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

Supporting Information for

Insight into the SEA amide thioester equilibrium. Application to the synthesis of thioesters at neutral pH

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1. General Methods

Reagents and solvents

N-[(dimethylamino)-1*H*-1,2,3-triazolo-[4,5-b]pyridin-1-ylmethylene]-*N*-methylmethanaminium hexafluorophosphate *N*-oxide (HATU) and *N*α-Fmoc protected amino acids were obtained from Iris Biotech GmbH. Side-chain protecting groups used for the amino acids were Fmoc-Ala-OH, Fmoc-Arg(Pbf)-OH, Fmoc-Asn(Trt)-OH, Fmoc-Asp(O*t*Bu)-OH, Fmoc-Gln(Trt)-OH, Fmoc-Glu(O*t*Bu)-OH, Fmoc-Gly-OH, Fmoc-His(Trt)-OH, Fmoc-Ile-OH, Fmoc-Leu-OH, Fmoc-Lys(Boc)-OH, Fmoc-Met-OH, Fmoc-Phe-OH, Fmoc-Pro-OH, Fmoc-Ser(*t*Bu)-OH, Fmoc-Thr(*t*Bu)-OH, Fmoc-Tyr(*t*Bu)-OH, Fmoc-Val-OH, Fmoc-Cys(S*t*Bu)-OH or Fmoc-Cys(Trt)-OH. Synthesis of *bis*(2-sulfanylethyl)aminotrityl polystyrene (SEA PS) resin was carried out as described elsewhere.[1] 4-mercaptophenylacetic acid (MPAA), 3-mercaptopropionic acid (MPA), *tris*(2-carboxyethyl)phosphine hydrochloride (TCEP) were purchased from Sigma-Aldrich. All other reagents were purchased from Acros Organics or Merck and were of the purest grade available.

Peptide synthesis grade *N,N*-dimethylformamide (DMF), dichloromethane (CH_2Cl_2), diethylether (Et_2O), acetonitrile (CH_3CN), heptane, LC–MS-grade acetonitrile (CH_3CN, 0.1% TFA and CH_3CN, 0.1% formic acid), LC–MS-grade water (H_2O, 0.1% TFA and H_2O, 0.1% formic acid), *N,N*-diisopropylethylamine (DIEA), acetic anhydride (Ac_2O) were purchased from Biosolve and Fisher-Chemical. Trifluoroacetic acid (TFA) was obtained from Biosolve. Water was purified with a Milli-Q Ultra Pure Water Purification System.

Analyses

The reactions were monitored by analytical LC–MS (Waters 2695 LC/ZQ 2000 quadripole) on an reverse phase column. The column, eluent system and gradient used are indicated in the figure legends. The column eluate was monitored by UV at 215 nm and by evaporative light scattering (ELS, Waters 2424). The peptide masses were measured by on-line LC–MS: Ionization mode: ES+, m/z range 350–2040, capillary voltage 3 kV, cone voltage 30 V, extractor voltage 3 V, RF lens 0.2 V, source temperature 120 °C, dessolvation temperature 350 °C. Samples were prepared using 10 µL aliquots of the reaction mixtures. The aliquots were diluted with water (90 µL), acidified with acetic acid (5 drops) and extracted four times with Et_2O to remove MPAA or MPA before analysis.

Column: Waters XBridgeTM BEH300 C18 3.5 µm, 4.6 x 150 mm; eluent A: TFA 0.05% by vol. in water; eluent B: TFA 0.05% by vol. in CH_3CN/water 4:1 by vol.; gradient: 0-100% B in 30 min; flow rate 1 mL/min; detection at 215 nm

MALDI-TOF mass spectra were recorded with a BrukerAutoflex Speed mass spectrometer. The matrix used for the analysis is indicated in the figure legends.

Peptide synthesis

Peptides **5a-f** and **16** were synthesized as described previously using SEA PS resin.[1,2]

Synthesis of Gly-MPA **14** was described previously.[3]

2. Kinetic studies

2.1 Effect of the pH on the SEA amide/thioester equilibrium (Fig. 1)

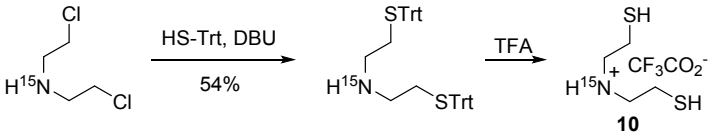
SEA peptides **5a-d** were dissolved at 2 mM in the presence of 100 mM TCEP. The pH was adjusted to 6.5 with 6 N NaOH. After 30 min at room temperature, the reduced peptides were diluted to 1 mM with the appropriate buffer (see below) and equilibrated at 37 °C under nitrogen atmosphere. The reactions were monitored by RP-HPLC (eluent A: TFA 0.1% by vol. in water; eluent B: TFA 0.1% by vol. in CH_3CN/water 4:1 by vol., gradient 0 to 100% B in 30 min., flow rate 0.3 ml/min, temperature 30 °C, Nucleosil C18 column).

Buffers :

0<pH<2	0.1 M HCl
2<pH<4	0.1 M sodium phosphate/sodium citrate buffer
4<pH<6	0.1 M sodium citrate buffer
6<pH<7	0.1 M sodium phosphate buffer

2.2 Exchange of the SEA group at neutral pH by 2,2'-(azanediyl-15N)*bis*(ethane-1-thiol) 10 (Fig. 2).

*Synthesis of compound **10***

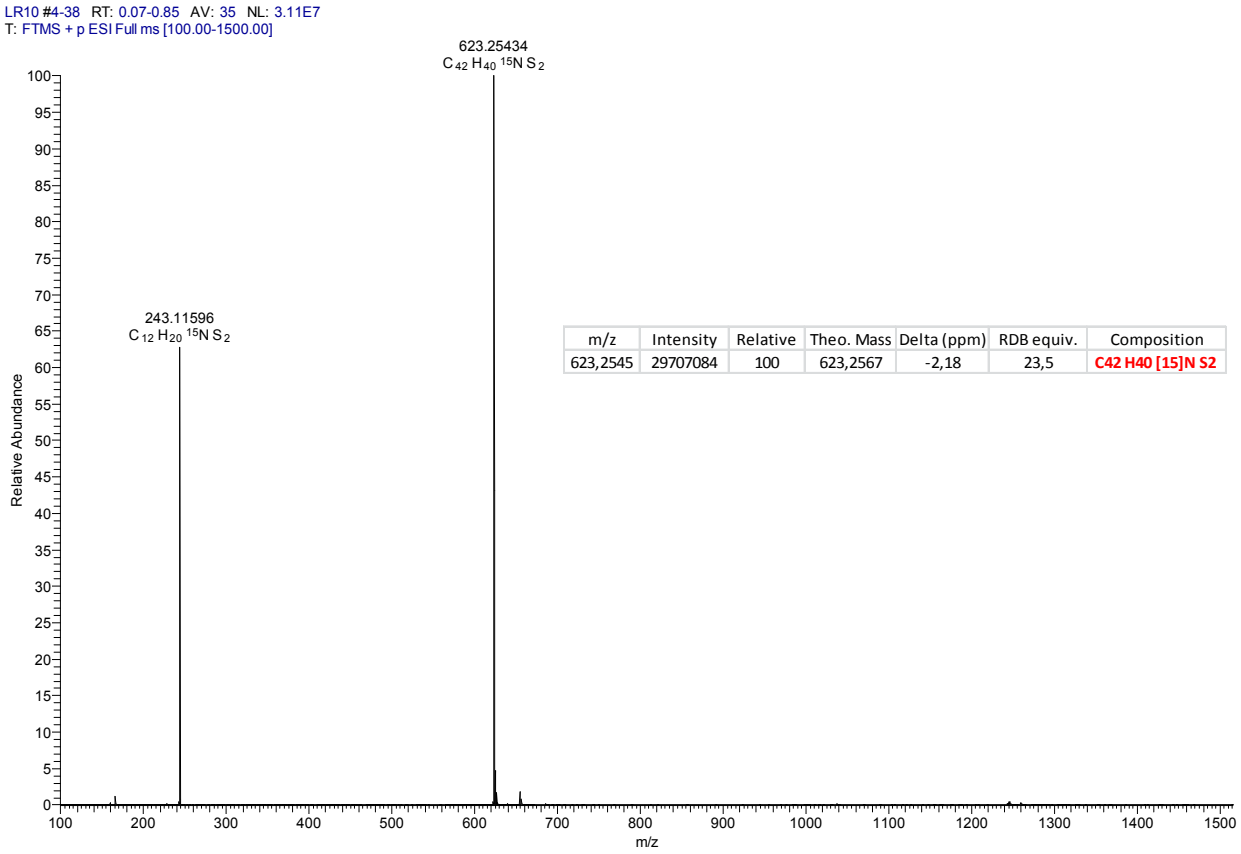


Scheme S1. Synthesis of compound **10**.

N,N-bis(2-chloroethyl)amine^{15}N hydrochloride was synthesized from benzylamine ^{15}N according to literature procedures (dialkylation with 2-bromoethanol, hydrogenolysis with Pd/C and chlorination with thionylchloride[4]).

Triphenylmethylmercaptan (1.7 g, 6.18 mmol, 2 equiv) was added to a stirred solution of *N,N-bis*(2-chloroethyl)amine^{15}N hydrochloride (550 mg, 3.00 mmol) dissolved in DMF (9 mL). Then, 1,8-diazabicyclo[5.4.0]undec-7-ene (1.85 mL, 12.3 mmol, 4 equiv) was added dropwise to the above ice-cooled mixture which was further stirred overnight at room temperature. After completion of the reaction, the solvent was evaporated and the residue was dissolved in CH_2Cl_2, washed with brine and dried over anhydrous sodium sulfate. The residue was purified by silica-gel column chromatography (cyclohexane / AcOEt / triethylamine : 8 / 2 / 0.1 by vol) to yield the *bis*(2-tritylsulfanylethyl)amine ^{15}N as a white powder (1.23 g, 64%).

Characterization of the bis(2-tritylsulfanylethyl)amine ^{15}N



m/z	Intensity	Relative	Theo. Mass	Delta (ppm)	RDB equiv.	Composition
623,2545	29707084	100	623,2567	-2,18	23,5	C42 H40 [15]N S2

Fig. S1. HR-MS analysis of the *bis*(2-tritylsulfanylethyl)amine ^{15}N.

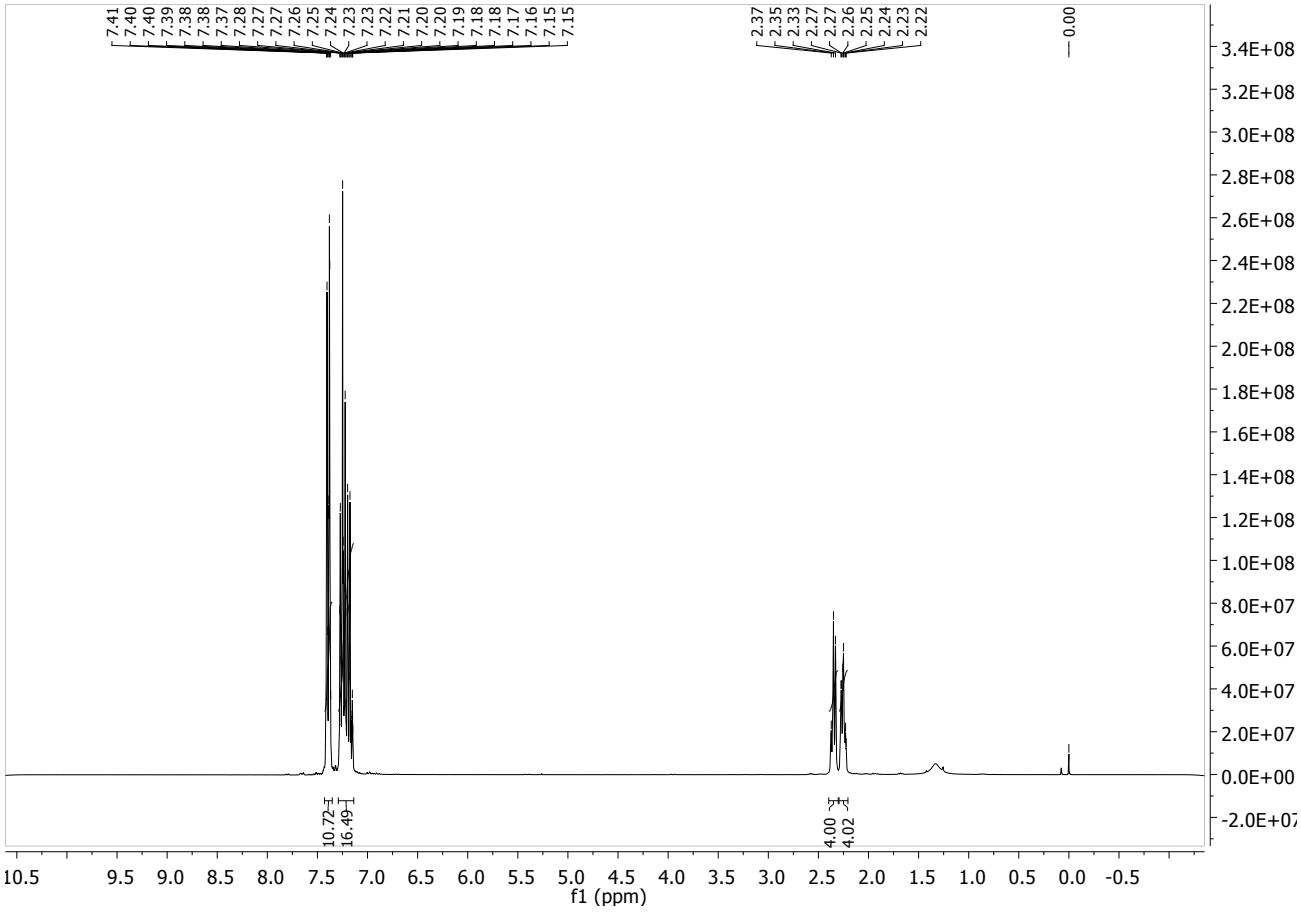


Fig. S2. ^{1}H NMR spectrum (300 MHz, $CDCl_3$) of the *bis*(2-tritylsulfanylethyl)amine ^{15}N. δ 7.43 – 7.35 (m, 11H), 7.29 – 7.14 (m, 16H), 2.40 – 2.30 (m, 4H), 2.29 – 2.21 (m, 4H).

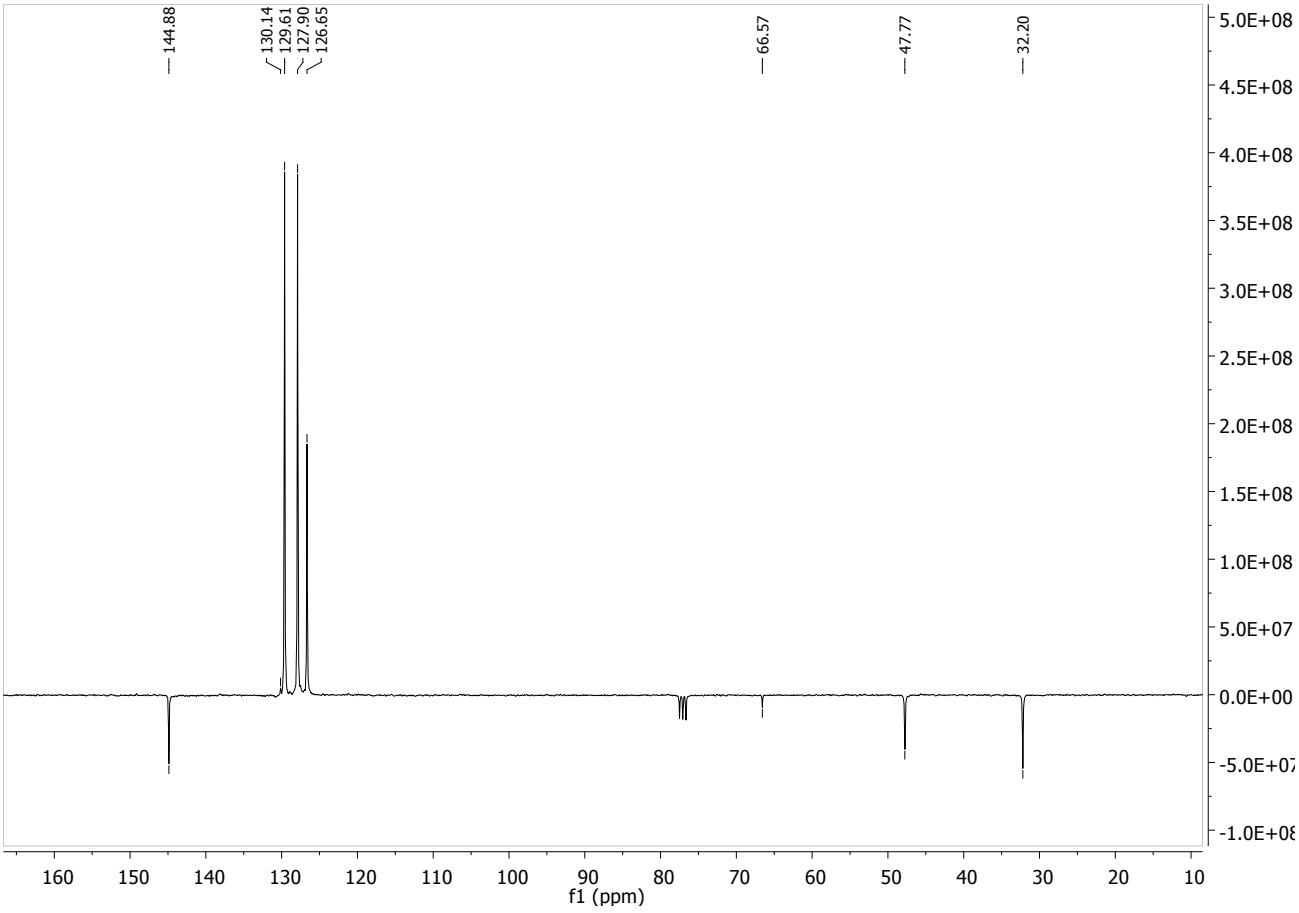


Fig. S3. ^{13}C NMR spectrum (75 MHz, $CDCl_3$) of the *bis*(2-tritylsulfanylethyl)amine ^{15}N. δ 144.88 (s), 130.18 – 129.04 (m), 127.90 (s), 126.65 (s), 66.57 (s), 47.77 (s), 32.20 (s).

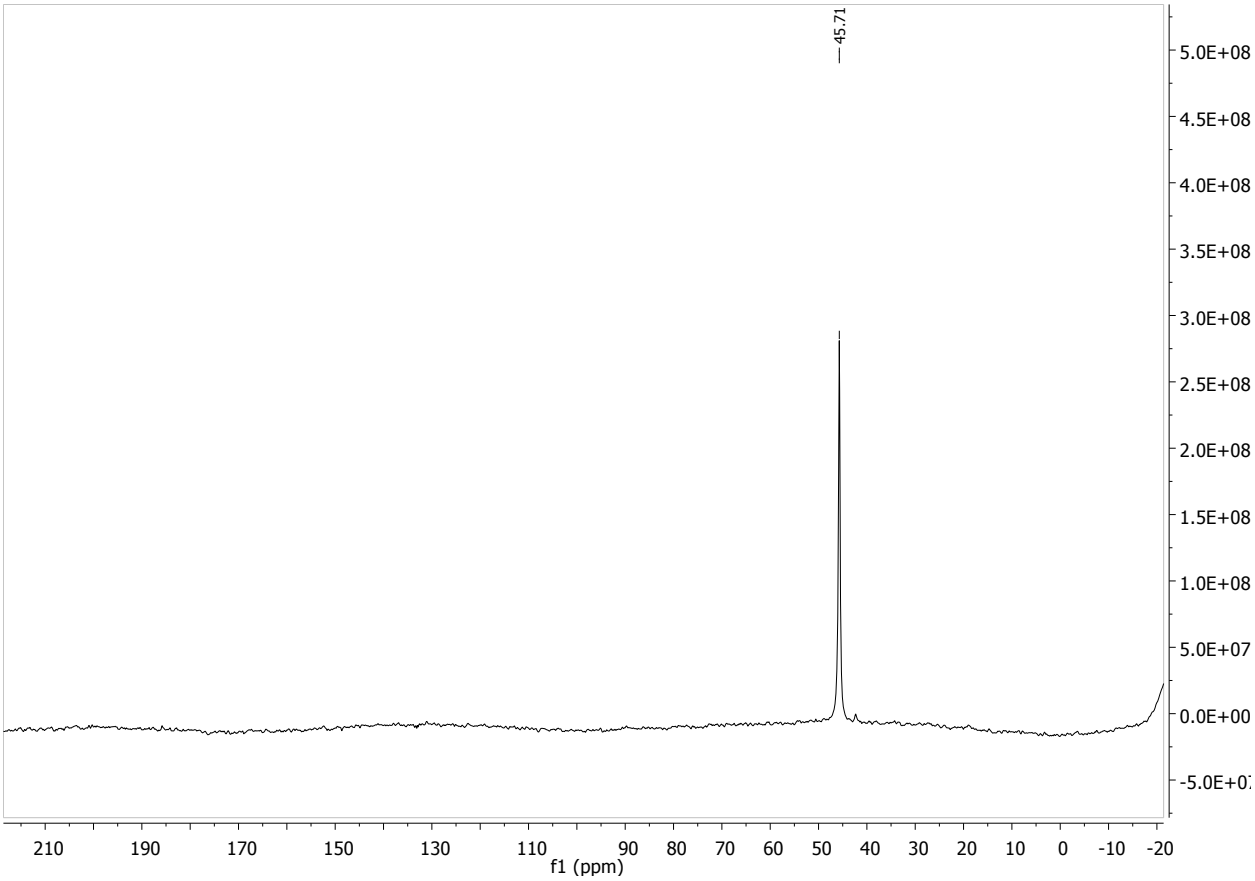


Fig. S4. ^{15}N NMR spectrum (30.4 MHz, $CDCl_3$) of the *bis*(2-tritylsulfanylethyl)amine ^{15}N.

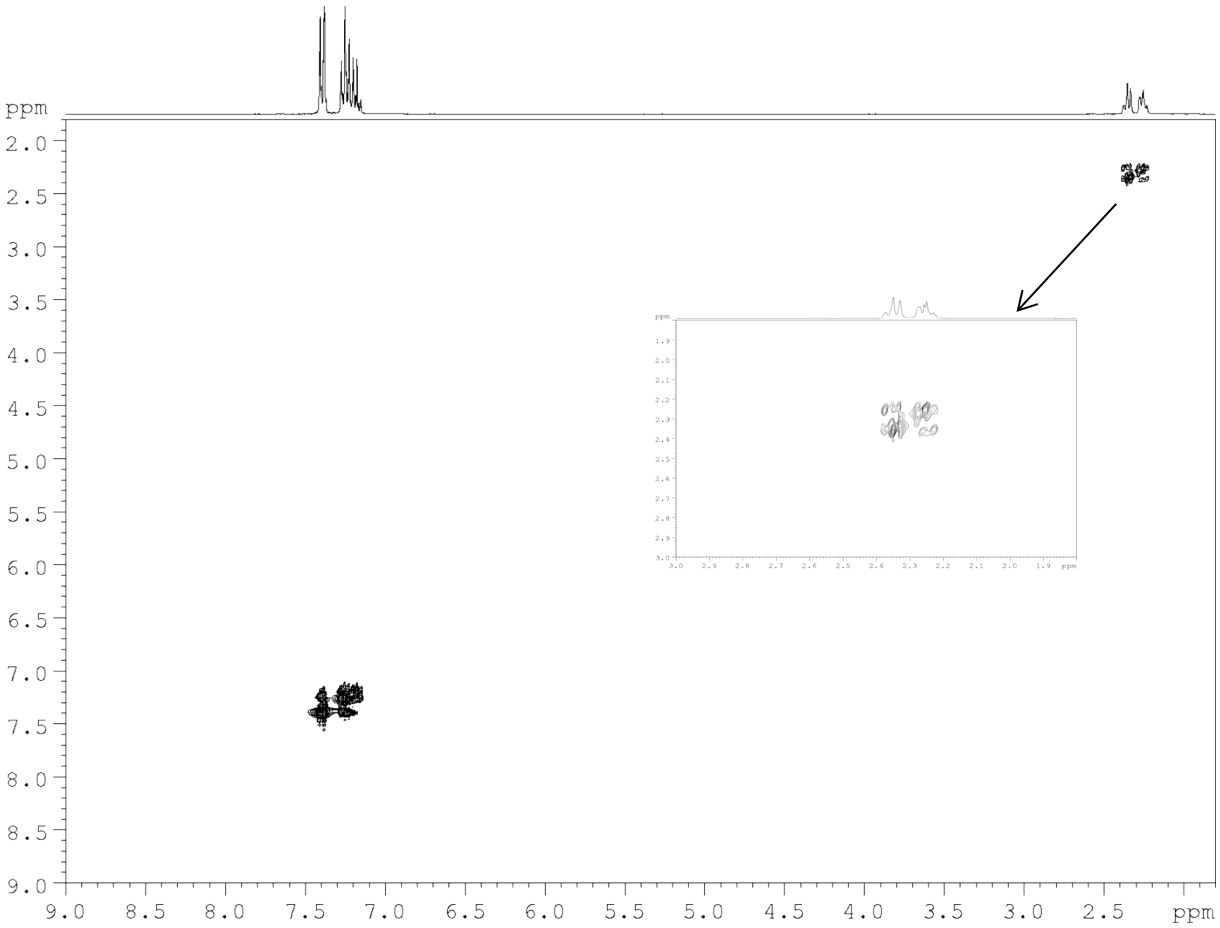


Fig. S5. ^{1}H-^{1}H COSY spectrum of the *bis*(2-tritylsulfanylethyl)amine ^{15}N.

