Electronic Supplementary Material (ESI) for ChemComm.

Electronic Supporting Information

Unearthing the unique stability of thiophosphonium-C-terminal cysteine adducts on peptides and proteins

Richard J. Spears,[a] Alina Chrzastek,[a] Steven Y. Yap,[a] Kersti Karu[a], Abil E. Aliev[a], James R. Baker*[a] and Vijay Chudasama*[a]

[a] UCL Department of Chemistry, 20 Gordon Street, London WC1H 0AJ, UK. E-mails: v.chudasama@ucl.ac.uk, j.r.baker@ucl.ac.uk

General Experimental

All chemical reagents were purchased from Sigma Aldrich, Alfa Aesar and Acros. Compounds and solvents were used as received. Petrol refers to petroleum ether (b.p. 40–60 °C). All 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). 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 300 MHz, 400 MHz, 500 MHz, 600 MHz or 700 MHz. ^{13}C NMR spectra were obtained at 125 MHz, 150 MHz, or 175 MHz. ^{31}P NMR spectra were obtained at 284 MHz. All results were obtained using Bruker NMR instruments, the models are as follows: Avance Neo 700, Avance III 600, Avance 500, Avance III 400, Avance 300. 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 has 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 JH-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.

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 H-Gly-2-ClTrt resin or an H-Cys(Trt)-2-ClTrt resin (Sigma) using Fmoc protected amino acids as described below:

Preloaded resin preparation. The preloaded 2-chlorotrityl resin was weighed out into a 2 mL SPPS cartridge fitted with 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 minutes. The resin was filtered off and repeated four more times, followed by washes with DMF (5 × 2 min with rotation).

Cleavage and Isolation. Resins containing full synthesised peptides were washed with DCM (3 × 2 min with rotation) and MeOH (3 × 2 min with rotation). The resin was dried on a vacuum manifold and further dried on a high vacuum line overnight. A solution of cleavage cocktail 95:2.5:2.5 (v/v) TFA:H_2O:triisopropylsilane containing 5% w/v 1,4-dithiothreitol (DTT) was then added to the resin, 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 until pelleted (*ca*. 5-10 min). The supernatant was carefully decanted and subsequently resuspended, centrifuged and supernatant decanted three

more times. The precipitated peptide pellet was then dissolved in 10% MeCN and lyophilised. Lyophilised peptides were then stored at -20 ^{o}C until required.

Procedure for sample clean up by solid phase extraction

Where required, sample clean up of peptides was carried out by the following procedure using a Supelclean™ LC-18 SPE Tube (Sigma-Aldrich): The C18 solid phase extraction cartridge was washed with 1 x column volume (CV) MeCN, 1 x CV H_2O. The sample was then loaded onto the column and then washed with 2 x CV H_2O. The product was then eluted with gradients of H_2O/MeCN.

LCMS analysis -Method 1

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: 214 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* = 100 – 1000 or 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.

LCMS analysis -Method 2

LCMS method 2 was identical to that of method 1 with the following exception: Gradient over 8 min (to 5:95 Water (0.1% Formic Acid): MeCN (0.1% Formic Acid). Flow Rate: 0.3 mL/min

MS for LTQ and Tandem MS

Tandem MS was performed on a ThermoScientific Finnigan LTQ fitted with a Hypersil Gold C18 column (100 mm x 2.1 mm). Flow rate was set at Solvent A is H_2O (0.1% formic acid), solvent B is MeCN (0.1% formic acid). Mobile phase: 95:5 A:B; gradient over 12 minutes to 5:95 A:B. Ionisation was performed in ES+ mode with a collision energy of 35% for the MS^2 spectra. An injection volume of 25 µL was employed. Spray voltage was 4.5 kV, capillary temperature 280 ^{o}C. MS scans consisted of three averaged “microscans”, each with a maximum injection time of 200 ms.

