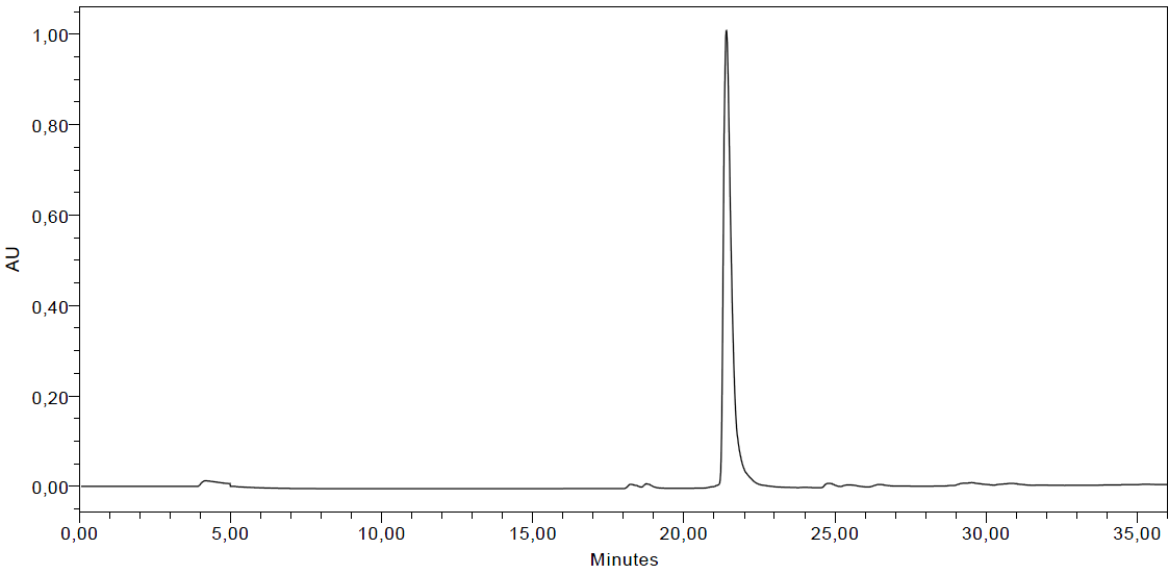




Ac-Nle(O-Bzl)-Met(O)2-Oic-Abu-ACC







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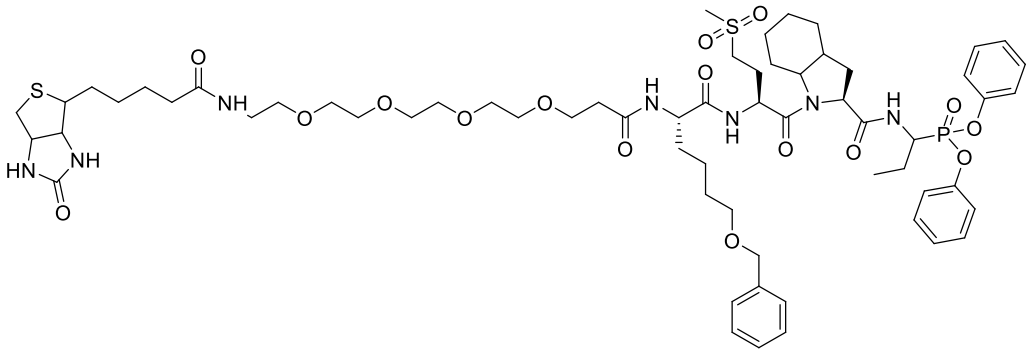