Exchange procedure

Compound **10** was obtained by treating the *bis*(2-tritylsulfanylethyl)amine ^{15}N (7.03 mg, 11.3 µmol) with TFA/TIS: 97.5/2.5 by vol (700 µL) for 30 min. The solvent was evaporated in vacuo. The residue was triturated with cyclohexane (500 µL) and the solvent was evaporated in vacuo (three times). Due to the high sensitivity of compound **10** to oxidation by molecular oxygen, the crude was used for the exchange reaction without further purification.

The exchange was carried out under nitrogen atmosphere in 6 M Gdn.HCl pH 7 0.1 M sodium phosphate buffer (555 µL) containing 200 mM TCEP, 30 mM of compound **10** (7.03 mg, 11.3 µmol) and 3 mM of peptide **8** (1.00 mg, 1.13 µmol).

2.3 Influence of the pH on the SEA/thiol exchange (Fig. 3)

In a glove box under nitrogen atmosphere, TCEP.HCl (8.7 mg), MPAA (97% purity; 11.4 mg), MPA (10 µL) and Gdn.HCl (574.6 mg) were dissolved in sodium phosphate buffer (0.1 M; 590 µL; pH 6.8) and the pH was adjusted to either 4.1, 5.0, 6.1 or 7.1 by adding a 6 M aqueous NaOH solution. The peptide H-ILKEPVHGA-SEA^{off} **5b** (1.0 mg, 0.70 µmol, 1 eq, 4.7 mM final concentration) was dissolved in the above solution (150 µL which leads to a final concentration of 29.9 mM eq for TCEP; 63 mM for MPAA; and 1% by vol for MPA). The reaction was stirred at 37 °C. The reaction was monitored by RP-HPLC as indicated in the General Methods.

2.4 SEA/thiol exchange with added thioester 14 (Fig. 4 & 5)

In a glove box under nitrogen atmosphere, TCEP.HCl (8.9 mg), MPAA (97% purity; 11.7 mg), MPA (10 µL) and Gdn.HCl (573.2 mg) were dissolved in sodium phosphate buffer (0.1 M; 590 µL; pH 6.8) and the pH was adjusted to 7.1 by adding a 6 M aqueous solution of NaOH (56 µL). Gly-MPA thioester **14** (2.08 mg, 7.50 µmol, 10.2 eq, final concentration 46.9 mM) was dissolved in the above solution (160 µL which leads to a final concentration of 29.6 mM for TCEP, 63 mM for MPAA and 1% by vol for MPA) and the resulting solution was added to the peptide 1 (1.05 mg, 0.74 µmol, 1 equiv, 4.6 mM final concentration). The reaction mixture was stirred at 37 °C and monitored by RP-HPLC.

General procedure for the synthesis of peptide thioesters 12

On approximately 10 µmol of peptide **5**:

In a glove box, TCEP.HCl (18.6 mg, 65 µmol, 6.5 equiv), MPAA (97% purity; 23.4 mg, 135 µmol, 13.5 equiv), MPA (22.5 µL, 1% by vol.) and Gdn.HCl (1.29 g) were dissolved in sodium phosphate buffer (0.1 M; 1.32 mL; pH 6.8) and the pH was adjusted to 7.0 by adding a 6 M aqueous NaOH solution (100-120 µL). The thioester **10** (27.7 mg, 100 µmol, 10 eq) was dissolved in the above solution and the resulting solution was added to the peptide 5 (10 µmol, 1 equiv). The reaction mixture was then stirred at 37 °C for 30-48 h under nitrogen atmosphere.

Work-up: The reaction mixture was diluted to a volume of 5 mL with desionized water and AcOH (0.75 mL) was added. The aqueous layer was extracted with Et_2O (4 × 5 mL) and lyophilised. The white solid obtained was purified by RP-HPLC.

Synthesis of peptide 12b

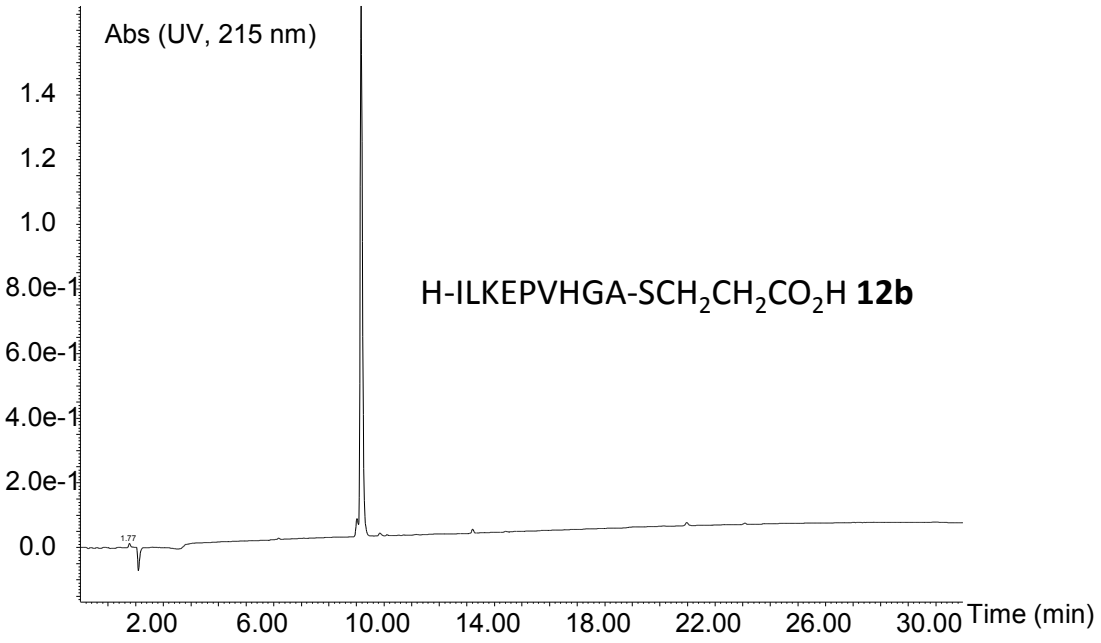
Scale: 10.1 mg (7.1 µmol) peptide **5b**.

Reaction time: 30 h.

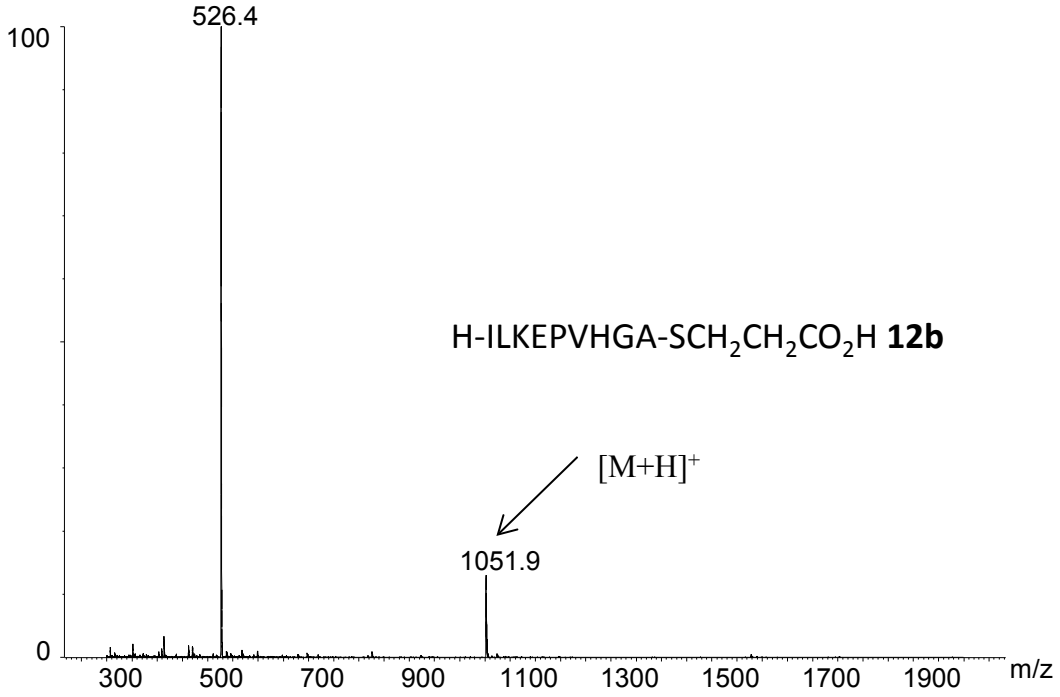
The crude product was purified by RP-HPLC (column: Waters XBridgeTM Prep C18 5 µm, 19 × 100 mm; eluent A: TFA 0.1% by vol. in water; eluent B: TFA 0.1% by vol. in CH3CN/water 4:1 by vol.; gradient: 0-30% B in 25 min; flow rate 25 mL/min; detection at 215 nm) to give the desired thioester (5.4 mg; 54%; 0.96% D-Ala by chiral GC-MS) as a white solid.

Characterization of peptide 12b

A)


Abs (UV, 215 nm)
1.4
1.2
1.0
8.0e-1
6.0e-1
4.0e-1
2.0e-1
0.0
1.77
H-ILKEPVHGA-SCH2CH2CO2H 12b
2.00
6.00
10.00
14.00
18.00
22.00
26.00
30.00
Time (min)

B)


526.4
100
H-ILKEPVHGA-SCH2CH2CO2H 12b
[M+H]+
1051.9
0
300
500
700
900
1100
1300
1500
1700
1900
m/z

C)

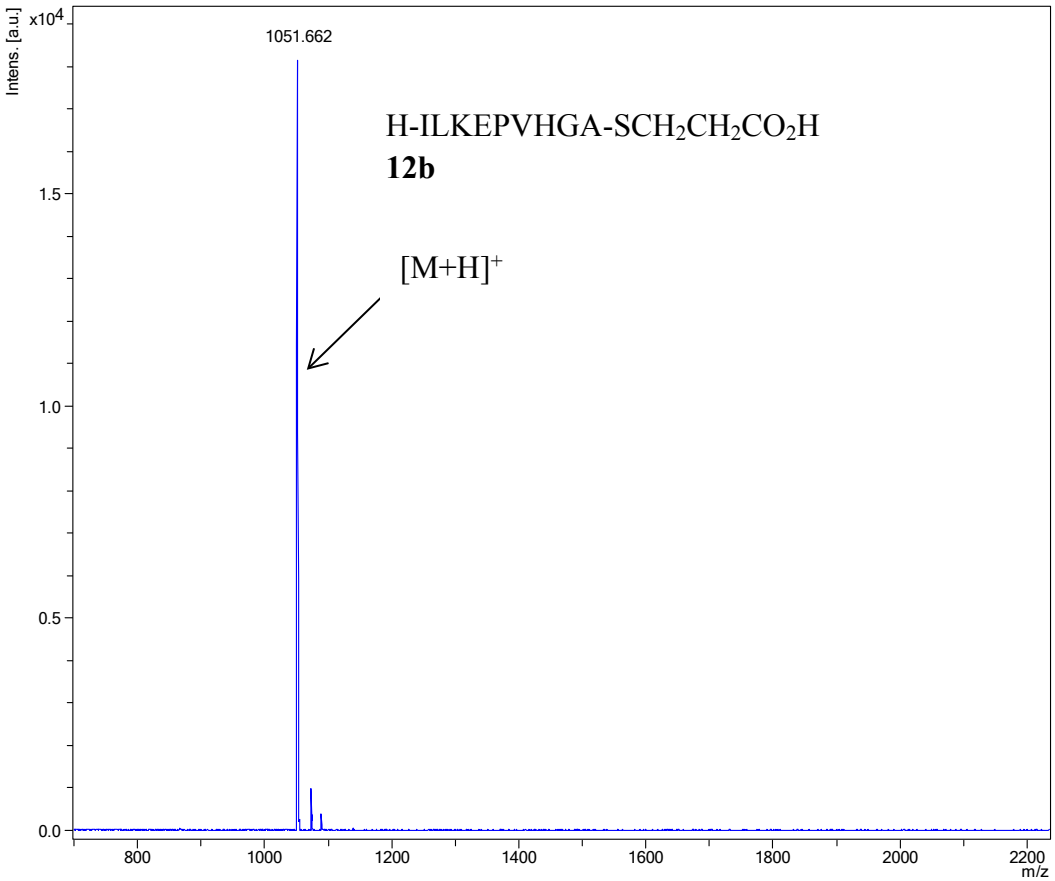
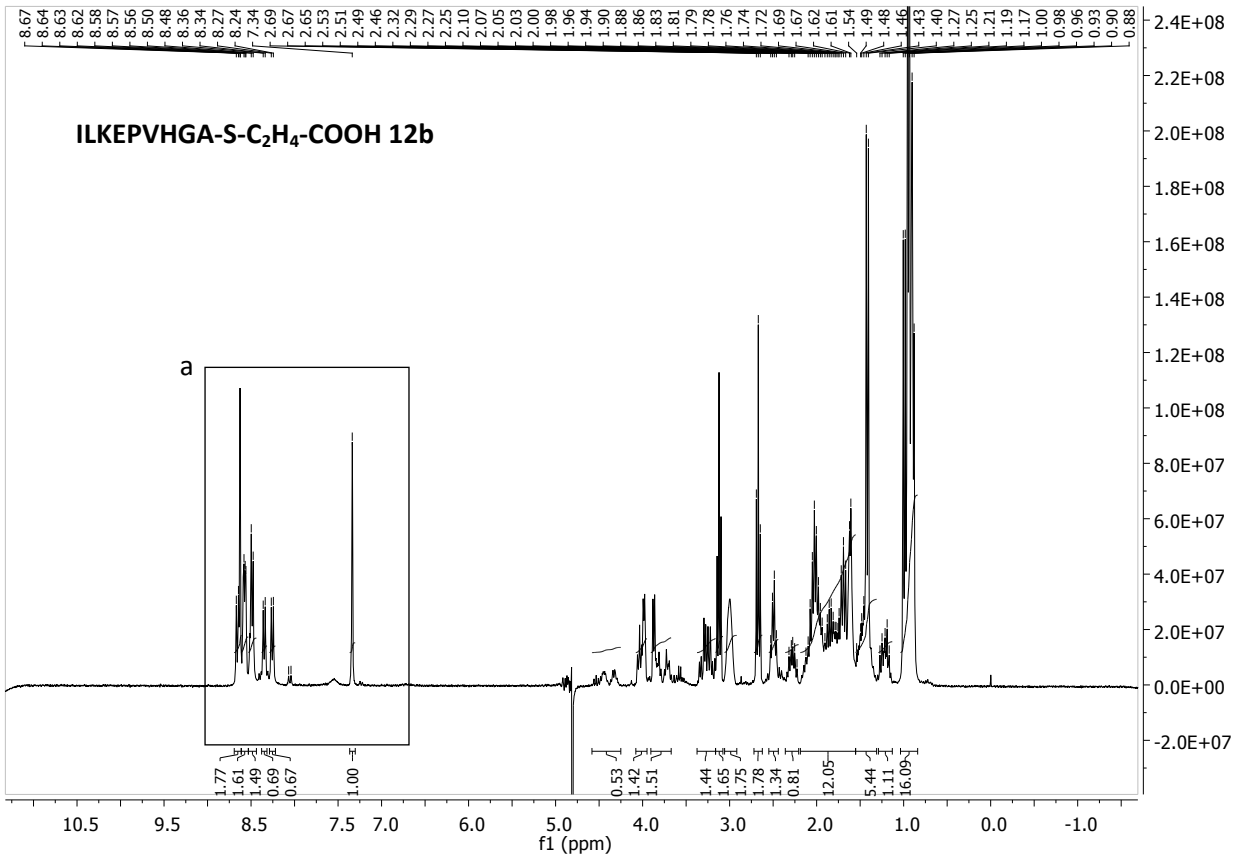
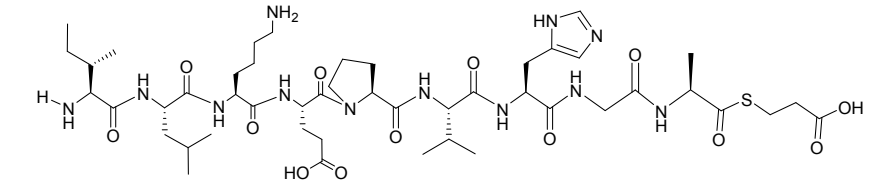


Fig. S6. Characterization of peptide **12b**. A) LC-MS, LC trace, column: Waters XBridgeTM BEH300 C18 3.5 µm (4.6 × 150 mm). Eluent A: TFA 0.1% by vol. in water; eluent B: TFA 0.1% by vol. in CH_3CN/water 4:1 by vol. Gradient: 0-100% B in 30 min; flow rate 1 mL/min; detection at 215 nm; B) LC-MS, MS trace, $[M+H]^+$ m/z calcd (monoisotopic) 1051.6 ; found 1051.9; C) MALDI-TOF analysis, matrix: α-cyano-4-hydroxycinnamic acid, $[M+H]^+$ m/z calcd (monoisotopic) 1051.6 ; found 1051.7.

NMR analysis of peptide 12b

^{1}H NMR (300 MHz, H_2O+D_2O : 9/1 (v/v)) δ 8.69 – 8.61 (m, 2H), 8.61 – 8.53 (m, 2H), 8.53 – 8.44 (m, 2H), 8.38 – 8.32 (m, 1H), 8.26 (d, *J* = 7.2 Hz, 1H), 7.34 (s, 1H), 4.58 – 4.25 (m, 4H), 4.08 – 3.95 (m, 3H), 3.91 – 3.67 (m, 3H), 3.37 – 3.16 (m, 2H), 3.12 (t, *J* = 6.8 Hz, 2H), 3.06 – 2.92 (m, 2H), 2.67 (t, *J* = 6.8 Hz, 2H), 2.50 (dd, *J* = 13.0, 6.6 Hz, 2H), 2.36 – 2.21 (m, 1H), 2.19 – 1.55 (m, 14H), 1.55 – 1.31 (m, 6H), 1.29 – 1.13 (m, 1H), 1.04 – 0.84 (m, 18H).
^{13}C NMR (75 MHz, H_2O+D_2O : 9/1 (v/v)) δ 205.45 ,180.22 , 179.74 – 179.50 , 176.77 , 176.60 , 176.30 , 176.04 , 174.77 , 174.11 , 173.87 , 171.99 , 131.18 , 120.21 , 62.98 , 62.55 , 60.45 , 58.61 , 56.16 , 55.28 , 53.62 , 50.77 , 45.17 , 42.50 , 42.18 , 39.24 , 36.80 , 33.11 , 32.85 , 32.57 , 32.09 , 29.16 , 28.60 , 27.38 , 27.05 , 26.75 , 26.54 , 24.77 , 24.67 , 23.96 , 21.11 , 20.68 , 19.58 , 16.83 , 13.27.


ILKEPVHGA-S-C2H4-COOH 12b
a
f1 (ppm)



a) I_1 L_2 K_3 E_4 P_5 V_6 H_7 G_8 A_9 10

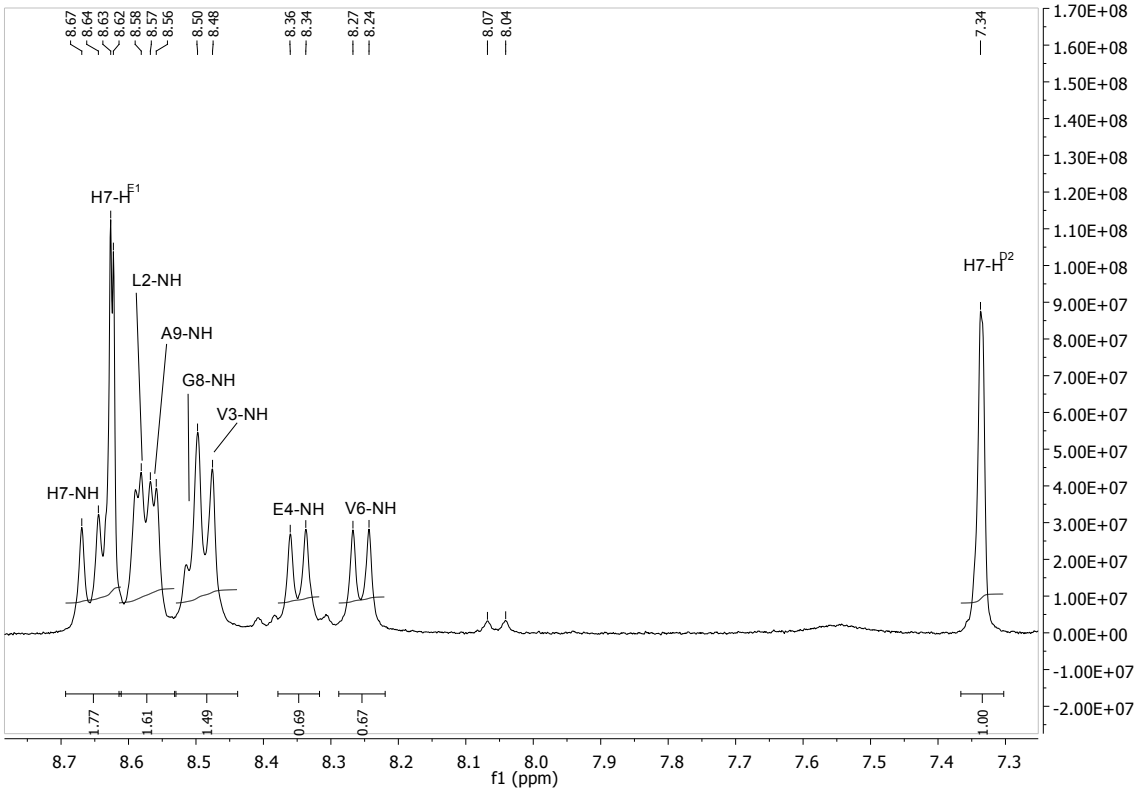


Fig. S7. ^{1}H NMR (300 MHz, H_2O+D_2O : 9/1 (v/v)) of peptide **12b**.

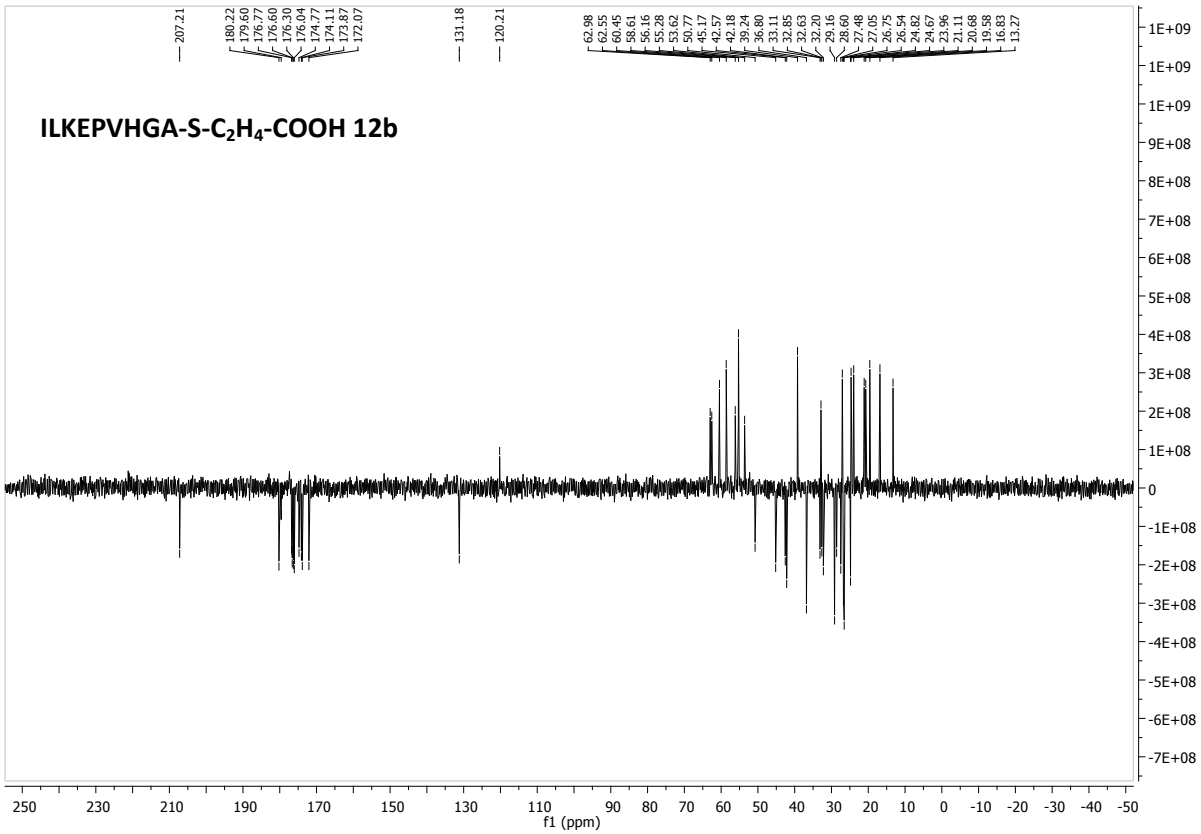


Fig. S8. ^{13}C NMR (75 MHz, H_2O+D_2O : 9/1 (v/v)) spectrum of peptide **12b**.