MS for Fab

Molecular masses of native and modified proteins were measured using an Agilent 6510 QTOF LC-MS system (Agilent, UK). Agilent 1200 HPLC system was equipped with an Agilent PLRPS, 1000A, 8 µM, 150 mm x 2.1 mm column. 10 µL of a protein sample (at ca. 2-4 µM) was separated on the column with an eluting gradient at a flow rate of 300 µl/min. Solvent A is H_2O (0.1% formic acid), solvent B is MeCN (0.1% formic acid). The eluting gradient used is as follows: 85:15 A:B for 2 min (2 min total),

68:32 A:B for 2 min (4 min total), 65:35 for 10 min (14 min total), 5:95 for 4 min (18 min total), 5:95 for 2 min (20 min total), 85:15 for 2 min (22 min total), followed by 85:15 for 3 min (25 min total). The oven temperature was maintained at 60 °C. Agilent 6510 QTOF mass spectrometer was operated in a positive polarity mode, coupled with an ESI ion source. The ion source parameters were set up with a VCap of 3500V, a gas temperature at 350 °C, a dry gas flow rate at 10 L/min and a nebulizer of 30 psig. MS Tof was acquired under conditions of a fragmentor at 350 V, a skimmer at 65 V and an acquisition rate at 0.5 spectra/s in a profile mode, within a scan range between 700 and 5000 m/z. The data was then analysed by deconvoluting a spectrum to a zero charge mass spectra using a maximum entropy deconvolution algorithm within the MassHunter software version B.07.00.

Semi-preparative HPLC – Small molecule

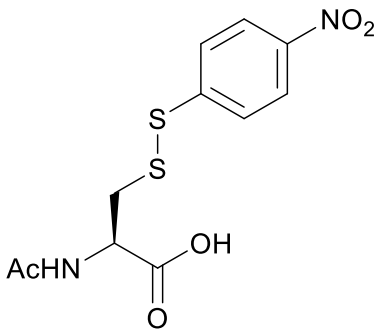
Semi-preparative HPLC was performed using a Dionex instrument with a PDA-100 photodiode array detector and an ASI-100 automated sample injector using a ZORBAX 300SB-C18 (5 µm, 9.4 × 250 mm) column (Agilent). Water with 0.1% (v/v) TFA (solvent A) and acetonitrile with 0.1 % (v/v) TFA (solvent B) were used as the mobile phase at a flow rate of 2 mL/min at room temperature (RT) with detection at 214 and 280 nm. Small molecules were separated using a multi-step gradient of 49 min and was programmed as follows: Gradient starts at 95% A (0 min), followed by a linear gradient to 30% B over 39.5 min (0->39.5 min), followed by a linear gradient to 95% B for1.5 min (39.5->41 min), followed by an additional 3 min at 95% B (41 -> 44 min). A linear gradient to 95% A over 2.2 min (44->46.2 min), followed by 95% A for an additional 2.8 min (46.2->49 min) was used to re-equilibrate the column. Fractions containing the desired product were pooled and lyophilised.