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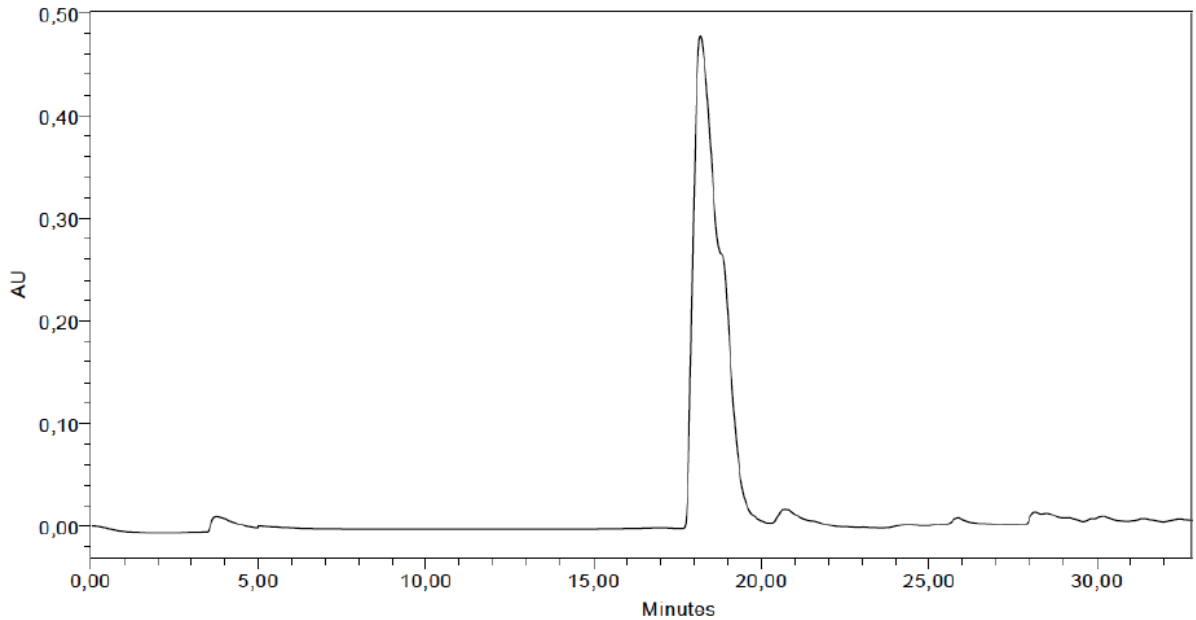
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