Supporting Information

Leveraging the Mukaiyama Oxidation-Reduction Reaction for On-Resin Thio-Esterification for Bio-Conjugation

Andrew Siow[†], Paul W.R. Harris[†,‡]

† School of Biological Sciences, School of Chemical Sciences and Maurice Wilkins Centre for Molecular Biodiversity
The University of Auckland
3b and 23 Symonds Street, Auckland 1010, New Zealand

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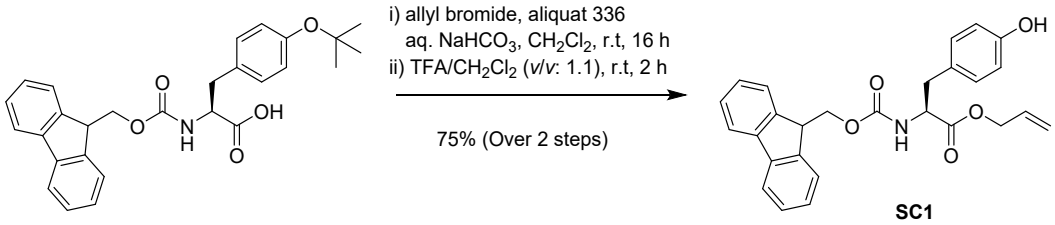
General Experimental

All reagents were purchased as reagent grade and used without further purification. Butane-1-thiol, *N*,*N*′-diisopropylcarbodiimide (DIC), *N*,*N*-diisopropylethylamine (DIPEA), 1,2-ethanedithiol (EDT), 3-mercaptopropionic acid (3-MPA), thiophenol, 4-methylmorpholine (NMM), tetrakis(triphenylphosphine)-palladium(0), triisopropylsilane (TIPS), and phenylsilane were purchased from Sigma-Aldrich (St. Louis, Missouri). *O*-(7-Azabenzotriazol-1-yl)-*N*,*N*,*N*′,*N*′-tetramethyluronium hexafluorophosphate (HATU), Fmoc-Ala-OH, Fmoc-Asn(Trt)-OH (Trt = triphenylmethane), Fmoc-Arg(Pbf)-OH (Pbf = 2,2,4,6,7-pentamethyIdlhydrobenzofuran-5-sulfonyl), Fmoc-Asp(*t*Bu)-OH (*t*Bu = *tert*-butyl), Fmoc-Cys(Trt)-OH, Fmoc-Gln(Trt)-OH, Fmoc-Glu(*t*Bu)-OH, Fmoc-Gly-OH, Fmoc-His(Trt)-OH, Fmoc-lle-OH, Fmoc-Leu-OH, Fmoc-Lys(Boc)-OH (Boc = *tert*-butoxycarbonyl), Fmoc-Phe-OH, Fmoc-Pro-OH, Fmoc-Thr(*t*Bu)-OH, Fmoc-Tyr(*t*Bu)-OH, Fmoc-Val-OH and 4-[(2,4-dimethoxyphenyl)(Fmoc-amino)methyl]phenoxyacetic acid (Rink amide linker) were purchased from CSBio (Shanghai, China). 2-Chlorotrityl chloride PS resin was purchased from ChemPep® (Wellington, Florida). 6-Chloro-1-hydroxybenzotriazole (6-Cl-HOBt) was purchased from Apptec (Louisville, Kentucky). 4-(4-hydroxymethyl-3-methoxyphenoxy)butyric acid (HMPB) was purchased from Novabiochem® (Darmstadt, Germany). TentaGel S NH_2 resin was purchased from RAPP polymere (Tübingen, Germany). Fmoc-(D)-Asp-Oallyl, Fmoc-(L)-Asp-Oallyl, Fmoc-Dab(Boc)-OH, 1,8-diazabicyclo(5.4.0)undec-7-ene (DBU), 2,2'-dithiodipyridine (DPS), Fmoc-(D)-Glu-Oallyl, Fmoc-(L)-Glu-Oallyl, guanidine hydrochloride (Gu·HCl), Fmoc-(L)-Lys-Oallyl, 4-mercaptophenylacetic acid (4-MPAA), tris(2-carboxyethyl)phosphine hydrochloride (TCEP·HCl) and triphenylphosphine (PPh_3) were purchased from AK Scientific (Union City, California). All reactions were performed under an oxygen atmosphere unless otherwise noted. Dimethylformamide (DMF), and dichloromethane (CH_2Cl_2), were dried using an LC Technology solvent purification system. High resolution mass spectra (HRMS) were obtained using the micrOTOF-Q spectrometer operating at a nominal accelerating voltage of 70 eV. Microwave SPPS reactions were carried out on a Biotage® Initiator+ Alstra™ (Uppsala, Sweden) automated peptide synthesizer. NMR spectra were recorded at room temperature in $CDCl_3$ on a Bruker DRX 400 spectrometer operating at 400 MHz for ^1H nuclei and 100 MHz for ^{13}C nuclei. Chemical shifts are reported in parts per million (ppm) calibrated relative to: TMS (δ_H 0.00 ppm), $CDCl_3$ (δ_H 7.26 ppm, δ_C 77.2 ppm). Multiplicities are reported as "s" (singlet), "d" (doublet), "dd" (doublet of doublets), "t" (triplet), "m" (multiplet), coupling constant (*J*, Hz), relative integral and structural assignment. ^{13}C NMR data were reported as position (δ), type and assignment of carbon resonance. Structural assignments were achieved with the aid of COSY, HSQC, HMBC and NOESY experiments where required. Infrared (IR) spectra were recorded as a thin film on a composite of zinc selenide and diamond crystal on a FT-IR system transform spectrometer expressed in wavenumbers (cm^{-1}). Optical rotations were measured with an automatic polarimeter using the sodium-D line (589 nm), with the concentration measured in grams per 100 mL. Melting points were determined on a Kofler hot-stage apparatus and are uncorrected. Semi-preparative RP-HPLC was performed on a Thermo Scientific (Waltham, MA) Dionex Ultimate 3000 HPLC equipped with a four channel UV Detector at 210, 225, 254 and 280 nm using either an analytical column (Waters (Milford, MA) XTerra® MS C18, (5 µm; 4.6 x 150 mm) at a flow rate of 1 mL min^{-1} or a semi-preparative column (Phenomenex® Gemini C18, (5 µm; 10 x 250 mm) at a flow rate of 4 mL min^{-1}. Analytical RP-HPLC was performed on Alliance® Waters (Milford, USA) e2695 HPLC using an Agilent TC-C18 column (5 µm;

4.6 × 250 mm) at wavelength 214 nm. A suitably adjusted gradient of 5% B to 95% B was used, where solvent A was 0.1% TFA in H_2O and B was 0.1% TFA in acetonitrile. LCMS spectra were acquired on an Agilent Technologies (Santa Clara, United States) 1260 Infinity LC equipped with an Agilent Technologies 6120 Quadrupole mass spectrometer at wavelength 214 nm. An analytical column (Agilent C3, 3.5 μm; 3.0 x 150 mm) was used at a flow rate of 0.3 mL min^{-1} using a linear gradient of 5% B to 95% B over 30 min, where solvent A was 0.1% formic acid in H_2O and B was 0.1% formic acid in acetonitrile.

Synthesis of allyl (((9H-fluoren-9-yl)methoxy)carbonyl)-L-tyrosinate (**SC1**)



To Fmoc-(L)-Tyr(*t*Bu)-OH (1 g, 2.17 mmol) and $NaHCO_3$ (0.18 g, 2.17 mmol) dissolved in H_2O (5.0 mL) is added with aliquat 336 (0.72 g, 1.83 mmol) and allyl bromide (0.98 mL, 11.4 mmol) in CH_2Cl_2 (4 mL) and stirred at room temperature overnight. The mixture was diluted with saturated $NaHCO_3$ (10 mL) and the aqueous phase was extracted with CH_2Cl_2 (2 × 10 mL) and further washed with brine (25 mL), dried with Na_2SO_4 and the combined organic layers were filtered and concentrated *in vacuo*. The crude product mixture was then treated with trifluoroacetic acid (TFA) in CH_2Cl_2 (20 mL, *v*/*v*: 1.1) at room temperature for 2 h, TFA is then evaporated under N_2 stream and resulting mixture was washed with H_2O (2 × 10 mL) and the aqueous phase was extracted with ether (2 × 10 mL) and then concentrated *in vacuo*. The crude residue was then purified by flash column chromatography (CH_2Cl_2-MeOH; 97:3) affording Fmoc-(L)-Tyr-Oallyl (**SC1**) (0.72 g, 75%) as a white powder; **^{1}H NMR** (400 MHz, $CDCl_3$, Me_4Si): *δ* 3.05 (dd, *J* = 14.1 Hz, *J* = 5.6 Hz, C*H*aCHb, CHaC*H*b, 2H), 4.20 (t, *J* = 7.1 Hz, 1H), 4.45-4.34 (m, 2H), 4.62 (d, *J* = 6.0 Hz, 2H), 4.69-4.65 (m, 1H), 5.35-5.25 (m, 3H), 5.79 (s, 1H), 5.93-5.83 (m, 1H), 6.72 (d, *J* = 8.2 Hz, 2H, 2 × Ar-*H*), 6.95 (d, *J* = 8.5 Hz, 2H, 2 × Ar-*H*), 7.30 (t, *J* = 7.4 Hz, 2H, 2 × Ar-*H*), 7.40 (t, *J* = 7.4 Hz, 2H, 2 × Ar-*H*), 7.57-7.55 (m, 2H, 2 × Ar-*H*), 7.76 (d, *J* = 7.5 Hz, 2H, 2 × Ar-*H*); **^{13}C{^{1}H} NMR** (100 MHz, $CDCl_3$, Me_4Si): *δ* 37.6, 47.3, 55.1, 66.3, 67.2, 115.7, 119.3, 120.1, 125.2, 127.2, 127.9, 130.6, 131.5, 141.4, 143.8, 143.9, 155.2, 155.9, 171.6; **$[\alpha]_D^{21}$** +51 (*c* 0.2, $CHCl_3$); **IR** ν_{max}(neat)/cm^{-1}: 3333, 1694, 1614, 1514, 1447, 1341, 1210, 1051, 738, 620; **m.p.**: 47-51 ˚C; **HRMS** (ESI+) $[M + Na]^+$ calcd: for $C_{27}H_{25}NO_5{}^{23}Na^+$: 466.1625; found 466.1624.

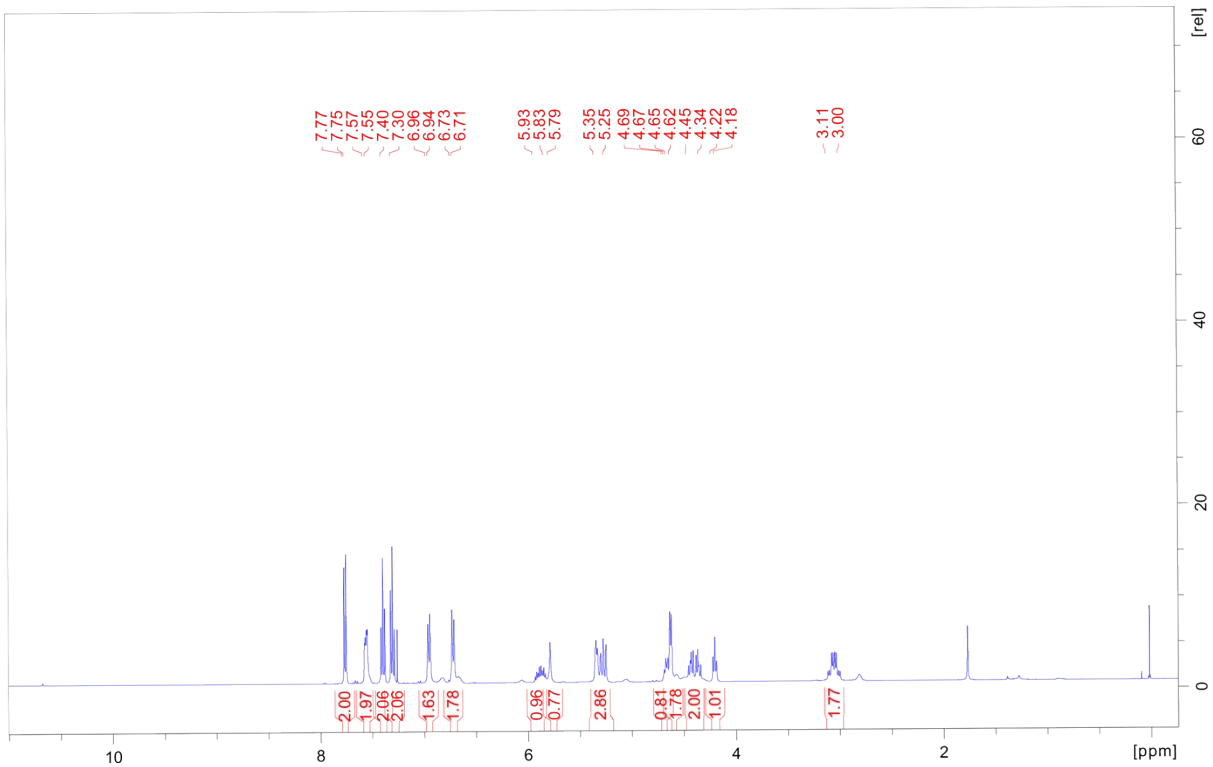


Figure S1: ^{1}H NMR ($CDCl_3$, 400 MHz): **SC1**.

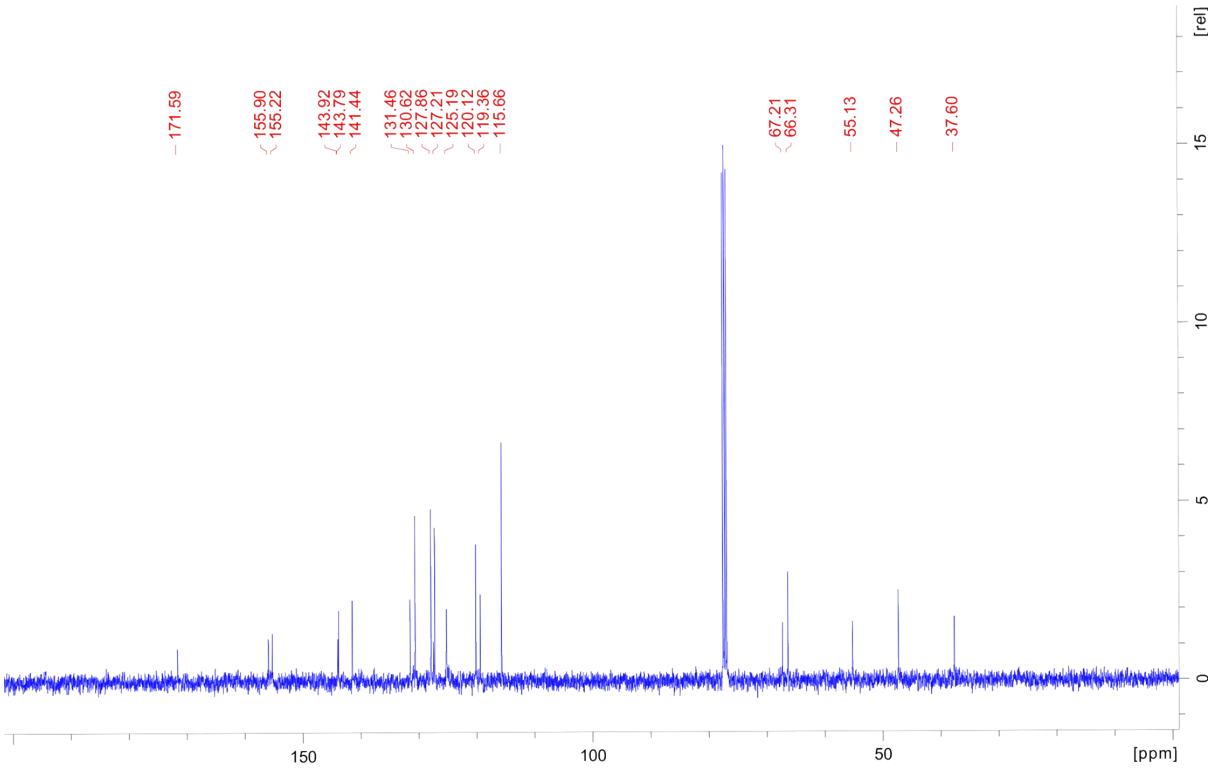


Figure S2: $^{13}C\{^{1}H\}$ NMR ($CDCl_3$, 100 MHz): **SC1**.

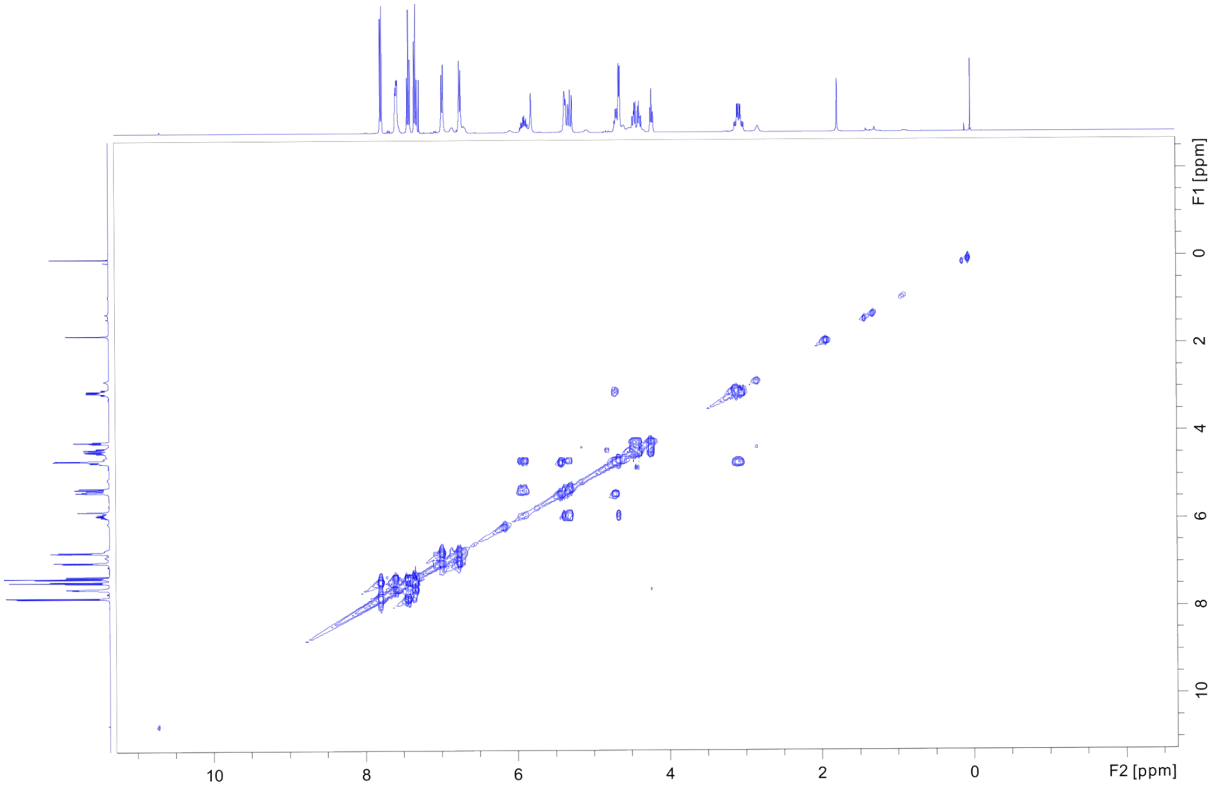


Figure S3. COSY spectrum for **SC1**.

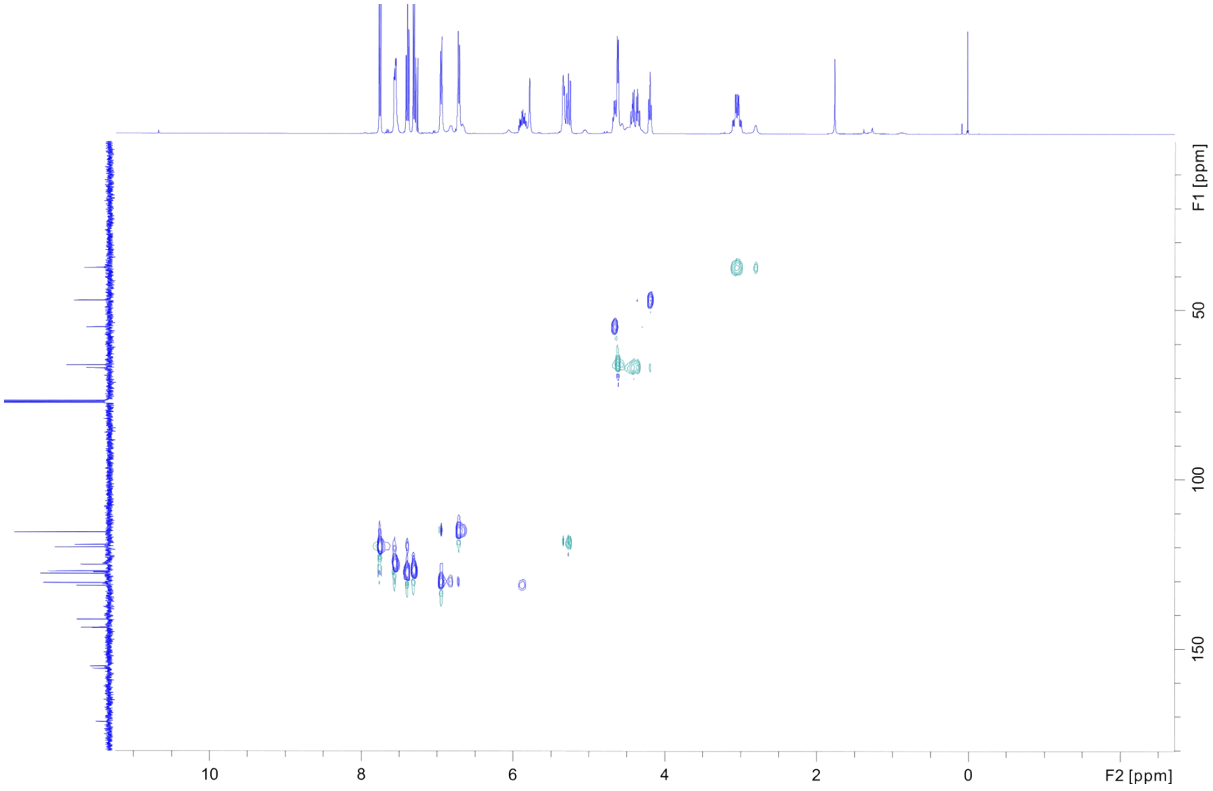


Figure S4. HSQC spectrum for **SC1**.

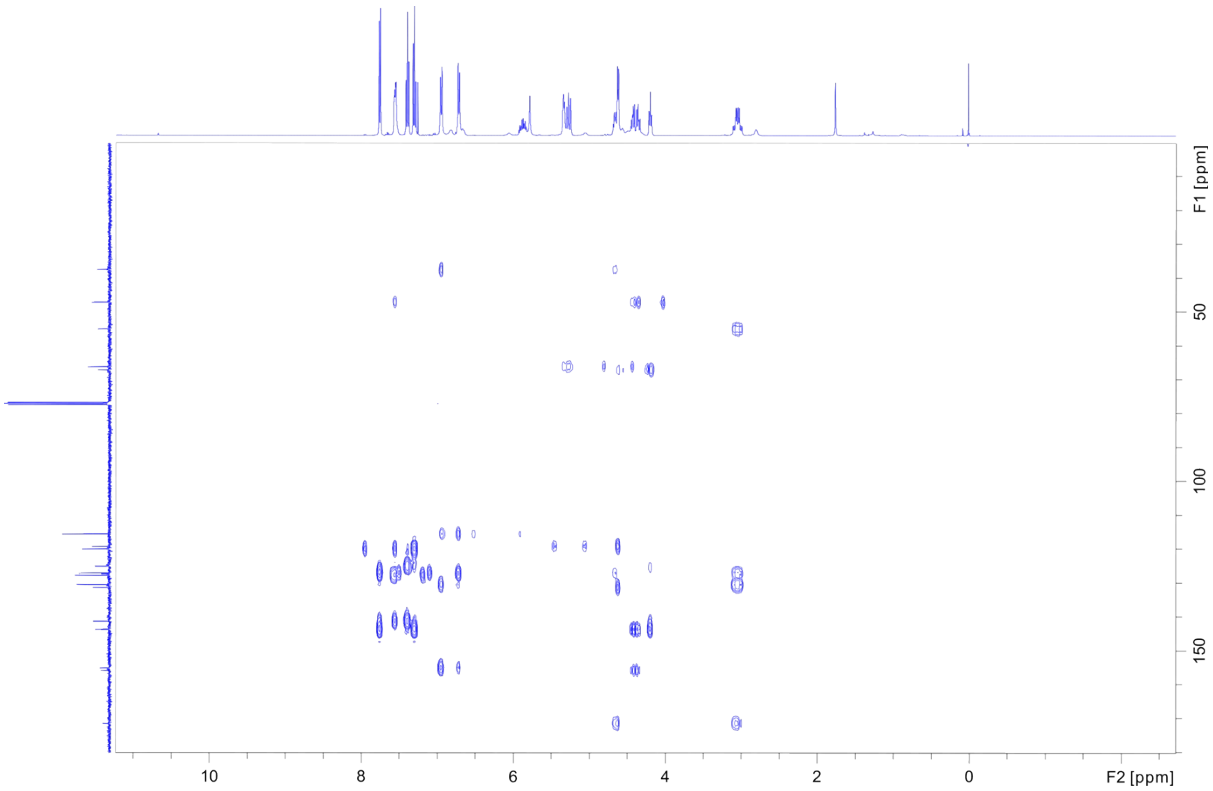


Figure S5. HMBC spectrum for **SC1**.

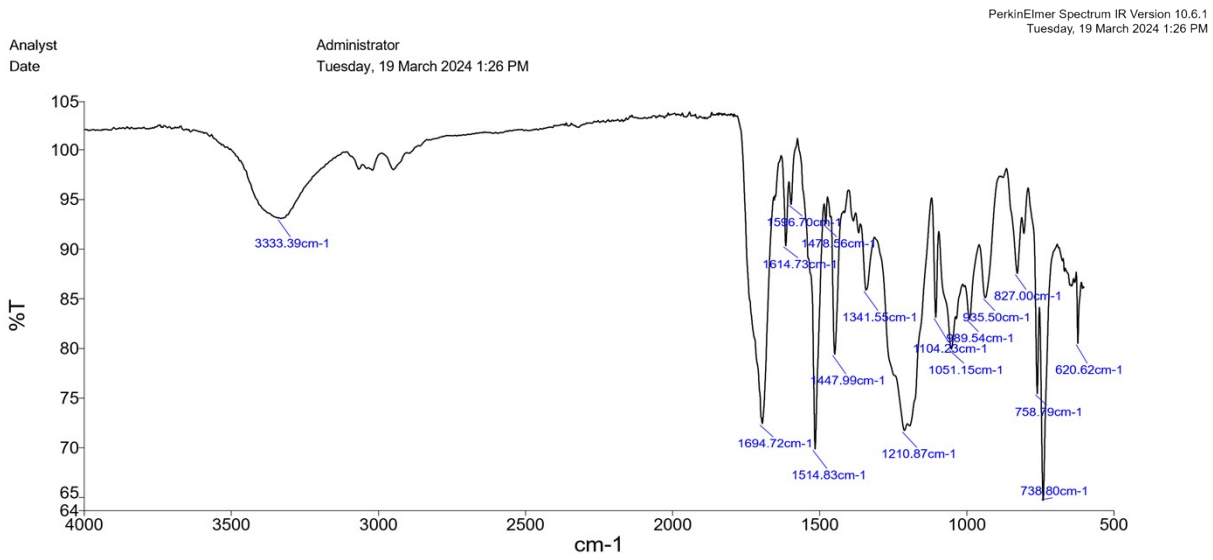
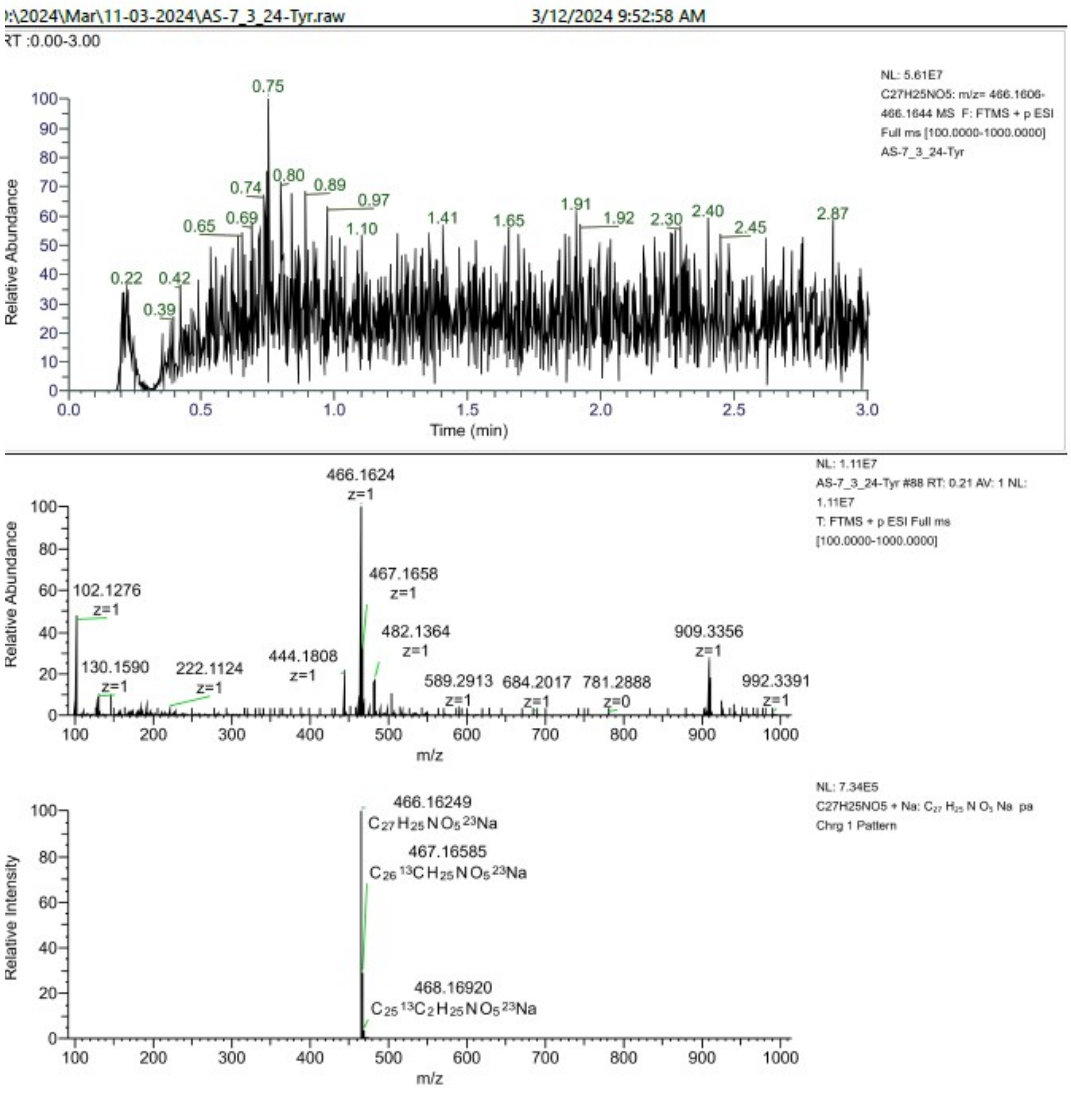


Figure S6: IR spectra for **SC1**.



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False					MDF Ranges			NaN

Figure S7: HRMS for **SC1**. HRMS (ESI+): (*m/z* [M + Na]$^+$ calcd: $C_{27}H_{25}NO_5{}^{23}Na^+$: 466.1624; found 466.1624.

General Methods

Method 1. General procedure for attachment of Fmoc-AA-Oall to 2-CTC resin:

To 2-CTC PS resin **S1** (112.0 mg, 0.1 mmol, loading: 0.64 mmol/g) pre swollen in CH_2Cl_2 (5 mL, 20 min), was added a solution Fmoc-AA-Oallyl (3.5 equiv., 0.35 mmol) and *i*-Pr_2NEt (70.0 µl, 4 equiv, 0.40 mmol) in DMF:CH_2Cl_2 (1:1, *v*/*v*, 4 mL). The reaction was agitated at room temperature for 24 h. The resin was washed with DMF (3 × 3 mL) and CH_2Cl_2 (3 × 3 mL). The resin is then treated with CH_2Cl_2:MeOH:*i*-Pr_2NEt (17:2:1, *v*/*v*/*v*, 4 mL) and agitated for 1 h at room temperature. The resin was washed with DMF (3 × 3 mL) and CH_2Cl_2 (3 × 3 mL).

Method 2a: General procedure for attachment of Fmoc Rink amide to the resin:

To TentaGel S resin **S5** (0.1 mmol, loading 0.27 mmol/g) pre-swollen in CH_2Cl_2 (5 mL, 20 min), was added 4-[(2,4-dimethoxyphenyl)(Fmoc-amino)methyl]phenoxyacetic acid (Rink amide linker) (220.2 mg, 4 equiv., 0.4 mmol) and 6-Cl-HOBt (70.0 mg, 3.5 equiv., 0.35 mmol) dissolved in DMF (1.5 mL) followed by addition of DIC (62 µL, 4 equiv., 0.4 mmol). The reaction mixture was gently agitated at room temperature for 24 h. The resin was filtered and washed with DMF (3 × 3 mL) after which a negative ninhydrin test confirmed successful coupling.

Method 2b: General procedure for attachment of 4-(4-hydroxy-3-methoxyphenoxy)butyric acid linker to the resin:

To TentaGel S resin **S5** (0.1 mmol, loading 0.27 mmol/g) pre-swollen in CH_2Cl_2 (5 mL, 20 min), was added 4-(4-hydroxy-3-methoxyphenoxy)butyric acid linker (HMPB) (100 mg, 4 equiv., 0.4 mmol) and 6-Cl-HOBt (70.0 mg, 3.5 equiv., 0.35 mmol) dissolved in DMF (1.5 mL) followed by addition of DIC (62 µL, 4 equiv., 0.4 mmol). The reaction mixture was gently agitated at room temperature for 24 h. The resin was filtered and washed with DMF (3 × 3 mL) after which a negative ninhydrin test confirmed successful coupling**.**

Method 2c. General procedure for amino acid loading on 4-(4-hydroxy-3-methoxyphenoxy)butyric acid linker loaded on resin (SPPS):

To the peptidyl resin (0.1 mmol) was added a mixture of Fmoc-AA-OH (2 equiv., 0.2 mmol), DIC (32 µL, 2 equiv., 0.2 mmol) and DMAP (5-10 mg, 0.01 equiv., 0.01 mmol) in DMF (1.5 mL). The reaction was agitated at room temperature for 1 h, filtered and repeated twice for a further 1 h with fresh reagents. The resin was washed with DMF (3 × 3 mL).

Method 3. General procedure for removal of N^{α}-Fmoc-protecting group:

The peptidyl resin was treated with a solution of 20 vol% piperidine in DMF (*v*/*v*, 4 mL) and the mixture agitated at room temperature for 5 min, filtered and repeated once for a further 5 min. The resin was washed with DMF (3 × 3 mL)

Method 4a. General procedure for r.t. HATU solid-phase peptide synthesis (SPPS):

To the peptidyl resin (0.1 mmol) was added a mixture of Fmoc-AA-OH (5 equiv., 0.5 mmol), HATU (180.0 mg, 4.7 equiv., 0.47 mmol) and *i*-Pr_2NEt (174.0 µL, 10 equiv., 1.0 mmol) in DMF (1.5 mL). The reaction was agitated at room temperature for 0.5 h. The resin was washed with DMF (3 × 3 mL).

Method 4b. General coupling procedure for microwave assisted solid-phase peptide synthesis (SPPS):

Couplings were performed using the Biotage® Initiator+ Alstra™ with the appropriate Fmoc-protected amino acid (0.2 M, DMF, 5 equiv.), HATU (0.5 M, DMF, 4.75 equiv.) and NMM in DMF (2 M, 8 equiv.) using a single coupling cycle at 75 °C, 110 W for 5 min. The resin was filtered and washed with DMF (3 × 3 mL).

Method 5: General coupling procedure for Fmoc-Arg(Pbf)-OH:

Double coupling cycles for Fmoc-Arg(Pbf)-OH was carried out with the Biotage® Initiator+ Alstra™ using Fmoc-Arg(Pbf)-OH (0.2 M, DMF, 5 equiv.), HATU (0.5 M, DMF, 4.75 equiv.) and NMM in DMF (2 M, 8 equiv.) with the

first coupling at room temperature for 25 min, followed by a second coupling with fresh reagents at 72 °C, 110 W for 5 min. The resin was filtered and washed with DMF (3 × 3 mL).

Method 6: General coupling procedure for Fmoc-Cys(Trt)-OH and Fmoc-His(Trt)-OH:

Double coupling cycles of Fmoc-Cys(Trt)-OH and Fmoc-His(Trt)-OH were carried out with the Biotage® Initiator Alstra using Fmoc protected amino acid (0.2 M, DMF, 5 equiv.), HATU (0.5 M, DMF, 4.75 equiv.) and NMM in DMF (2 M, 8 equiv.) with first coupling at room temperature for 15 min, followed by a second coupling with fresh reagents at 43 °C, 110 W for 10 min. The resin was filtered and washed with DMF (3 × 3 mL).

Method 7. General method for capping the free amino group:

Peptidyl resin (0.1 mmol) was treated with di-*tert*-butyl decarbonate (550.0 mg, 25 equiv., 2.5 mmol) and *i*-Pr_2NEt (174.0 µL, 10 equiv., 1.0 mmol) in DMF (4 mL) and the mixture was agitated at room temperature for 0.5 h. The resin was filtered and washed with DMF (3 × 3 mL).

Method 8. General method for removal of 4-allyl ester protecting group:

Peptidyl resin (0.1 mmol) was treated with $Pd(PPh_3)_4$ (231.0 mg, 2 equiv., 0.2 mmol), phenylsilane (119 µL, 9.7 equiv., 0.97 mmol) in DMF:CH_2Cl_2 (1:1, *v*/*v*, 3.5 mL) for 3 h at room temperature. The resin was washed with DMF (3 × 3 mL). The resin is then treated with sodium diethyldithiocarbamate 0.5% w/v (500 mg/ 10 mL) and agitated for 5 min at room temperature and washed with DMF (3 × 3 mL) and CH_2Cl_2 (3 × 3 mL).

Method 9. General method DPS/PPh_3 activation:

In a dry inert vessel 2,2'-dipyridyldisulfide (90.0 mg, 4 equiv., 0.4 mmol) and PPh_3 (104 mg, 4 equiv., 0.4 mmol) in anhydrous CH_2Cl_2 (1.0 mL) is agitated at room temperature for 1 h under argon.

Method 10. General method for synthesis of *in-situ* C^{α}-terminal 2-pyridinethiol ester:

To free carboxylate C^{α}-terminal peptidyl resin (0.1 mmol) preswollen in anhydrous CH_2Cl_2 is added 0.5 mL anhydrous DMF and 6-Cl-HOBt (51.0 mg, 2.5 equiv., 0.25 mmol) followed by addition of pre-activated 1 mL solution **2** from **Method 9** and agitated at room temperature for 1 h under argon.

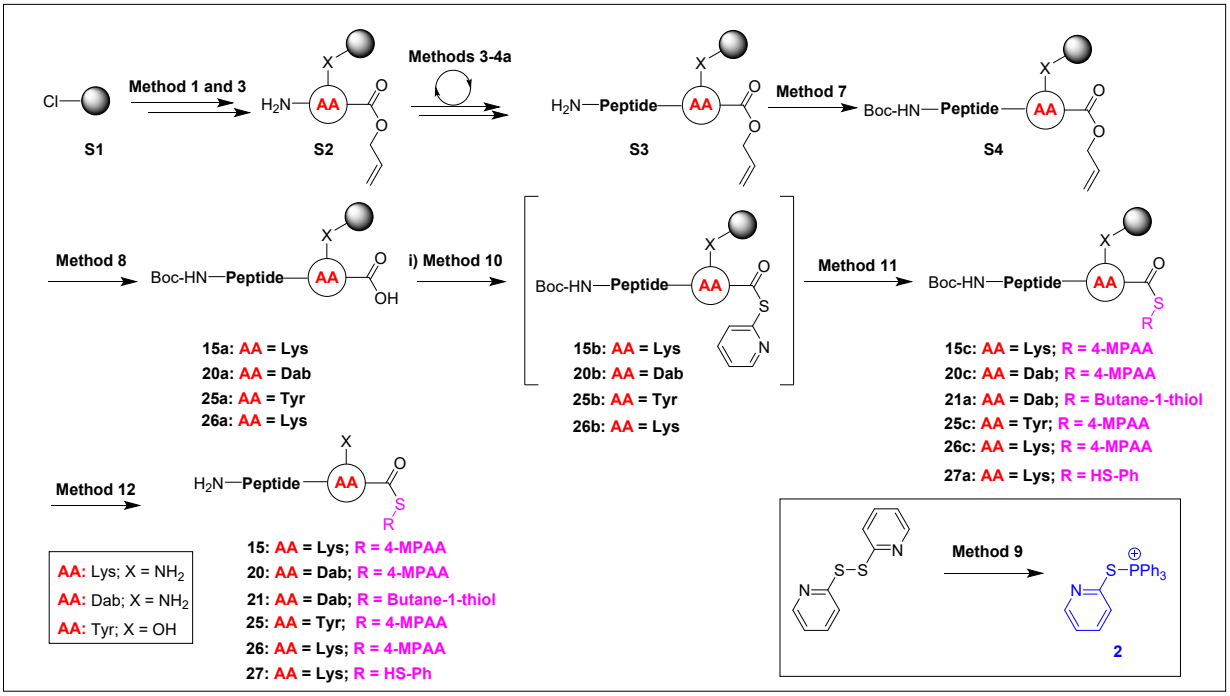
Method 11. General method for C^{α}-terminal thioesterification:

To peptidyl solution (0.1 mmol) is added thiol (4 equiv., 0.4 mmol) and *i*-Pr_2NEt (140.0 µL, 8 equiv., 0.4 mmol) and agitated at room temperature for 16 h under argon. The resin was filtered and washed with DMF (3 × 3 mL) and CH_2Cl_2 (3 × 3 mL). Analysis of reaction may necessitate double coupling under same **Methods 9**-**11**.

Method 12: General procedure for TFA-mediated resin cleavage and global deprotection:

Peptidyl resin was treated with a mixture of TFA/H_2O/TIPS/EDT (94:2.5:2.5:1, *v*/*v*/*v*/*v*, 10 mL) for 2 h. The filtrate was partially concentrated under a gentle stream of N_2, then cold diethyl ether was then added to form a precipitate. The mixture was centrifuged, and the solution was carefully decanted off and discarded, before dissolving the solid pellet in H_2O:acetonitrile (1:1, *v*/*v*, 25 mL) containing 0.1% TFA and lyophilised.

Synthesis of C^{α}-thioesters on 2-CTC Resin Using Fmoc-SPPS



Scheme S1. General synthetic scheme for C^{α}-thioesterification on 2-CTC.

Fmoc-(L)-Lys-Oallyl, Fmoc-(L)-Dab-Oallyl or in house synthesised Fmoc-(L)-Tyr-Oallyl (**SC1**) was attached to 2-chlorotrityl chloride PS resin (2-CTC) resin **S1** using **Method 1** followed by Fmoc-removal using **Method 3** affording **S2**. Direct attachment of second amino acid to resin bound (2-CTC) **S2** was achieved using **Method 4a**. **Method 3** was used for all subsequent N^{α}-Fmoc removals. Linear elongation of the peptide chain was achieved by coupling appropriate Fmoc-amino acids up to final target *N*-terminal position **S3** indicated in **Scheme S1** using **Method 4a**. Boc protection of free amino group **S3** at *N*-terminus was conducted using **Method 7**. Selective allyl ester removal of **S4** was achieved using **Method 8** affording (**15a**, **20a**, **25a**, **26a**; **Figure S8/Figure S9/Figure S11-S12/Figure S13-14**). Peptide (**15a**, **20a**, **25a**, **26a**) was then subjected **Method 10** followed by addition of preactivated DPS/PPh_3 complex **2** generated from **Method 9** affording *in-situ* (**15b**, **20b**, **25b**, **26b**). Peptide (**15b**, **20b**, **25b**, **26b**) is treated with **Method 11** affording (**15c**, **20c**, **21a**, **25c**, **26c**, **27a**) followed by acidolytic cleavage using **Method 12** affording crude (**15**, **20**, **21**, **25**, **26**, **27**; **Figure S15-16/Figure S17/Figure S18/Figure S19-20/Figure S21-22/Figure S23-24**). Crude materials are purified batchwise by semi-preparative RP-HPLC on a Xterra® Prep MS C18 column, (19 × 300 mm, 10 µm) using a linear gradient of 5% to 95% over 90 min (*ca.* 1% B/min) with a flow rate of 4 mL/min. Fractions were collected at 0.2 min intervals and analysed by ESI-MS and RP-HPLC. Fractions identified with correct *m*/*z* were combined and lyophilised to afford the *title compounds* as white amorphous solids.