Semi-preparative HPLC – Peptides

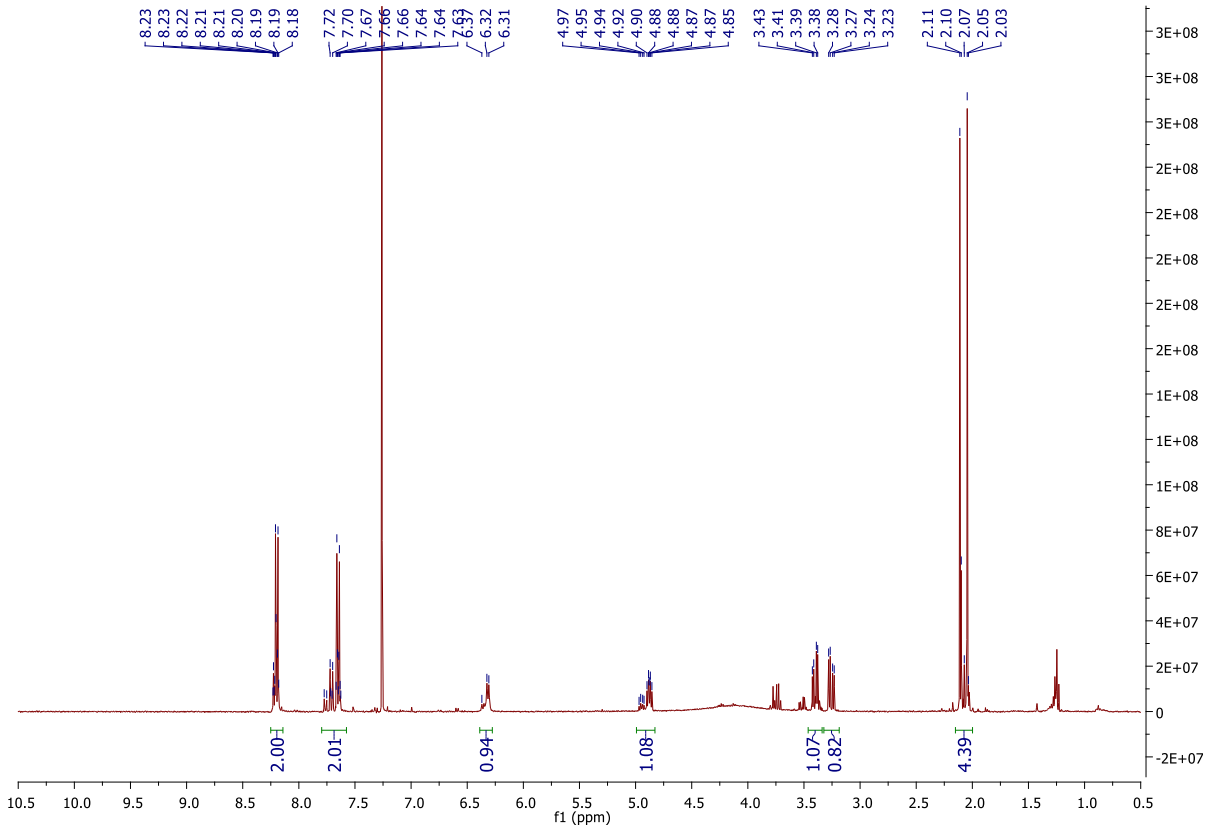
Semi-preparative HPLC for peptides was performed in an identical manner to that for small molecules, with the following exception: Peptides were separated using a multi-step gradient of 43 min and was programmed as follows: Gradient starts at 95% A (0 min), followed by a linear gradient to 70% B over 30.5 min (0->30.5 min), followed by a linear gradient to 95% B for 2.5 min (30.5-> 33 min) followed by 95% B for an additional 3 min (33->36 min). A linear gradient to 95% A over 3.2 min (36->39.2 min), followed by 95% A for an additional 2.8 min (39.2->43 min) was used to re-equilibrate the column