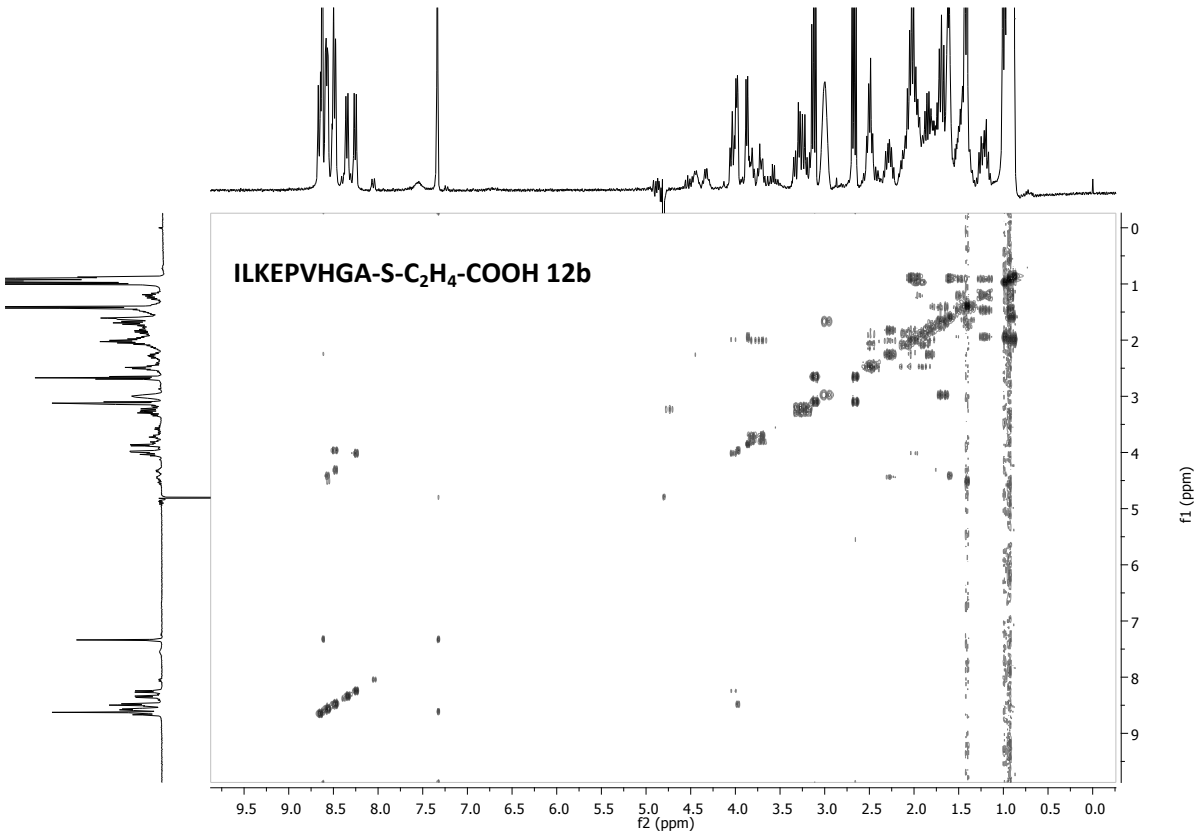


Fig. S9. ^{1}H-^{1}H COSY (H_2O+D_2O : 9/1 (v/v)) spectrum of peptide **12b**.

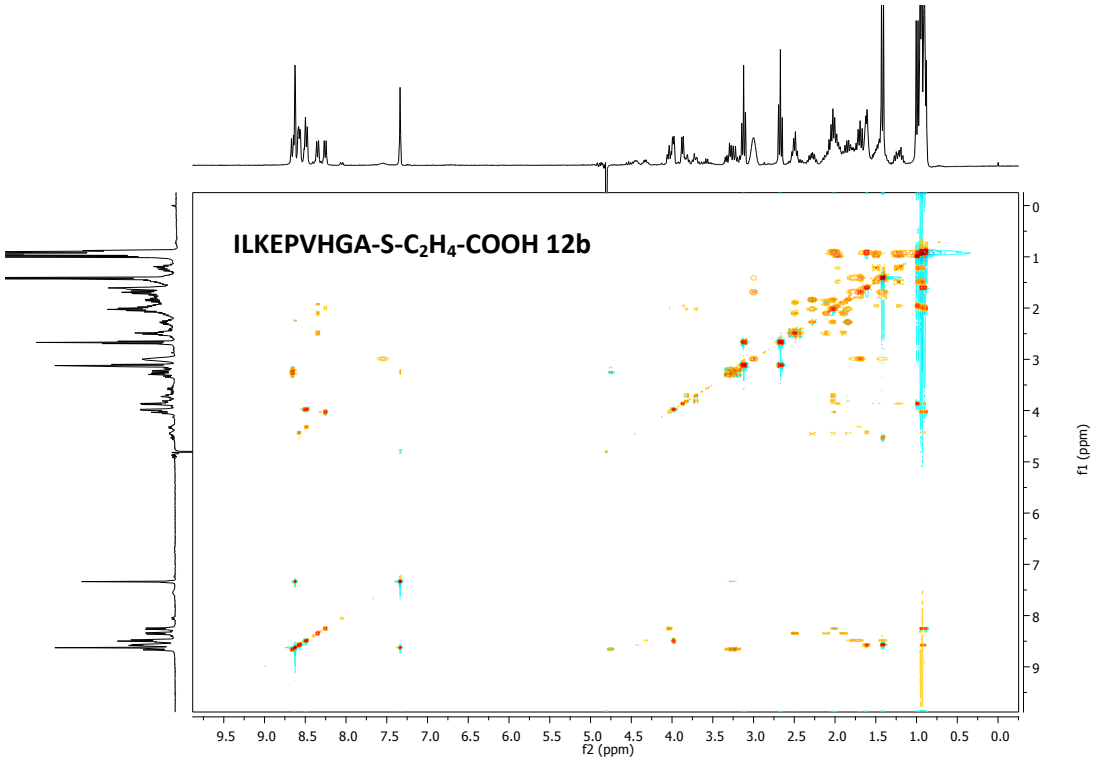


Fig. S10. ^{1}H-^{1}H DIPSI (H_2O+D_2O : 9/1 (v/v)) spectrum of peptide **12b**.

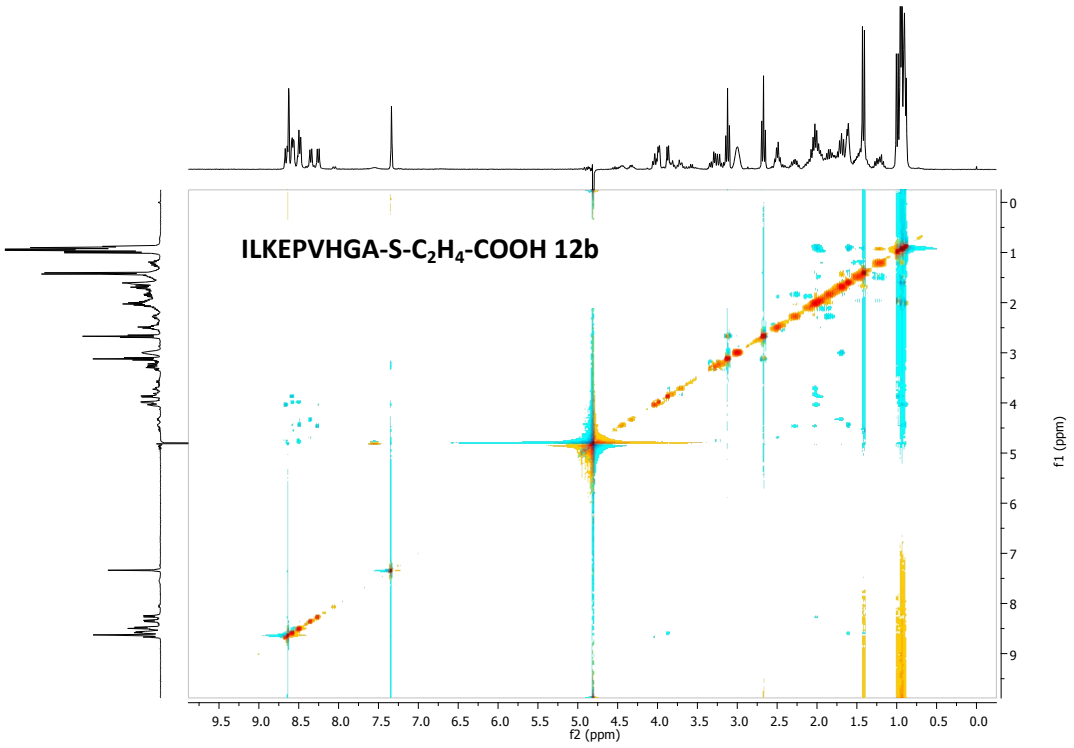
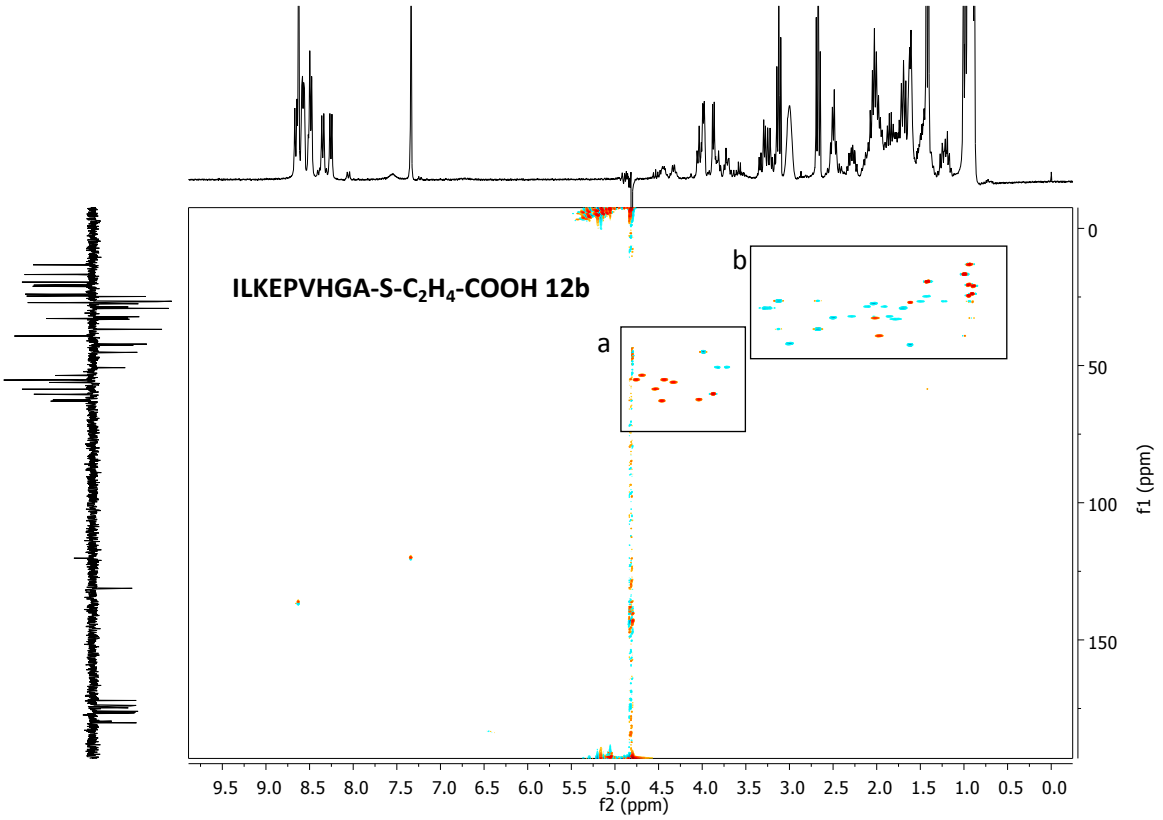
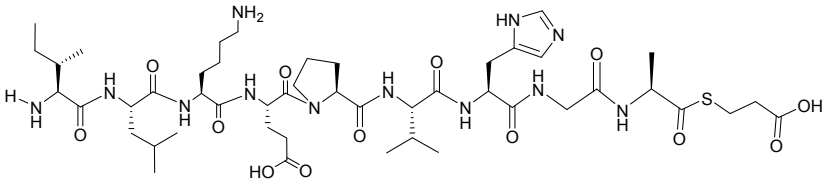
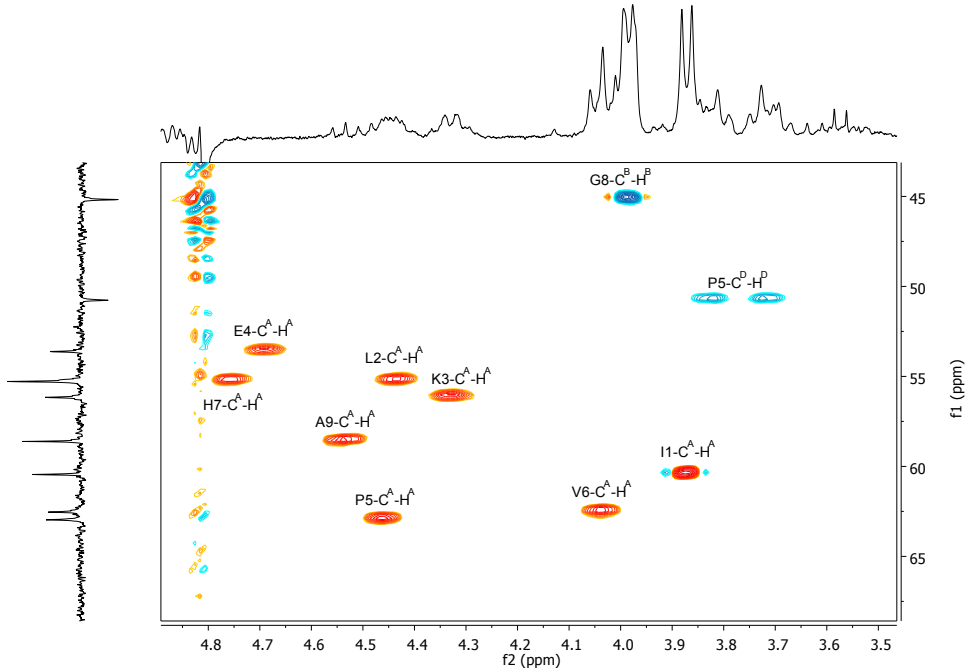


Fig. S11. ^{1}H-^{1}H ROESY (H_2O+D_2O : 9/1 (v/v)) spectrum of peptide **12b**.





a) I_1 L_2 K_3 E_4 P_5 V_6 H_7 G_8 A_9 10



b)

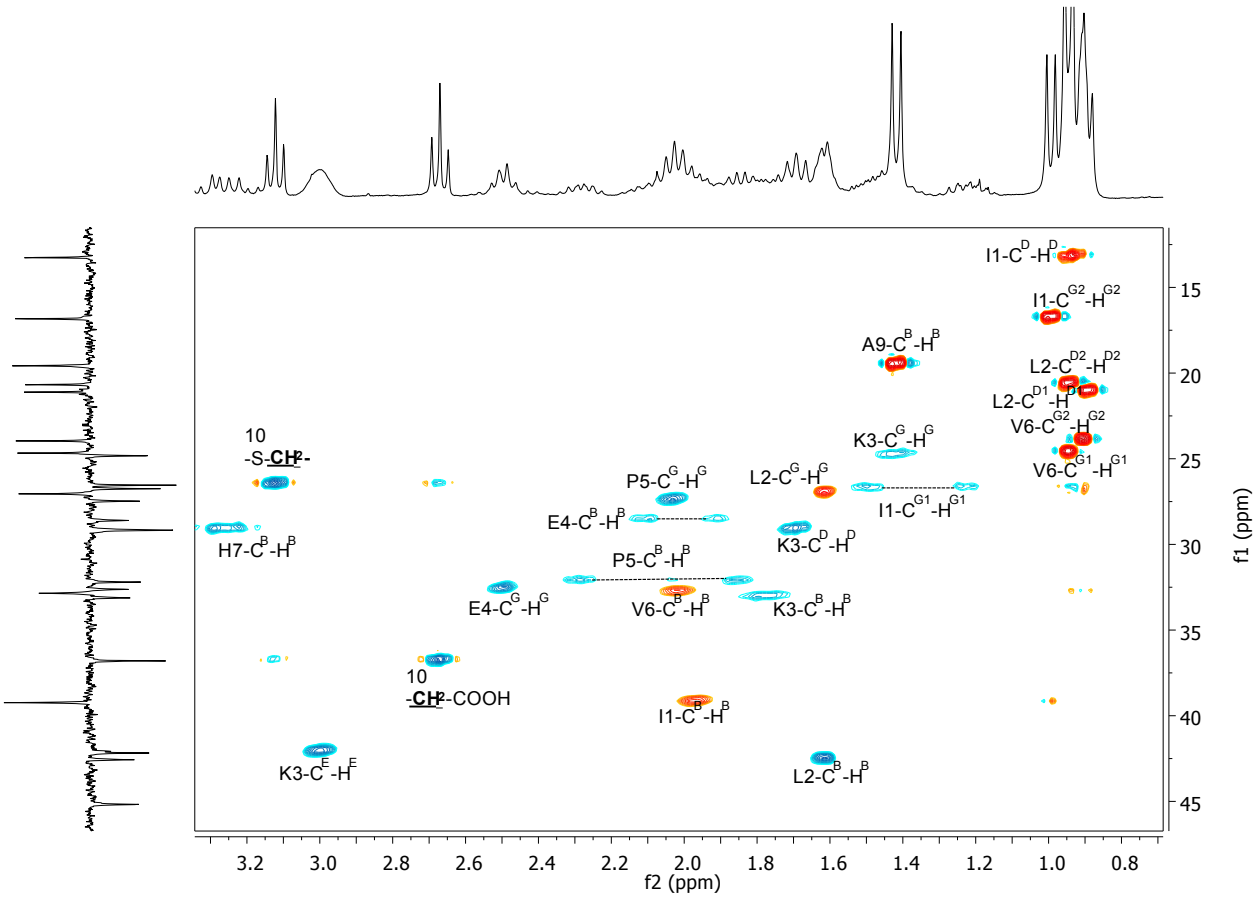


Fig. S12. ^{1}H-^{13}C HSQC (H_2O+D_2O : 9/1 (v/v)) spectrum of peptide **12b**.

Synthesis of peptide 12c

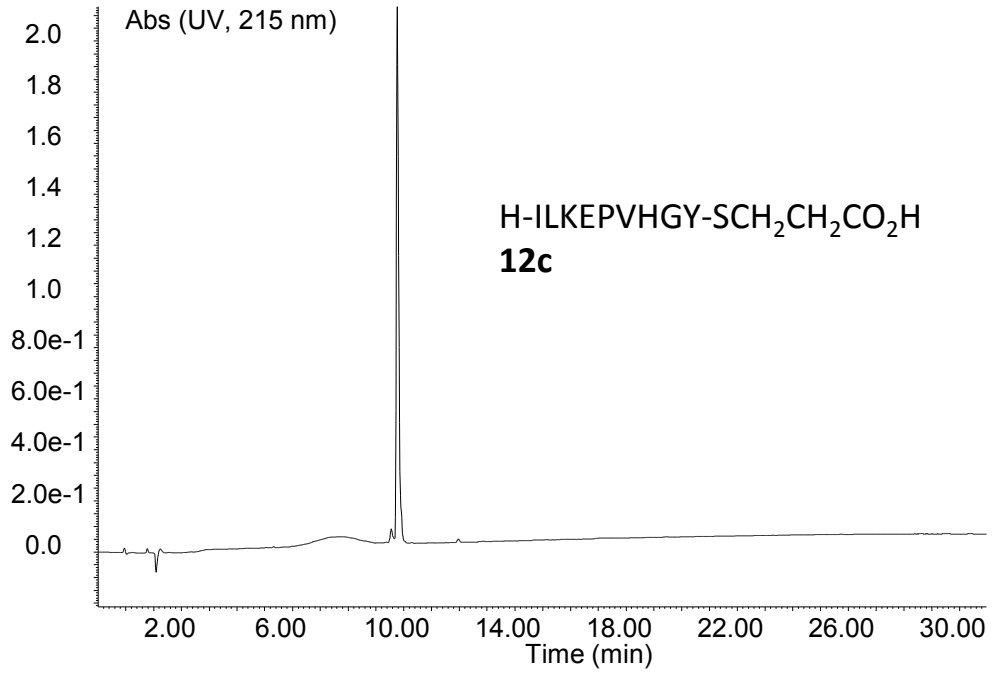
Scale: 15.5 mg (10.2 µmol).

Reaction time: 48 h.

The crude product was purified by RP-HPLC (column: Waters XBridgeTM Prep C18 5 µm, 19 × 100 mm; eluent A: TFA 0.1% by vol. in water; eluent B: TFA 0.1% by vol. in CH_3CN/water 4:1 by vol.; gradient: 10-30% B in 25 min; flow rate 25 mL/min; detection at 215 nm) to give the desired thioester (8 mg; 53%; 0.74% D-Tyr) as a white solid.