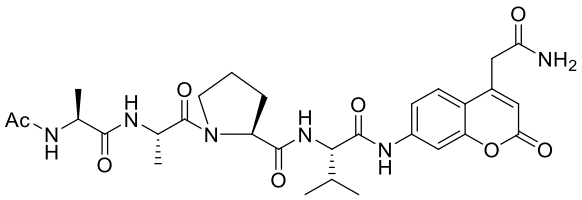
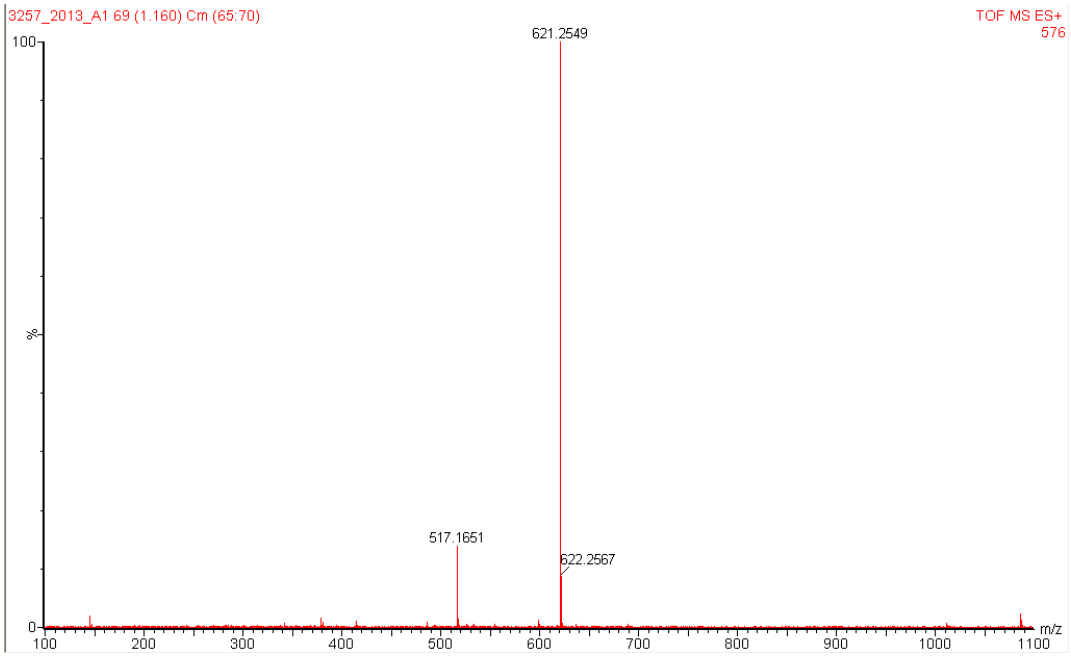
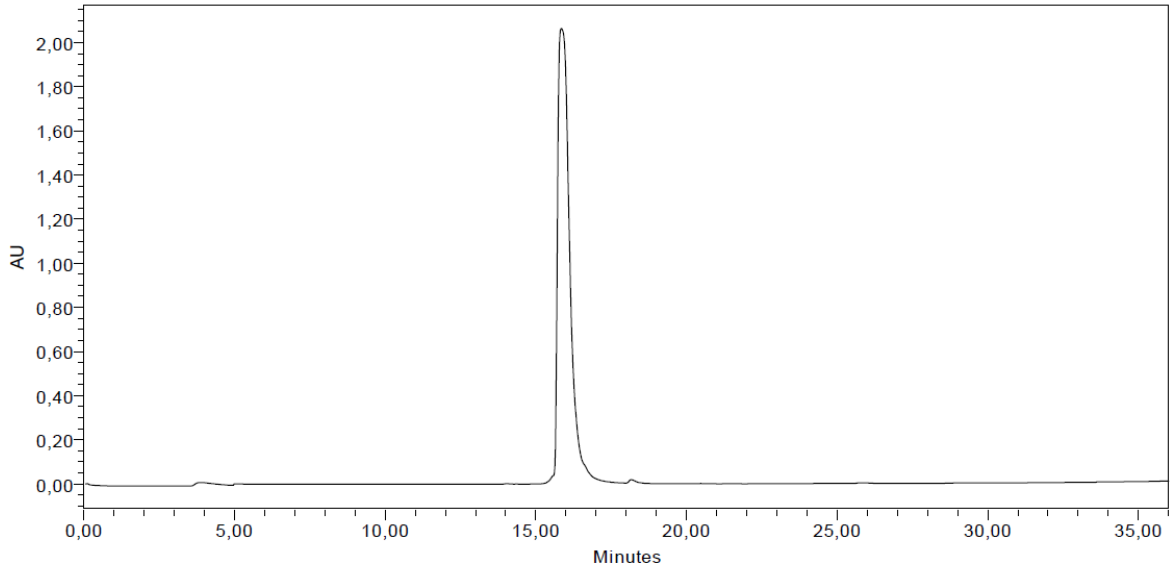
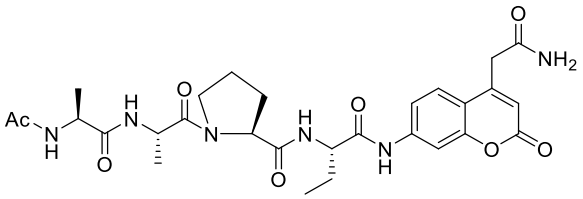