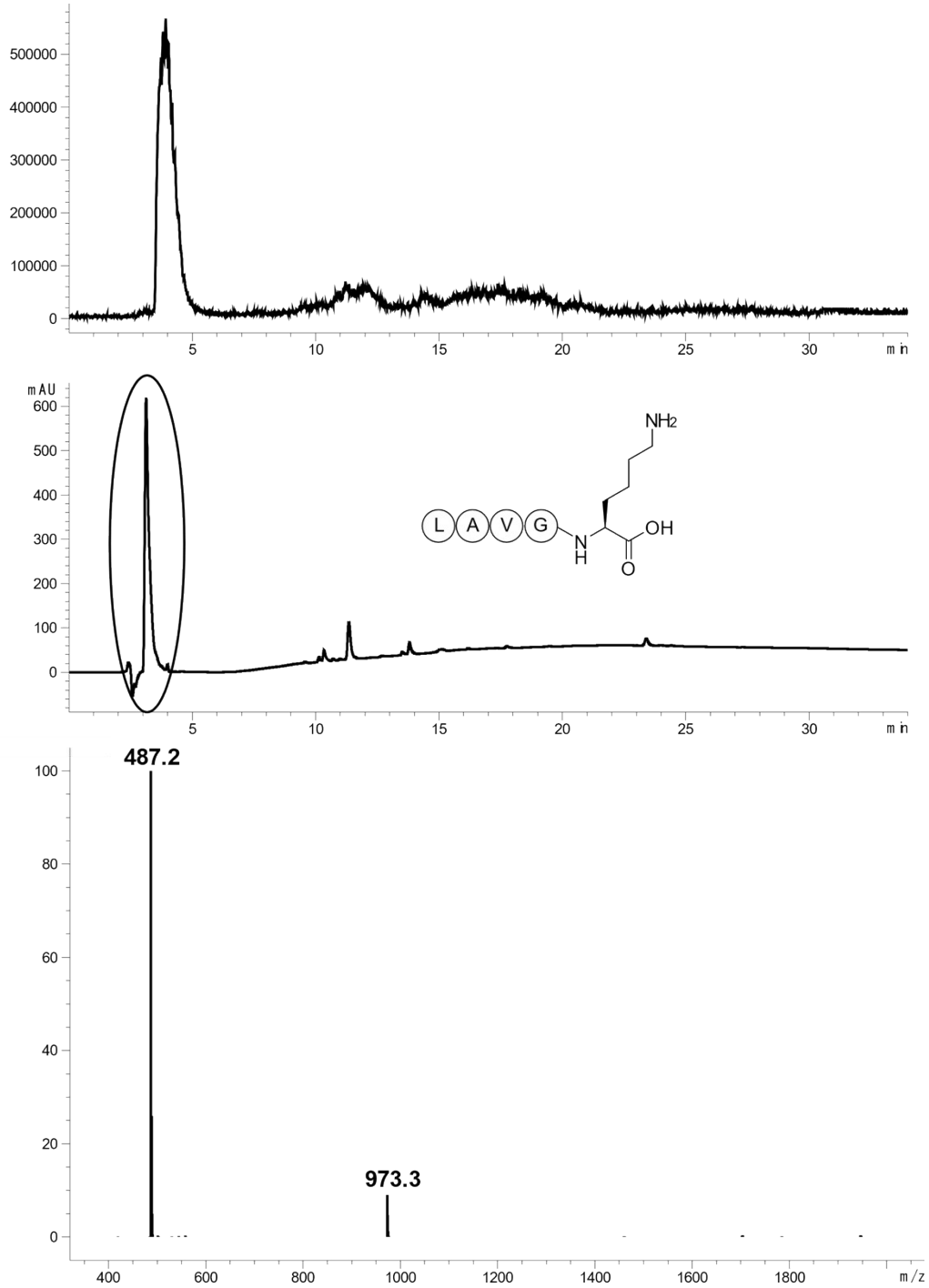


Figure S8: LC-MS profile of crude deprotected peptide LAVG-(L)-Lys-COOH (**15a**); ion polarity positive operating at a nominal accelerating voltage of 70 eV. ESI-MS *m/z* $[M+1H]^{1+}$ calcd: 487.6 Da; found: 487.2 Da; $[2M+1H]^{1+}$ calcd: 974.1 Da; found 973.3 Da. Theoretical molecular weight: 486.6 Da. Chemical formula: $C_{22}H_{42}N_6O_6$.

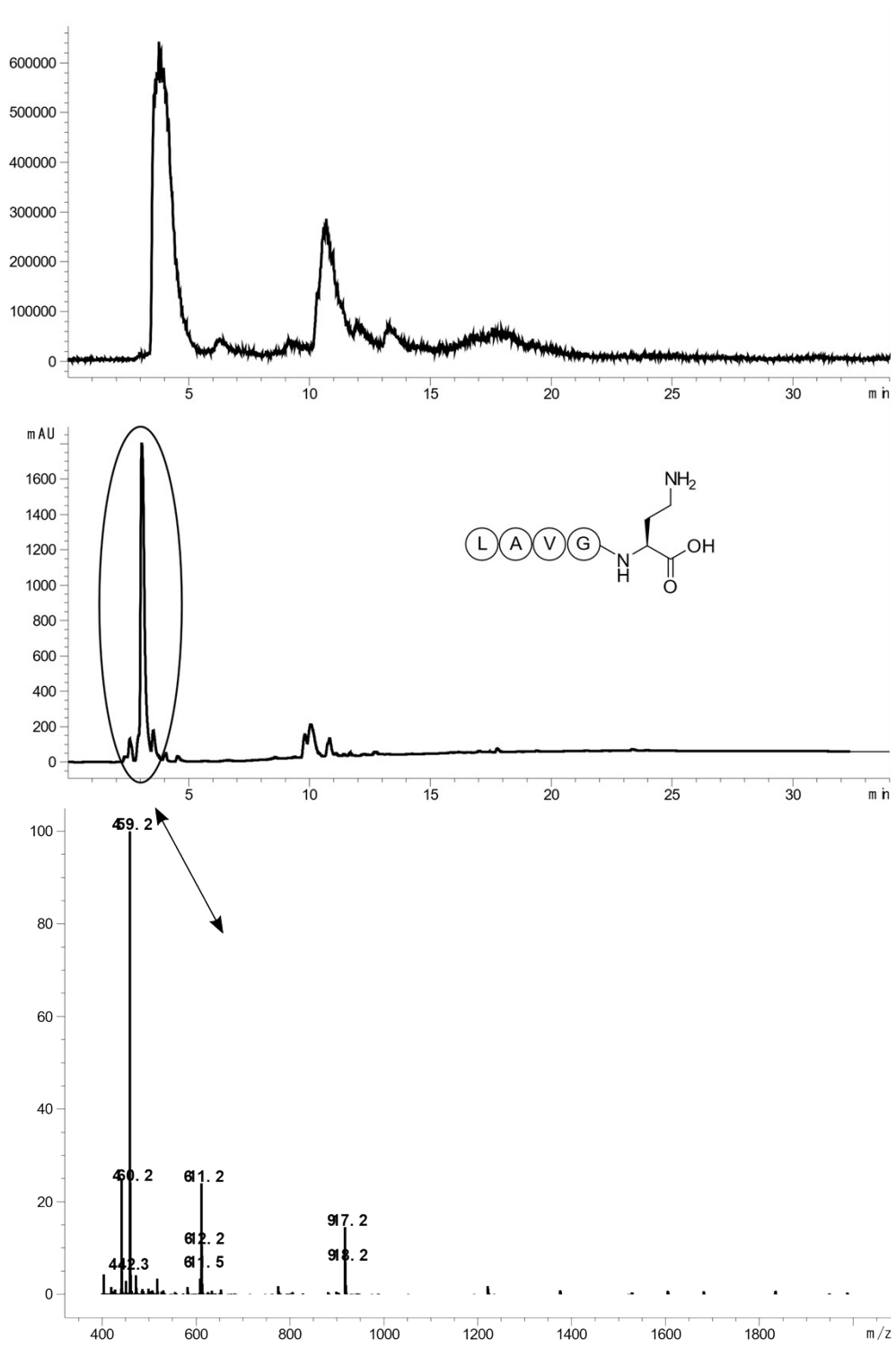


Figure S9: LC-MS profile of crude deprotected peptide LAVG-(L)-Dab-COOH (**20a**); ion polarity positive operating at a nominal accelerating voltage of 70 eV. ESI-MS *m/z* $[M+1H]^{1+}$ calcd: 459.5 Da; found: 459.2 Da; $[2M+1H]^{1+}$ calcd: 918.1 Da; found 917.2 Da. Theoretical molecular weight: 458.6 Da. Chemical formula: $C_{20}H_{38}N_6O_6$.

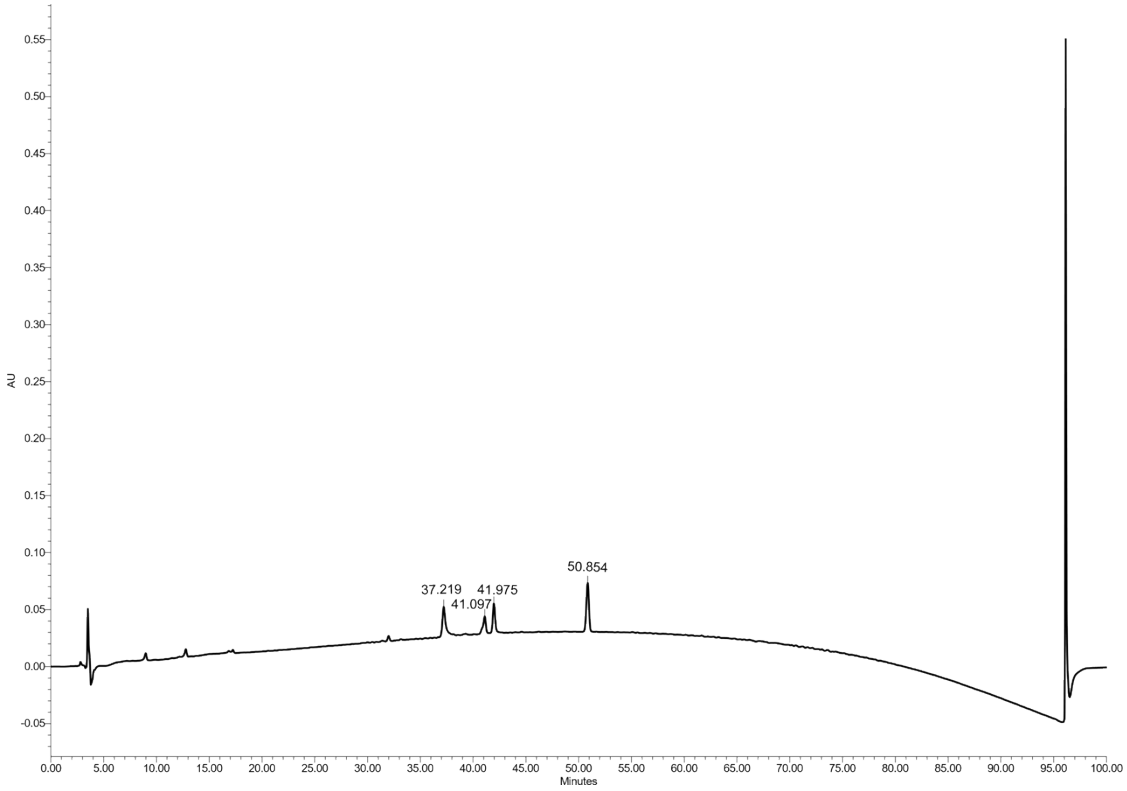


Figure S10: Analytical RP-HPLC chromatogram of **Blank**. Chromatographic separations were performed on a Waters e2695 HPLC using a Agilent TC-C18 column (5 µm; 4.6 × 250 mm) and a linear gradient of 5-95% B in 90 min at room temperature, *ca*. 1% B per min at a flow rate of 1.0 mL/min. Buffer A: H_2O containing 0.1% TFA (*v/v*); Buffer B: acetonitrile containing 0.1% TFA (*v/v*).

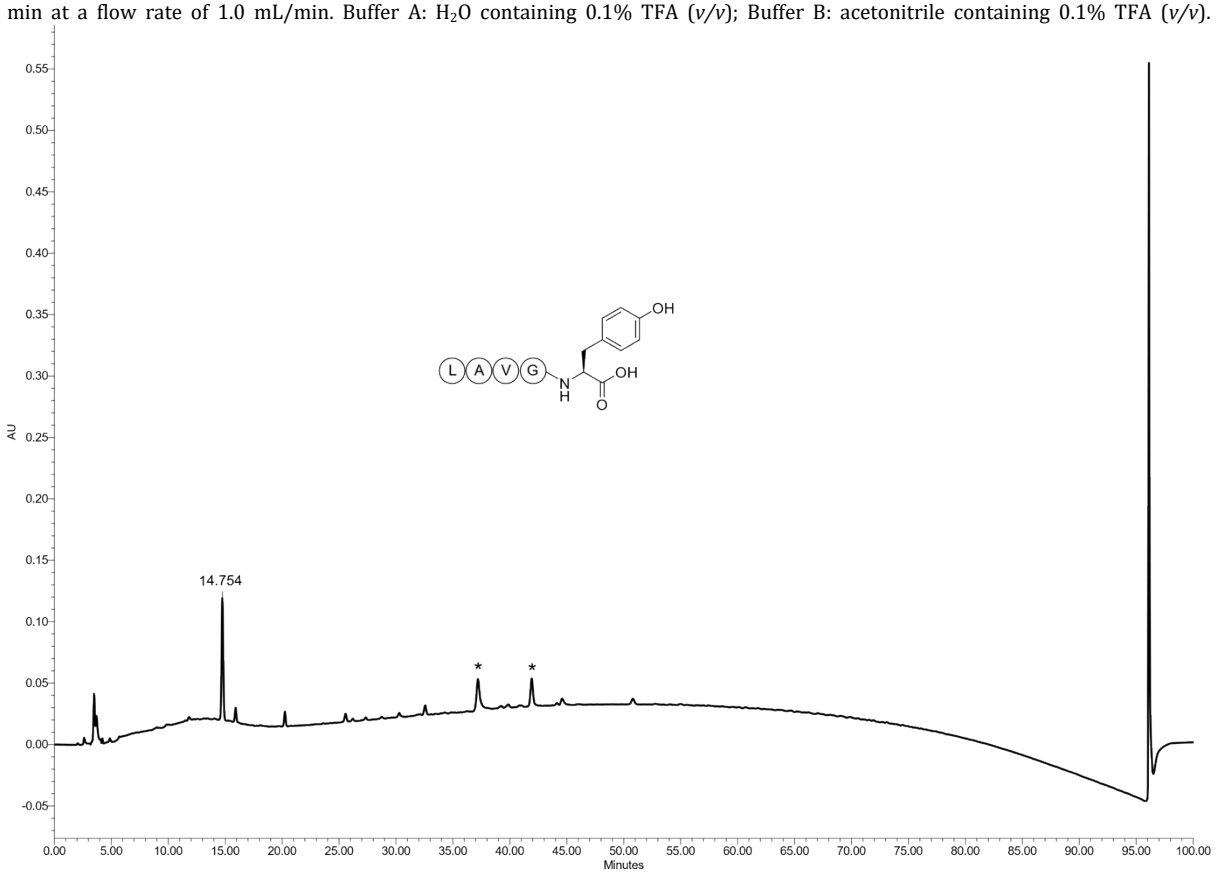


Figure S11: Analytical RP-HPLC chromatogram of crude deprotected peptide LAVG-(L)-Tyr-COOH **25a,** t_R = 14.7 min. Chromatographic separations were performed on a Waters e2695 HPLC using a Agilent TC-C18 column (5 µm; 4.6 × 250 mm) and a linear gradient of 5-95% B in 90 min at room temperature, *ca*. 1% B per min at a flow rate of 1.0 mL/min. Buffer A: H_2O containing 0.1% TFA (*v/v*); Buffer B: acetonitrile containing 0.1% TFA (*v/v*).* Peaks present in blank.

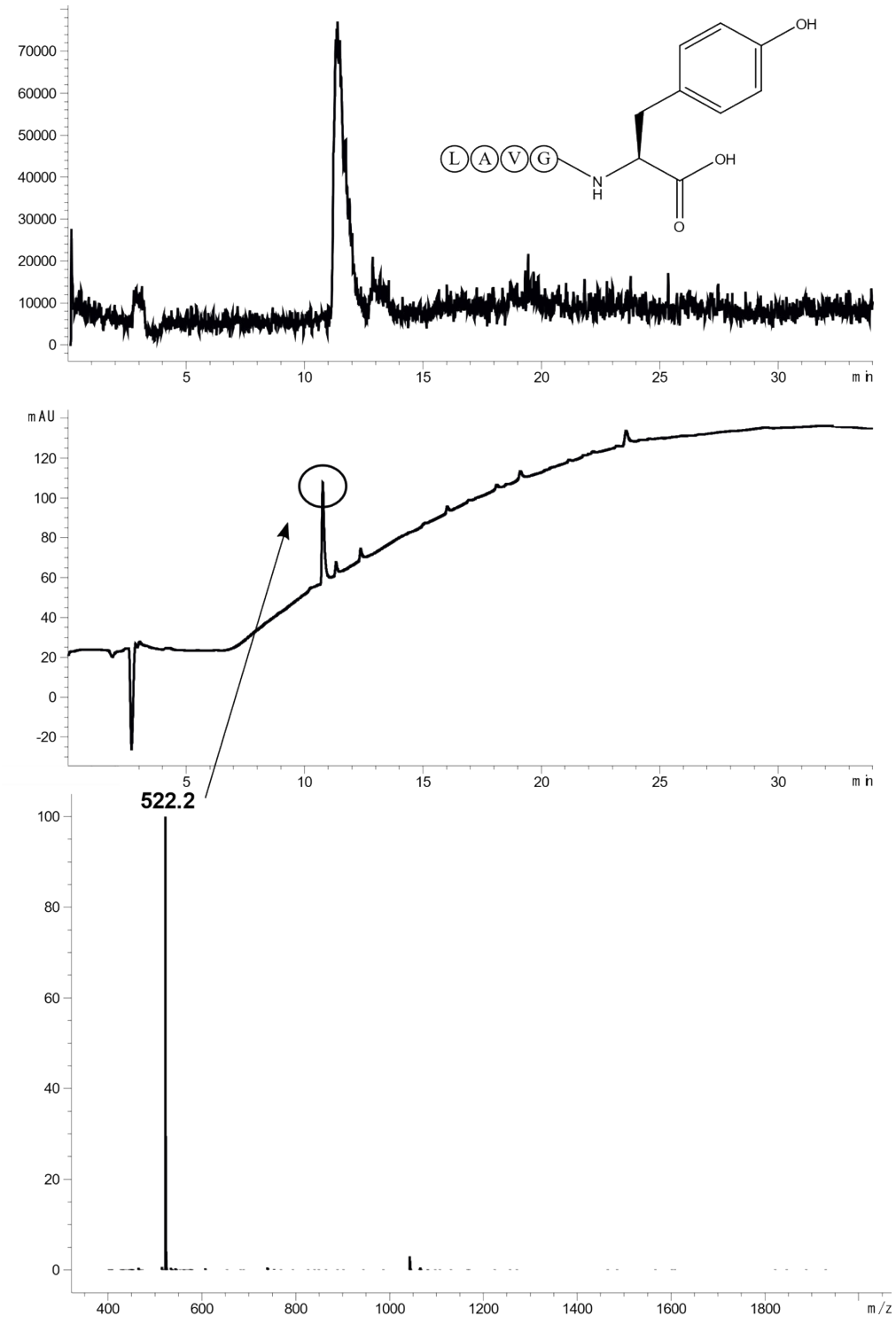


Figure S12: LC-MS profile of crude deprotected peptide LAVG-(L)-Tyr-COOH **25a**; ion polarity positive operating at a nominal accelerating voltage of 70 eV. ESI-MS *m/z* $[M+1H]^{1+}$ calcd: 522.6 Da; found: 522.2 Da. Theoretical molecular weight: 521.6 Da. Chemical formula: $C_{25}H_{39}N_5O_7$.

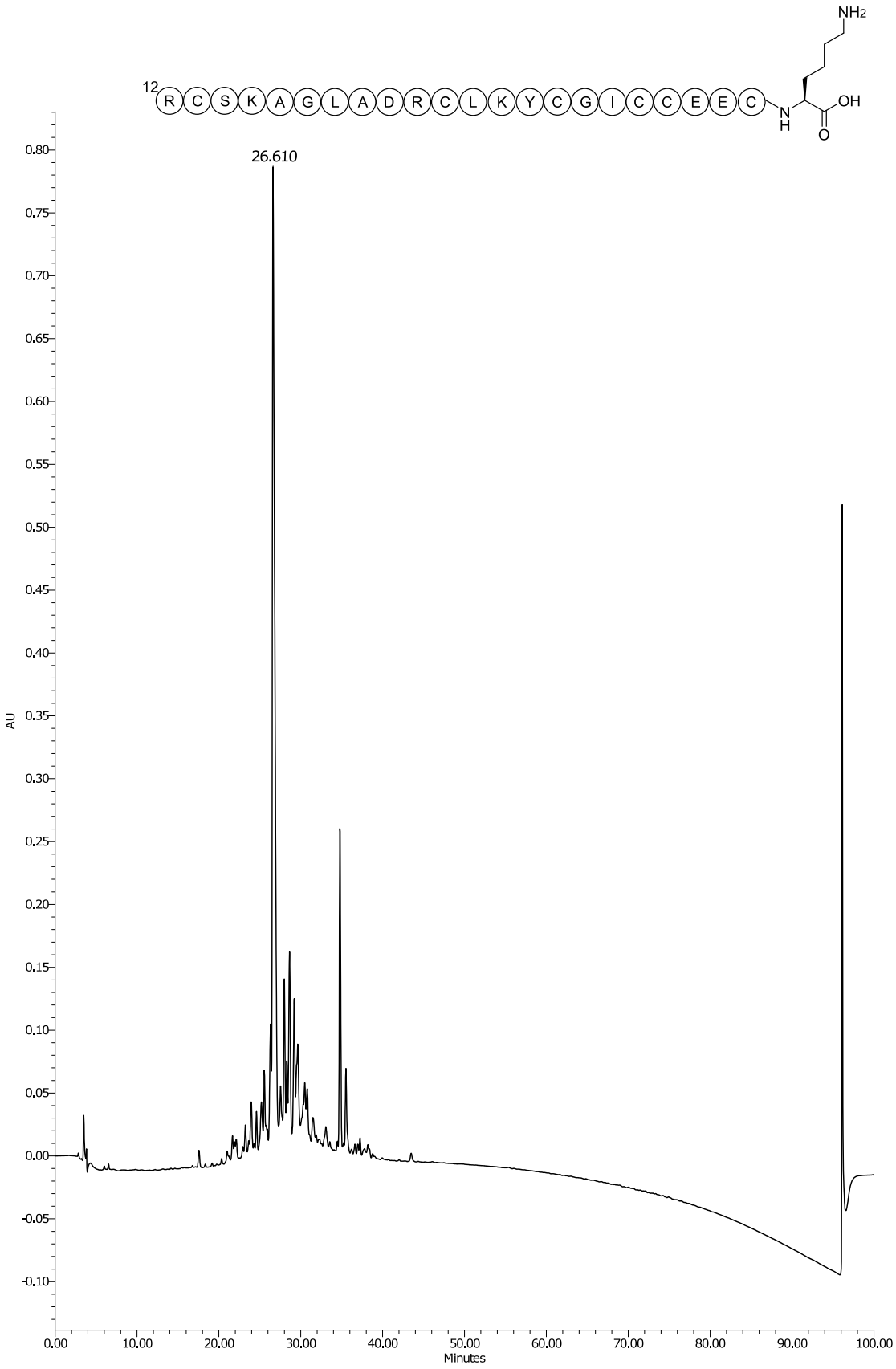


Figure S13: Analytical RP-HPLC chromatogram of crude deprotected peptide Snakin-1 (12-33)-Lys-COOH (**26a**), t_R = 26.6 min. Chromatographic separations were performed on a Waters e2695 HPLC using a Agilent TC-C18 column (5 µm; 4.6 × 250 mm) and a linear gradient of 5-95% B in 90 min at room temperature, *ca*. 1% B per min at a flow rate of 1.0 mL/min. Buffer A: H_2O containing 0.1% TFA (*v/v*); Buffer B: acetonitrile containing 0.1% TFA (*v/v*).

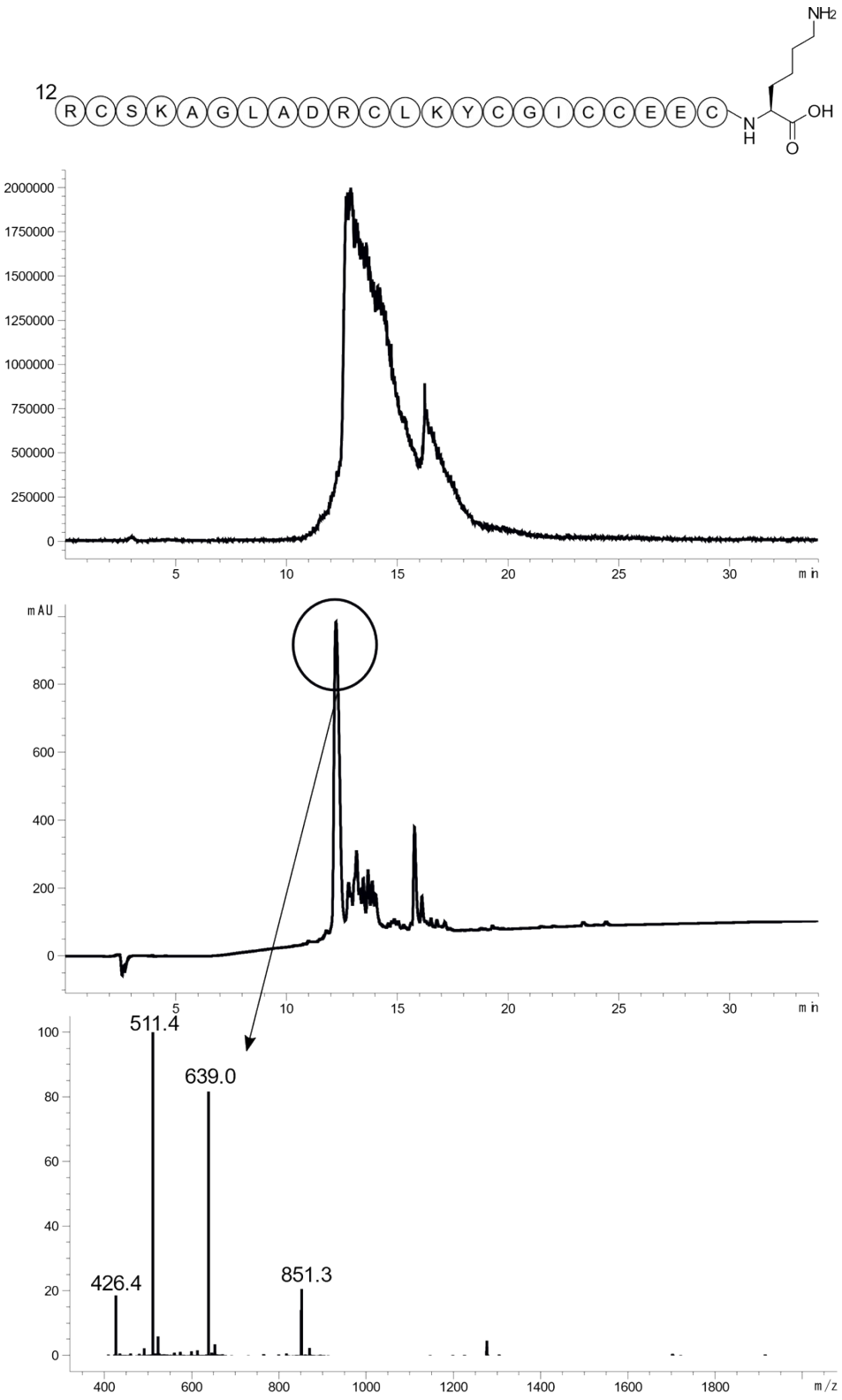


Figure S14: LC-MS profile of crude deprotected peptide Snakin-1 (12-33)-Lys-COOH (**26a**); ion polarity positive operating at a nominal accelerating voltage of 70 eV. ESI-MS *m/z* $[M+3H]^{3+}$ calcd: 852.0 Da; found: 851.3 Da; $[M+4H]^{4+}$ calcd: 639.3 Da; found: 639.0 Da; $[M+5H]^{5+}$ calcd: 511.6 Da; found: 511.4 Da; $[M+6H]^{6+}$ calcd: 426.5 Da; found: 426.4 Da. Theoretical molecular weight: 2553.1 Da. Chemical formula: $C_{102}H_{174}N_{32}O_{32}S_6$.

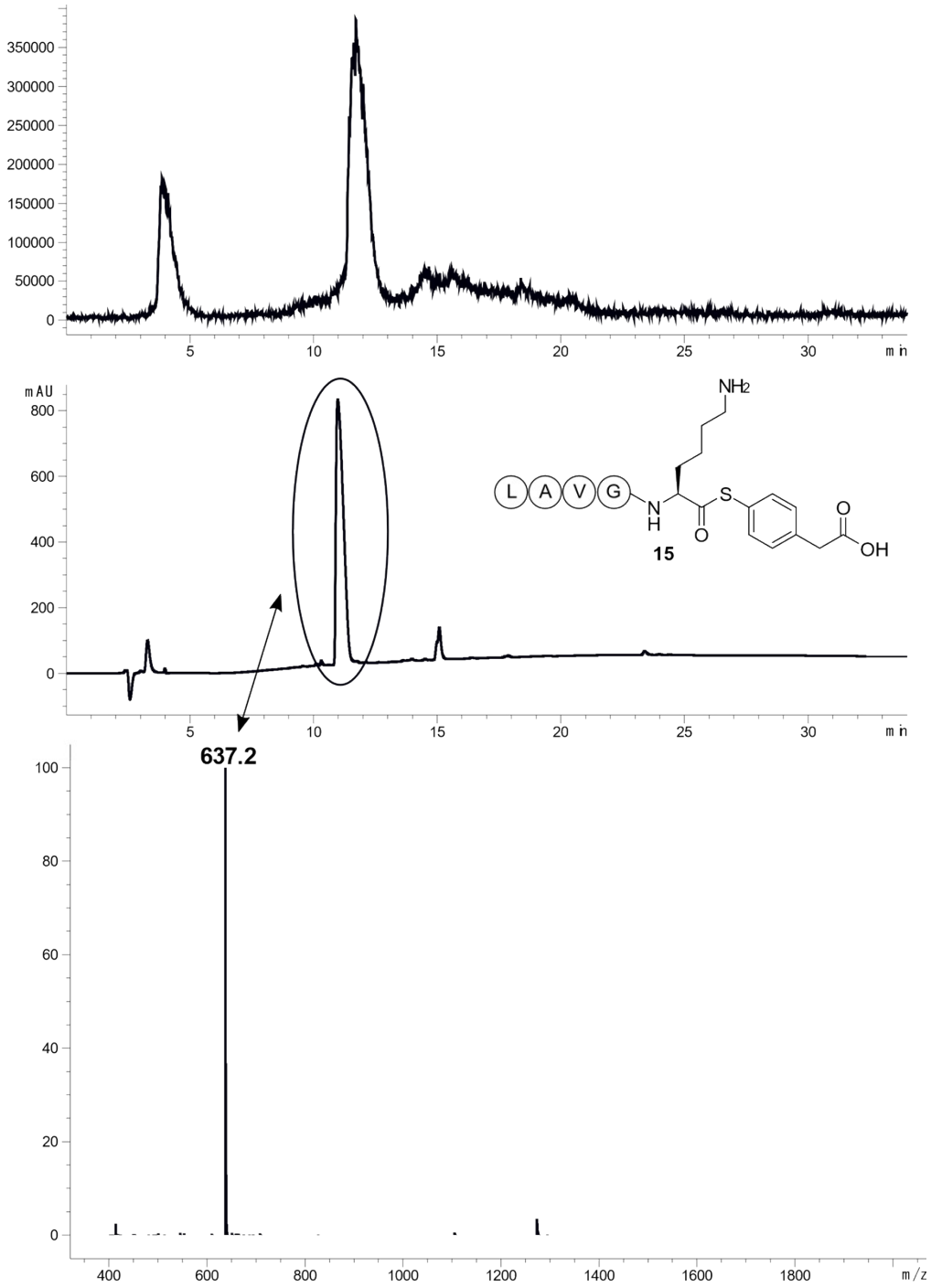


Figure S15: LC-MS profile of crude peptide LAVG-(L)-Lys-4-MPAA (**15**); ion polarity positive operating at a nominal accelerating voltage of 70 eV. ESI-MS *m/z* $[M+1H]^{1+}$ calcd: 637.8 Da; found: 637.2 Da. Theoretical molecular weight: 636.8 Da. Chemical formula: $C_{30}H_{48}N_6O_7S$.

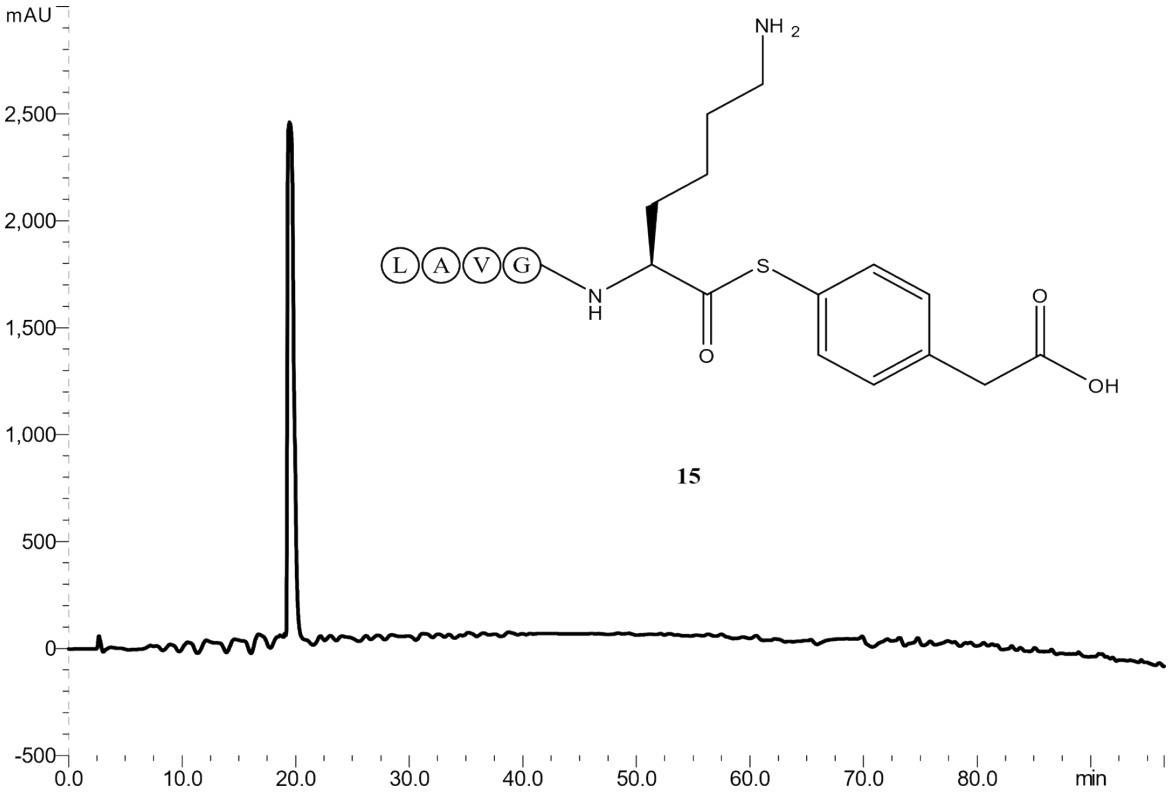


Figure S16: Analytical RP-HPLC chromatogram of purified peptide LAVG-(L)-Lys-4-MPAA (**15**), t_R = 19.4 min. Chromatographic separations were performed on a Thermo Scientific Dionex Ultimate 3000 HPLC using a Phenomenex® Gemini MS C-18 column (5 µm; 4.6 × 150 mm) and a linear gradient of 5-95% B in 90 min at room temperature, *ca*. 1% B per min at a flow rate of 1.0 mL/min. Buffer A: H_2O containing 0.1% TFA (*v/v*); Buffer B: acetonitrile containing 0.1% TFA (*v/v*).

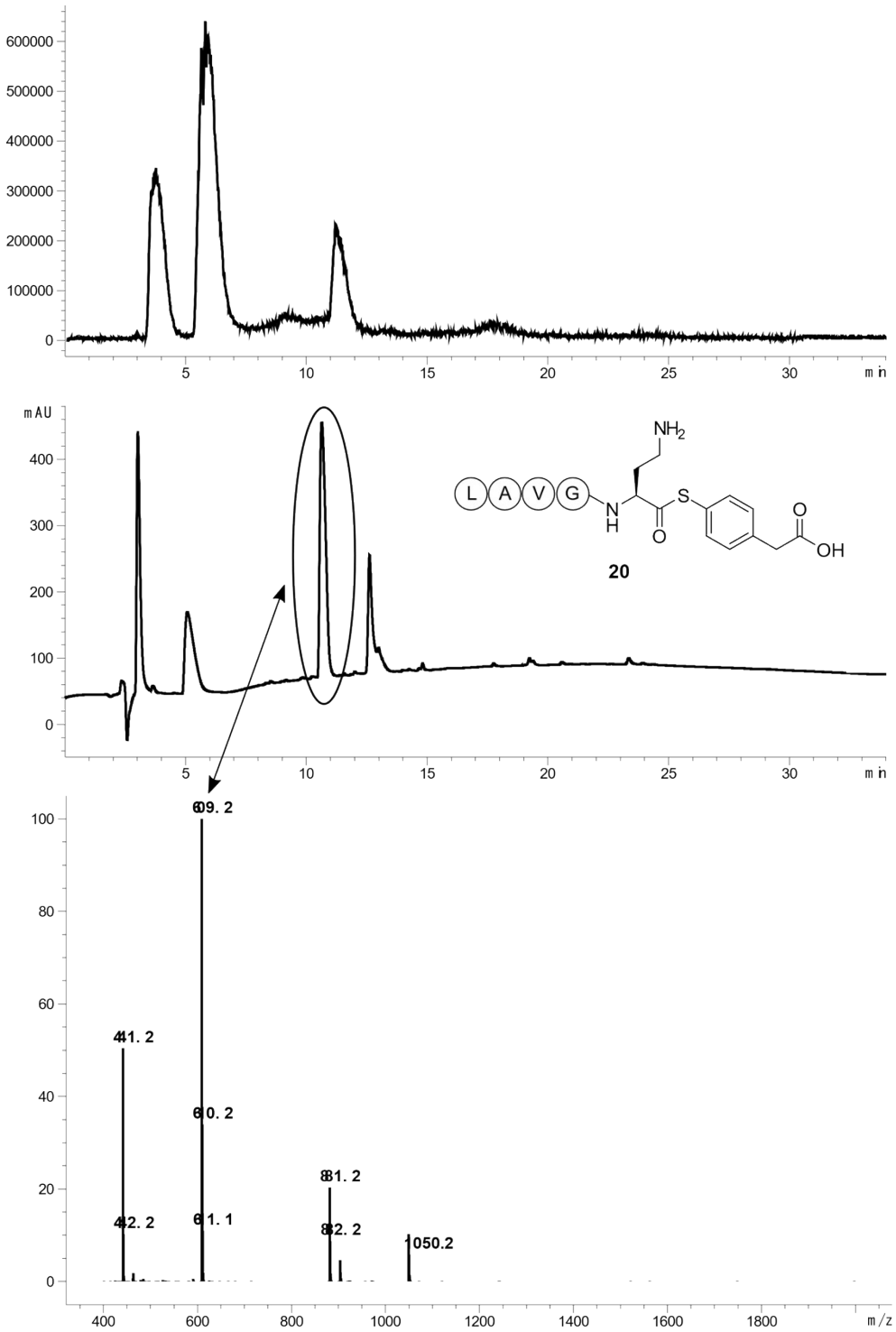


Figure S17: LC-MS profile of crude peptide LAVG-(L)-Dab-4-MPAA (**20**); ion polarity positive operating at a nominal accelerating voltage of 70 eV. ESI-MS *m/z* $[M+1H]^{1+}$ calcd: 609.7 Da; found: 609.2 Da. Theoretical molecular weight: 608.7 Da. Chemical formula: $C_{28}H_{44}N_6O_7S$. Peak at 441.2 Da corresponds to fragmentation product $C_{20}H_{37}N_6O_5^{\cdot}$ calcd: at 441.5 Da, 882.2 Da corresponds to dimer product of $C_{20}H_{37}N_6O_5^{\cdot}$ calcd: 883 Da.

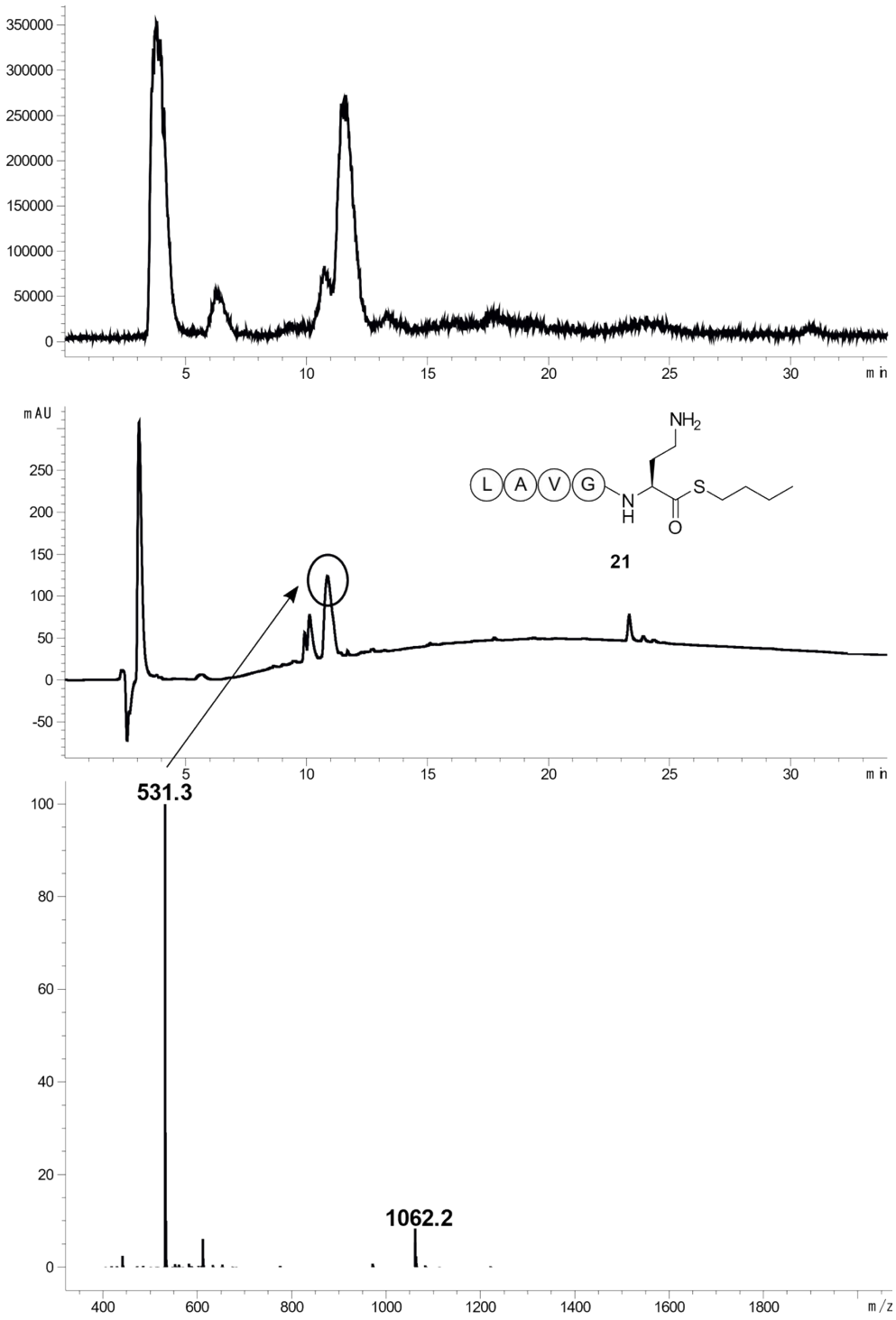


Figure S18: LC-MS profile of crude peptide LAVG-(L)-Dab-butane-1-thiol (**21**); ion polarity positive operating at a nominal accelerating voltage of 70 eV. ESI-MS *m/z* $[M+1H]^{1+}$ calcd: 531.7 Da; found: 531.3 Da; $[2M+1H]^{1+}$ calcd: 1062.4 Da; found 1062.2 Da. Theoretical molecular weight: 530.7 Da. Chemical formula: $C_{24}H_{46}N_6O_5S$. * Peak at t_R = 10 min column artifact.