Characterization of peptide 12c

A)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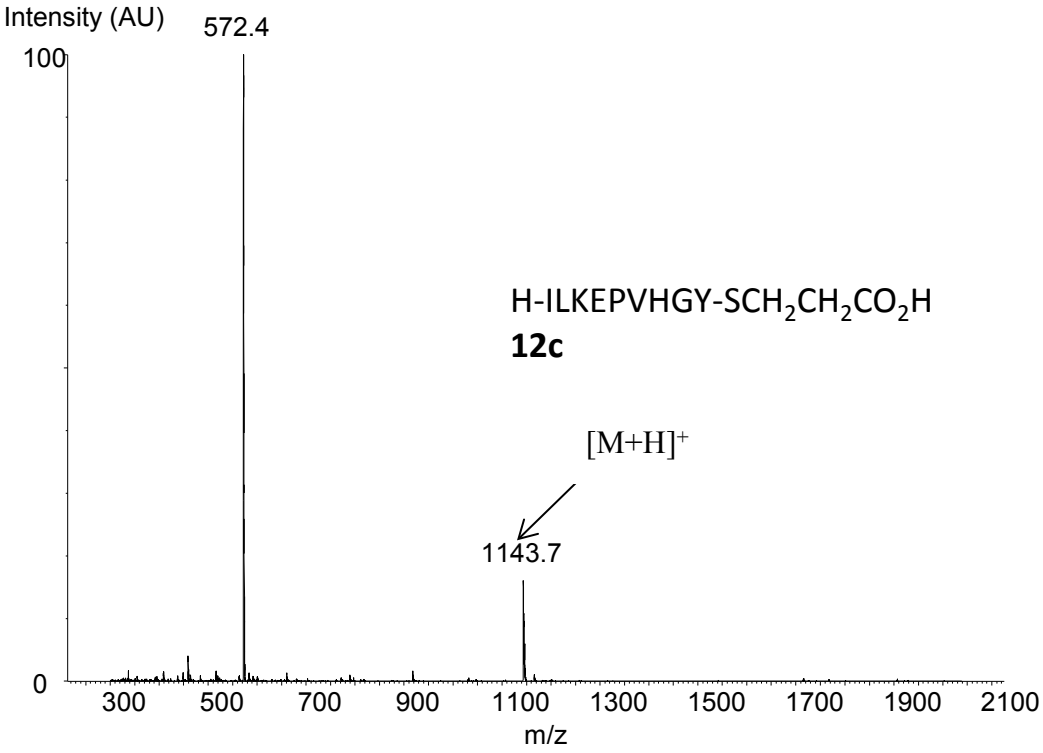
2.0
1.8
1.6
1.4
1.2
1.0
8.0e-1
6.0e-1
4.0e-1
2.0e-1
0.0
2.00
6.00
10.00
14.00
18.00
22.00
26.00
30.00
Time (min)
H-ILKEPVHGY-SCH2CH2CO2H
12c

B)


Intensity (AU)
572.4
100
0
300
500
700
900
1100
1300
1500
1700
1900
2100
m/z
H-ILKEPVHGY-SCH2CH2CO2H
12c
[M+H]+
1143.7

C)

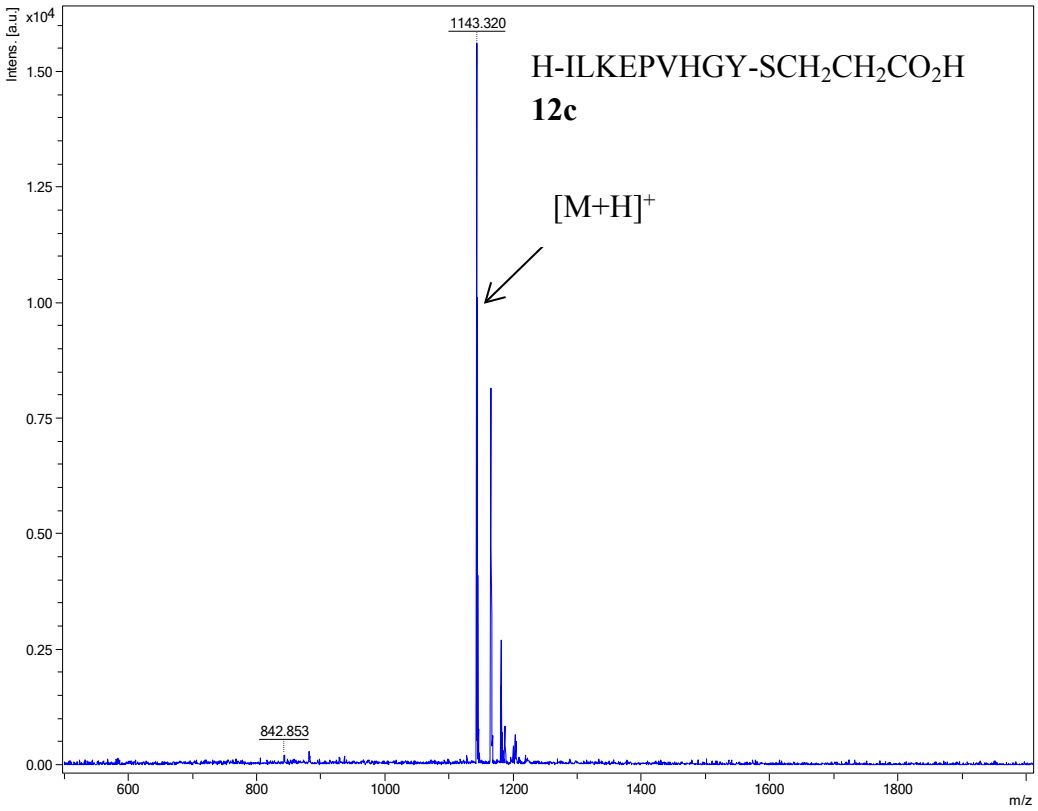
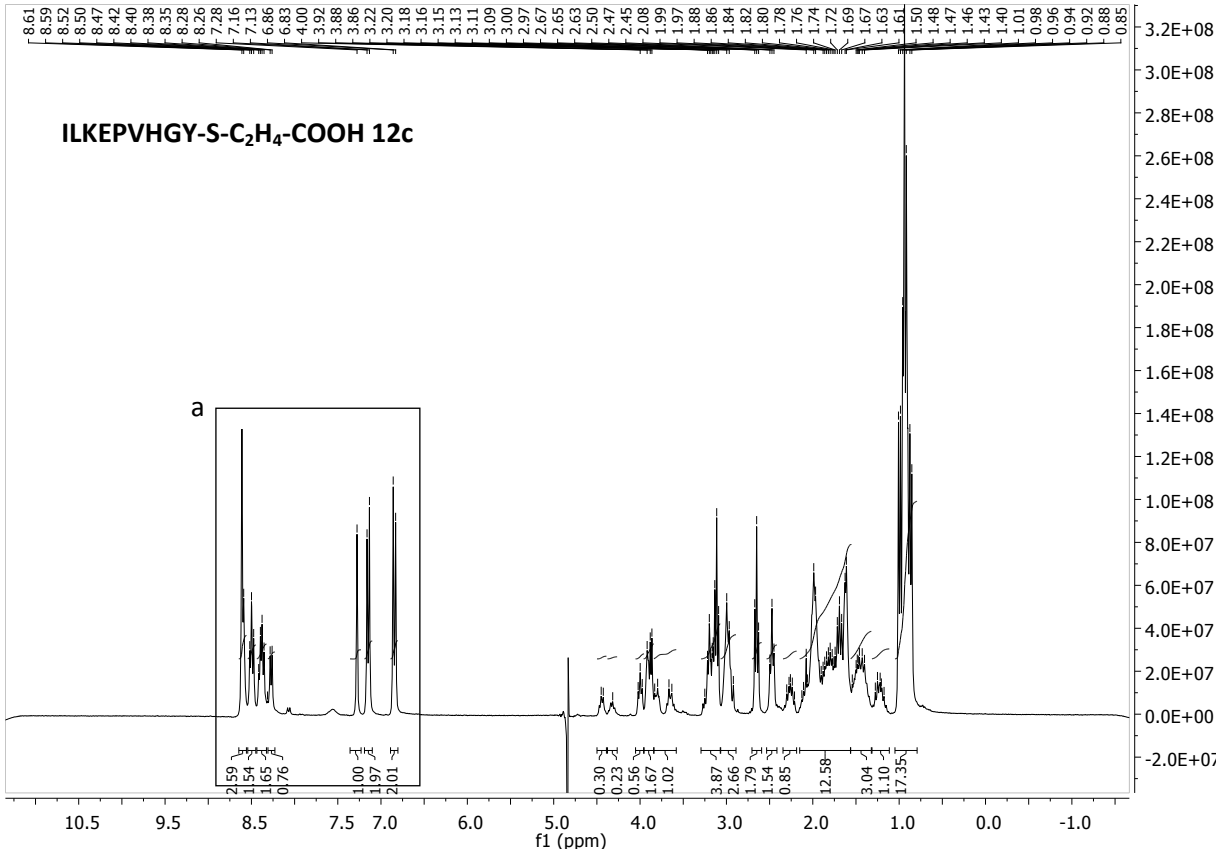


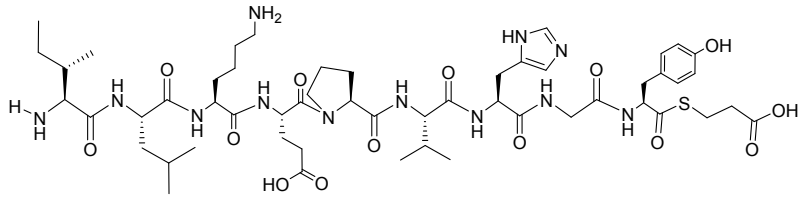
Fig. S13. Characterization of peptide **12c**. A) LC-MS, LC trace, column: Waters XBridgeTM BEH300 C18 3.5 µm (4.6 × 150 mm). Eluent A: TFA 0.1% by vol. in water; eluent B: TFA 0.1% by vol. in CH3CN/water 4:1 by vol. Gradient: 0-100% B in 30 min; flow rate 1 mL/min; detection at 215 nm; B) LC-MS, MS trace, $[M+H]^+$ m/z calcd (monoisotopic) 1143.6; found 1143.7; C) MALDI-TOF analysis, matrix: α-cyano-4-hydroxycinnamic acid, $[M+H]^+$ m/z calcd (monoisotopic) 1143.6; found 1143.32.

NMR analysis of peptide 12c

^{1}H NMR (300 MHz, H_2O+D_2O : 9/1 (v/v)) δ 8.65 – 8.56 (m, 3H), 8.55 – 8.45 (m, 2H), 8.44 – 8.33 (m, 2H), 8.27 (d, *J* = 6.7 Hz, 1H), 7.28 (s, 1H), 7.15 (d, *J* = 8.3 Hz, 2H), 6.84 (d, *J* = 8.3 Hz, 2H), 4.51 – 4.39 (m, 2H), 4.39 – 4.26 (m, 1H), 4.00 (t, *J* = 7.2 Hz, 1H), 3.96 – 3.84 (m, 3H), 3.84 – 3.58 (m, 2H), 3.29 – 3.07 (m, 5H), 3.06 – 2.89 (m, 3H), 2.65 (t, *J* = 6.7 Hz, 2H), 2.47 (t, *J* = 7.2 Hz, 2H), 2.34 – 2.19 (m, 1H), 2.16 – 1.55 (m, 14H), 1.56 – 1.32 (m, 3H), 1.31 – 1.12 (m, 1H), 1.05 – 0.78 (m, 18H).

^{13}C NMR (75 MHz, H_2O+D_2O : 9/1 (v/v)) δ 205.51, 180.24, 179.55, 176.83, 176.66 – 176.49, 176.33 – 176.20, 176.00, 174.58, 174.08, 173.85, 172.05, 157.38, 133.39, 131.20, 130.58, 118.26, 63.86, 62.90, 62.78 – 62.58, 60.41, 56.14, 55.15, 53.62, 50.73, 45.02, 42.53, 42.14, 39.20, 39.04 – 38.82, 36.73, 33.10, 32.72, 32.63, 32.14, 29.13, 28.58, 27.44, 27.01, 26.71, 24.80, 24.63, 23.92, 21.04, 20.68, 16.81, 13.23.


ILKEPVHGY-S-C2H4-COOH 12c
a
f1 (ppm)



a) I_1 L_2 K_3 E_4 P_5 V_6 H_7 G_8 Y_9 10

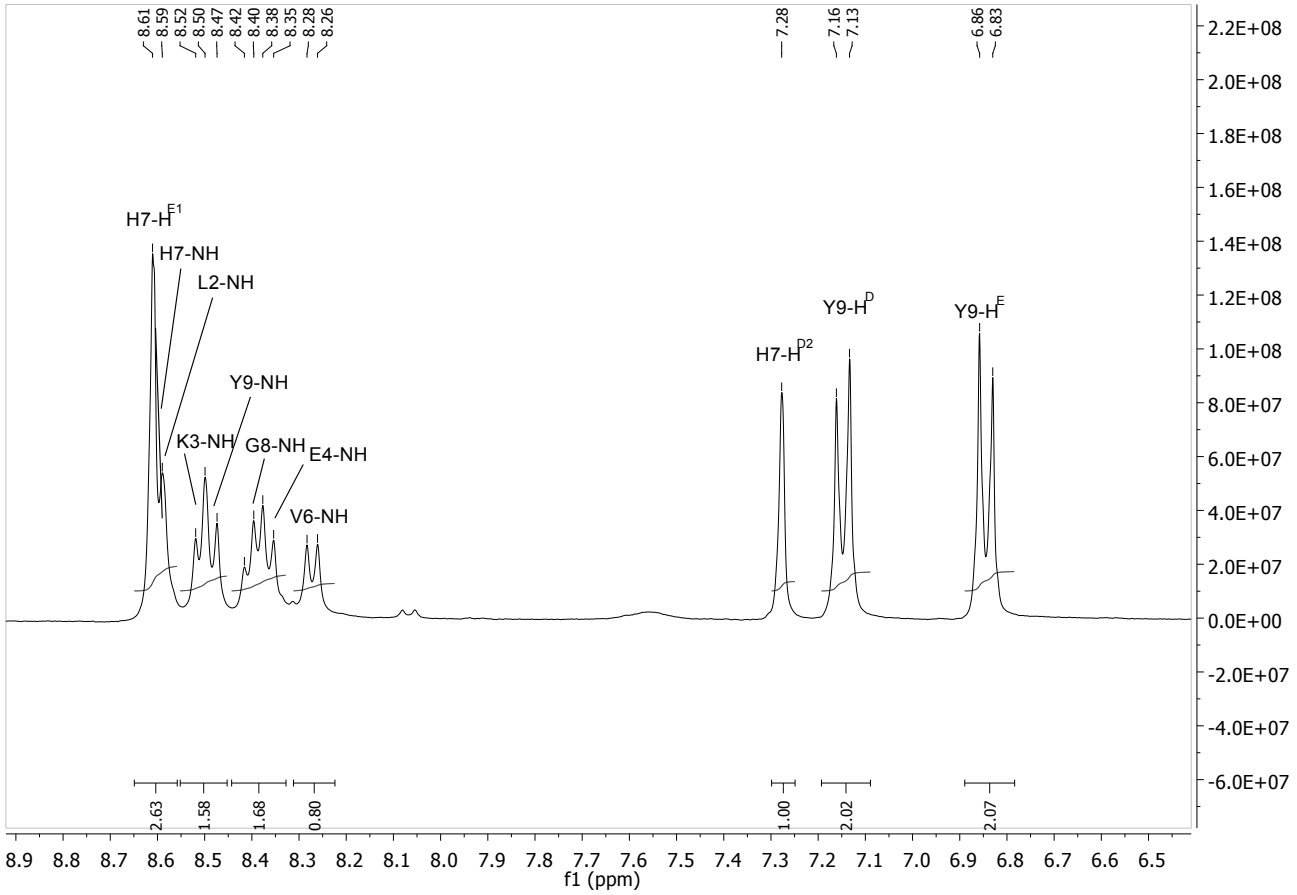


Fig. S14. ^{1}H NMR (H_2O+D_2O : 9/1 (v/v)) spectrum of peptide **12c**.

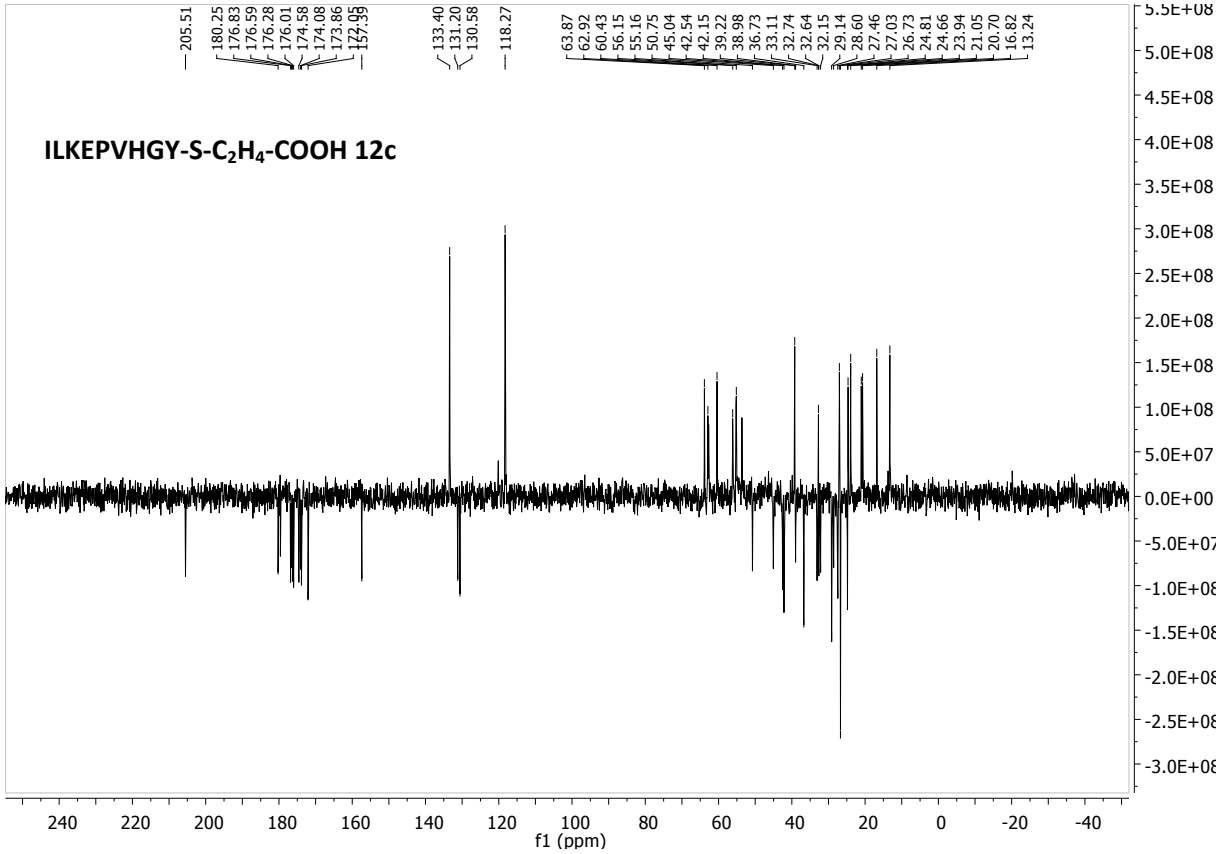


Fig. S15. ^{13}C NMR (75 MHz, H_2O+D_2O : 9/1 (v/v)) spectrum of peptide **12c**.

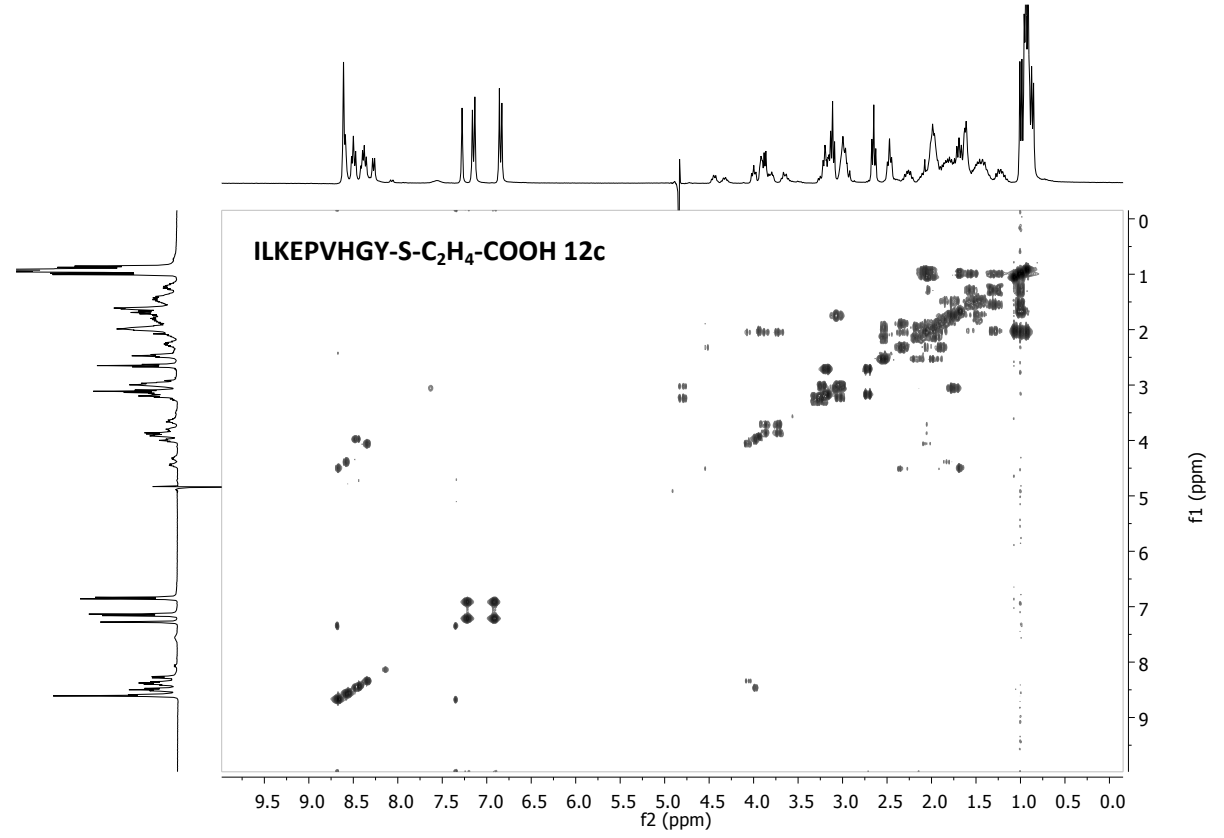


Fig. S16. ^{1}H-^{1}H COSY NMR (H_2O+D_2O : 9/1 (v/v)) spectrum of peptide **12c**.

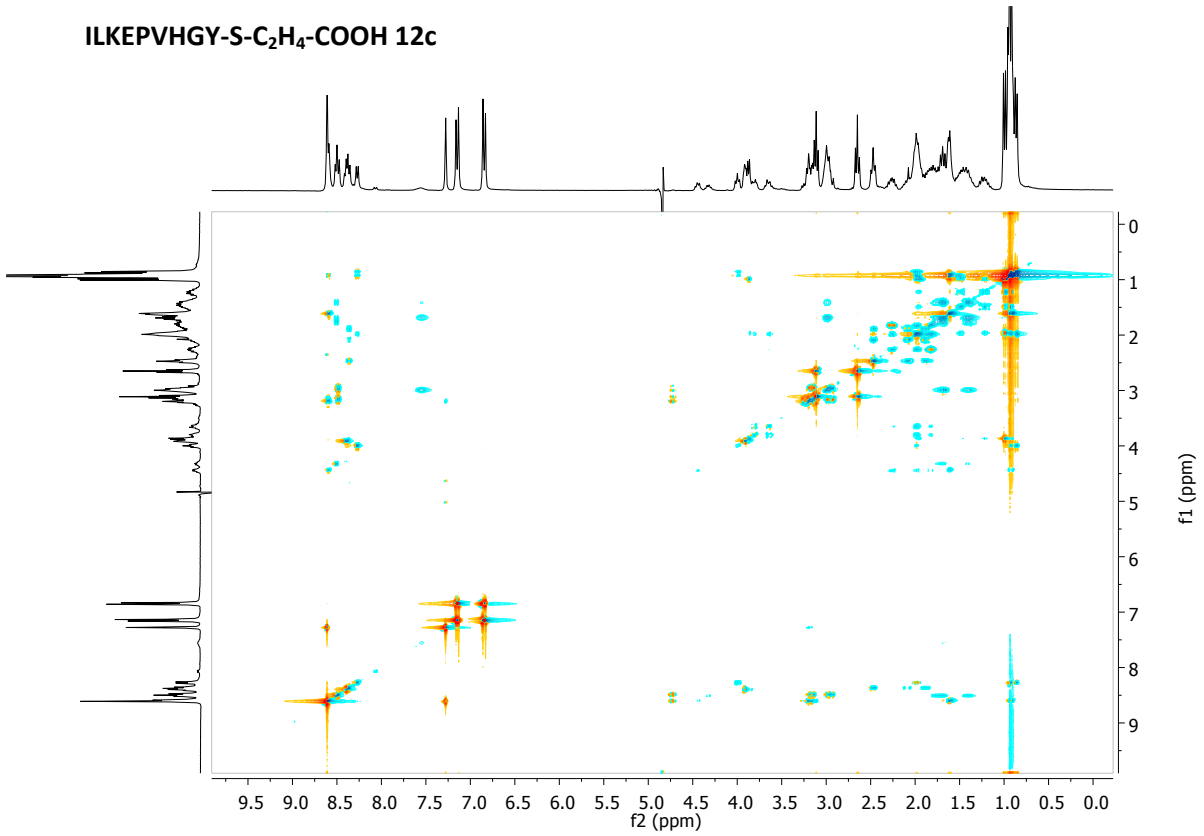


Fig. S17. ^{1}H-^{1}H DIPSY NMR (H_2O+D_2O : 9/1 (v/v)) spectrum of peptide **12c**.