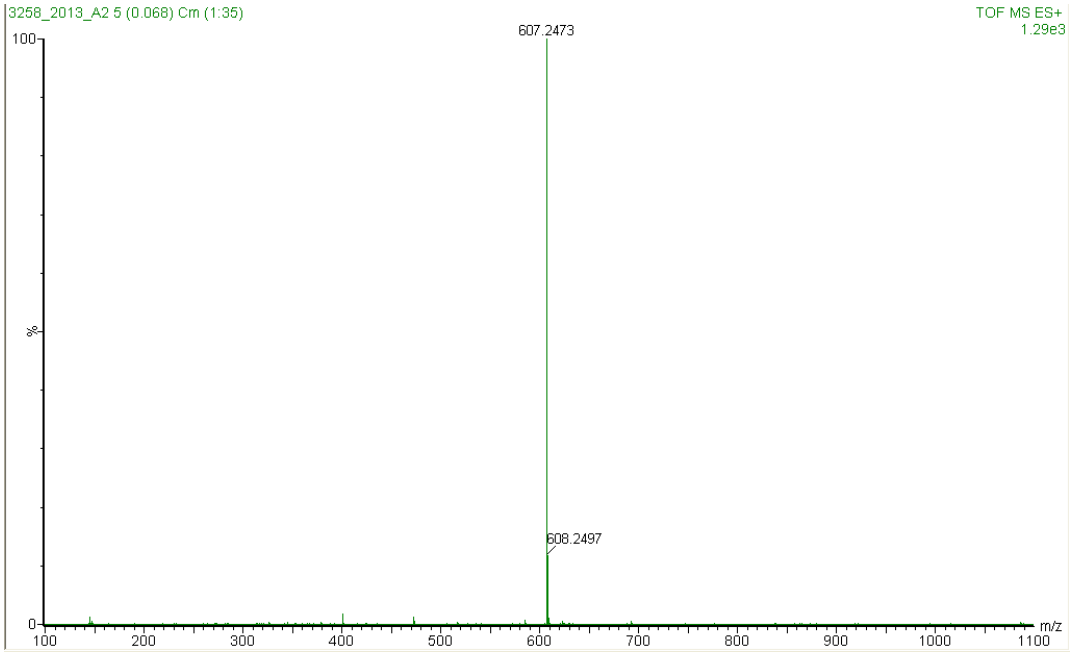


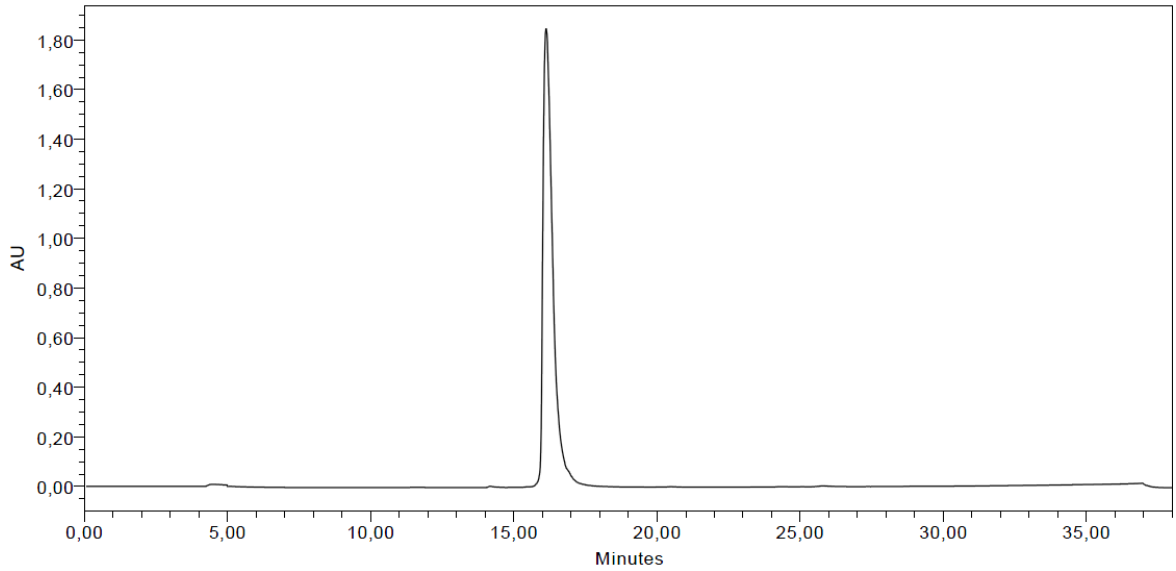




Ac-Ala-Ala-Pro-Abu-ACC







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