

Supporting information

Tetrahydropyranyl (Thp), a non-aromatic acid-labile Cys protecting group for Fmoc peptide chemistry

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Table of contents

Materials and methods	S2
Experimental Section	S3
Preparation of Fmoc-Cys-OH (1).....	S3
Preparation of Fmoc-Cys(Thp)-OH (2)	S3
Lability experiments	S3
C-terminal amide: Ala-Cys-Leu-NH ₂ tripeptides synthesis	
A.1-Sieber amide resin (SA).....	S4
A.2- Rink amide Resin (RA)	S10
C-terminal carboxylic acid: Ala-Cys-Leu-OH tripeptides synthesis	
B. 2-Chlorotrityl chloride resin (2-CTC).....	S13
C-terminal carboxylic acid: Ala-Leu-Cys-OH tripeptides synthesis	
2-Chlorotrityl chloride resin	S16
Wang Resin	S17
C-Terminal Cys Racemization study	
2-CTC resin	S20
Wang resin	S24
NMR Spectra	S29

Materials and Methods

Pruducts and comercial source:

The commercial products and solvents were used as received without further purification. All the Fmoc and Boc-amino acids, the Fmoc-Rink amide PS and 2-chloro trityl chloride resins were purchased from Iris Biotech (Marktredwitz, Germany). Fmoc-Sieber amide resin was from Merck (Darmstadt, Germany). Dihydropyran (DHP), *p*-Toluen sulfonic acid (*p*-TsOH), *N,N'*-diisopropyl carbodimide (DIPCDI), diisopropylethyl amine (DIEA) and Trifluoroacetic acid (TFA) were acquired from Sigma-Aldrich (Milwaukee, WI), Oxyma Pure was obtained from Luxembourg Industries Ltd. (Tel Aviv, Israel). *N,N*-Dimethyl formamide (DMF), dichloromethane (DCM), diethyl ether (Et₂O), Dimethyl sulfoxide (DMSO), piperidine and acetonitrile (MeCN) (HPLC grade) were purchased from Scharlau SDS (Peypin, France).

Solid-phase peptide synthesis (SPPS):

2, 5, 10 and 20 mL polypropylene syringes with a porous disc-containing were used for the solid-phase peptide synthesis as reactors. At short reaction times (2 – 5 min), the reactions were manually stirred with a Teflon stick, meanwhile for long reactions times (>30 min) the reactions were stirred on a Unimax 1010 shaker from Heidolph instruments. Solvents were removed from the reactor by vacuum suction. All the reactions were carried out at room temperature (~25 °C). The Fmoc protecting group elimination was performed by three treatments of piperidine/DMF (1:4) (1×1 min + 2×5 min). Every reaction step was followed of washings of the peptide-resin with DMF (4×1 min) and DCM (4×1min). Peptides were precipitated by ultracentrifugation using an Allegra™ 21 R centrifuge from Beckman Coulter™.

HPLC reversed-phase column Xbridge™ BEH130 C18 3.5 μm (4.6×100 mm), from Waters (Ireland) was used to analyze the synthesized peptides. The analytical HPLC was carried out on Waters instrument comprising two solvent-delivery pumps (Waters 1525), an automatic injector (Waters 717 auto sampler), diode array wavelength detector (Waters 2487) and linear gradients of MeCN (+0.036% TFA) into H₂O (+0.045% TFA) were run at flow rate of 1 mL/min. The average in the chromatograms was determined by the area integration of the chromatographic peaks at λ = 220 nm.

HPLC-ESMS reversed-phase column SunFire™ C18 3.5 μm (2.1×100 mm) was from Waters (Ireland) and it was performed on a Waters instrument comprising two solvent-delivery pumps (Waters 2695 separation module) with an automatic injector and photodiode array wavelength detector (Waters 2998) and Waters Micromass ZQ spectrometer. Linear gradients of MeCN (+0.07% formic acid) into H₂O (+0.1% formic acid) were run at flow rate of 0.3 mL/min.

The solvents for HPLC were H₂O Milli-Q, which was filtered through a Milli-Q Plus (Millipore) system with 0.45 μm of pore (resistivity over to 18 MΩ·cm⁻¹), and MeCN (HPLC quality).

NMR spectra was recorded on a Varian Mercury-400 (¹H 400 MHz and ¹³C 100 MHz). ¹H is reported as follows: chemical shift (δ ppm), [integration, multiplicity (s = singlet, d = doublet, t = triplet, q = quartet, m = multiplet), coupling constant (*J* in Hz)]. Data for ¹³C NMR are reported in terms of chemical shift. NMR spectra were referenced by tetramethylsilane (TMS) and it took as an internal reference the signal for the deuterated solvent that was used in each particular case.

The automatic purification was performed on CombiFlash® Rf TELEDYNE ISCO by using pre-packed Redisep® Rf Gold 50 g HPC18 column from Teledyne Technology Company. Solid samples

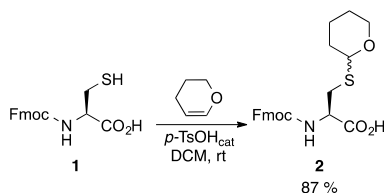
were charged into C18 silica and eluted into the column by linear gradients of MeCN (+0.036% TFA) into H₂O (+0.045% TFA) which were run at a flow rate of 40 mL/min. UV detection was at 220 nm.

Experimental section

Preparation of Fmoc-Cys-OH (1)

To a mixture of Fmoc-Cys(Trt)-OH (2.0 g, 3.4 mmol) in DCM (45 mL), TIS (0.75 mL) and TFA (5 mL) was added. The reaction mixture was stirred at room temperature for 1h. Then the solvent was removed under reduced pressure and the crude was washed and precipitated with hexane (6×40 mL). Afterwards the solid was lyophilized obtaining 1.1 g of pure Fmoc-Cys-OH (95% yield) as a white powder. **HPLC** (H₂O/MeCN from 95:5 to 0:100 over 8 min) t_R : 6.8 min). m/z calculated for C₁₈H₁₇NO₄S = 343.4; found = 344.1 [M+H]⁺, being M the MW of Fmoc-Cys-OH (1). **¹H NMR** (400 MHz, DMSO-*d*₆) δ 12.86 (s, 1H), 7.90 (dt, J = 7.6, 1.0 Hz, 2H), 7.74 (d, J = 7.3 Hz, 1H), 7.69 (d, J = 8.2 Hz, 1H), 7.42 (t, J = 7.2 Hz, 1H), 7.34 (td, J = 7.5, 1.2 Hz, 2H), 4.34 – 4.30 (m, 2H), 4.24 (t, J = 7.0 Hz, 1H), 4.13 (td, J = 8.4, 4.4 Hz, 1H), 2.94 – 2.85 (m, 1H), 2.79 – 2.69 (m, 1H), 2.54 – 2.47 (m, 1H). **¹³C NMR** (100 MHz, DMSO-*d*₆): δ 171.8, 156.0, 143.8, 143.7, 140.7, 127.6, 127.1, 125.3, 120.1, 65.7, 56.6, 46.6, 25.4.

Preparation of Fmoc-Cys(Thp)-OH (2):



To a suspension of Fmoc-Cys-OH (1.8 g, 5.2 mmol) and 3,4-dihydro-2H-pyran (DHP) (575 μ L, 6.2 mmol) in DCM (40 mL), *p*-toluenesulfonic acid (99.7 mg, 0.52 mmol) was added. Then the mixture was left to react at room temperature for 1h under N₂. Afterwards the organic layer was washed with brine (3×50 mL) and water (3×50 mL)

then it was dried over Na₂SO₄, filtered and the solvent was evaporated under reduced pressure, furnishing a white solid (2.2 g) which was automatically purified on a pre-packed Redisep Rf Gold C18 43g column by using H₂O/MeCN from 90:10 to 0:100 over 30 min. The collected fractions were lyophilized to obtain Fmoc-Cys(Thp)-OH as a white powder (1.95 g, 87% of yield). **HPLC** (H₂O/MeCN from 95:5 to 0:100 over 8 min): t_R : 7.6 min. m/z calculated for C₂₃H₂₅NO₅S = 427.1; found = 428.0 [M+H]⁺, being M the MW of Fmoc-Cys(Thp)-OH. **¹H NMR** (400 MHz, CDCl₃) δ 7.78 – 7.73 (m, 2H), 7.63 – 7.59 (m, 2H), 7.43 – 7.36 (m, 2H), 7.34 – 7.28 (m, 2H), 6.97 (d, J = 8.3 Hz, 1H), 6.40 (d, J = 6.8 Hz, 0.6H), 5.44 (s, 1.7H), 4.77 – 4.64 (m, 1.6H), 4.54 – 4.39 (m, 2H), 4.28 – 4.18 (m, 1H), 4.04 – 3.89 (m, 1H), 3.46 – 3.38 (m, 1H), 3.20 (dd, J = 14.6, 5.1 Hz, 0.4H), 3.06 (dd, J = 14.5, 6.8 Hz, 0.4H), 2.89 (dd, J = 14.7, 3.9 Hz, 1H), 1.99 – 1.86 (m, 1H), 1.85 – 1.77 (m, 1H), 1.74 – 1.65 (m, 1H), 1.62 – 1.50 (m, 3H). **¹³C NMR** (100 MHz, CDCl₃) δ 174.3, 156.8, 144.1, 143.9, 143.9, 141.5, 141.5, 141.4, 127.9, 127.8, 127.2, 127.2, 125.2, 125.2, 120.1, 120.0, 84.7, 83.6, 67.3, 67.0, 66.5, 65.9, 54.2, 47.3, 47.3, 35.2, 32.9, 31.6, 31.3, 25.3, 25.2, 22.6, 22.3, 15.3.

Lablity experiments:

The lablity experiments were performed by using the protected Fmoc-Cys(Thp)-OH aminoacid (1 mg/mL) into mixtures which contains different percentage of acids [2-(*N*-morpholino)ethanesulfonic acid (MES), trifluoroacetic acid (TFA) and HCl], scavengers (such as water and TIS) and DCM at room temperature. The table below indicates the acidic proportion of scavenger used in the acidolytic cocktail. Moreover it indicates the % of Fmoc-Cys-OH determined by the HPLC chromatographic peak area. The acidolytic reaction was monitored by RP-HPLC until the complete elimination of the Thp protecting group by using a linear gradient of H₂O/MeCN (from 95:5 to 0:100) over 8 min. t_R = 6.87 min; m/z calculated for C₁₈H₁₇NO₄S, 343.1; found, 344.0 [M+H]⁺.

C-terminal amide: Ala-Cys-Leu-NH₂ tripeptides synthesis:

The resins used for the manual preparation of C-Terminal amide tripeptide were Fmoc-Sieber-amide AM-polystyrene resin ($f = 0.69$ mmol/g) and Fmoc-Rink-amide AM-polystyrene resin ($f = 0.70$ mmol/g). Initially, both resins were washed with DCM (4×5 mL) and DMF (4×5 mL) and the Fmoc protecting group was eliminated by treating the resins with DMF/piperidine (4:1) (1×5 mL×1 min + 2×5 mL×5 min), and washed with DCM (4×5 mL) and DMF (4×5 mL). For both resins, Fmoc-Leu-OH (3 eq.) was incorporated by using DIPCDI (3 eq.) and Oxyme Pure (3 eq.) in DMF, after pre-activation of 5 min. The mixtures were left to react for 1 h and 30 min. Afterwards, the resins were washed with DMF (4×5 mL) and DCM (4×5 mL).

A.1-Sieber amide resin (SA):

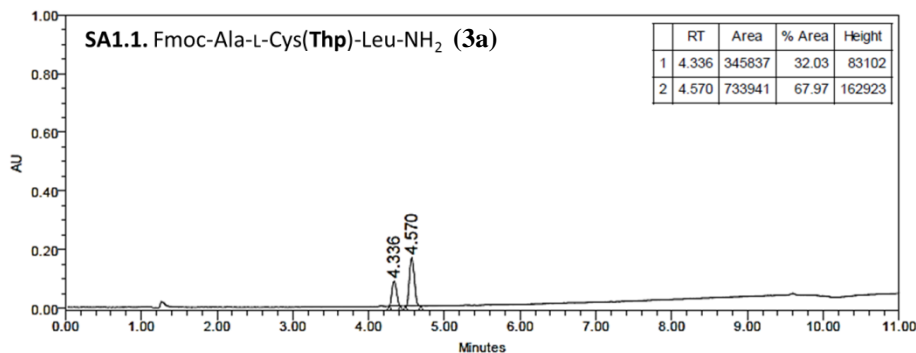
The Fmoc-Leu-Sieber amide resin was divided into five fractions (SA1-SA5), which were solvated with DCM (3×5 mL) and DMF (3×5 mL). The following Cys protected aminoacid (2 eq.) –Fmoc-L-Cys(Thp)-OH (SA1), Fmoc-L-Cys(Trt)-OH (SA2), Fmoc-D-Cys(Trt)-OH (SA3), Fmoc-L-Cys(Dpm)-OH (SA4), Fmoc-L-Cys(Acm)-OH (SA5) – were coupled using DICPDI (2 eq.) and Oxyma Pure (2 eq.) in DMF with a 5 min pre-activation and 1 h and 30 min of coupling at 25 °C. Re-coupling of Cys(Trt) (A2 and A3) and Cys(Dpm) (A4) were needed as the Kaiser test determined after 1 h and 30 min of reaction. For the re-coupling the same conditions as the coupling was used for SA2-SA4 (5 min of pre-activation + 1 h of coupling at 25 °C). The resins were washed with DMF (4×5 mL), DCM (4×5 mL) and DMF (2×5 mL). Fmoc protecting group was eliminated by treating the resins with DMF/piperidine (4:1) (1×5 mL×1 min + 2×5 mL×5 min). The resins were washed with DMF (4×5 mL), DCM (4×5 mL) and DMF (2×5 mL) and then Alanine was incorporated to the peptide chain using Fmoc-Ala-OH (3 eq.), DIPCDI (3 eq.) and Oxyme Pure (3 eq.): 5 min pre-activation + 1 h 30 min coupling at 25 °C. The resins were washed with DMF (4 × 5 mL) and DCM (4 × 5 mL).

Cleavage

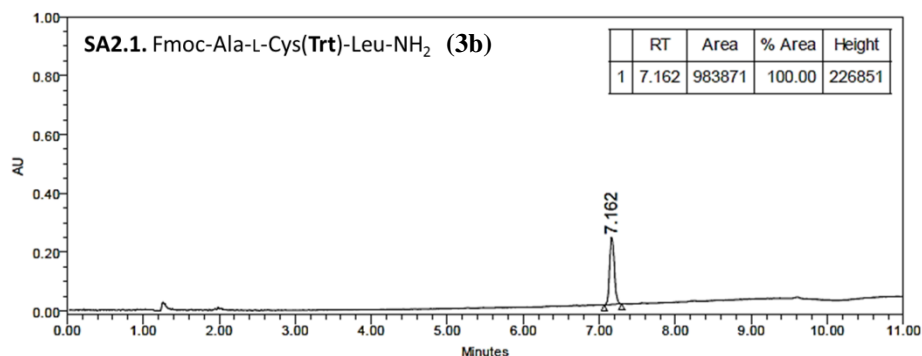
For each cleavage studies, 10 mg of the Fmoc-Ala-Cys(PG)-Leu-NH-Resin were used.

- Fmoc-Ala-Cys(PG)-Leu-NH₂ tripeptides: Tripeptides-containing resins were treated with TFA/DCM (1:99) (3×0.5 mL×1 min) and washed with DCM (2×1 mL). Then the filtered washes were evaporated and precipitated with Et₂O. The resulting peptides were dissolved into 2 mL H₂O/MeCN (1:1) for their analysis by RP-HPLC.