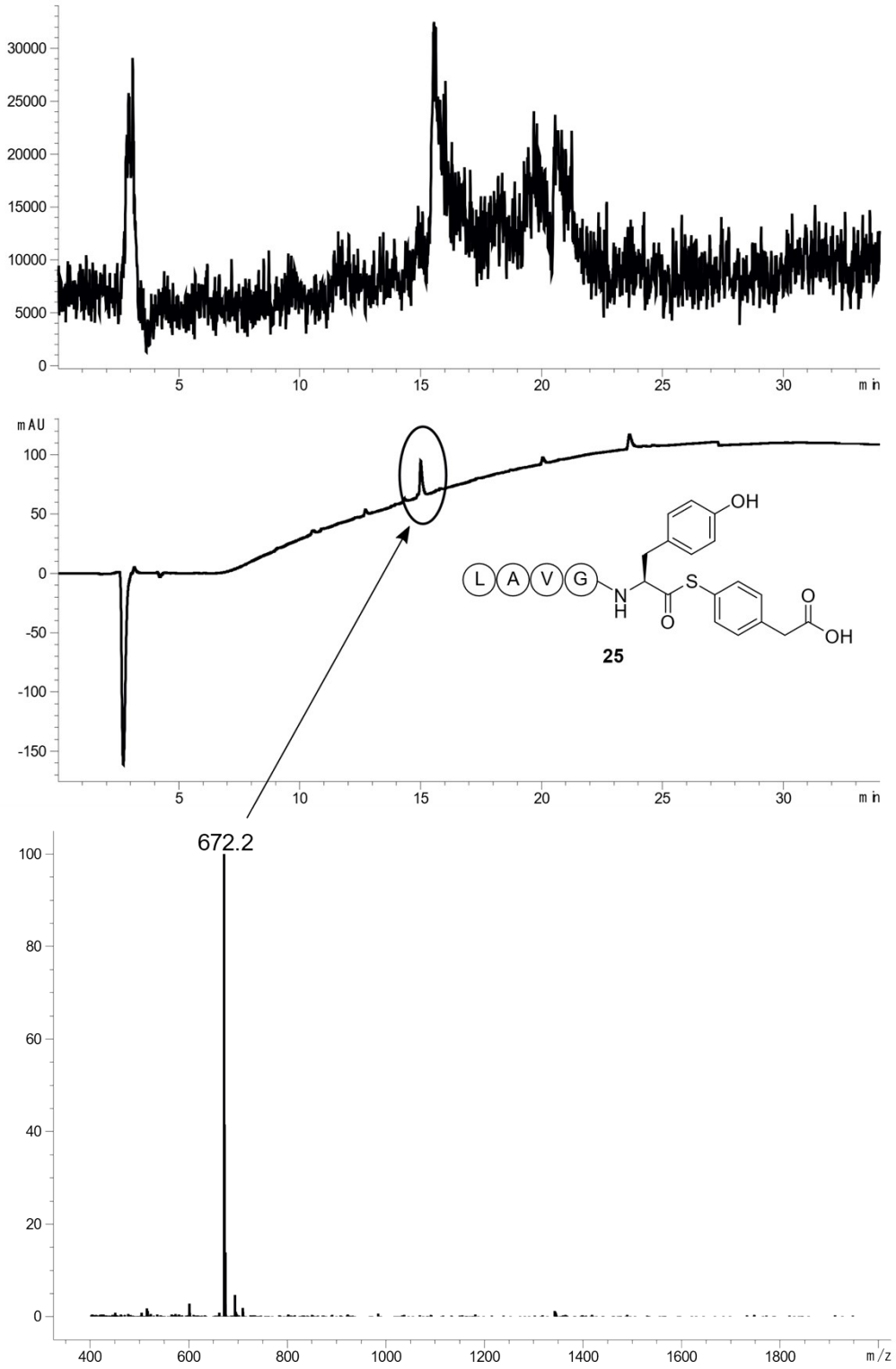


Figure S19: LC-MS profile of crude peptide LAVG-(L)-Tyr-4-MPAA (**25**); ion polarity positive operating at a nominal accelerating voltage of 70 eV. ESI-MS *m/z* $[M+1H]^{1+}$ calcd: 672.8 Da; found: 672.2 Da. Theoretical molecular weight: 671.8 Da. Chemical formula: $C_{33}H_{45}N_5O_8S$.

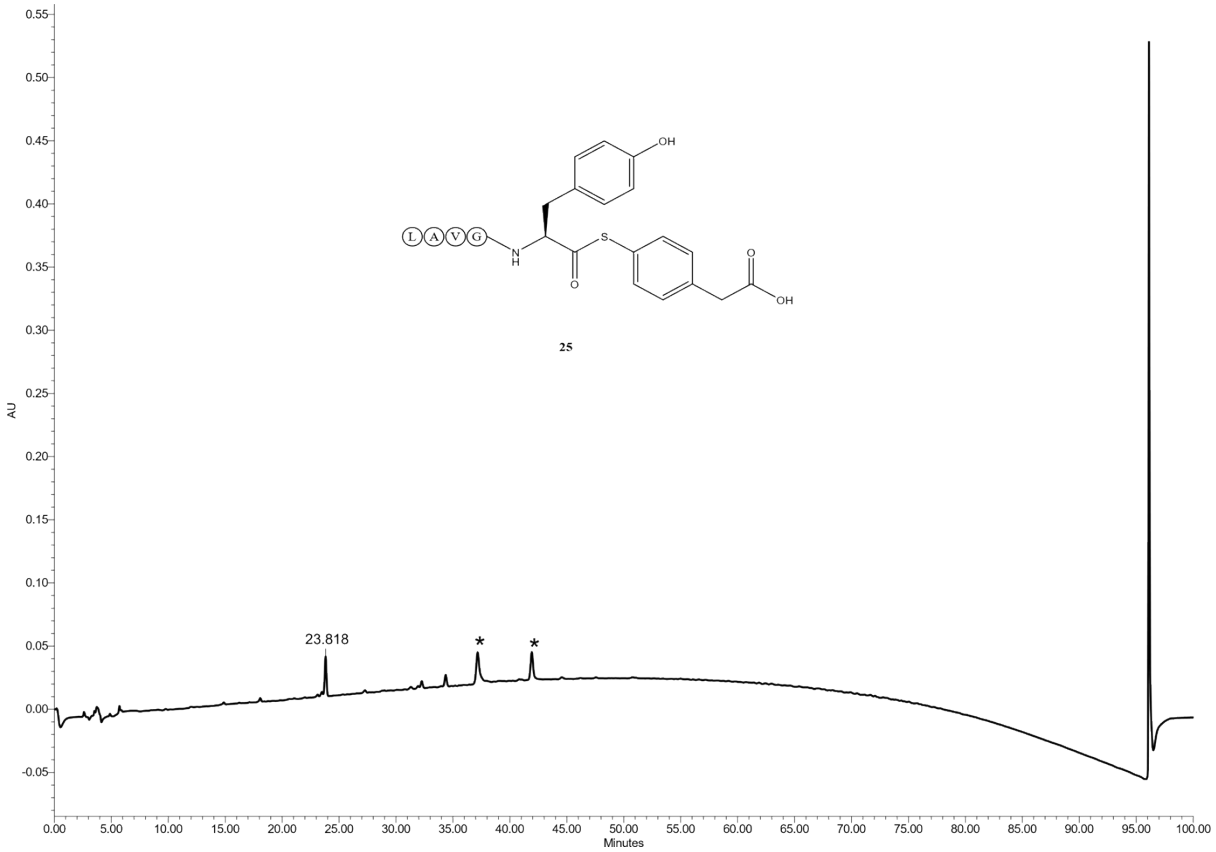


Figure S20: Analytical RP-HPLC chromatogram of crude peptide LAVG-(L)-Tyr-4-MPAA (**25**), t_R = 23.8 min. Chromatographic separations were performed on a Waters e2695 HPLC using a Agilent TC-C18 column (5 µm; 4.6 × 250 mm) and a linear gradient of 5-95% B in 90 min at room temperature, *ca*. 1% B per min at a flow rate of 1.0 mL/min. Buffer A: H_2O containing 0.1% TFA (*v/v*); Buffer B: acetonitrile containing 0.1% TFA (*v/v*). * Peaks present in blank Figure S10 and Figure S11

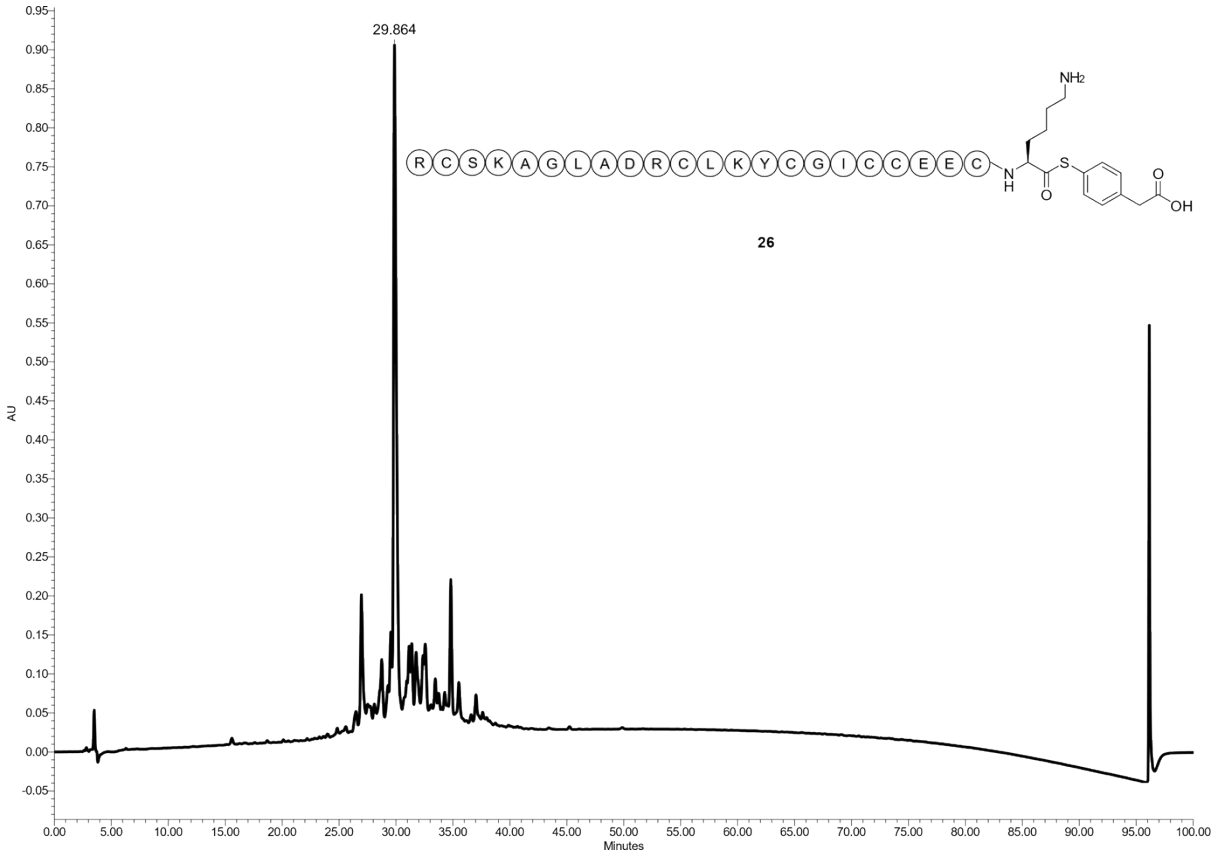


Figure S21: Analytical RP-HPLC chromatogram of crude peptide Snakin-1 (12-33)-Lys-4-MPAA (**26**) from table 1 in main manuscript trial 16[b], t_R = 29.8 min. Chromatographic separations were performed on a Waters e2695 HPLC using a Agilent TC-C18 column (5 µm; 4.6 × 250 mm) and a linear gradient of 5-95% B in 90 min at room temperature, *ca*. 1% B per min at a flow rate of 1.0 mL/min. Buffer A: H_2O containing 0.1% TFA (*v/v*); Buffer B: acetonitrile containing 0.1% TFA (*v/v*).

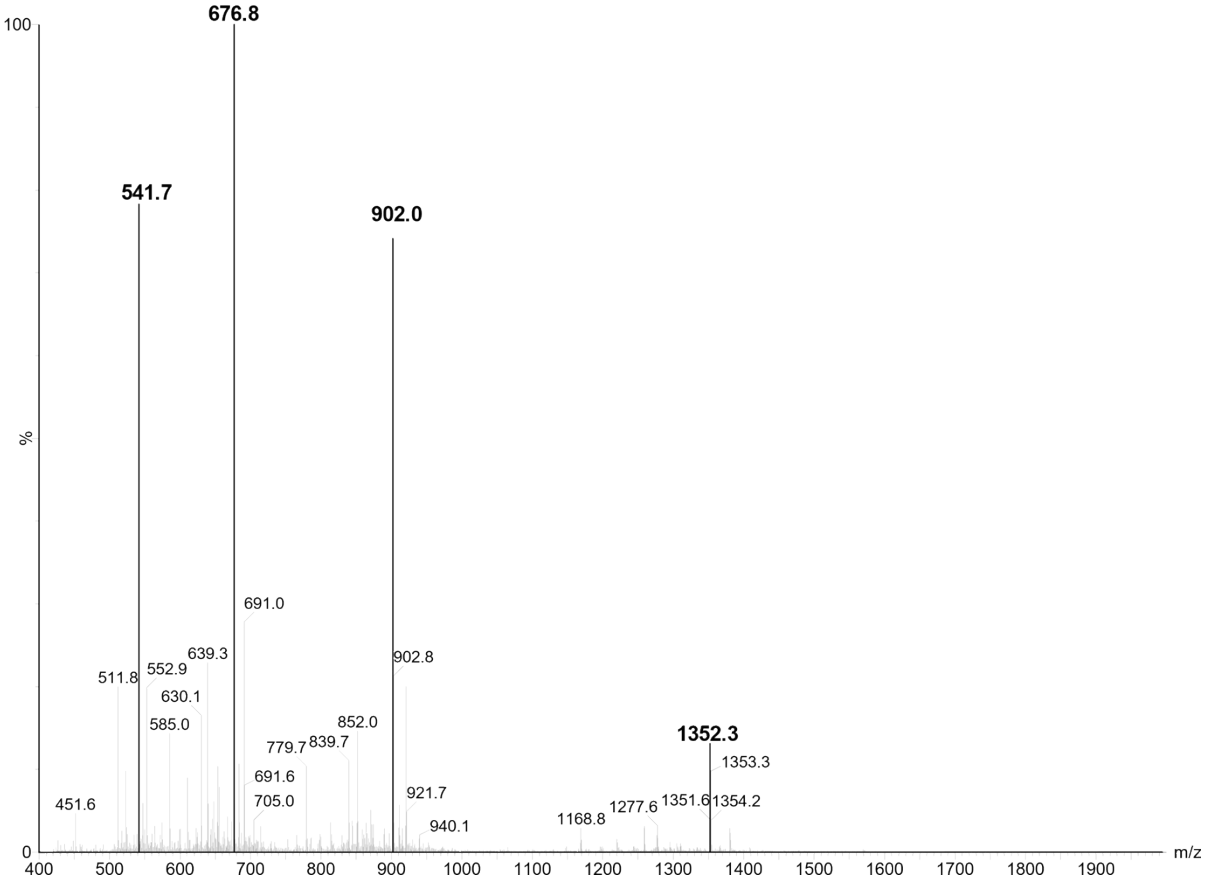


Figure S22: ESI-MS of crude Snakin-1 (12-33)-Lys-4-MPAA (**26**). m/z $[M+2H]^{2+}$ calcd: 1352.6 Da; found: 1352.3 Da; $[M+3H]^{3+}$ calcd: 902.1 Da; found: 902.0 Da; $[M+4H]^{4+}$ calcd: 676.8 Da; found: 676.8 Da; $[M+5H]^{5+}$ calcd: 541.6 Da; found: 541.7 Da. Theoretical molecular weight: 2703.26 Da. Chemical formula: $C_{110}H_{180}N_{32}O_{33}S_7$.

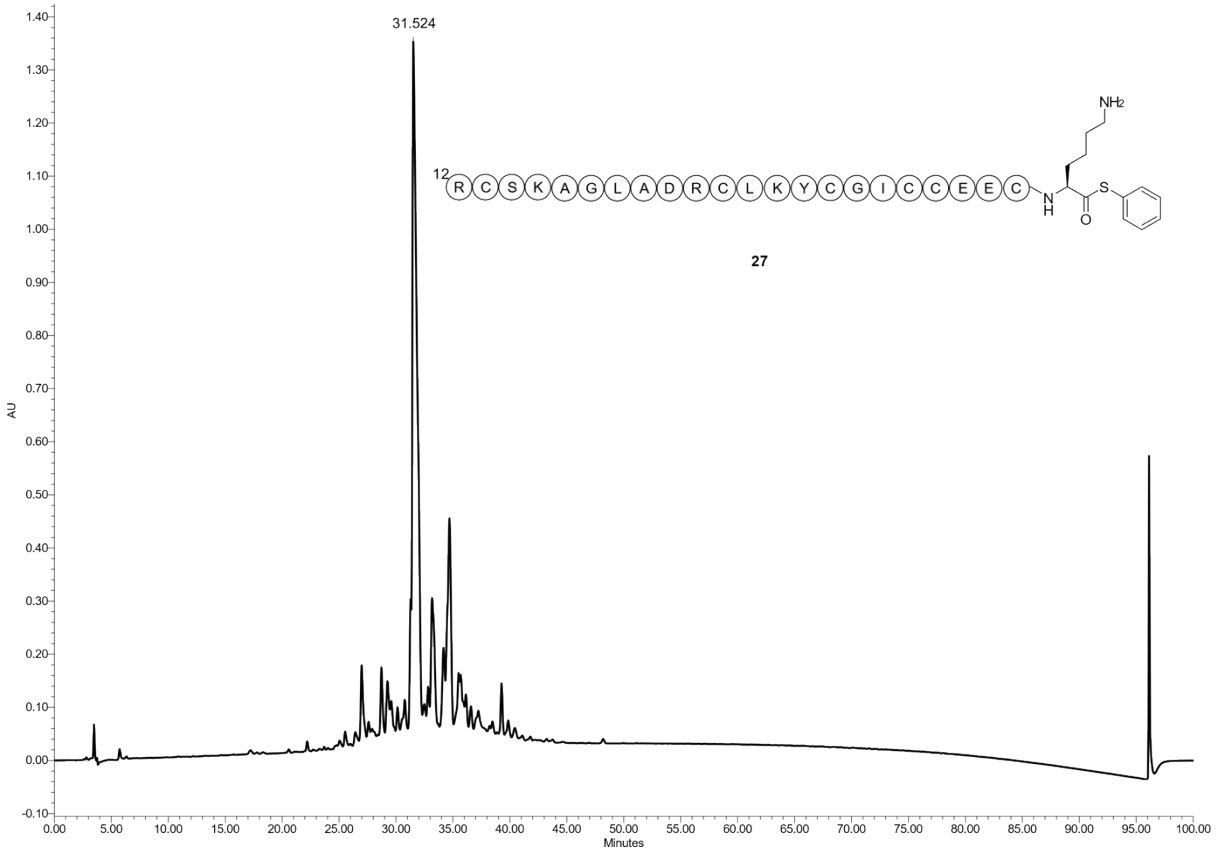


Figure S23: Analytical RP-HPLC chromatogram of crude peptide Snakin-1 (12-33)-Lys-thiophenol (**27**) from table 1 in main manuscript trial 17[b], t_R = 31.5 min. Chromatographic separations were performed on a Waters e2695 HPLC using a Agilent TC-C18 column (5 µm; 4.6 × 250 mm) and a linear gradient of 5-95% B in 90 min at room temperature, *ca*. 1% B per min at a flow rate of 1.0 mL/min. Buffer A: H_2O containing 0.1% TFA (*v/v*); Buffer B: acetonitrile containing 0.1% TFA (*v/v*).

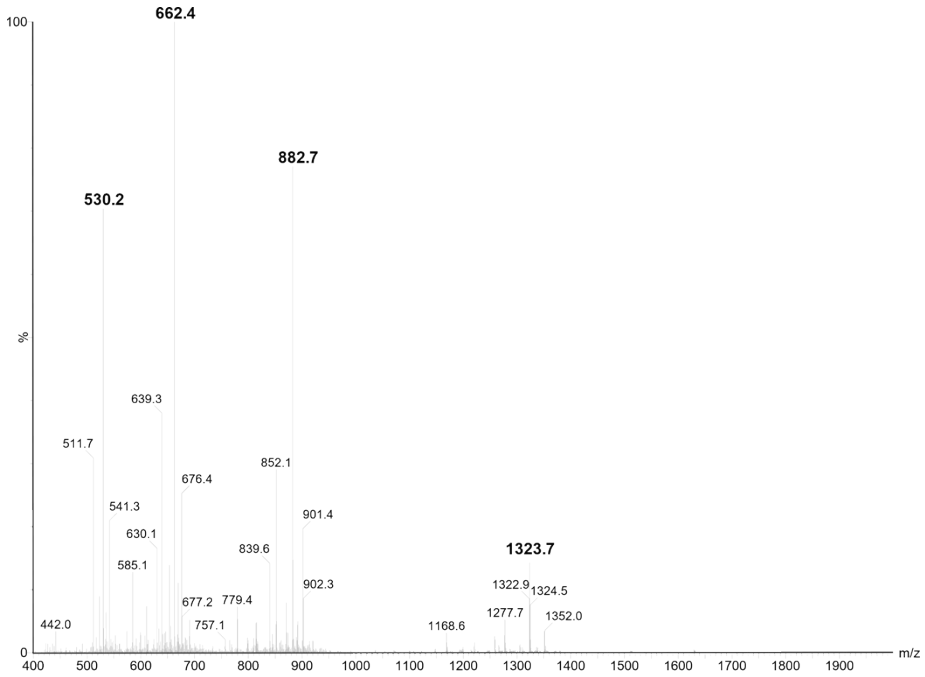


Figure S24: ESI-MS of crude Snakin-1 (12-33)-Lys-thiophenol (**27**). *m/z* $[M+2H]^{2+}$ calcd: 1323.6 Da; found: 1323.7 Da; $[M+3H]^{3+}$ calcd: 882.7 Da; found: 882.7 Da; $[M+4H]^{4+}$ calcd: 662.3 Da; found: 662.4 Da; $[M+5H]^{5+}$ calcd: 530.0 Da; found: 530.2 Da. Theoretical molecular weight: 2645.2 Da. Chemical formula: $C_{108}H_{178}N_{32}O_{31}S_7$.

Snakin-1 RP-HPLC Diastereomer Analysis

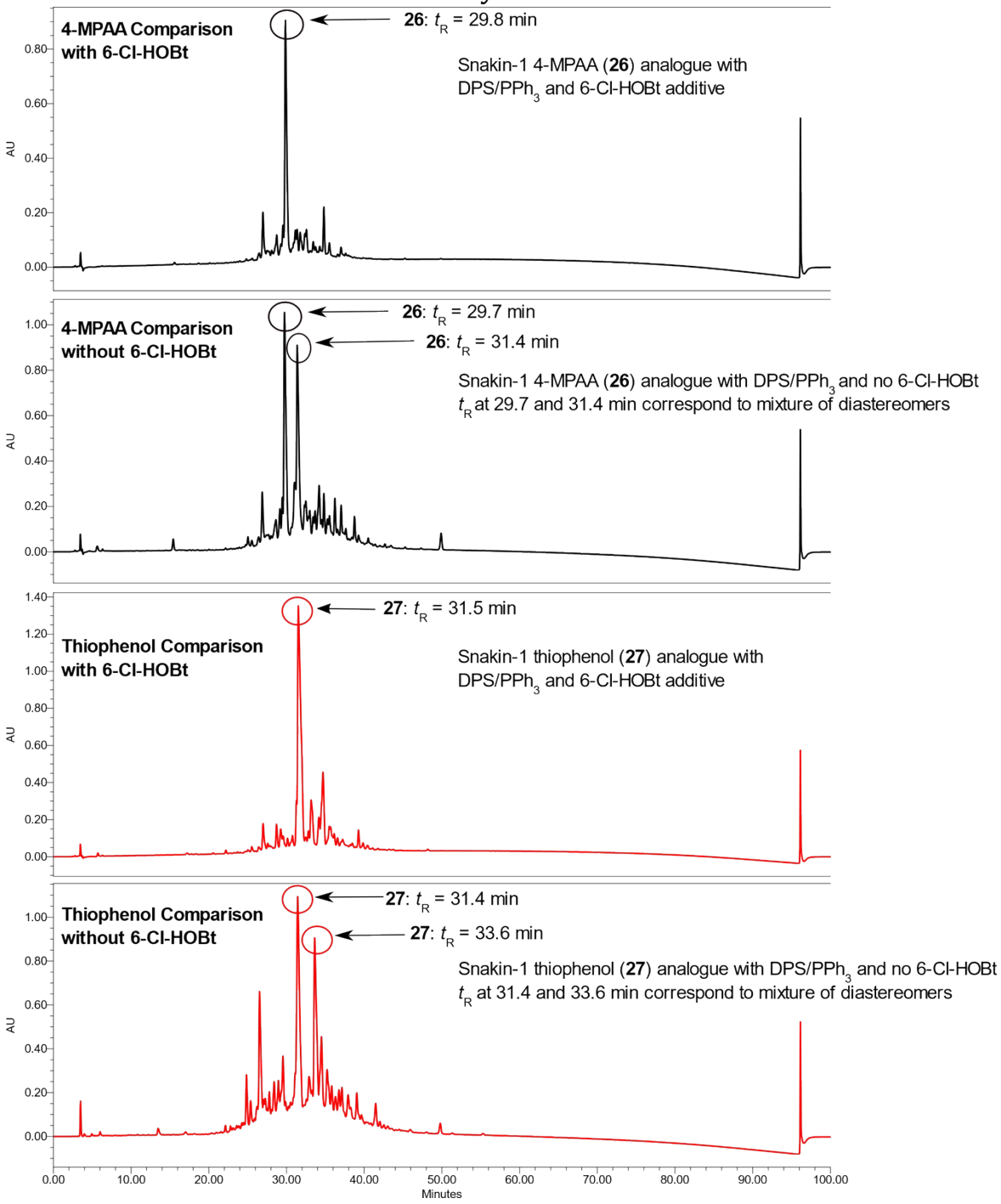
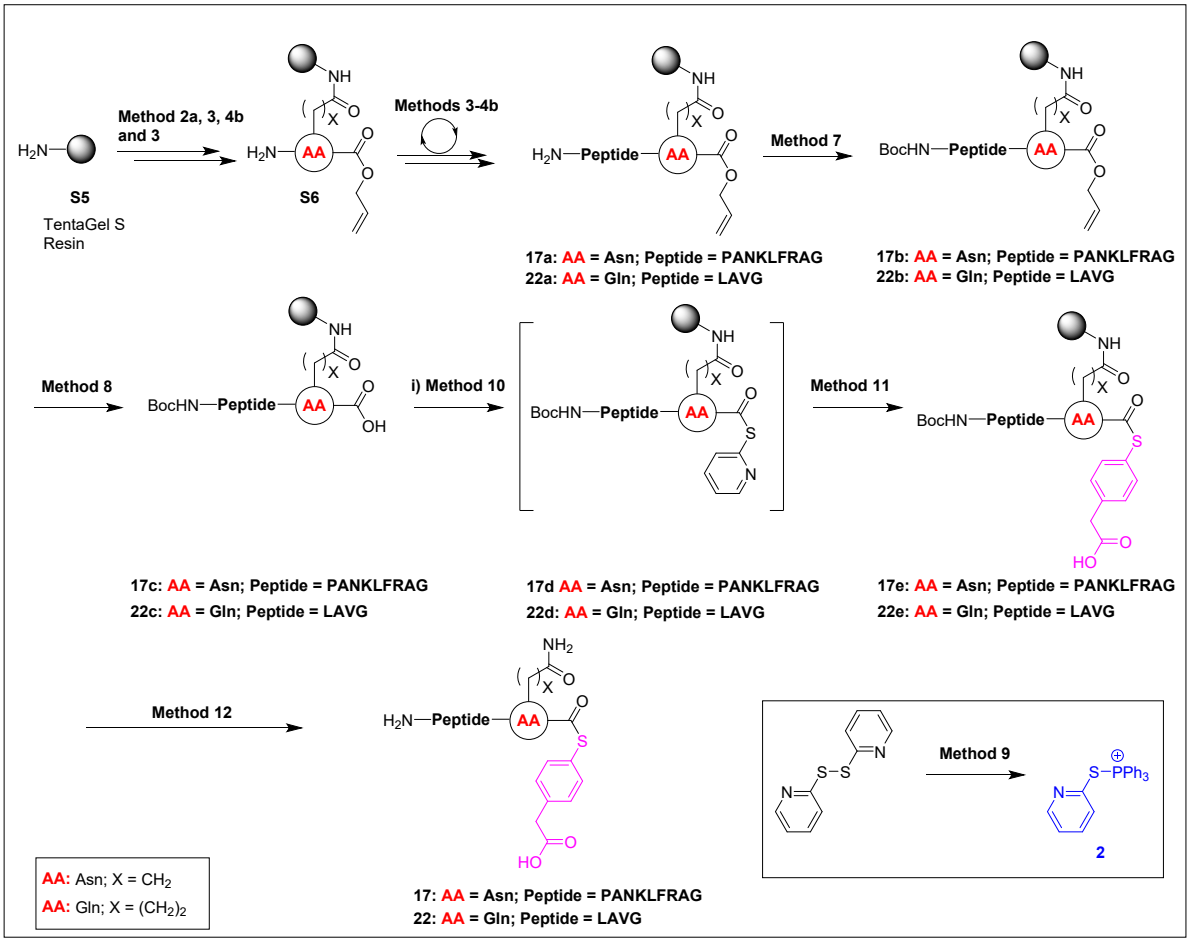


Figure S25: Analytical RP-HPLC chromatograms of overlaid crude peptides **26** and **27**. Chromatographic separations were performed on a Waters e2695 HPLC using a Agilent TC-C18 column (5 µm; 4.6 × 250 mm) and a linear gradient of 5-95% B in 90 min at room temperature, *ca.* 1% B per min at a flow rate of 1.0 mL/min. Buffer A: H_2O containing 0.1% TFA (*v*/*v*); Buffer B: acetonitrile containing 0.1% TFA (*v*/*v*).

Synthesis of C^{α}-thioesters on Rink Amide Linker via Fmoc-SPPS



Scheme S2. General synthetic scheme for C^{α}-thioesterification on Rink amide resin.

Fmoc-(L)-Asp-Oallyl or Fmoc-(L)-Glu-Oallyl was attached to Rink amide loaded on Tentagel S resin **S5** using **Method 2a** followed by Fmoc-removal using **Method 3** followed by direct attachment of Fmoc-Gly-OH to resin using **Method 4b** and **Method 3** affording **S6**. **Method 3** was used for all subsequent N^{α}-Fmoc removals. Linear elongation of the peptide chain was achieved by coupling appropriate Fmoc-amino acids in **Scheme S2** using microwave assisted **Method 4b**. Boc protection of free amino group at *N*-terminus of (**17a** or **22a**) was conducted using **Method 7**. Selective allyl ester removal of (**17b** or **22b**) was achieved using **Method 8** affording (**17c**, **22c**; **Figure S26/Figure S28**). Peptide (**17c** or **22c**) was then subjected **Method 10** followed by addition of preactivated DPS/PPh_3 complex **2** generated from **Method 9** affording *in-situ* (**17d** or **22d**). Peptide (**17d** or **22d**) is treated with **Method 11** affording (**17e or 22e**) followed by acidolytic cleavage using **Method 12** affording crude (**17** or **22**) **(Figure S29/Figure S32-33)**.

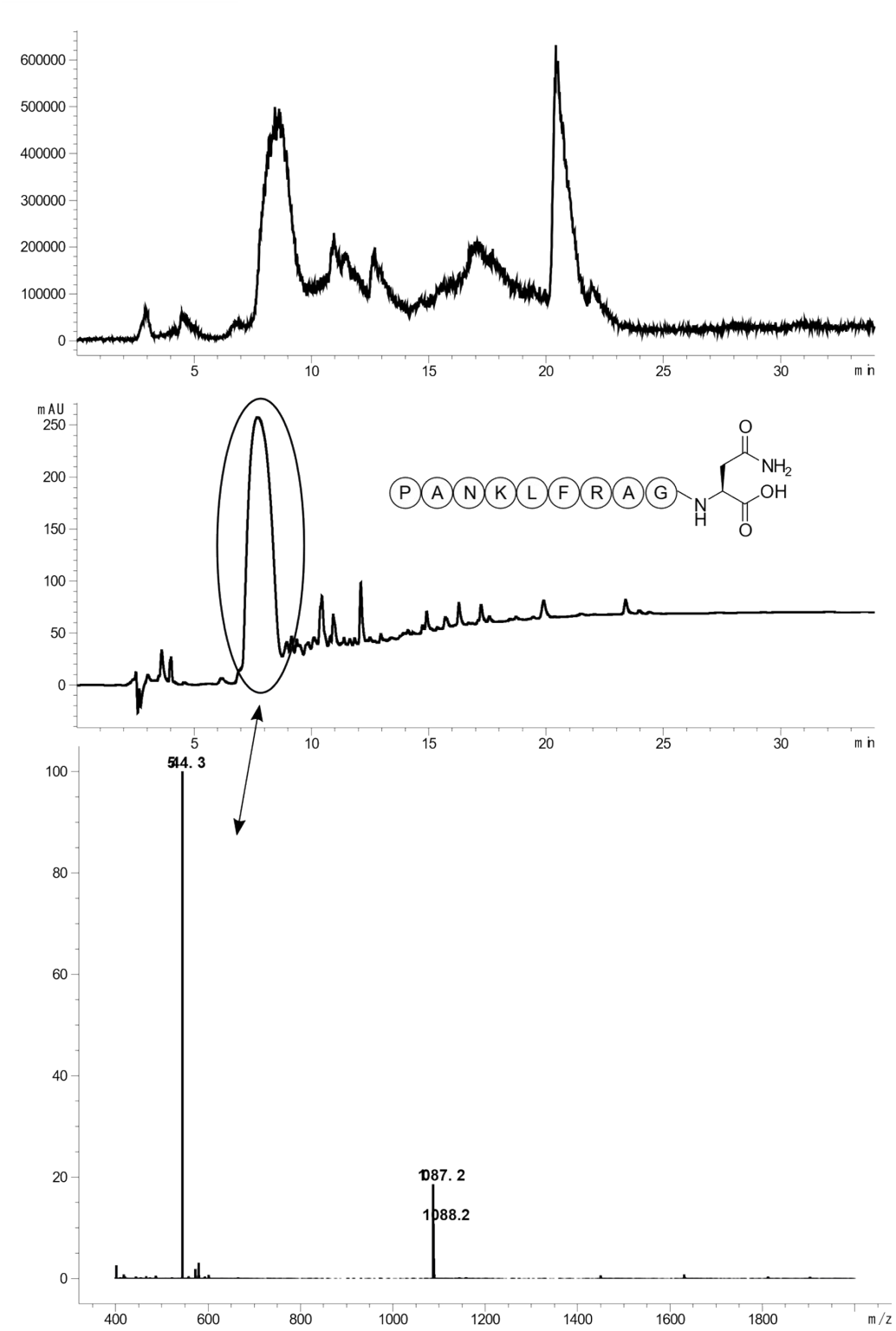


Figure S26: LC-MS profile of crude deprotected peptide **17c**; ion polarity positive operating at a nominal accelerating voltage of 70 eV. ESI-MS *m/z* $[M+1H]^{1+}$ calcd: 1088.3 Da; found: 1087.2 Da; $[M+2H]^{2+}$ calcd: 544.6 Da; found 544.3 Da. Theoretical molecular weight: 1087.3 Da. Chemical formula: $C_{48}H_{78}N_{16}O_{13}$.

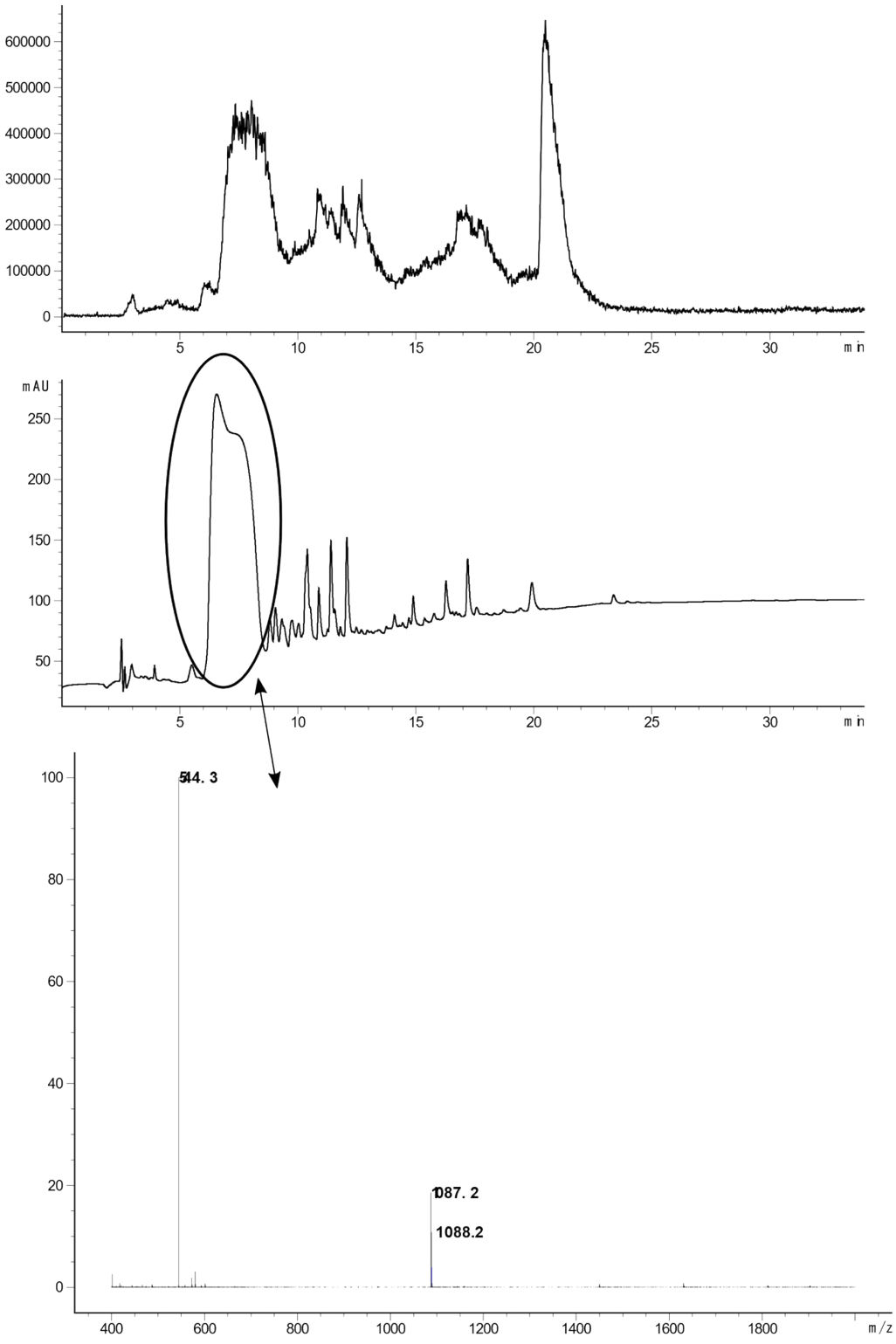


Figure S27: LC-MS profile of test reaction without DPS and PPh_3 addition for **17c** thioesterification; ion polarity positive operating at a nominal accelerating voltage of 70 eV. ESI-MS *m/z* $[M+1H]^{1+}$ calcd: 1088.2 Da; found: 1087.2 Da; $[M+1H]^{2+}$ calcd: 544.6 Da; found 544.3 Da. Theoretical molecular weight: 1087.3 Da.

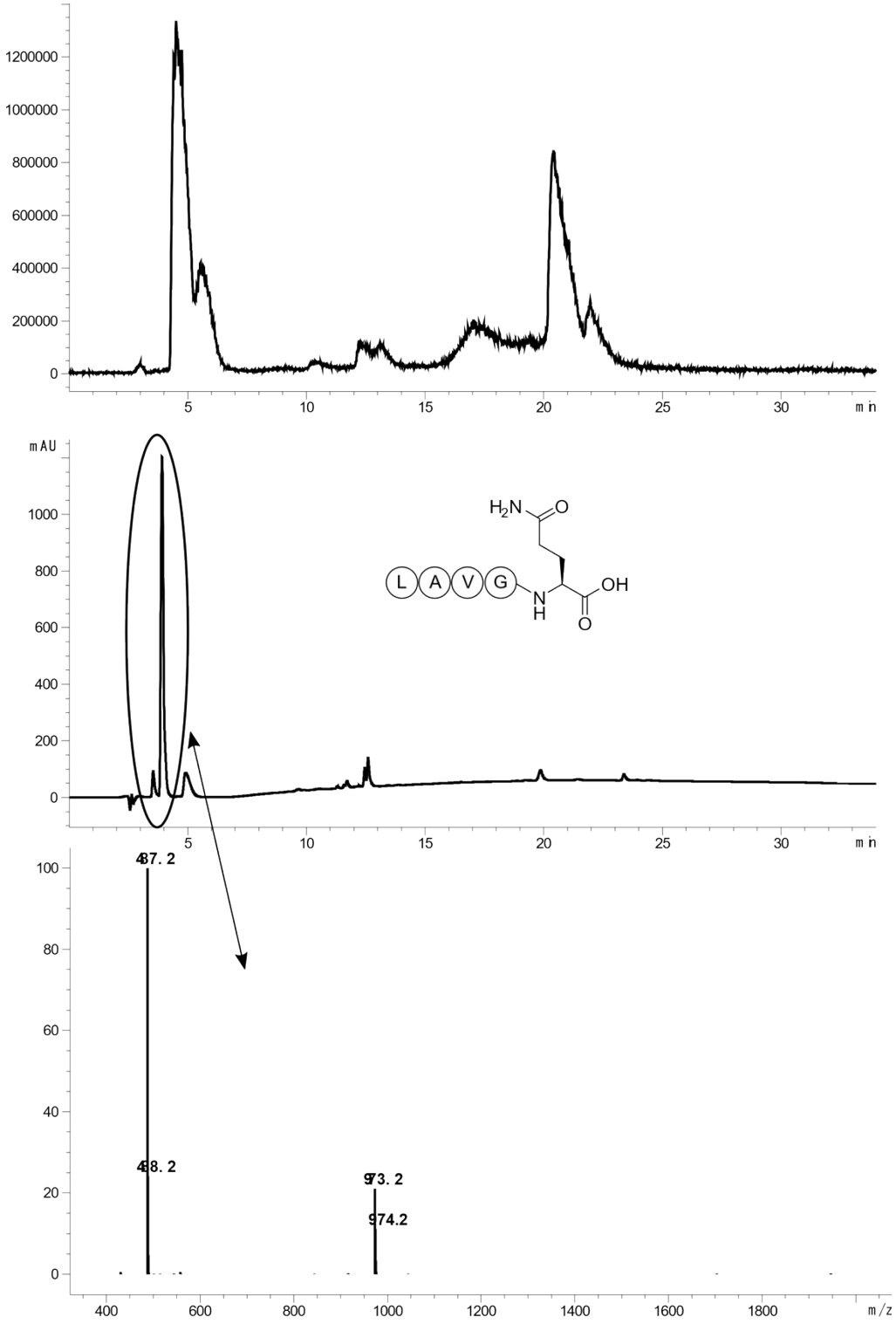


Figure S28: LC-MS profile of crude deprotected peptide **22c**; ion polarity positive operating at a nominal accelerating voltage of 70 eV. ESI-MS *m/z* $[M+1H]^{1+}$ calcd: 487.6 Da; found: 487.2 Da; $[2M+1H]^{1+}$ calcd: 974.2 Da; found 973.2 Da. Theoretical molecular weight: 486.6 Da. Chemical formula: $C_{21}H_{38}N_6O_7$.

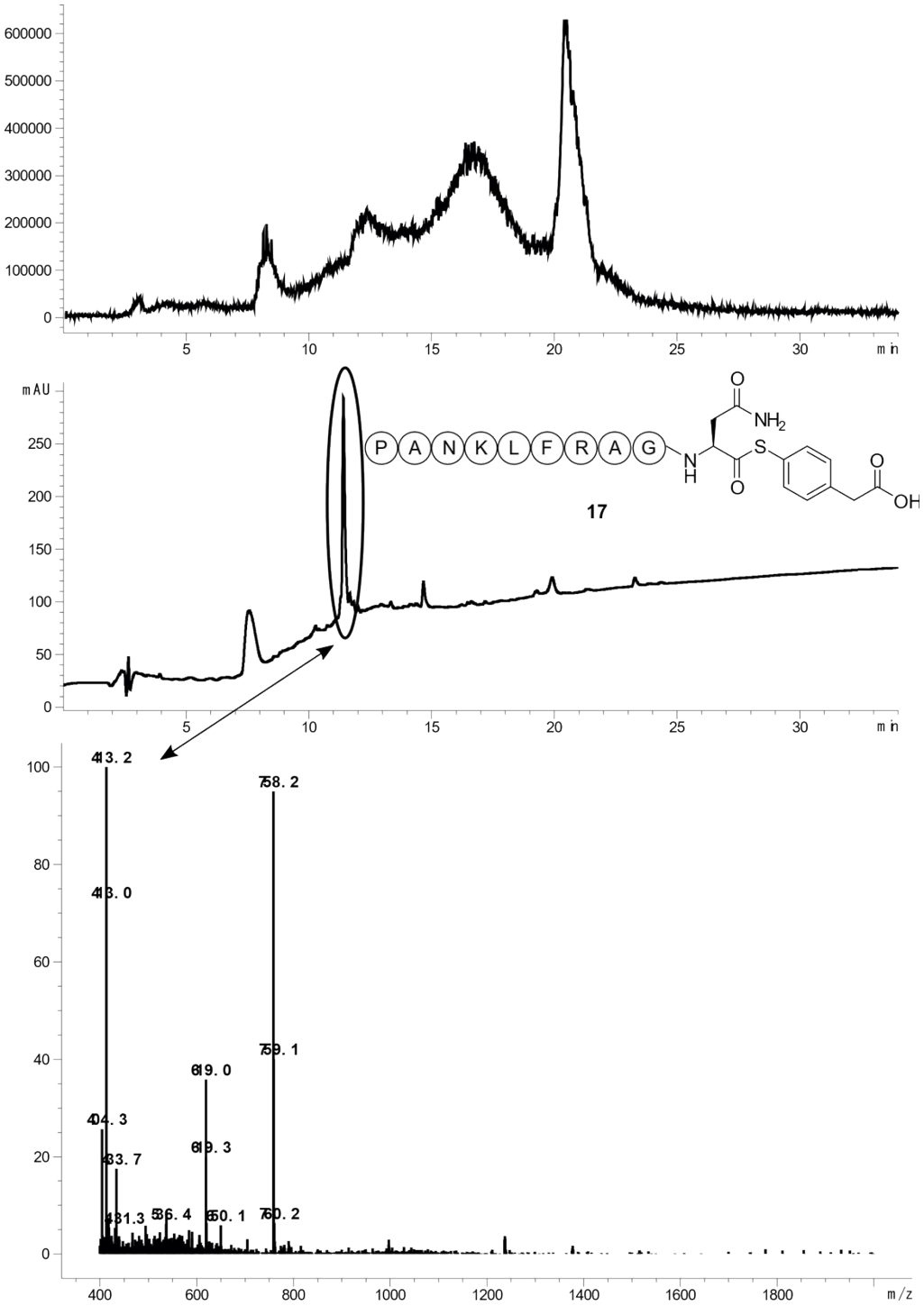


Figure S29: LC-MS profile of crude peptide **17**; ion polarity positive operating at a nominal accelerating voltage of 70 eV. ESI-MS *m/z* $[M+2H]^{2+}$ calcd: 619.7 Da; found: 619.0 Da; $[M+3H]^{3+}$ calcd: 413.5 Da; found 413.2 Da. Theoretical molecular weight: 1237.4 Da. Chemical formula: $C_{56}H_{84}N_{16}O_{14}S$. *Unknown fragmentation species at 758.2 Da not present in ensuing NCL ligation ESI-MS analysis.

