Electronic Supplementary Material (ESI) for ChemComm.

Electronic Supporting Information

A novel thiol-labile cysteine protecting group for peptide synthesis based on a pyridazinedione (PD) scaffold

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General Experimental

All chemical reagents were purchased from Sigma Aldrich, Alfa Aesar, Acros and Fluorochem. Compounds and solvents were used as received. Petrol refers to petroleum ether (b.p. 40–60 °C). All small molecule reactions were carried out under positive pressure of argon, unless stated otherwise, and were monitored using thin layer chromatography (TLC) on pre-coated silica gel plates (254 μm). Peptide manipulations, reactions and conjugations were performed under ambient conditions. Flash column chromatography was carried out with pre-loaded GraceResolv™ Silica Flash cartridges (Grace™) or FlashPure EcoFlex catridges (Büchi) on a Biotage® Isolera Spektra One flash chromatography system (Biotage®). ^{1}H NMR spectra were obtained at 700 MHz. ^{13}C NMR spectra were obtained at 176 MHz. All results were obtained using a Bruker NMR instrument (Avance Neo 700). Unless otherwise specified, all samples were run at 25 °C. Chemical shifts (δ) for ^{1}H NMR and ^{13}C NMR are quoted on a parts per million (ppm) scale relative to tetramethylsilane (TMS), calibrated using residual signals of the solvent. Where amide rotamers are the case, and when possible, only the chemical shifts of the major rotamer have been assigned and areas underneath all rotameric peaks have been considered for the integral intensity calculations. Coupling constants (J values) are reported in Hertz (Hz) and are reported as J*H-H* couplings between protons. Infrared spectra were obtained on a Perkin Elmer Spectrum 100 FTIR spectrometer operating in ATR mode. Mass spectra were obtained, for synthetic products, from the UCL mass spectroscopy service on a Waters LCT Premier XE (ES) mass spectrometer. Melting points were measured with Gallenkamp apparatus and are uncorrected. UV-Vis spectroscopy was performed using either a Jenway 7305 spectrophotomer (Jenway) or a NanoDrop OneC spectrophotometer (ThermoScientific) operating at 21 °C unless stated otherwise.

Methods for manual Solid Phase Peptide Synthesis (SPPS)

Peptides were synthesised *via* manual solid phase peptide synthesis (SPPS) using an *in situ* neutralisation/HCTU activation procedure for Fmoc chemistry on an Fmoc-Gly-2-ClTrt resin (0.19 mmol/g, Sigma) or an Fmoc-Gly Rink Amide AM resin (0.32 mmol/g, Fluorochem) using Fmoc protected amino acids. The procedures used for Fmoc SPPS are outlined below:

Preloaded resin preparation. The preloaded resin was weighed out into a 5 mL syringe fitted with frit and a PTFE stopcock, swollen in DMF for 30 min and then filtered.

Amino acid coupling. DIPEA (11.0 eq.) was added to a solution of amino acid (5.0 eq.) and HCTU (5.0 eq.) dissolved in the minimum volume of DMF and the solution added to the resin. The reaction mixture was gently agitated by rotation for 1 h, and the resin filtered off and washed with DMF (3 × 2 min with rotation).

Fmoc deprotection. A solution of 20% piperidine in DMF was added to the resin and gently agitated by rotation for 2 min. The resin was filtered off and repeated four more times, followed by washes with DMF (5 × 2 min with rotation).

On-resin deprotection of PD protecting group

Following the final **Fmoc deprotection** of the resin-bound peptide, a 5-10 mg portion of resin was washed with 1 mL of thiol-containing deprotection solution (3 × 5 min for the synthesis of CSLYRAG **5**

and SLYCRAG **7**, 9 × 5 min for the synthesis of oxytocin **10**). Deprotection was monitored by mixing 0.5 mL of wash solution used to treat the resin with 0.5 mL DMF and then subjecting this solution to UV-Vis analysis. The PD deprotection washes were repeated until a reading of A_{301} *ca.* 0.1 was reached.* The resin was then washed with DMF (3 × 2 min) and then either subjected to **Cleavage and Isolation** for the synthesis of crude CSLYRAG **5** and crude SLYCRAG **7** or subjected to **On-resin disulfide formation** for the synthesis crude oxytocin **10**.

*A_{301} was selected due to the wash solutions showing the highest UV absorbance at this value. All UV-Vis analyses were taken using a 1 mL 1:1 solution of DMF:Deprotection solution as a blank for baseline correction.

Cleavage and Isolation

Resins containing full synthesised peptides were washed with DCM (3 × 2 min) with rotation. The resin was dried on a vacuum manifold and further dried on a high vacuum line. A microcleavage was then performed thus: to 5-10 mg of dry resin was added a solution of cleavage cocktail 95:2.5:2.5 (v/v) TFA:H_2O:triisopropylsilane, and the resulting mixture was gently agitated by rotation for 60 min. The reaction mixture was drained into ice-cold Et_2O and centrifuged at 6000 rpm at 4 °C for 5 min. The supernatant was carefully decanted and subsequently resuspended, centrifuged and supernatant decanted three more times. The precipitated peptide pellet was then dried under vacuum and dissolved in H_2O:MeCN (1:1) for crude analysis, purification, or lyophilisation.

Large scale Cleavage and Isolation of C(PD)SLYRAG 4

Large scale resin cleavage and subsequent isolation of C(PD)SLYRAG **4** for analysis/downstream *Native chemical ligation experiments* was performed in an identical manner to that described in "**Cleavage and Isolation**" using 100 mg (as opposed to 5-10 mg) of dry resin.

On-resin disulfide formation

On-resin disulfide formation was carried in a manner as described previously.[1] Following **On-resin deprotection of PD protecting group**, the resin was incubated with a solution containing NCS (2 mol equiv., calculated using initial resin loading) in DMF, 100 µL/µmol resin-bound peptide, calculated using initial resin loading) for 15 min with gentle rotation. The resin was then washed with DMF (3 × 2 min) and then subjected to **Cleavage and Isolation** for the synthesis of crude oxytocin **10**.

HPLC analysis

Analytical HPLC was performed on an Agilent 1260 Infinity instrument with a Reprosil Gold 200 C8 (5 µm, 4.6 × 250 mm) column (Dr. Maisch GmbH) and equipped with a 10 mm guard column. Water with 0.1 % (v/v) TFA (solvent A) and acetonitrile with 0.1 % (v/v) TFA (solvent B) were used as the mobile phase at a flow rate of 1 mL/min at room temperature (RT) with detection at 214 nm. A multi-step gradient of 35 min was programmed as follows: 95% A for 2 min (0->2 min), followed by a

linear gradient to 95% B over 18 min (2->20 min), followed by 95% B for an additional 5 min (20->25 min). A linear gradient to 95% A over 5 min (25->30 min), followed by 95% A for an additional 5 min (30->35 min) was used to re-equilibrate the column.

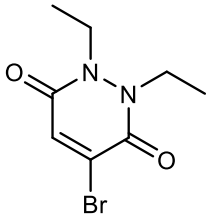
Semi-preparative HPLC

Semi-preparative HPLC was performed using a Dionex instrument with a PDA-100 photodiode array detector and an ASI-100 automated sample injector using a ZORBAX 300SB-C18 (5 µm, 9.4 × 250 mm) column (Agilent). Water with 0.1% (v/v) TFA (solvent A) and acetonitrile with 0.1 % (v/v) TFA (solvent B) were used as the mobile phase at a flow rate of 2 mL/min at room temperature (RT) with detection at 214 and 280 nm. Peptides were separated using a multi-step gradient of 38.5 min and was programmed as follows: 95% A for 3 min (0->3 min), followed by a linear gradient to 95% B over 30 min (3->33 min), followed by 95% B for an additional 2 min (33->35 min). A linear gradient to 95% A over 0.5 min (35->35.5 min), followed by 95% A for an additional 3 min (35.5->38.5 min) was used to re-equilibrate the column. Fractions containing the desired peptide were pooled and lyophilised.

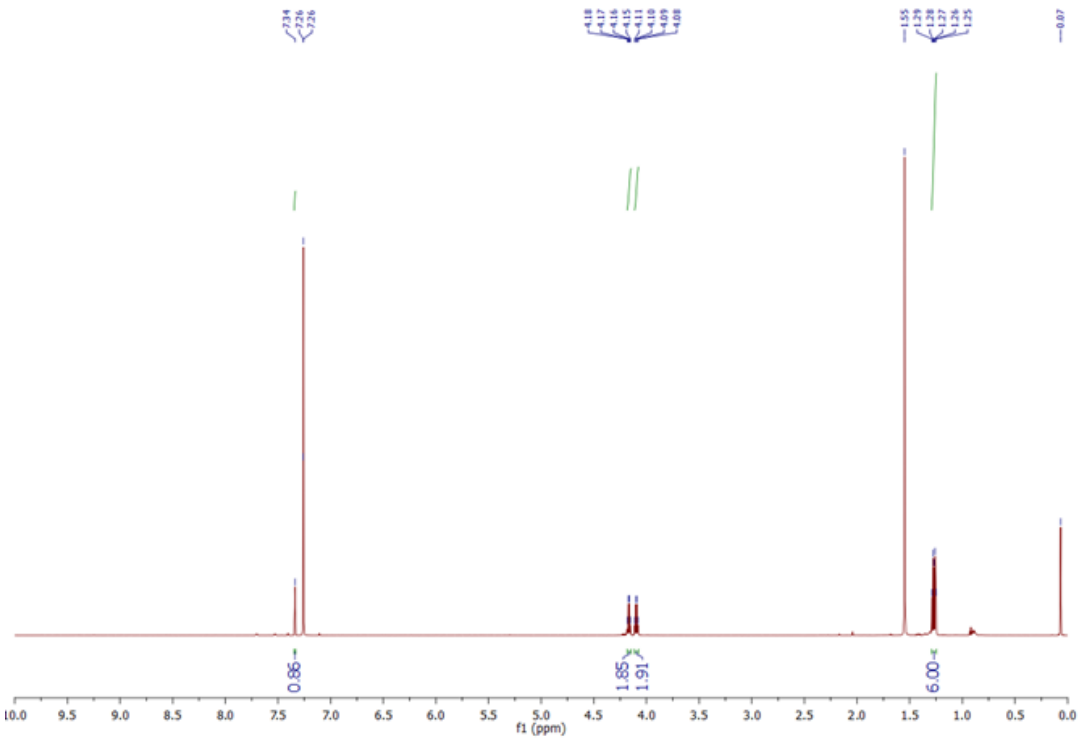
LCMS analysis

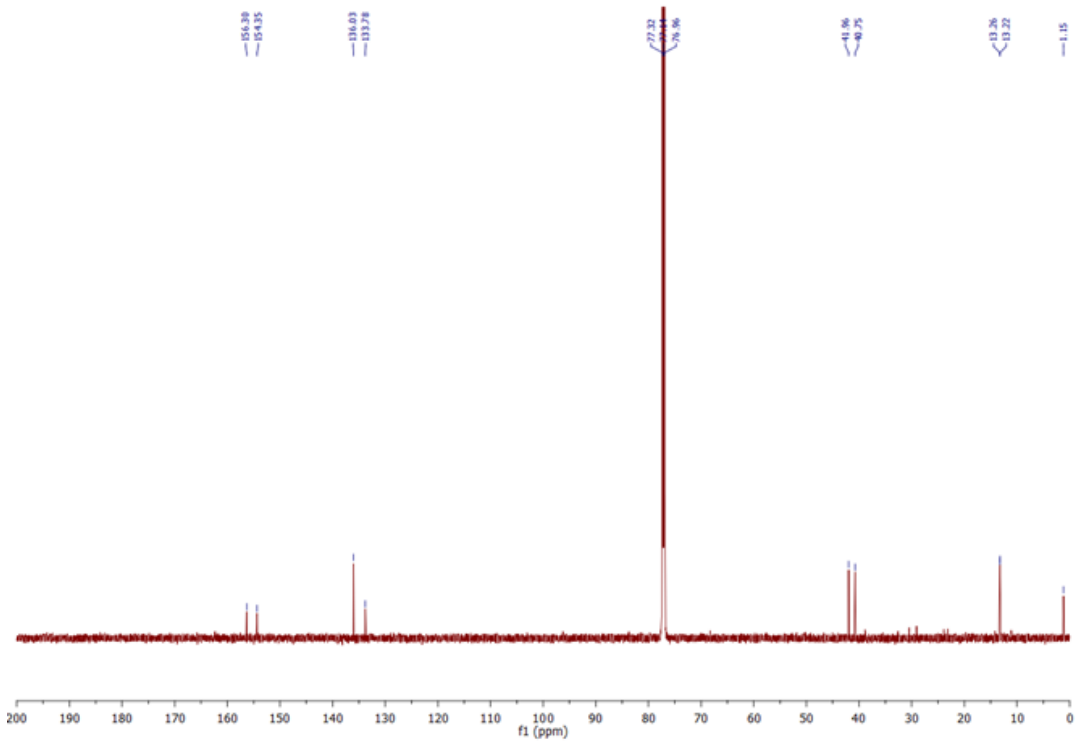
LCMS was performed using a Waters Acquity uPLC connected to Waters Acquity Single Quad Detector (SQD). All samples were run with the following parameters. Column: Hypersil Gold C4, 1.9 µm, 2.1 µm × 50 µm. Wavelength: 254 nm. Mobile Phase: 95:5 Water (0.1% Formic Acid): MeCN (0.1% Formic Acid) Gradient over 4 min (to 5:95 Water (0.1% Formic Acid): MeCN (0.1% Formic Acid). Flow Rate: 0.6 mL/min. MS Mode: ES+. Scan Range: m/z = 250 – 2000. Scan time: 0.25 s. Data obtained in continuum mode. The electrospray source of the MS was operated with a capillary voltage of 3.5 kV and a cone voltage of 50 V. Nitrogen was used as the nebulizer and desolvation gas at a total flow of 600 L/h. Ion series were generated by integration of the total ion chromatogram (TIC) over the appropriate range.

Bromo-1,2-diethyl-1,2-dihydropyridazine-3,6-dione ***2***[2]

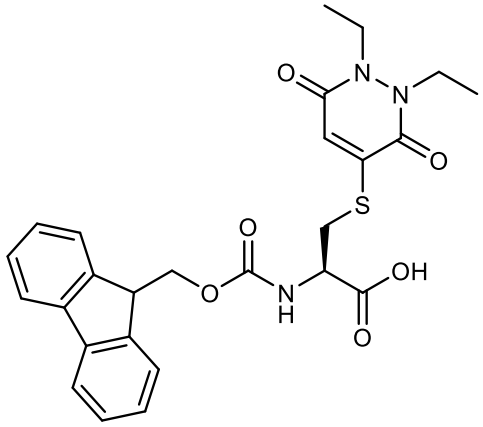


Bromomaleic anhydride (80 µL, 0.86 mmol) was added to a stirred solution of di-*tert*-butyl-1,2-diethyl hydrazine[3] (270 mg, 0.95 mmol) in glacial acetic acid (5 mL) and the reaction mixture was stirred under reflux (*ca.* 130 °C) for 24 h. After this time, the solvent was removed *in vacuo* with toluene co-evaporation (3 × 20 mL) as an azeotrope and then with chloroform co-evaporation (3 × 30 mL) as an azeotrope. The crude residue was purified by column chromatography (20-80% hexane/EtOAc) to afford 4-bromo-1,2-diethyl-1,2-dihydropyridazine-3,6-dione **2** (180 mg, 0.77 mmol, 89% yield) as a yellow oil. ^{1}H NMR (700 MHz, $CDCl_3$) δ 7.34 (s, 1H), 4.17 (q, *J* = 7.0 Hz, 2H), 4.10 (q, *J* = 7.0 Hz, 2H), 1.29-1.25 (m, 6H); ^{13}C NMR (176 MHz, $CDCl_3$) δ 156.3 (C), 154.4 (C), 136.0 (CH), 133.8 (C), 42.0 (CH_2), 40.8 (CH_2), 13.4 (CH_3), 13.2 (CH_3); IR (thin film) 3046, 2982, 1712, 1615, 1591 cm^{-1}.