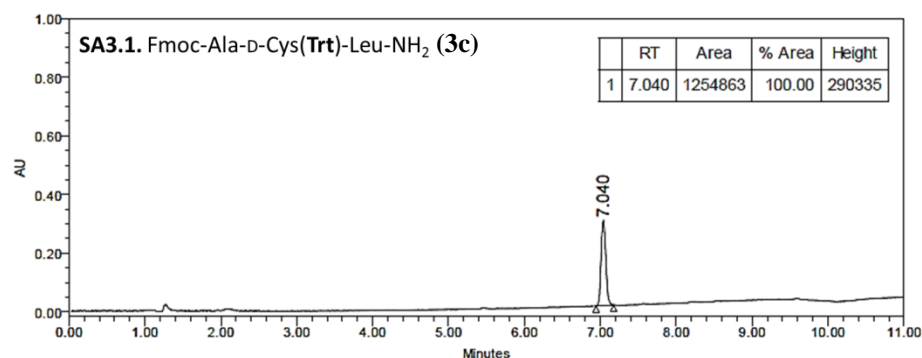
SA1.1. *Fmoc-Ala-L-Cys(Thp)-Leu-NH₂ (3a)*: RP-HPLC [linear gradient H₂O/MeCN (50:50) to (0:100) over 8 min] $t_R = 4.34 + 4.57$ min; Purity = 32 % and 68 % respectively (diastereoisomers). m/z calculated for C₃₂H₄₂N₄O₆S = 610.3; found = 611.2 [M+H]⁺, being M the MW of Fmoc-Ala-L-Cys(Thp)-Leu-NH₂.



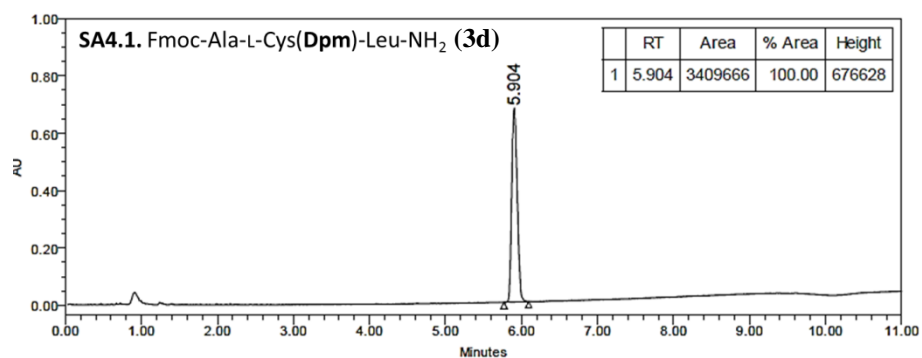
SA2.1. *Fmoc-Ala-L-Cys(Trt)-Leu-NH₂* (**3b**): RP-HPLC [linear gradient H₂O/MeCN (50:50) to (0:100) over 8 min] t_R = 7.16 min; Purity = >99 %. m/z calculated for C₄₆H₄₈N₄O₅S = 768.3; found = 769.3 [M+H]⁺, being M the MW of *Fmoc-Ala-L-Cys(Trt)-Leu-NH₂*.



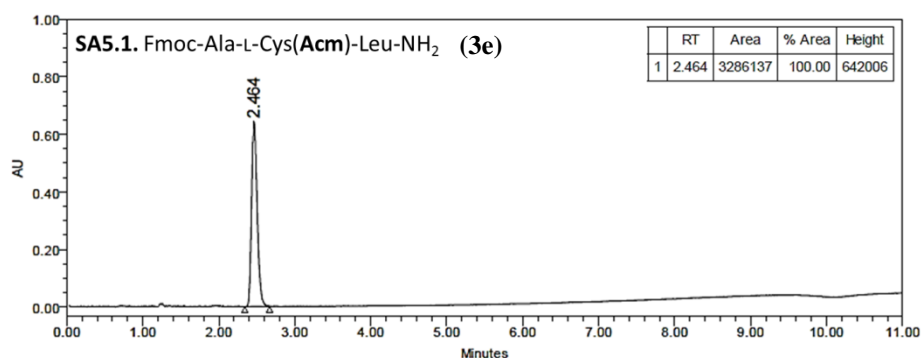
SA3.1. *Fmoc-Ala-D-Cys(Trt)-Leu-NH₂* (**3c**): RP-HPLC [linear gradient H₂O/MeCN (50:50) to (0:100) over 8 min] t_R = 7.04 min; Purity = >99 %. m/z calculated for C₄₆H₄₈N₄O₅S = 768.3; found = 769.3 [M+H]⁺, being M the MW of *Fmoc-Ala-D-Cys(Trt)-Leu-NH₂*.



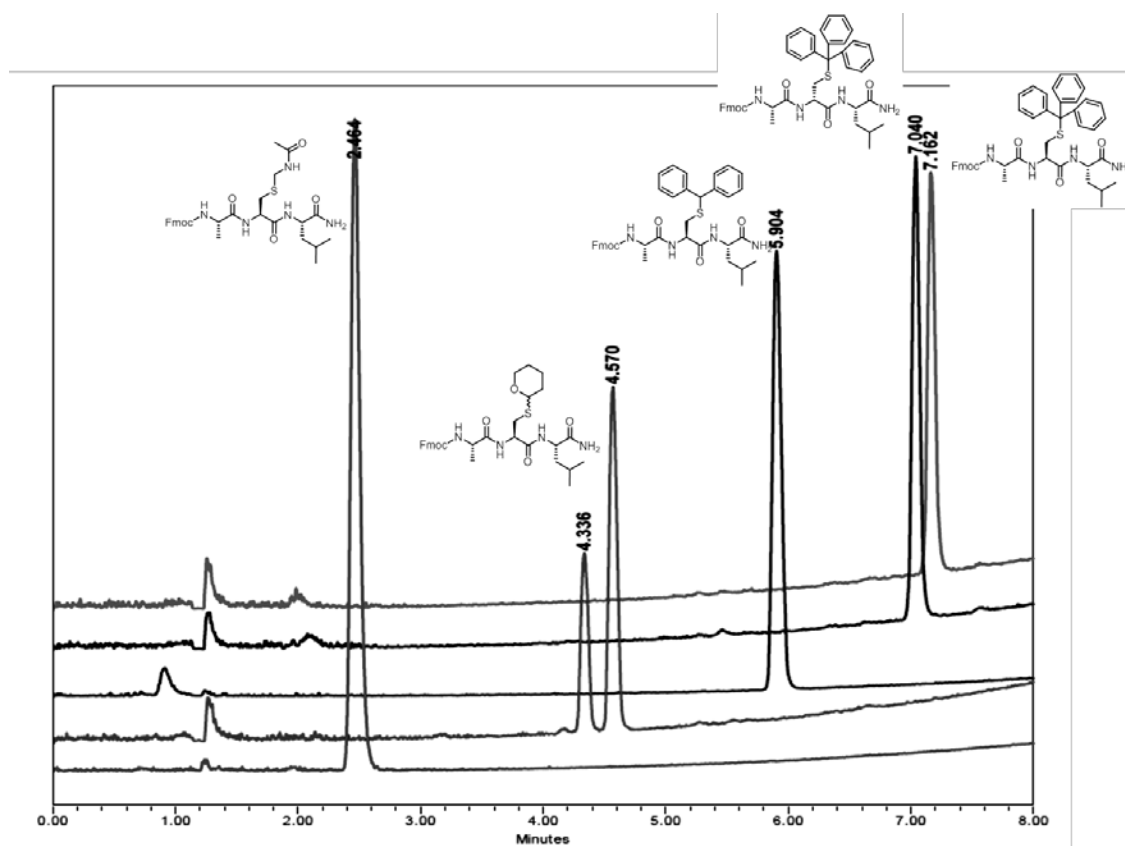
SA4.1. *Fmoc-Ala-L-Cys(Dpm)-Leu-NH₂* (**3d**): RP-HPLC [linear gradient H₂O/MeCN (50:50) to (0:100) over 8 min] t_R = 5.90 min; Purity = >99 %. m/z calculated for C₄₀H₄₄N₄O₅S = 692.3; found = 693.2 [M+H]⁺, being M the MW of *Fmoc-Ala-L-Cys(Dpm)-Leu-NH₂*.



SA5.1. *Fmoc-Ala-L-Cys(Acm)-Leu-NH₂* (**3e**): RP-HPLC [linear gradient H₂O/MeCN (50:50) to (0:100) over 8 min] t_R = 2.46 min; Purity = >99 %. m/z calculated for C₃₀H₃₉N₅O₆S = 597.3; found = 598.2 [M+H]⁺, being M the MW of *Fmoc-Ala-L-Cys(Acm)-Leu-NH₂*.



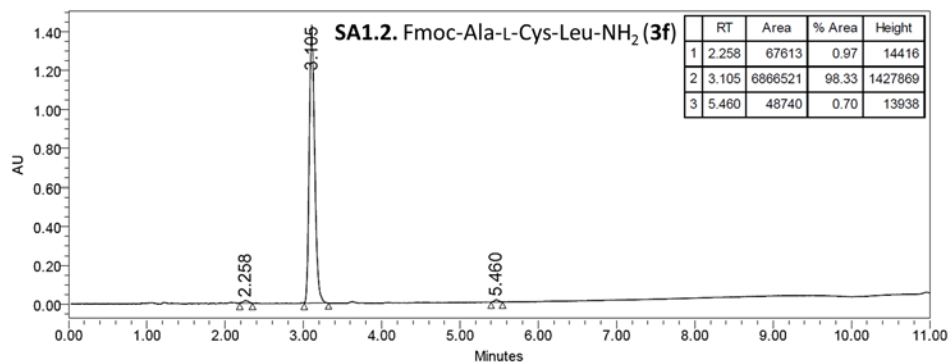
Overlay chromatograms for SA1-SA5 (**3a-3e**)



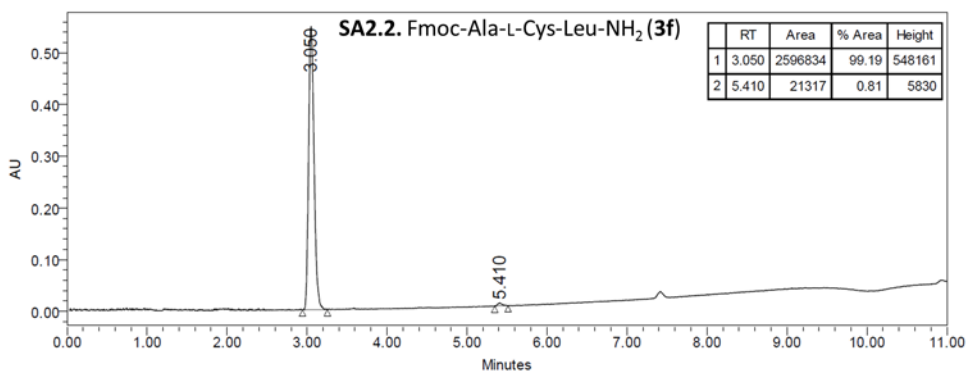
RP-HPLC [linear gradient H₂O/MeCN (50:50) to (0:100) over 8 min]

- *Fmoc-Ala-L-Cys-Leu-NH₂* tripeptide (**3f**): The fully-protected tripeptides resins were treated with TFA/TIS/DCM (95:2.5:2.5) (1×2 mL×30 min) and washed with DCM (2×1 mL). Then the filtered washes were evaporated and precipitated with Et₂O. The resulting peptides were dissolved into 2 mL H₂O/MeCN (1:1) for their analysis by RP-HPLC.

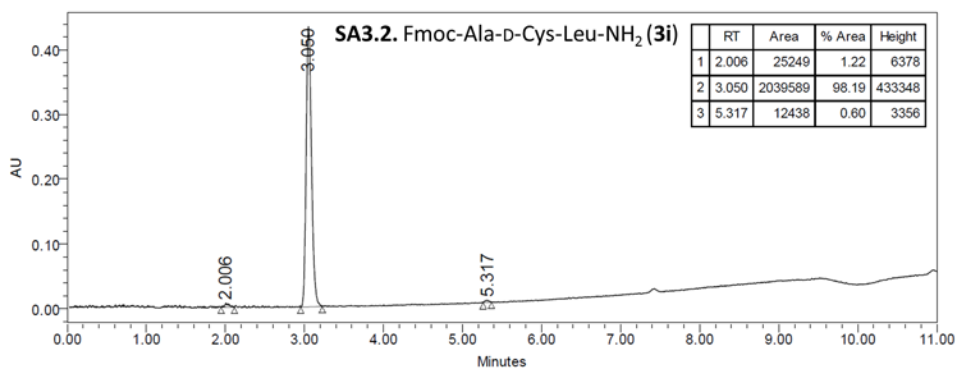
SA1.2. *Fmoc-Ala-L-Cys-Leu-NH₂*: RP-HPLC [linear gradient H₂O/MeCN (50:50) to (0:100) over 8 min] $t_R = 3.10$ min; Purity = 98 %. Cleavage from **3a** (Thp).



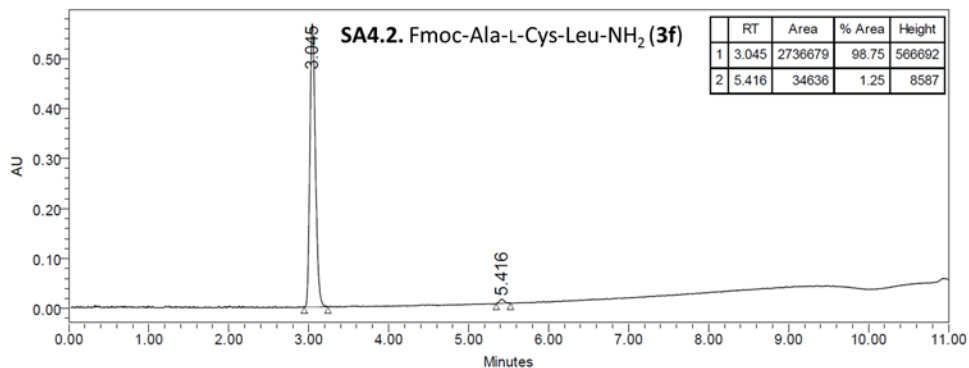
SA2.2. *Fmoc-Ala-L-Cys-Leu-NH₂*: RP-HPLC [linear gradient H₂O/MeCN (50:50) to (0:100) over 8 min] $t_R = 3.05$ min; Purity = 99 %. Cleavage from **3b** (Trt).



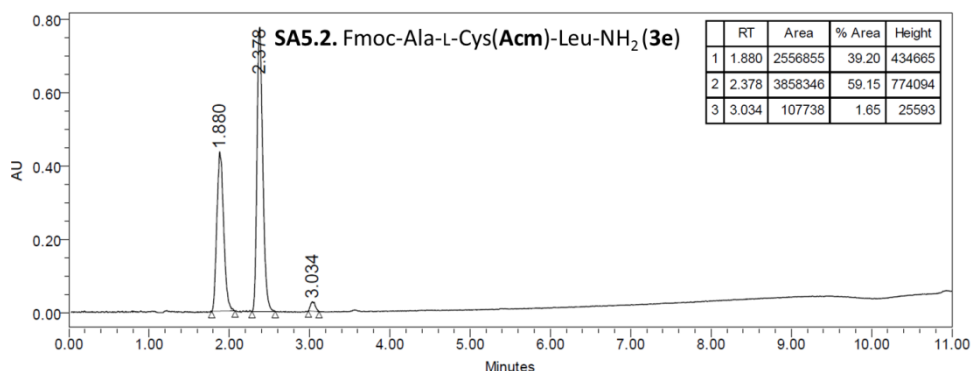
SA3.2. *Fmoc-Ala-D-Cys-Leu-NH₂*: RP-HPLC [linear gradient H₂O/MeCN (50:50) to (0:100) over 8 min] $t_R = 3.05$ min; Purity = 98 %. Cleavage from **3c** (Trt).



SA4.2. *Fmoc-Ala-L-Cys-Leu-NH₂*: RP-HPLC [linear gradient H₂O/MeCN (50:50) to (0:100) over 8 min] $t_R = 3.05$ min; Purity = 99 %. Cleavage from 3d (Dpm).

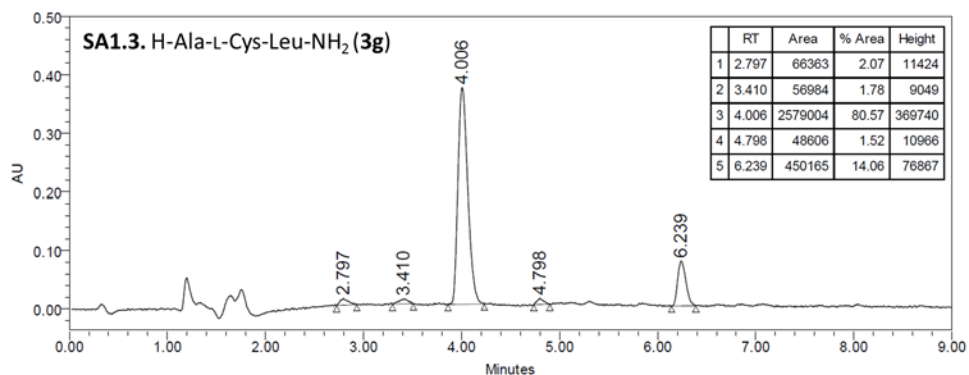


SA5.2. *Fmoc-Ala-L-Cys(Acm)-Leu-NH₂*: RP-HPLC [linear gradient H₂O/MeCN (50:50) to (0:100) over 8 min] $t_R = 2.38$ min; Purity = 60 %.