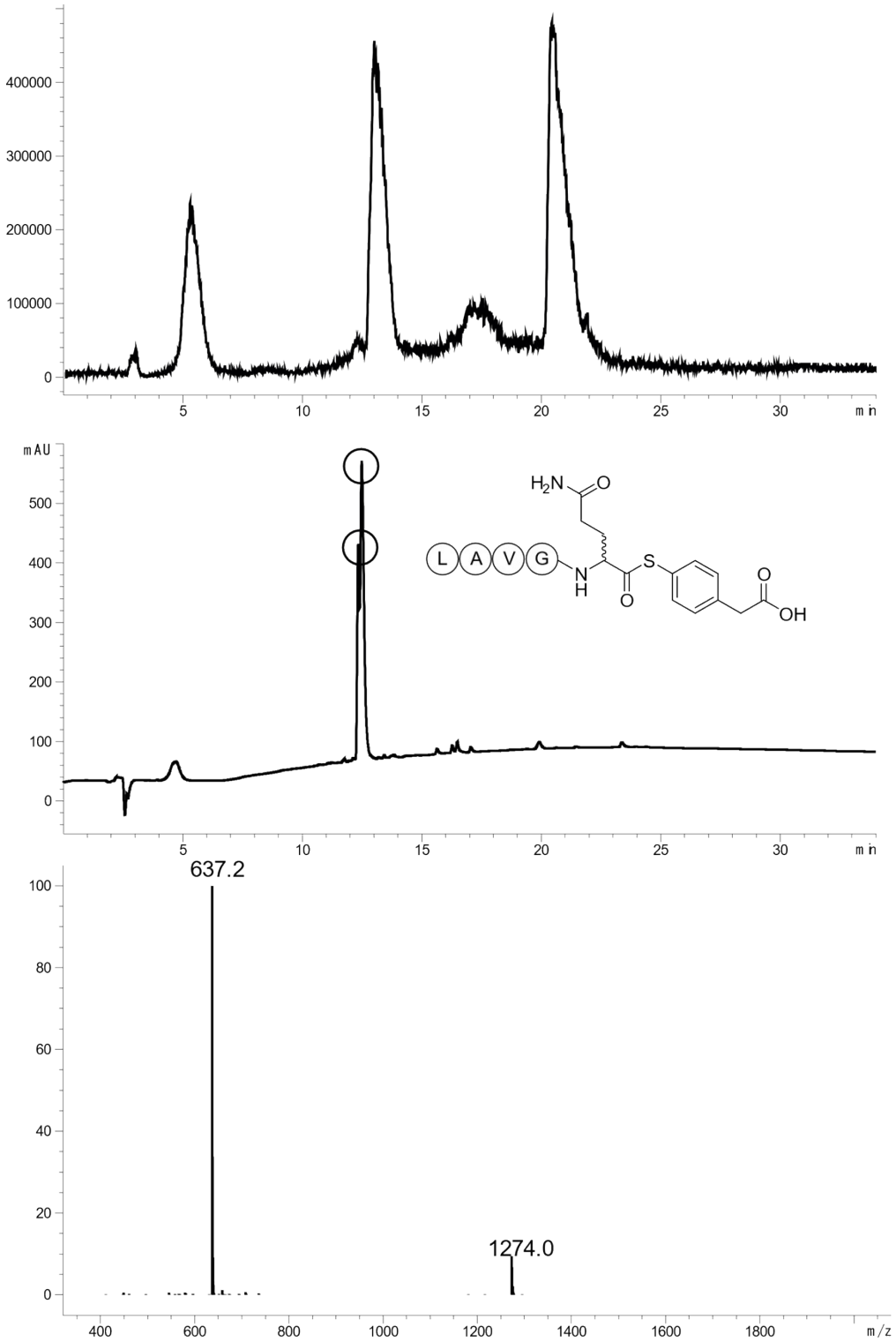


Figure S30: LC-MS profile of crude peptide **22** from table 1 trial 7[a] in main manuscript; ion polarity positive operating at a nominal accelerating voltage of 70 eV. ESI-MS *m/z* $[M+1H]^{1+}$ calcd: 637.8 Da; found: 637.2 Da; $[2M+1H]^{1+}$ calcd: 1274.6 Da; found 1274.0 Da. Theoretical molecular weight: 636.8 Da. Chemical formula: $C_{29}H_{44}N_6O_8S$. *Presence of diastereomers in LC-MS trace with no 6-Cl-HOBt additive.

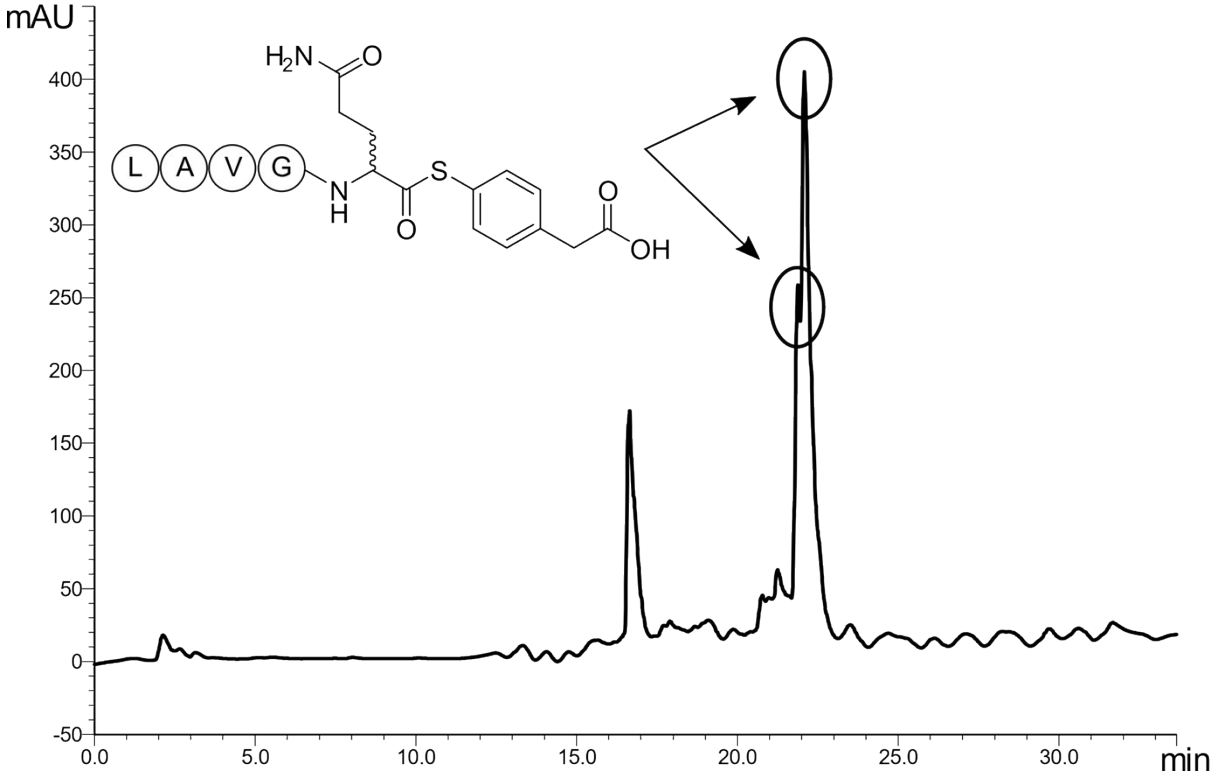


Figure S31: Analytical RP-HPLC chromatogram of crude peptide **22** from table 1 trial 7[a] in main manuscript, t_R = 21.8 and 22.1 min. Chromatographic separations were performed on a Thermo Scientific Dionex Ultimate 3000 HPLC using a Pheneoemex® Gemini MS C-18 column (5 µm; 4.6 × 150 mm) and a linear gradient of 5-95% B in 90 min at room temperature, *ca*. 1% B per min at a flow rate of 1.0 mL/min. Buffer A: H_2O containing 0.1% TFA (*v/v*); Buffer B: acetonitrile containing 0.1% TFA (*v/v*).

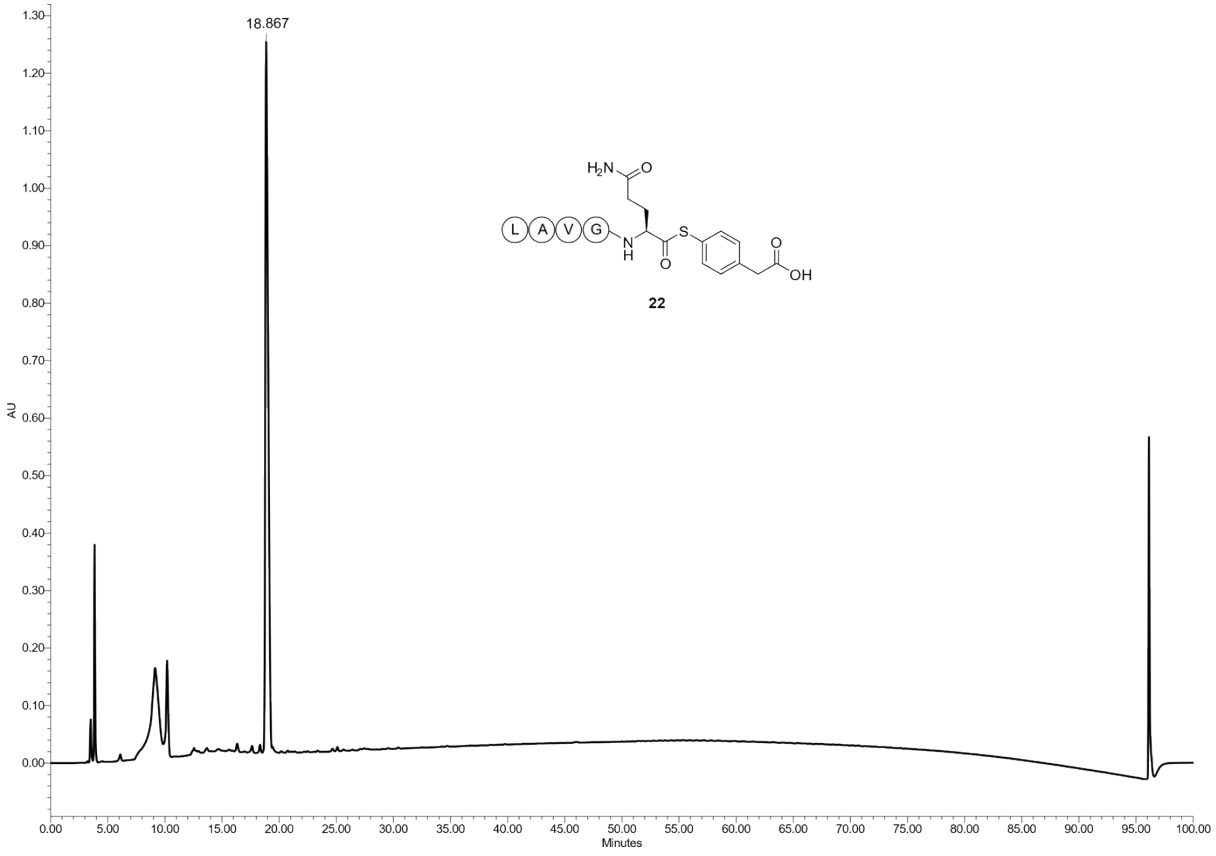


Figure S32: Analytical RP-HPLC chromatogram of crude peptide **22** from table 1 trial 13[b] in main manuscript, t_R = 18.8 min. Chromatographic separations were performed on a Waters e2695 HPLC using a Agilent TC-C18 column (5 µm; 4.6 × 250 mm) and a

linear gradient of 5-95% B in 90 min at room temperature, *ca*. 1% B per min at a flow rate of 1.0 mL/min. Buffer A: H_2O containing 0.1% TFA (*v/v*); Buffer B: acetonitrile containing 0.1% TFA (*v/v*).

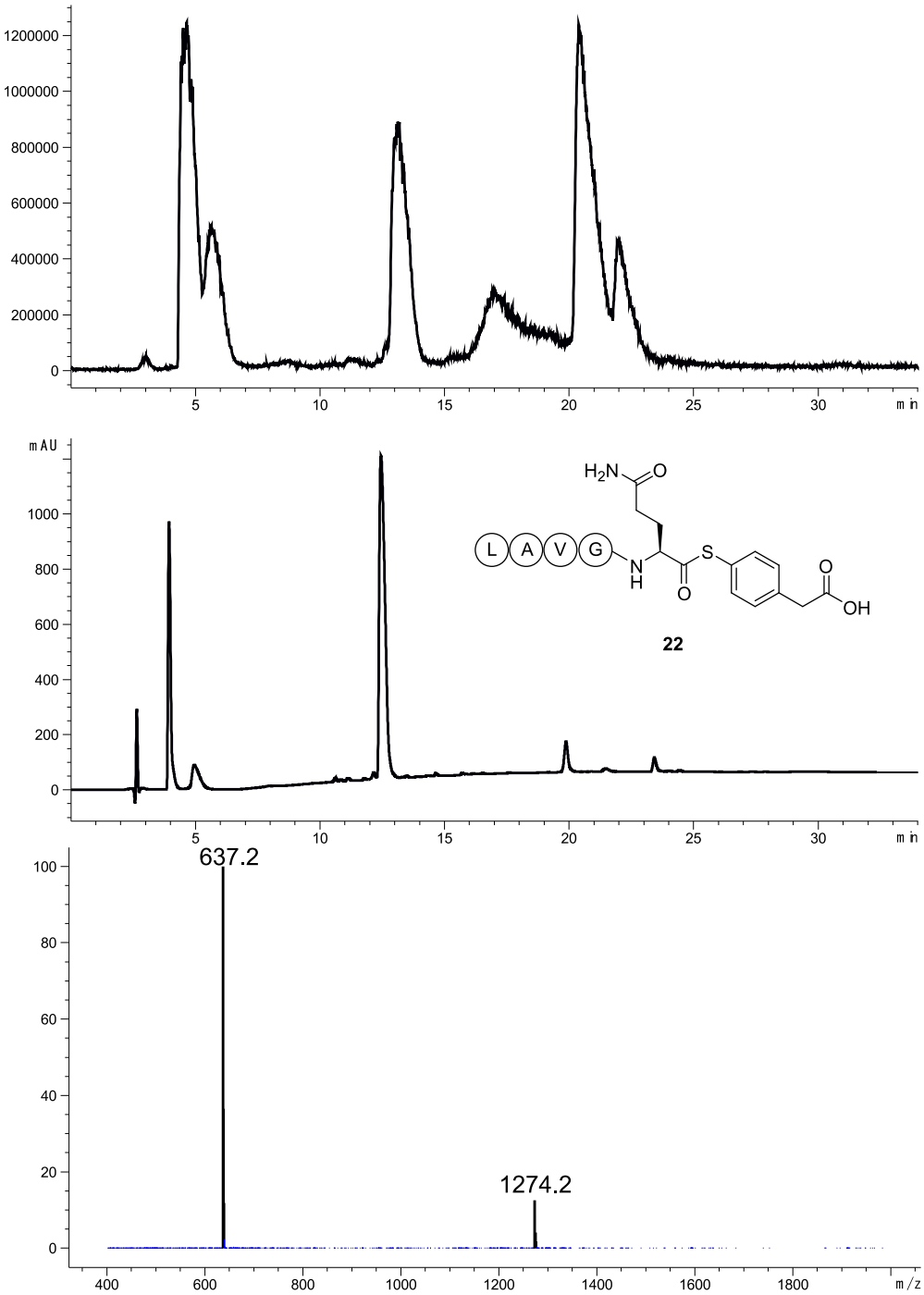
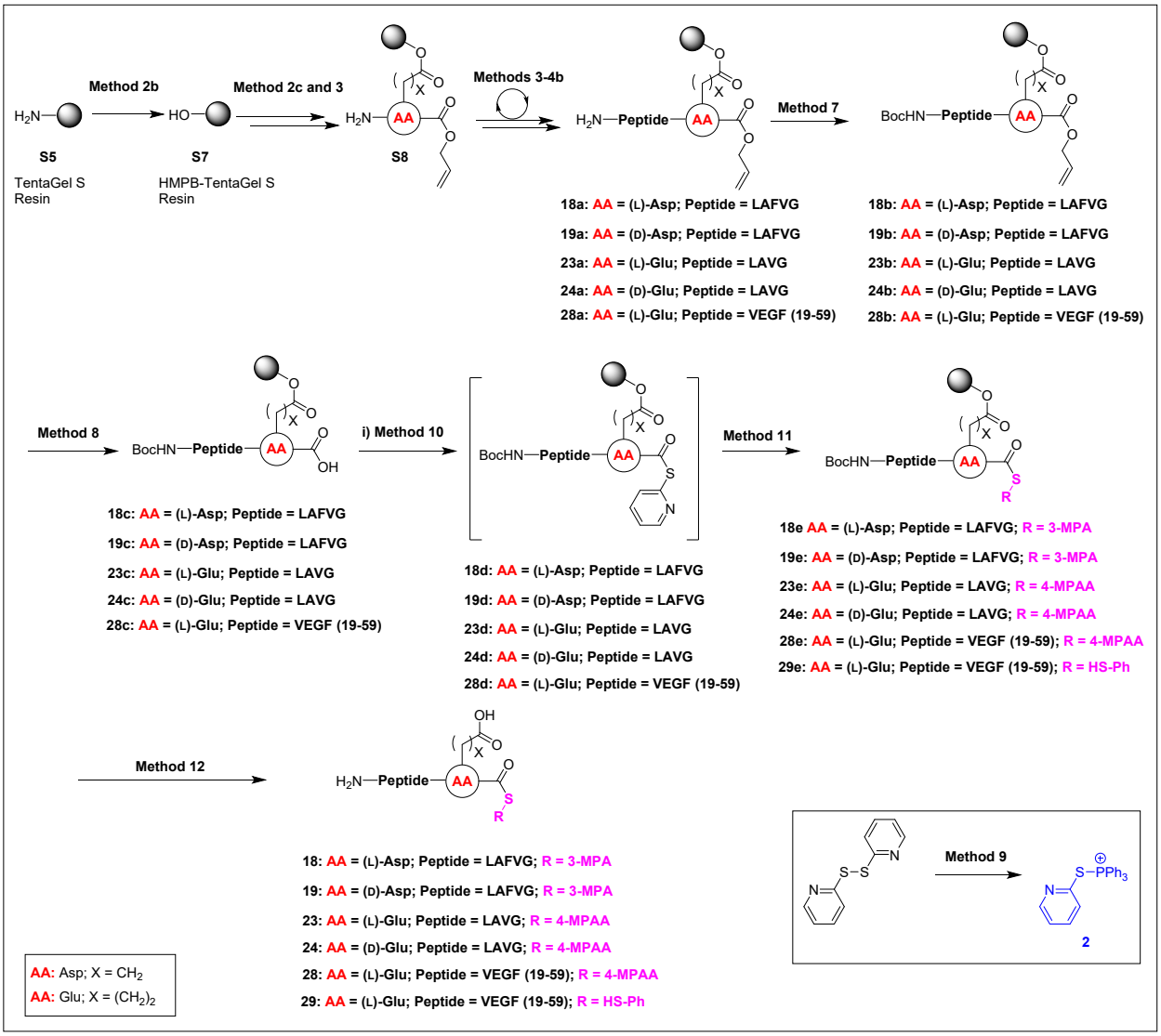


Figure S33: LC-MS profile of crude peptide **22** from table trial 13[b] in main manuscript; ion polarity positive operating at a nominal accelerating voltage of 70 eV. ESI-MS *m/z* $[M+1H]^{1+}$ calcd: 637.8 Da; found: 637.2 Da; $[2M+1H]^{1+}$ calcd: 1274.6 Da; found 1274.2 Da. Theoretical molecular weight calcd: 636.8 Da. Chemical formula: $C_{29}H_{44}N_6O_8S$.

Synthesis of C^{α}-thioesters on HMPB Linker via Fmoc-SPPS



Scheme S3. General synthetic scheme for C^{α}-thioesterification on HMPB resin.

HMPB linker was loaded to Tentagel S resin **S5** using **Method 2b** affording **S7.** Fmoc-(L)-Asp-Oallyl, Fmoc-(L)-Glu-Oallyl or Fmoc-(D)-Glu-Oallyl was attached to HMPB resin **S7** using **Method 2c** followed by Fmoc-removal using **Method 3** affording **S8**. Direct attachment of desired amino acid to resin bound **S8** was achieved using **Method 4b**. **Method 3** was used for all subsequent N^{α}-Fmoc removals. Boc protection of free amino group at *N*-terminus was conducted using **Method 7**. Selective allyl ester removal of (**18b**, **19b**, **23b**, **24b**, **28b**) was achieved using **Method 8** affording (**18c**, **19c**, **23c**, **24c**, **28c**; **Figure S34/Figure S35/Figure S36/Figure S37/Figure S38-39**). Peptide (**18c**, **19c**, **23c**, **24c**, **28c**) was then subjected **Method 10** followed by addition of preactivated DPS/PPh_3 complex **2** generated from **Method 9** affording *in-situ* (**18d**, **19d**, **23d**, **24d**, **28d**). Peptide (**18d**, **19d**, **23d**, **24d**, **28d**) is treated with **Method 11** affording (**18e**, **19e**, **23e**, **24e**, **28e, 29e**) followed by acidolytic cleavage using **Method 12** affording (**18**, **19**, **23**, **24**, **28**, **29**) **(Figure S40-41/Figure S42-43/Figure S46-47/Figure S48-49/Figure S50-S52/Figure S53-S55)**. Crude **S28-S29** was purified batchwise by semi-preparative RP-HPLC on a Xterra® Prep MS C18 column, (19 × 300 mm, 10 µm) using a linear gradient of 5% to 95% over 90 min (*ca.* 1% B/min) with a flow rate of 4 mL/min. Fractions were collected at 0.2 min intervals and analysed by ESI-MS and RP-HPLC. Fractions identified with correct *m/z* were combined and lyophilised to afford the *title compound* **S28-S29** as a white amorphous solid (**Figure S51-52/Figure S54-55**).

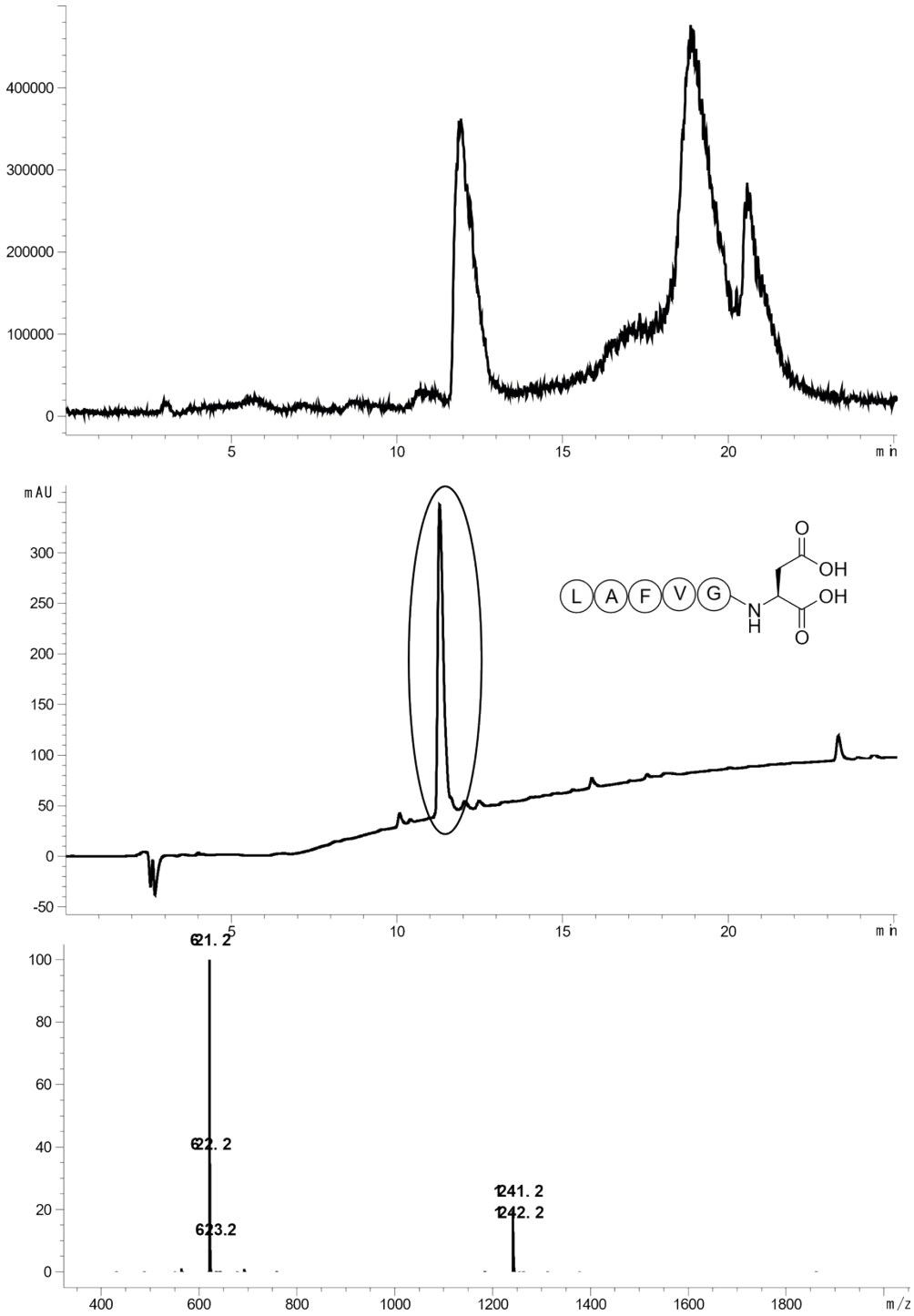


Figure S34: LC-MS profile of crude peptide deprotected **18c**; ion polarity positive operating at a nominal accelerating voltage of 70 eV. ESI-MS *m/z* $[M+1H]^{1+}$ calcd: 621.7 Da; found: 621.2 Da; $[2M+1H]^{1+}$ calcd: 1242.4 Da; found 1242.2 Da. Theoretical molecular weight calcd: 620.7 Da. Chemical formula: $C_{29}H_{44}N_6O_9$.

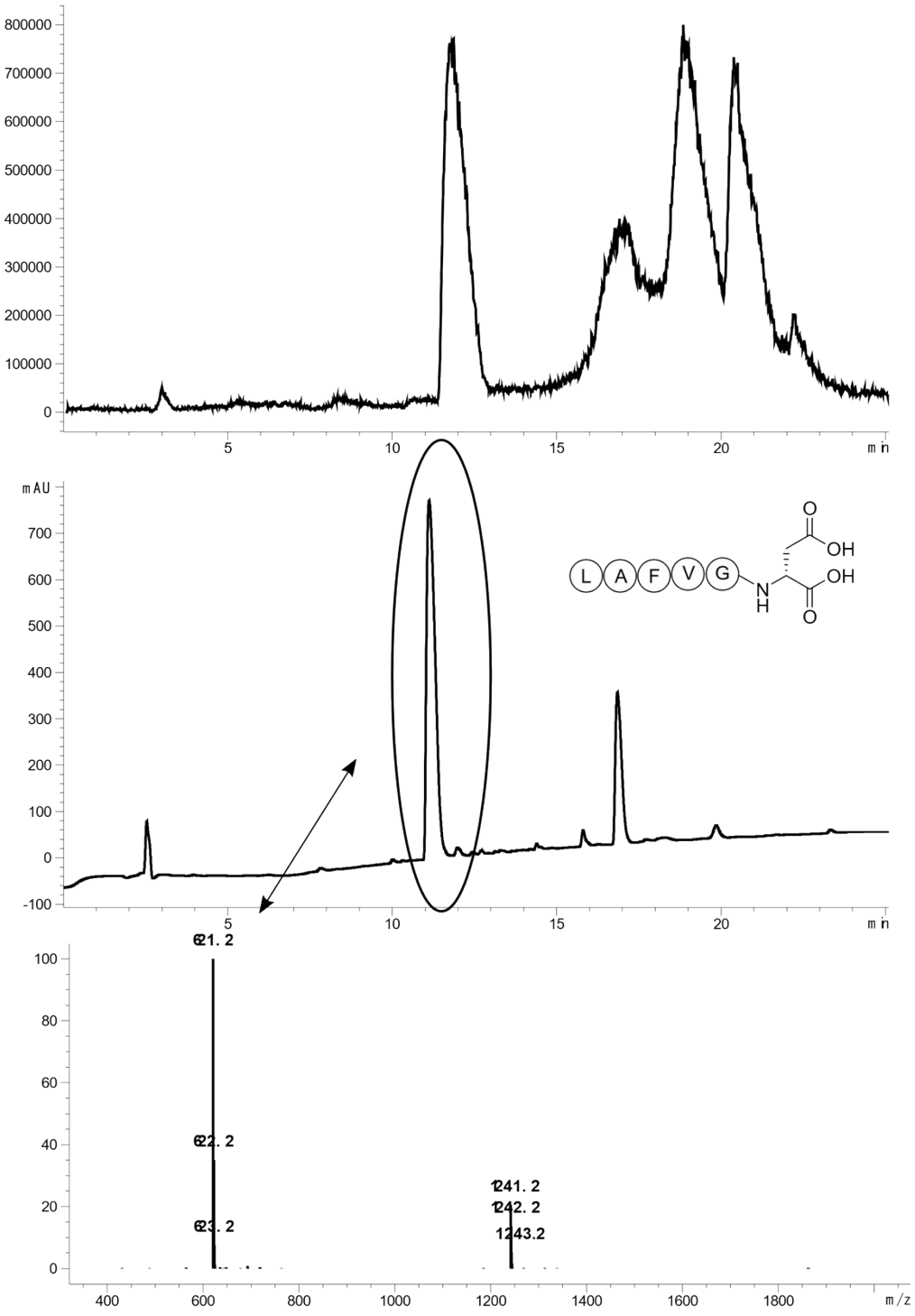


Figure S35: LC-MS profile of crude deprotected peptide **19c**; ion polarity positive operating at a nominal accelerating voltage of 70 eV. ESI-MS *m/z* $[M+1H]^{1+}$ calcd: 621.7 Da; found: 621.2 Da; $[2M+1H]^{1+}$ calcd: 1242.4 Da; found 1241.2 Da. Theoretical molecular weight calcd: 620.7 Da. Chemical formula: $C_{29}H_{44}N_6O_9$.

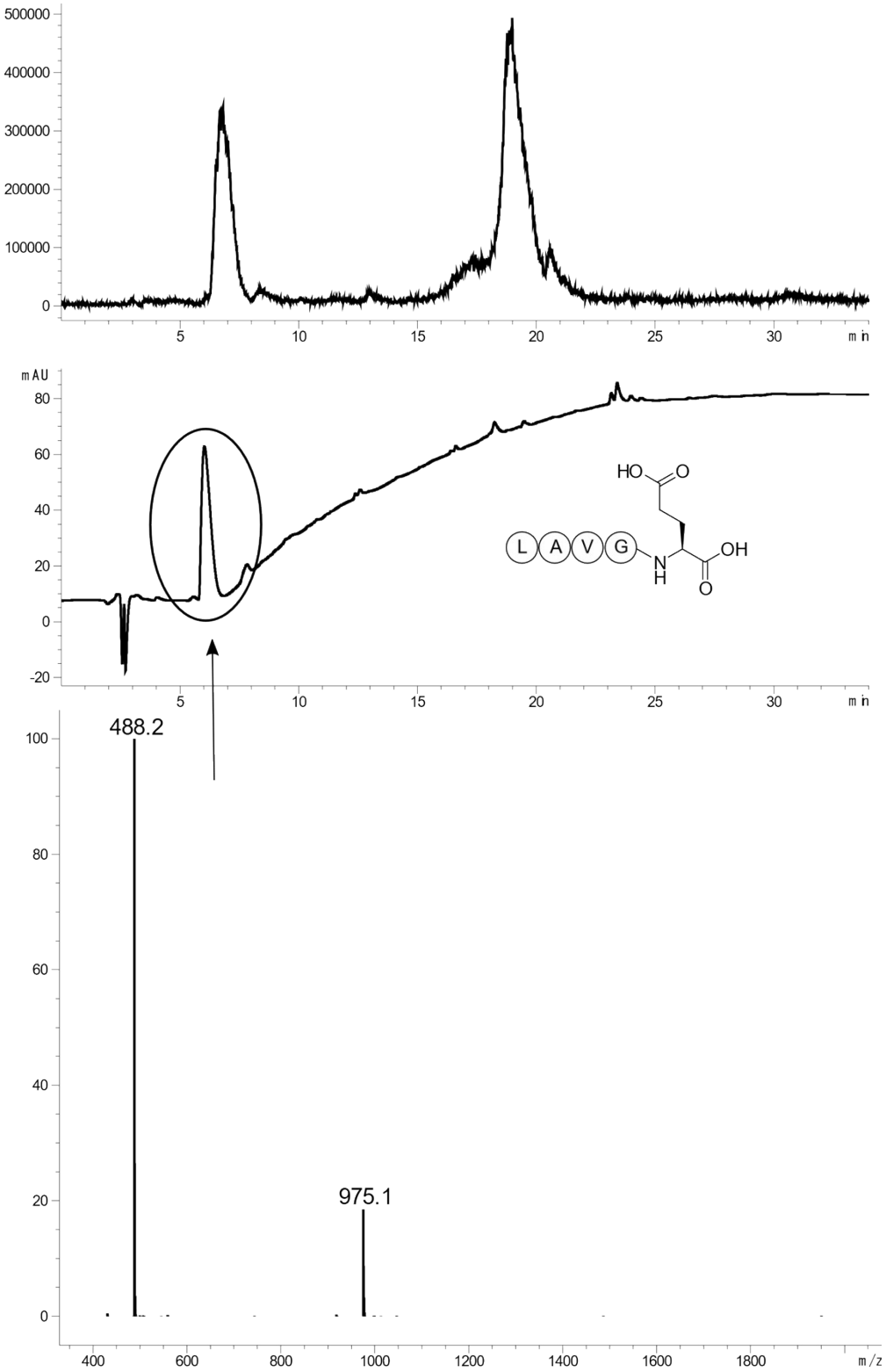


Figure S36: LC-MS profile of crude deprotected peptide **23c**; ion polarity positive operating at a nominal accelerating voltage of 70 eV. ESI-MS *m/z* $[M+1H]^{1+}$ calcd: 488.6 Da; found: 488.2 Da. corresponds to unreacted starting material. $[2M+1H]^{1+}$ calcd: 976.2 Da; found 975.1 Da. Theoretical molecular weight calcd: 487.6 Da. Chemical formula: $C_{21}H_{37}N_5O_8$.

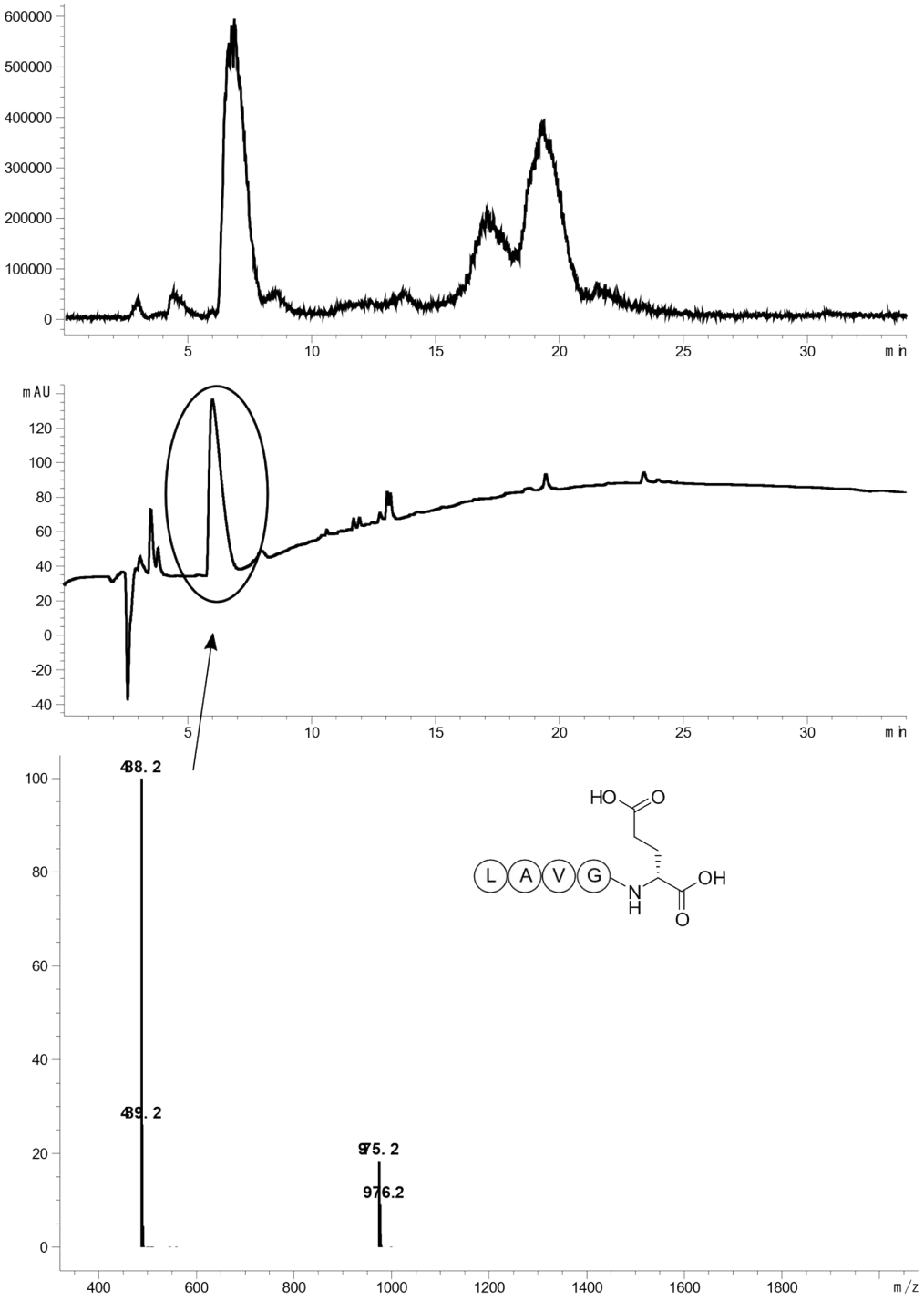


Figure S37: LC-MS profile of crude deprotected peptide **24c**; ion polarity positive operating at a nominal accelerating voltage of 70 eV. ESI-MS *m/z* $[M+1H]^{1+}$ calcd: 488.6 Da; found: 488.2 Da. corresponds to unreacted starting material. $[2M+1H]^{1+}$ calcd: 976.2 Da; found 975.2 Da. Theoretical molecular weight calcd: 487.6 Da. Chemical formula: $C_{21}H_{37}N_5O_8$.

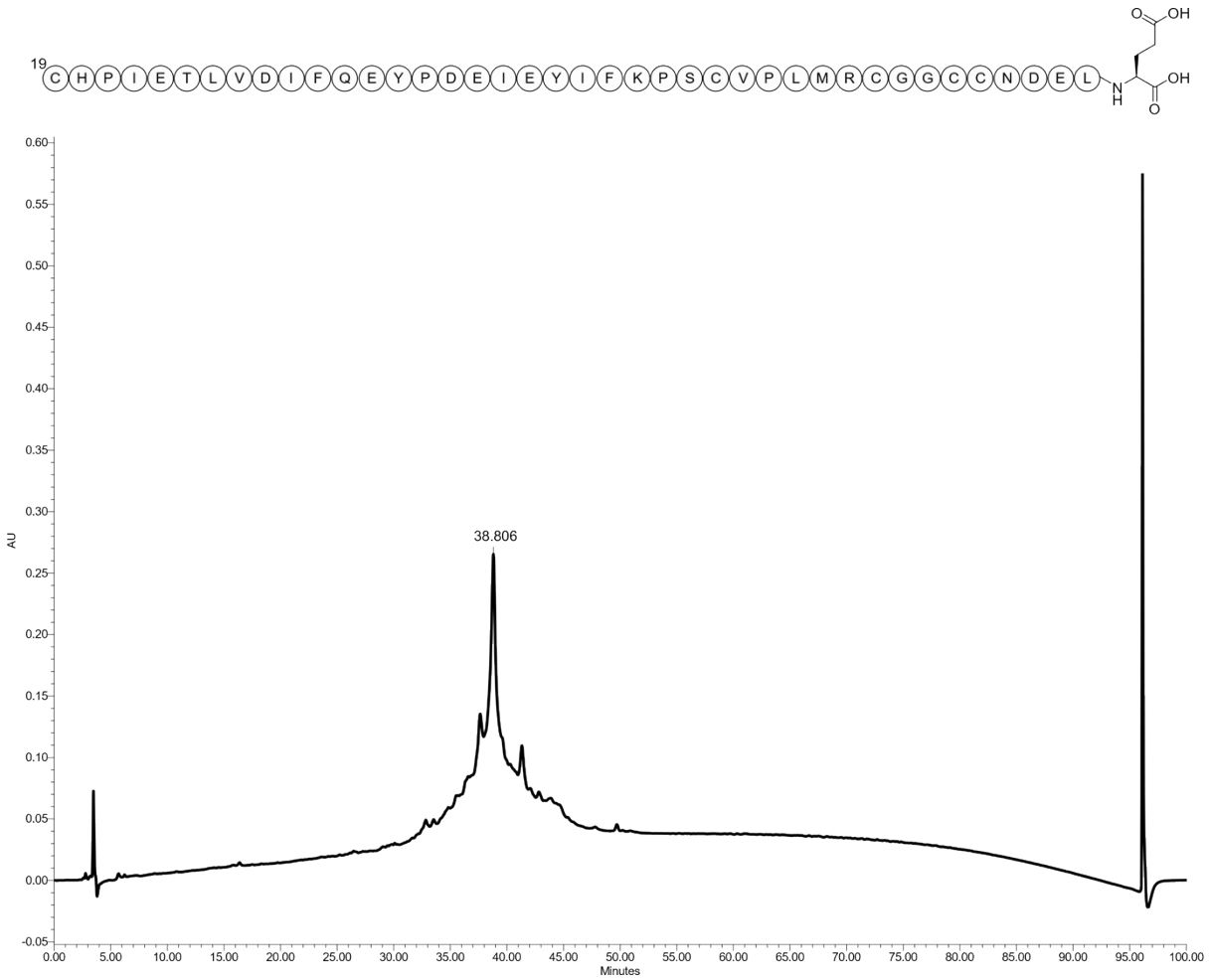


Figure S38: Analytical RP-HPLC chromatogram of crude deprotected peptide **28c**, t_R = 38.8 min. Chromatographic separations were performed on a Waters e2695 HPLC using a Agilent TC-C18 column (5 µm; 4.6 × 250 mm) and a linear gradient of 5-95% B in 90 min at room temperature, *ca*. 1% B per min at a flow rate of 1.0 mL/min. Buffer A: H_2O containing 0.1% TFA (*v/v*); Buffer B: acetonitrile containing 0.1% TFA (*v/v*).