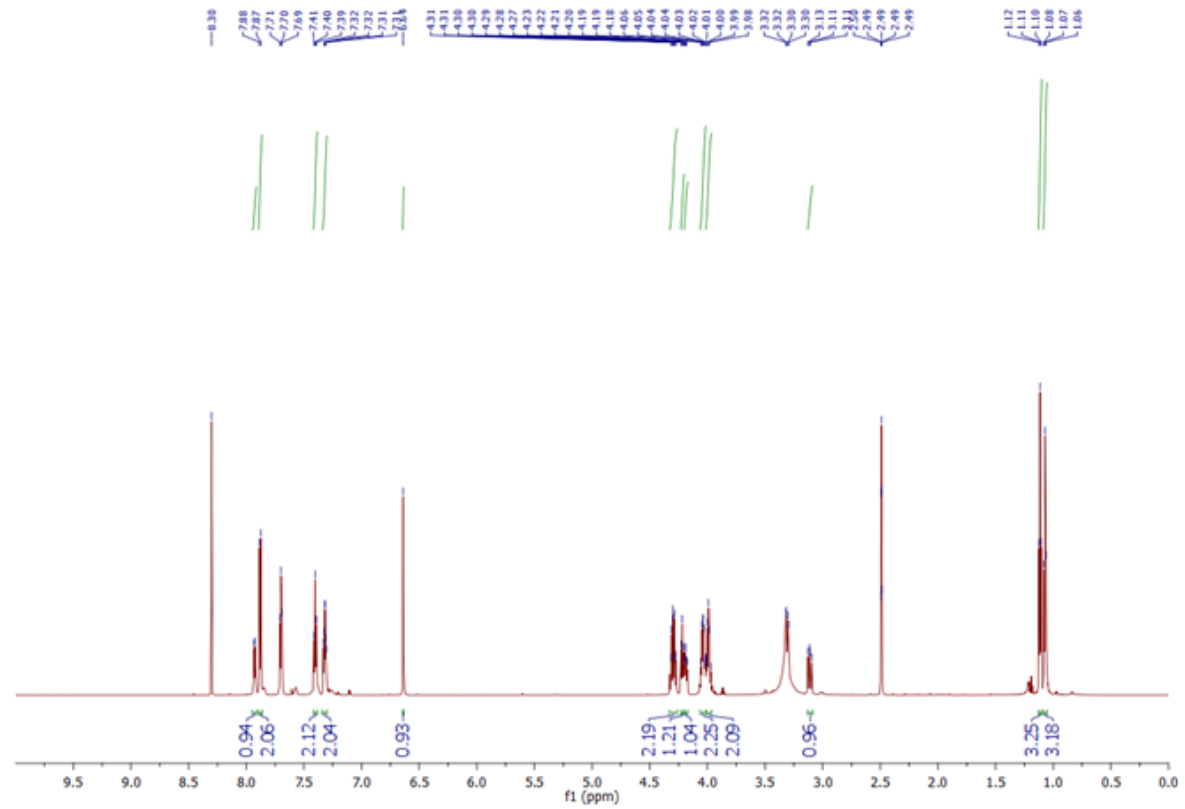


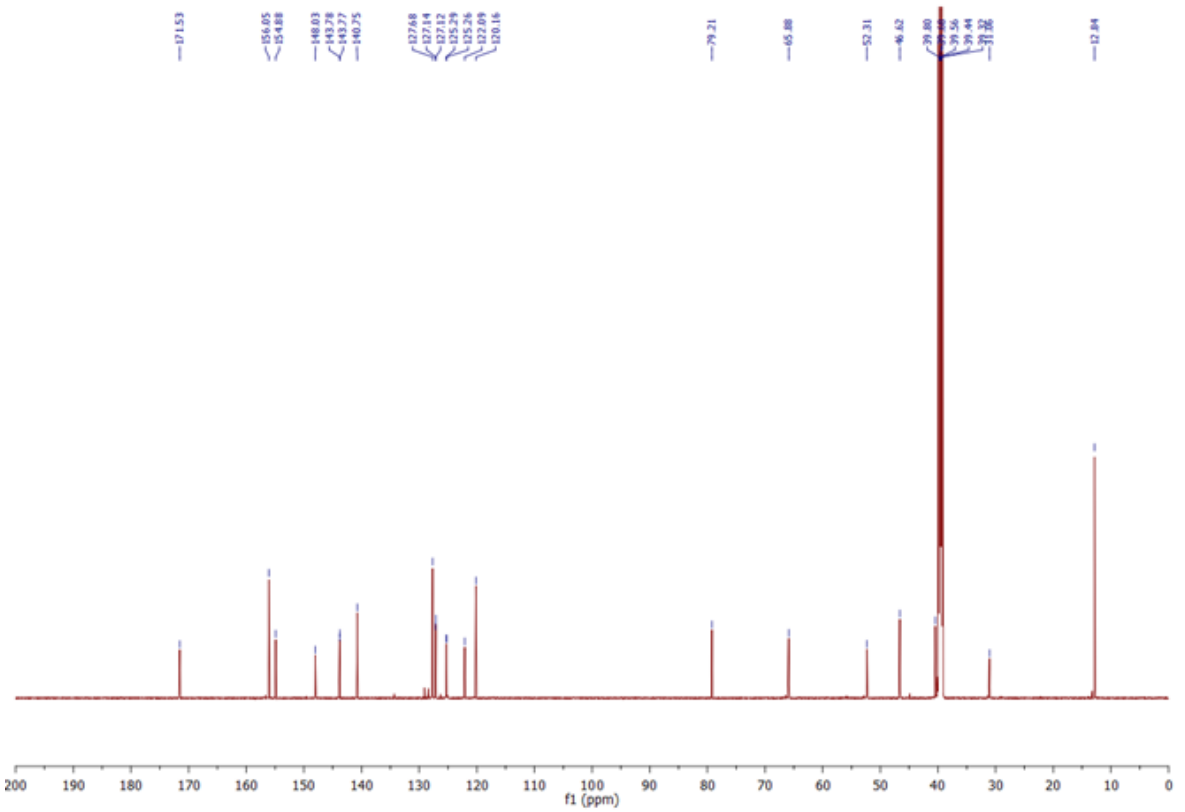

156.39
154.35
136.03
133.78
77.32
77.00
76.96
41.96
40.75
13.26
13.22
1.15
200 190 180 170 160 150 140 130 120 110 100 90 80 70 60 50 40 30 20 10 0
f1 (ppm)

*Fmoc-Cys(PD)-OH **3***

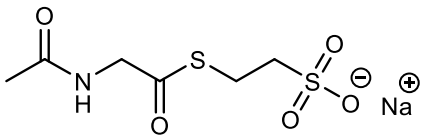


Fmoc-Cys-OH **1** [4] (451 mg, 1.31 mmol) and NaOAc (292 mg, 3.65 mmol) were dissolved in MeOH (6 mL) and stirred for 20 min (monitored by TLC) under argon. Bromopyridazinedione **2** (327 mg, 1.32 mmol) was added and the solution was stirred for 2 h at 21 °C. After this, all solvents were removed *in vacuo*, the crude material was suspended in EtOAc (25 mL) and washed with 10% citric acid (10 mL). The organic layer was dried over $MgSO_4$ and the solvent removed *in vacuo*. The crude residue was purified by column chromatography (0-10% MeOH, 1% AcOH in DCM) to afford Fmoc-Cys(PD)-OH **1** as a white solid: m.p. 158 °C; (312 mg, 0.61 mmol, 71%). ^{1}H NMR (700 MHz, DMSO) δ 7.92 (d, *J* = 7.0 Hz, 1H), 7.88 (d, *J* = 7.0 Hz, 2H), 7.70 (t, *J* = 7.0 Hz, 2H), 7.40 (t, *J* = 7.0 Hz, 2H), 7.33-7.30 (m, 2H), 6.64 (s, 1H), 4.30 (q, 2H), 4.22 (s, 1H), 4.19 (s, 1H), 4.04 (q, 2H), 3.99 (q, 2H), 3.11 (t, 1H), 1.10 (t, *J* = 7.0, 3H) 1.07 (t, *J* = 7.0 Hz, 3H); ^{13}C NMR (176 MHz, DMSO) δ 171.5 (C), 156.0 (C), 154.9 (C), 148.0 (C), 143.8 (C), 140.8 (C), 127.7 (CH), 127.1 (CH), 125.3 (CH), 122.1 (CH), 120.2 (CH), 65.9 (CH_2), 52.3 (CH), 46.6 (CH), 40.5 (CH_2), 31.0 (CH_2), 12.8 (CH_3). LRMS (ES+) 510 (100), $[M+H]^+$); HRMS (ES+) calcd for $C_{26}H_{27}N_3O_6S^+$ $[M+H]^+$ 510.1693, observed 510.1692.

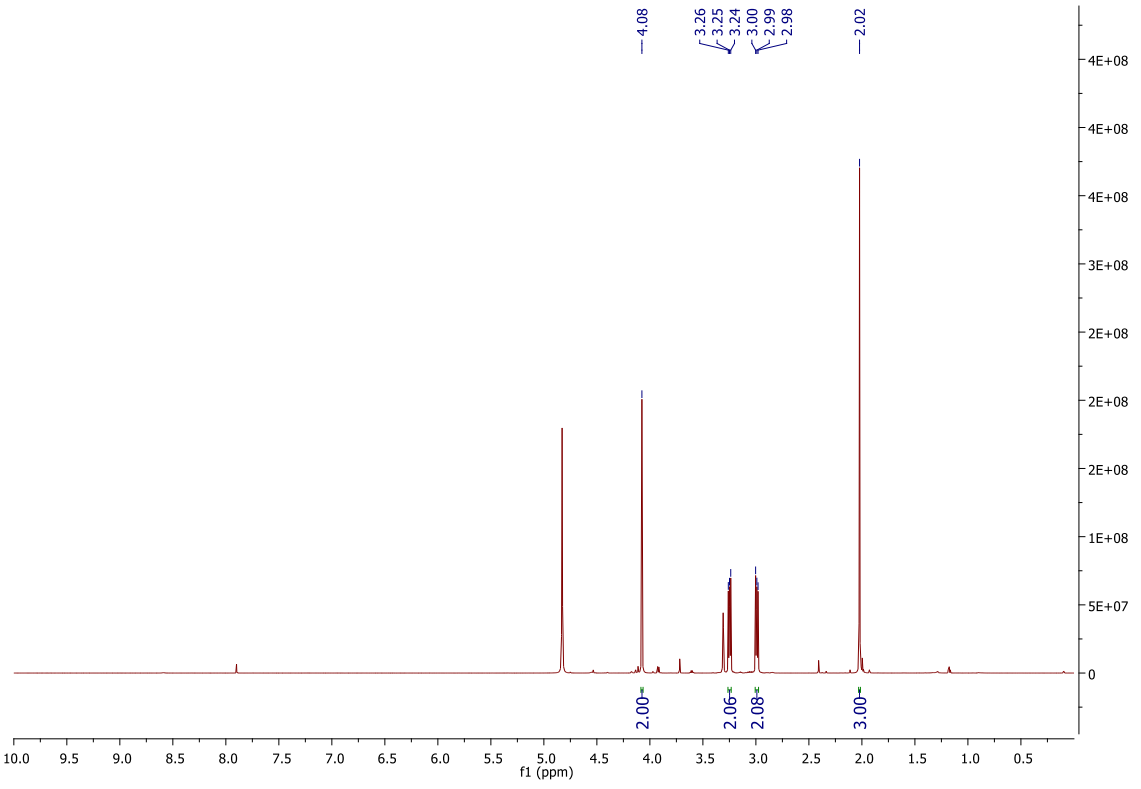

0.94
2.06
2.12
2.04
0.93
2.19
1.21
1.04
2.25
2.09
0.96
3.25
3.18
f1 (ppm)

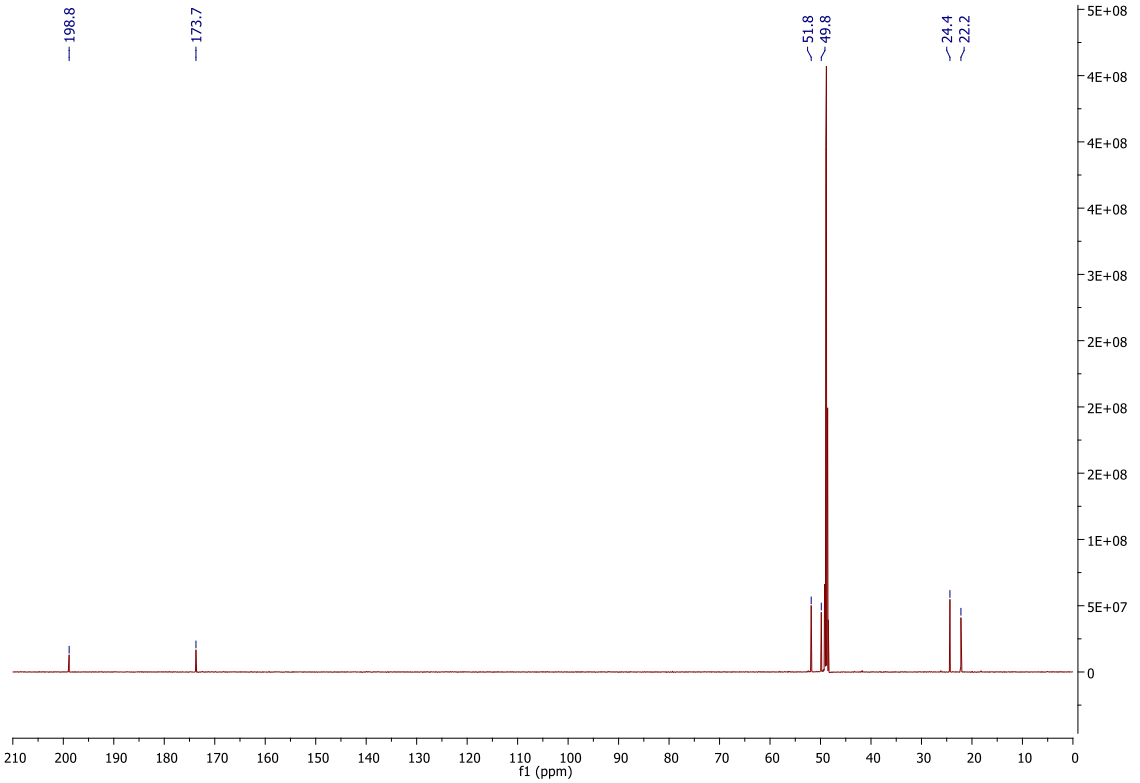

f1 (ppm)

N-Acetylglycine thioester ***11***



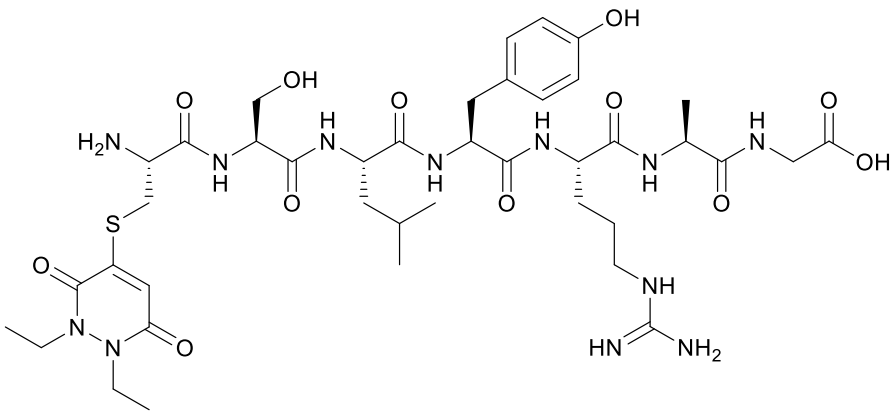
N-Acetylglycine (239 mg, 2.04 mmol) and 2-ethoxy-1-ethoxycarbonyl-1,2-dihydroquinoline (604 mg, 2.45 mmol) were dissolved in MeCN (12 mL) and DMF (2 mL) and the reaction mixture was stirred at 21 °C for 30 min. To this mixture, after this time, was added sodium 2-mercaptoethanesulfonate (335 mg, 2.04 mmol) and the resultant reaction mixture was stirred at 75 °C for 18 h. All solvents were then removed *in vacuo*. The crude residue was purified by column chromatography (0-20% MeOH in DCM) to afford sodium 2-((acetylglycyl)thio)ethane-1-sulfonate 11 (139 mg,0.53 mmol, 37%) as a white solid: m.p. 240 °C (decomposition); ^{1}H NMR (700 MHz, CD_3OD) δ 4.08 (s, 2H), 3.26-3.24 (m, 2H), 3.00-2.98 (m, 2H), 2.02 (s, 3H); ^{13}C NMR (176 MHz, CD_3OD) δ 198.8 (C), 173.7 (C), 51.8 (CH_2), 49.8 (CH_2), 24.4 (CH_2), 22.2 (CH_3); IR (solid) 3266, 3071, 2928 cm^{-1}; LRMS (ES+) 144 (100), 242 (12) $[M+H]^+$); HRMS (ES+) calcd for $C_6H_{12}NO_5S_2^+$ $[M+H]^+$ 242.0151, observed 242.0153.