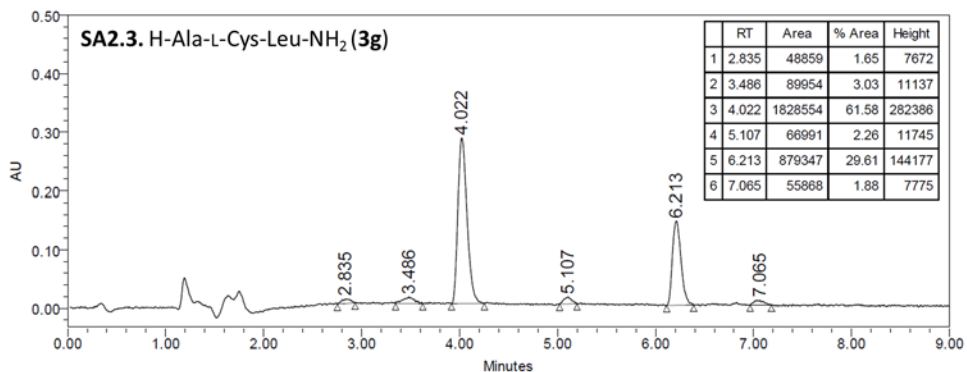


- H-Ala-Cys-Leu-NH₂ tripeptide (3g): The resins were solved with DCM (3×2 mL×5 min) and DMF (3×2 mL×5 min). Fmoc protecting group was eliminated by treating the resins with DMF/piperidine (4:1) (1×2 mL×1 min + 2×2 mL×5 min). Then the resins were washed with DCM (4×5 mL). The complete cleavage from the resins were performed with TFA/TIS/DCM (95:2.5:2.5) (1×2 mL×30 min) then the resins were washed with DCM (2×1 mL). Then the filtered washes were evaporated and precipitated with Et₂O. The resulting peptides were dissolved into 2 mL of TCEP 2 mM in H₂O/MeCN (1:1) for their analysis by RP-HPLC.

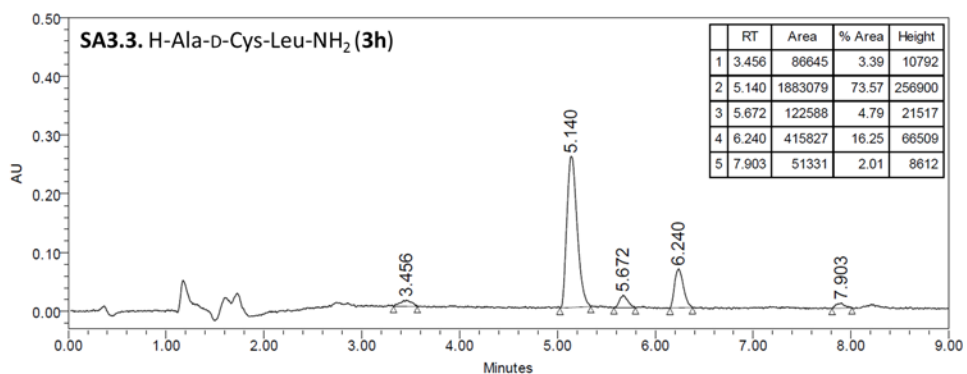
SA1.3. *H-Ala-L-Cys-Leu-NH₂*: RP-HPLC [linear gradient H₂O/MeCN (95:5) to (50:50) over 8 min] $t_R = 4.01$ min; Purity = 81 %. Cleavage from 3a (Thp).



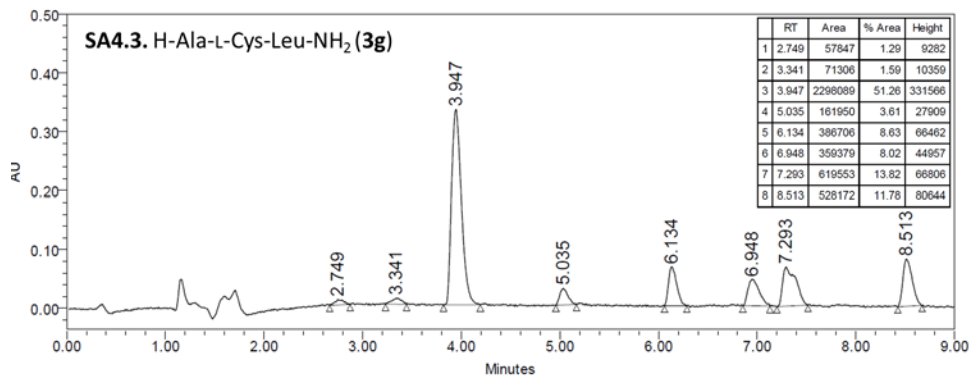
SA2.3. *H-Ala-L-Cys-Leu-NH₂*: RP-HPLC [linear gradient H₂O/MeCN (95:5) to (50:50) over 8 min]
 $t_R = 4.02$ min; Purity = 63 %. Cleavage from **3b** (Trt).



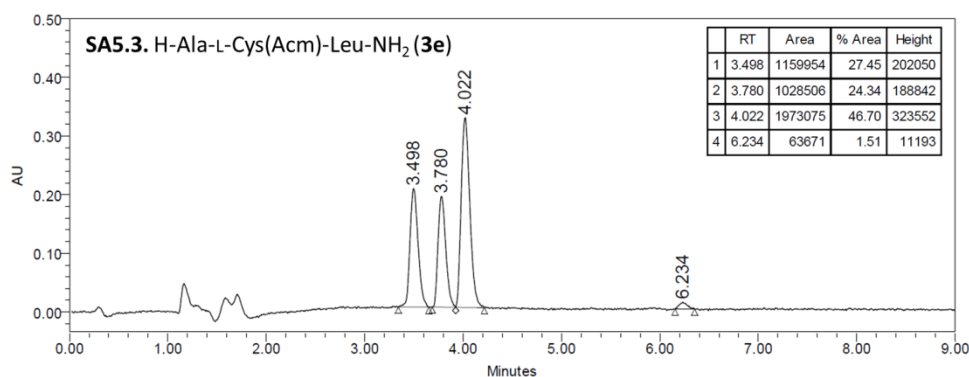
SA3.3. *H-Ala-D-Cys-Leu-NH₂*: RP-HPLC [linear gradient H₂O/MeCN (95:5) to (50:50) over 8 min]
 $t_R = 5.18$ min; Purity = 75 %. Cleavage from **3c** (Trt).



SA4.3. *H-Ala-L-Cys-Leu-NH₂*: RP-HPLC [linear gradient H₂O/MeCN (95:5) to (50:50) over 8 min]
 $t_R = 3.95$ min; Purity = 52 %. Cleavage from **3d** (Dpm).



SA5.3. *H-Ala-L-Cys(Acm)-Leu-NH₂*: RP-HPLC [linear gradient H₂O/MeCN (95:5) to (50:50) over 8 min] $t_R = 3.50 + 3.78 + 4.02$ [*H-Ala-L-Cys-Leu-NH₂*] min; Purity = 27 % + 24 % + 47 % respectively.



A.2- Rink amide Resin (RA):

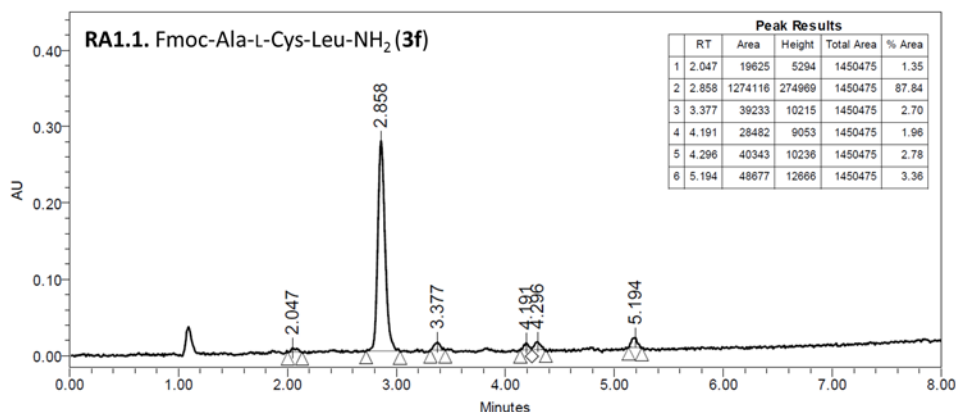
The Fmoc-Leu-Rink amide resin was divided into three fractions (RA1-RA3), which were solvated with DCM (3×5 mL) and DMF (3×5 mL). The following Cys protected aminoacid (2 eq.) –Fmoc-L-Cys(Thp)-OH (RA1), Fmoc-L-Cys(Trt)-OH (RA2), Fmoc-L-Cys(Dpm)-OH (RA3)– were coupled using DICPDI (2 eq.) and Oxyma Pure (2 eq.) in DMF with a 5 min pre-activation and 1 h and 30 min of coupling at 25 °C. The resins were washed with DMF (4×5 mL), DCM (4×5 mL) and DMF (2×5 mL). Fmoc protecting group was eliminated by treating the resins with DMF/piperidine (4:1) (1×5 mL×1 min + 2×5 mL×5 min). The resins were washed with DMF (4×5 mL), DCM (4×5 mL) and DMF (2×5 mL) and then Alanine was incorporated to the peptide chain using Fmoc-Ala-OH (3 eq.), DIPCDI (3 eq.) and Oxyme Pure (3 eq.): 5 min pre-activation + 1 h 30 min coupling at 25 °C. The resins were washed with DMF (4×5 mL) and DCM (4×5 mL).

Cleavage

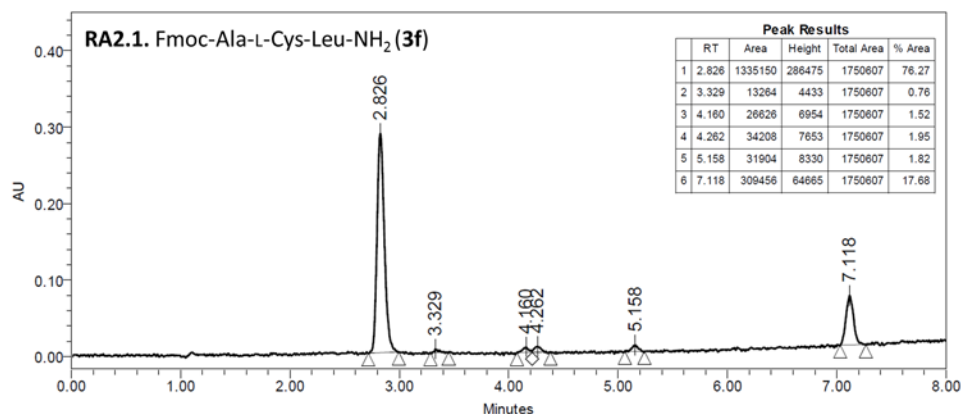
For each cleavage studies, 10 mg of the Fmoc-Ala-Cys(PG)-Leu-NH-Resin were used.

- Fmoc-Ala-Cys-Leu-NH₂ tripeptide (**3f**): Tripeptides-containing resins were treated with TFA/TIS/H₂O (95:2.5:2.5) (1×2 mL×1 h) and washed with DCM (2×1 mL). Then the filtered washes were evaporated and precipitated with Et₂O. The resulting peptides were dissolved into 2 mL H₂O/MeCN (1:1) for their analysis by RP-HPLC.

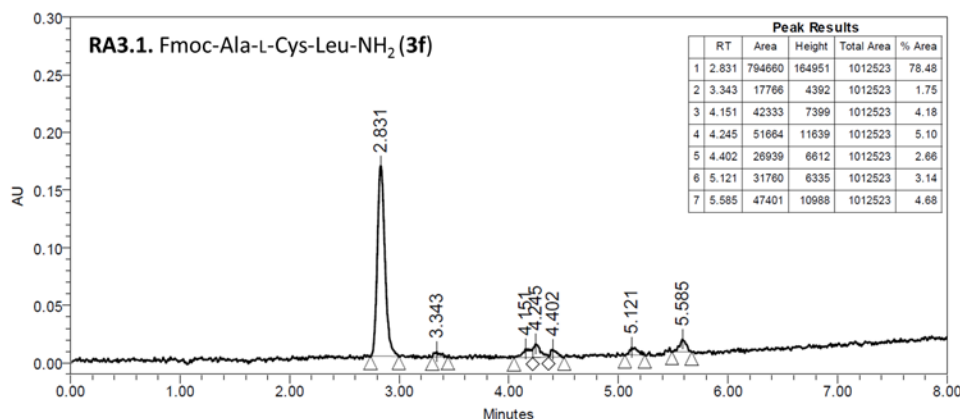
RA1.1. *Fmoc-Ala-L-Cys-Leu-NH₂*: RP-HPLC [linear gradient H₂O/MeCN (50:50) to (0:100) over 8 min] $t_R = 2.86$ min; Purity = 88 %. Synthesized with Thp as Cys PG



RA2.1. *Fmoc-Ala-L-Cys-Leu-NH₂*: RP-HPLC [linear gradient H₂O/MeCN (50:50) to (0:100) over 8 min] *t_R* = 2.83 min; Purity = 76 %. Synthesized with Trt as Cys PG

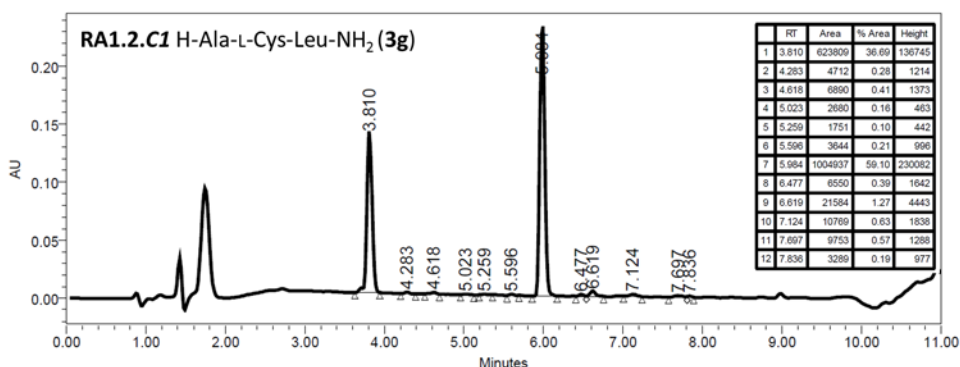


RA3.1. *Fmoc-Ala-L-Cys-Leu-NH₂*: RP-HPLC [linear gradient H₂O/MeCN (50:50) to (0:100) over 8 min] *t_R* = 2.83 min; Purity = 78 %. Synthesized with Dpm as Cys PG

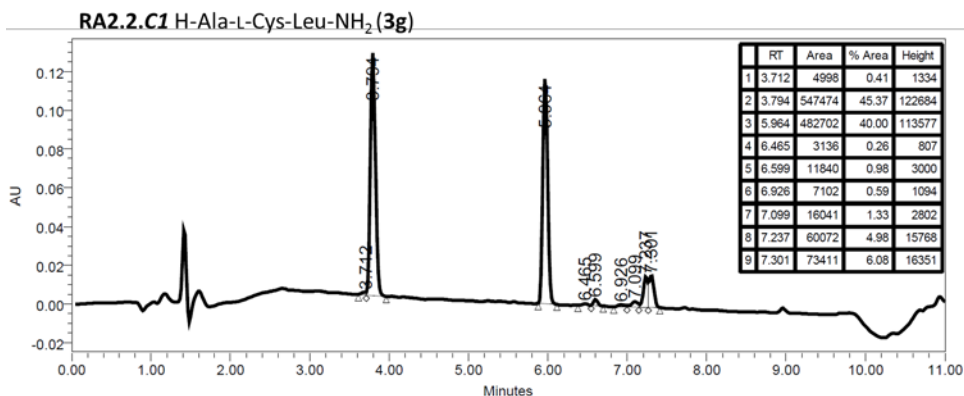


- H-Ala-L-Cys-Leu-NH₂ tripeptide (**3g**): The resins were solved with DCM (3×2 mL×5 min) and DMF (3×2 mL×5 min). Fmoc protecting group was eliminated by treating the resins with DMF/piperidine (4:1) (1×2 mL×1 min + 2×2 mL×5 min). Then the resins were washed with DCM (4×5 mL). The complete cleavage from the resins were performed with **TFA/TIS/H₂O (95:2.5:2.5)** (1×2 mL×1 h) then the resins were washed with DCM (2×1 mL). Then the filtered washes were evaporated and precipitated with Et₂O. The resulting peptides were dissolved into 2 mL of TCEP 2 mM in H₂O/MeCN (1:1) for their analysis by RP-HPLC.