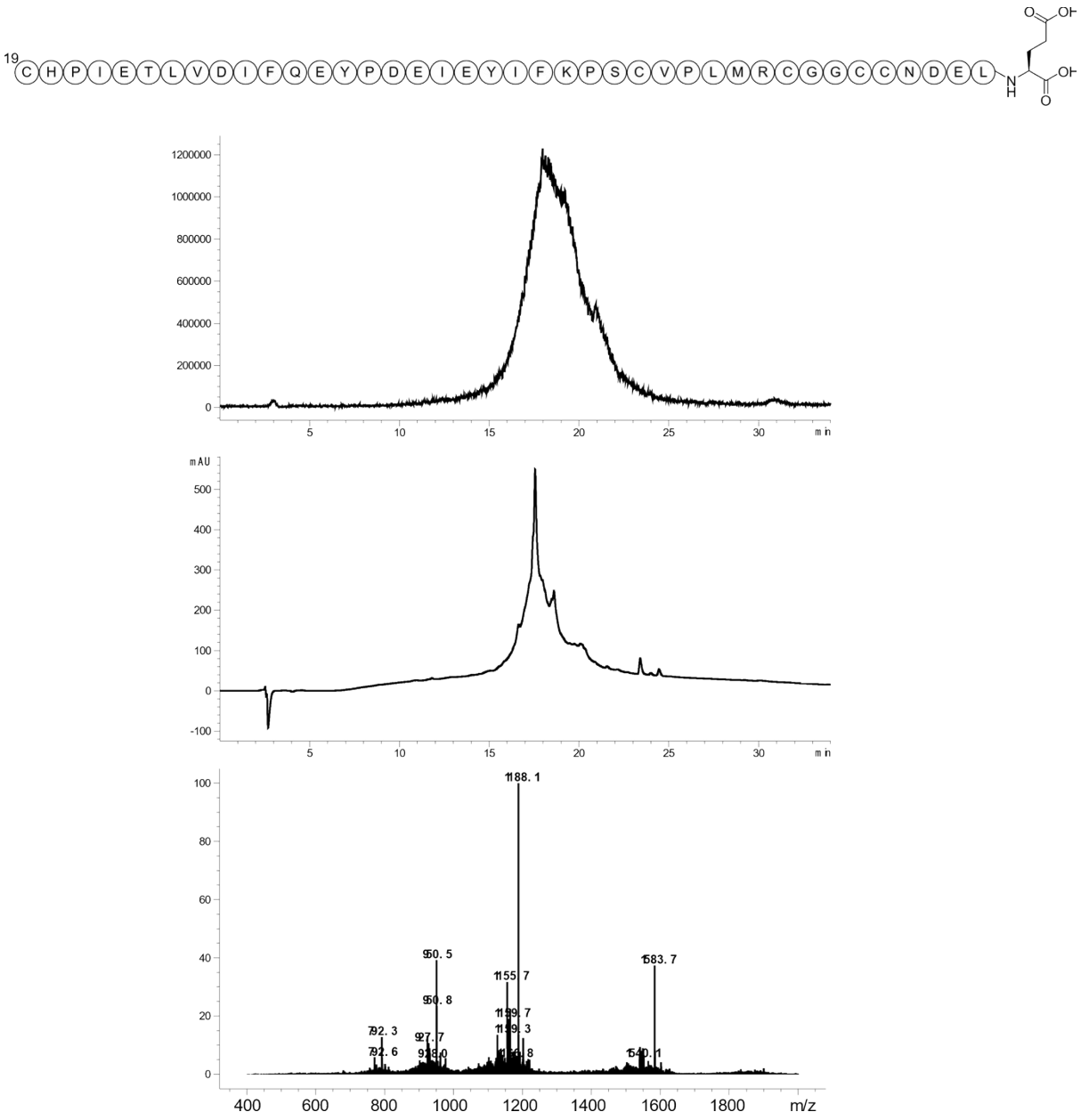
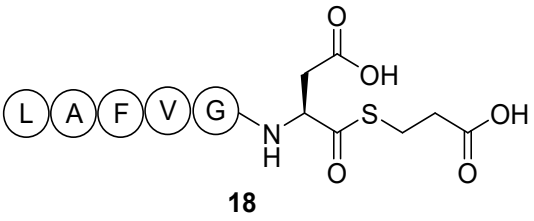


Figure S39: LC-MS profile of crude peptide **28c**; ion polarity positive operating at a nominal accelerating voltage of 70 eV. ESI-MS *m/z* $[M+3H]^{3+}$ calcd: 1584.5 Da; found: 1583.7 Da; $[M+4H]^{4+}$ calcd: 1188.6 Da; found: 1188.1 Da; $[M+5H]^{5+}$ calcd: 951.1 Da; found: 950.5 Da; $[M+6H]^{6+}$ calcd 792.7 Da; found: 792.3 Da. Theoretical molecular weight calcd: 4750.4 Da. Chemical formula: $C_{208}H_{315}N_{49}O_{66}S_6$.



18

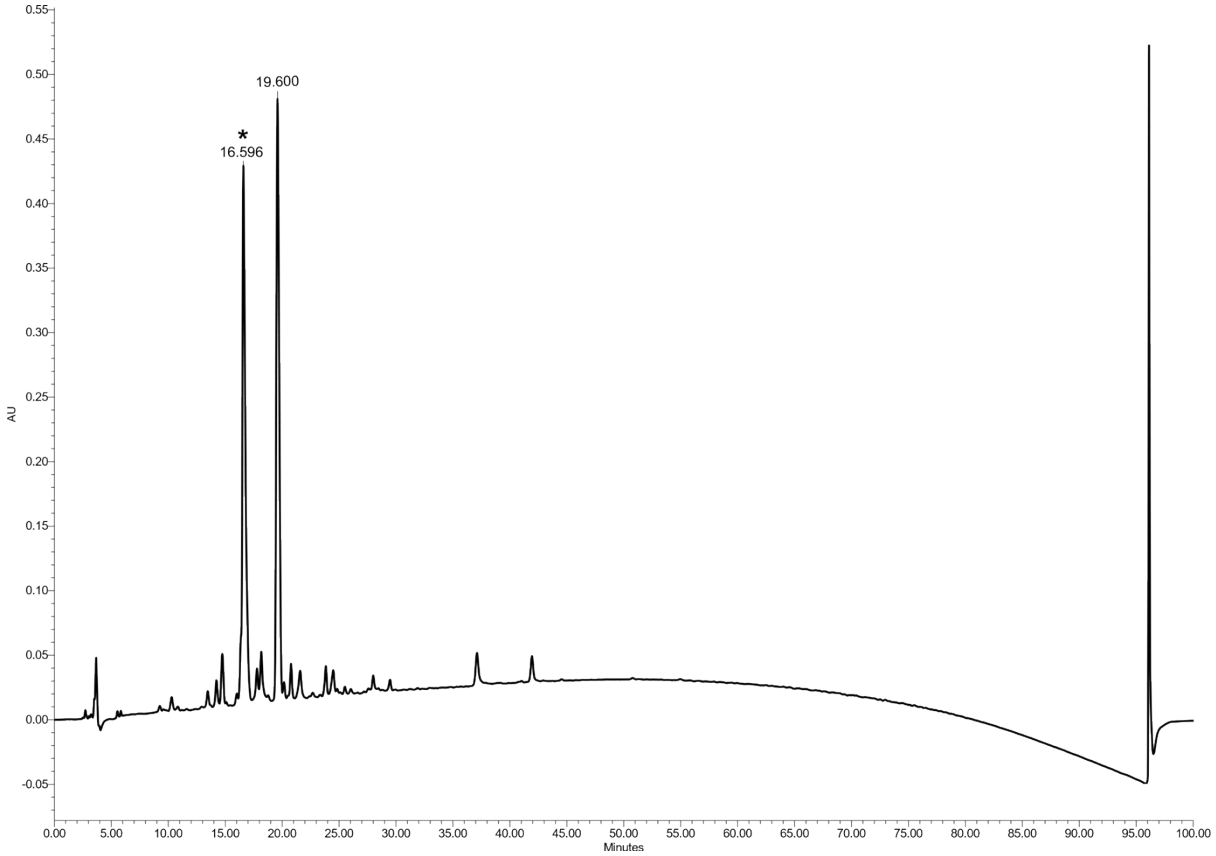


Figure S40: Analytical RP-HPLC chromatogram of crude peptide **18**, t_R = 19.6 min. Chromatographic separations were performed on a Waters e2695 HPLC using a Agilent TC-C18 column (5 µm; 4.6 × 250 mm) and a linear gradient of 5-95% B in 90 min at room temperature, *ca*. 1% B per min at a flow rate of 1.0 mL/min. Buffer A: H_2O containing 0.1% TFA (*v/v*); Buffer B: acetonitrile containing 0.1% TFA (*v/v*). *Peak at t_R = 16.5 min corresponds to unreacted starting material **18c**.

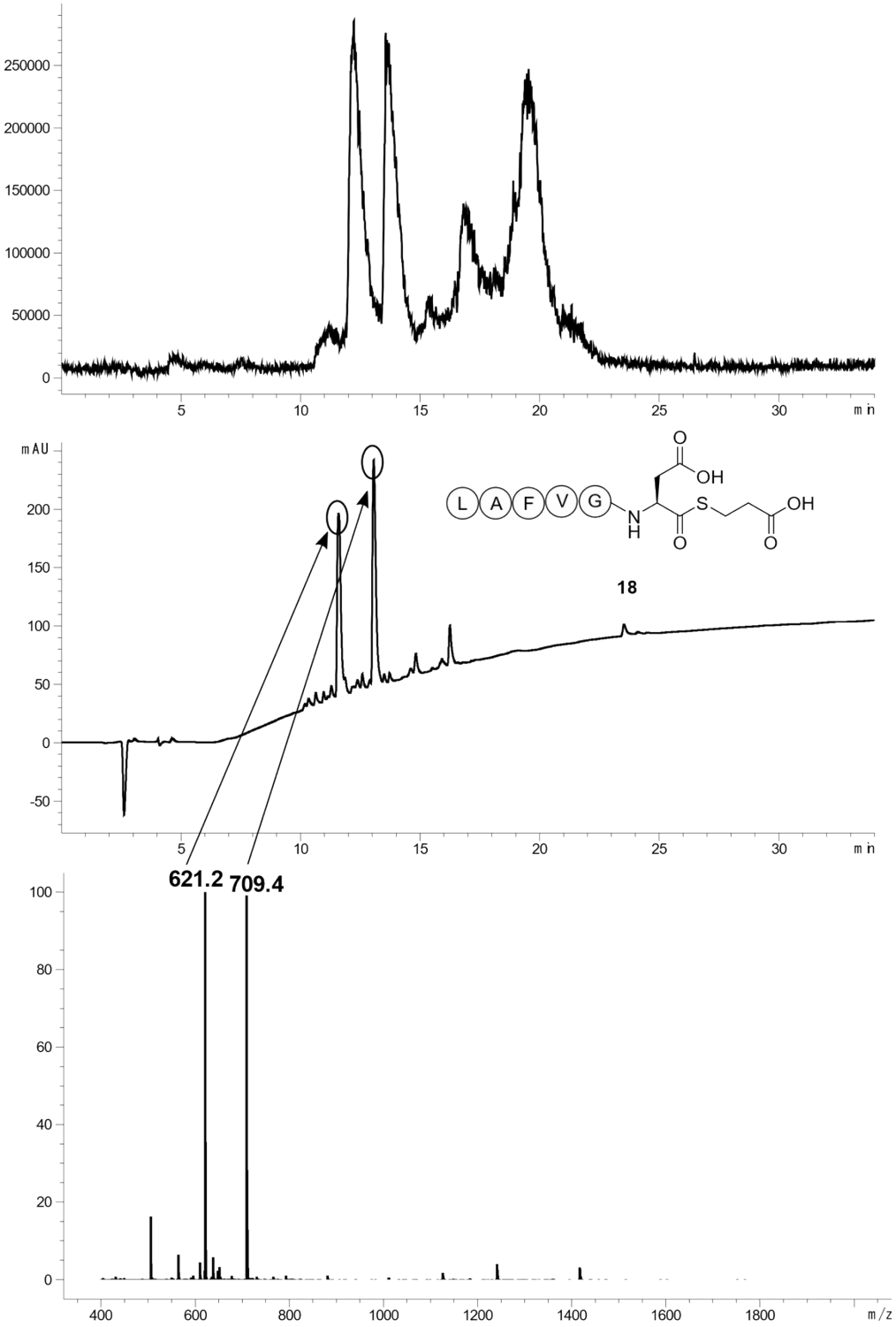


Figure S41: LC-MS profile of crude peptide **18**; ion polarity positive operating at a nominal accelerating voltage of 70 eV. ESI-MS *m/z* $[M+1H]^{1+}$ calcd: 709.8 Da; found: 709.4 Da. Theoretical weight calcd: 708.8 Da. *$[M+1H]^{1+}$ calcd: 621.7 Da; found: 621.2 Da corresponds to unreacted starting material. Chemical formula: $C_{32}H_{48}N_6O_{10}S$.

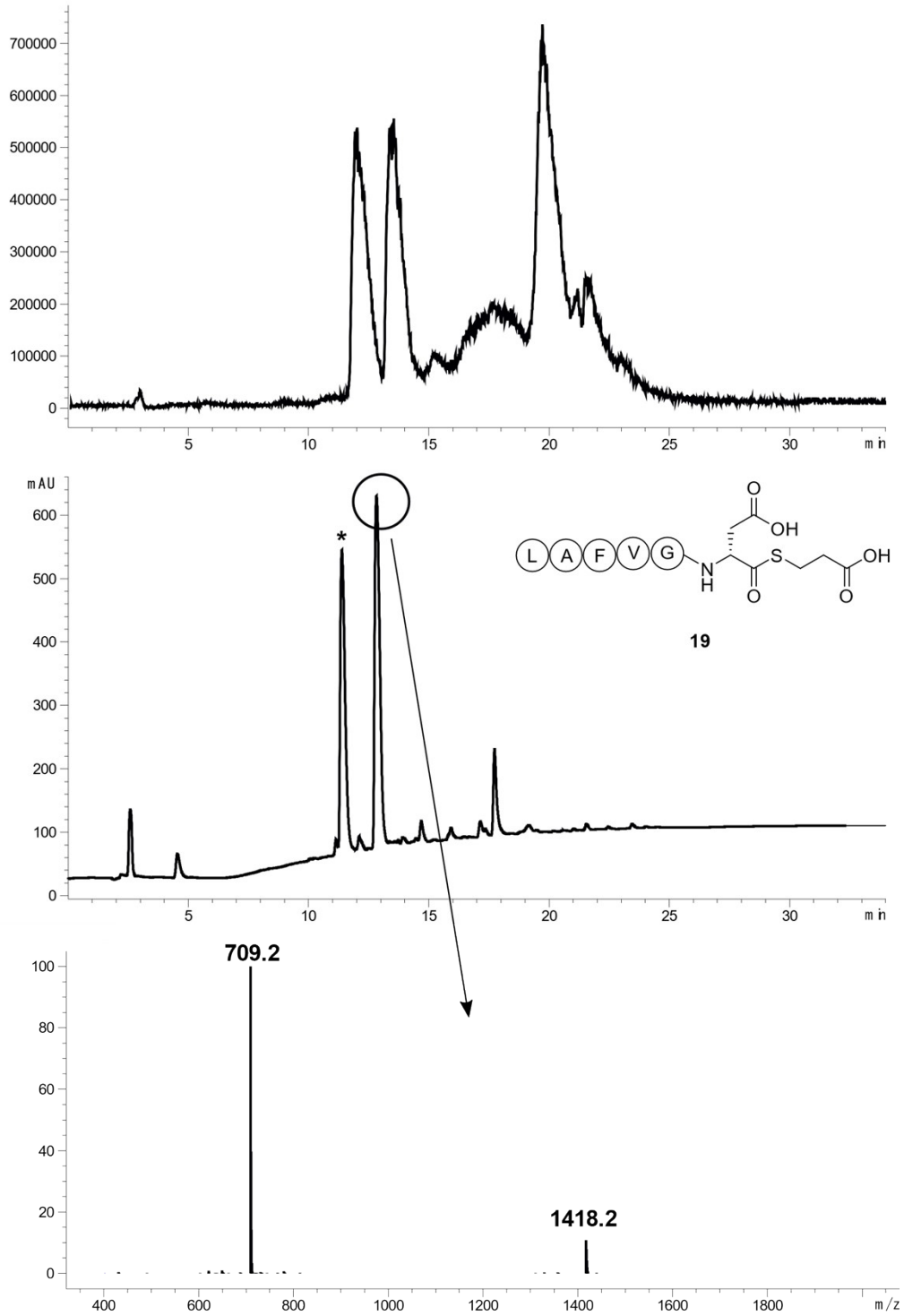
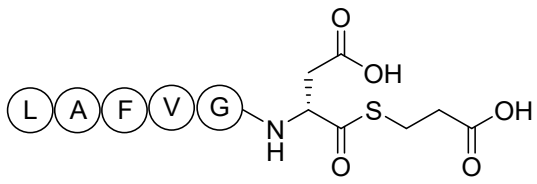


Figure S42: LC-MS profile of crude peptide **19**; ion polarity positive operating at a nominal accelerating voltage of 70 eV. ESI-MS *m/z* $[M+1H]^{1+}$ calcd: 709.8 Da; found: 709.2 Da. Theoretical weight calcd: 708.8 Da. Chemical formula: $C_{32}H_{48}N_6O_{10}S$. *Corresponds to unreacted starting material **19c**.



19

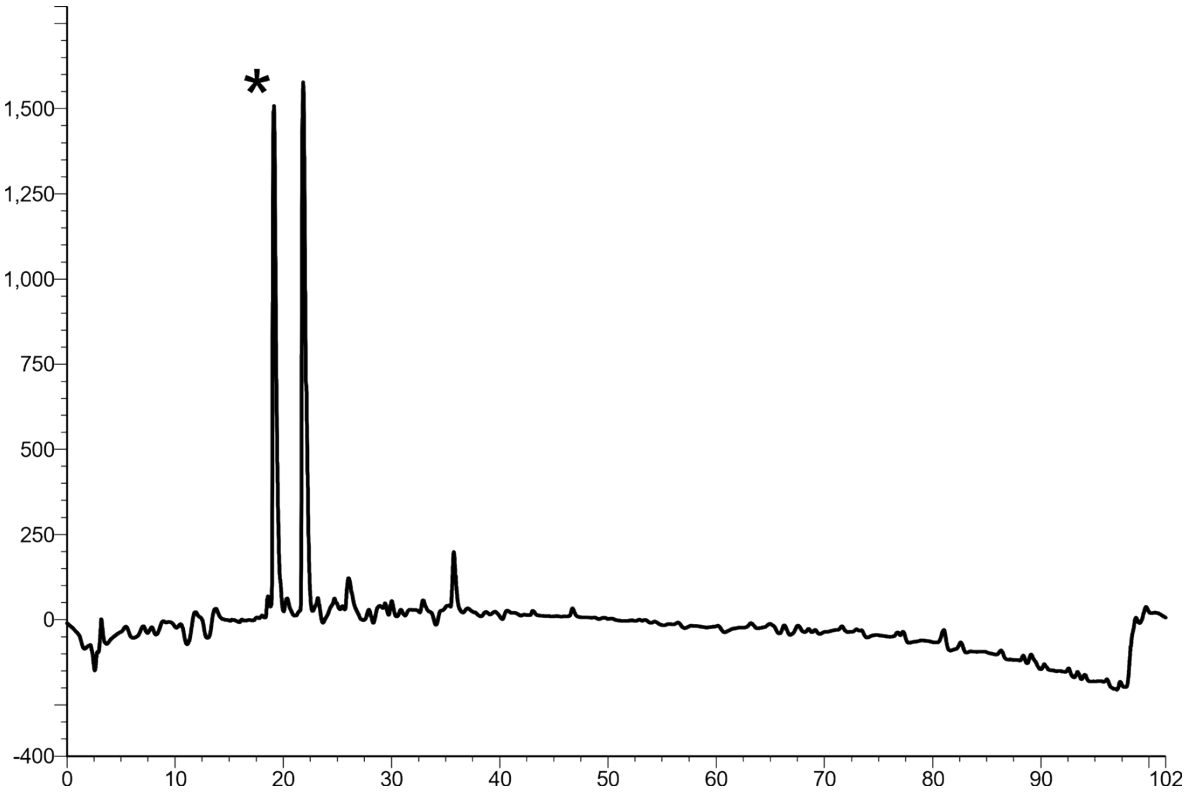


Figure S43: Analytical RP-HPLC chromatogram of crude peptide **19**, t_R = 21.8 min. Chromatographic separations were performed on a Thermo Scientific Dionex Ultimate 3000 HPLC using a Phenomenex® Gemini MS C-18 column (5 µm; 4.6 × 150 mm)and a linear gradient of 5-95% B in 90 min at room temperature, *ca*. 1% B per min at a flow rate of 1.0 mL/min. Buffer A: H_2O containing 0.1% TFA (*v/v*); Buffer B: acetonitrile containing 0.1% TFA (*v/v*). *Peak at t_R = 20.0 min corresponds to unreacted starting material **19c**.

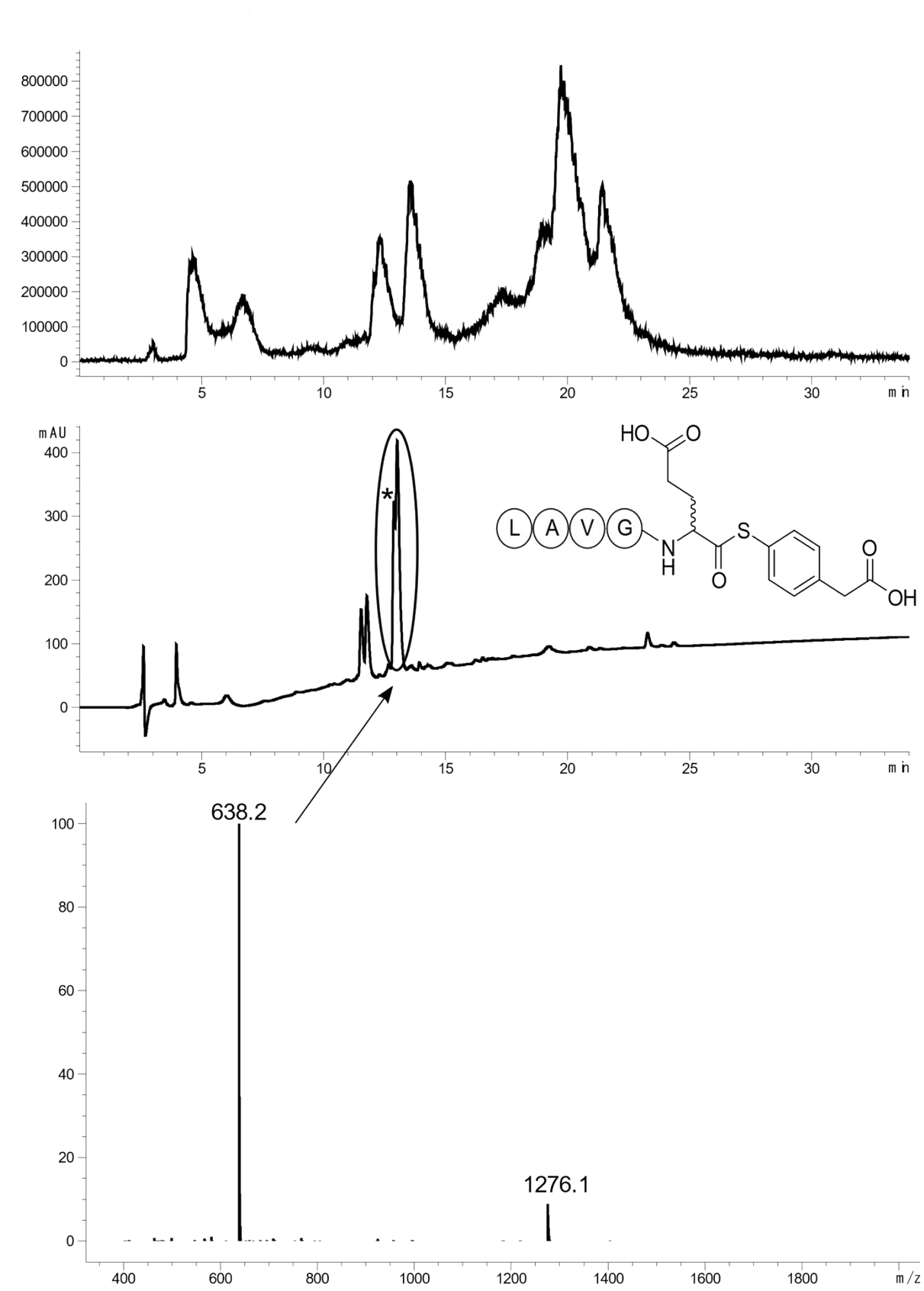


Figure S44: LC-MS profile of crude peptide **23** from table 1 in main manuscript trial 8[a]; ion polarity positive operating at a nominal accelerating voltage of 70 eV. ESI-MS *m/z* $[M+1H]^{1+}$ calcd: 638.7 Da; found: 638.2 Da. corresponds to unreacted starting material. $[2M+1H]^{1+}$ calcd: 1275.6 Da; found 1276.1 Da. Theoretical molecular weight calcd: 637.8 Da. Chemical formula: $C_{29}H_{43}N_5O_9S$. *Two peaks correspond to mixture of diastereomers.

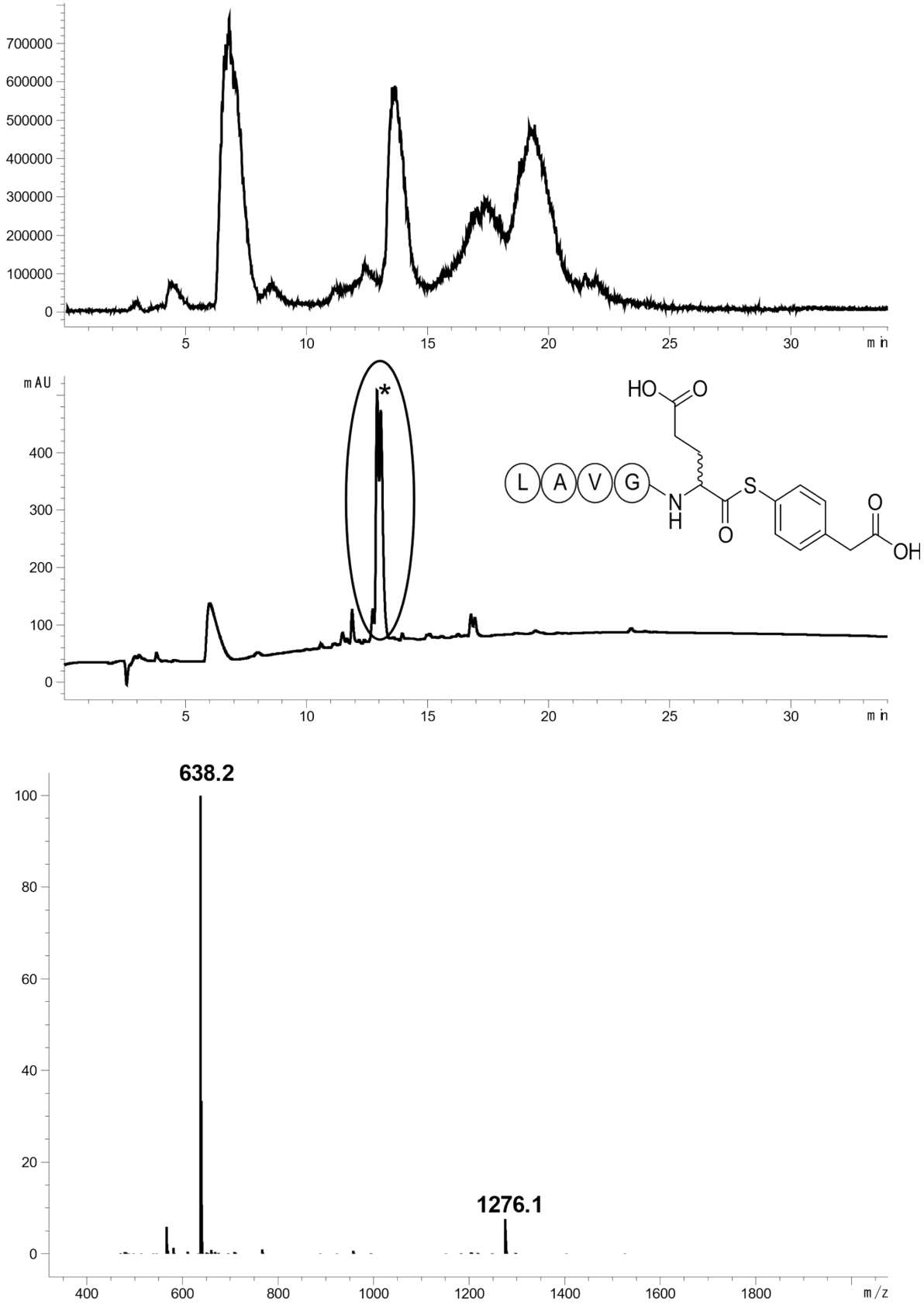


Figure S45: LC-MS profile of crude peptide **24** from table 1 in main manuscript trial 9[a]; ion polarity positive operating at a nominal accelerating voltage of 70 eV. ESI-MS *m/z* $[M+1H]^{1+}$ calcd: 638.7 Da; found: 638.2 Da; $[2M+1H]^{1+}$ calcd: 1275.6 Da; found 1276.1 Da. Theoretical molecular weight calcd: 637.8 Da. Chemical formula: $C_{29}H_{43}N_5O_9S$. *Two peaks correspond to mixture of diastereomers.

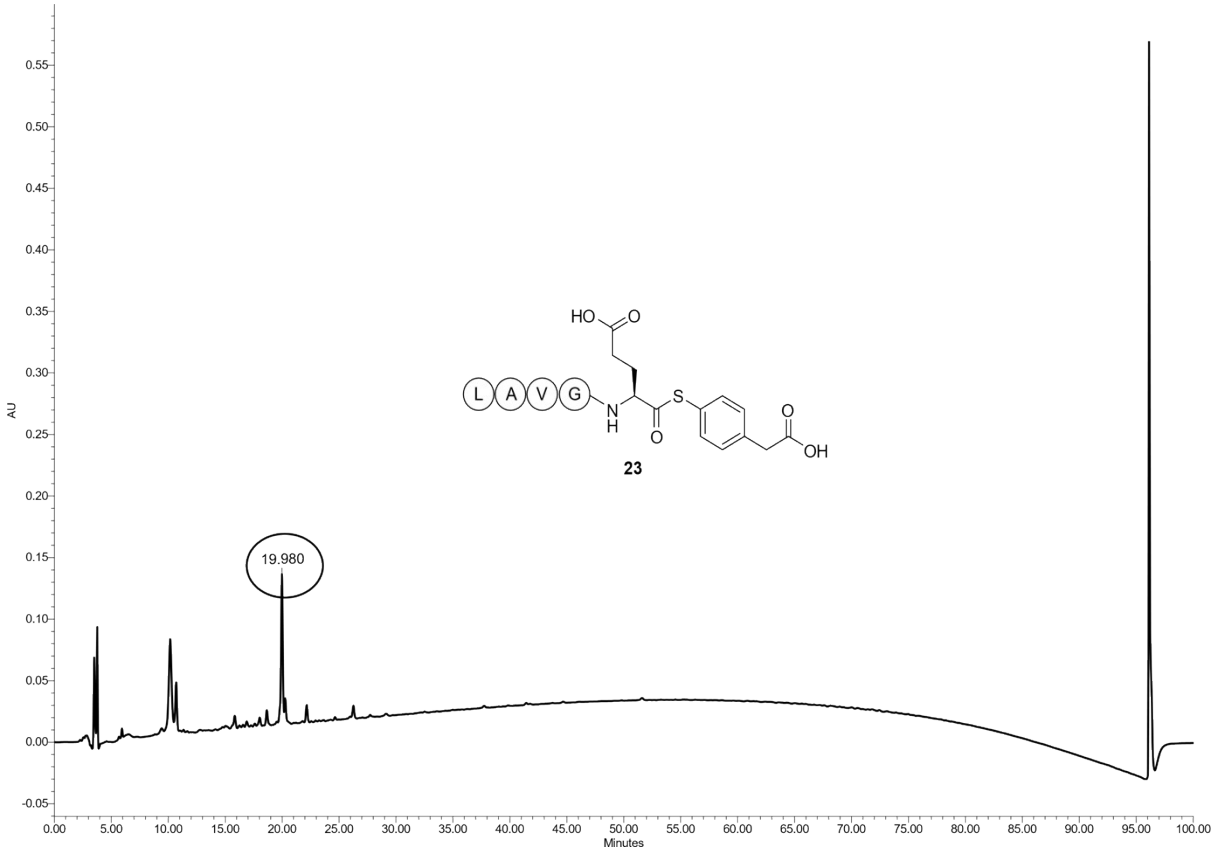


Figure S46: Analytical RP-HPLC chromatogram of crude peptide **23** from table 1 in main manuscript trial 14^{b}, t_R = 19.9 min. Chromatographic separations were performed on a Waters e2695 HPLC using a Agilent TC-C18 column (5 µm; 4.6 × 250 mm) and a linear gradient of 5-95% B in 90 min at room temperature, *ca*. 1% B per min at a flow rate of 1.0 mL/min. Buffer A: H_2O containing 0.1% TFA (*v/v*); Buffer B: acetonitrile containing 0.1% TFA (*v/v*).

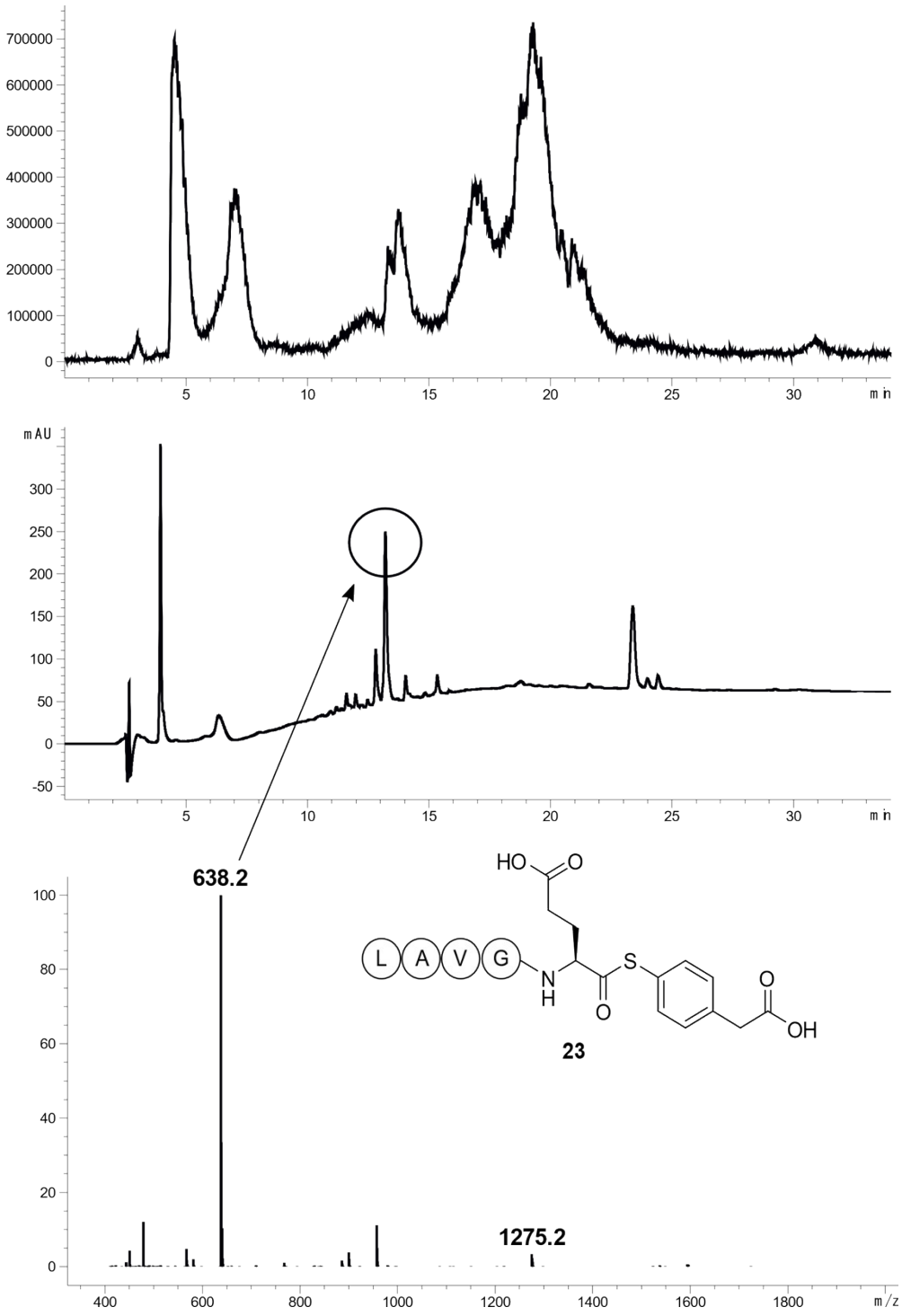


Figure S47: LC-MS profile of crude peptide **23** from table 1 in main manuscript trial 14[b]; ion polarity positive operating at a nominal accelerating voltage of 70 eV. ESI-MS *m/z* $[M+1H]^{1+}$ calcd: 638.7 Da; found: 638.2 Da; $[2M+1H]^{1+}$ calcd: 1275.6 Da; found 1275.2 Da. Theoretical molecular weight calcd: 637.8 Da. Chemical formula: $C_{29}H_{43}N_5O_9S$.

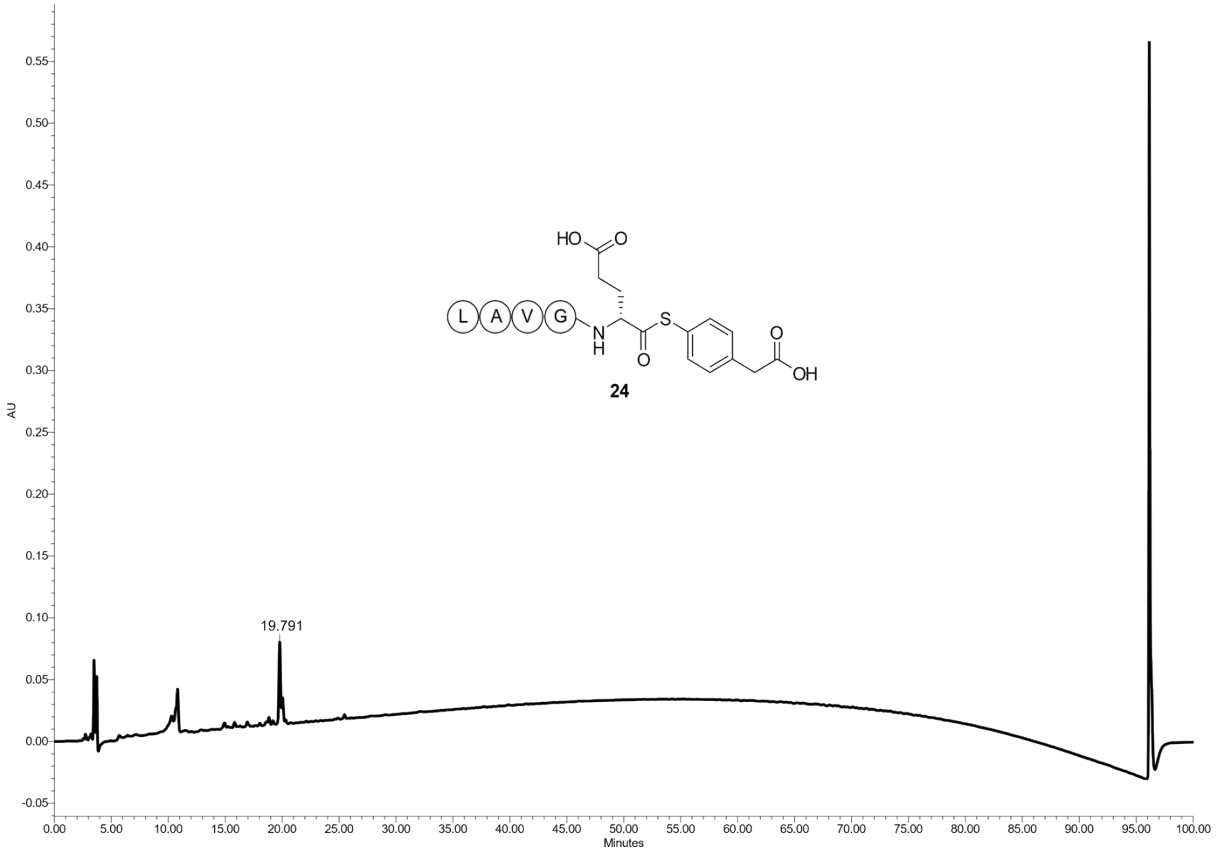


Figure S48: Analytical RP-HPLC chromatogram of crude peptide **24** from table 1 in main manuscript trial 15[b], t_R = 19.8 min. Chromatographic separations were performed on a Waters e2695 HPLC using an Agilent TC-C18 column (5 µm; 4.6 × 250 mm) and a linear gradient of 5-95% B in 90 min at room temperature, *ca*. 1% B per min at a flow rate of 1.0 mL/min. Buffer A: H_2O containing 0.1% TFA (*v/v*); Buffer B: acetonitrile containing 0.1% TFA (*v/v*).

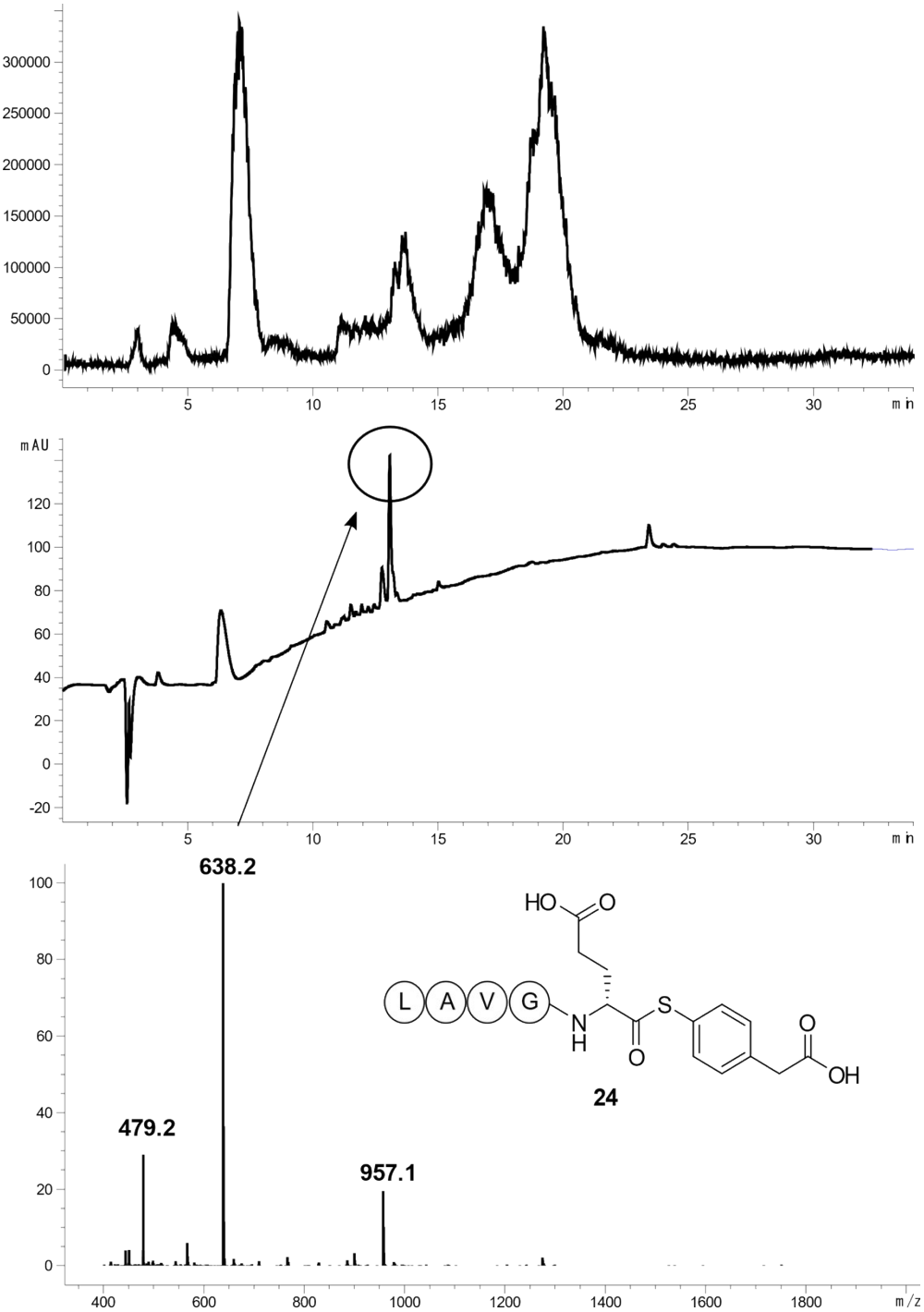


Figure S49: LC-MS profile of crude peptide **24** from table 1 in main manuscript trial 15[b]; ion polarity positive operating at a nominal accelerating voltage of 70 eV. ESI-MS *m/z* $[M+1H]^{1+}$ calcd: 638.7 Da; found: 638.2 Da. Theoretical molecular weight calcd: 637.8 Da. Chemical formula: $C_{29}H_{43}N_5O_9S$.