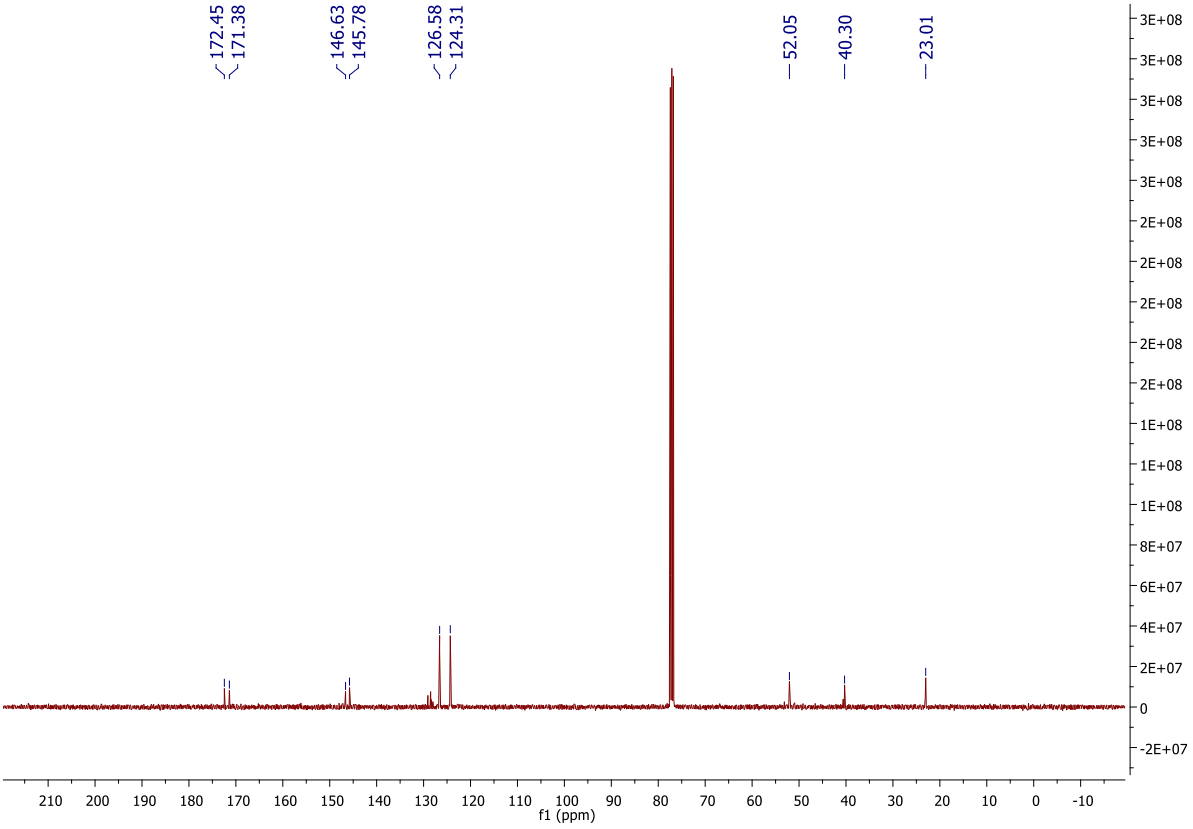
Synthesis

*Small molecule disulfide **4***

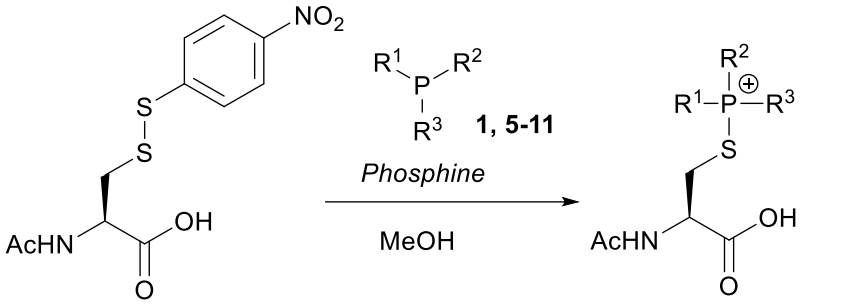


Synthesis of disulfide **4** was adapted from a previous reported protocol:[1] To a solution of 4-nitrophenyl disulfide **3** (943 mg, 3.06 mmol) in THF (100 mL) was added dropwise a solution of *N*-acetyl cysteine **2** (250 mg, 1.53 mmol) dissolved in MeOH (25 mL), followed by addition of two drops of 4 M NaOH. The resulting dark red solution was allowed to stir at 21 °C for 1 h. After this time, the solvent was removed *in vacuo*, and the residue was dissolved in EtOAc (100 mL). The solution was then washed with 1M HCl (3 × 30 mL), and the organic layer was then dried ($MgSO_4$) and then concentrated *in vacuo*. The crude residue was then purified by flash chromatography (DCM:MeOH, 100:0 -> 85:15), yielding disulfide **4** (353 mg, 34%) as an orange syrup. ^{1}H NMR (400 MHz, $CDCl_3$, potential rotamers) δ 8.23-8.18 (m, 2H), 7.77-7.63 (m, 2H), 6.37-6.31 (m, 1H), 4.97-4.85 (m, 1H), 3.43-3.38 (m, 1H), 3.28-3.23 (m, 1H), 2.11-2.03 (m, 3H); ^{13}C NMR (100 MHz, $CDCl_3$) δ 172.4 (C), 171.4 (C), 146.6 (C), 145.8 (C), 126.6 (CH), 124.3 (CH), 52.1 (CH), 40.3 (CH_2), 23.01 (CH_3); IR (thin film) 3340, 3093, 2499, 1509, 1333 cm^{-1}; LRMS (ESI) 318 (100, $[M+H]^+$); HRMS (ESI) calcd for $C_{11}H_{12}N_2O_5S_2$ $[M+H]^+$ 317.02604; observed 317.02603.