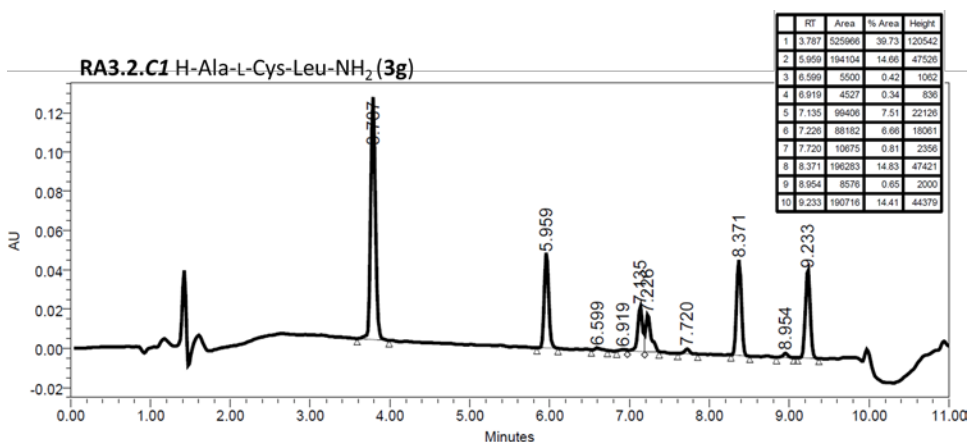
RA1.2.C1. *H-Ala-L-Cys-Leu-NH₂*: RP-HPLC [linear gradient H₂O/MeCN (95:5) to (50:50) over 8 min] *t_R* = 3.81 min; Purity = 37%. Synthesized with Thp as Cys PG



RA2.2.C1. *H-Ala-L-Cys-Leu-NH₂*: RP-HPLC [linear gradient H₂O/MeCN (95:5) to (50:50) over 8 min] *t_R* = 3.79 min; Purity = 45 %. Synthesized with Trt as Cys PG

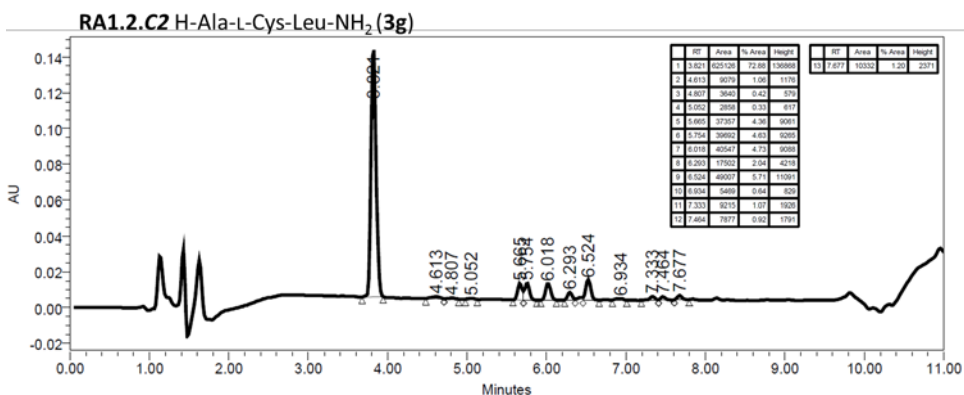


RA3.2.C1. *H-Ala-L-Cys-Leu-NH₂*: RP-HPLC [linear gradient H₂O/MeCN (95:5) to (50:50) over 8 min] *t_R* = 3.79 min; Purity = 40 %. Synthesized with Dpm as Cys PG

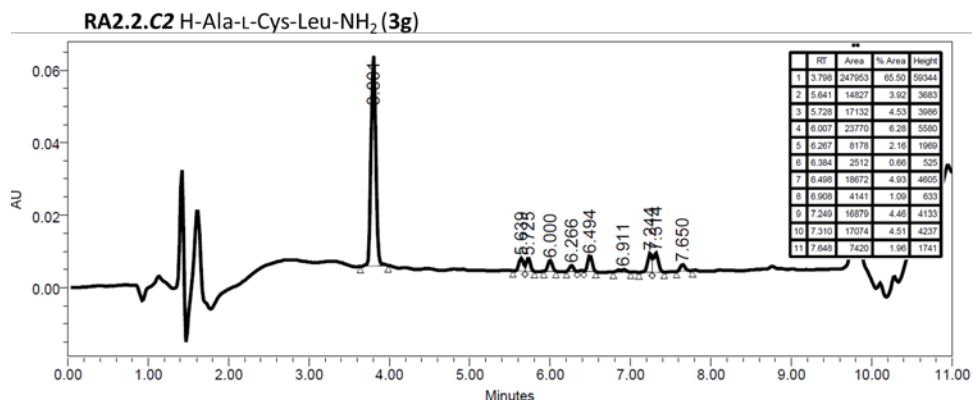


- *H-Ala-L-Cys-Leu-NH₂* tripeptide (**3g**): The resins were solved with DCM (3×2 mL×5 min) and DMF (3×2 mL×5 min). Fmoc protecting group was eliminated by treating the resins with DMF/piperidine (4:1) (1×2 mL×1 min + 2×2 mL×5 min). Then the resins were washed with DCM (4×5 mL). The complete cleavage from the resins were performed with **TFA/TIS/DMB (92.5:2.5:5)** (1×2 mL×1 h) then the resins were washed with DCM (2×1 mL). Then the filtered washes were evaporated and precipitated with Et₂O. The resulting peptides were dissolved into 2 mL of TCEP 2 mM in H₂O/MeCN (1:1) for their analysis by RP-HPLC.

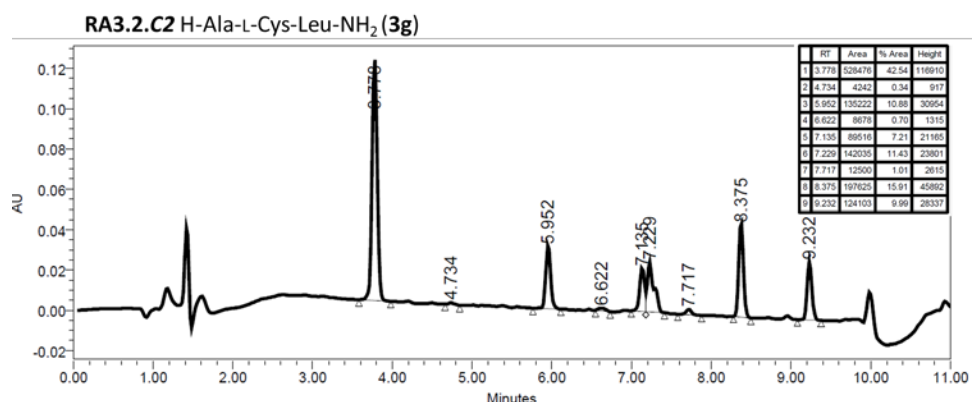
RA1.2.C2. *H-Ala-L-Cys-Leu-NH₂*: RP-HPLC [linear gradient H₂O/MeCN (95:5) to (50:50) over 8 min] *t_R* = 3.76 min; Purity = 73 %. Synthesized with Thp as Cys PG



RA2.2.C2. *H-Ala-L-Cys-Leu-NH₂*: RP-HPLC [linear gradient H₂O/MeCN (95:5) to (50:50) over 8 min] *t_R* = 3.80 min; Purity = 67 %. Synthesized with Trt as Cys PG



RA3.2.C2. *H-Ala-L-Cys-Leu-NH₂*: RP-HPLC [linear gradient H₂O/MeCN (95:5) to (50:50) over 8 min] *t_R* = 3.78 min; Purity = 43 %. Synthesized with Dpm as Cys PG



C-terminal carboxylic acid: Ala-Cys-Leu-OH tripeptides synthesis:

B. 2-Chlorotrityl chloride resin (2-CTC):

The resin used for the manual preparation of C-Terminal carboxylic acid tripeptide was 2-Chlorotrityl chloride resin (*f* = 1.60 mmol/g). Initially, the resin was washed with DCM (4×5 mL) and DMF (10×5 mL) and DCM (4×5 mL) again. The attachment of the first amino acid was carried out by the treatment of the resin with Fmoc-Leu-OH (1 eq.) and DIEA (3 eq.) in DCM during 10 min at 25 °C. Then, more DIEA (7 eq.) was added to the mixture, which was left to react for 40 min at 25 °C. Thereafter, the resin was capped by adding MeOH (10 eq.) to the mixture, which was left for 10 min at 25°C. Then the resin was washed with DCM (4×5 mL) and DMF (5×5 mL) and the Fmoc protecting group was eliminated by treating the resins with DMF/piperidine (4:1) (1×2 mL×1 min + 2×2 mL×5 min). Being, 0.97 mmol/g the loading for the H-Leu-2CTrityl as the UV quantification Fmoc group determined. The resins were washed with DMF (4×5 mL), DCM (4×5 mL).

The H-Leu-O-2-CTC Resin was divided into three fractions (CT1-CT3), which were solvated with DCM (3×5 mL) and DMF (3×5 mL). The following Cys protected aminoacid (2 eq.) –Fmoc-L-Cys(Thp)-OH (CT1), Fmoc-L-Cys(Trt)-OH (CT2), Fmoc-D-Cys(Trt)-OH (CT3)– were coupled using DICPDI (2 eq.) and Oxyma Pure (2 eq.) in DMF with a 5 min pre-activation and 1 h and 30 min of coupling at 25 °C. The resins were washed with DMF (4×5 mL), DCM (4×5 mL) and DMF (2×5 mL). Fmoc protecting group was eliminated by treating the resins with DMF/piperidine (4:1) (1×5 mL×1 min

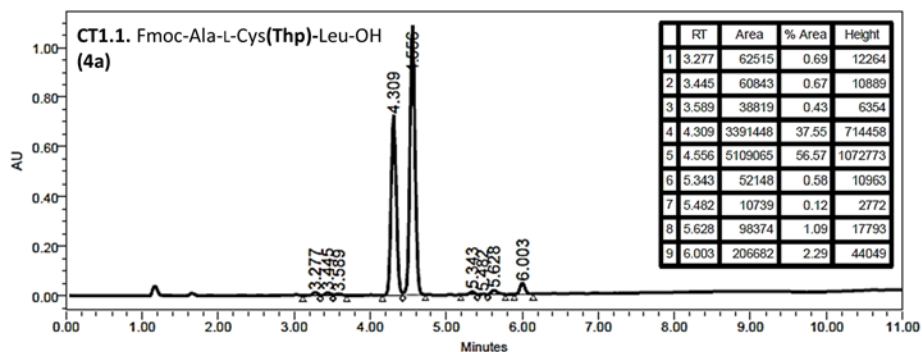
+ 1×5 mL×3 min). The resins were washed with DMF (4×5 mL), DCM (4×5 mL) and DMF (2×5 mL) and then Alanine was incorporated to the peptide chain using Fmoc-Ala-OH (3 eq.), DIPCDI (3 eq.) and Oxyme Pure (3 eq.): 5 min pre-activation + 1 h 30 min coupling at 25 °C. The resins were washed with DMF (4×5 mL) and DCM (4×5 mL).

Cleavage

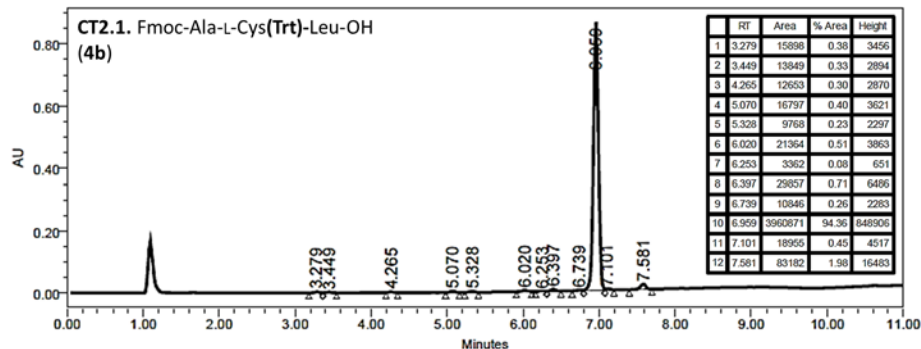
For each cleavage studies, 10 mg of the Fmoc-Ala-Cys(PG)-Leu-O-2CTC Resin were used.

- Fmoc-Ala-Cys(**PG**)-Leu-OH tripeptides (**4a-b**): Tripeptides-containing resins were treated with TFE/AcOH/DCM (20:20:60) (1×2 mL×1 h 30 min) and washed with the cleavage cocktail (2×2 mL) and with DCM (2×2 mL). Then the filtered washes were evaporated and precipitated with Et₂O. The resulting peptides were dissolved into 2 mL H₂O/MeCN (1:1) for their analysis by RP-HPLC.

2-CTC1.1. Fmoc-Ala-L-Cys(**Thp**)-Leu-OH (**4a**): RP-HPLC [linear gradient H₂O/MeCN (50:50) to (0:100) over 8 min] $t_R = 4.31 + 4.56$ min; Purity = 38 % and 57 % respectively (diastereoisomers).

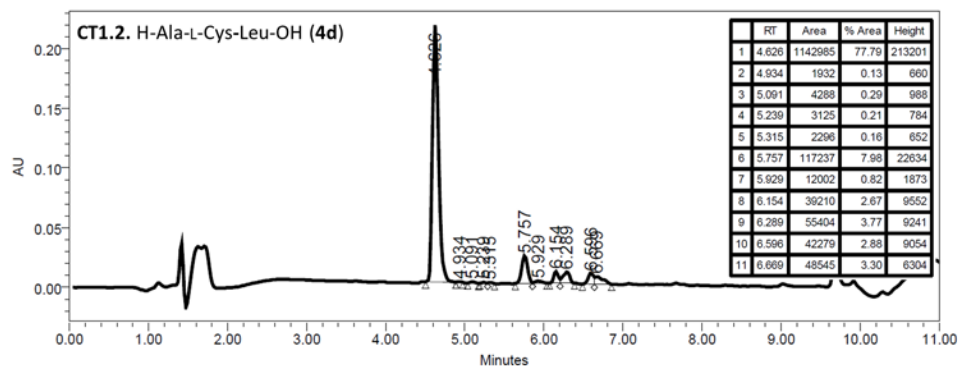


2-CTC2.1. Fmoc-Ala-L-Cys(**Trt**)-Leu-OH (**4b**): RP-HPLC [linear gradient H₂O/MeCN (50:50) to (0:100) over 8 min] $t_R = 6.96$ min; Purity = 94 %.

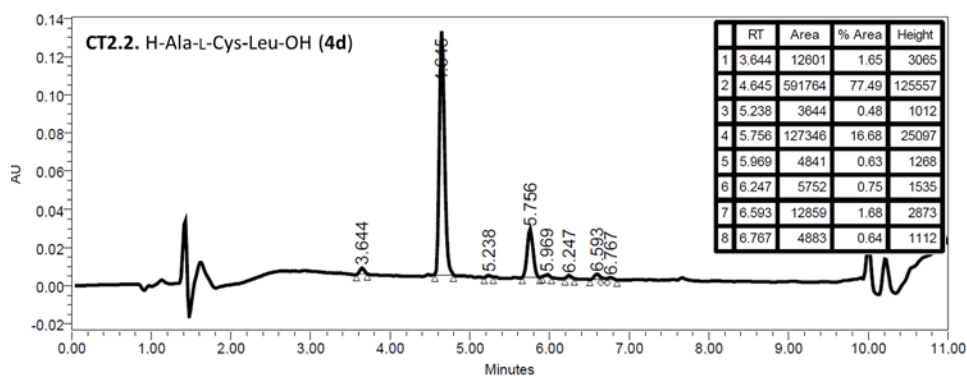


- H-Ala-Cys-Leu-OH tripeptide (**4d**): The resins were solved with DCM (3×2 mL×5 min) and DMF (3×2 mL×5 min). Fmoc protecting group was eliminated by treating the resins with DMF/piperidine (4:1) (1×2 mL×1 min + 2×2 mL×5 min). Then the resins were washed with DCM (4×5 mL). The complete cleavage from the resins were performed with TFA/TIS/DCM (95:2.5:2.5) (1×2 mL×30 min) and washed with DCM (2×1 mL). Then the filtered washes were evaporated and precipitated with Et₂O. The resulting peptides were dissolved into 2 mL of H₂O/MeCN (1:1) for their analysis by RP-HPLC.