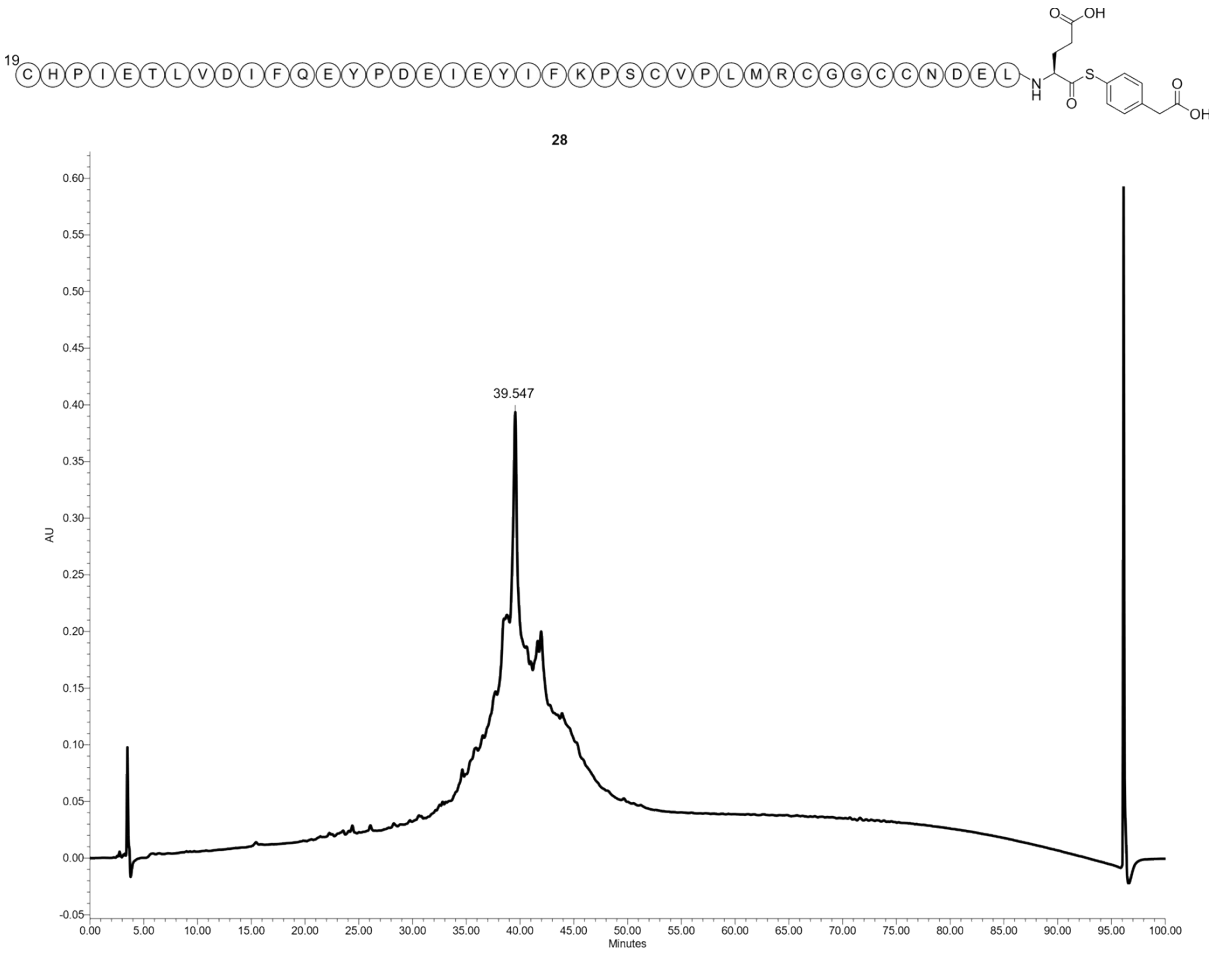


Figure S50: Analytical RP-HPLC chromatogram of crude peptide **28**, t_R = 39.5 min. Chromatographic separations were performed on a Waters e2695 HPLC using an Agilent TC-C18 column (5 µm; 4.6 × 250 mm) and a linear gradient of 5-95% B in 90 min at room temperature, *ca*. 1% B per min at a flow rate of 1.0 mL/min. Buffer A: H_2O containing 0.1% TFA (*v/v*); Buffer B: acetonitrile containing 0.1% TFA (*v/v*).

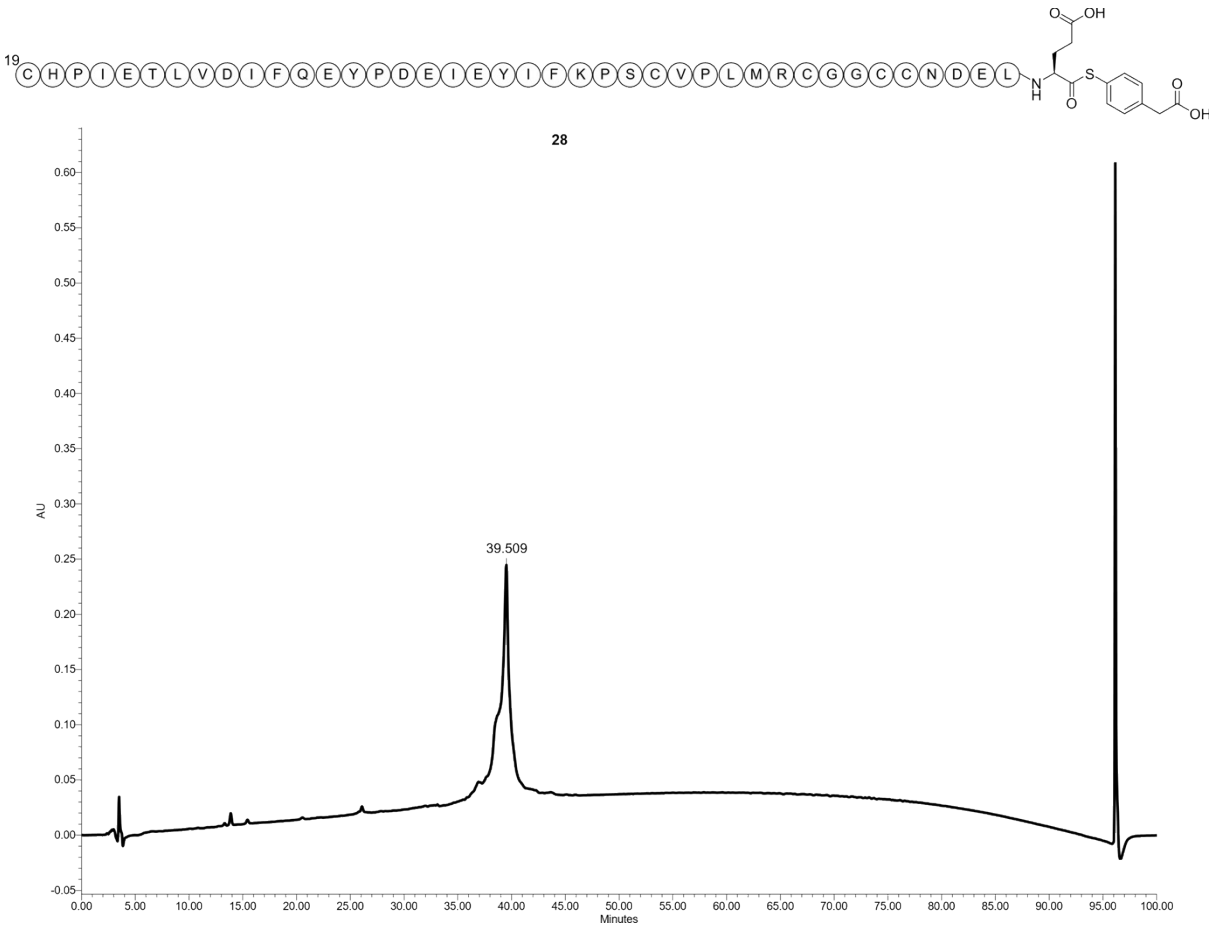


Figure S51: Analytical RP-HPLC chromatogram of purified peptide **28**, t_R = 39.5 min. Chromatographic separations were performed on a Waters e2695 HPLC using an Agilent TC-C18 column (5 µm; 4.6 × 250 mm) and a linear gradient of 5-95% B in 90 min at room temperature, *ca*. 1% B per min at a flow rate of 1.0 mL/min. Buffer A: H_2O containing 0.1% TFA (*v/v*); Buffer B: acetonitrile containing 0.1% TFA (*v/v*).

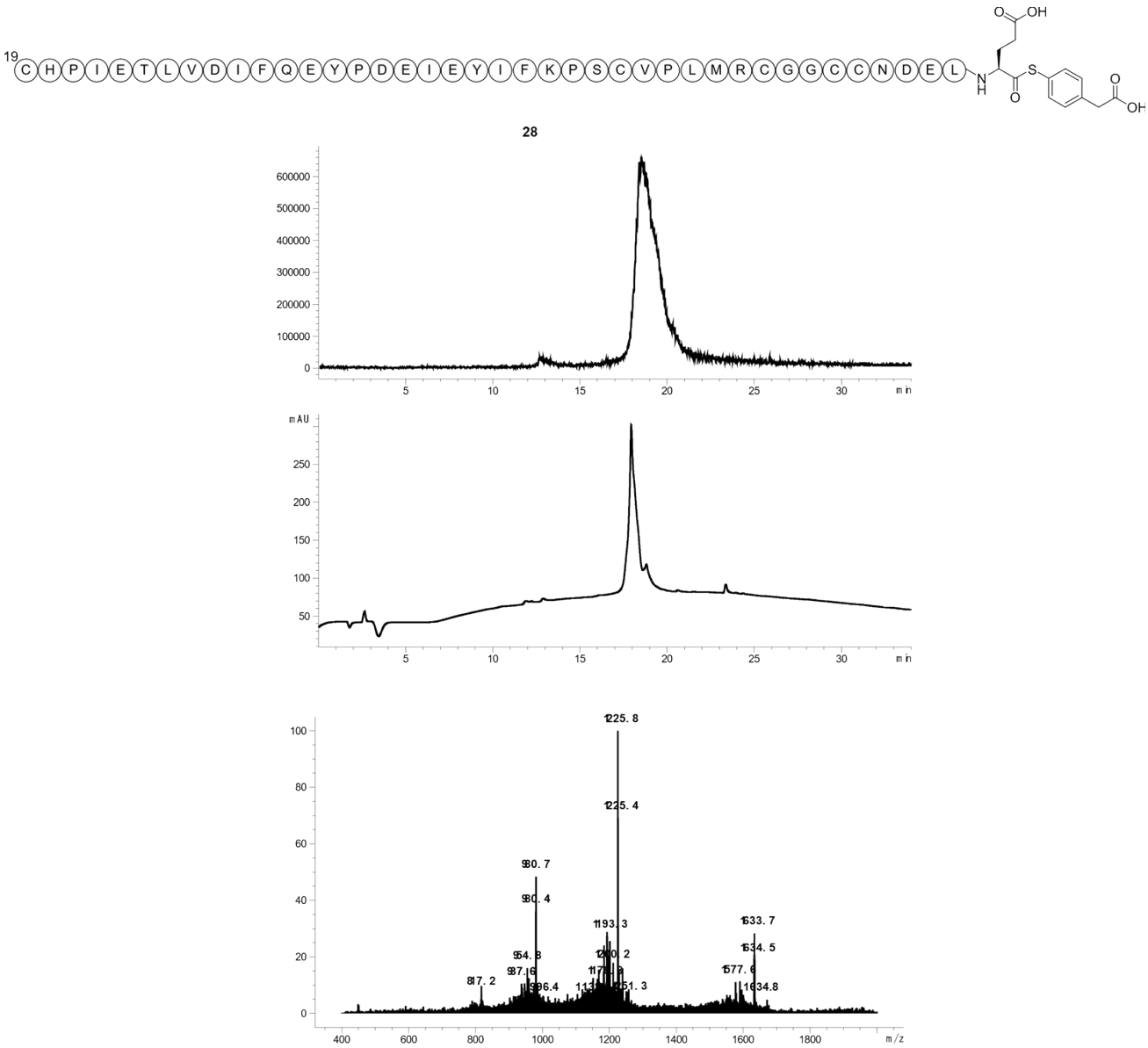


Figure S52: LC-MS profile of purified peptide **28**; ion polarity positive operating at a nominal accelerating voltage of 70 eV. ESI-MS *m/z* $[M+3H]^{3+}$ calcd: 1634.5 Da; found: 1633.7 Da; $[M+4H]^{4+}$ calcd: 1226.1 Da; found:1225.8 Da; $[M+5H]^{5+}$ calcd: 981.1 Da; found: 980.7 Da. Mass deconvolution calculated at 4898.60 Da with std deviation of 0.56. Theoretical molecular weight calcd: 4900.6 Da. Chemical formula: $C_{216}H_{321}N_{49}O_{67}S_7$.

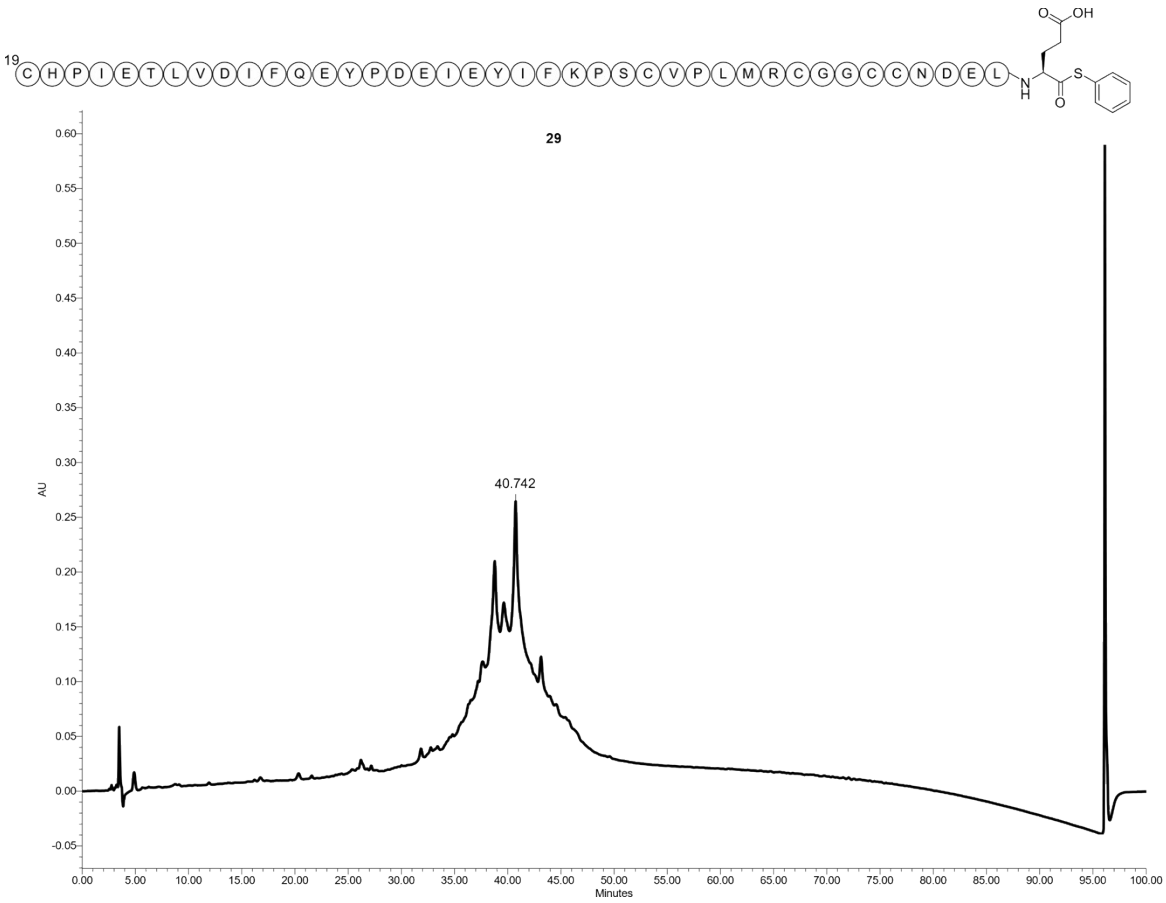


Figure S53: Analytical RP-HPLC chromatogram of crude peptide **29**, t_R = 40.7 min. Chromatographic separations were performed on a Waters e2695 HPLC using an Agilent TC-C18 column (5 µm; 4.6 × 250 mm) and a linear gradient of 5-95% B in 90 min at room temperature, *ca*. 1% B per min at a flow rate of 1.0 mL/min. Buffer A: H_2O containing 0.1% TFA (*v/v*); Buffer B: acetonitrile containing 0.1% TFA (*v/v*).

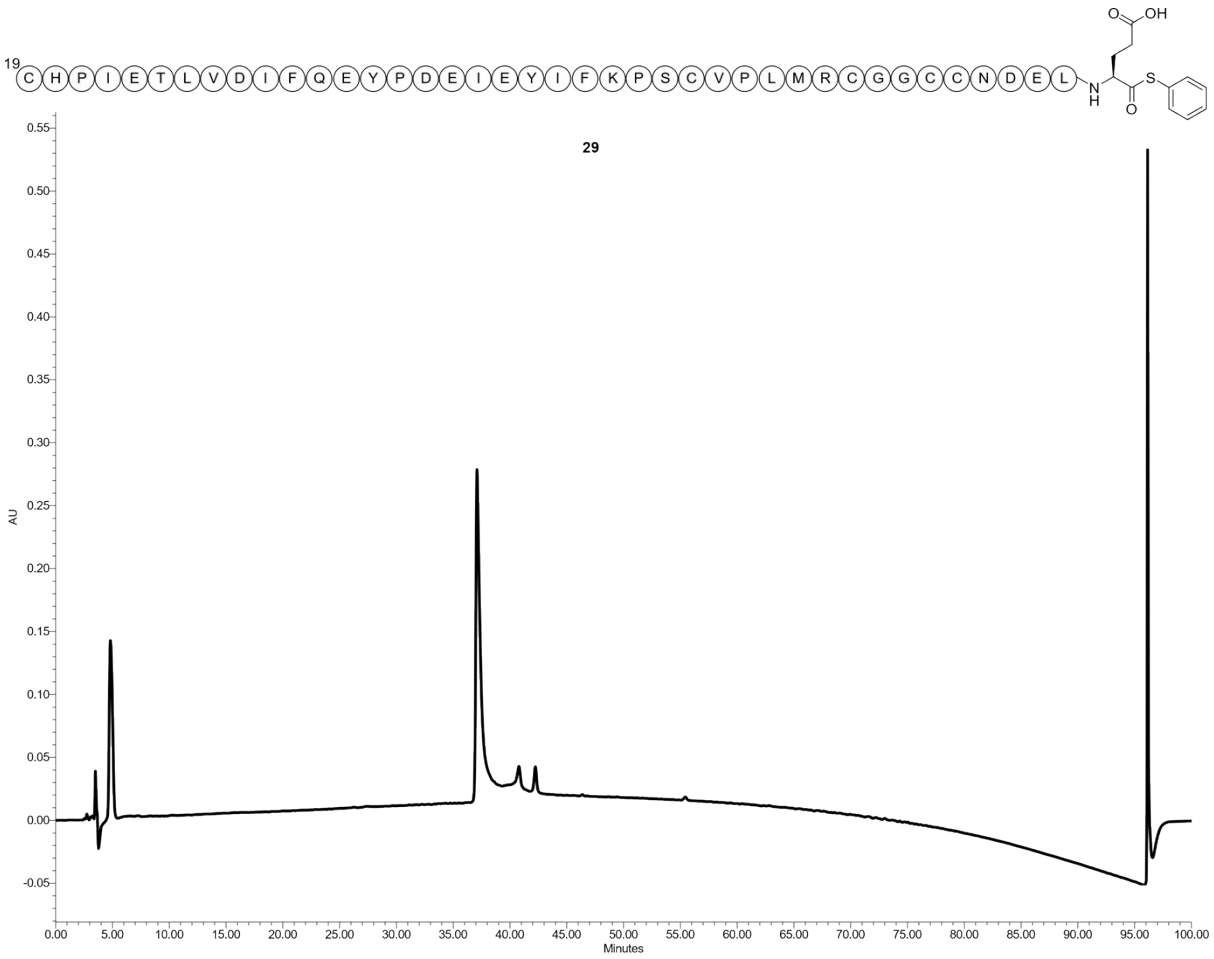


Figure S54: Analytical RP-HPLC chromatogram of purified peptide **29**, t_R = 37.1 min. Chromatographic separations were performed on a Waters e2695 HPLC using an Agilent TC-C18 column (5 µm; 4.6 × 250 mm) and a linear gradient of 5-95% B in 90 min at room temperature, *ca*. 1% B per min at a flow rate of 1.0 mL/min. Buffer A: H_2O containing 0.1% TFA (*v/v*); Buffer B: acetonitrile containing 0.1% TFA (*v/v*).

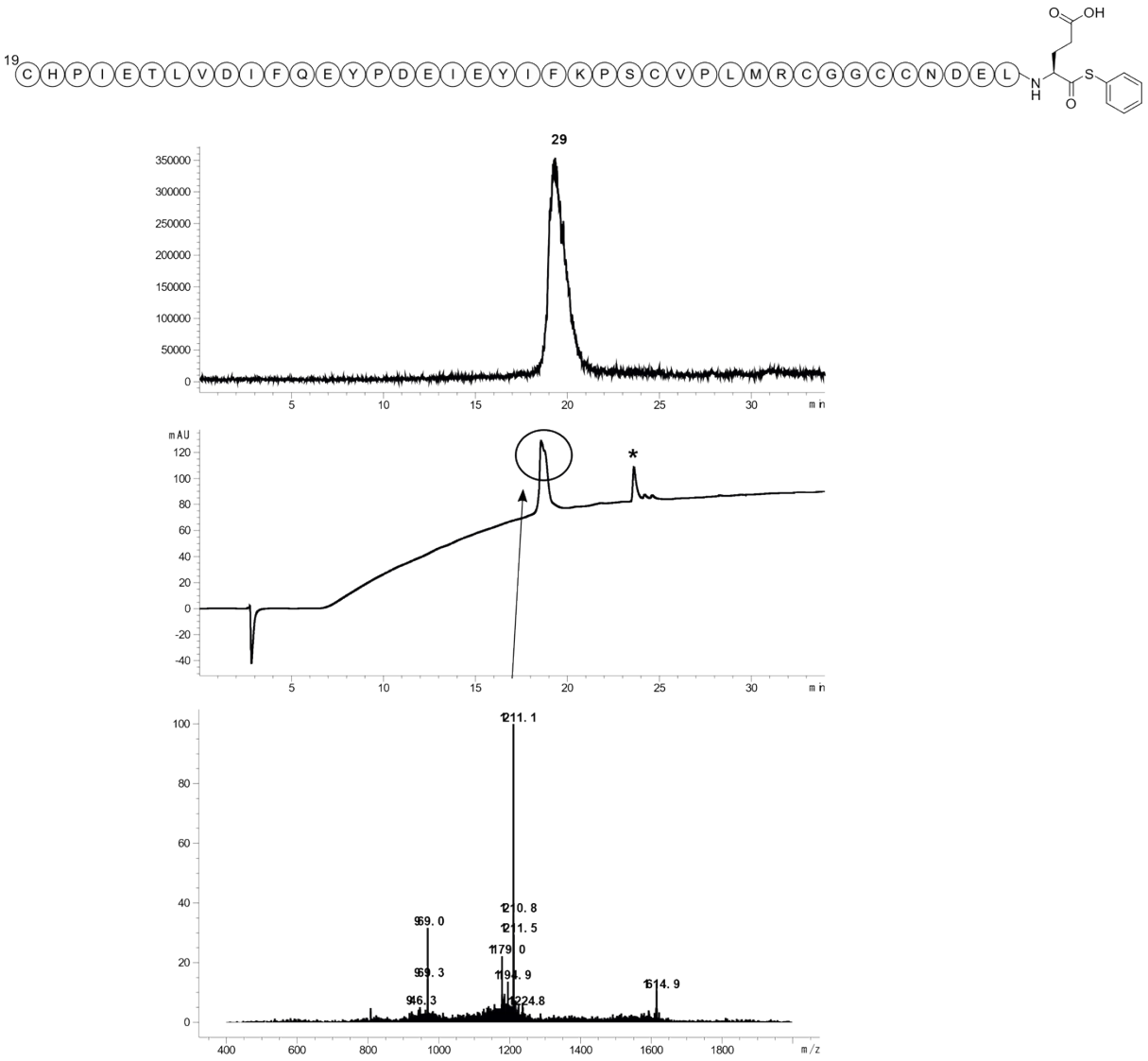


Figure S55: LC-MS profile of purified peptide **29**; ion polarity positive operating at a nominal accelerating voltage of 70 eV. ESI-MS *m/z* $[M+3H]^{3+}$ calcd: 1615.2 Da; found: 1614.9 Da; $[M+4H]^{4+}$ calcd: 1211.6 Da; found:1211.1 Da; $[M+5H]^{5+}$ calcd: 969.5 Da; found: 969.0 Da. Mass deconvolution calculated at 4840.70 Da with std deviation of 0.89. Theoretical molecular weight calcd: 4842.6 Da. Chemical formula: $C_{214}H_{319}N_{49}O_{65}S_7$. *Column artifact.

Diastereomer RP-HPLC/LCMS Co-injection Analysis

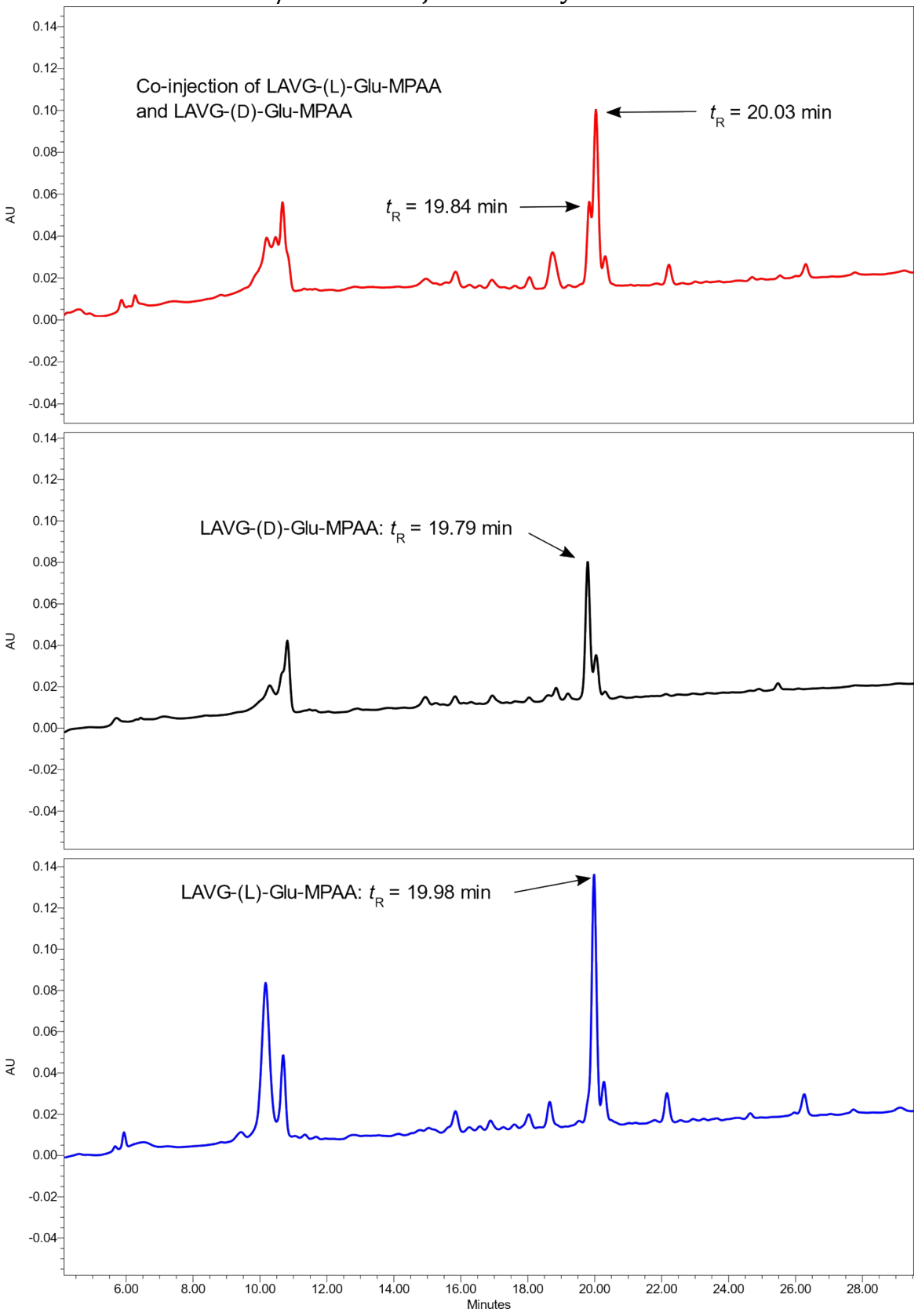


Figure S56: Analytical RP-HPLC chromatogram of crude peptide co-injection of LAVG-(L)-Glu-MPAA (**23**) + LAVG-(D)-Glu-MPAA (**24**) diastereomers. Chromatographic separations were performed on a Waters e2695 HPLC using an Agilent TC-C18 column (5 µm; 4.6 × 250 mm) and a linear gradient of 5-95% B in 90 min at room temperature, *ca.* 1% B per min at a flow rate of 1.0 mL/min. Buffer A: H_2O containing 0.1% TFA (*v/v*); Buffer B: acetonitrile containing 0.1% TFA (*v/v*).

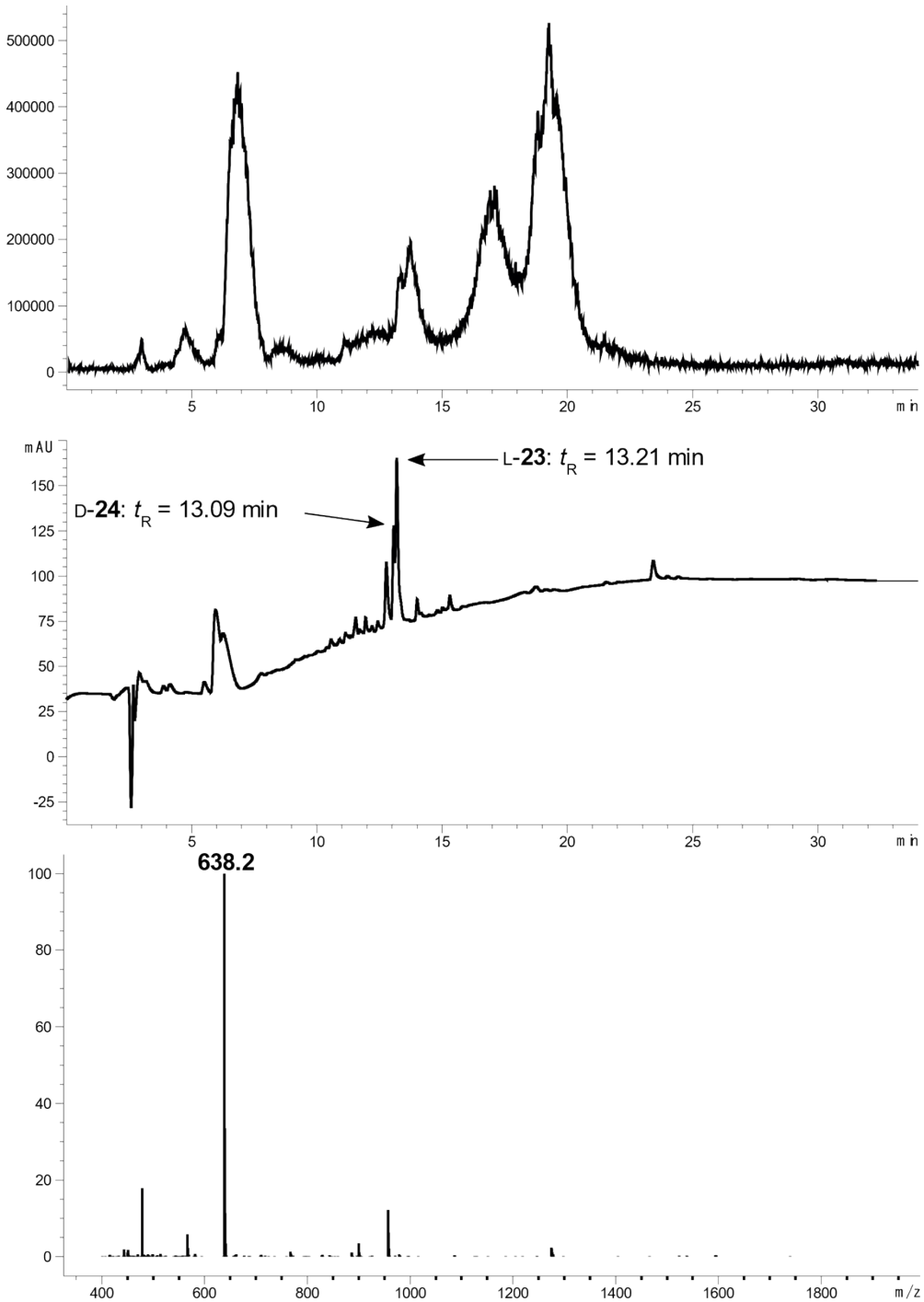
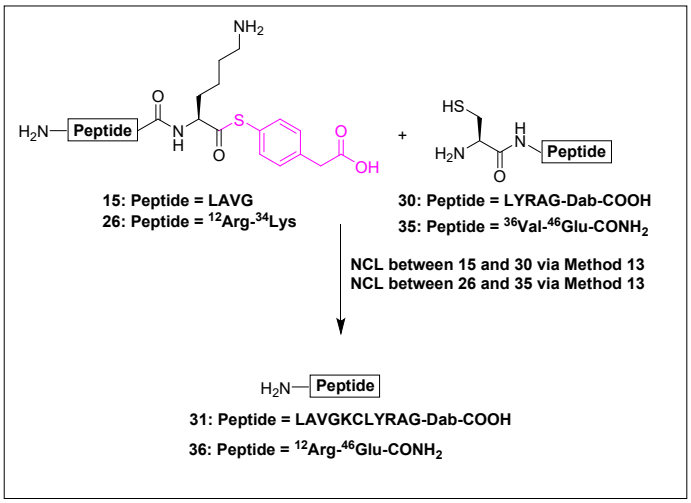


Figure S57: LC-MS profile of crude peptide co-injection of LAVG-(L)-Glu-MPAA (**23**) + LAVG-(D)-Glu-MPAA (**24**) diastereomers from trial 17[b] and 18[b] in main manuscript; ion polarity positive operating at a nominal accelerating voltage of 70 eV. ESI-MS *m/z* $[M+1H]^{1+}$ calcd: 638.8 Da; found: 638.2 Da. Theoretical molecular weight calcd: 637.8 Da. Chemical formula: $C_{29}H_{43}N_5O_9S$.

Method 13: General procedure for native chemical ligations:

To thioester derived peptide (2 mM) and cysteinyl fragment (2 mM) was added a solution of (6 M Gu·HCl/0.2 M Na_2HPO_4/50 mM TCEP·HCl) in MQ H_2O degassed with argon and pH adjusted using a non-glass S2K922 ISFET pH sensor to7.35 - 7.41 using 5 M HCl and 10 M NaOH. The resulting solution was sonicated for 1 min then agitated at room temperature for 1-3 h. The crude peptide solution was immediately purified batchwise by semi-preparative RP-HPLC.

Lysine C^{α}-thioester NCL Studies



Scheme S4. General synthetic scheme for lysine fragment NCL.

LC-MS of purified cysteine intermediate **30** for use in NCL

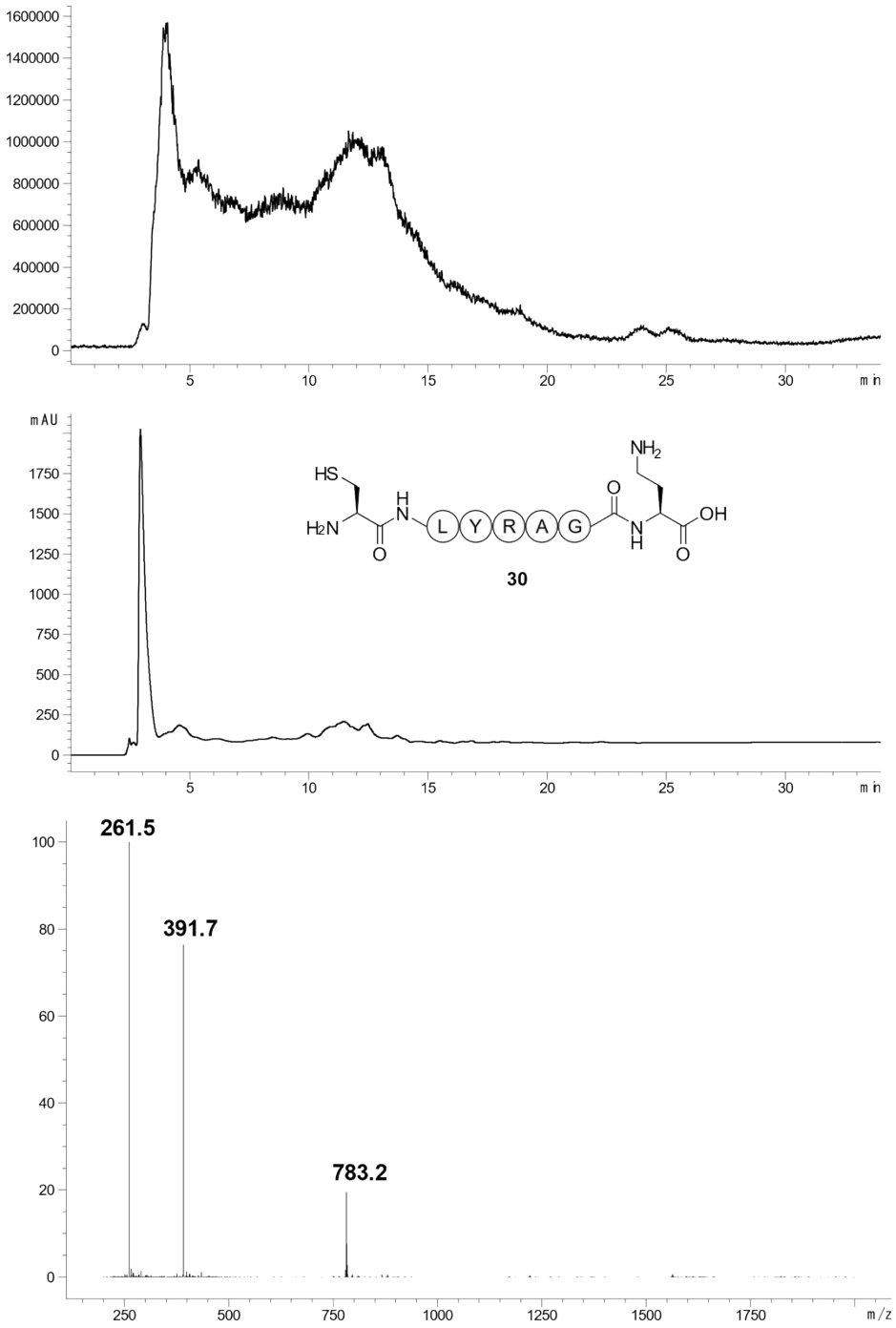


Figure S58: LC-MS profile of purified peptide **30**; ion polarity positive operating at a nominal accelerating voltage of 70 eV. ESI-MS *m/z* $[M+1H]^{1+}$ calcd: 782.9 Da; found: 782.2 Da; $[M+2H]^{2+}$ calcd: 391.9 Da; found: 391.7 Da; $[M+3H]^{3+}$ calcd: 261.6 Da; found: 261.5 Da. Theoretical molecular weight calcd: 781.9 Da. Chemical formula: $C_{33}H_{55}N_{11}O_9S$. *Compound **30** synthesised *via* general methods.

LC-MS of purified cysteine intermediate **35** for use in NCL

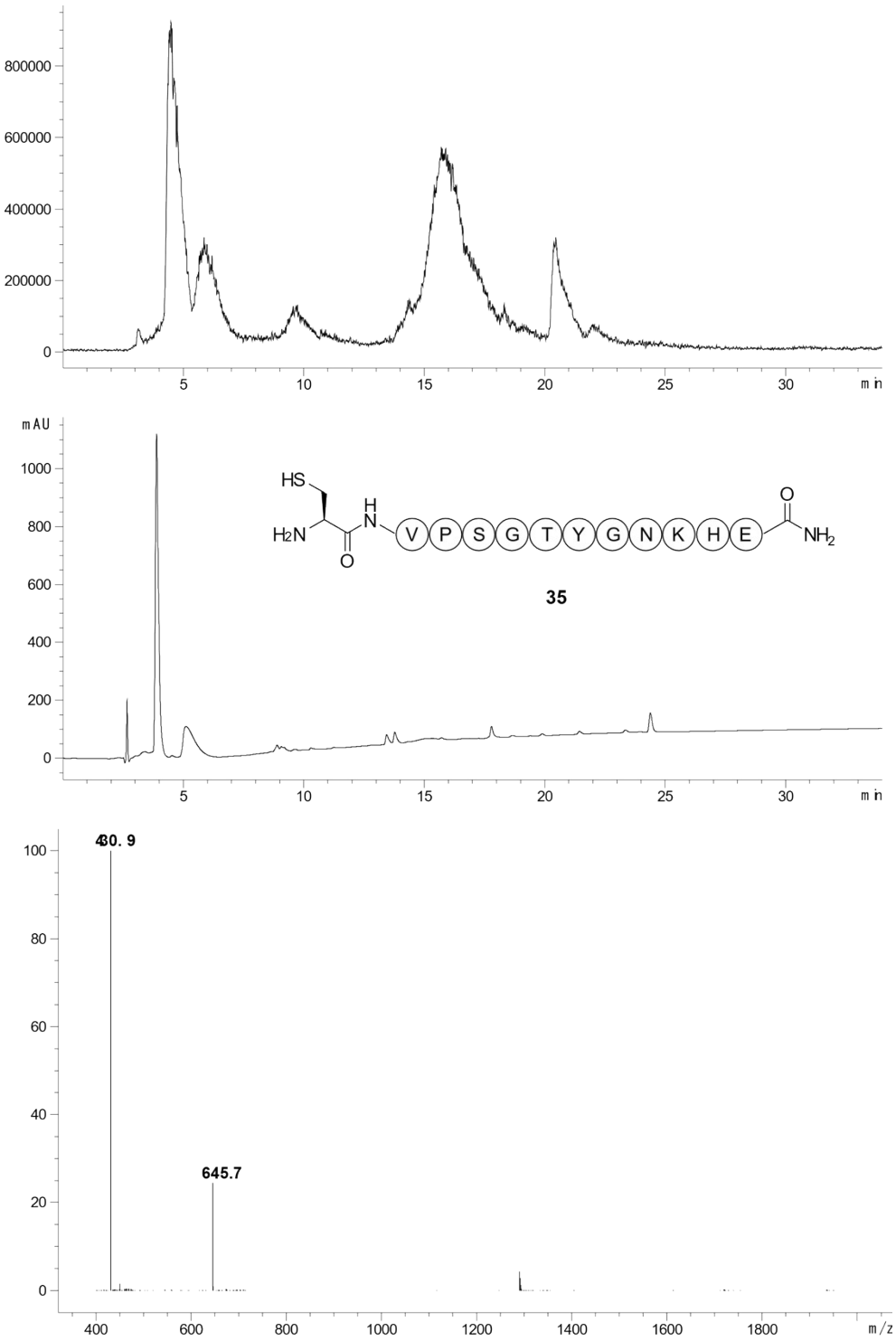


Figure S59: LC-MS profile of purified peptide **35**; ion polarity positive operating at a nominal accelerating voltage of 70 eV. ESI-MS *m/z* $[M+2H]^{2+}$ calcd: 646.2 Da; found: 645.7 Da; $[M+3H]^{3+}$ calcd: 431.1 Da; found: 430.9 Da. Theoretical molecular weight calcd: 1290.4 Da. Chemical formula: $C_{54}H_{83}N_{17}O_{18}S$. *Compound **35** synthesised *via* general methods.

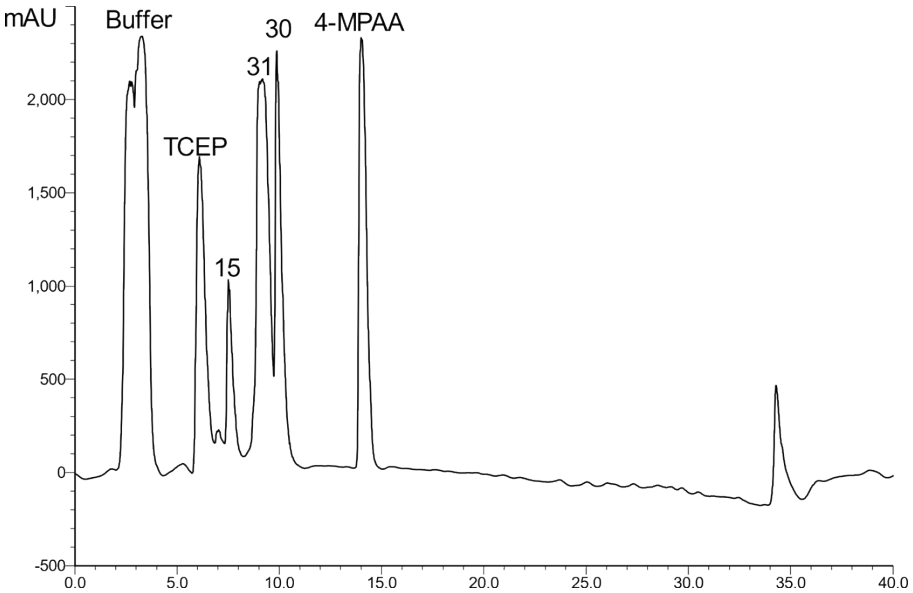


Figure S60: Analytical RP-HPLC chromatogram of NCL between Lys **15** and cysteine **30** at t = 0 h. Chromatographic separations were performed on a Thermo Scientific Dionex Ultimate 3000 HPLC using a Phenomenex® Gemini MS C-18 column (5 µm; 4.6 × 150 mm) and a linear gradient of 5-95% B in 40 min at room temperature, *ca*. 3% B per min at a flow rate of 1.0 mL/min. Buffer A: H_2O containing 0.1% TFA (*v/v*); Buffer B: acetonitrile containing 0.1% TFA (*v/v*).