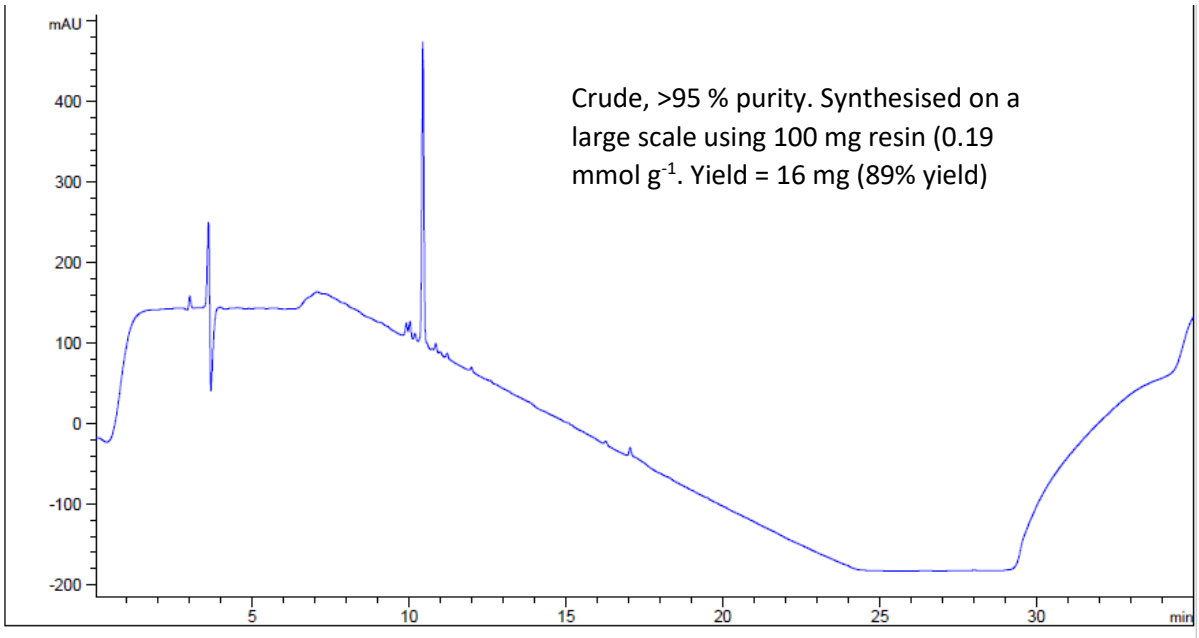



198.8
173.7
51.8
49.8
24.4
22.2
5E+08
4E+08
4E+08
4E+08
3E+08
2E+08
2E+08
2E+08
1E+08
5E+07
0
210
200
190
180
170
160
150
140
130
120
110
100
90
80
70
60
50
40
30
20
10
0
f1 (ppm)

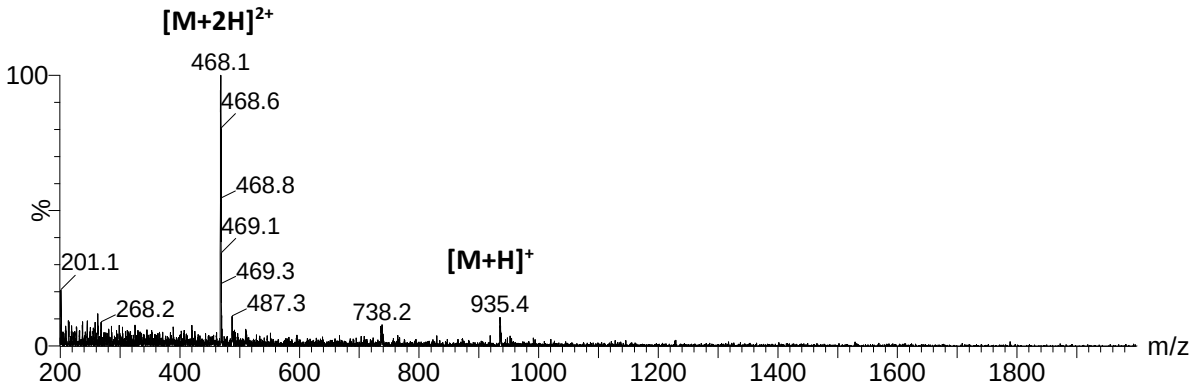
Peptides

C(PD)SLYRAG ***4***

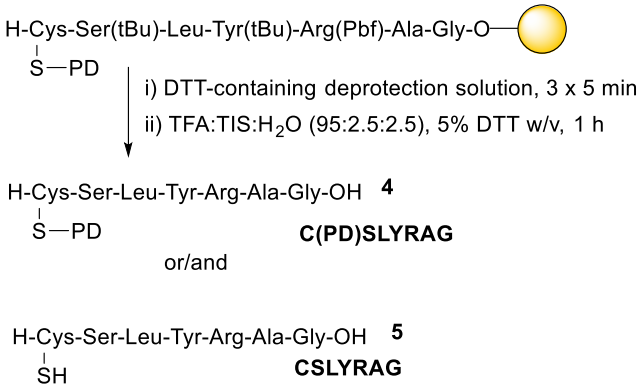




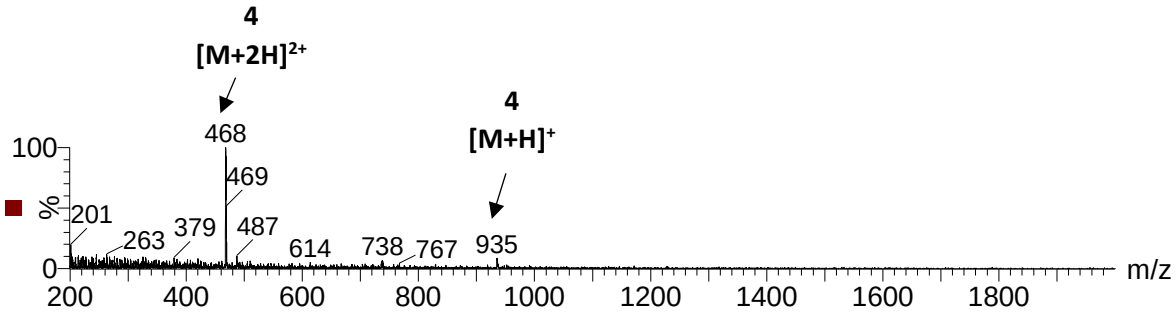
Crude, >95 % purity. Synthesised on a large scale using 100 mg resin (0.19 mmol g^{-1}. Yield = 16 mg (89% yield)



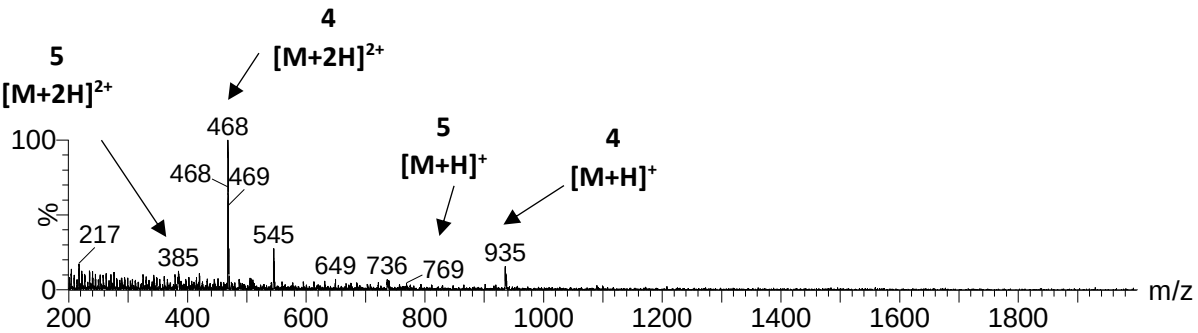
*Deprotection studies for the synthesis CSLYRAG **5***



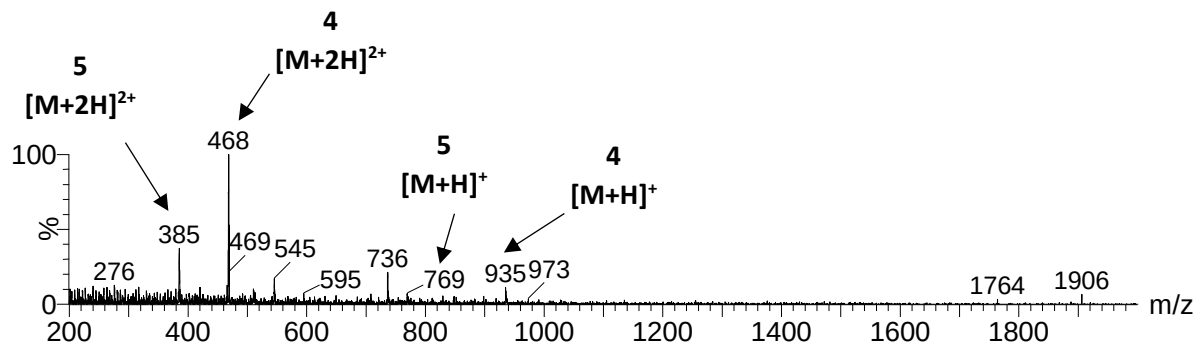
No DTT-containing deprotection solution used



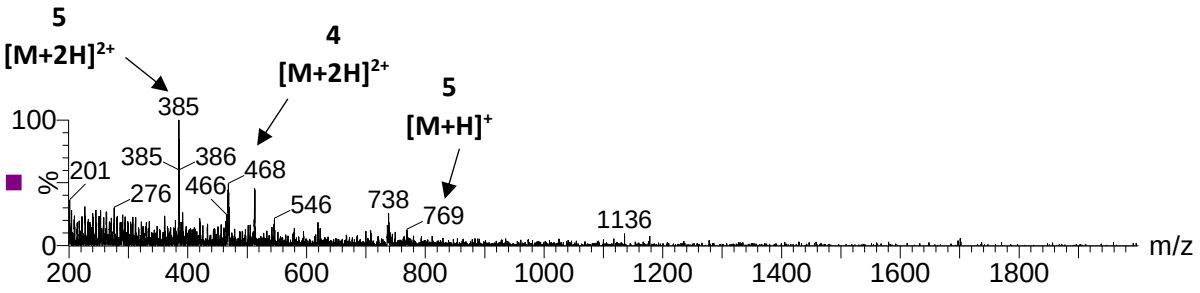
PD deprotection using 5% DTT w/v in DMF, 0.1M NMM



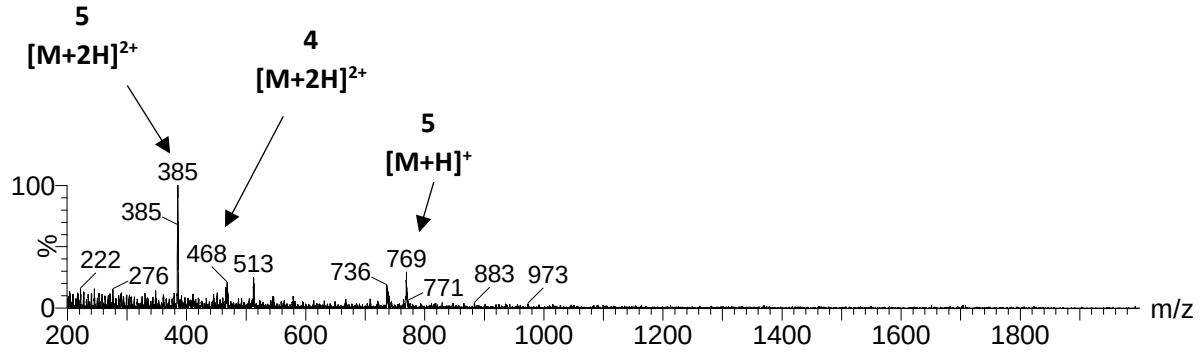
PD deprotection using 5% DTT w/v in 19:1 DMF:PB



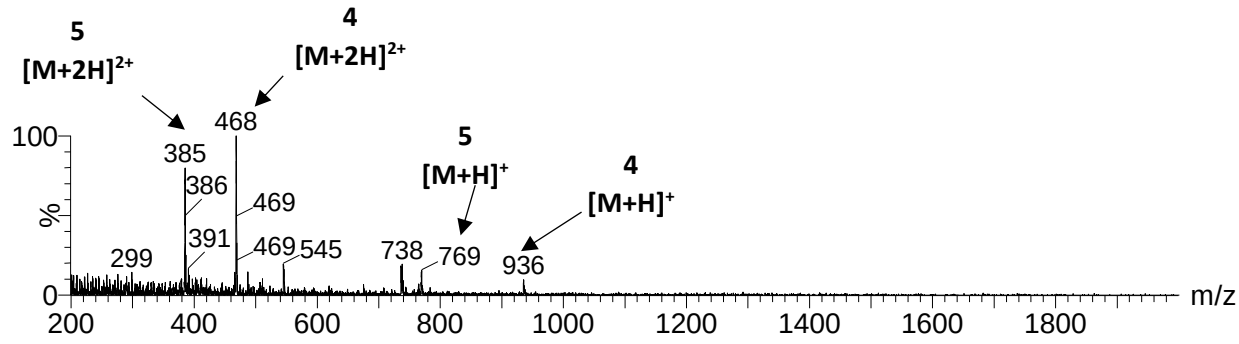
PD deprotection using 5% DTT w/v in 9:1 DMF:PB