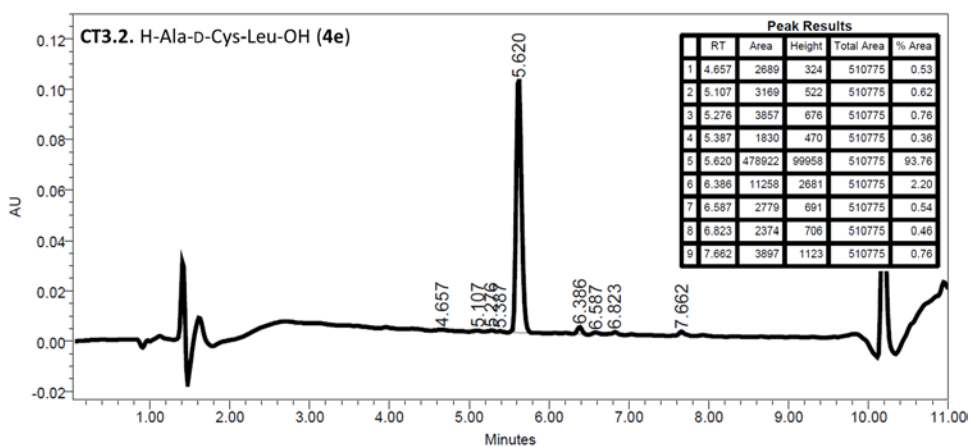
2-CTC1.2. *H-Ala-L-Cys-Leu-OH*: RP-HPLC [linear gradient $H_2O/MeCN$ (95:5) to (50:50) over 8 min] $t_R = 4.63$ min; Purity = 78 %. Cleavage from **4a** (Thp).



2-CTC2.2. *H-Ala-L-Cys-Leu-OH*: RP-HPLC [linear gradient $H_2O/MeCN$ (95:5) to (50:50) over 8 min] $t_R = 4.64$ min; Purity = 78 %. Cleavage from **4b** (Trt).



2-CTC3.2. *H-Ala-D-Cys-Leu-OH* (**4e**): RP-HPLC [linear gradient $H_2O/MeCN$ (95:5) to (50:50) over 8 min] $t_R = 5.82$ min; Purity = 94 %. Synthesized with Trt as Cys PG



C-terminal carboxylic acid: Ala-Leu-Cys-OH tripeptides synthesis.

2-Chlorotrityl chloride resin

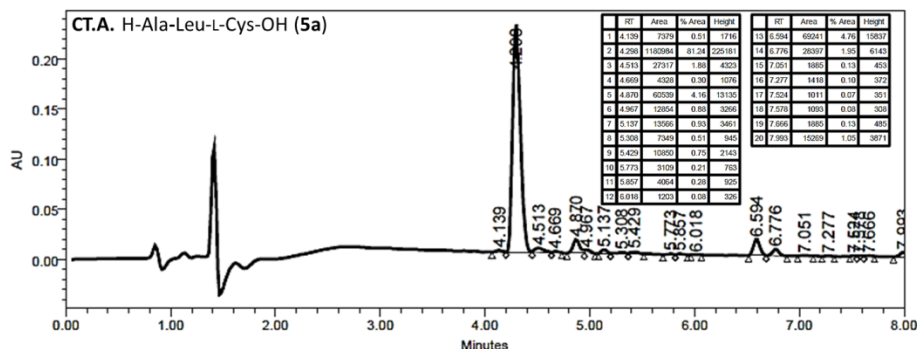
The resin used for the manual preparation of C-Terminal carboxylic acid tripeptide was 2-Chlorotrityl chloride resin ($f = 1.60$ mmol/g). Initially, the resin was washed with DCM (4×5 mL), DMF (10×5 mL) and DCM (4×5 mL). The attachment of the first amino acid was carried out by the treatment of the resin with the appropriate Fmoc-Cys-(PG)-OH (1 eq.) and DIEA (3 eq.) in DCM during 10 min at 25 °C. Then, more DIEA (7 eq.) was added to the mixture, and it was left to react for 40 min at 25 °C. Thereafter, the resin was capped by adding MeOH (10 eq.) to the mixture, which was left for 10 min at 25°C. Then the resin was washed with DCM (4×5 mL) and DMF (5×5 mL) and the Fmoc protecting group was eliminated by treating the resins with DMF/piperidine (4:1) (1×5 mL \times 1 min + 2×5 mL \times 5 min). Being, 0.60, 0.57 and 0.60 mmol/g the loading for the H-L-Cys(Thp)-O-2ClTrityl, H-L-Cys(Trt)-O-2ClTrityl and H-D-Cys(Trt)-O-2ClTrityl resins respectively, as the UV quantification Fmoc group determined. The resins were washed with DMF (4×5 mL), DCM (4×5 mL) and DMF (4×5 mL). Then leucine was incorporated into each resin using Fmoc-Leu-OH (3 eq.), DICPDI (3 eq.) and Oxyma Pure (3 eq.) in DMF with a 5 min pre-activation and 1 h and 30 min of coupling at 25 °C. Then the resins were washed with DMF (4×5 mL), DCM (4×5 mL) and DMF (2×5 mL). Fmoc protecting group was eliminated by treating the resins with DMF/piperidine (4:1) (1×5 mL \times 1 min + 1×5 mL \times 3 min). The resins were washed with DMF (4×5 mL), DCM (4×5 mL) and DMF (2×5 mL) and then Alanine was incorporated to the peptide chain using Boc-Ala-OH (3 eq.), DIPCPI (3 eq.) and Oxyma Pure (3 eq.): 5 min pre-activation + 1 h 30 min coupling at 25 °C. The resins were washed with DMF (4×5 mL) and DCM (4×5 mL).

Cleavage

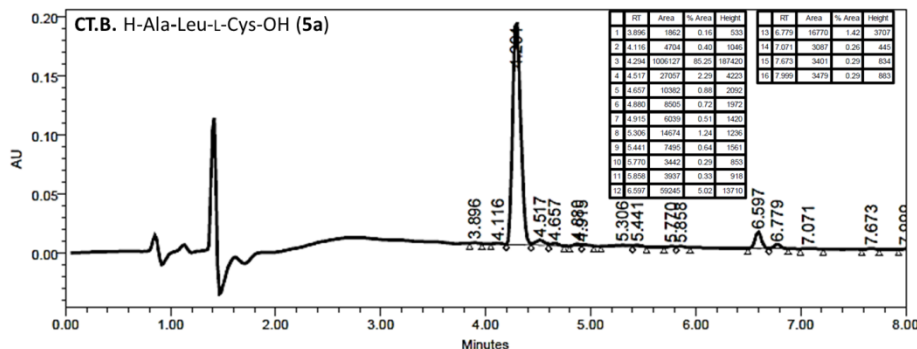
For each cleavage studies, 10 mg of the Boc-Ala-Leu-Cys(PG)-O-2ClTrityl Resins were used.

- H-Ala-Leu-Cys-OH tripeptide (**5a**): The resins were solved with DCM (3×2 mL \times 5 min) and its complete cleavage were performed with TFA/TIS/DCM (95:2.5:2.5) (1×2 mL \times 1 h) followed by washes with DCM (3×1 mL). Then the filtered washes were evaporated and precipitated with Et₂O. The resulting peptides were dissolved into 2 mL of H₂O/MeCN (1:1) for their analysis by RP-HPLC.

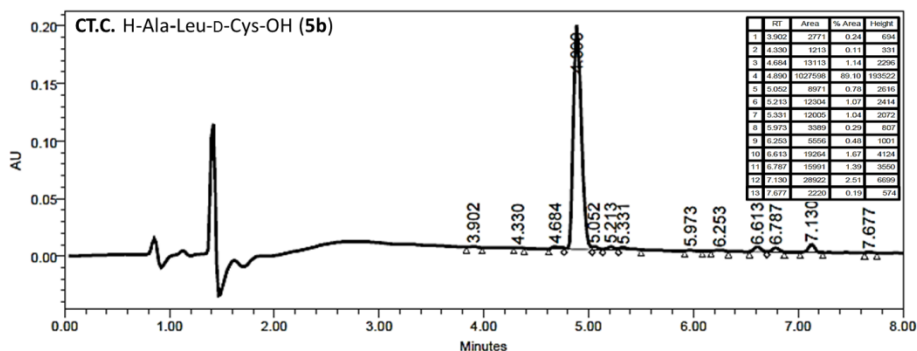
CT.A. H-Ala-Leu-L-Cys-OH: RP-HPLC [linear gradient H₂O/MeCN (95:5) to (50:50) over 8 min] $t_R = 4.30$ min; Purity = 81 %.



CT.B. H-Ala-Leu-L-Cys-OH: RP-HPLC [linear gradient H₂O/MeCN (95:5) to (50:50) over 8 min] *t_R* = 4.30 min; Purity = 85 %.



CT.C. H-Ala-Leu-D-Cys-OH: RP-HPLC [linear gradient H₂O/MeCN (95:5) to (50:50) over 8 min] *t_R* = 4.89 min; Purity = 89 %.



Wang Resin

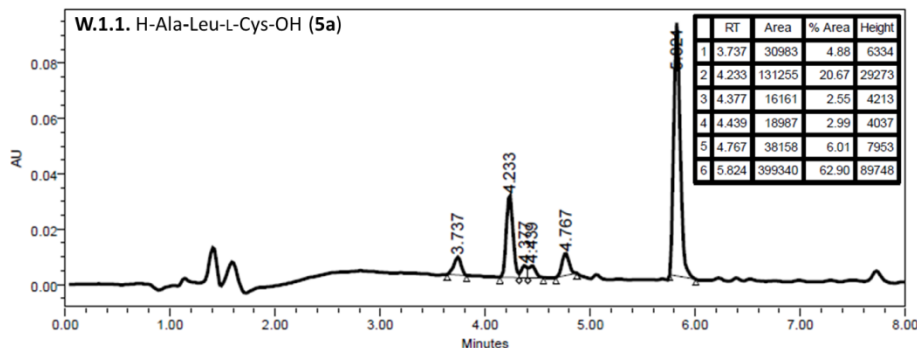
The resin used for the manual preparation of C-Terminal carboxylic acid tripeptides was Wang resin ($f = 1.2$ mmol/g). Initially, the resins were washed with DCM (4×5 mL), DMF (10×5 mL) and DCM (4×5 mL). The attachment of the first amino acid was carried out by the treatment of the resins with the appropriate Fmoc-Cys(PG)-OH (5 eq.) derivative, DIPCDI (2.5 eq.), Oxyme Pure (5 eq.) and DMAP (0.1 eq.) in DCM:DMF (9:1) (5 mL) during 3 h at 25 °C. Then, the resins were washed with DCM (5×5 mL) and it were capped with acetic anhydride (2 eq.) and DIEA (2 eq.) during 30 min at 25 °C. Thereafter, the resins were washed with DCM (4×5 mL) and DMF (4×5 mL) and the Fmoc protecting group was eliminated by treating the resins with DMF/piperidine (4:1) (1×5 mL×1 min + 2×5 mL×5 min). Being, 1.0, 1.1 and 0.9 mmol/g the loading for the H-L-Cys(Thp)-O-Wang, H-L-Cys(Trt)-O-Wang and H-L-Cys(*St*Bu)-O-Wang resins respectively, as the UV quantification Fmoc group determined. Then the resin was washed with DCM (4×5 mL) and DMF (5×5 mL). Leucine was incorporated into each resin using Fmoc-Leu-OH (3 eq.), DICPDI (3 eq.) and Oxyma Pure (3 eq.) in DMF with a 5 min pre-activation and 1 h and 30 min of coupling at 25 °C. Then the resins were washed with DMF (4×5 mL), DCM (4×5 mL) and DMF (2×5 mL). Fmoc protecting group was eliminated by treating the resins with DMF/piperidine (4:1) (1×5 mL×1 min + 1×5 mL×3 min). The resins were washed with DMF (4×5 mL), DCM (4×5 mL) and DMF (2×5 mL) and immediately Alanine was incorporated to the peptide chain using Boc-Ala-OH (3 eq.), DIPCDI (3 eq.) and Oxyme Pure (3 eq.): 5 min pre-activation + 1 h 30 min coupling at 25 °C. The resins were washed with DMF (4×5 mL) and DCM (4×5 mL).

Cleavage

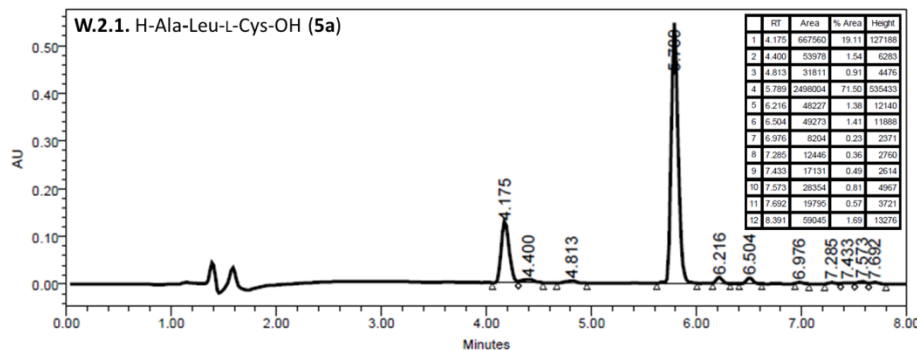
For each cleavage studies, 10 mg of the Boc-Ala-Leu-Cys(PG)-O-Wang Resins were used.

- H-Ala-Leu-Cys-OH tripeptide: The resins were solved with DCM (3×2 mL× 5 min) and its complete cleavage were performed with **TFA/TIS/H₂O (95:2.5:2.5)** (1×2 mL×1 h) followed by washes with DCM (3×1 mL). Then the filtered washes were evaporated and precipitated with Et₂O. The resulting peptides were dissolved into 2 mL of H₂O/MeCN (1:1) for their analysis by RP-HPLC.

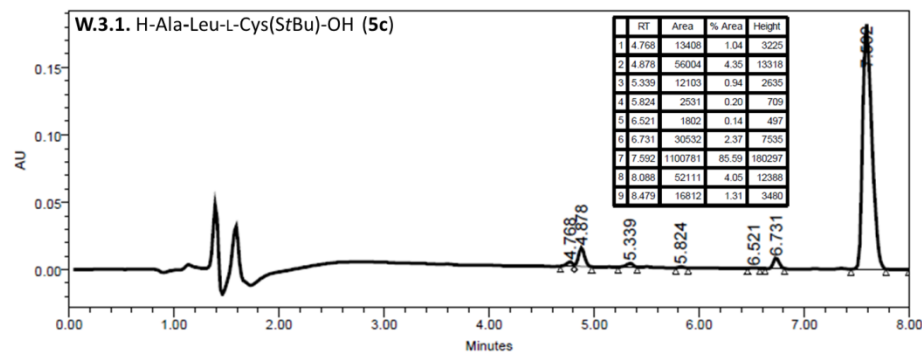
W.1.1 H-Ala-Leu-L-Cys-OH: RP-HPLC [linear gradient H₂O/MeCN (95:5) to (50:50) over 8 min] t_R = 4.23 min; Purity = 21 %.



W.2.1 H-Ala-Leu-L-Cys-OH: RP-HPLC [linear gradient H₂O/MeCN (95:5) to (50:50) over 8 min] t_R = 4.18 min; Purity = 19 %.