8.23
8.23
8.22
8.21
8.21
8.20
8.19
8.19
8.18
7.72
7.70
7.67
7.66
7.66
7.64
7.64
7.63
6.32
6.31
4.97
4.95
4.94
4.92
4.90
4.88
4.88
4.87
4.87
4.85
3.43
3.41
3.39
3.38
3.28
3.27
3.24
3.23
2.11
2.10
2.07
2.05
2.03
2.00
2.01
0.94
1.08
1.07
0.82
4.39
f1 (ppm)


172.45
171.38
146.63
145.78
126.58
124.31
52.05
40.30
23.01
f1 (ppm)

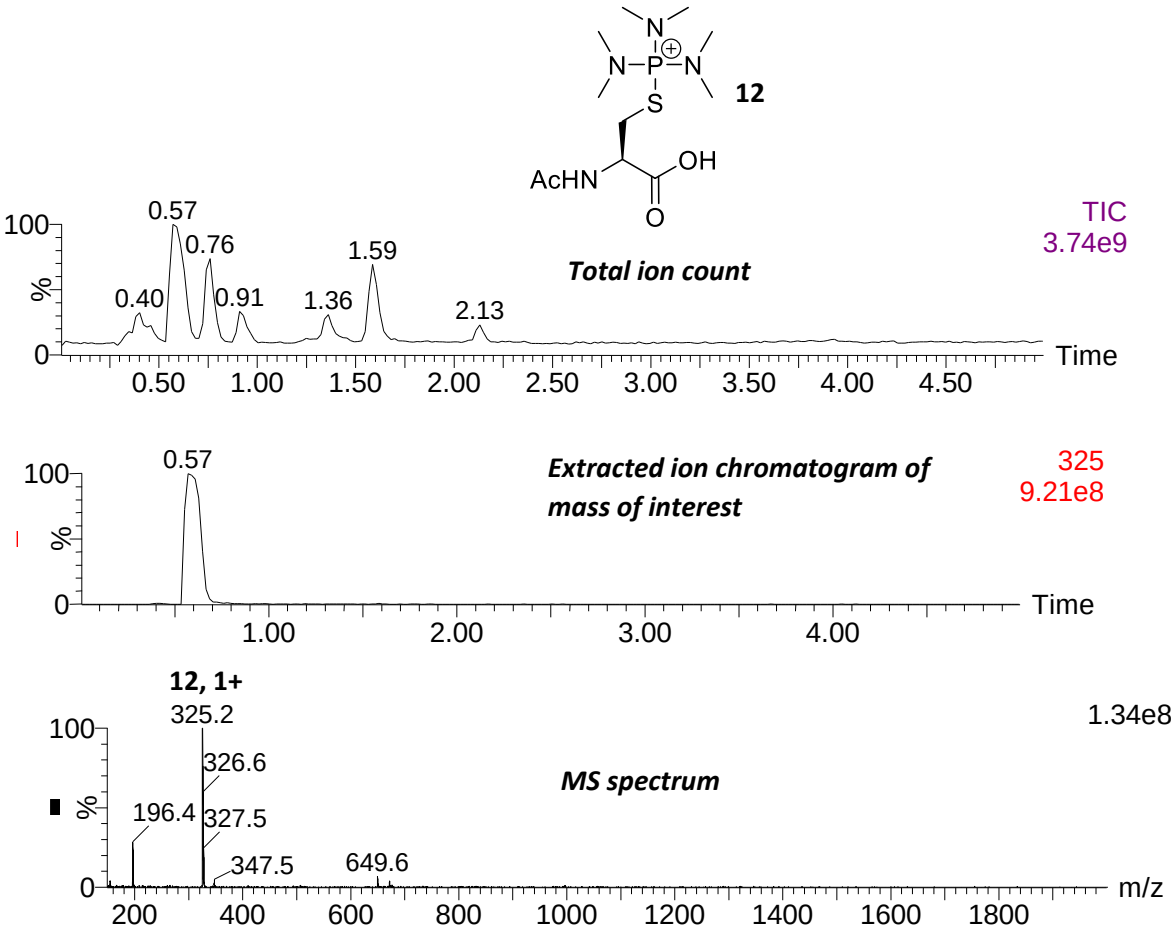
Small molecule thiophosphonium formation