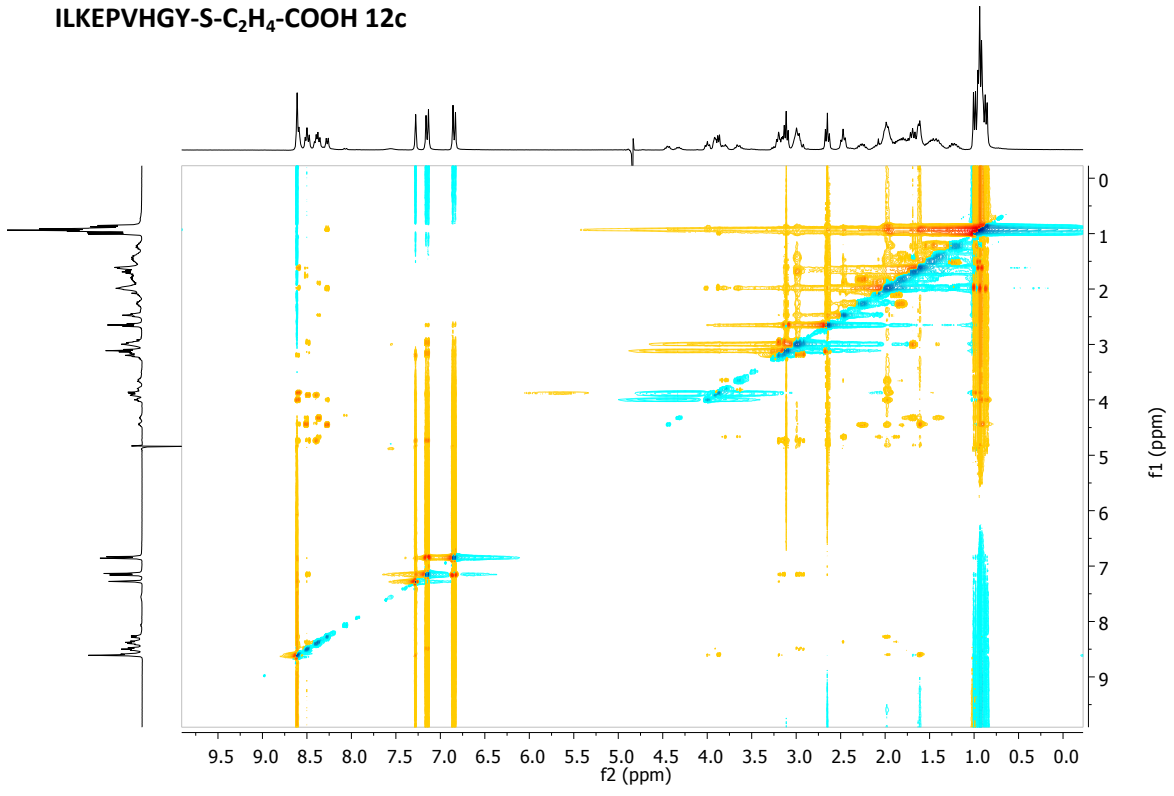
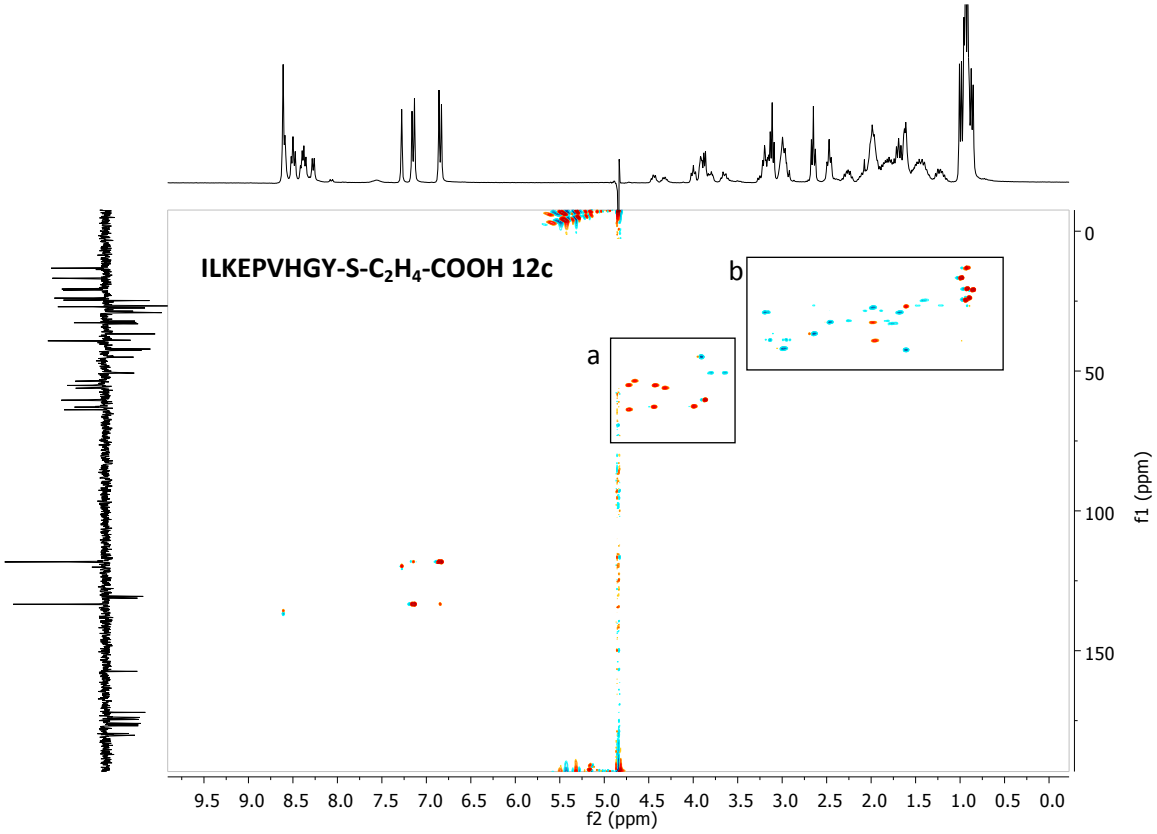
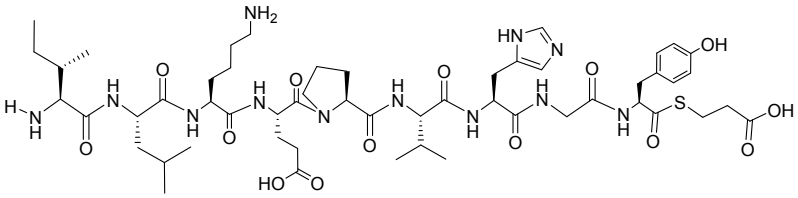
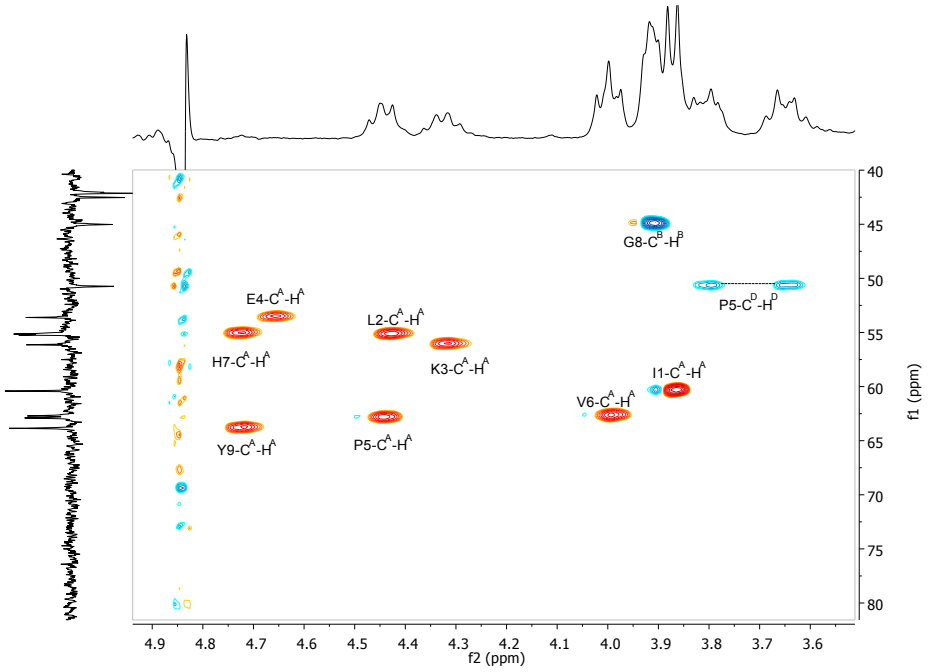


Fig. S18. ^{1}H-^{1}H ROESY NMR (H_2O+D_2O : 9/1 (v/v)) spectrum of peptide **12c**.





a) I_1 L_2 K_3 E_4 P_5 V_6 H_7 G_8 Y_9 10



b)

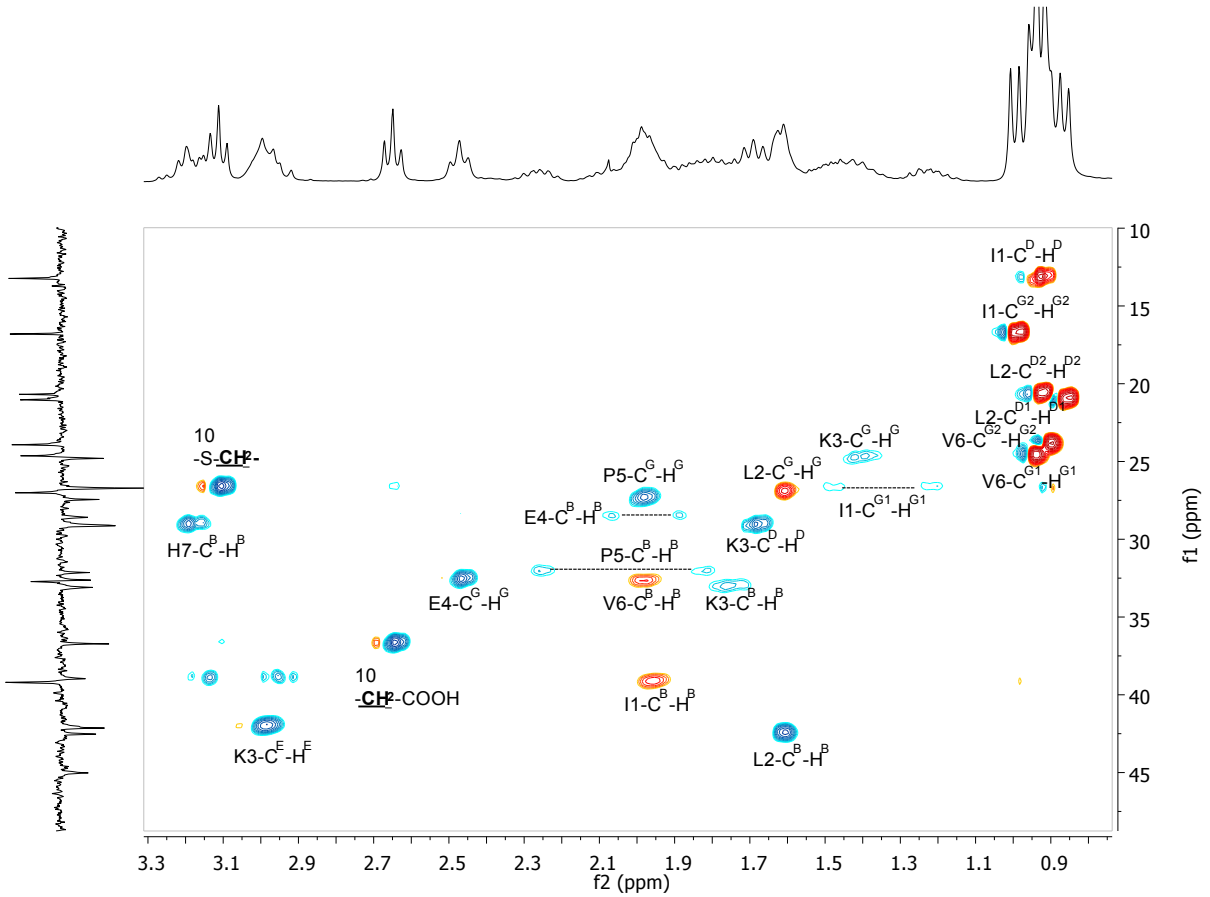


Fig. S19. ^{1}H-^{13}C HSQC NMR (H_2O+D_2O : 9/1 (v/v)) spectrum of peptide **12c**.

Synthesis of peptide 12e

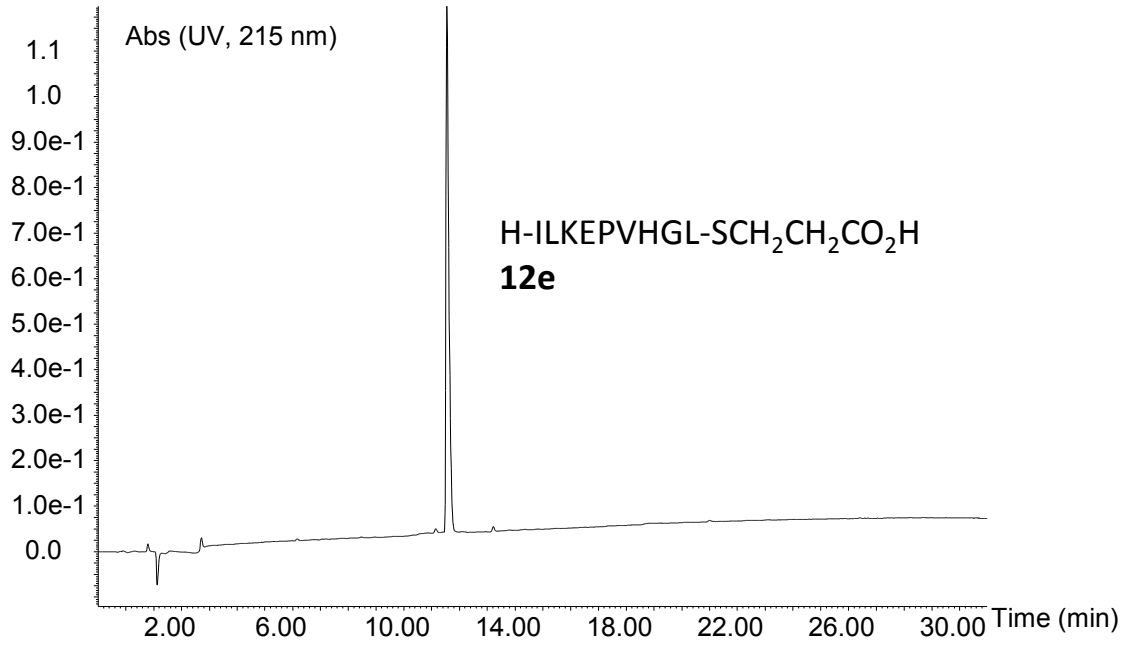
Scale: 15.5 mg (10.6 µmol).

Reaction time: 30 h.

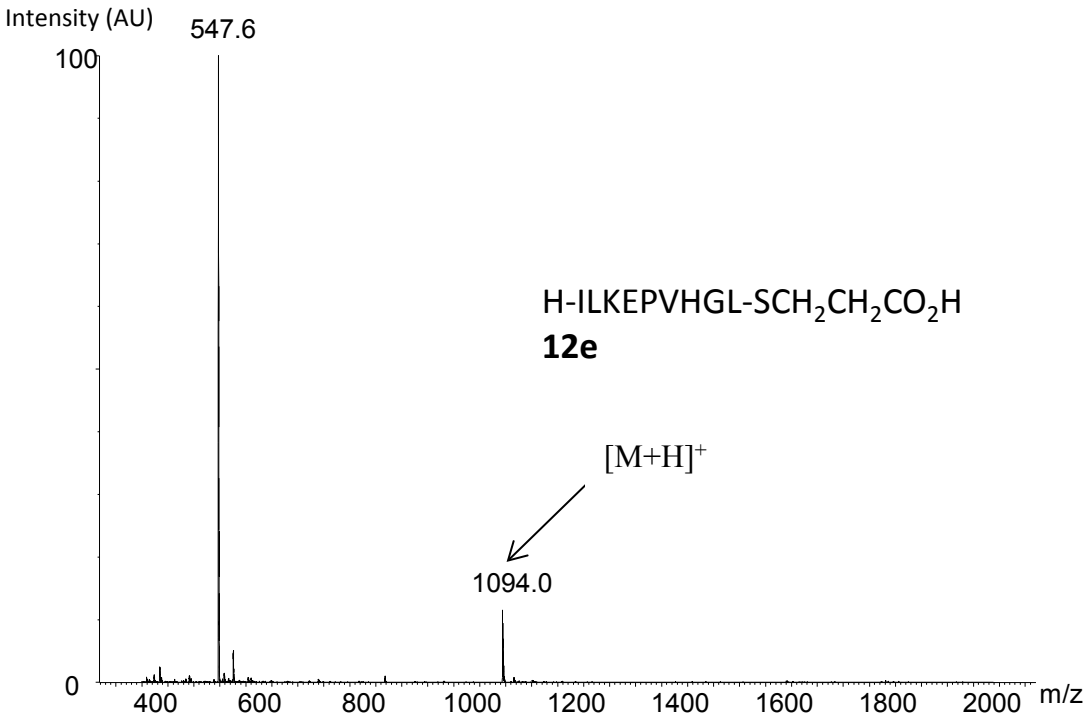
The crude product was purified by RP-HPLC (column: Waters XBridgeTM Prep C18 5 µm, 19 × 100 mm; eluent A: TFA 0.1% by vol. in water; eluent B: TFA 0.1% by vol. in CH_3CN/water 4:1 by vol.; gradient: 0-30% B in 25 min; flow rate 25 mL/min; detection at 215 nm) to give the desired thioester (9.1 mg; 60%; 0.22% D-Leu) as a white solid.

Characterization of peptide 12e

A)


Abs (UV, 215 nm)
1.1
1.0
9.0e-1
8.0e-1
7.0e-1
6.0e-1
5.0e-1
4.0e-1
3.0e-1
2.0e-1
1.0e-1
0.0
H-ILKEPVHGL-SCH2CH2CO2H
12e
2.00
6.00
10.00
14.00
18.00
22.00
26.00
30.00
Time (min)

B)


Intensity (AU)
547.6
100
H-ILKEPVHGL-SCH2CH2CO2H
12e
[M+H]+
1094.0
0
400
600
800
1000
1200
1400
1600
1800
2000
m/z

C)

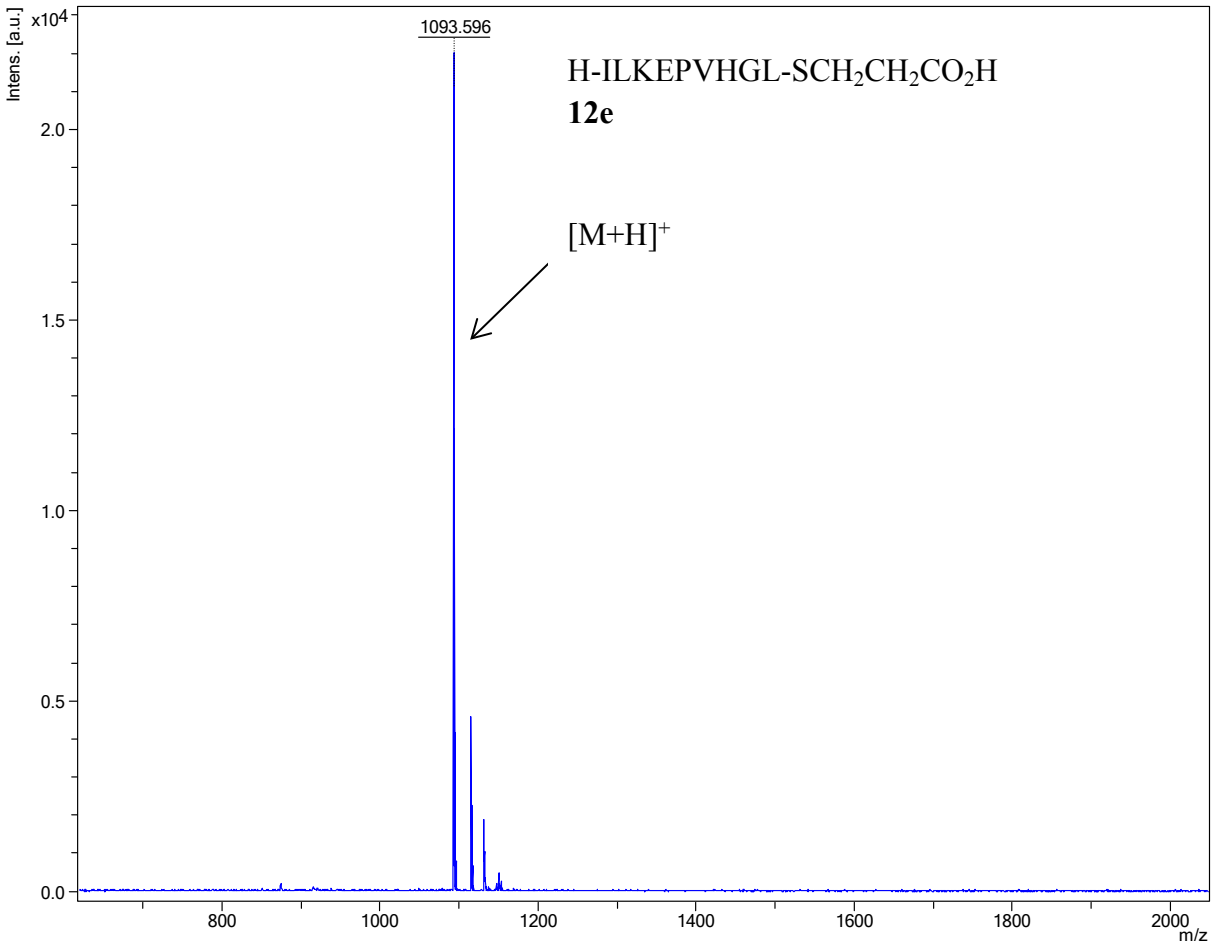
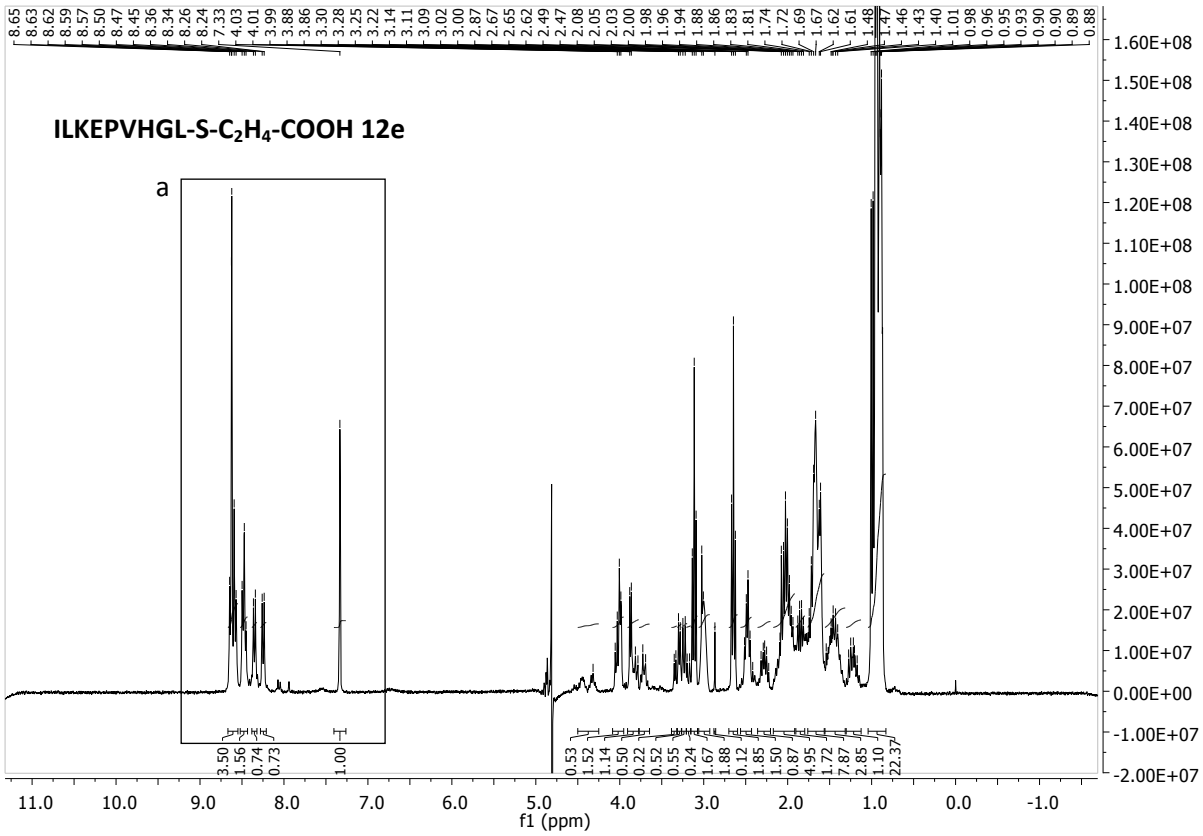


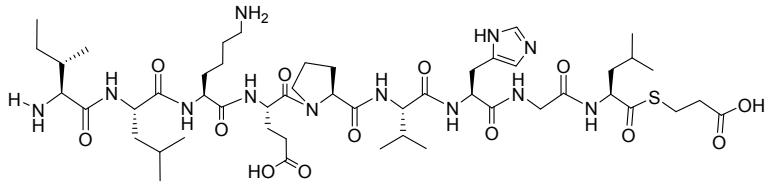
Fig. S20. Characterization of peptide **12e**. A) LC-MS, LC trace, column: Waters XBridgeTM BEH300 C18 3.5 µm (4.6 × 150 mm). Eluent A: TFA 0.1% by vol. in water; eluent B: TFA 0.1% by vol. in CH3CN/water 4:1 by vol. Gradient: 0-100% B in 30 min; flow rate 1 mL/min; detection at 215 nm; B) LC-MS, MS trace, $[M+H]^+$ m/z calcd (monoisotopic) 1093.6; found 1094.0; C) MALDI-TOF analysis, matrix: α-cyano-4-hydroxycinnamic acid, $[M+H]^+$ m/z calcd (monoisotopic) 1193.6; found 1093.56.