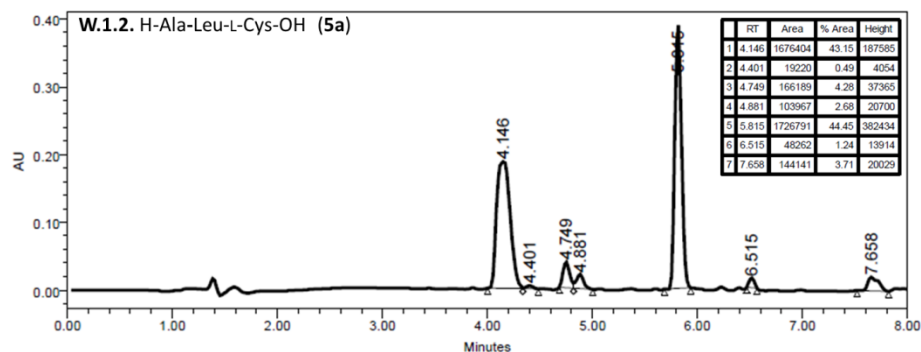


W.3.1 H-Ala-Leu-L-Cys(StBu)-OH: RP-HPLC [linear gradient H₂O/MeCN (95:5) to (50:50) over 8 min] t_R = 7.59 min; Purity = 86 %.

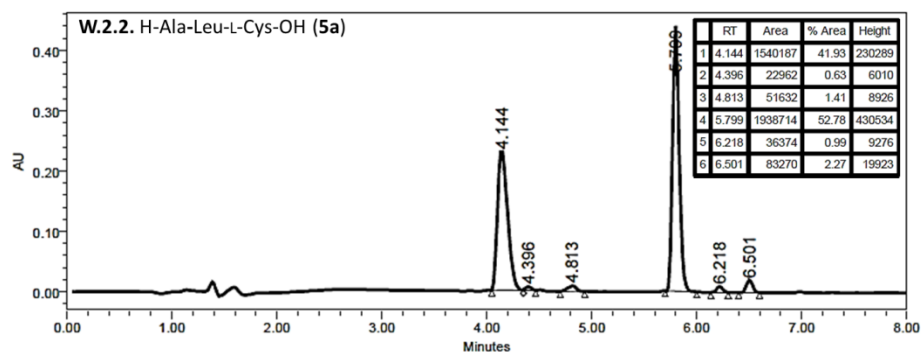


- H-Ala-Leu-Cys-OH tripeptide: The resins were solved with DCM (3×2 mL×5 min) and its complete cleavage were performed with **TFA/TIS/DMB (92.5:2.5:5)** (1×2 mL×1 h) followed by washes with DCM (3×1 mL). Then the filtered washes were evaporated and precipitated with Et₂O. The resulting peptides were dissolved into 2 mL of H₂O/MeCN (1:1) for their analysis by RP-HPLC.

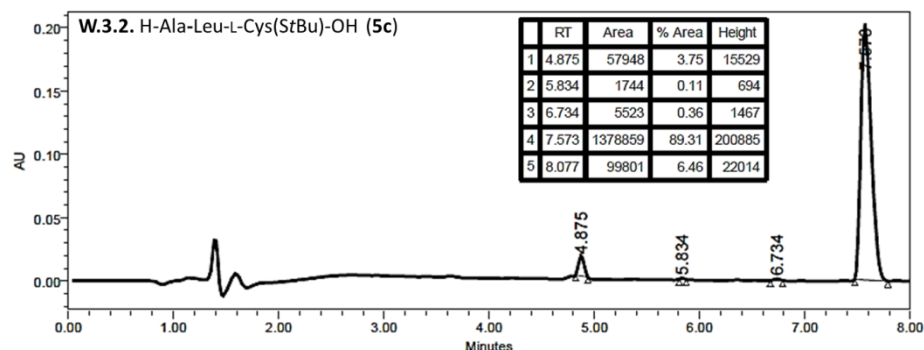
W.1.2 H-Ala-Leu-L-Cys-OH: RP-HPLC [linear gradient H₂O/MeCN (95:5) to (50:50) over 8 min] t_R = 4.15 min; Purity = 43 %.



W.2.2 H-Ala-Leu-L-Cys-OH: RP-HPLC [linear gradient H₂O/MeCN (95:5) to (50:50) over 8 min] t_R = 4.14 min; Purity = 42 %.



W.3.2 H-Ala-Leu-L-Cys(StBu)-OH: RP-HPLC [linear gradient H₂O/MeCN (95:5) to (50:50) over 8 min] t_R = 7.57 min; Purity = 89 %.

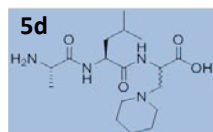


C-Terminal Cys Racemization study:

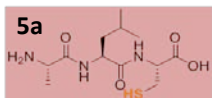
For C-terminal Cysteine racemization studies 100 mg of each Boc-Ala-Leu-Cys(PG)-O-2-CTC and Boc-Ala-Leu-Cys(PG)-O-Wang Resins were used.

The following products were observed into the analyzed cleavage mixtures.

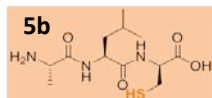
3-(1-piperidinyl)alanine adduct



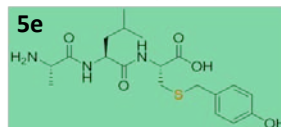
H-Ala-Leu-L-Cys-OH



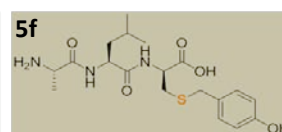
H-Ala-Leu-D-Cys-OH



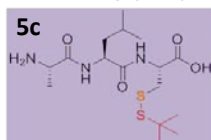
H-Ala-Leu-L-Cys(Phb)-OH



H-Ala-Leu-D-Cys(Phb)-OH

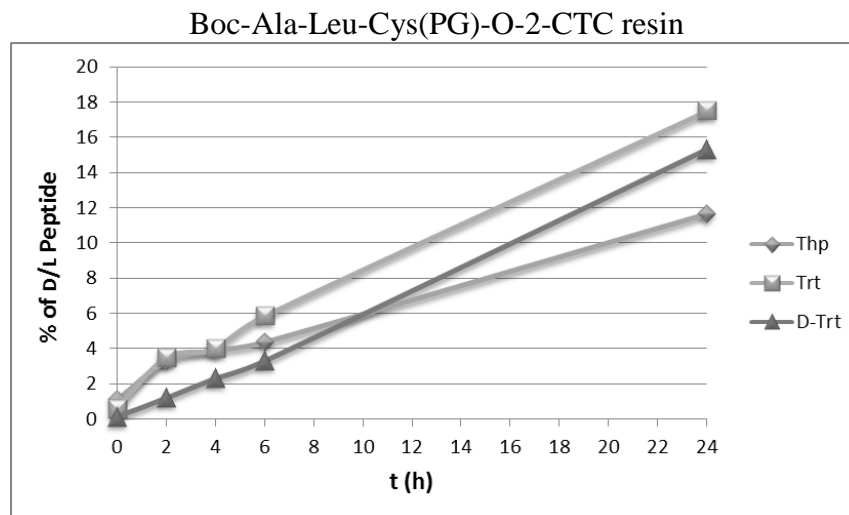


H-Ala-Leu-L-Cys(StBu)-OH

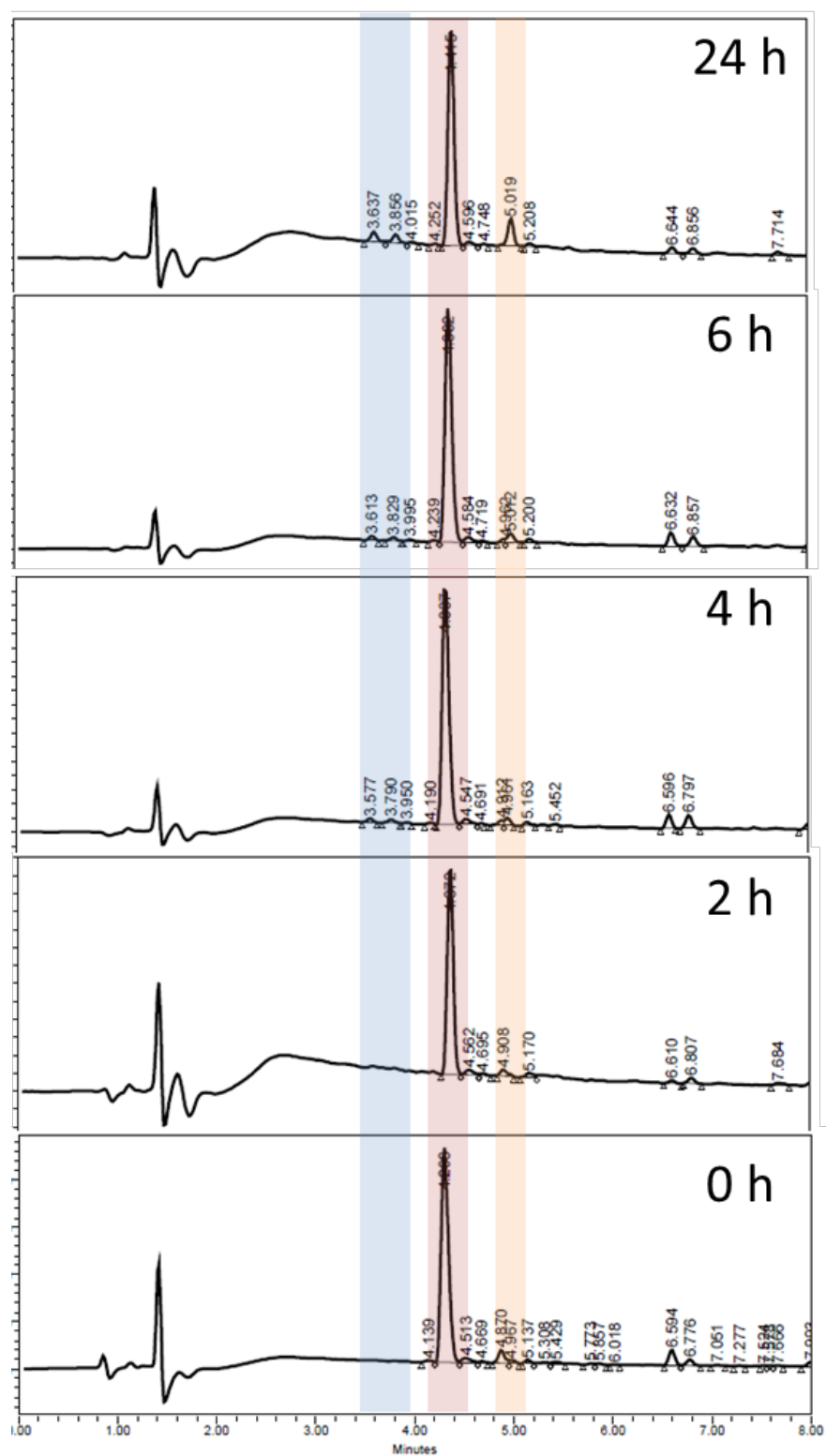


2-CTC resin

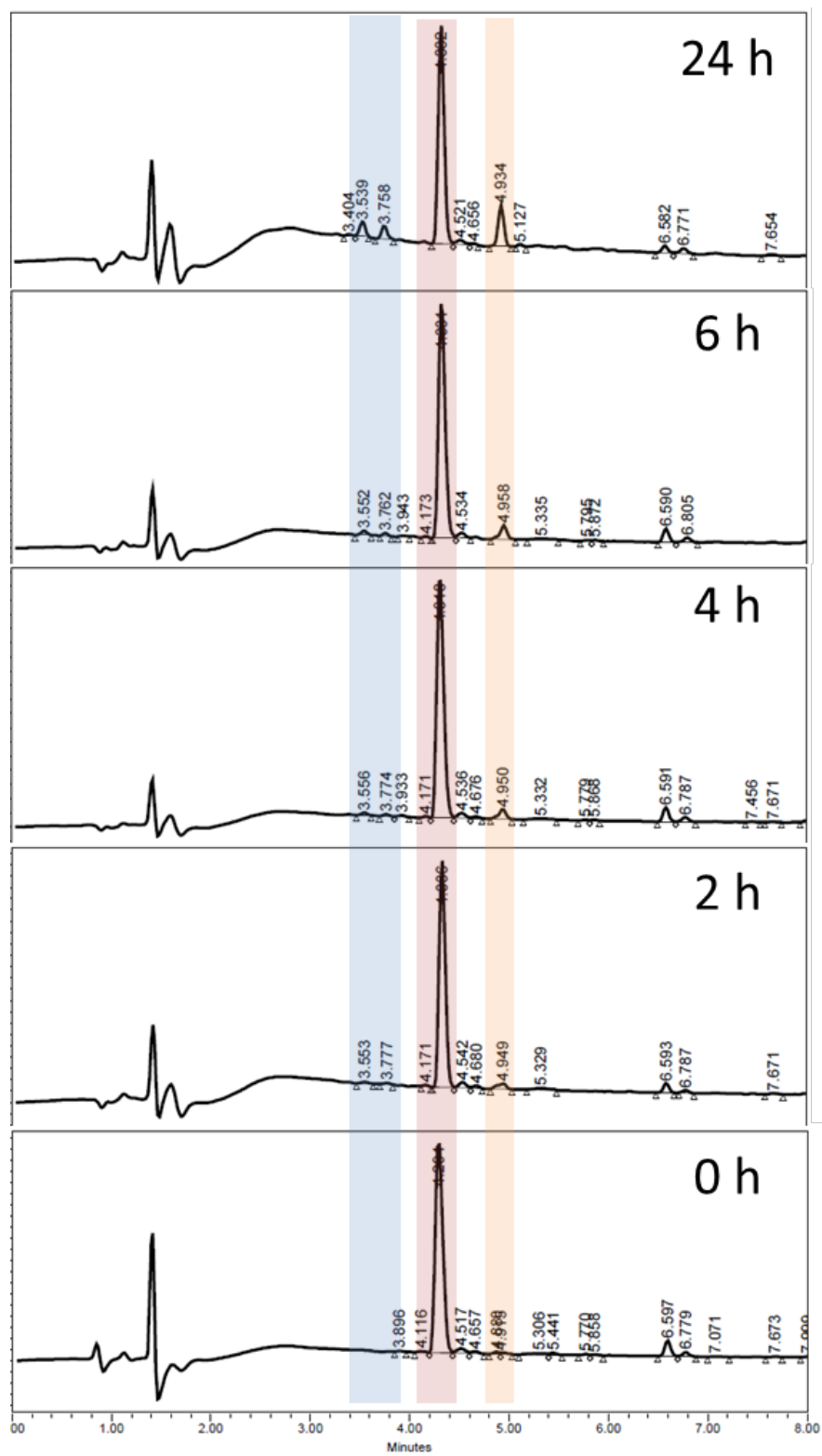
The corresponding Boc-Ala-Leu-Cys(PG)-O-2-CTC resins [for PG: Thp, L- and D-Cys(Trt)] were introduced into glass vial and it were treated with DMF/piperidine (4:1) (3 mL) for 24 h at 25 °C. At 2, 4, 6 and 24 h, a small aliquot of resin were separated and it was introduced into a polypropylene syringe and after washes with DMF (5×2 mL) and DCM (4×2 mL). The peptides were cleaved from the resin using TFA/TIS/H₂O (95:2.5:2.5) (1×2 mL×1 h) followed by washes with DCM (3×1 mL). Then the filtered washes were evaporated and precipitated with Et₂O. The resulting peptides were dissolved into 1 mL of H₂O/MeCN (1:1) for their analysis by RP-HPLC.