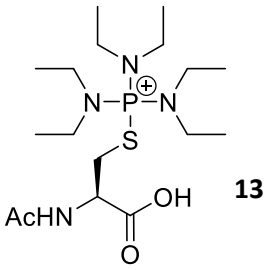


Thiophosphonium cation

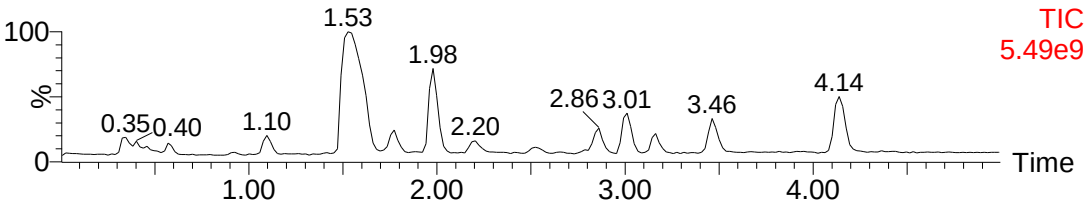
12-19

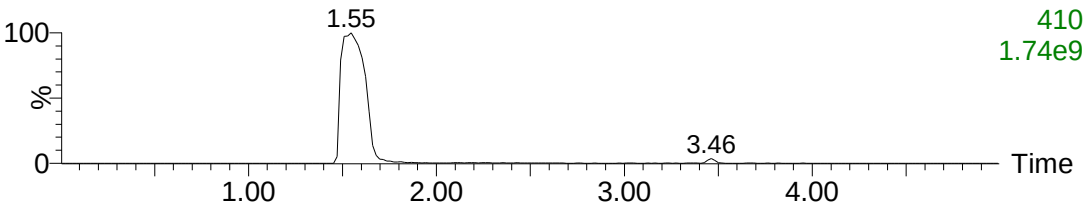
To a solution of disulfide **4** (10 mg, 0.0316 mmol) in MeOH (3 mL) was added phosphine reagent (0.174 mmol, 5.5 equiv.). The reaction was mixed thoroughly, and allowed to incubate at 21 °C for 1 h. Samples were then subjected to LCMS analysis to appraise the presence of thiophosphonium salt.



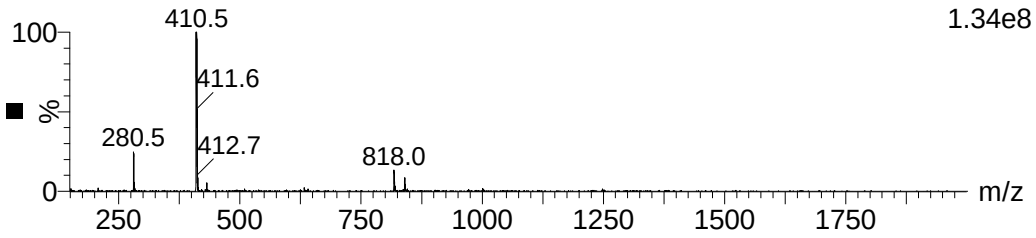

N
N
P
N
S
AcHN
OH
O

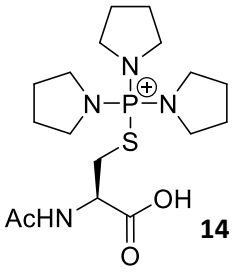
13


TIC
5.49e9
100
%
0
1.53
1.98
0.35
0.40
1.10
2.20
2.86
3.01
3.46
4.14
Time
1.00
2.00
3.00
4.00