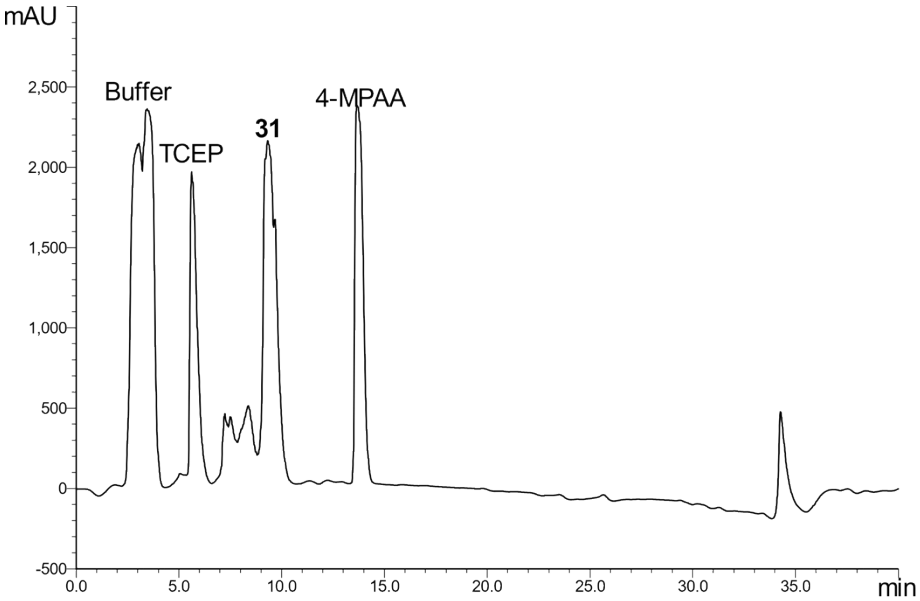


Figure S61: Analytical RP-HPLC chromatogram of NCL between Lys **15** and cysteine **30** at t = 1 h. Chromatographic separations were performed on a Thermo Scientific Dionex Ultimate 3000 HPLC using a Phenomenex® Gemini MS C-18 column (5 µm; 4.6 × 150 mm) and a linear gradient of 5-95% B in 40 min at room temperature, *ca*. 3% B per min at a flow rate of 1.0 mL/min. Buffer A: H_2O containing 0.1% TFA (*v/v*); Buffer B: acetonitrile containing 0.1% TFA (*v/v*).

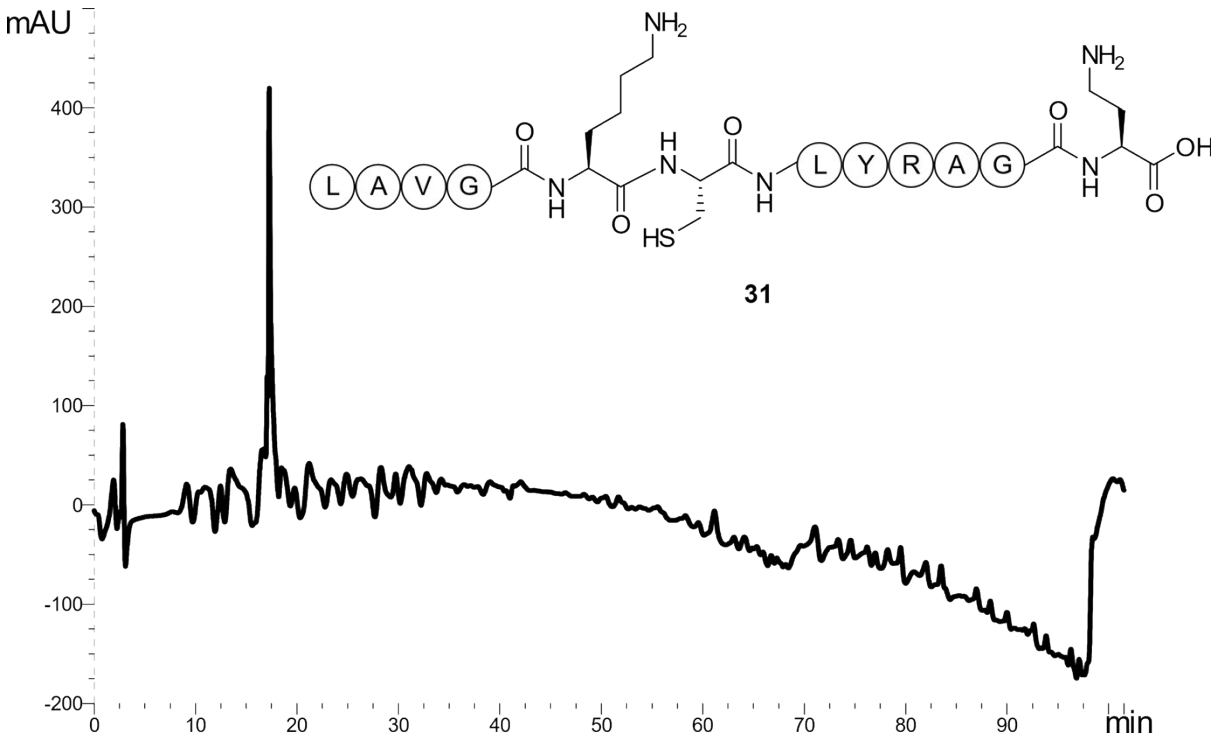


Figure S62: Analytical RP-HPLC chromatogram of purified **31**, t_R = 17.3 min. Chromatographic separations were performed on a Thermo Scientific Dionex Ultimate 3000 HPLC using a Phenomenex® Gemini MS C-18 column (5 µm; 4.6 × 150 mm) and a linear gradient of 5-95% B in 90 min at room temperature, *ca*. 1% B per min at a flow rate of 1.0 mL/min. Buffer A: H_2O containing 0.1% TFA (*v/v*); Buffer B: acetonitrile containing 0.1% TFA (*v/v*).

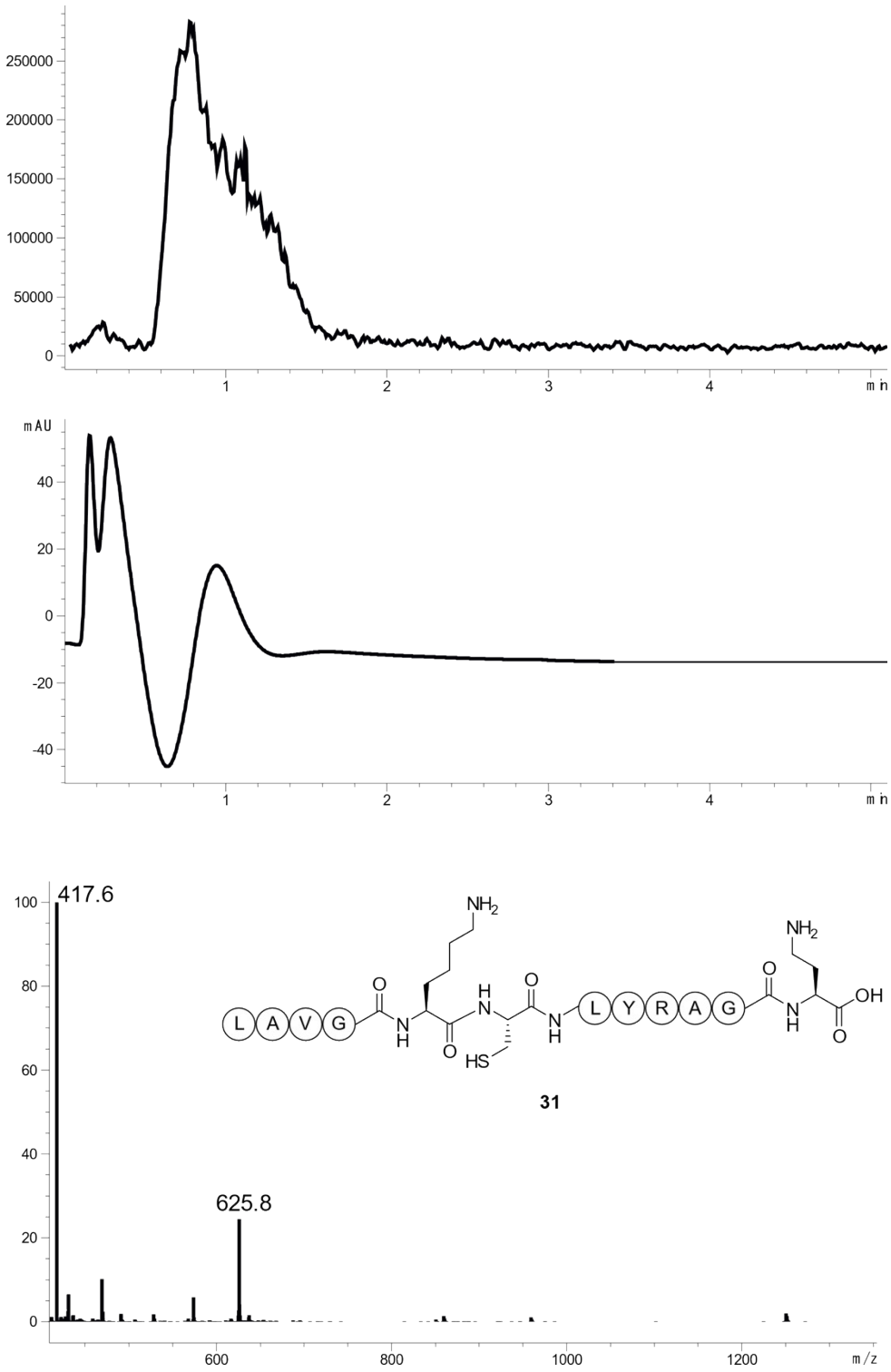


Figure S63: ESI-MS profile of purified peptide **31**; ion polarity positive operating at a nominal accelerating voltage of 70 eV. ESI-MS *m/z* $[M+2H]^{2+}$ calcd: 626.2 Da; found: 625.8 Da; $[M+3H]^{3+}$ calcd: 417.8 Da; found:417.6 Da. Theoretical molecular weight calcd: 1250.5 Da. Chemical formula: $C_{55}H_{95}N_{17}O_{14}S$.

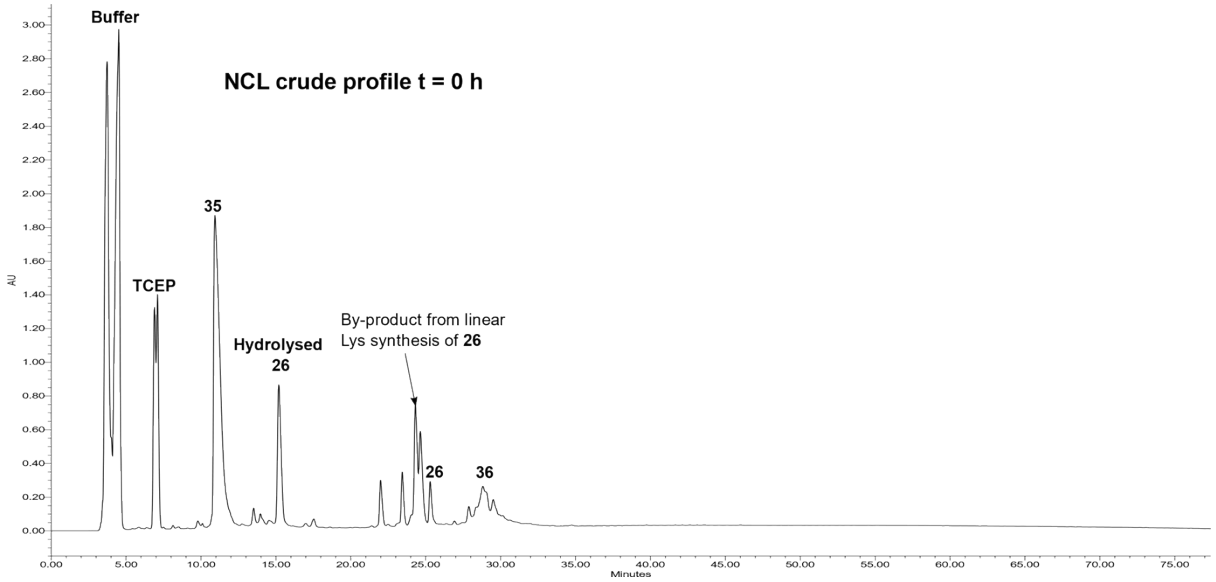


Figure S64: Analytical RP-HPLC chromatogram of crude peptide NCL between **26** and **35** at t = 0 h. Chromatographic separations were performed on a Waters e2695 HPLC using a Agilent TC-C18 column (5 µm; 4.6 × 250 mm) and a linear gradient of 5-95% B in 90 min at room temperature, *ca.* 1% B per min at a flow rate of 1.0 mL/min. Buffer A: H_2O containing 0.1% TFA (*v/v*); Buffer B: acetonitrile containing 0.1% TFA (*v/v*).

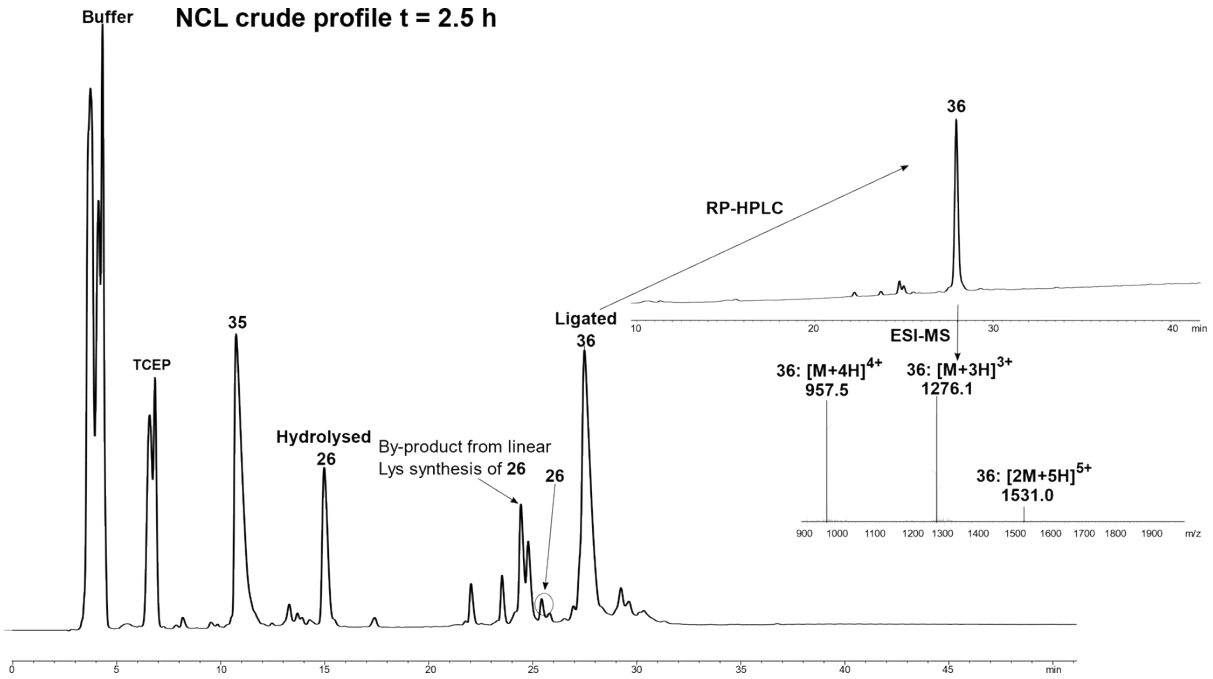


Figure S65: Analytical RP-HPLC chromatogram of crude peptide NCL between **26** and **35** at t = 2.5 h. Chromatographic separations were performed on a Waters e2695 HPLC using a Agilent TC-C18 column (5 µm; 4.6 × 250 mm) and a linear gradient of 5-95% B in 90 min at room temperature, *ca.* 1% B per min at a flow rate of 1.0 mL/min. Buffer A: H_2O containing 0.1% TFA (*v/v*); Buffer B: acetonitrile containing 0.1% TFA (*v/v*).

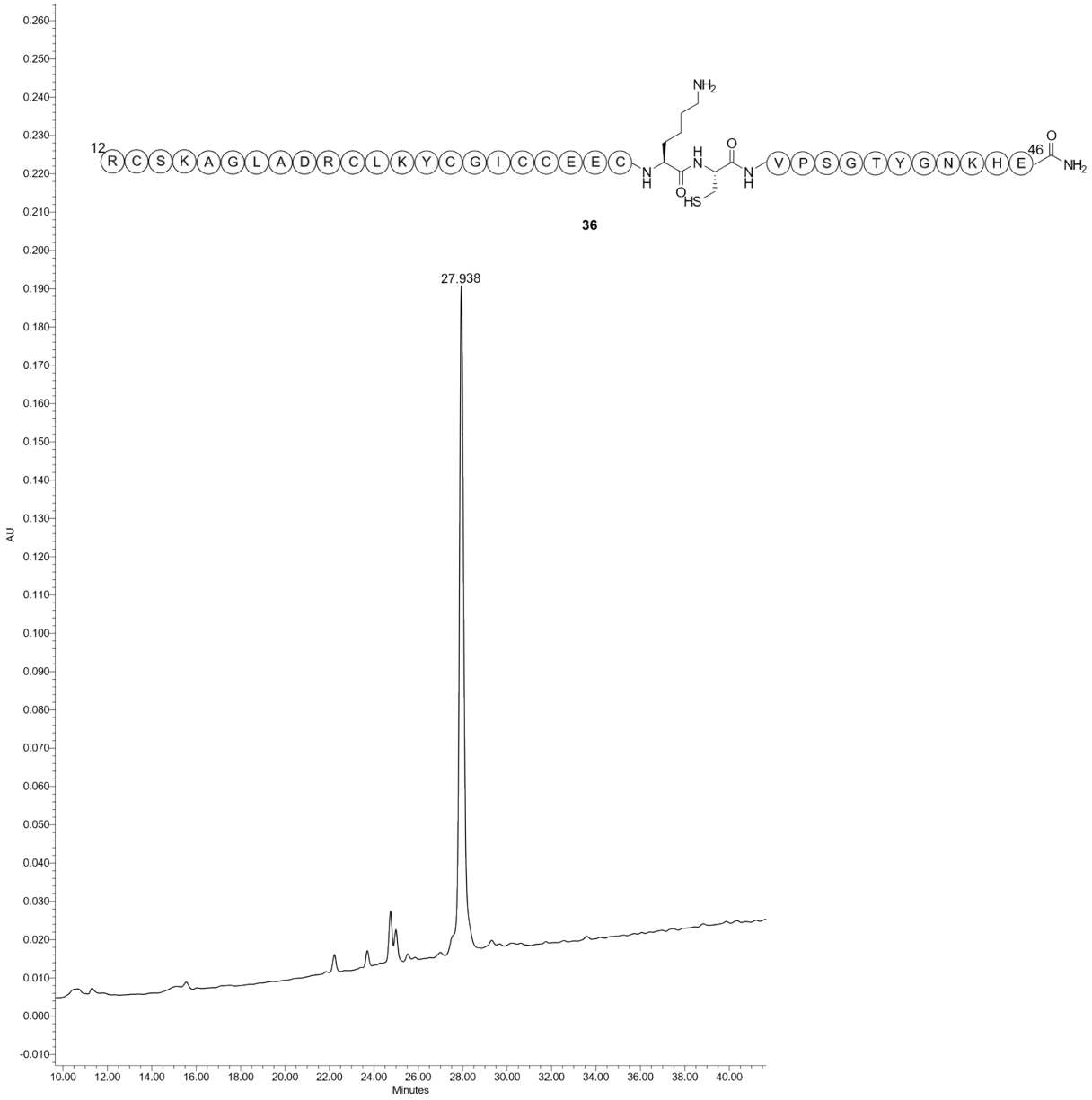


Figure S66: Analytical RP-HPLC chromatogram of purified peptide **36**, t_R = 27.9 min. Chromatographic separations were performed on a Waters e2695 HPLC using a Agilent TC-C18 column (5 µm; 4.6 × 250 mm) and a linear gradient of 5-95% B in 45 min at room temperature, *ca*. 1% B per min at a flow rate of 1.0 mL/min. Buffer A: H_2O containing 0.1% TFA (*v/v*); Buffer B: acetonitrile containing 0.1% TFA (*v/v*).

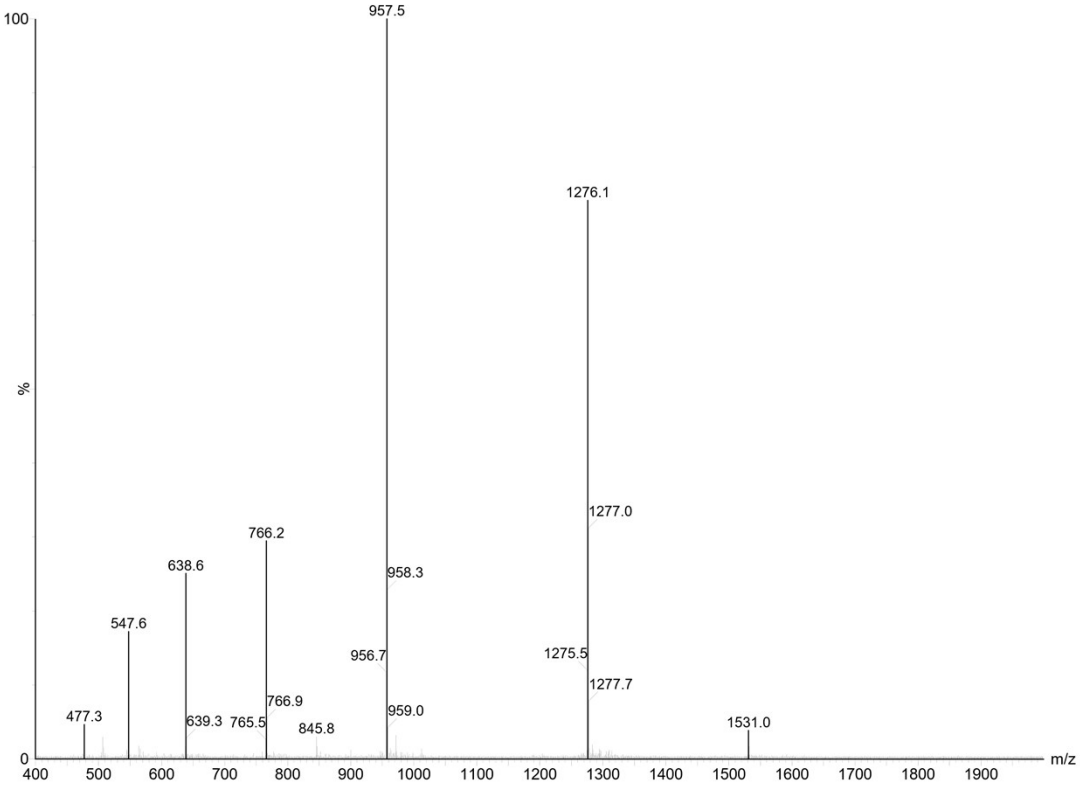
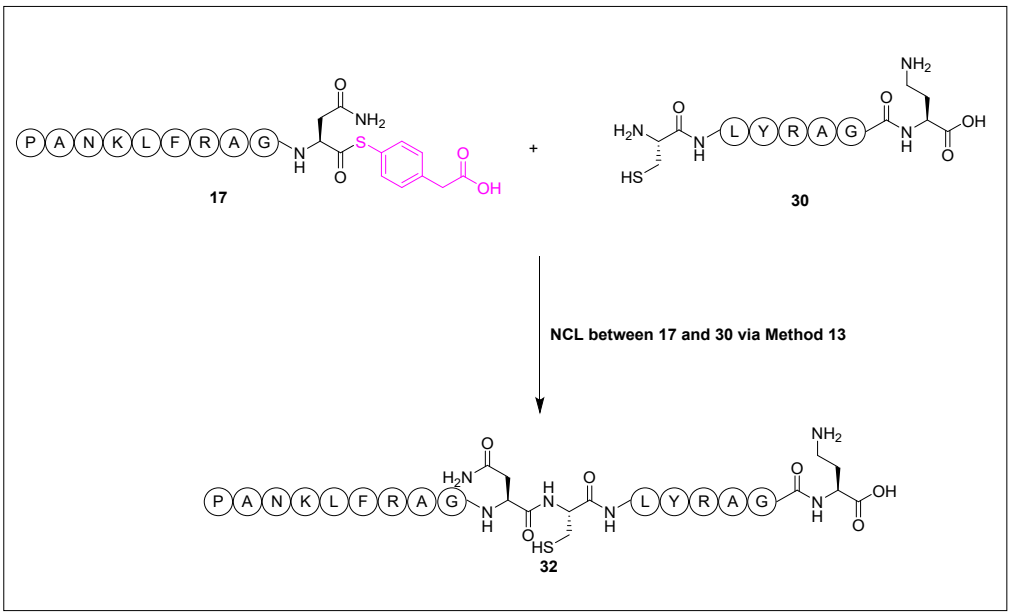


Figure S67: ESI-MS profile of purified peptide **36**; ion polarity positive operating at a nominal accelerating voltage of 70 eV. ESI-MS *m/z* $[2M+5H]^{5+}$ calcd: 1531.2 Da; found: 1531.0 Da; $[M+3H]^{3+}$ calcd: 1276.2 Da; found: 1276.1 Da; $[M+4H]^{4+}$ calcd: 957.4 Da; found: 957.5 Da; $[M+5H]^{5+}$ calcd: 766.1 Da; found: 766.2 Da; $[M+6H]^{6+}$ calcd: 638.6 Da; found: 638.6 Da; $[M+7H]^{7+}$ calcd: 547.5 Da; found: 547.6 Da; $[M+8H]^{8+}$ calcd: 479.2 Da; found: 477.3 Da. Theoretical molecular weight calcd: 3825.5 Da.[1] Chemical formula: $C_{156}H_{255}N_{49}O_{49}S_7$.

Asparagine C^{α}-thioester NCL Studies



Scheme S5. General synthetic scheme for asparagine fragment NCL.

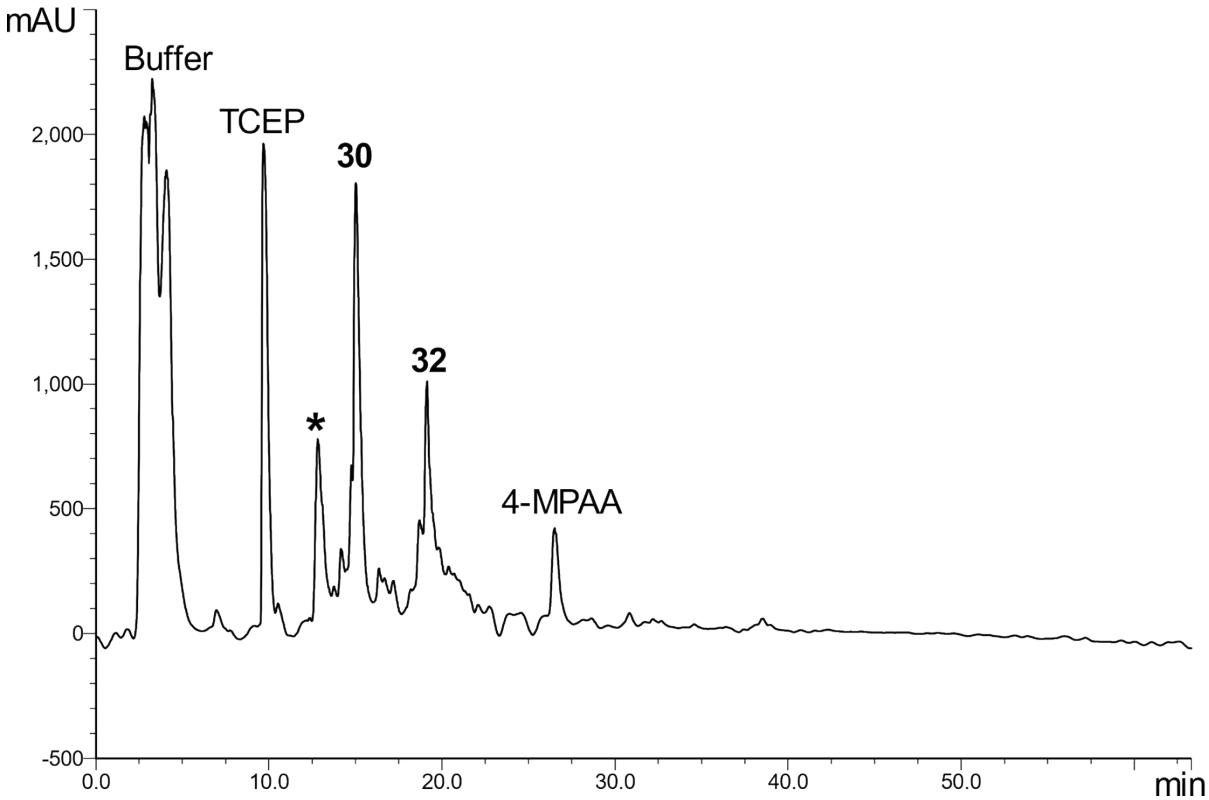


Figure S68: Analytical RP-HPLC chromatogram of NCL between Asn **17** and cysteine **30** at t = 3 h. Chromatographic separations were performed on a Thermo Scientific Dionex Ultimate 3000 HPLC using a Phenomenex® Gemini MS C-18 column (5 µm; 4.6 × 150 mm) and a linear gradient of 5-95% B in 90 min at room temperature, *ca*. 1% B per min at a flow rate of 1.0 mL/min. Buffer A: H_2O containing 0.1% TFA (*v/v*); Buffer B: acetonitrile containing 0.1% TFA (*v/v*).*Corresponds to hydrolysed **17**.

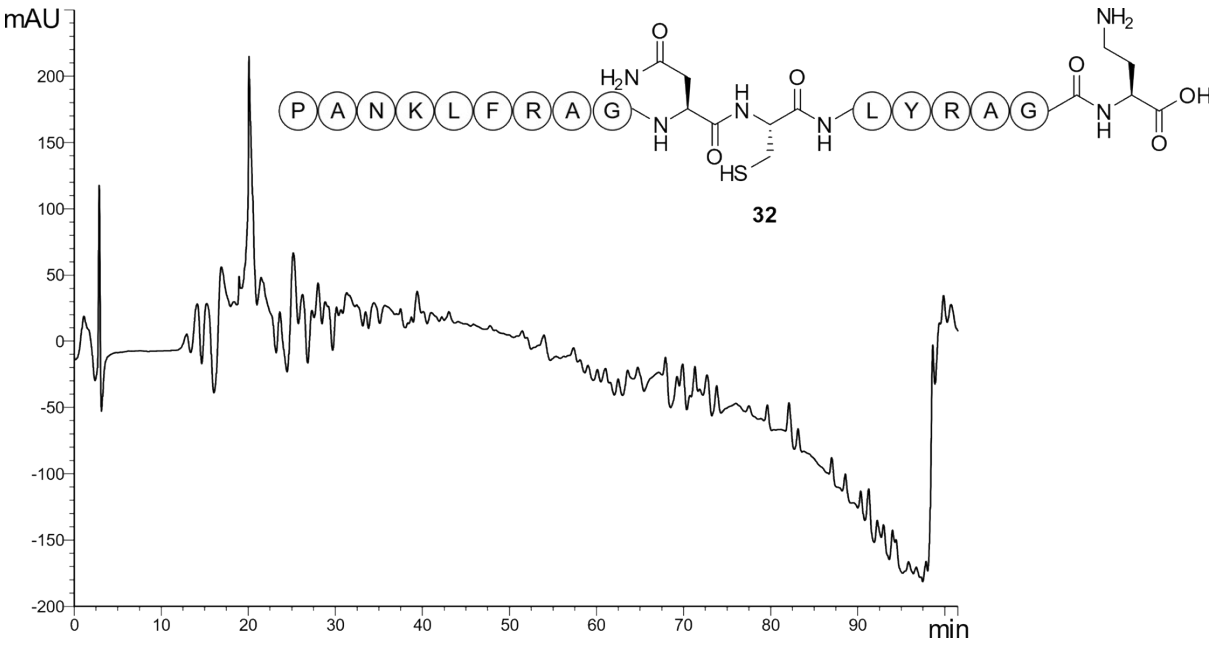


Figure S69: Analytical RP-HPLC chromatogram of purified **32**, t_R = 20.1 min. Chromatographic separations were performed on a Thermo Scientific Dionex Ultimate 3000 HPLC using a Phenomenex® Gemini MS C-18 column (5 µm; 4.6 × 150 mm) and a linear gradient of 5-95% B in 90 min at room temperature, *ca*. 1% B per min at a flow rate of 1.0 mL/min. Buffer A: H_2O containing 0.1% TFA (*v/v*); Buffer B: acetonitrile containing 0.1% TFA (*v/v*).

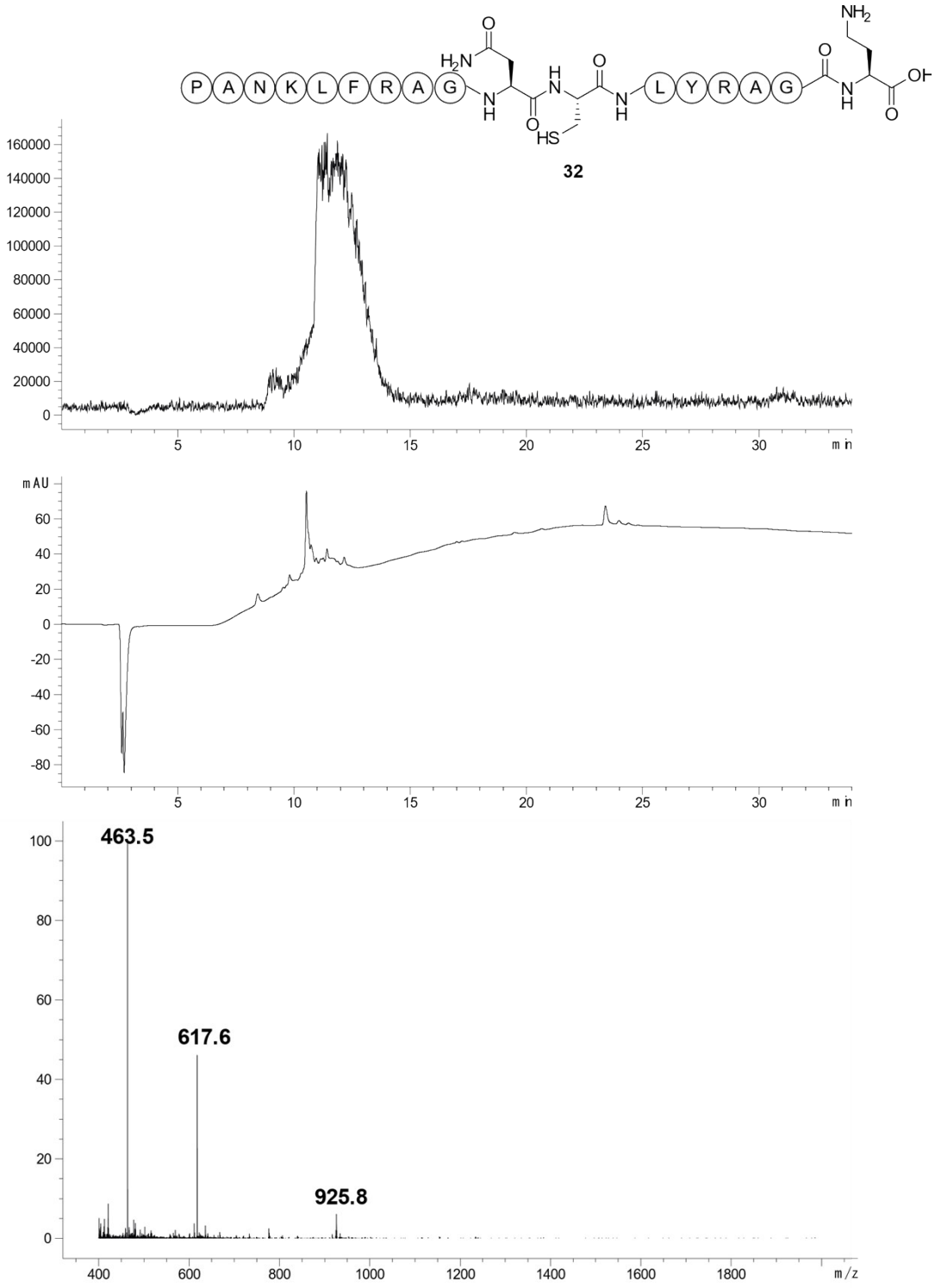
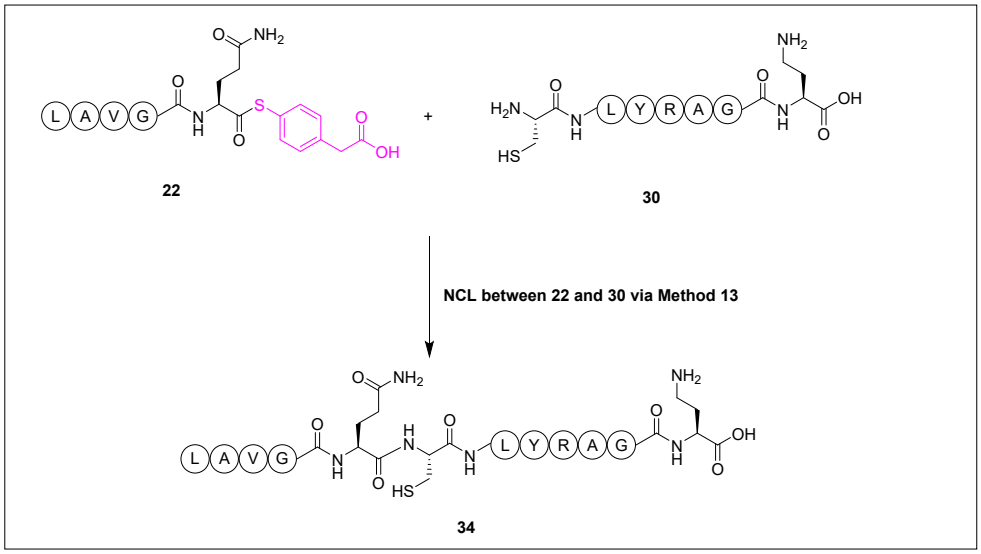


Figure S70: LC-MS profile of purified peptide **32**; ion polarity positive operating at a nominal accelerating voltage of 70 eV. ESI-MS *m/z* $[M+2H]^{2+}$ calcd: 926.6 Da; found: 925.8 Da; $[M+3H]^{3+}$ calcd: 618.1 Da; found: 617.6 Da; $[M+4H]^{4+}$ calcd: 463.8 Da; found: 463.5 Da. Theoretical molecular weight calcd: 1851.2 Da. Chemical formula: $C_{81}H_{131}N_{27}O_{21}S$.

Glutamine C^{α}-thioester NCL Studies



Scheme S6. General synthetic scheme for glutamine fragment NCL.

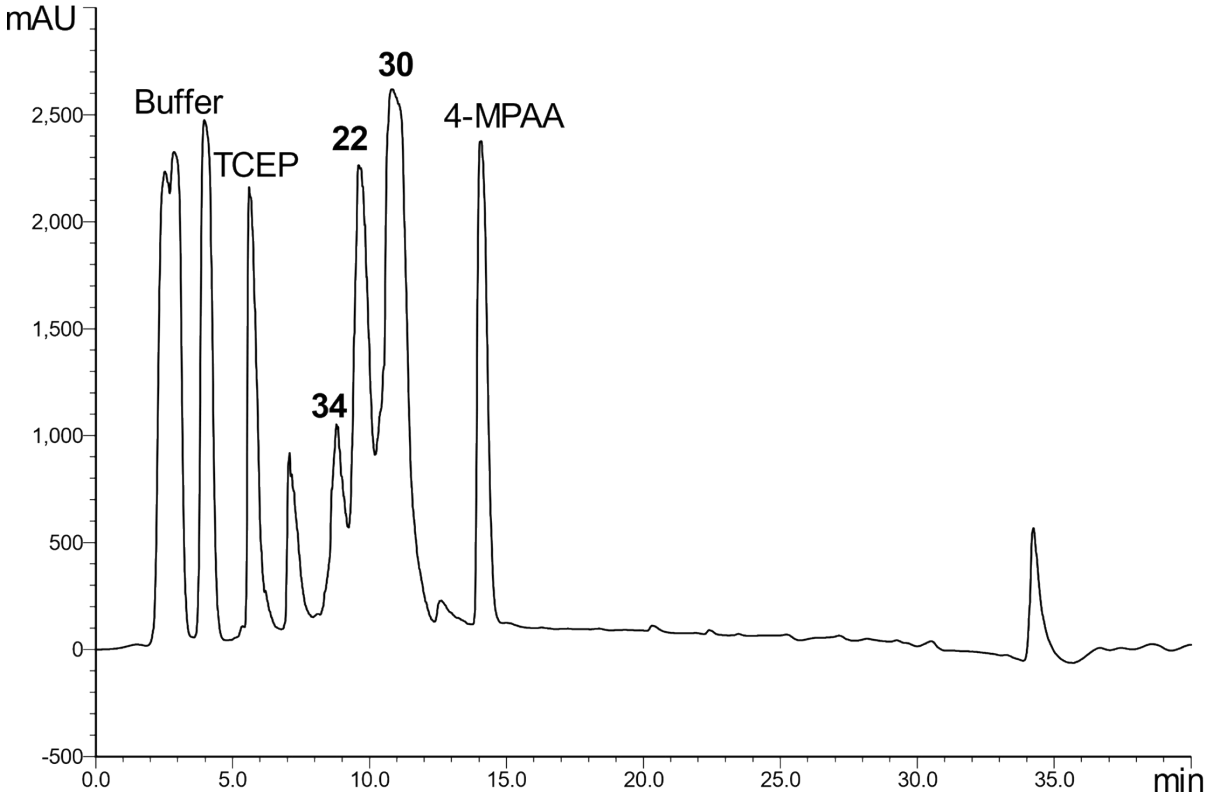


Figure S71: Analytical RP-HPLC chromatogram of NCL between **22** and **30** at t = 0 h. Chromatographic separations were performed on a Thermo Scientific Dionex Ultimate 3000 HPLC using a Phenomenex® Gemini MS C-18 column (5 µm; 4.6 × 150 mm) and a linear gradient of 5-95% B in 40 min at room temperature, *ca*. 3% B per min at a flow rate of 1.0 mL/min. Buffer A: H_2O containing 0.1% TFA (*v/v*); Buffer B: acetonitrile containing 0.1% TFA (*v/v*).

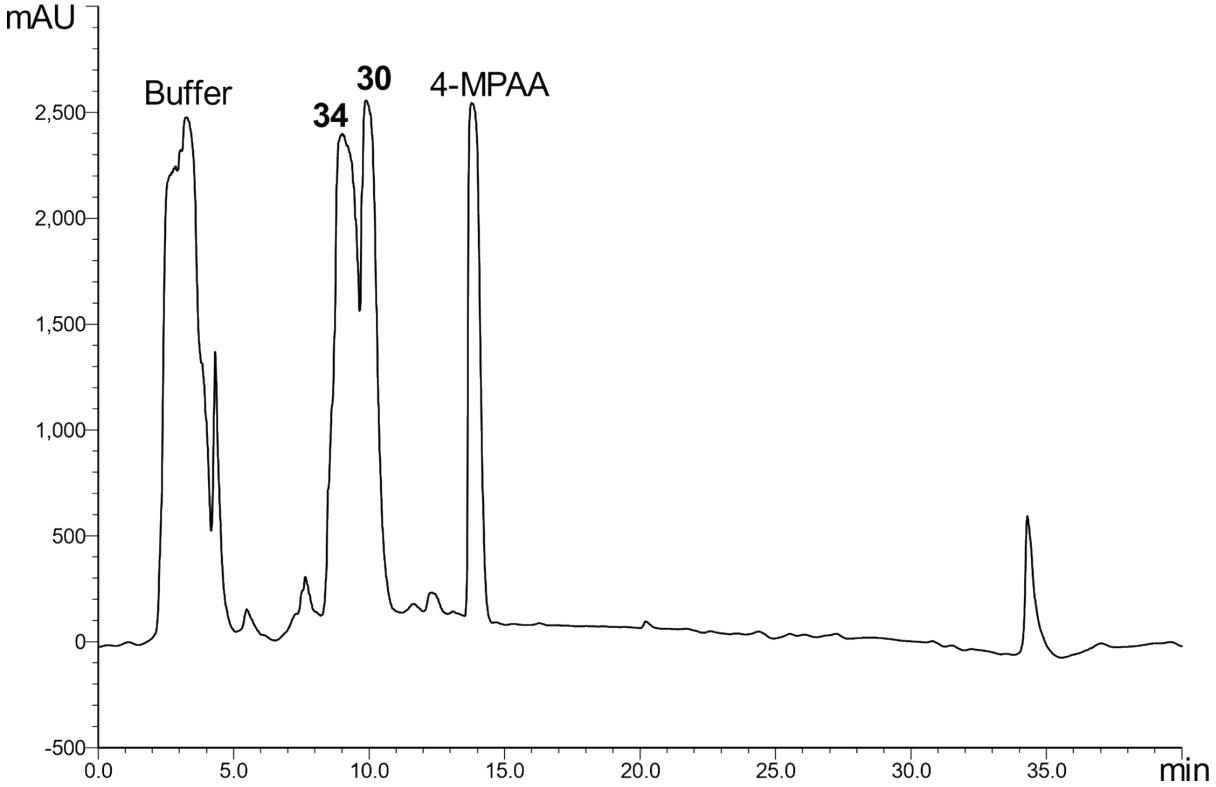


Figure S72: Analytical RP-HPLC chromatogram of NCL between Gln **22** and cysteine **30** at t = 1 h. Chromatographic separations were performed on a Thermo Scientific Dionex Ultimate 3000 HPLC using a Phenomenex® Gemini MS C-18 column (5 µm; 4.6 × 150 mm) and a linear gradient of 5-95% B in 40 min at room temperature, *ca*. 3% B per min at a flow rate of 1.0 mL/min. Buffer A: H_2O containing 0.1% TFA (*v/v*); Buffer B: acetonitrile containing 0.1% TFA (*v/v*).