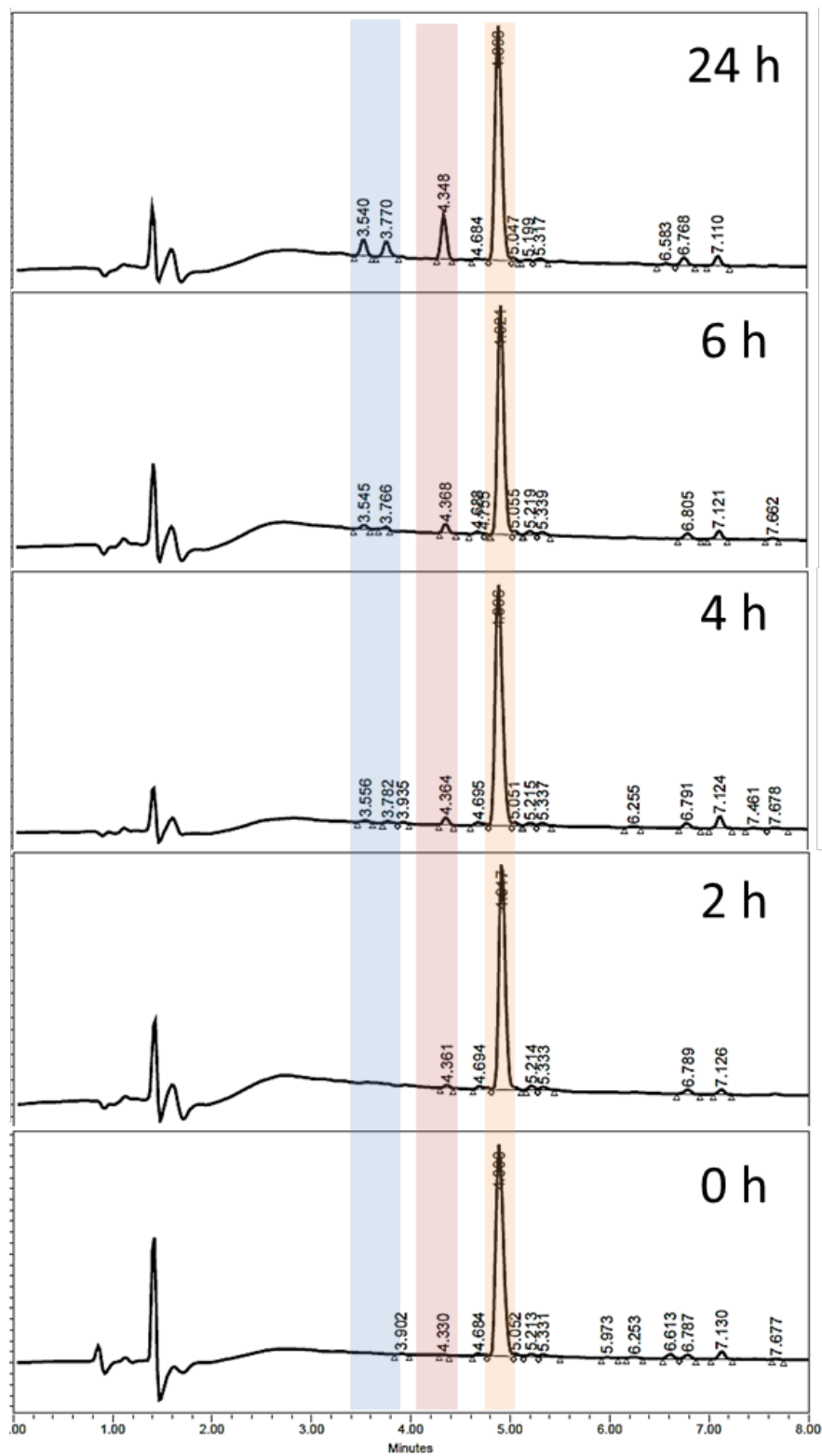
CTA.1. 2-CTC resin with Cys(Thp).



CTB.1. 2-CTC resin with L-Cys(Trt)

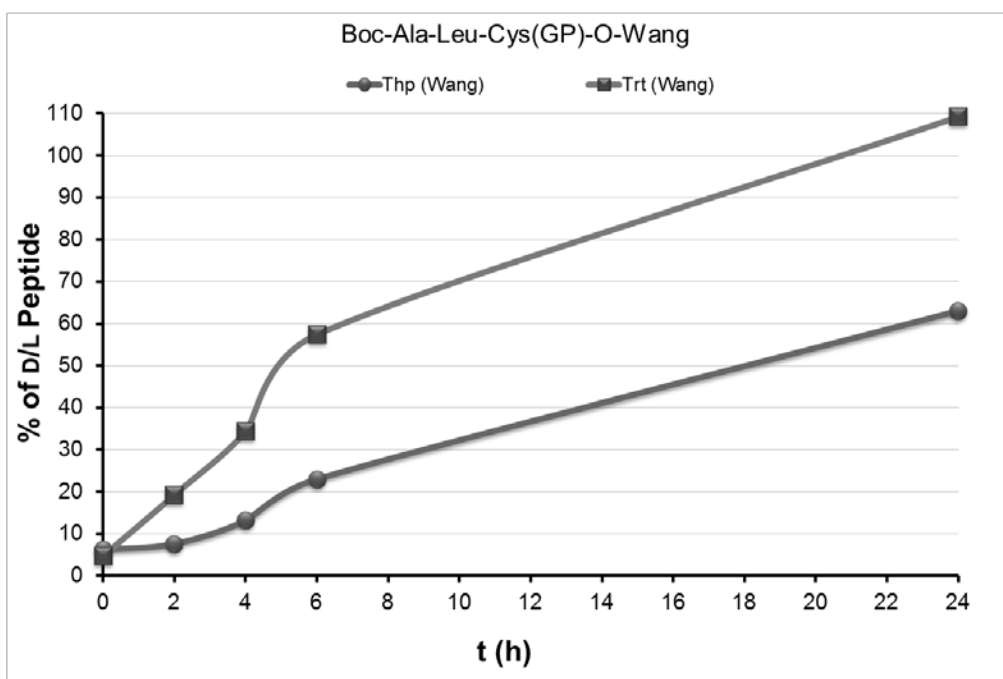


CTC.1. 2-CTC resin with D-Cys(Trt)

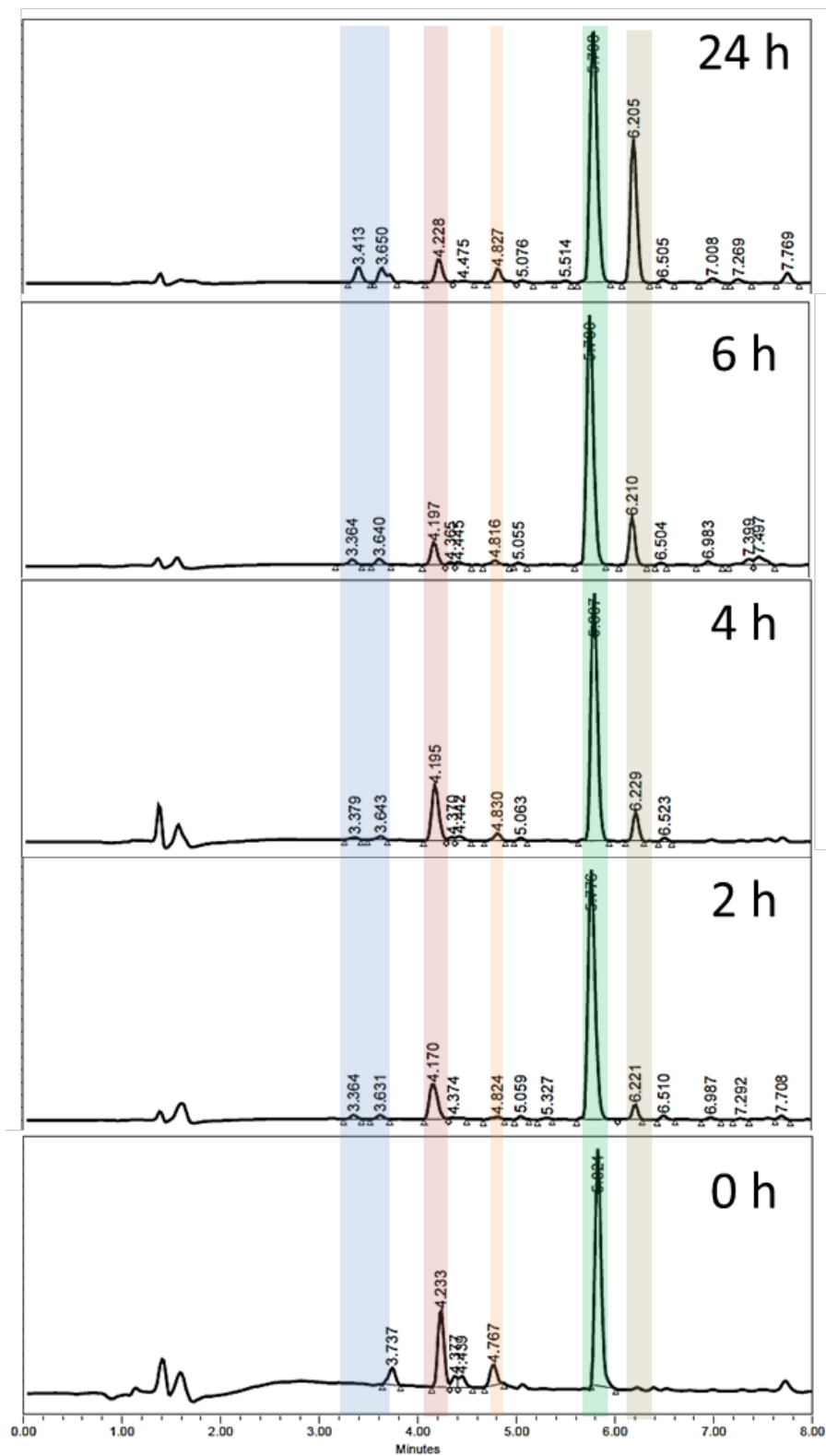


Wang Resin

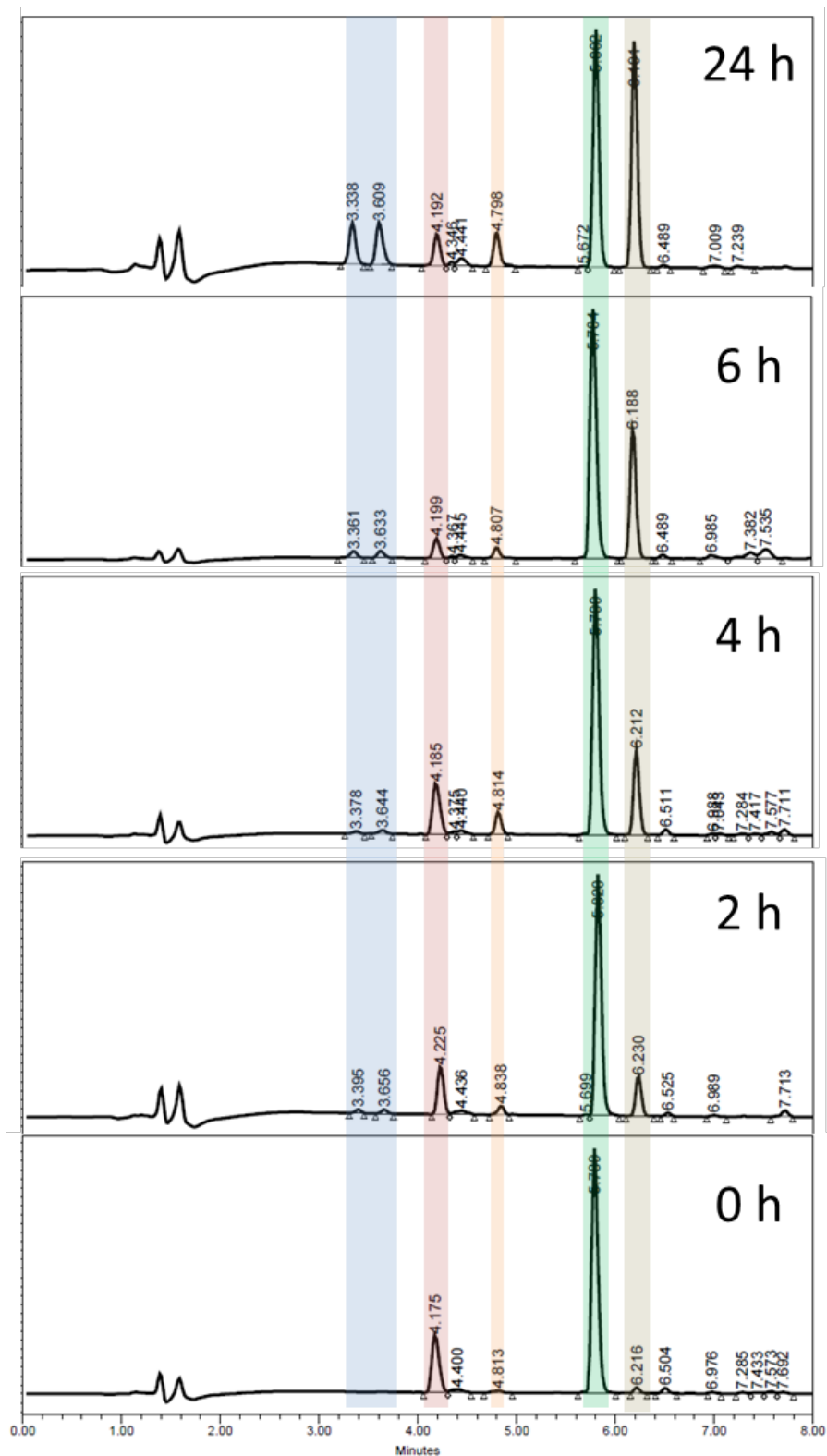
The corresponding Boc-Ala-Leu-Cys(PG)-O-Wang resins [for PG: Thp, L- and D-Cys(Trt)] were introduced into glass vial and it were treated with DMF/piperidine (4:1) (3 mL) for 24 h at 25 °C. At 2, 4, 6 and 24 h, a small aliquot of resin were separated and it was introduced into a polypropylene syringe and after washes with DMF (5×2 mL) and DCM (4×2 mL). The peptides were cleaved from the resin using **TFA/TIS/H₂O (95:2.5:2.5)** (1×2 mL×1 h) followed by washes with DCM (3×1 mL). Then the filtered washes were evaporated and precipitated with Et₂O. The resulting peptides were dissolved into 1 mL of H₂O/MeCN (1:1) for their analysis by RP-HPLC.



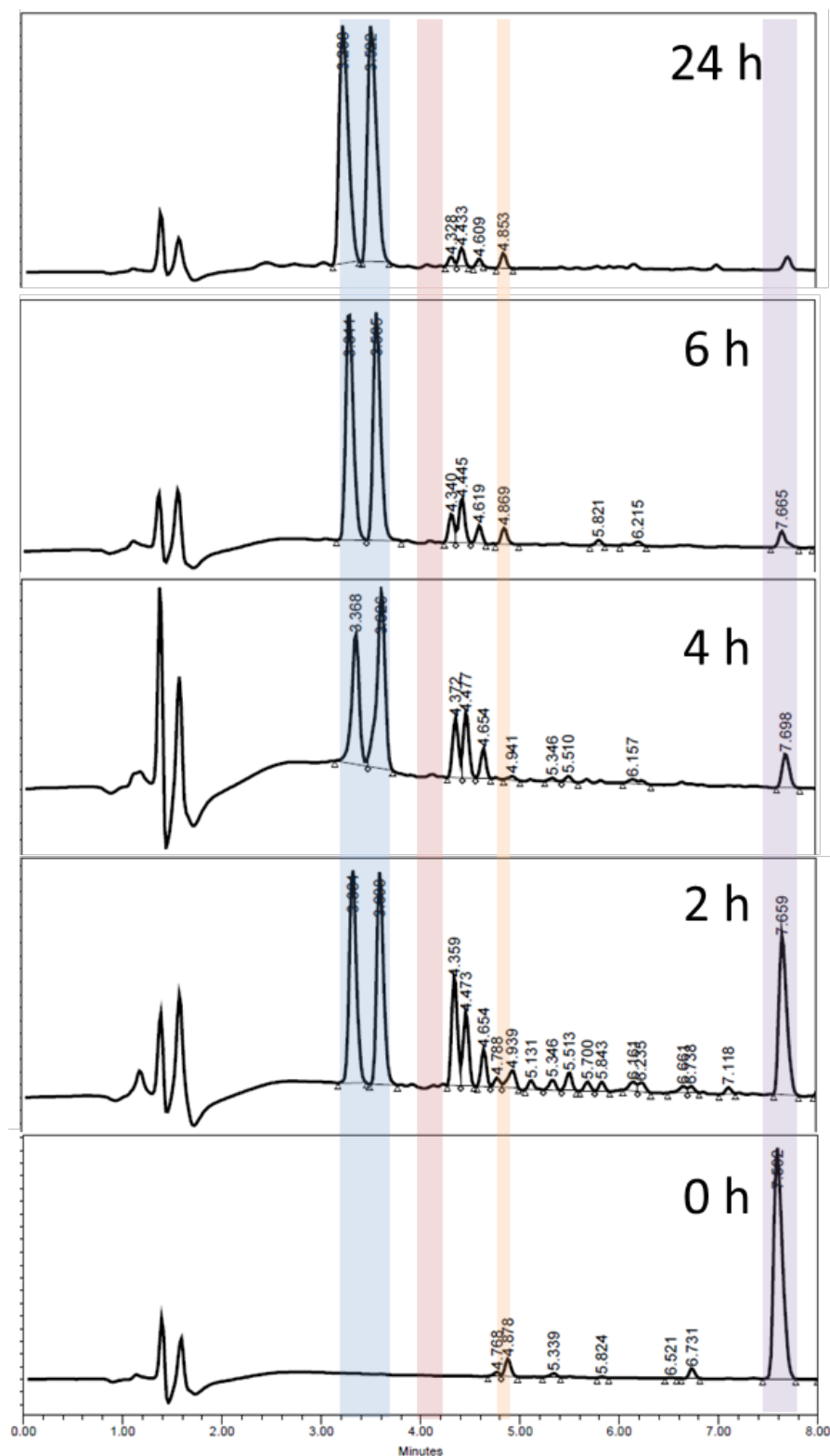
W1.1. Wang resin with Cys(Thp)