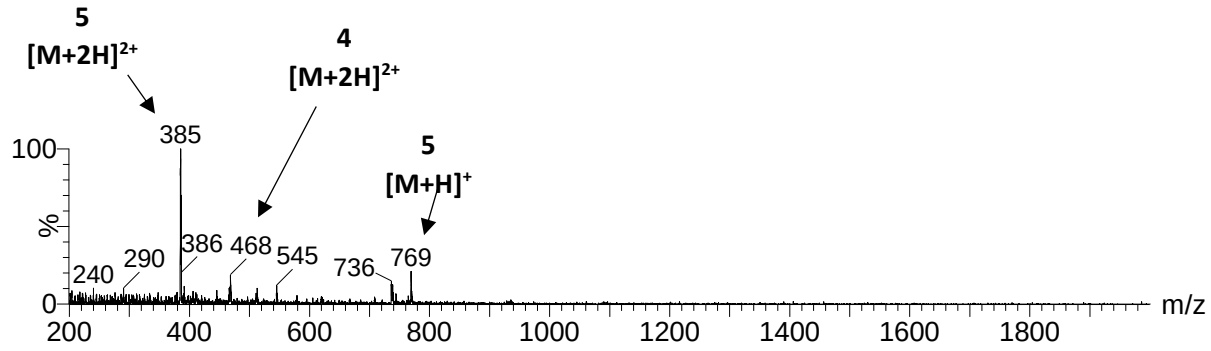
PD deprotection using 5% DTT w/v in 8:2 DMF:PB



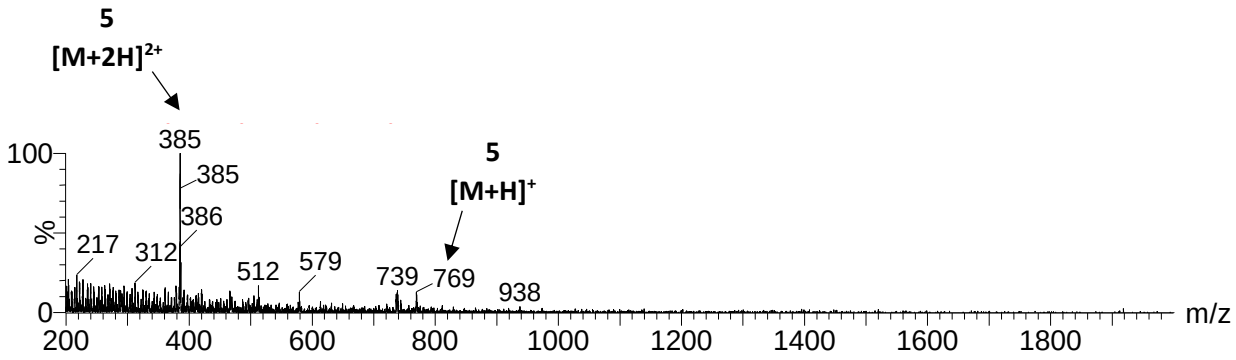
PD deprotection using 10% DTT w/v in 19:1 DMF:PB



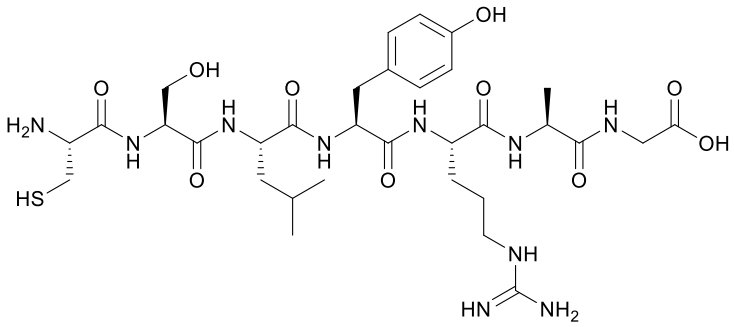
PD deprotection using 10% DTT w/v in 9:1 DMF:PB

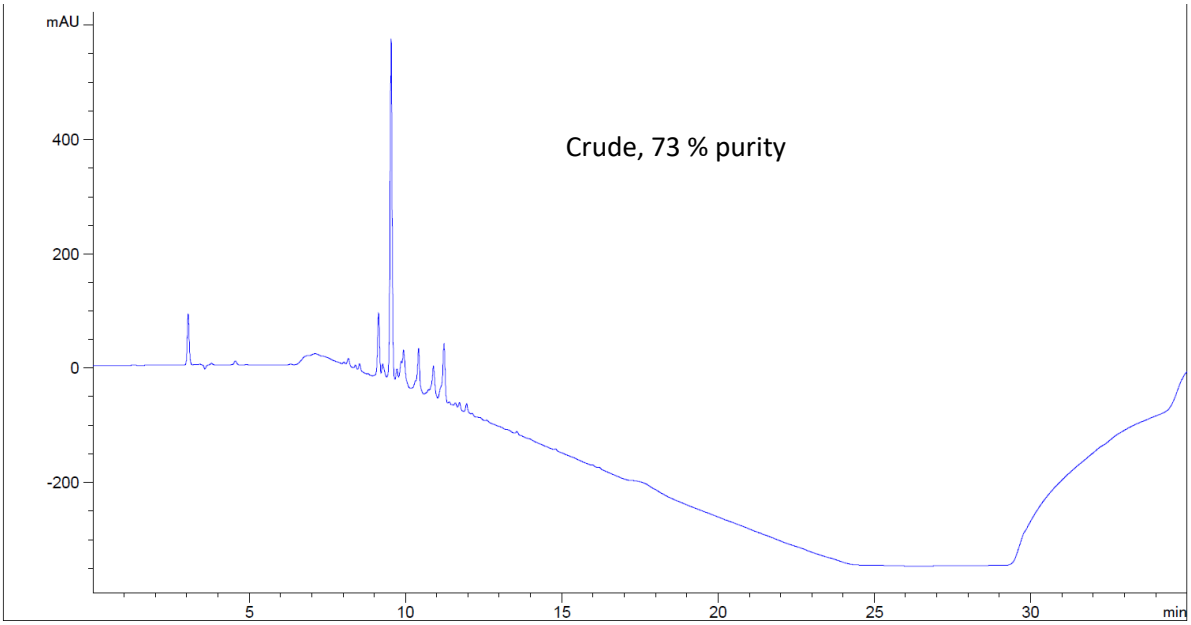


PD deprotection using 10% DTT w/v in 8:2 DMF:PB

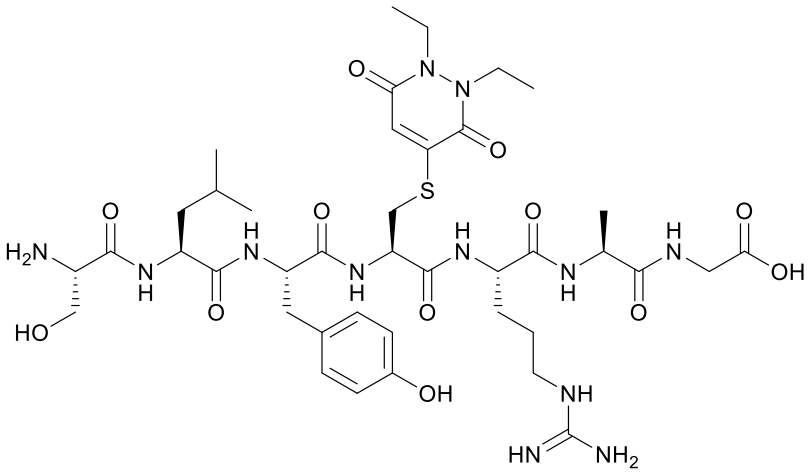


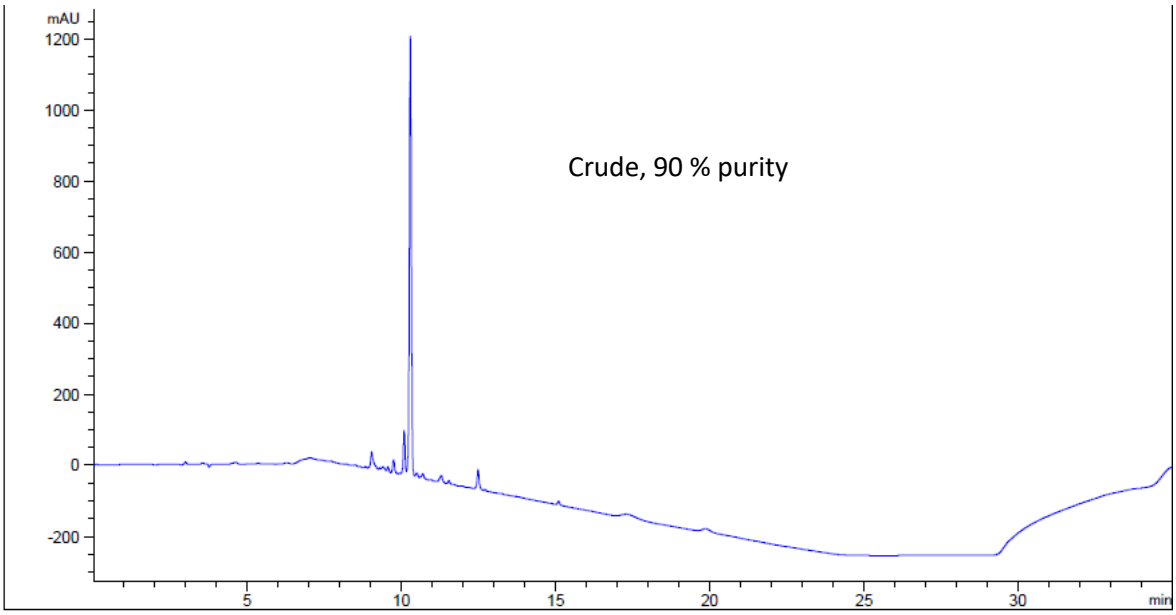
CSLYRAG ***5***

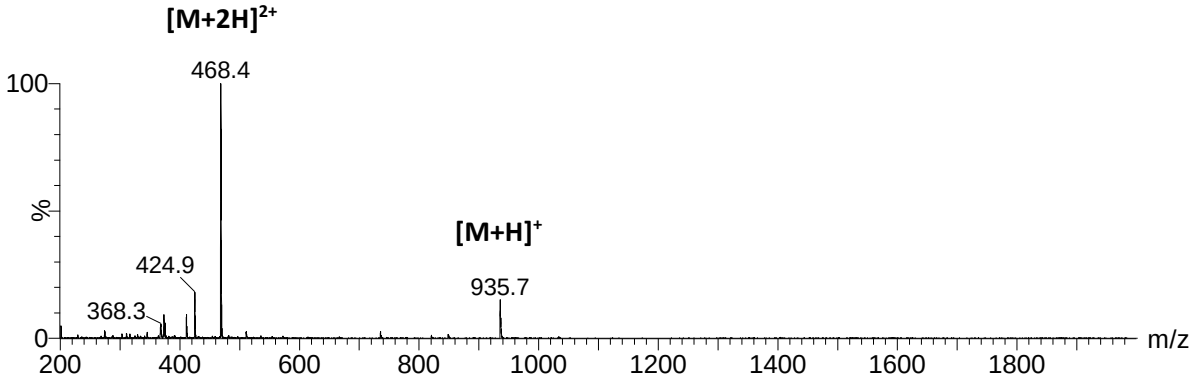

OH
OH
O
O
O
O
O
O
H_2N
N
H
N
H
N
H
N
H
N
H
OH
HS
NH
HN
NH_2


mAU
400
200
0
-200
Crude, 73 % purity
5
10
15
20
25
30
min

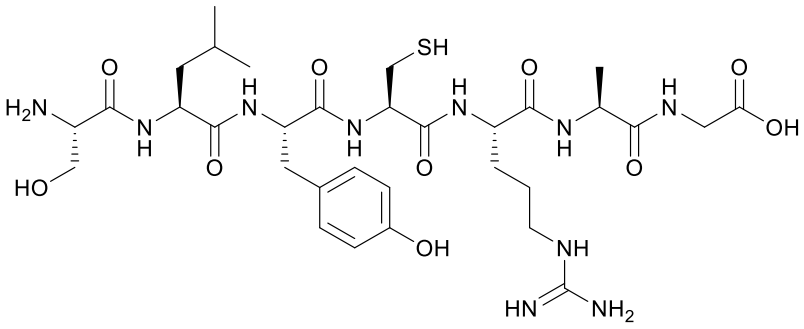
SLYC(PD)RAG ***6***

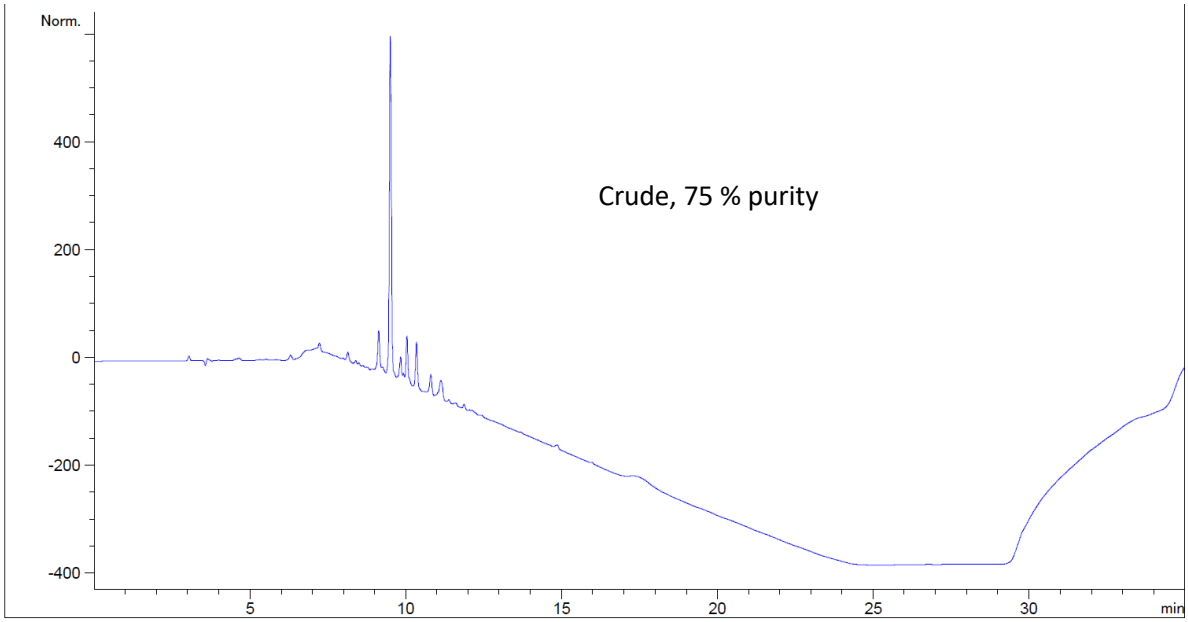


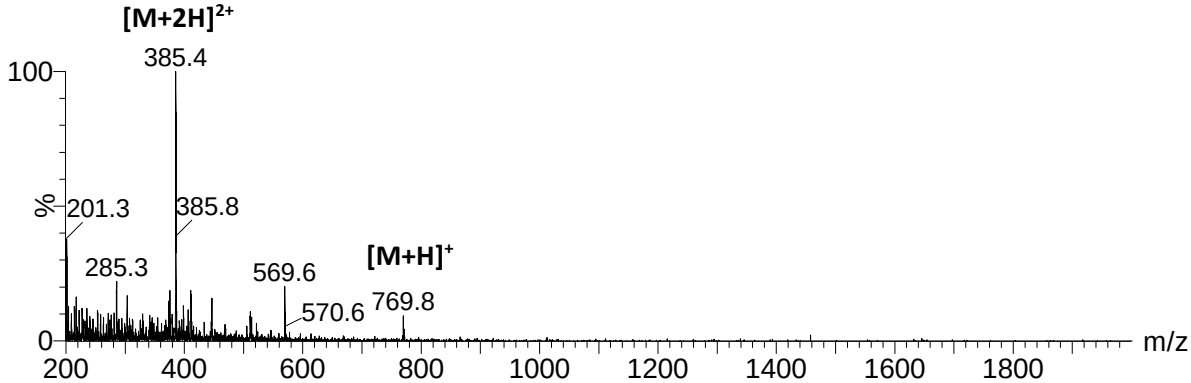

mAU
1200
1000
800
600
400
200
0
-200
5
10
15
20
25
30
min
Crude, 90 % purity


[M+2H]2+
468.4
100
%
[M+H]+
424.9
935.7
368.3
0
200
400
600
800
1000
1200
1400
1600
1800
m/z