NMR analysis of peptide 12e

^{1}H NMR (300 MHz, H_2O+D_2O : 9/1 (v/v)) δ 8.67 – 8.55 (m, 3H), 8.52 – 8.43 (m, 2H), 8.38 – 8.32 (m, 1H), 8.28 – 8.21 (m, 1H), 7.33 (s, 1H), 4.32 (s, 1H), 4.09 – 3.95 (m, 2H), 3.91 – 3.77 (m, 1H), 3.77 – 3.65 (m, 1H), 3.38 – 3.32 (m, 1H), 3.31 – 3.27 (m, 1H), 3.26 – 3.21 (m, 1H), 3.21 – 3.16 (m, 1H), 3.15 – 3.07 (m, 2H), 3.06 – 2.93 (m, 2H), 2.87 (s, 1H), 2.70 – 2.60 (m, 2H), 2.56 – 2.43 (m, 2H), 2.36 – 2.20 (m, 1H), 2.17 – 1.92 (m, 5H), 1.90 – 1.80 (m, 2H), 1.76 – 1.56 (m, 8H), 1.56 – 1.31 (m, 3H), 1.30 – 1.13 (m, 1H), 1.04 – 0.83 (m, 22H).

^{13}C NMR (75 MHz, H_2O+D_2O : 9/1 (v/v)) δ 207.25 (s), 180.53 (s), 176.79 (s), 176.60 (s), 176.28 (s), 176.02 (s), 174.67 (s), 174.15 (s), 172.07 (s), 131.24 (s), 50.77 (s), 45.19 (s), 42.59 (s), 42.18 (s), 39.24 (s), 37.06 (s), 33.13 (s), 32.91 (s), 32.87 (d, *J* = 5.7 Hz), 32.20 (s), 29.18 (s), 27.48 (s), 27.07 (s), 26.75 (s), 24.97 (s), 24.82 (s), 24.67 (s), 23.96 (s), 23.23 (s), 21.13 (s), 20.70 (s), 16.84 (s), 13.27 (s).


ILKEPVHGL-S-C2H4-COOH 12e
a
f1 (ppm)



a) I_1 L_2 K_3 E_4 P_5 V_6 H_7 G_8 L9 10

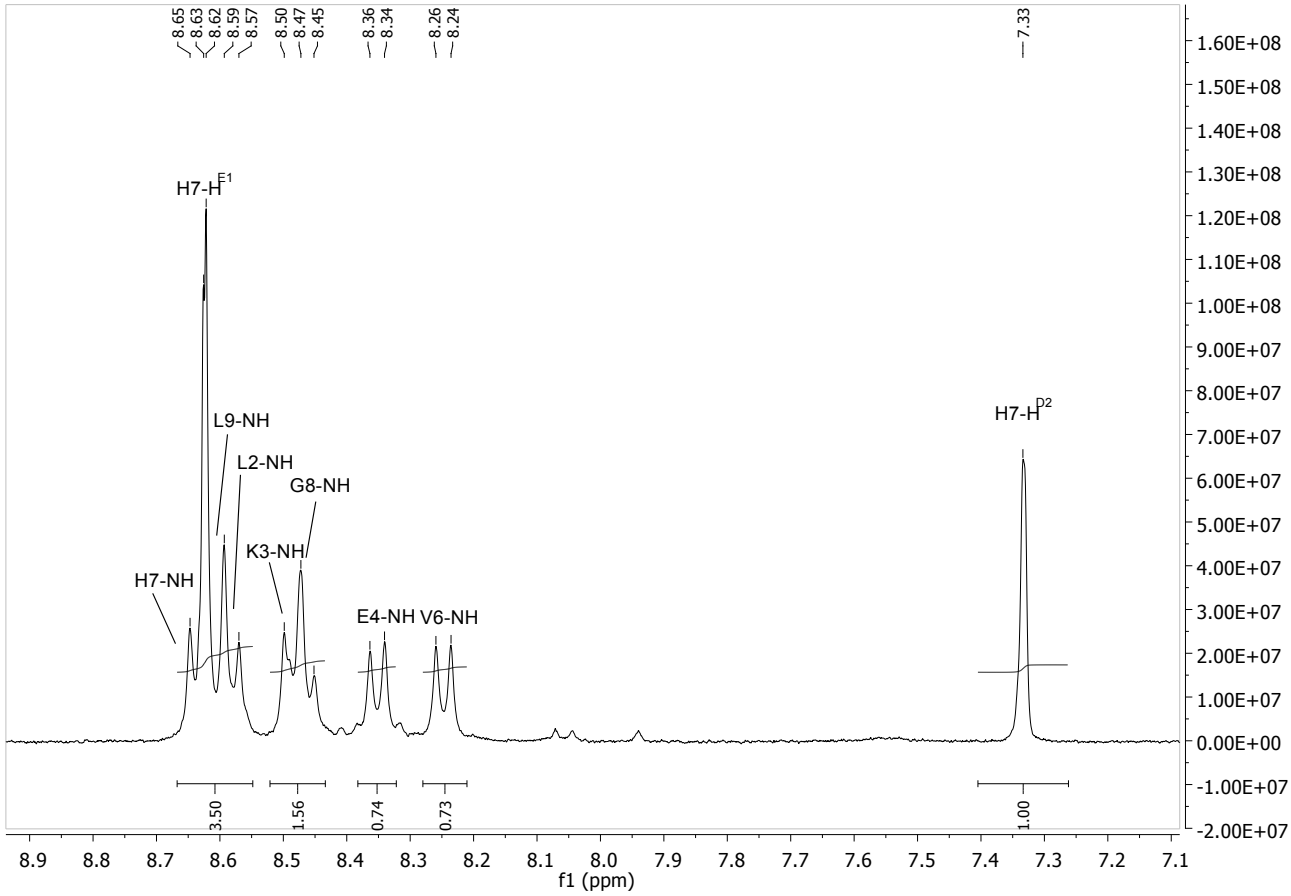


Fig. S21. ^{1}H NMR (H_2O+D_2O : 9/1 (v/v)) spectrum of peptide **12e**.

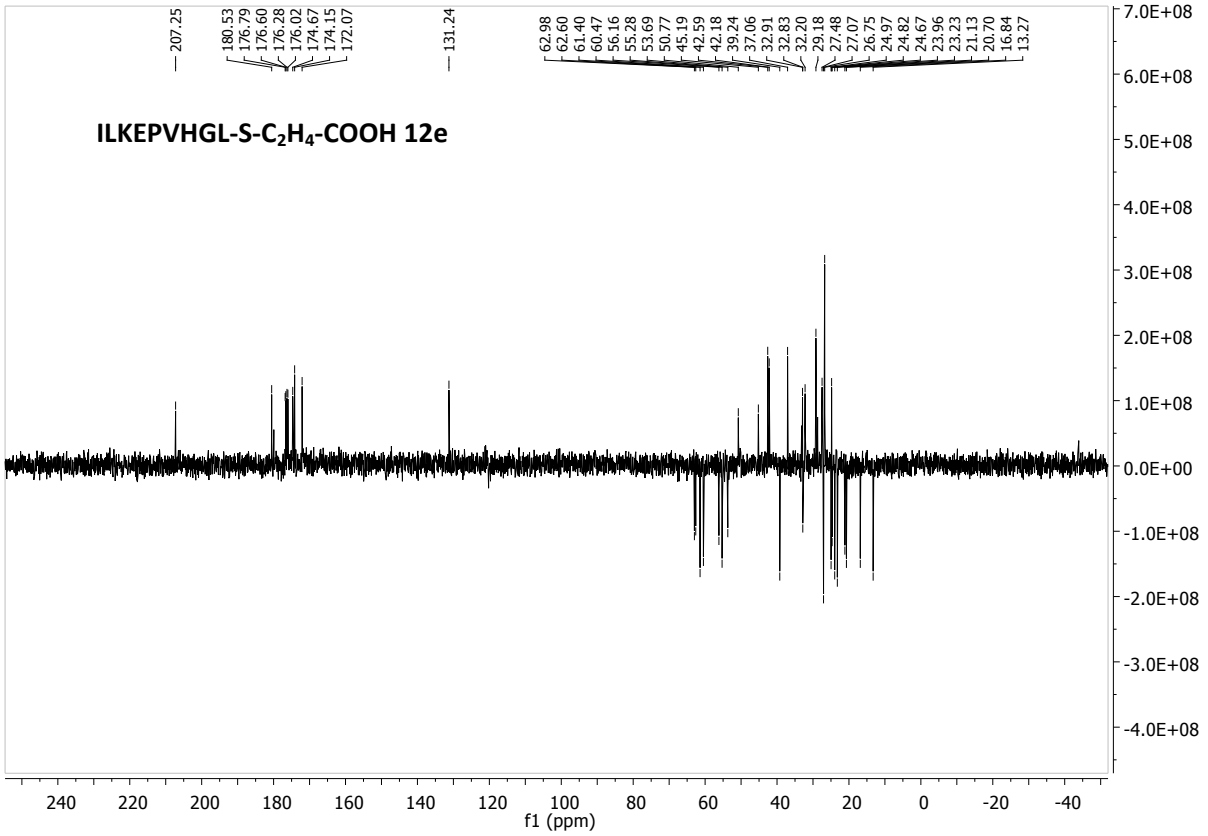


Fig. S22. ^{13}C NMR (75 MHz, H_2O+D_2O : 9/1 (v/v)) spectrum of peptide **12e**.

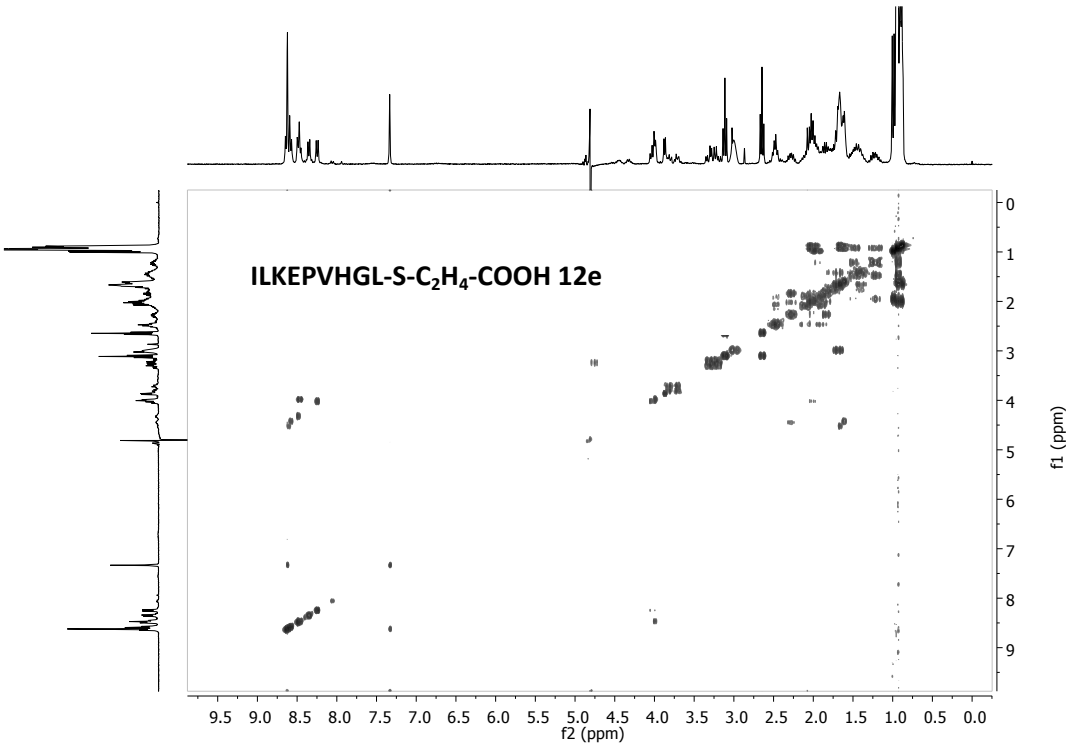


Fig. S23. ^{1}H-^{1}H COSY NMR (H_2O+D_2O : 9/1 (v/v)) spectrum of peptide **12e**.

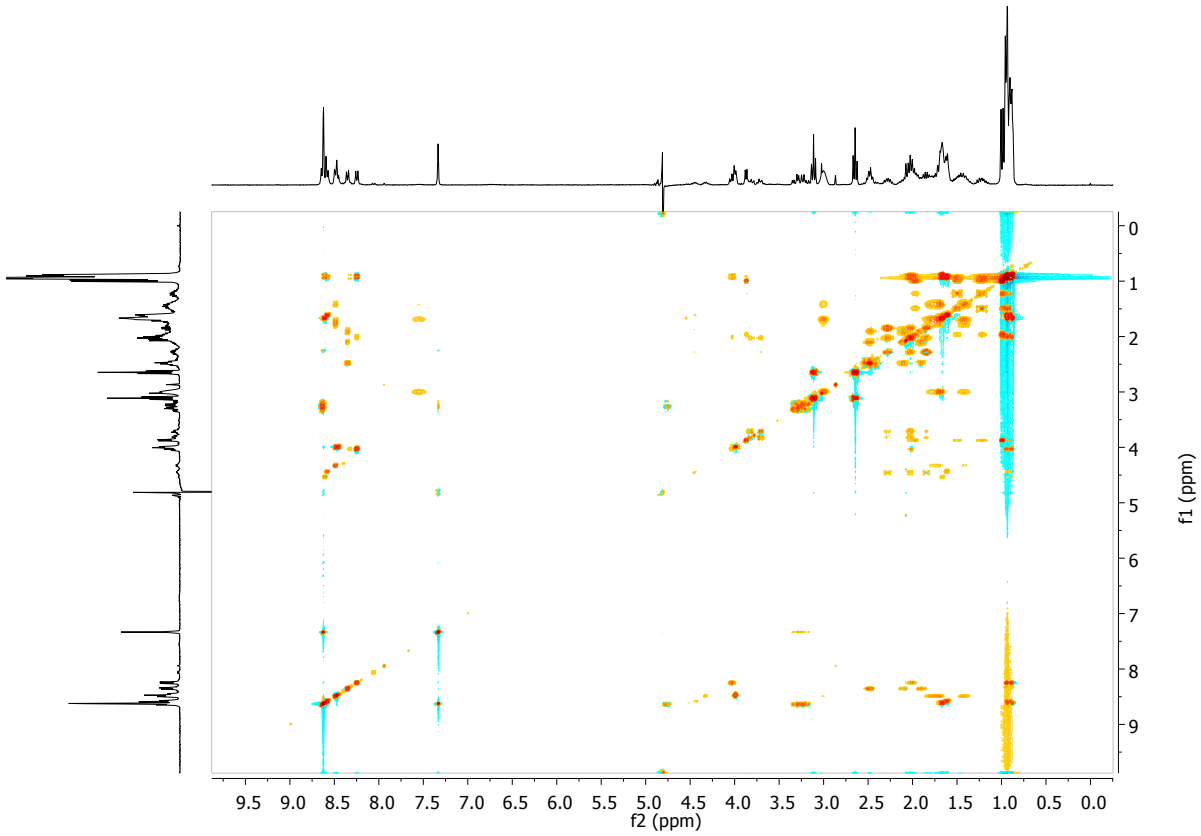


Fig. S24. ^{1}H-^{1}H DIPSY NMR (H_2O+D_2O : 9/1 (v/v)) spectrum of peptide **12e**.

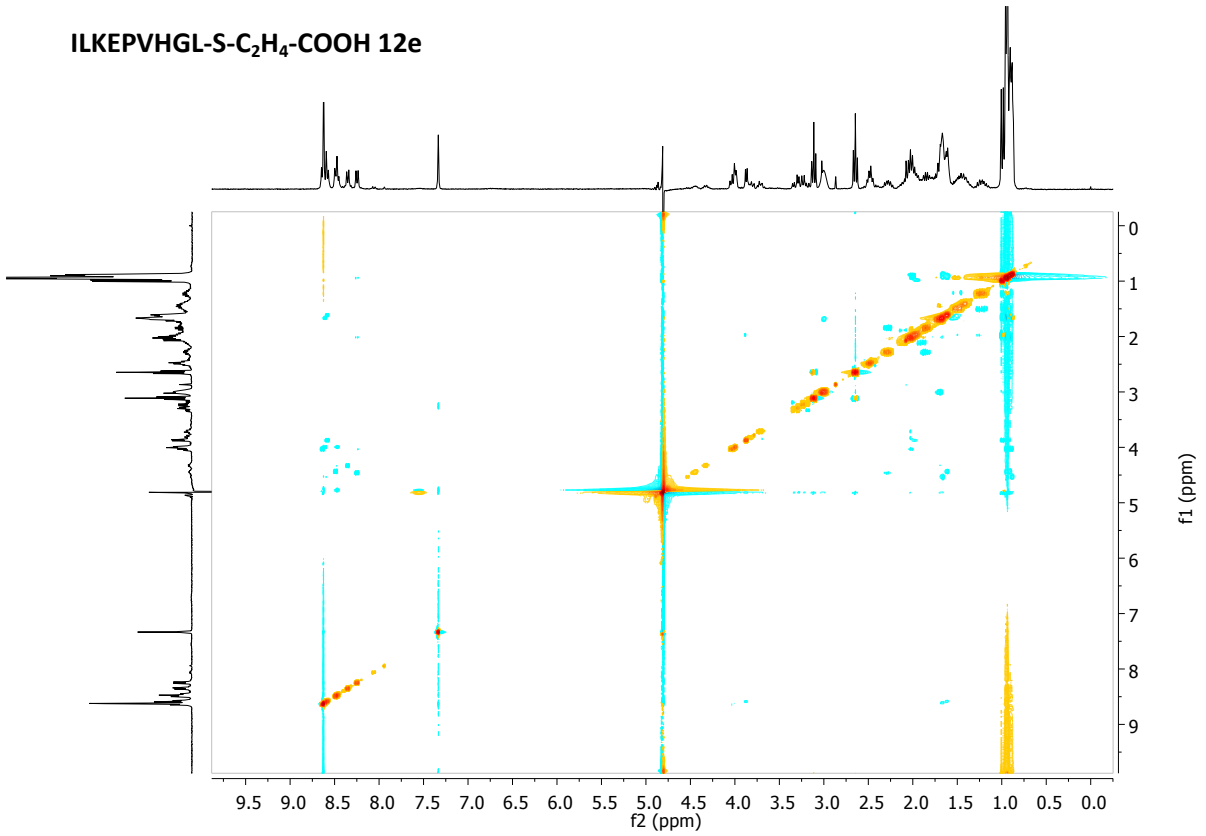
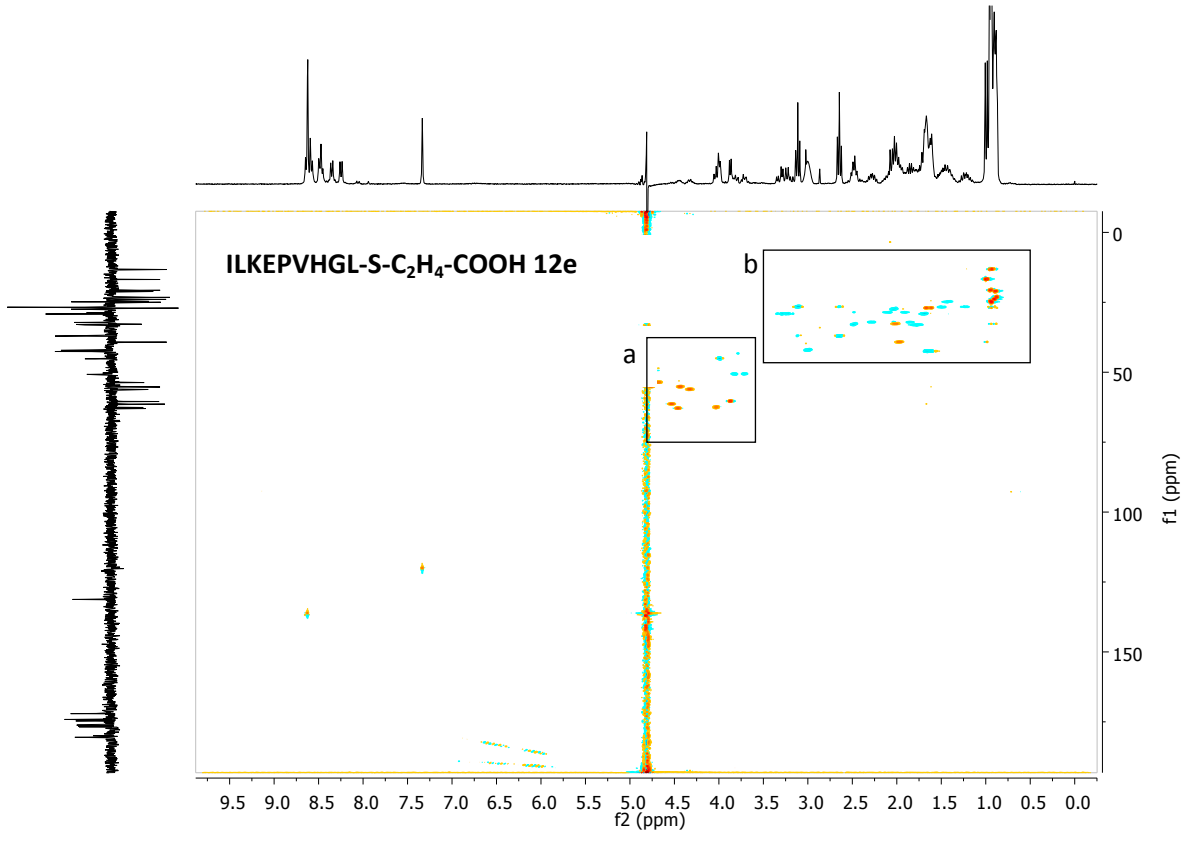
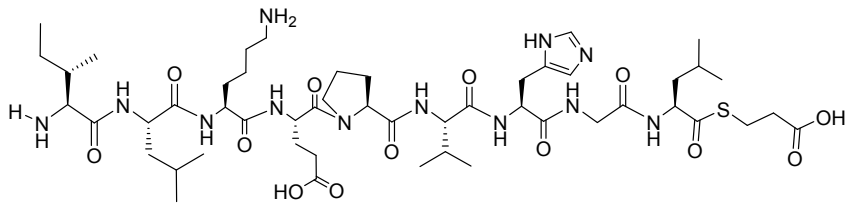
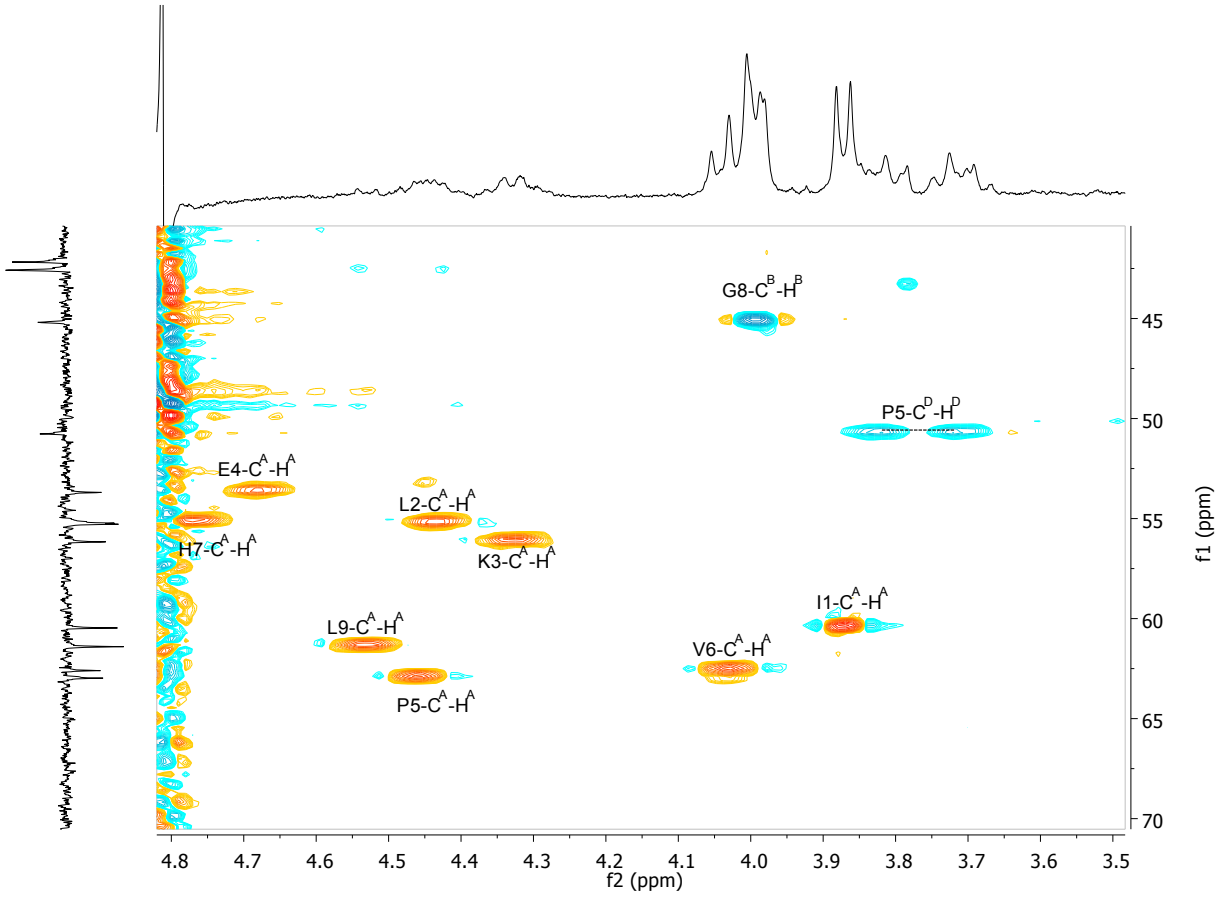