W2.1. Wang resin with Cys(Trt)

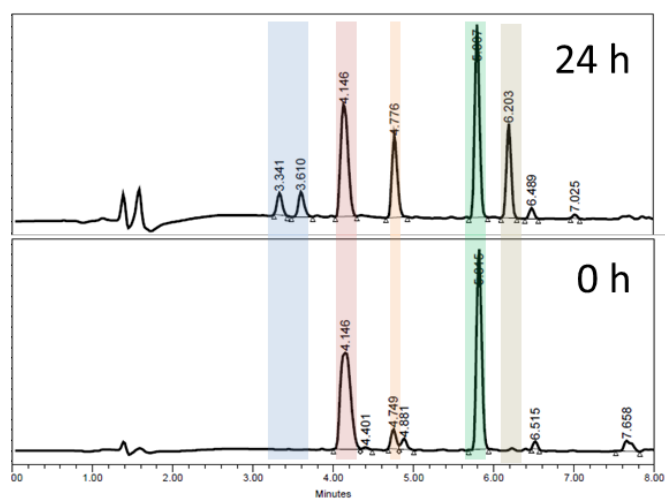


W3.1. Wang resin with Cys(*St*Bu)

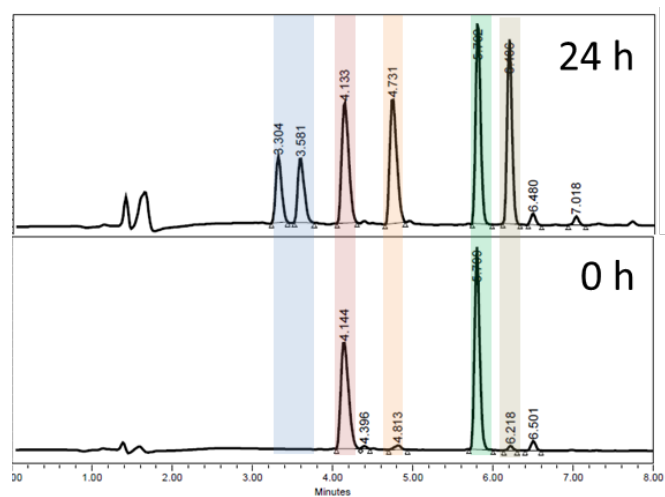


Alternatively, the aliquots after 24 h were cleaved using **TFA/TIS/DMB (92.5:2.5:5)** (1×2 mL×1 h) followed by washes with DCM (3×1 mL). Then the filtered washes were evaporated and precipitated with Et₂O. The resulting peptides were dissolved into 1 mL of H₂O/MeCN (1:1) for their analysis by RP-HPLC.

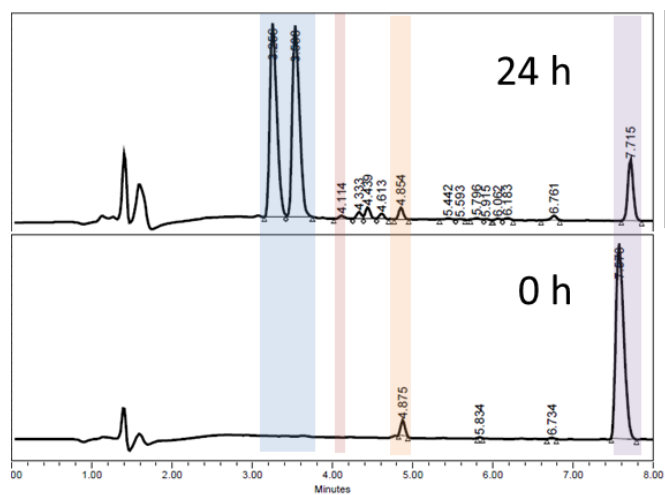
W1.2. Wang resin with Cys(Thp)



W2.2. Wang resin with Cys(Trt)



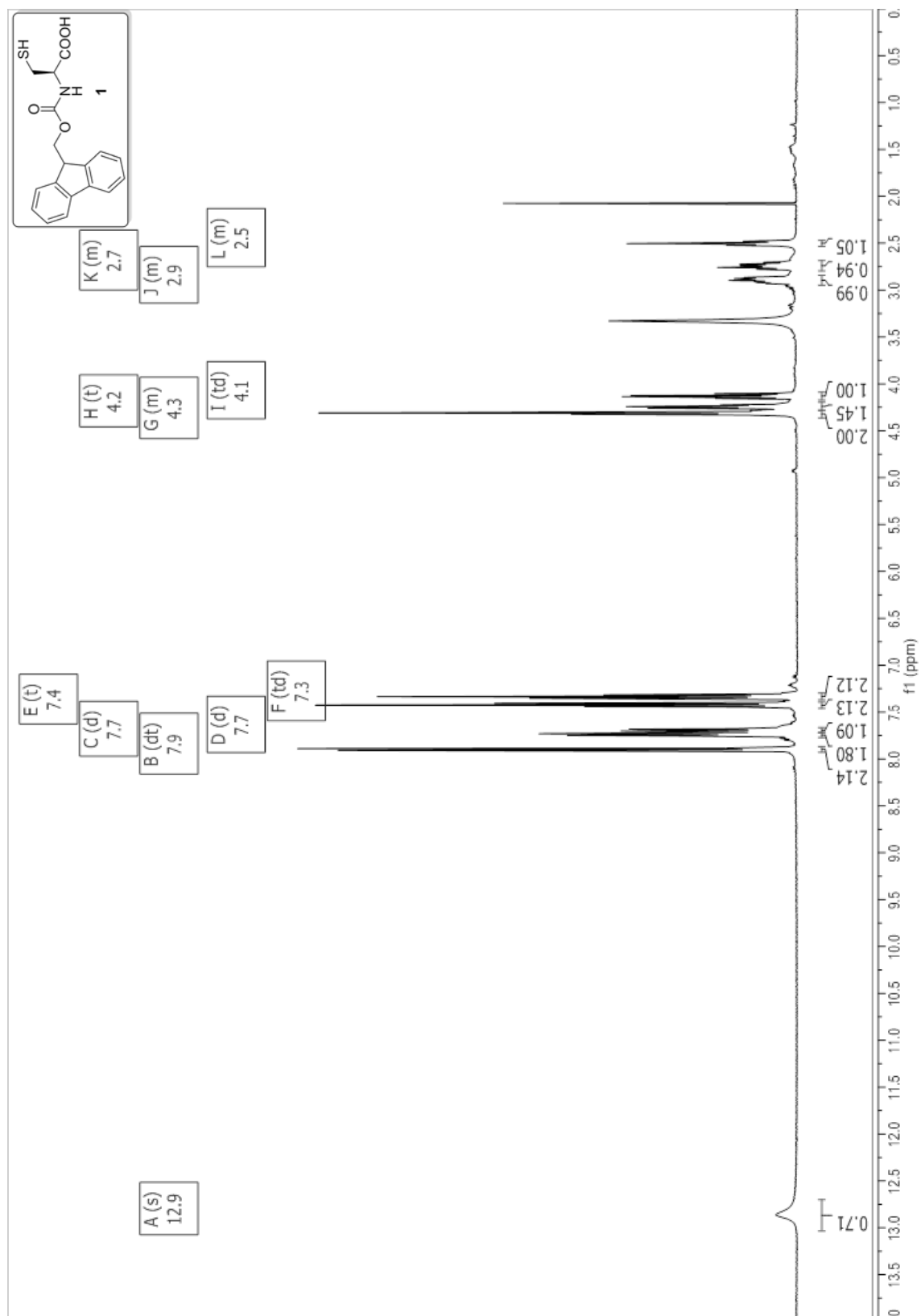
W3.2. Wang resin with Cys(*St*Bu)



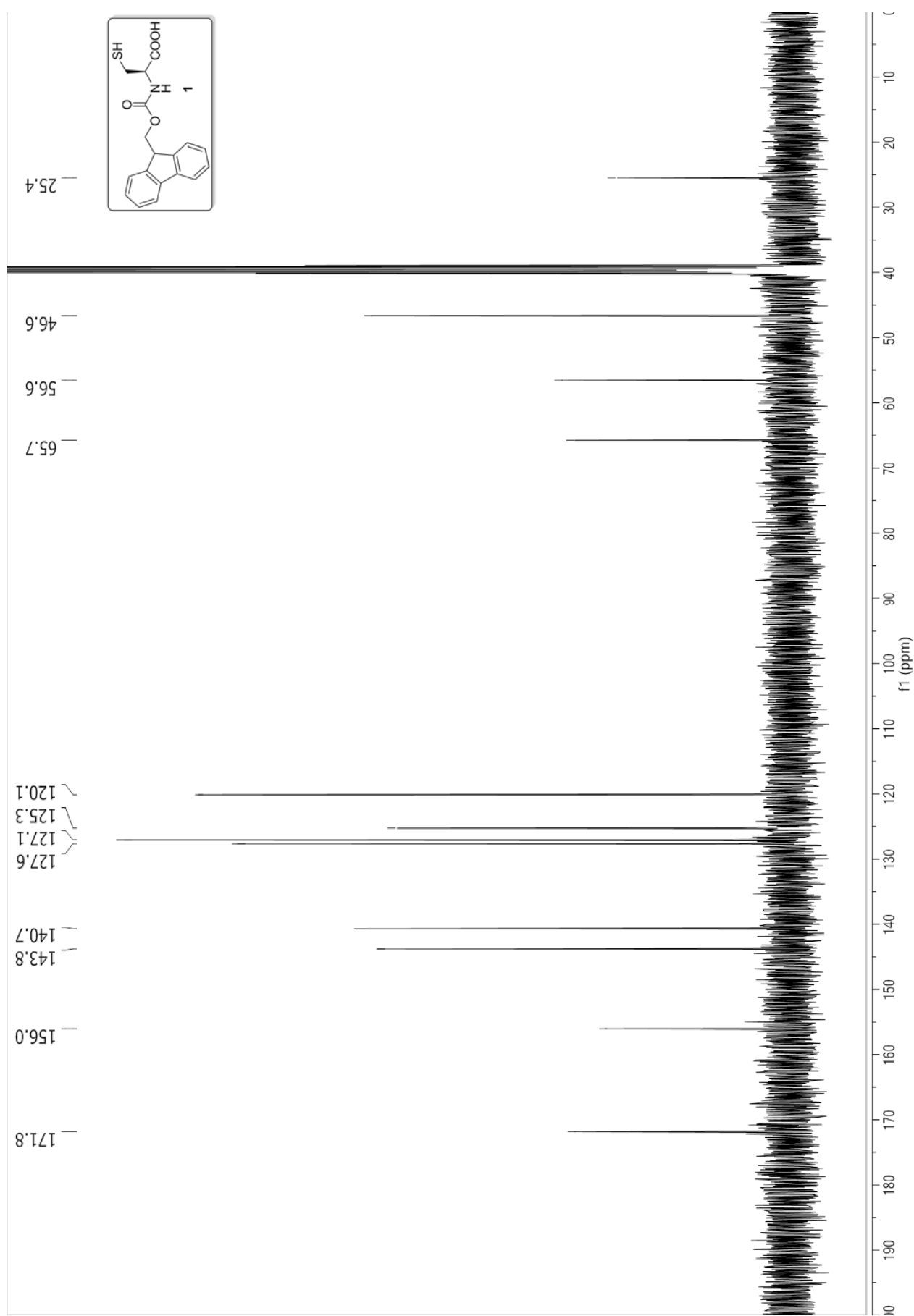
NMR Spectra

1. Fmoc-Cys-OH

^1H NMR (400 MHz, DMSO- d_6):

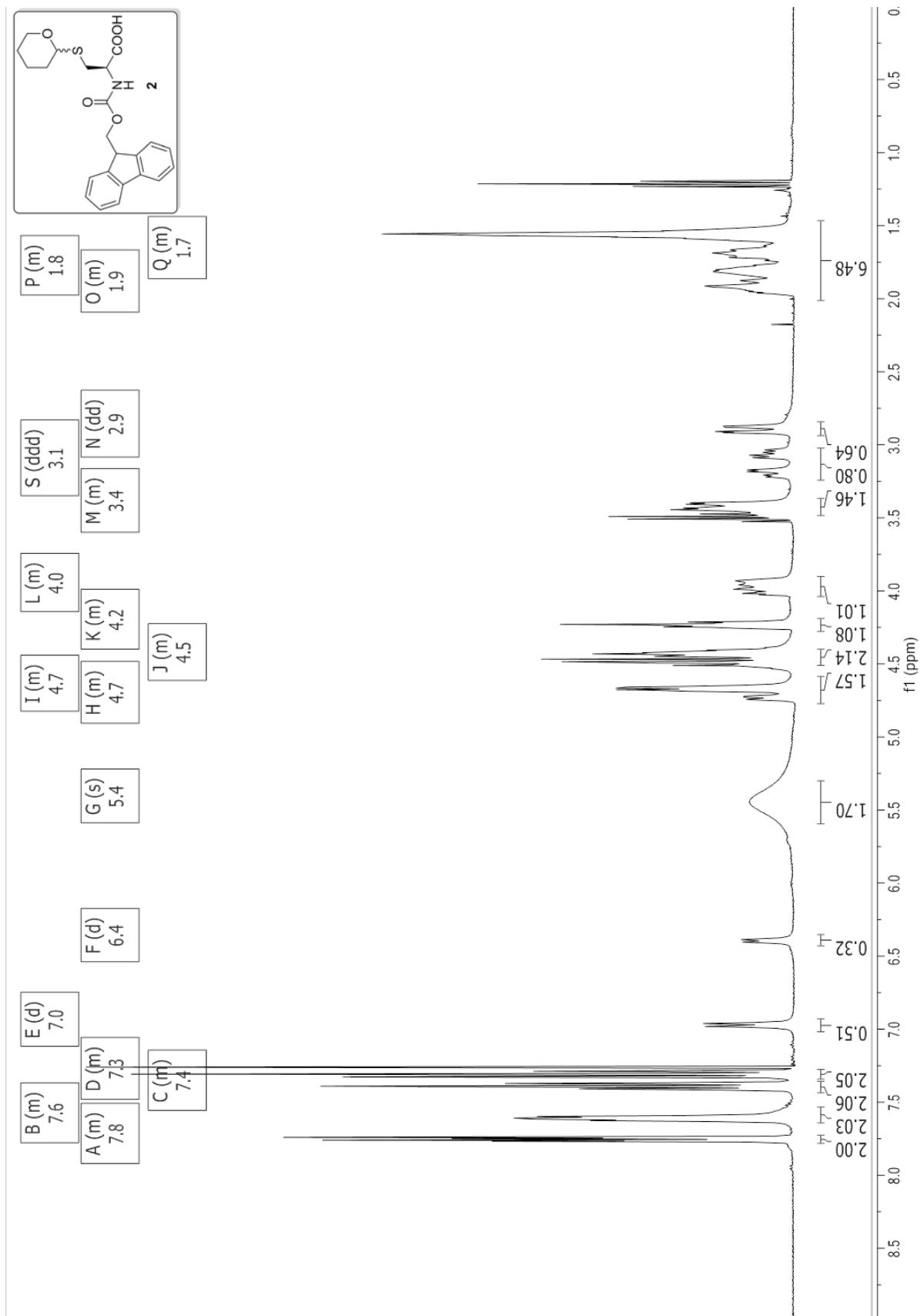


¹³C NMR (100 MHz, DMSO-*d*₆):



2. Fmoc-Cys(Thp)-OH

^1H NMR (400 MHz, CDCl_3):



^{13}C NMR (100 MHz, CDCl_3):