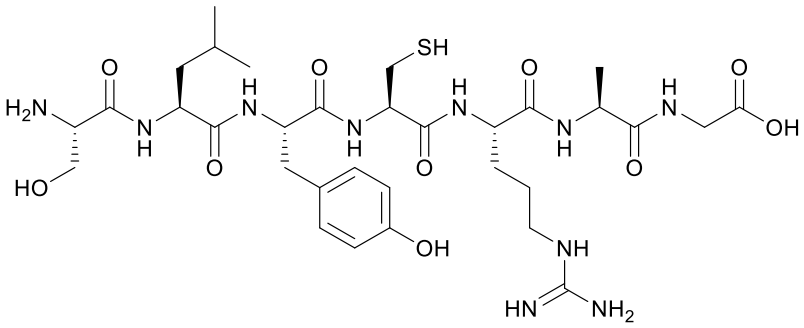
*SLYCRAG **7** synthesised using Fmoc-Cys(PD)-OH **3***

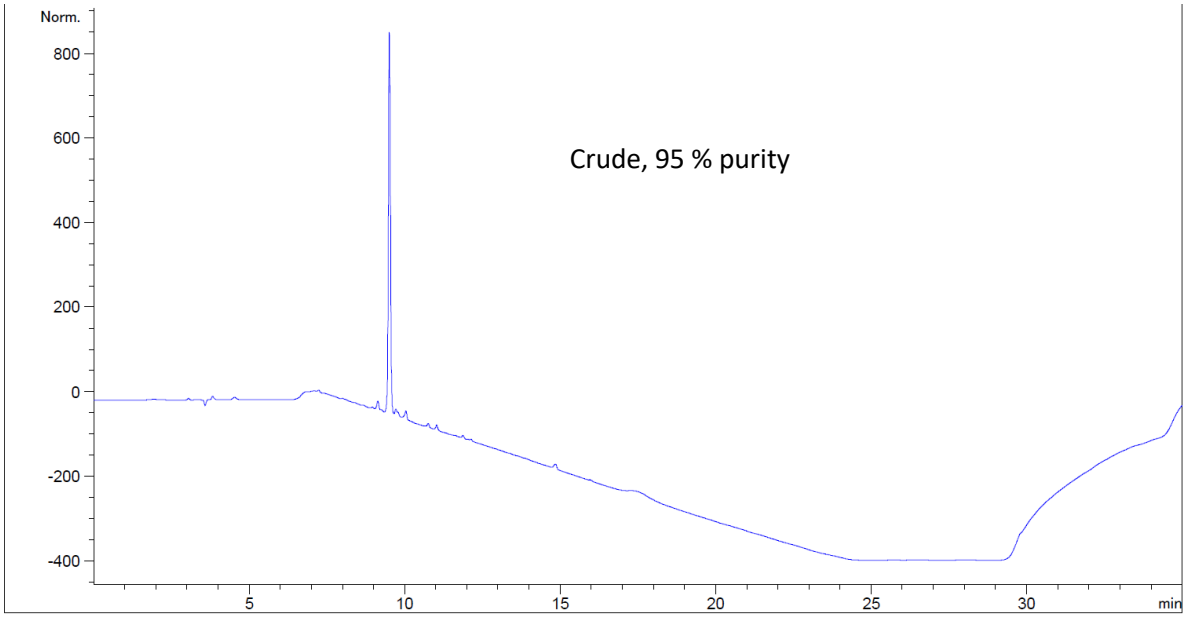


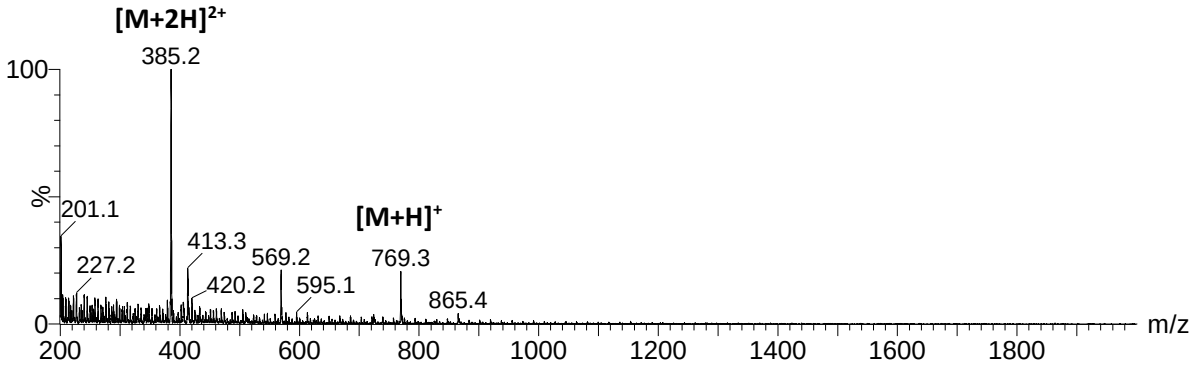




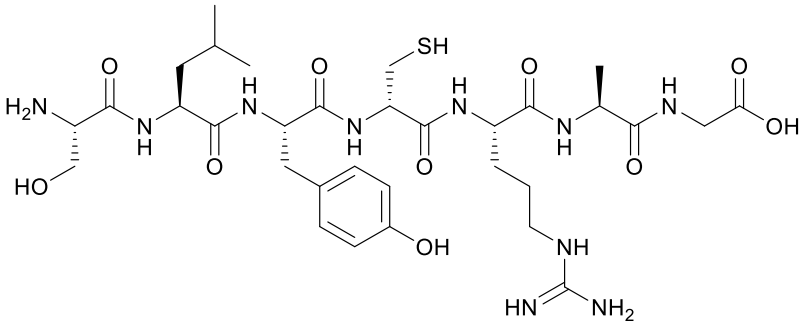
*SLYCRAG **7** synthesised using Fmoc-L-Cys(Trt)-OH*

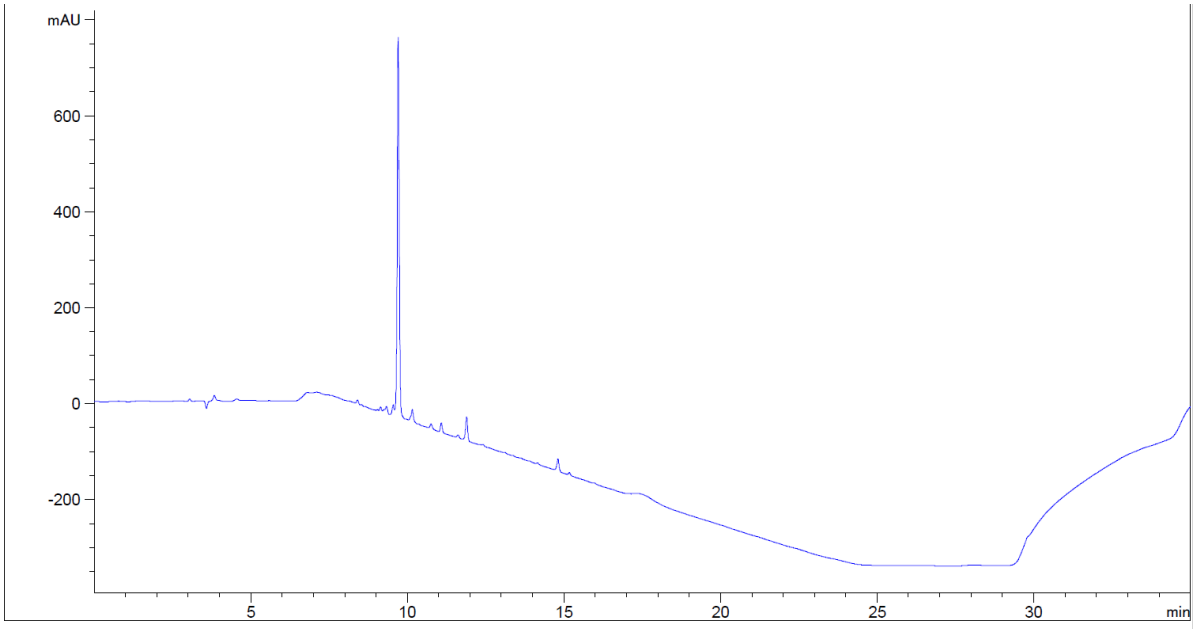


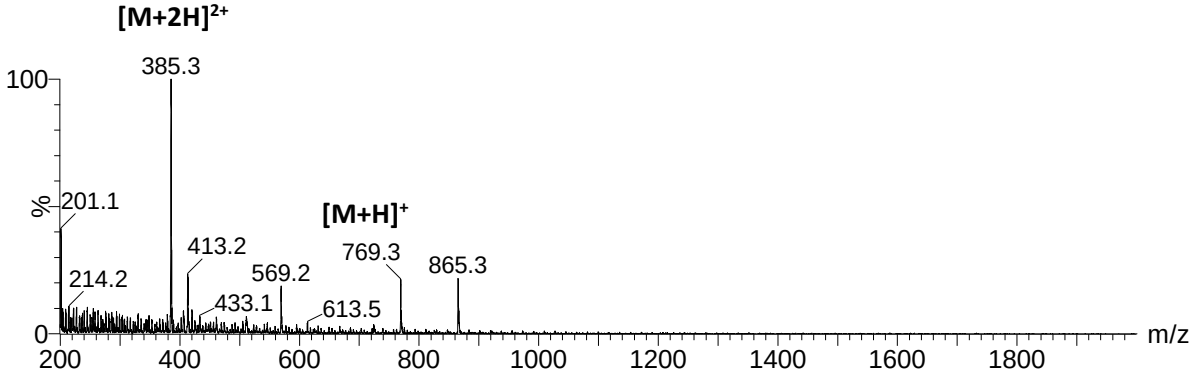




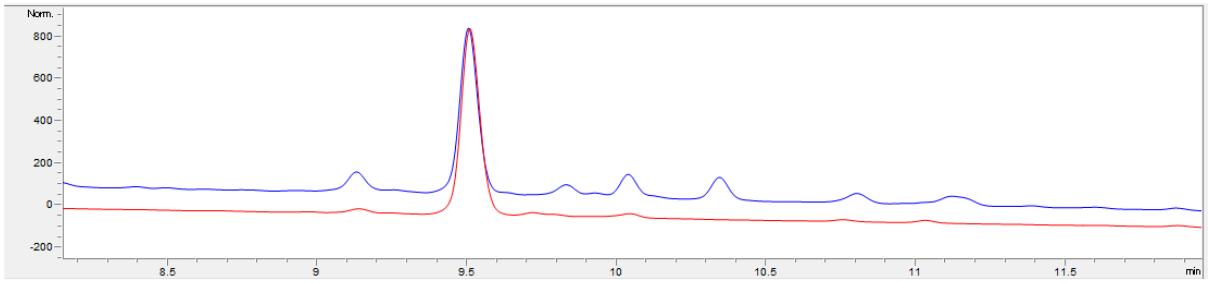
*SLYCRAG **7-D** synthesised using Fmoc-D-Cys(Trt)-OH*





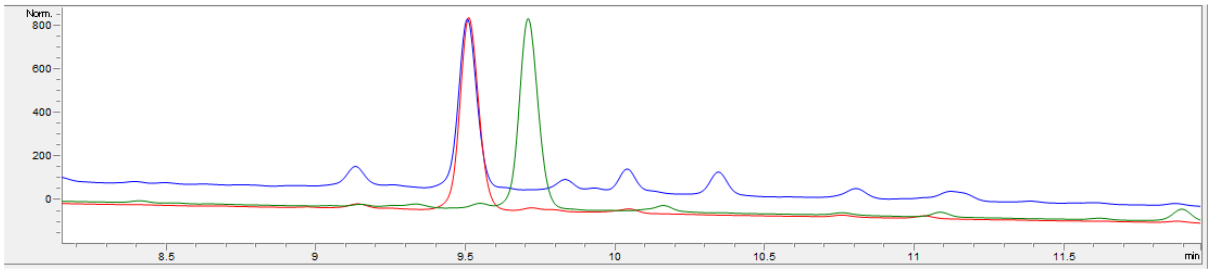


Comparison of HPLC data for racemisation study



Blue = SLYCRAG synthesised using Fmoc-Cys(PD)-OH

Red = SLYCRAG synthesised using Fmoc-L-Cys(Trt)-OH

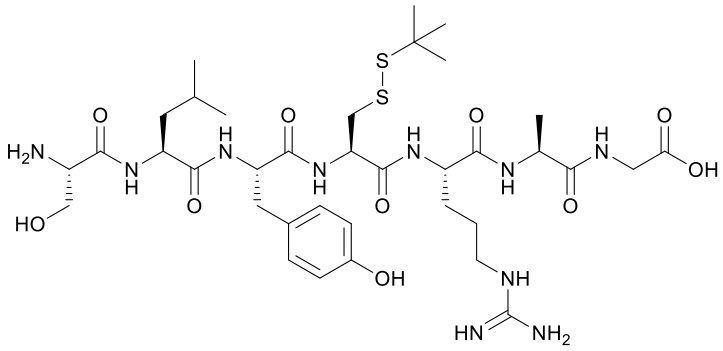


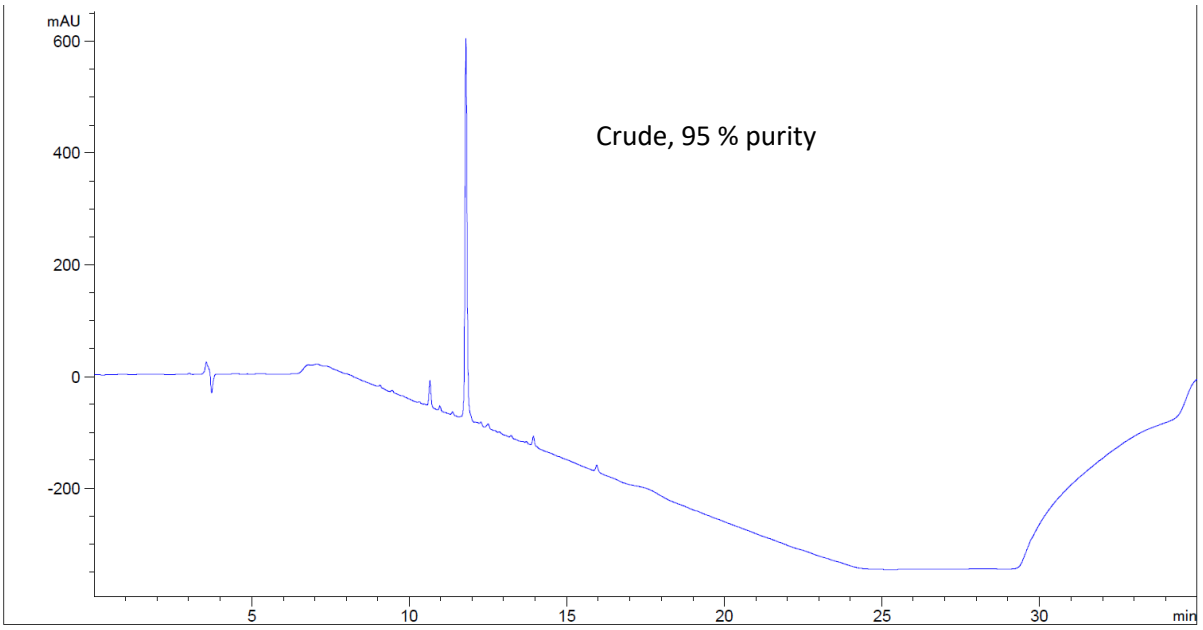
Blue = SLYCRAG synthesised using Fmoc-Cys(PD)-OH