Fig. S25. ^{1}H-^{1}H ROESY NMR (H_2O+D_2O : 9/1 (v/v)) spectrum of peptide **12e**.





a) I_1 L_2 K_3 E_4 P_5 V_6 H_7 G_8 L9 10



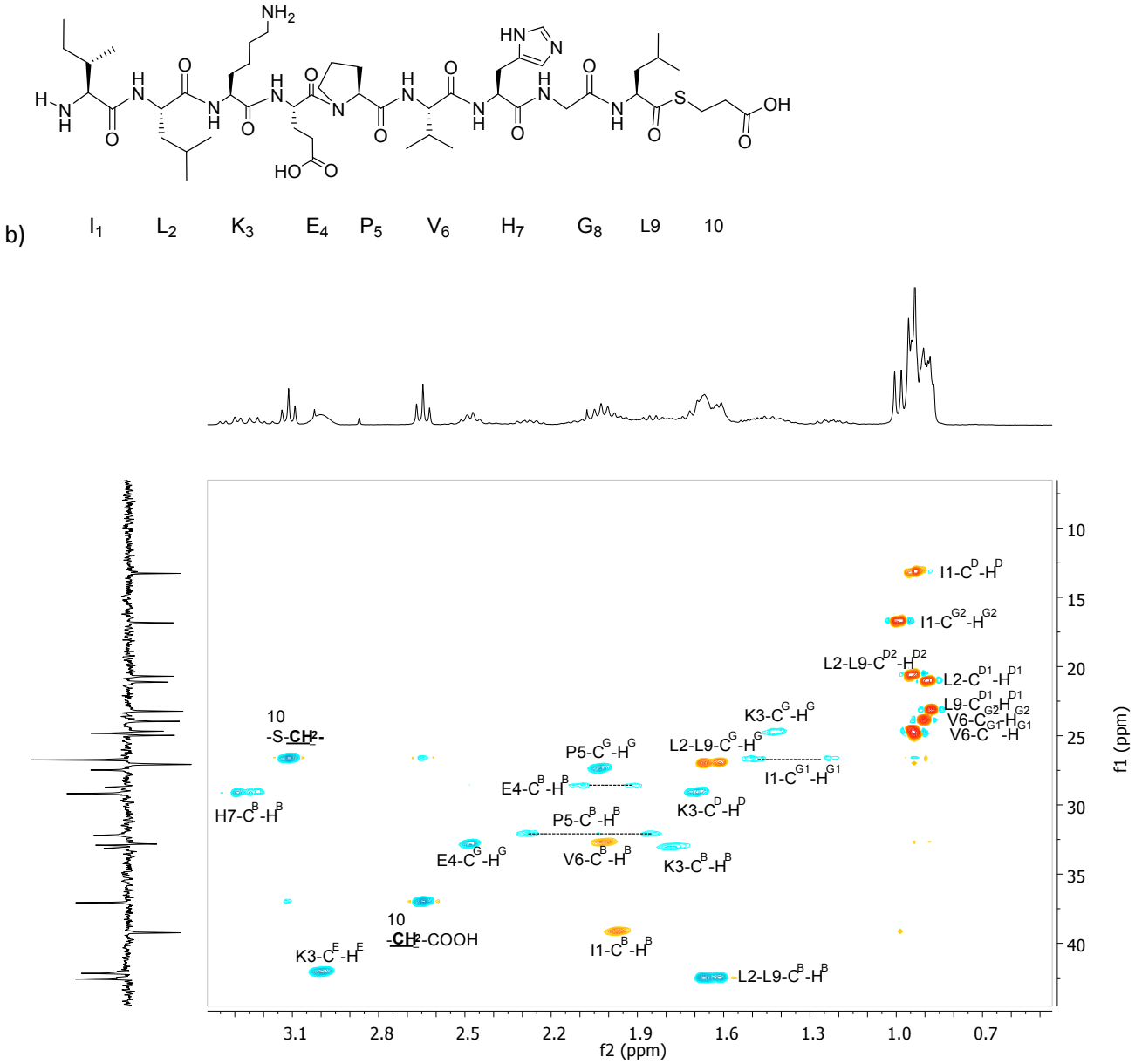


Fig. S26. ^{1}H-^{13}C HSQC NMR (H_2O+D_2O : 9/1 (v/v)) spectrum of peptide **12e**.

Synthesis of peptide 12f

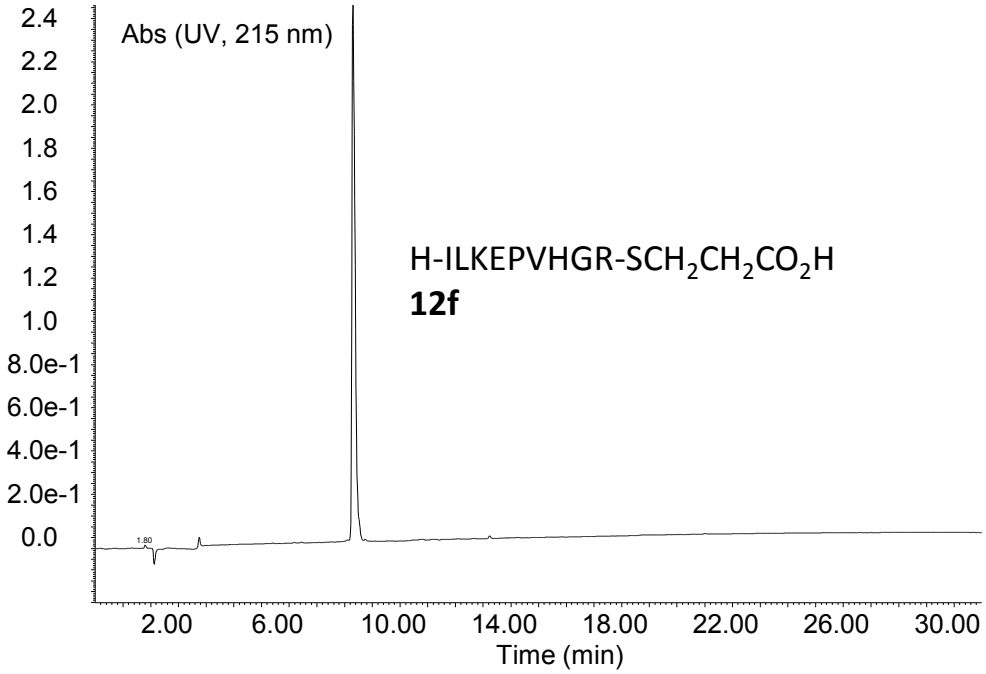
Scale: 15 mg (9.2 µmol).

Reaction time: 30 h.

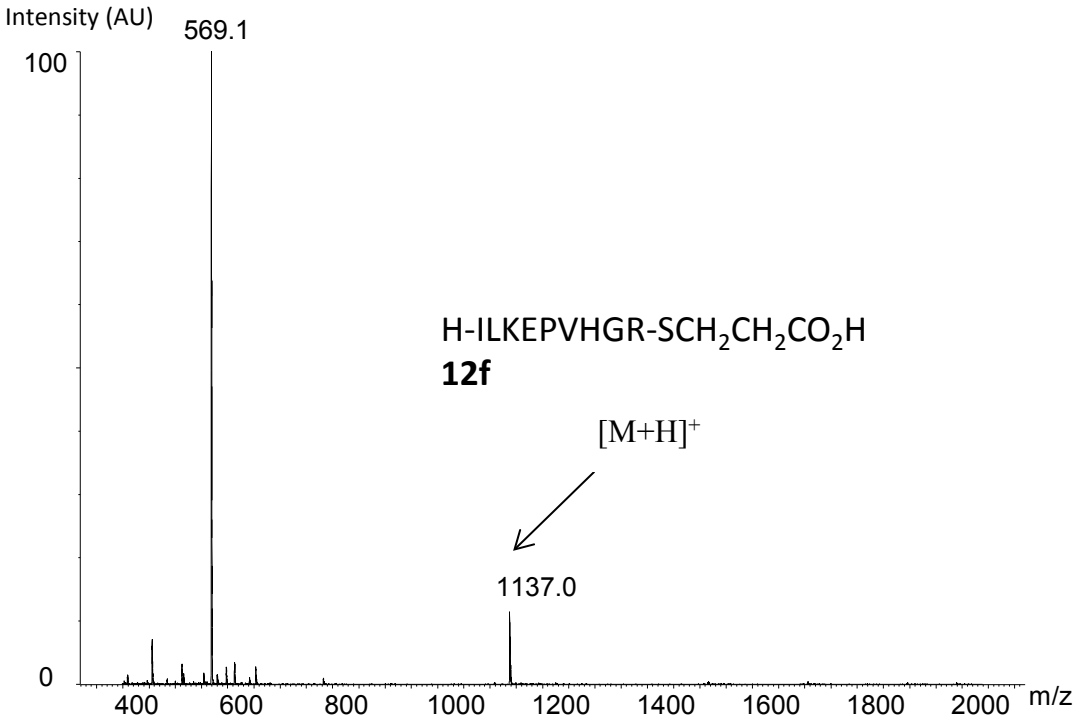
The crude product was purified by RP-HPLC (column: Waters XBridgeTM Prep C18 5 µm, 19 × 100 mm; eluent A: TFA 0.1% by vol. in water; eluent B: TFA 0.1% by vol. in CH_3CN/water 4:1 by vol.; gradient: 0-30% B in 25 min; flow rate 25 mL/min; detection at 215 nm) to give the desired thioester (9.4 mg; 64%; 0.50% D-Arg) as a white solid.

Characterization of peptide 12f

A)



B)



C)

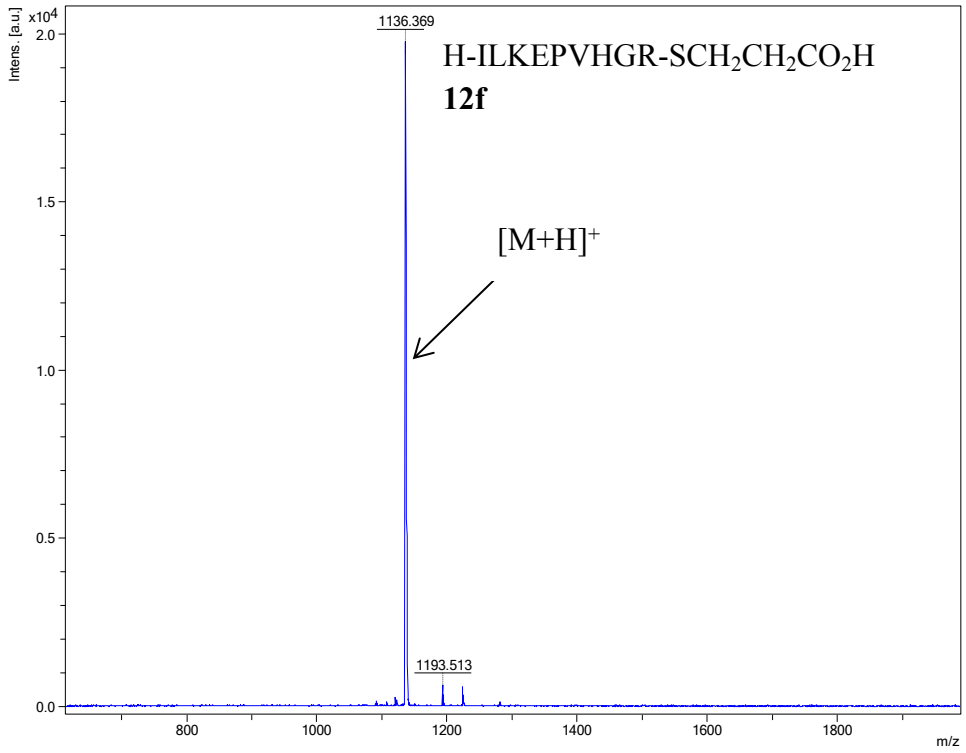
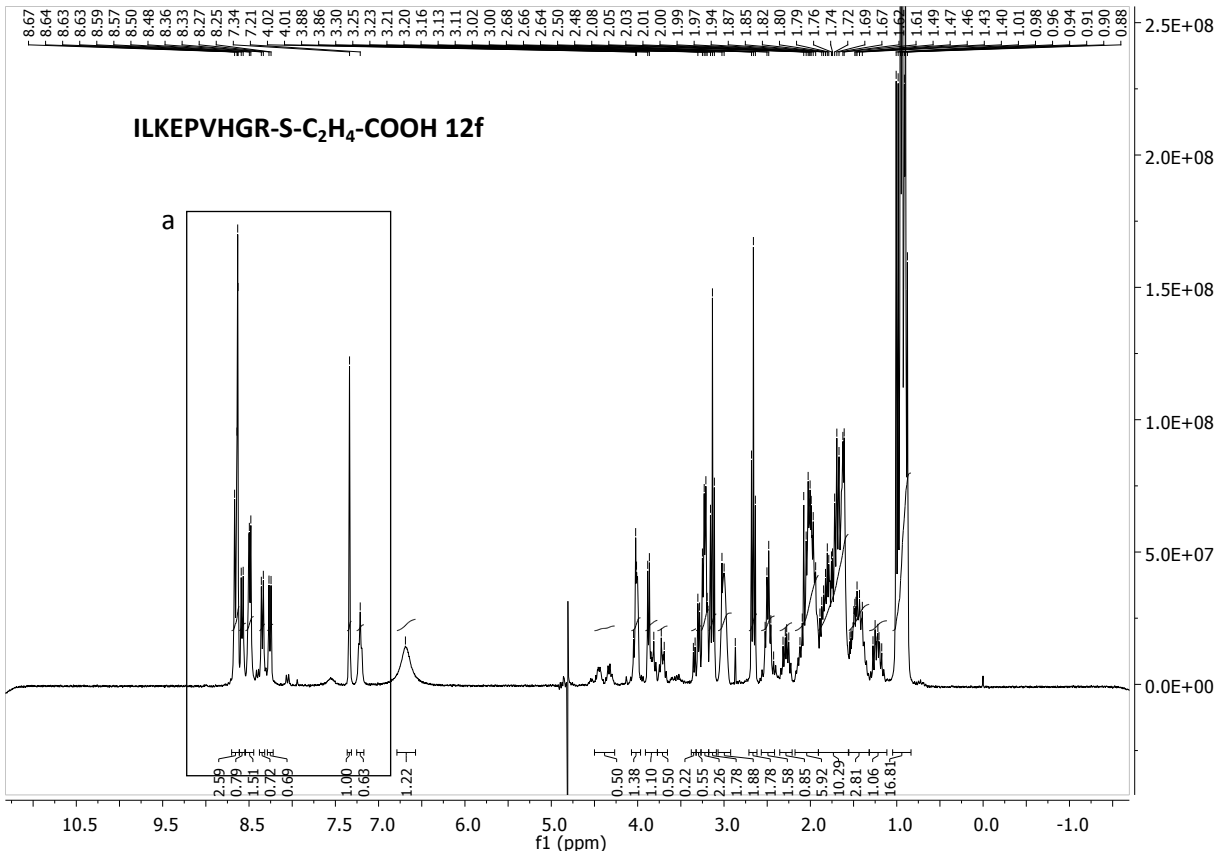
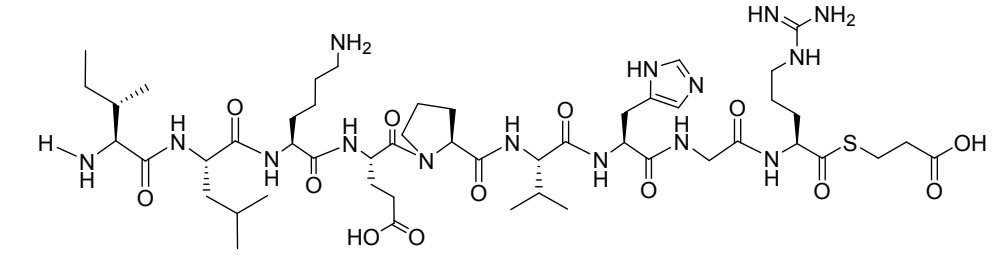


Fig. S27. Characterization of peptide **12f**. A) LC-MS, LC trace, column: Waters XBridgeTM BEH300 C18 3.5 µm (4.6 × 150 mm). Eluent A: TFA 0.1% by vol. in water; eluent B: TFA 0.1% by vol. in CH3CN/water 4:1 by vol. Gradient: 0-100% B in 30 min; flow rate 1 mL/min; detection at 215 nm; B) LC-MS, MS trace, $[M+H]^+$ m/z calcd (monoisotopic) 1136.6; found 1037.0; C) MALDI-TOF analysis, matrix: α-cyano-4-hydroxycinnamic acid, $[M+H]^+$ m/z calcd (monoisotopic) 1136.6; found 1036.4.

NMR analysis of peptide 12f

^{1}H NMR (300 MHz, H_2O+D_2O : 9/1 (v/v)) δ 8.70 – 8.61 (m, 3H), 8.61 – 8.55 (m, 1H), 8.49 (d, *J* = 5.2 Hz, 2H), 8.38 – 8.32 (m, 1H), 8.29 – 8.22 (m, 1H), 7.34 (s, 1H), 7.21 (s, 1H), 6.69 (s, 1H), 4.50 – 4.27 (m, 1H), 4.07 – 3.97 (m, 1H), 3.91 – 3.77 (m, 1H), 3.77 – 3.65 (m, 1H), 3.38 – 3.32 (m, 1H), 3.32 – 3.27 (m, 1H), 3.22 (dd, *J* = 11.1, 5.2 Hz, 2H), 3.18 – 3.09 (m, 2H), 3.07 – 2.92 (m, 2H), 2.71 – 2.62 (m, 2H), 2.57 – 2.41 (m, 2H), 2.35 – 2.21 (m, 1H), 2.18 – 1.91 (m, 6H), 1.91 – 1.56 (m, 10H), 1.55 – 1.32 (m, 3H), 1.32 – 1.11 (m, 1H), 1.05 – 0.83 (m, 17H).


ILKEPVHGR-S-C2H4-COOH 12f
a
f1 (ppm)



a) I1 L2 K3 E4 P5 V6 H7 G8 R9 10

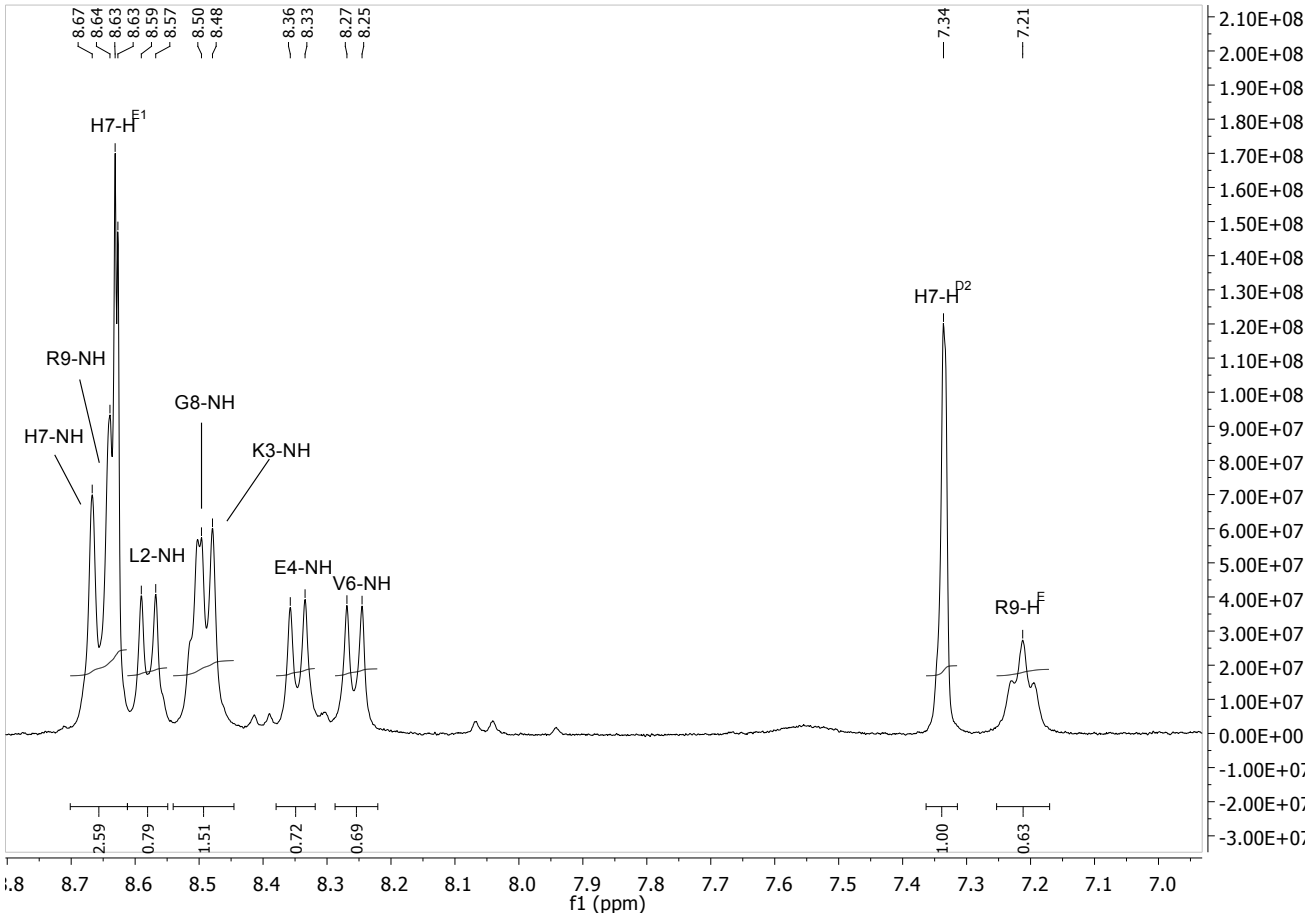


Fig. S28. ^{1}H NMR (H_2O+D_2O : 9/1 (v/v)) spectrum of peptide **12f**.

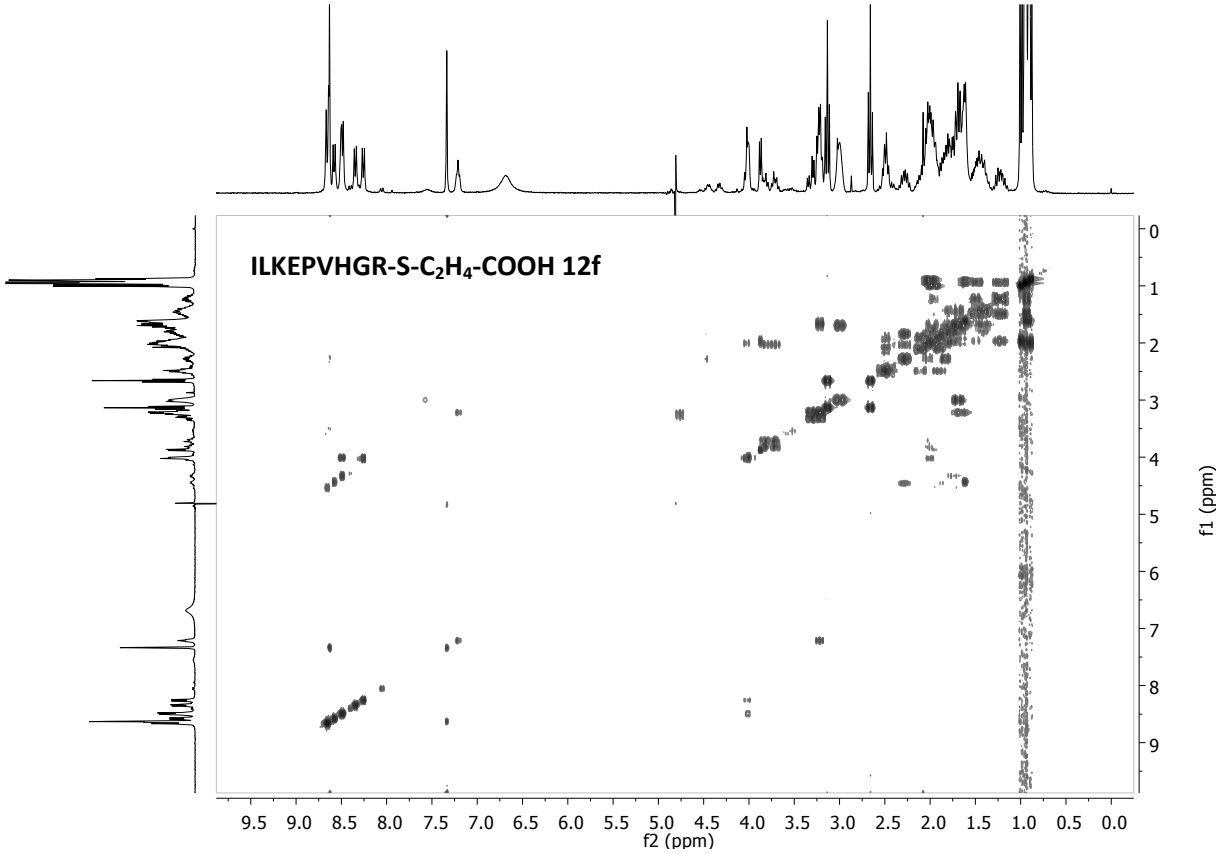


Fig. S29. ^{1}H-^{1}H COSY NMR (H_2O+D_2O : 9/1 (v/v)) spectrum of peptide **12f**.