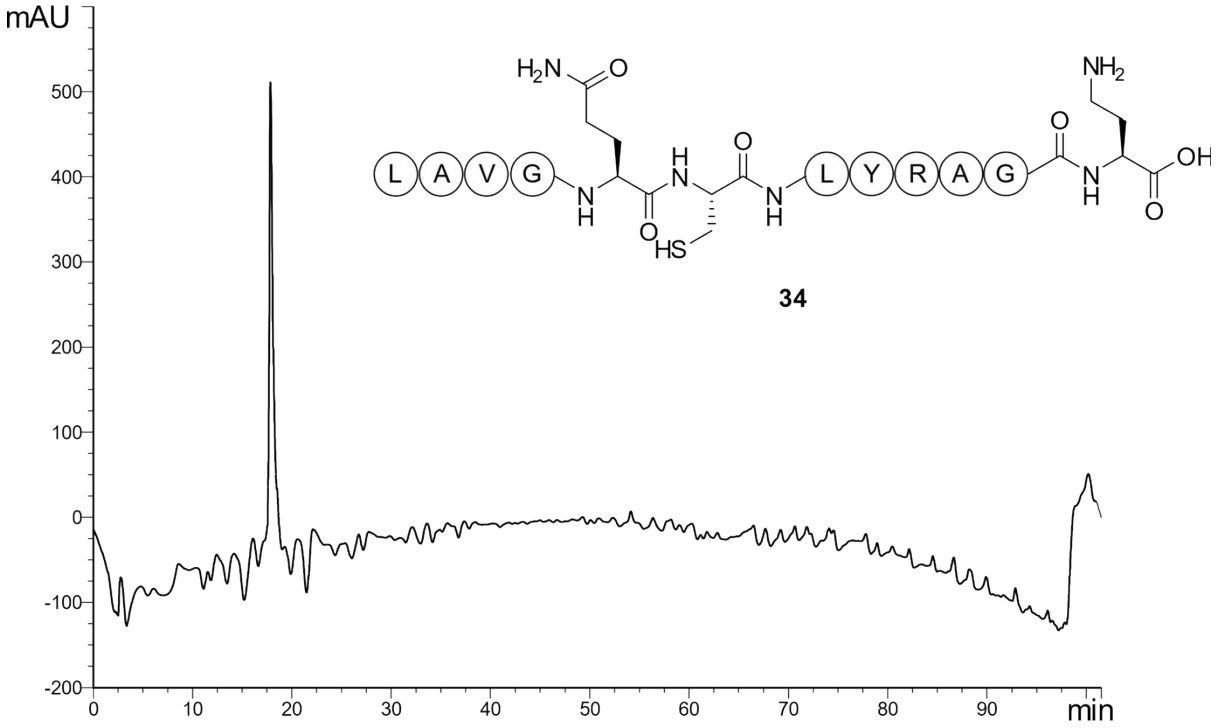


Figure S73: Analytical RP-HPLC chromatogram or purified **34**, t_R = 19.9 min. Chromatographic separations were performed on a Thermo Scientific Dionex Ultimate 3000 HPLC using a Phenomenex® Gemini MS C-18 column (5 µm; 4.6 × 150 mm) and a linear gradient of 5-95% B in 90 min at room temperature, *ca*. 1% B per min at a flow rate of 1.0 mL/min. Buffer A: H_2O containing 0.1% TFA (*v/v*); Buffer B: acetonitrile containing 0.1% TFA (*v/v*).

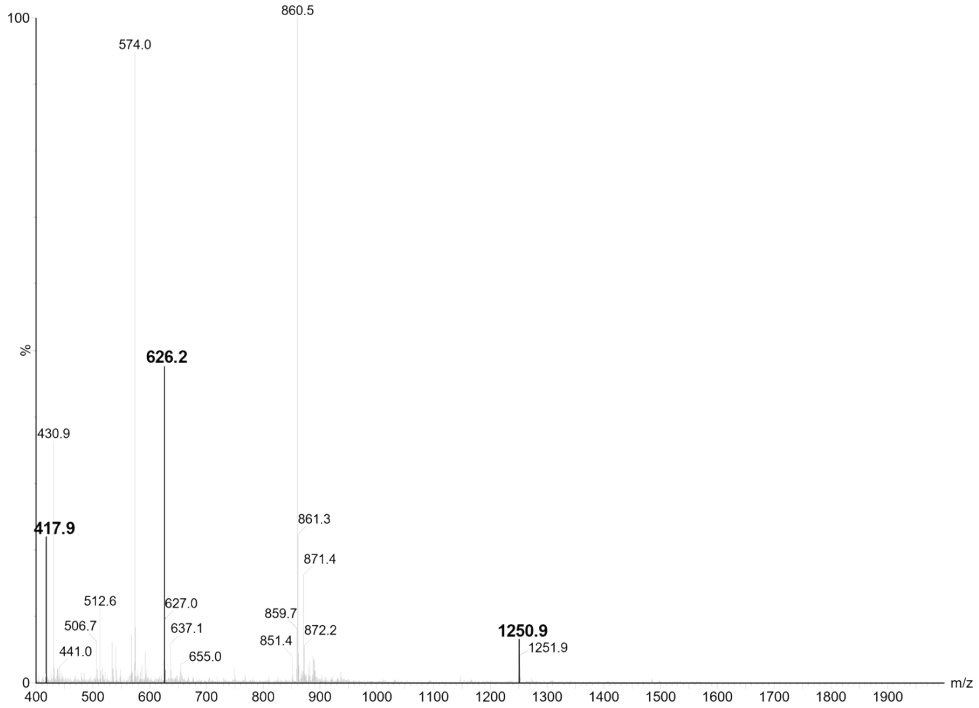
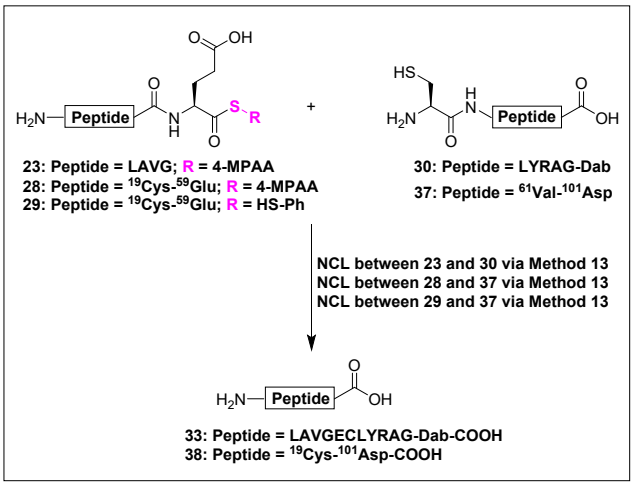


Figure S74: ESI-MS profile of purified peptide **34**; ion polarity positive operating at a nominal accelerating voltage of 70 eV. ESI-MS *m/z* $[M+1H]^{1+}$ calcd: 1251.5 Da; found: 1250.9 Da; $[M+2H]^{2+}$ calcd: 626.3 Da; found: 626.2 Da; $[M+3H]^{3+}$ calcd: 417.8 Da; found: 417.9 Da. Theoretical molecular weight calcd: 1250.5 Da. Chemical formula: $C_{54}H_{91}N_{17}O_{15}S$.

Glutamic Acid C^{α}-thioester NCL Studies



Scheme S7. General synthetic scheme for glutamic acid fragment NCL.

RP-HPLC of purified cysteine intermediate **37** for use in NCL

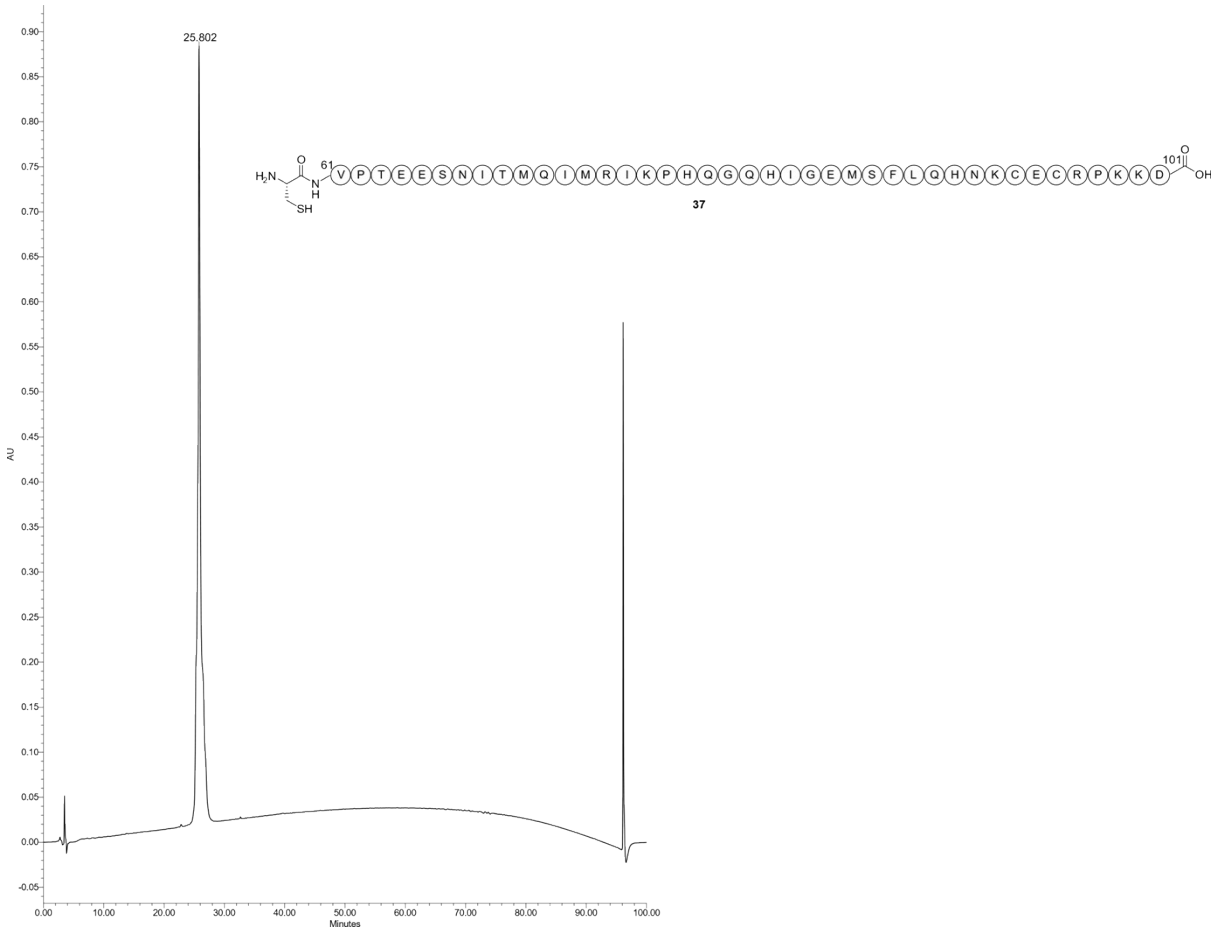


Figure S75: Analytical RP-HPLC chromatogram of purified **37**, t_R = 25.8 min. Chromatographic separations were performed on a Waters e2695 HPLC using a Agilent TC-C18 column (5 µm; 4.6 × 250 mm) and a linear gradient of 5-95% B in 90 min at room temperature, *ca*. 1% B per min at a flow rate of 1.0 mL/min. Buffer A: H_2O containing 0.1% TFA (*v/v*); Buffer B: acetonitrile containing 0.1% TFA (*v/v*).

ESI-MS of purified cysteine intermediate **37** for use in NCL

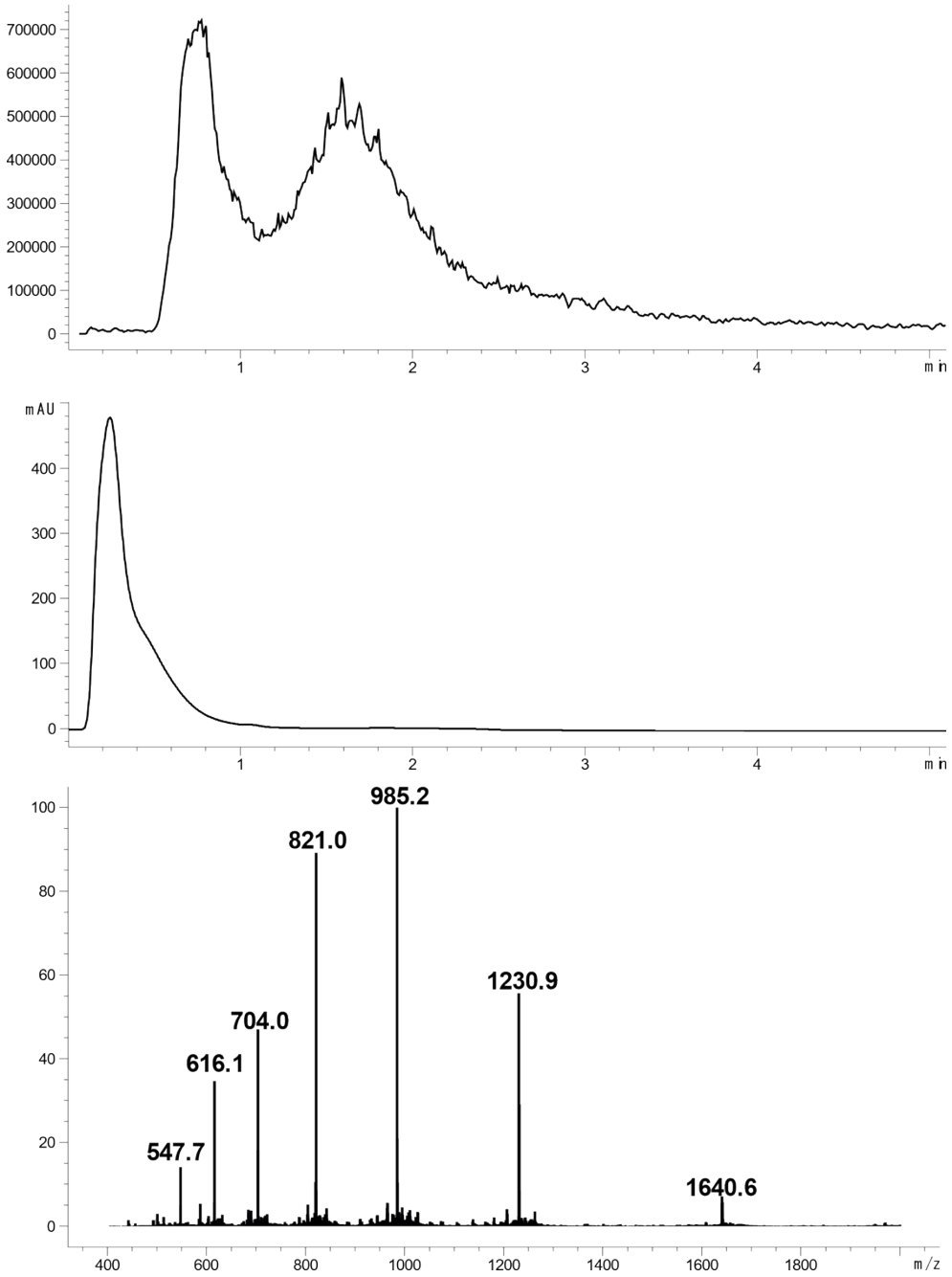


Figure S76: ESI-MS profile of purified peptide **37**; ion polarity positive operating at a nominal accelerating voltage of 70 eV. ESI-MS *m/z* $[M+3H]^{3+}$ calcd: 1642.2 Da; found: 1640.6 Da; $[M+4H]^{4+}$ calcd: 1231.9 Da; found: 1230.9 Da; $[M+5H]^{5+}$ calcd: 985.7 Da; found: 985.2 Da; $[M+6H]^{6+}$ calcd: 821.6 Da; found: 821.0 Da; $[M+7H]^{7+}$ calcd: 704.4 Da; found: 704.0 Da; $[M+8H]^{8+}$ calcd: 616.4 Da; found: 616.1 Da; $[M+9H]^{9+}$ calcd: 548.1 Da; found: 547.7 Da. Theoretical molecular weight calcd: 4923.7 Da. Chemical formula: $C_{207}H_{338}N_{64}O_{63}S_6$.

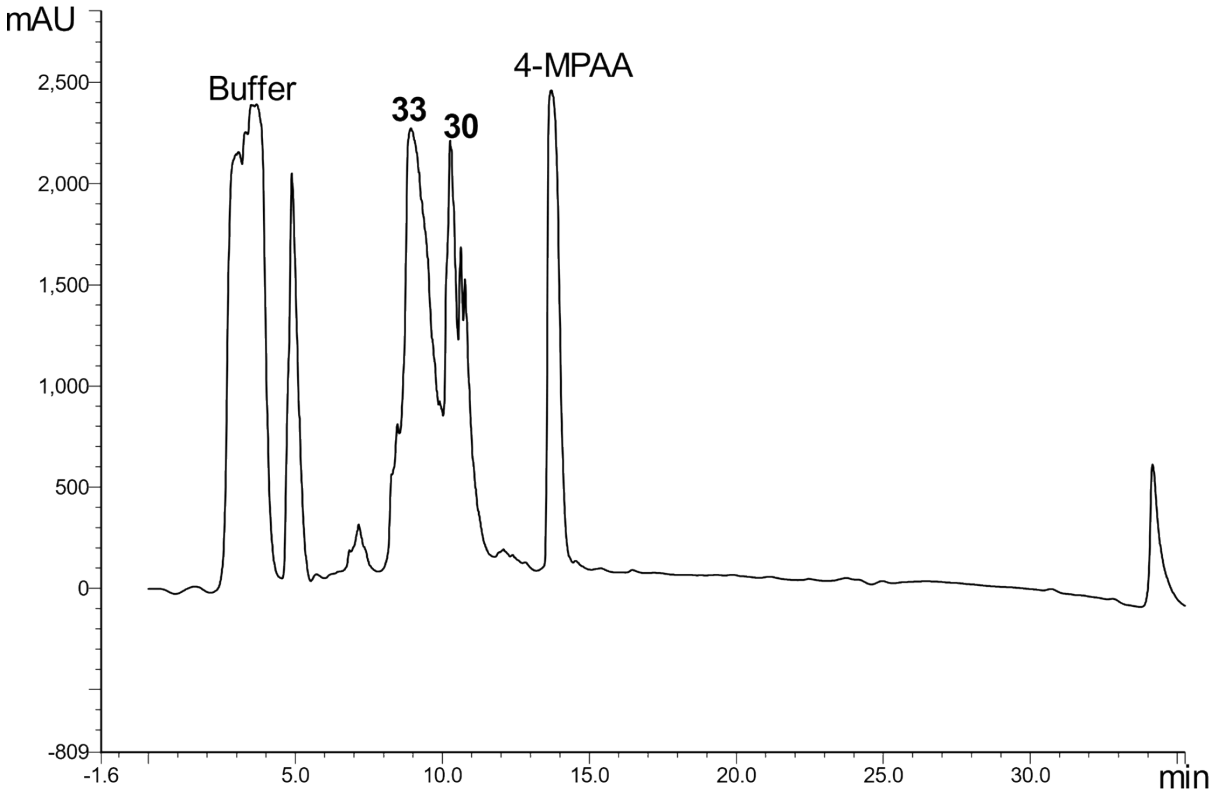


Figure S77: Analytical RP-HPLC chromatogram of NCL between Glu **23** and cysteine **30** at t = 1 h. Chromatographic separations were performed on a Thermo Scientific Dionex Ultimate 3000 HPLC using a Phenomenex® Gemini MS C-18 column (5 µm; 4.6 × 150 mm) and a linear gradient of 5-95% B in 40 min at room temperature, *ca*. 3% B per min at a flow rate of 1.0 mL/min. Buffer A: H_2O containing 0.1% TFA (*v/v*); Buffer B: acetonitrile containing 0.1% TFA (*v/v*).

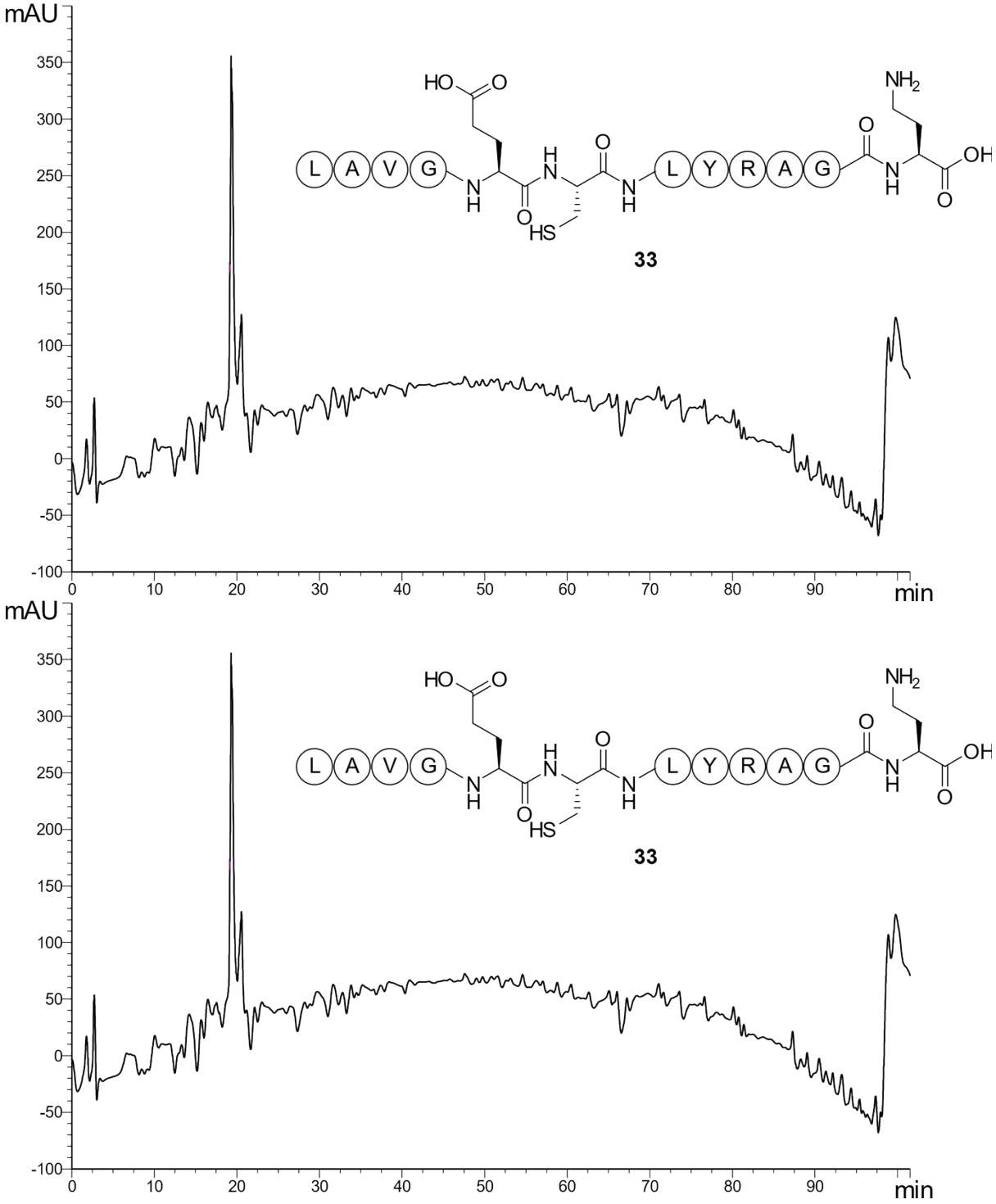


Figure S78: Analytical RP-HPLC chromatogram of purified **33**, t_R = 19.2 min. Chromatographic separations were performed on a Thermo Scientific Dionex Ultimate 3000 HPLC using a Phenomenex® Gemini MS C-18 column (5 µm; 4.6 × 150 mm) and a linear gradient of 5-95% B in 90 min at room temperature, *ca*. 1% B per min at a flow rate of 1.0 mL/min. Buffer A: H_2O containing 0.1% TFA (*v/v*); Buffer B: acetonitrile containing 0.1% TFA (*v/v*).

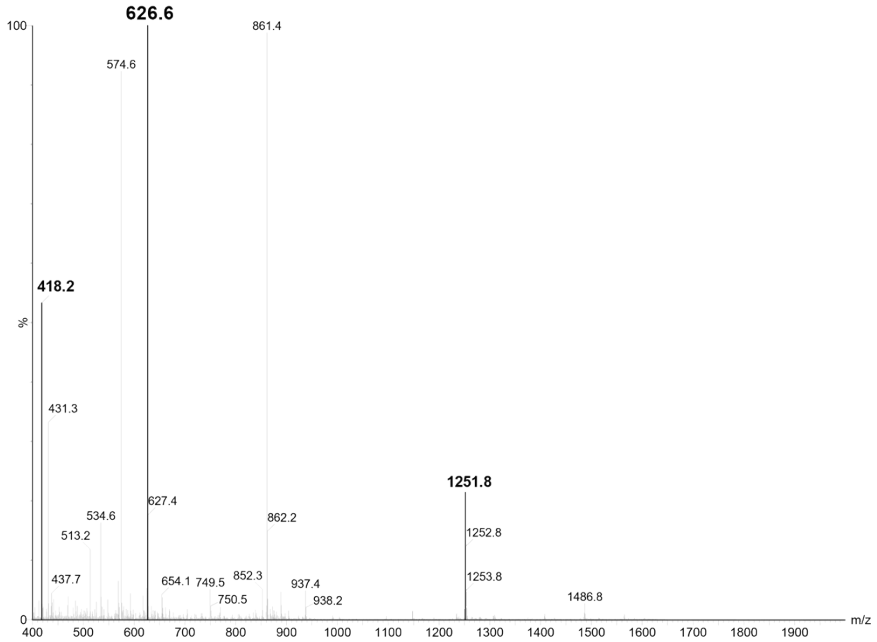


Figure S79: ESI-MS profile of purified peptide **33**; ion polarity positive operating at a nominal accelerating voltage of 70 eV. ESI-MS *m/z* $[M+1H]^{1+}$ calcd: 1252.5 Da; found: 1251.8 Da; $[M+2H]^{2+}$ calcd: 626.8 Da; found: 626.6 Da; $[M+3H]^{3+}$ calcd: 418.2 Da; found: 418.2 Da. Theoretical molecular weight calcd: 1251.5 Da. Chemical formula: $C_{54}H_{90}N_{16}O_{16}S$.

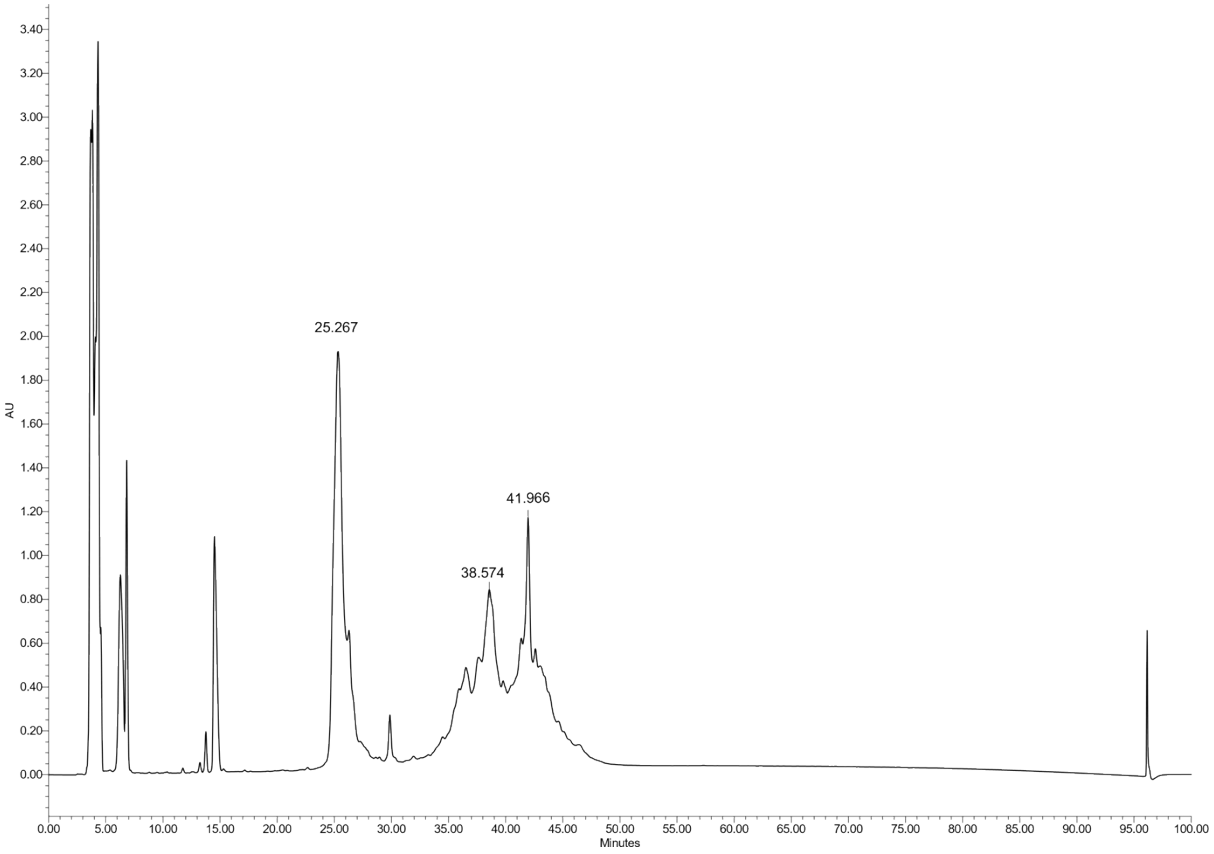


Figure S80: Analytical RP-HPLC chromatogram of crude NCL between **28** and **37** at t = 2 h, t_R = 38.5 min corresponds to **38**. Chromatographic separations were performed on a Waters e2695 HPLC using a Agilent TC-C18 column (5 µm; 4.6 × 250 mm) and a linear gradient of 5-95% B in 90 min at room temperature, *ca*. 1% B per min at a flow rate of 1.0 mL/min. Buffer A: H_2O containing 0.1% TFA (*v/v*); Buffer B: acetonitrile containing 0.1% TFA (*v/v*). *t_R = 25.2 min corresponds to **37**, peak at t_R = 41.9 min corresponds to intramolecular *N-C* terminal cyclisation of **28**.

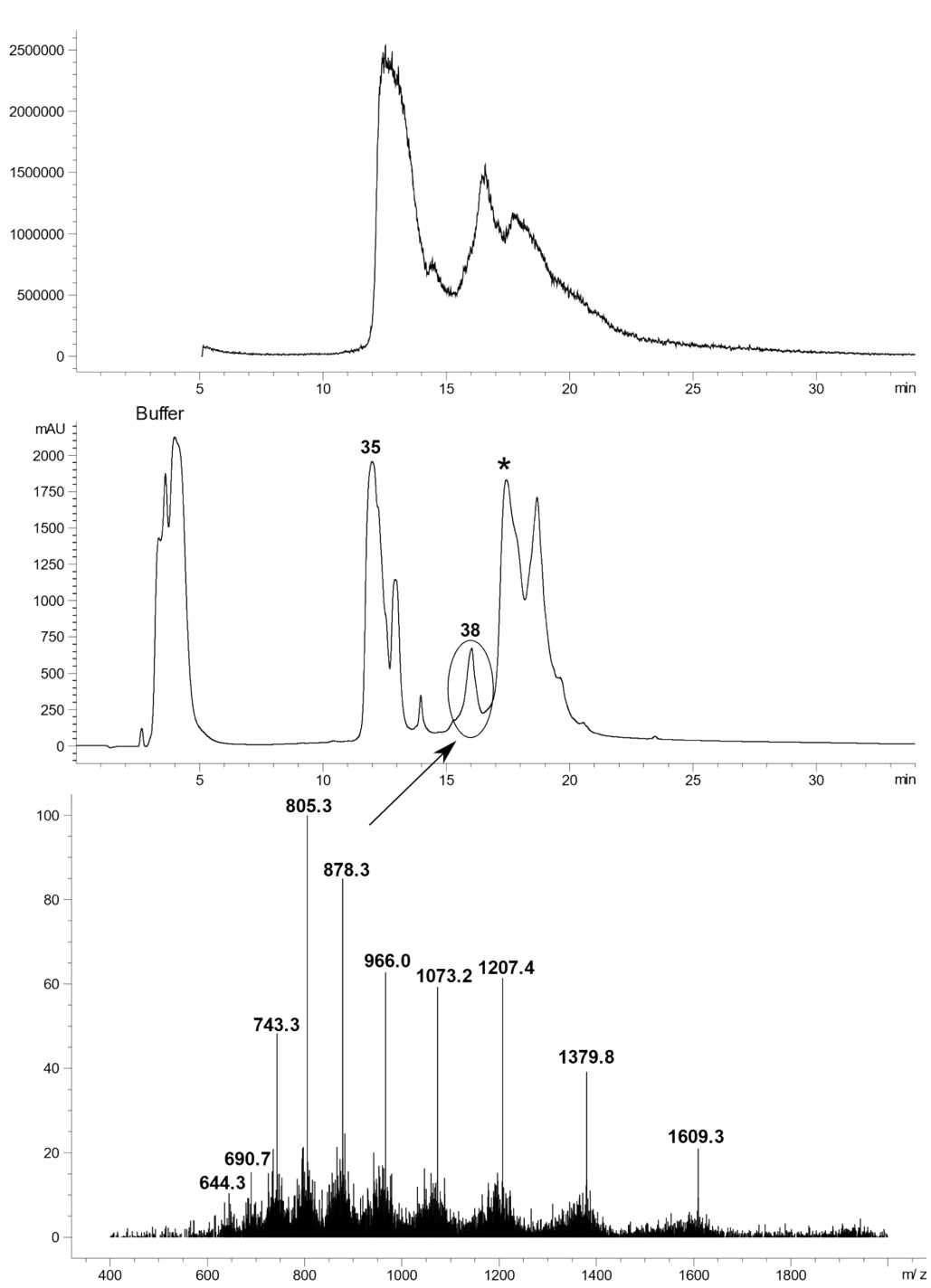


Figure S81: LC-MS profile of crude NCL between **28** and **37** at t = 2 h; ion polarity positive operating at a nominal accelerating voltage of 70 eV. ESI-MS of **33** *m/z* $[M+6H]^{6+}$ calcd: 1610.3 Da; found: 1609.3 Da; $[M+7H]^{7+}$ calcd: 1380.4 Da; found: 1379.8 Da; $[M+8H]^{8+}$ calcd: 1208.0 Da; found: 1207.4 Da; $[M+9H]^{9+}$ calcd: 1073.9 Da; found: 1073.2 Da; $[M+10H]^{10+}$ calcd: 966.6 Da; found: 966.0 Da; $[M+11H]^{11+}$ calcd: 878.8 Da; found: 878.3 Da; $[M+12H]^{12+}$ calcd: 805.7 Da; found: 805.3 Da; $[M+13H]^{13+}$ calcd: 743.8 Da; found: 743.3 Da; $[M+14H]^{14+}$ calcd: 690.7 Da; found: 690.7 Da; $[M+15H]^{15+}$ calcd: 644.7 Da; found: 644.3 Da. Theoretical molecular weight calcd: 9656.15 Da.[2] Chemical formula: $C_{415}H_{651}N_{113}O_{128}S_{12}$. *Peak corresponds to intramolecular *N-C* terminal cyclisation of **28**.

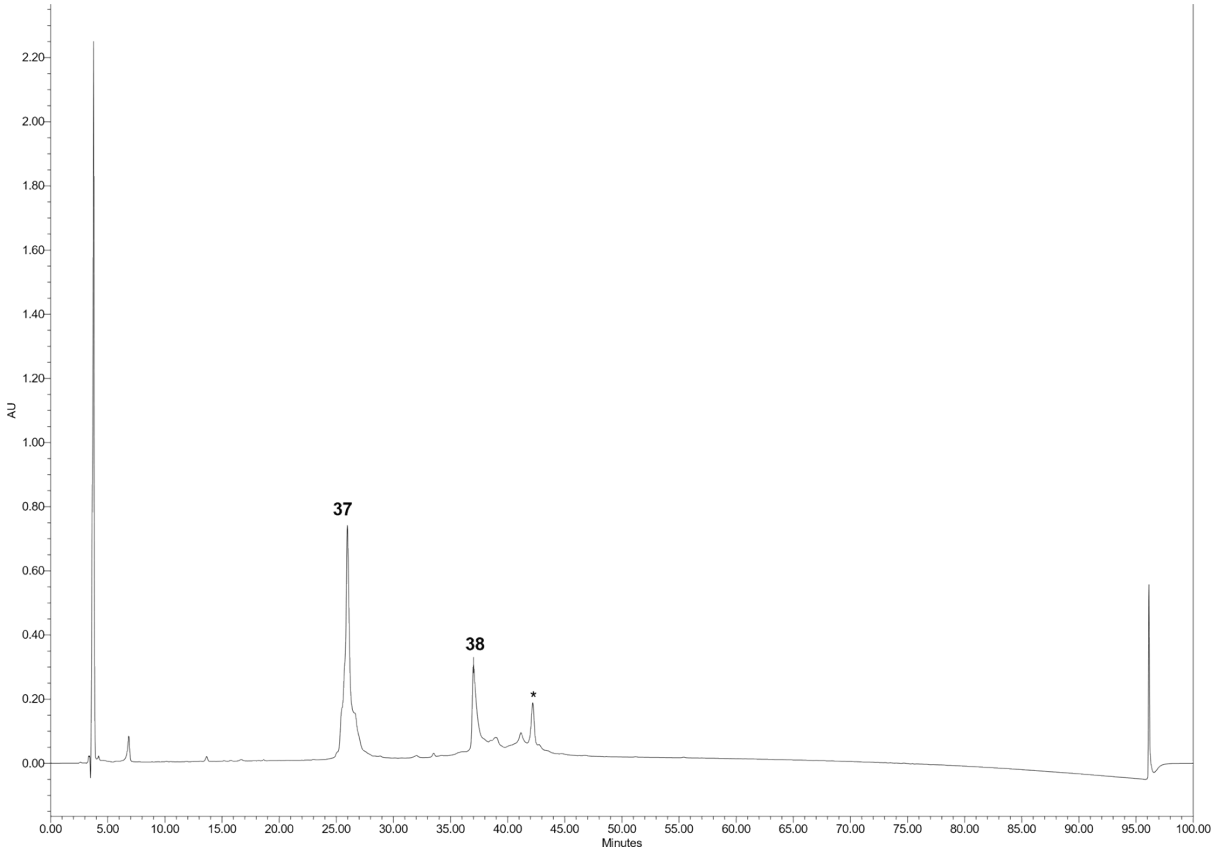


Figure S82: Analytical RP-HPLC chromatogram of crude NCL between **29** and **37** at t = 2 h, t_R = 37.0 min corresponds to **38**. Chromatographic separations were performed on a Waters e2695 HPLC using an Agilent TC-C18 column (5 µm; 4.6 × 250 mm) and a linear gradient of 5-95% B in 90 min at room temperature, *ca*. 1% B per min at a flow rate of 1.0 mL/min. Buffer A: H_2O containing 0.1% TFA (*v/v*); Buffer B: acetonitrile containing 0.1% TFA (*v/v*). *Peak corresponds to intramolecular *N-C* terminal cyclisation of **29**.

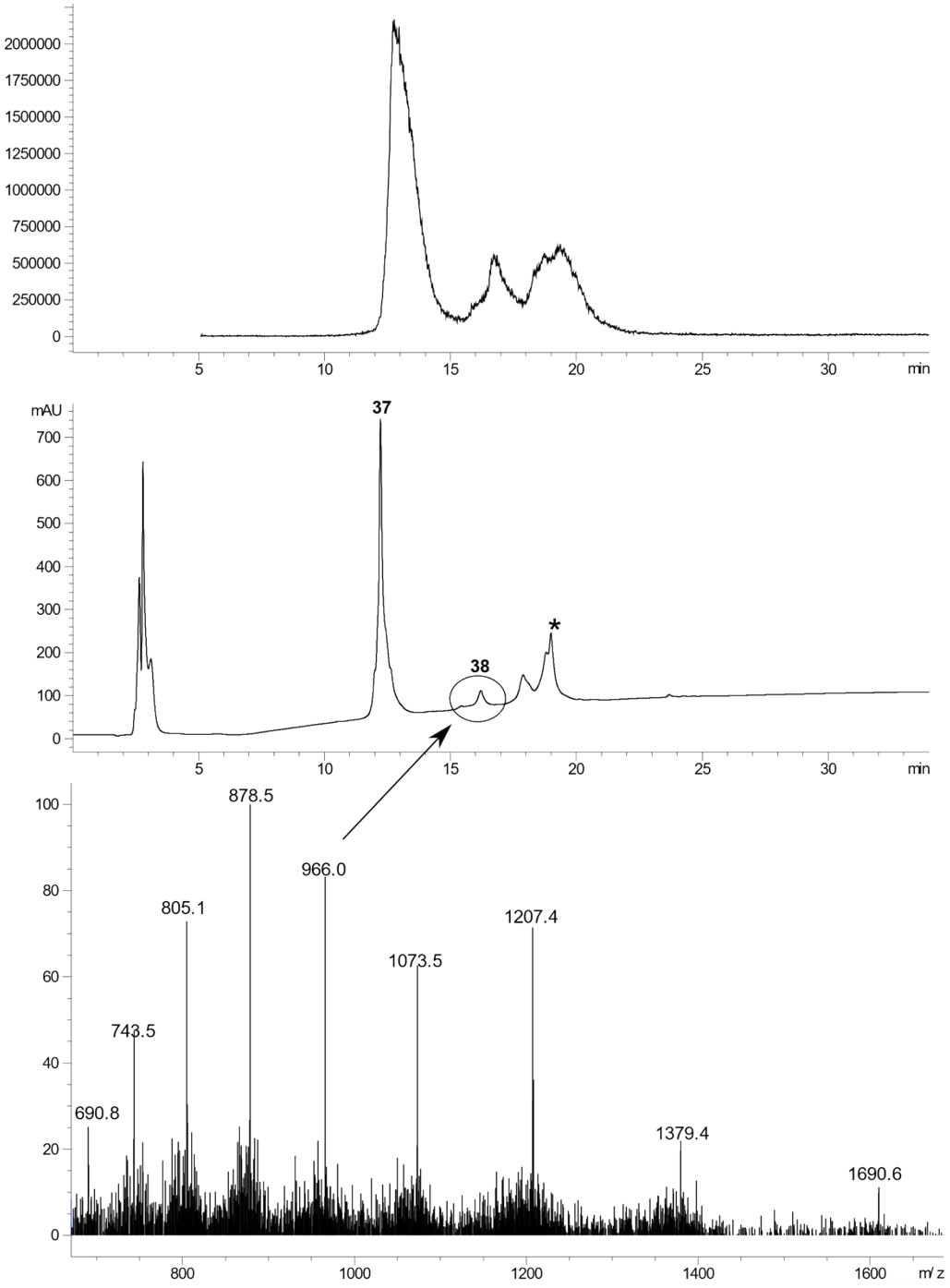


Figure S83: LC-MS profile of crude NCL between **29** and **37** at t = 2 h; ion polarity positive operating at a nominal accelerating voltage of 70 eV. ESI-MS of **33** *m/z* $[M+6H]^{6+}$ calcd: 1610.3 Da; found: 1609.6 Da; $[M+7H]^{7+}$ calcd: 1380.4 Da; found: 1379.4 Da; $[M+8H]^{8+}$ calcd: 1208.0 Da; found: 1207.4 Da; $[M+9H]^{9+}$ calcd: 1073.9 Da; found: 1073.5 Da; $[M+10H]^{10+}$ calcd: 966.6 Da; found: 966.0 Da; $[M+11H]^{11+}$ calcd: 878.8 Da; found: 877.5 Da; $[M+12H]^{12+}$ calcd: 805.7 Da; found: 805.1 Da; $[M+13H]^{13+}$ calcd: 743.8 Da; found: 743.5 Da; $[M+14H]^{14+}$ calcd: 690.7 Da; found: 690.8 Da. Theoretical Molecular weight calcd: 9656.15 Da. Chemical formula: $C_{415}H_{651}N_{113}O_{128}S_{12}$. *Peak corresponds to intramolecular *N-C* terminal cyclisation of **29**.

References

[1] H. Yeung, C. J. Squire, Y. Yosaatmadja, S. Panjikar, G. López, A. Molina, E. N. Baker, P. W. R. Harris, M. A. Brimble, *Angewandte Chemie International Edition,* 2016, ***55***, 7930.
[2] K. Mandal, S. B. H. Kent, *Angewandte Chemie International Edition,* 2011, ***50***, 8029.