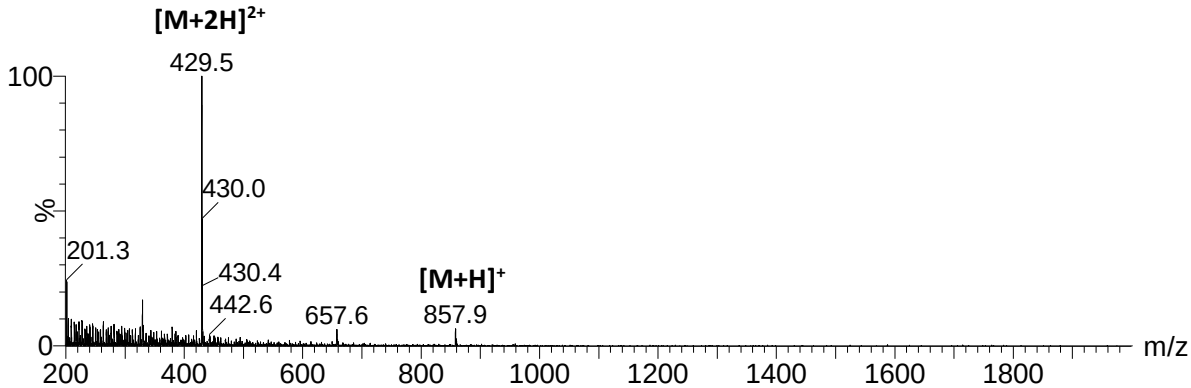
Red = SLYCRAG synthesised using Fmoc-L-Cys(Trt)-OH

Green = SLYCRAG synthesised using Fmoc-D-Cys(Trt)-OH

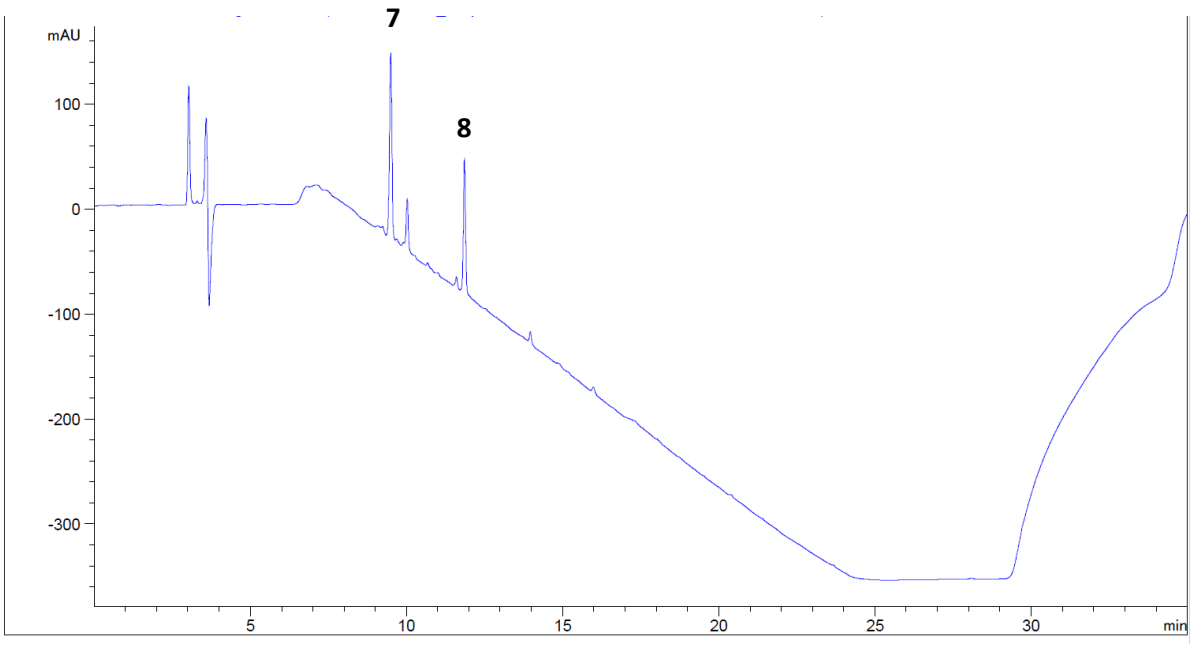
SLYC(StBu)RAG ***8***

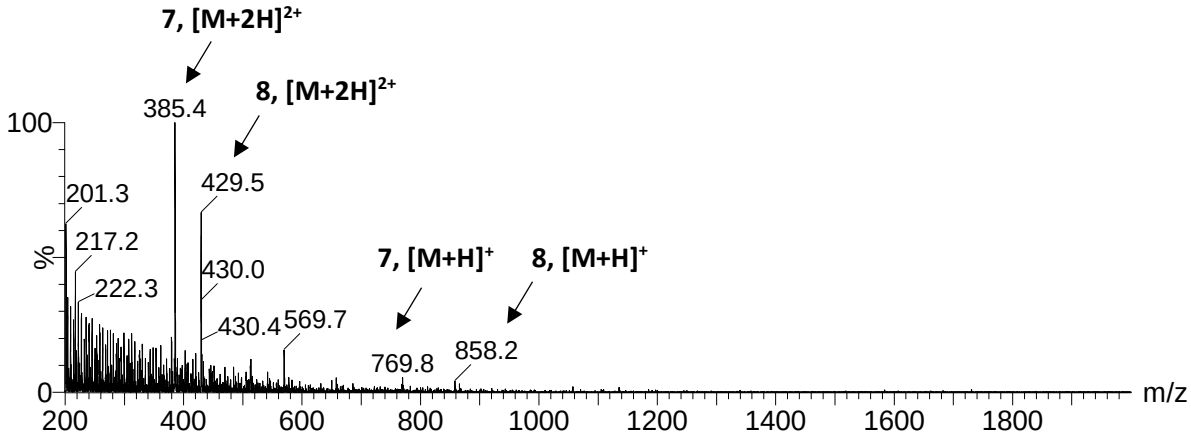




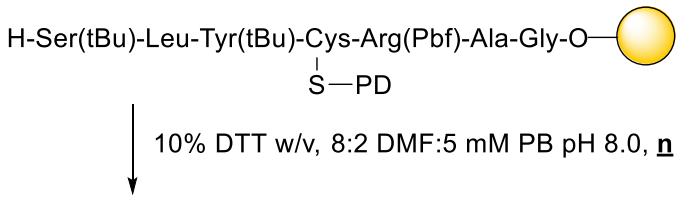


Attempted deprotection of SLYC(StBu) ***8***

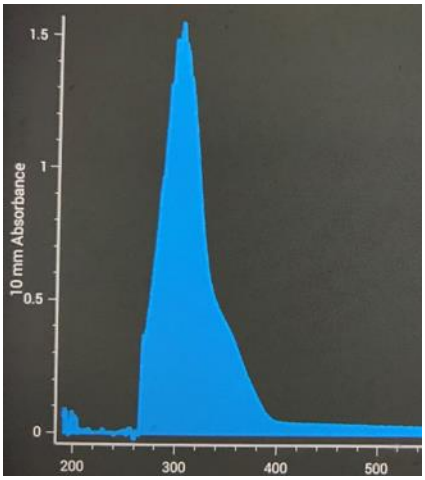




Example of UV-Vis analysis of wash solutions for monitoring on-resin PD deprotection



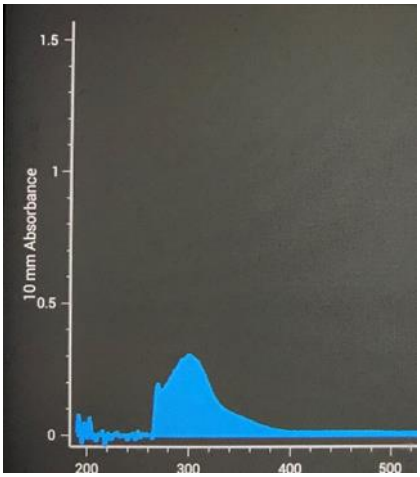
UV-Vis analysis of wash solution



n = 1

A_{280} = 0.71 A_{301} = 1.49

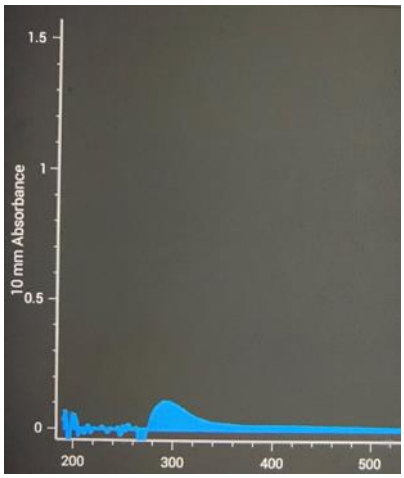
A_{330} = 0.60 A_{345} = 0.43



n = 2

A_{280} = 0.20 A_{301} = 0.30

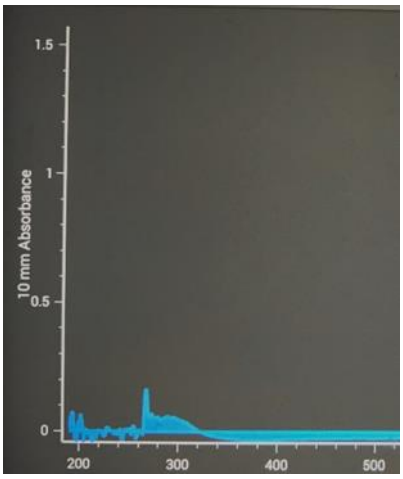
A_{330} = 0.11 A_{345} = 0.08



n = 3

A_{280} = 0.06 A_{301} = 0.10

A_{330} = 0.03 A_{345} = 0.02

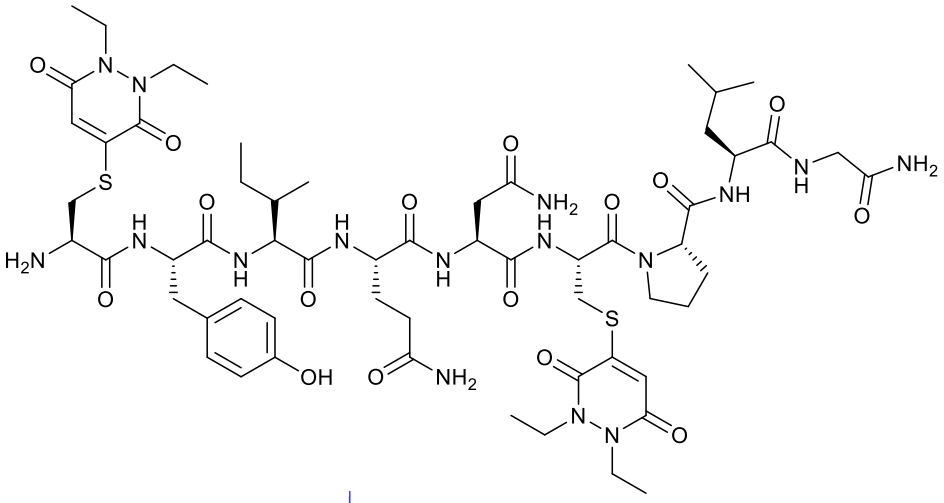


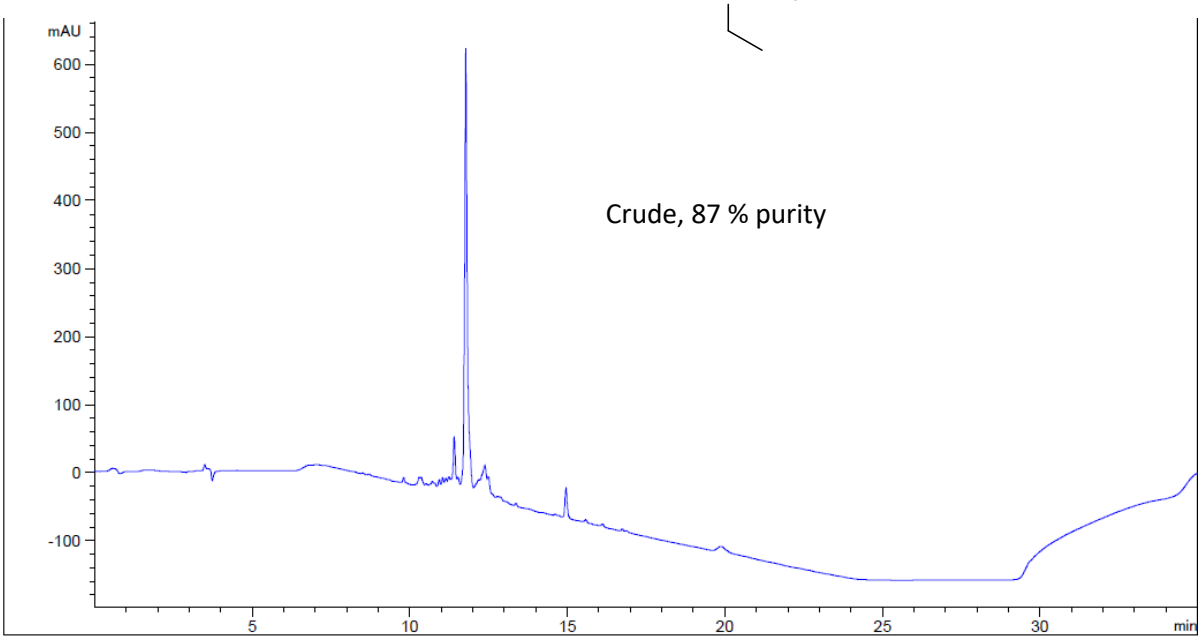
Example of additional wash (n = 4)