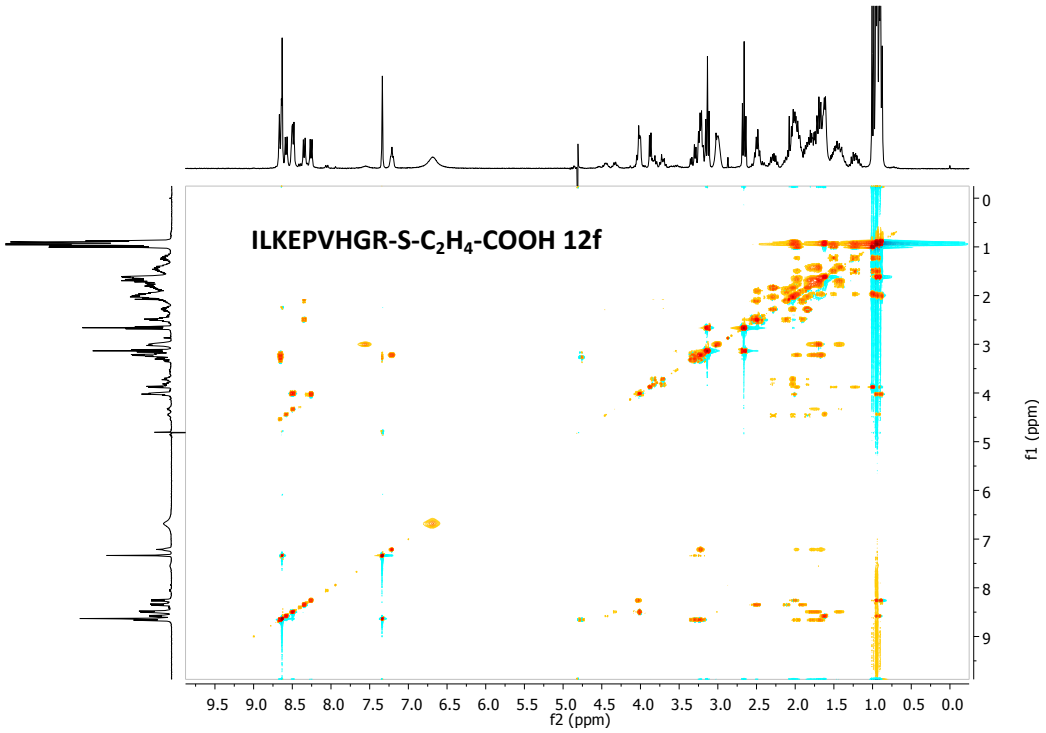
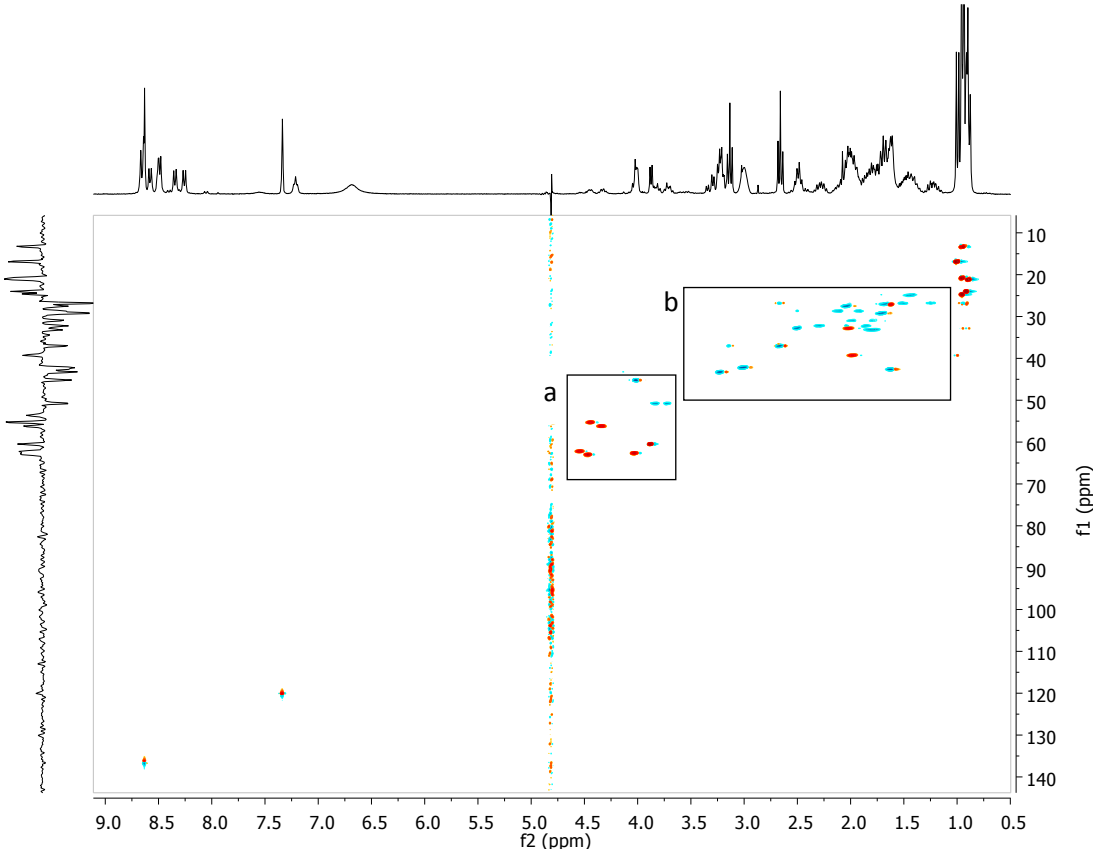
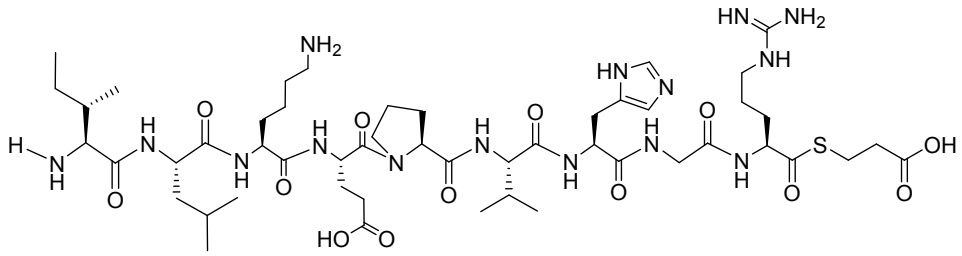
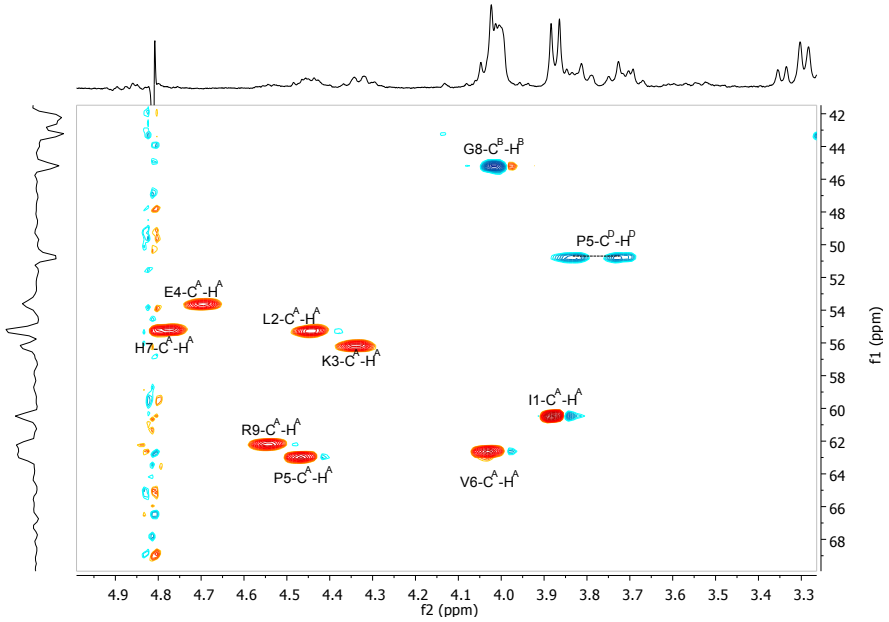


Fig. S30. ^{1}H-^{1}H DIPSY NMR (H_2O+D_2O : 9/1 (v/v)) spectrum of peptide **12f**.


a
b
9.0
8.5
8.0
7.5
7.0
6.5
6.0
5.5
5.0
4.5
4.0
3.5
3.0
2.5
2.0
1.5
1.0
0.5
f2 (ppm)
10
20
30
40
50
60
70
80
90
100
110
120
130
140
f1 (ppm)



a) I1 L2 K3 E4 P5 V6 H7 G8 R9 10



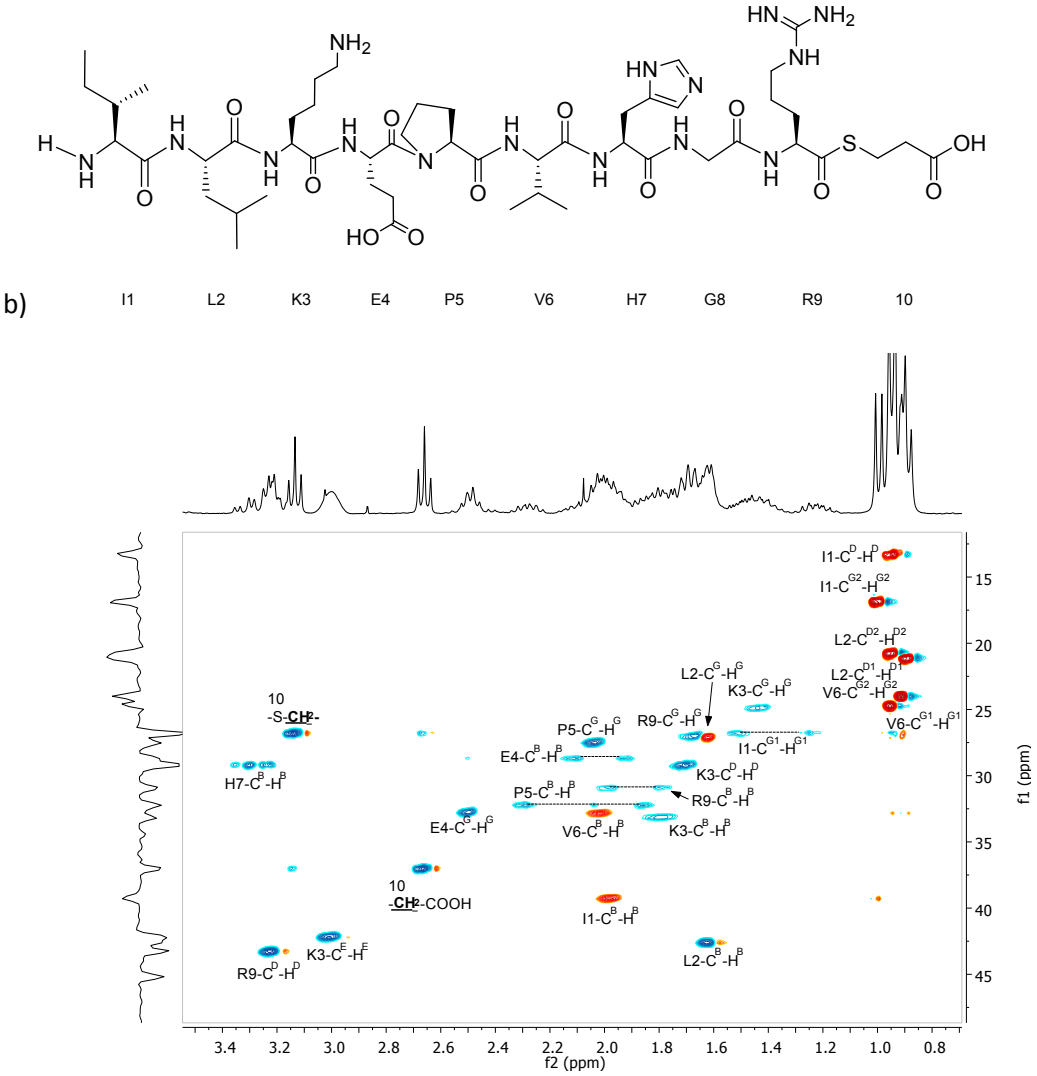


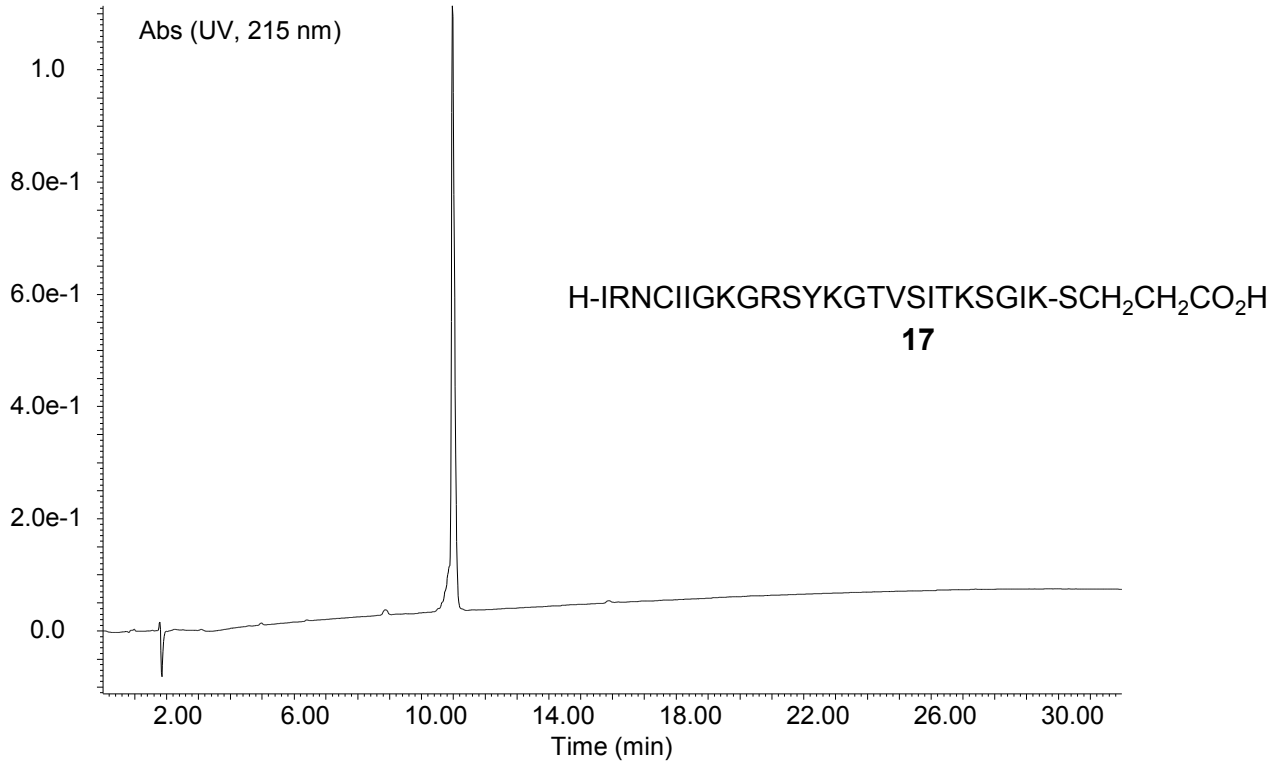
Fig. S31. ^{1}H-^{13}C HSQC NMR (H_2O+D_2O : 9/1 (v/v)) spectrum of peptide **12f**.

Synthesis of 17

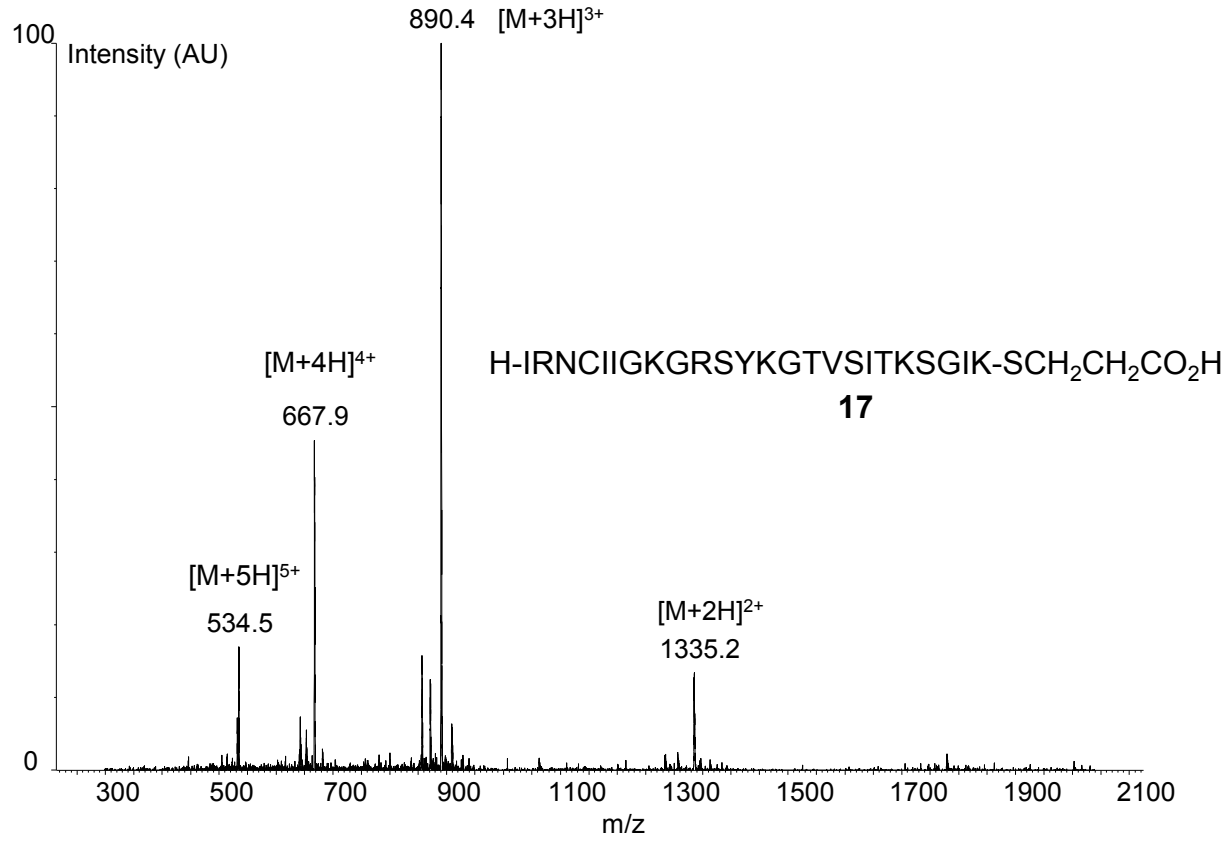
In a glove box, TCEP.HCl (8.5 mg, 29.6 µmol, 6.2 eq), MPAA (10.8 mg, 62.3 µmol, 13 eq), MPA (10 µL, 1% by vol.) and Gdn.HCl (573.9 mg) were dissolved in sodium phosphate buffer (0.1 M; 590 µL; pH 6.8) and the pH was adjusted to 7.1 by adding a 6 M aqueous NaOH solution (53 µL). The thioester **14** (14 mg, 50.5 µmol, 10.5 eq) was dissolved in the above solution and the resulting solution was added to the peptide **16** (17.2 mg, 4.8 µmol, 1 eq). The reaction mixture was then stirred at 37 °C for 46 h. The reaction mixture was diluted to a volume of 3 mL with water and AcOH (0.5 mL) was added. The aqueous layer was extracted with Et_2O (4 x 3 mL) and injected (after bubbling argon through the aqueous layer to remove any traces of diethyl ether) in the RP-HPLC system for purification (column: Waters XBridgeTM Prep C18 5 µm, 19 x 100 mm; eluent A: TFA 0.1% by vol. in water; eluent B: TFA 0.1% by vol. in CH_3CN/water 4:1 by vol.; gradient: 0-30% B in 25 min; flow rate 25 mL/min; detection at 215 nm) to give the desired thioester **17** (5.0 mg; 30 %) as a white solid.

Characterization of peptide 17

A)



B)



C)

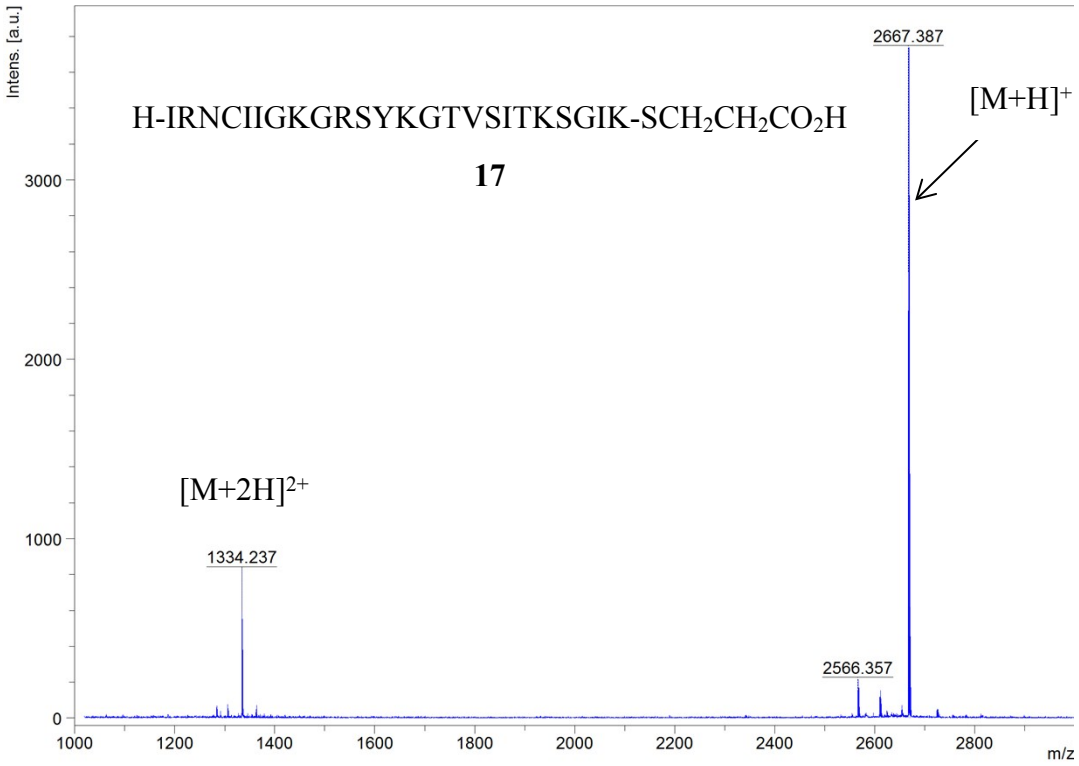


Fig. S32. Characterization of peptide **17**. A) LC-MS, LC trace, column: Agilent ZORBAX 300SB-C3 3.5 µm (4.6 x 150 mm). Eluent A: TFA 0.1% by vol. in water; eluent B: TFA 0.1% by vol. in CH3CN/water 4:1 by vol. Gradient: 0-100% B in 30 min; flow rate 1 mL/min; detection at 215 nm; B) LC-MS, MS trace, $[M+2H]^{2+}$ m/z calcd (monoisotopic); 1334.2 found 1335.2, $[M+3H]^{3+}$ m/z calcd (monoisotopic) 889.8 ; found 890.4, $[M+4H]^{4+}$ m/z calcd (monoisotopic) 667.6 ; found 667.9, $[M+5H]^{5+}$ m/z calcd (monoisotopic) 534.3; found 534.5; C) MALDI-TOF analysis, matrix: α-cyano-4-hydroxycinnamic acid, $[M+H]^{+}$ m/z calcd (monoisotopic) 2667.5; found 2667.4.

References

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