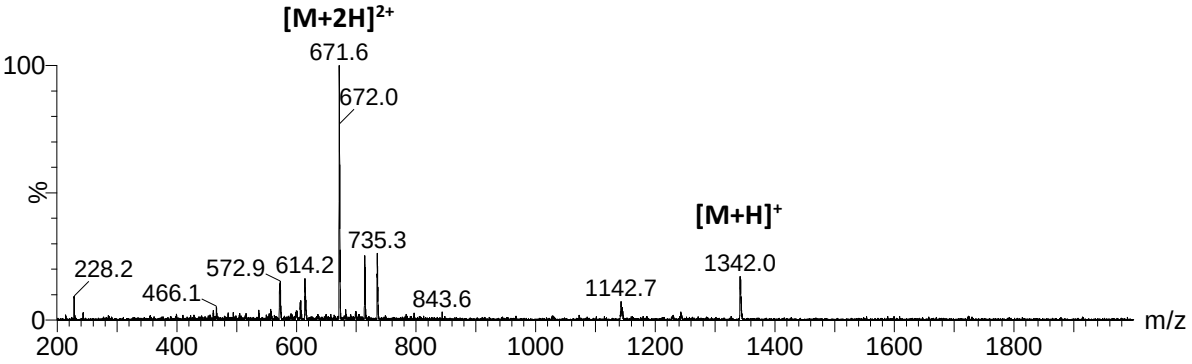
A_{280} = 0.06 A_{301} = 0.05

A_{330} = 0.00 A_{345} = 0.00

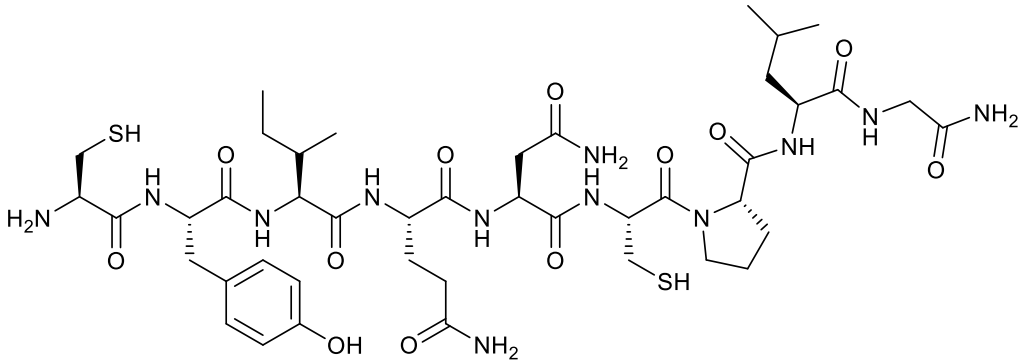
*PD protected oxytocin **9***

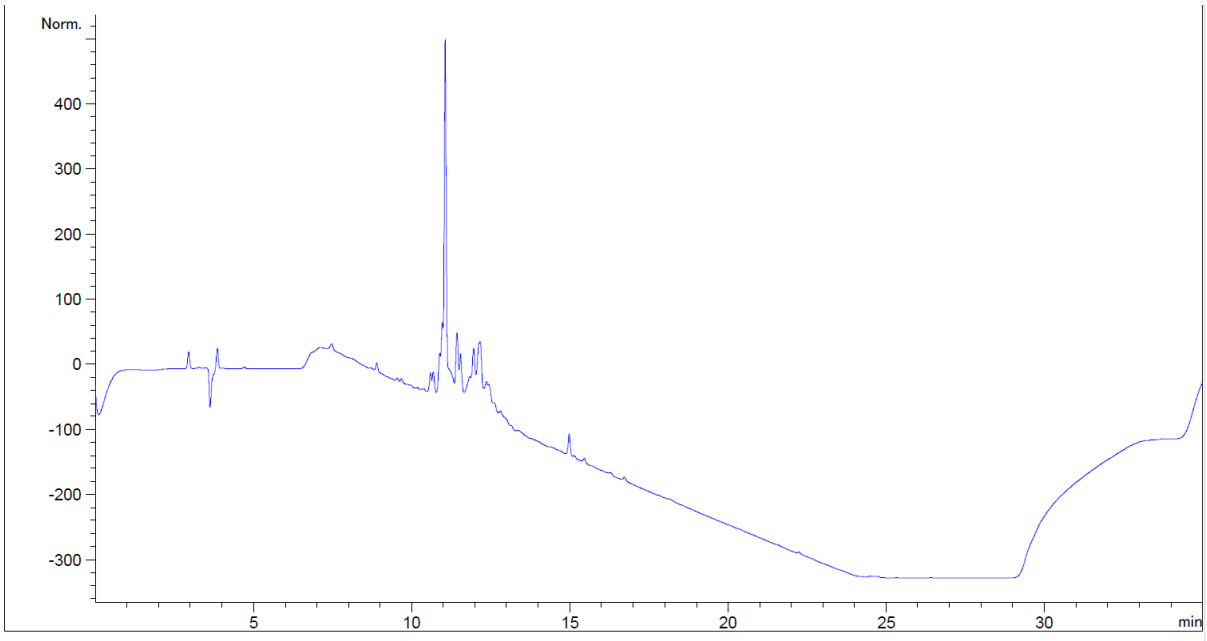


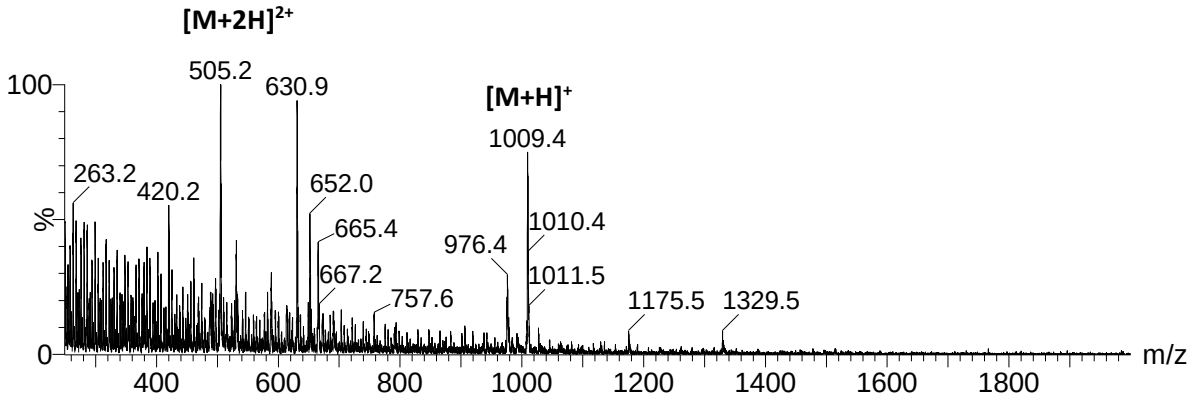




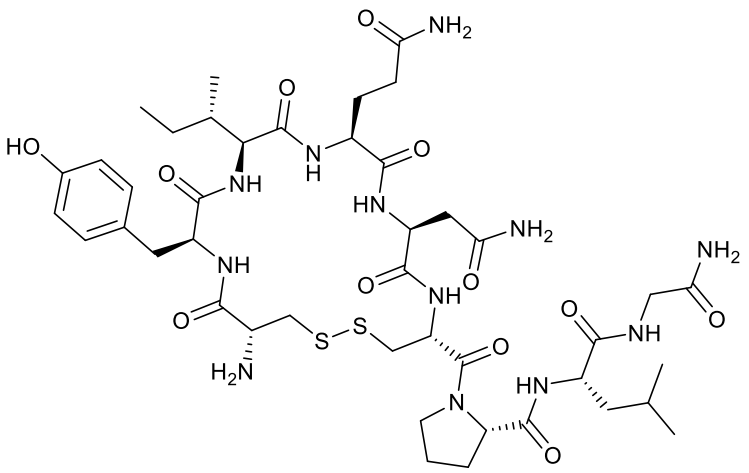
Linear deprotected oxytocin

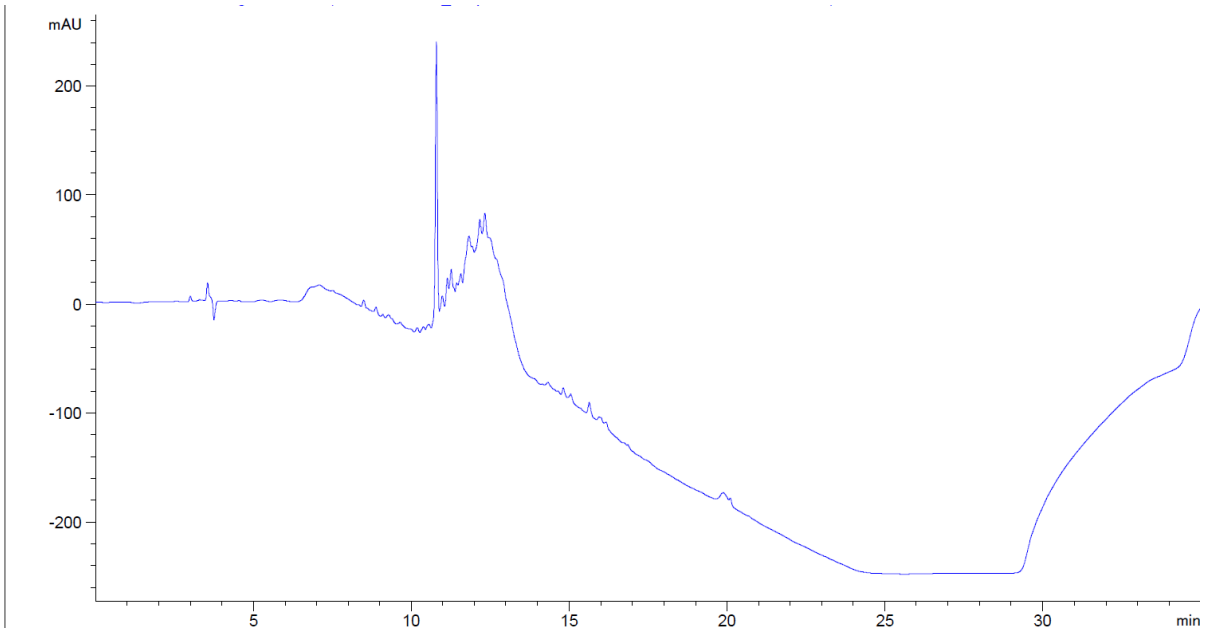




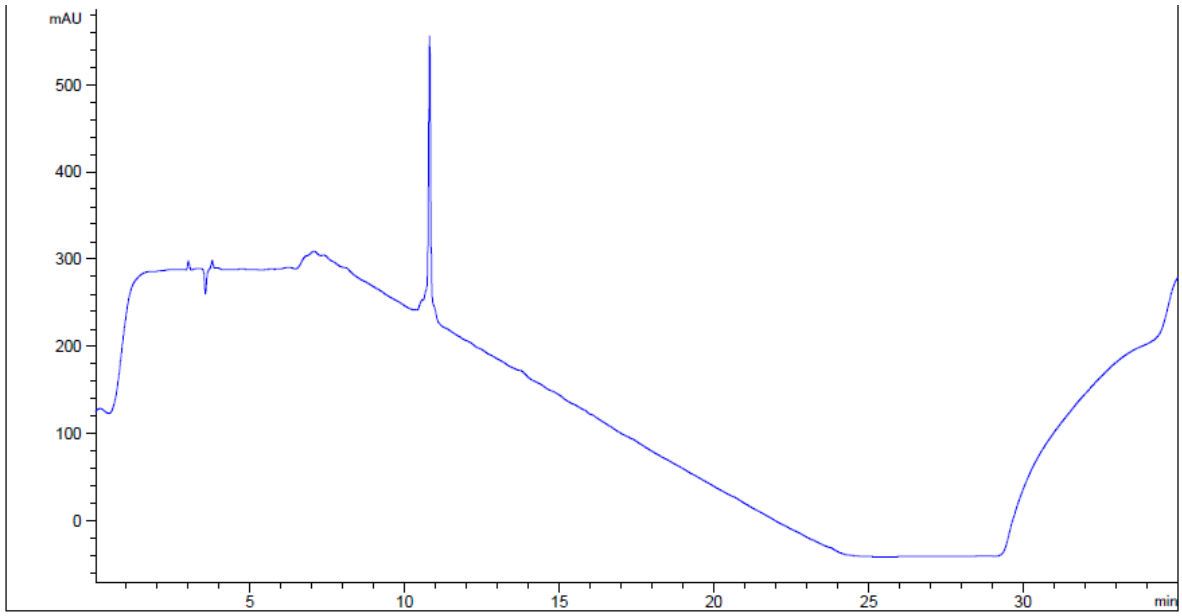


Oxytocin ***10***

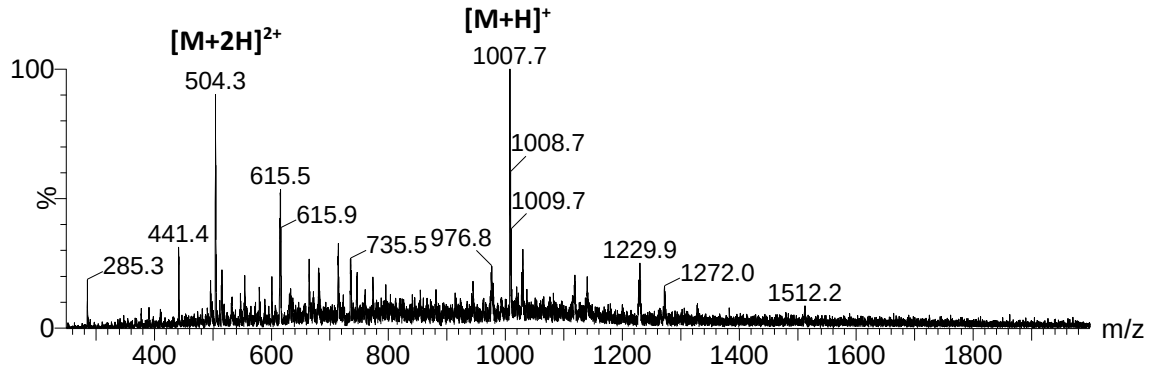




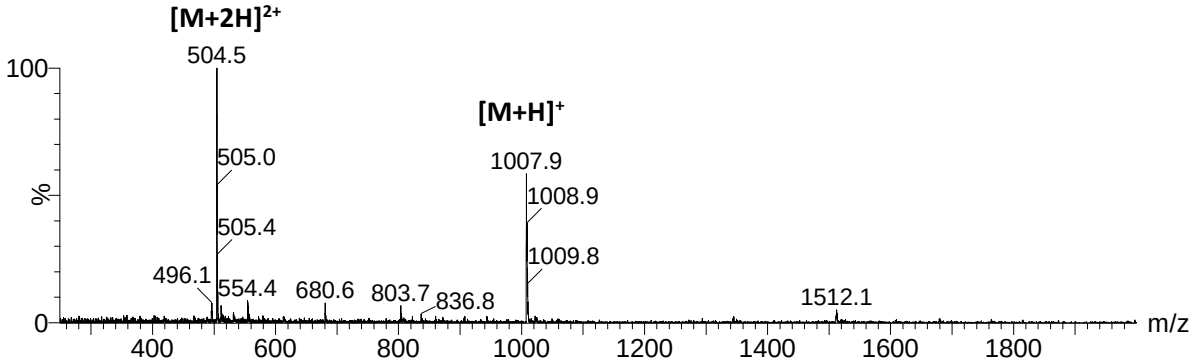
Purified by semi-preparative HPLC as described previously. Obtained 0.5 mg (42%, calculated based on initial resin loading).



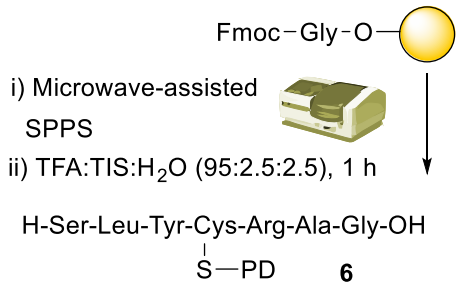
Crude



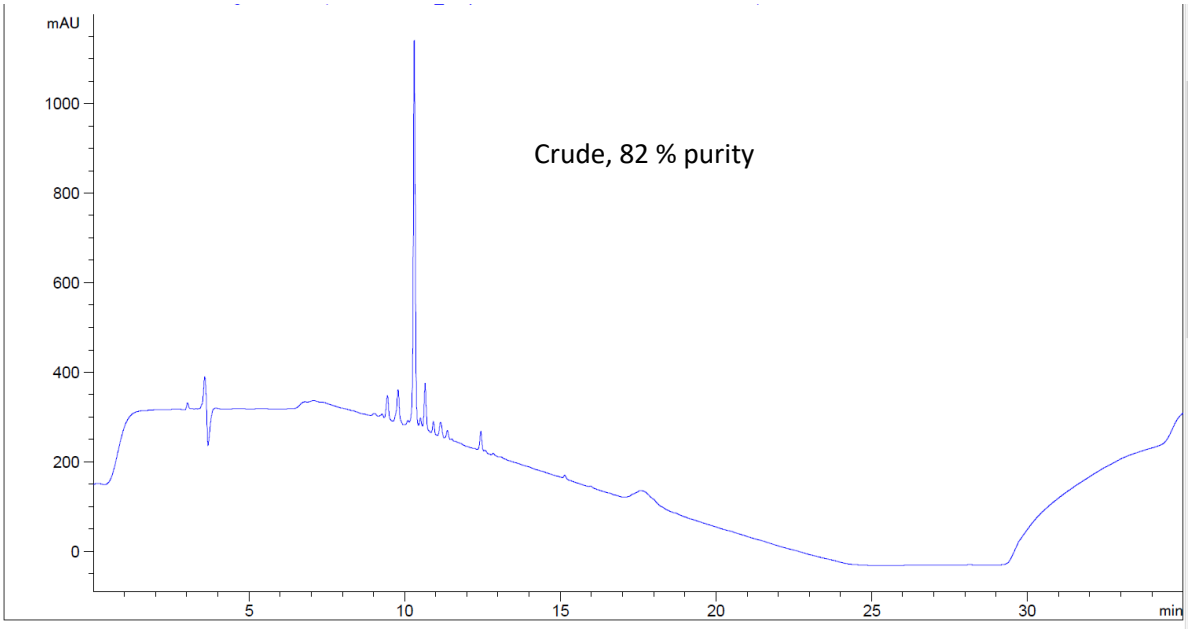
Purified

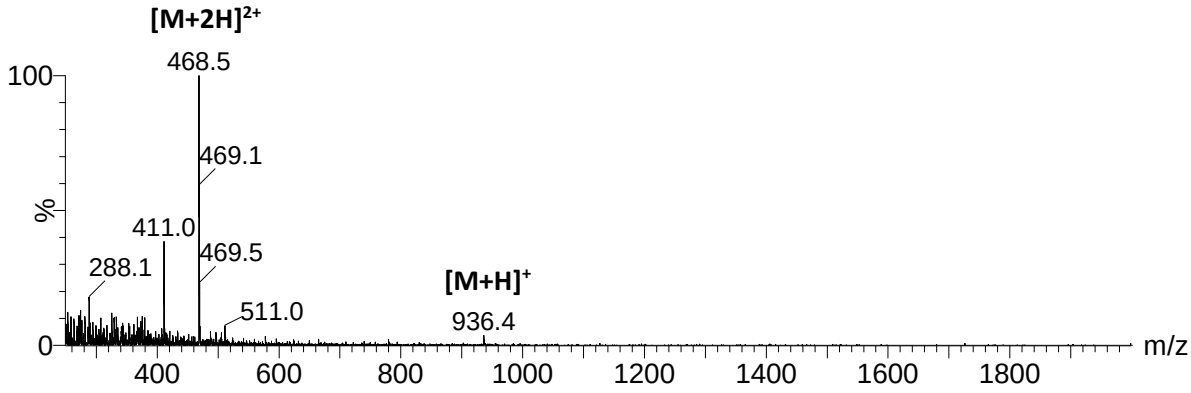


Microwave-assisted synthesis of SLYC(PD)RAG ***6***



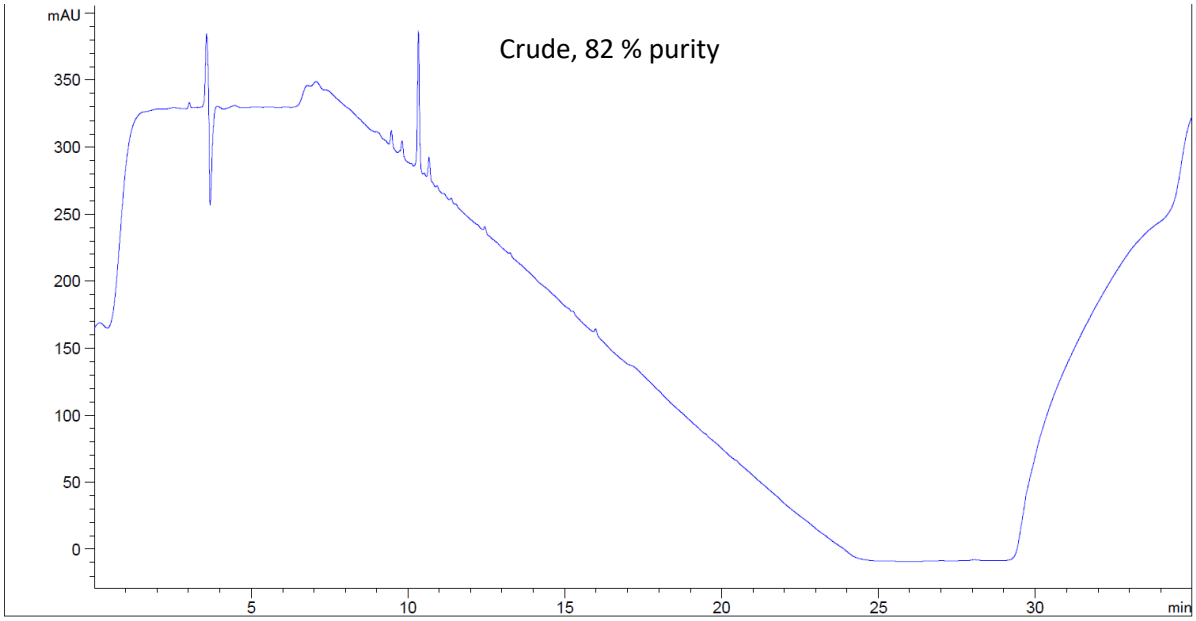
Microwave reactions were performed using a Personal Chemistry Smith Creator microwave-assisted organic synthesiser system in 5 mL reaction vials, with maximum 300 W power. Microwave-assisted peptide synthesis was performed in a similar manner to that described in the **Methods for manual Solid Phase Peptide Synthesis (SPPS)** section, with the **Amino acid coupling** step performed as follows: DIPEA (11.0 eq.) was added to a solution of amino acid (5.0 eq.) and HCTU (5.0 eq.) dissolved in *ca.* 3 mL of DMF and the solution added to the resin. The solution was then subjected to microwave-assisted conditions at 75 °C for 15 min. **Fmoc deprotection** was performed without the use of microwave assisted conditions.

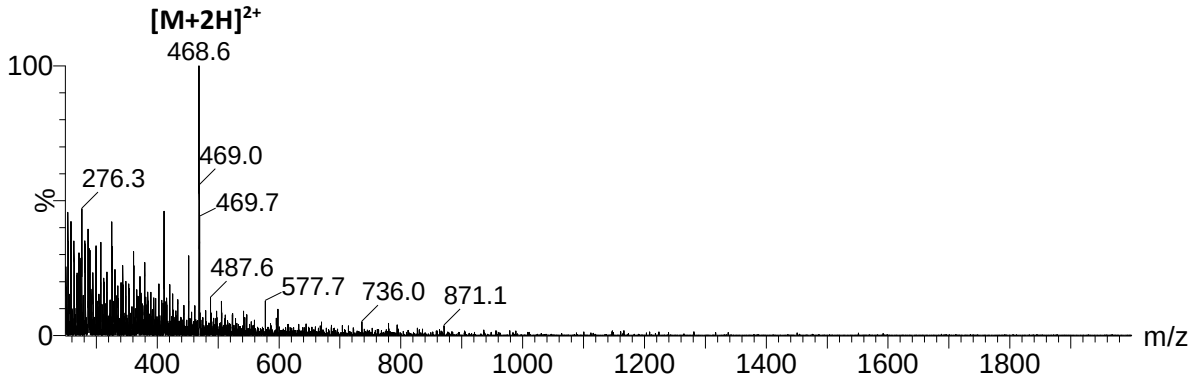




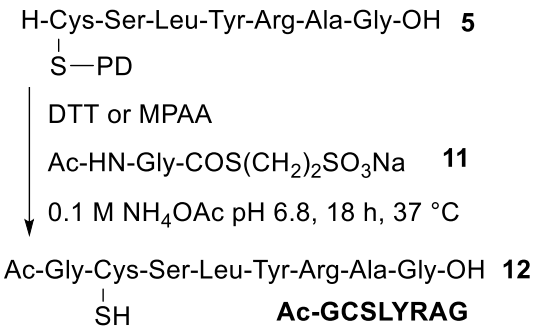
Stability test under microwave-assisted conditions

Following completion of peptide synthesis in the *Microwave-assisted synthesis of SLYC(PD)RAG* ***6*** and prior to **Cleavage and Isolation**, a 5 mg portion of resin bound peptide was incubated in *ca*. 3 mL of 20% piperidine in DMF and subjected to microwave-assisted conditions at 75 °C for 15 min. **Cleavage and Isolation** of the peptide was then performed as described earlier.



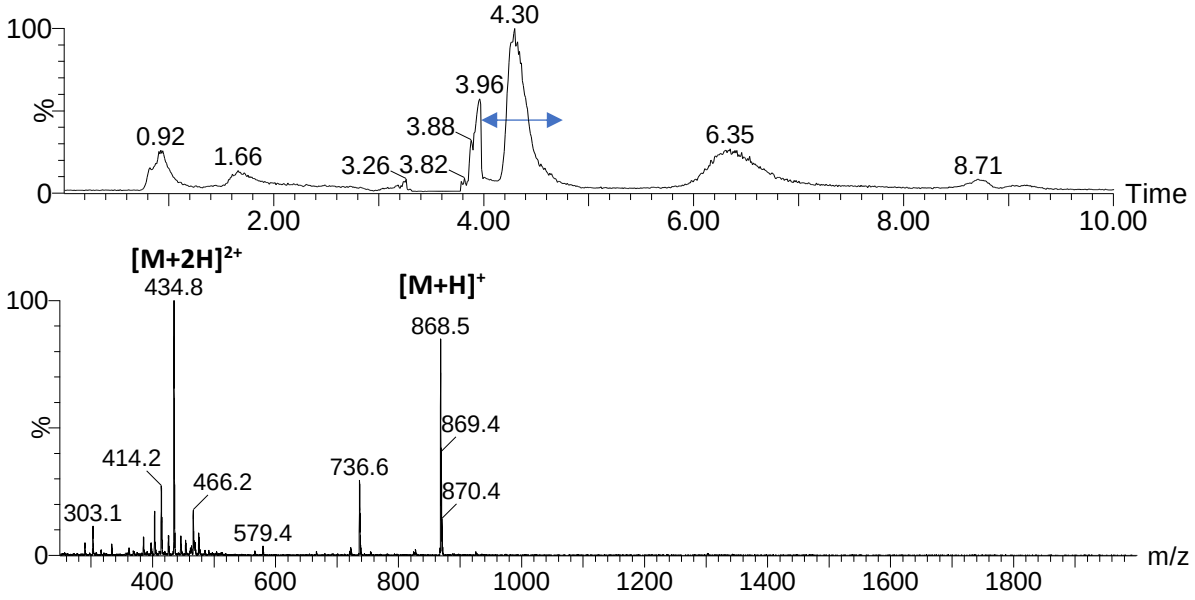


Native chemical ligation experiments

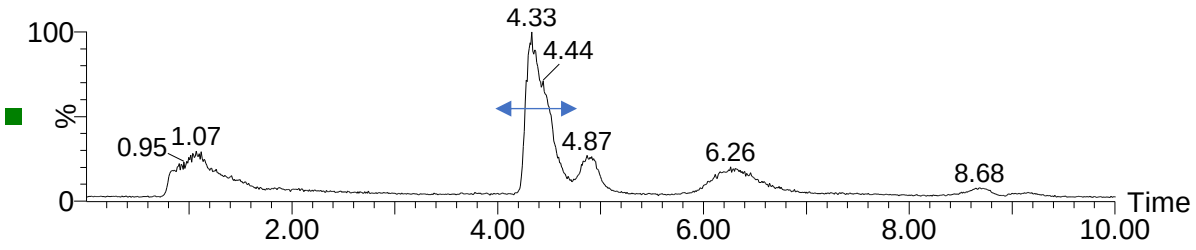


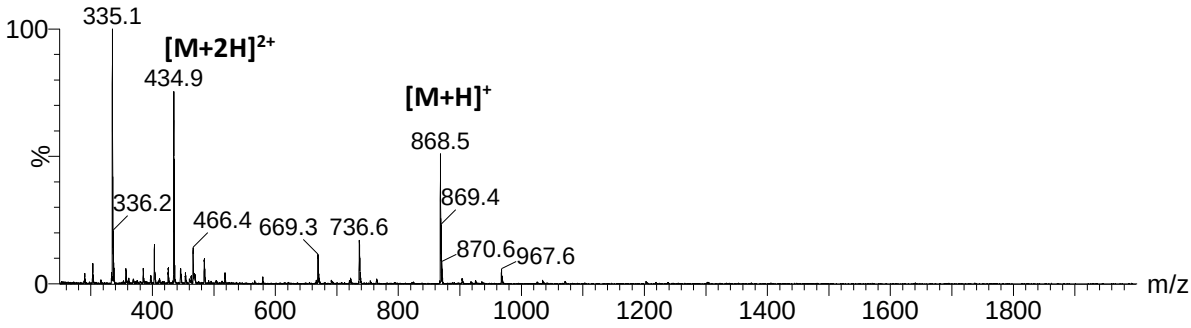
A 50 µL aliquot of 5 mM C(PD)SLYRAG **2** in 0.1 M NH_4OAc pH 6.8 was charged with 25 µL of a 20 mM thioester **9** solution in 0.1 M NH_4OAc and with 150 µL of 0.1 M NH_4OAc pH 6.8. The solution was then charged with 25 µL of a 1 M solution of DTT or MPAA in 0.1 M NH_4OAc. Following mixing by pipetting, the reaction was kept at 37 °C for 18 h. The conversion of peptide **2** to Ac-GCSLYRAG **9** was assessed by LCMS analysis, with total consumption of peptide **2** observed.

DTT



MPAA


100
%
0
4.33
4.44
0.95
1.07
4.87
6.26
8.68
Time
2.00
4.00
6.00
8.00
10.00


100
%
0
335.1
[M+2H]²⁺
434.9
[M+H]⁺
868.5
336.2
466.4
669.3
736.6
869.4
870.6
967.6
m/z
400
600
800
1000
1200
1400
1600
1800

References

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2 M. Fernández, A. Shamsabadi and V. Chudasama, Fine-tuning thio-pyridazinediones as SMDC scaffolds (with intracellular thiol release via a novel self-immolative linker), *Chem. Commun.*, 2020, **56**, 1125–1128.

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4 D. A. Richards, S. A. Fletcher, M. Nobles, H. Kossen, L. Tedaldi, V. Chudasama, A. Tinker and J. R. Baker, Photochemically re-bridging disulfide bonds and the discovery of a thiomaleimide mediated photodecarboxylation of C-terminal cysteines, *Org. Biomol. Chem.*, 2016, **14**, 455–459.