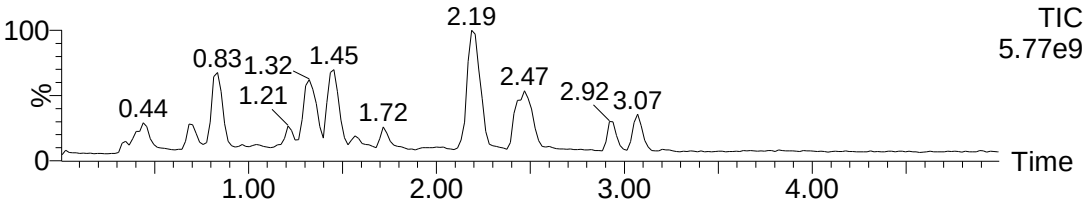

410
1.74e9
100
%
0
1.55
3.46
Time
1.00
2.00
3.00
4.00

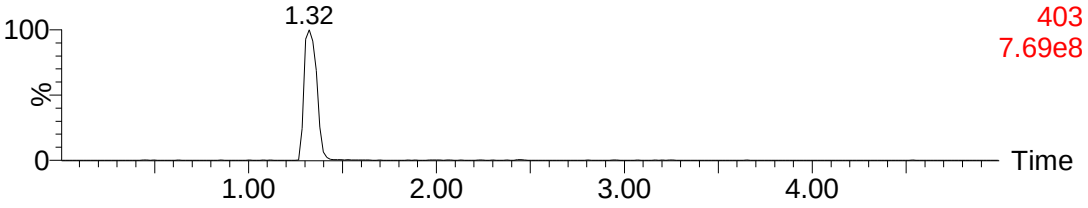
13, 1+

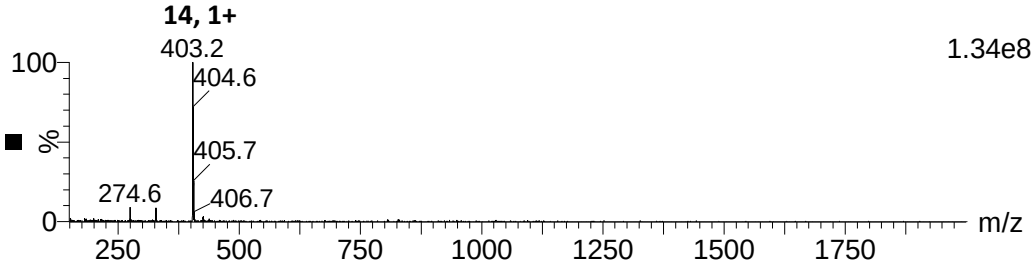

1.34e8
100
%
0
410.5
411.6
412.7
280.5
818.0
m/z
250
500
750
1000
1250
1500
1750

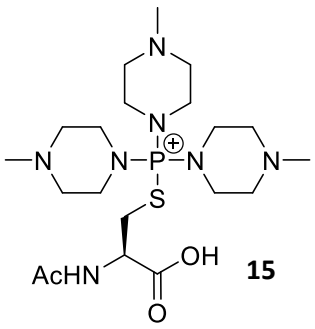

N
N
P
N
S
AcHN
OH
O

14

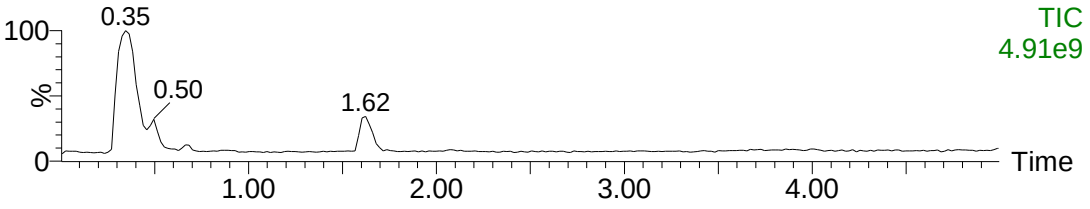

100
%
0
0.44
0.83
1.21
1.32
1.45
1.72
2.19
2.47
2.92
3.07
1.00
2.00
3.00
4.00
Time
TIC
5.77e9

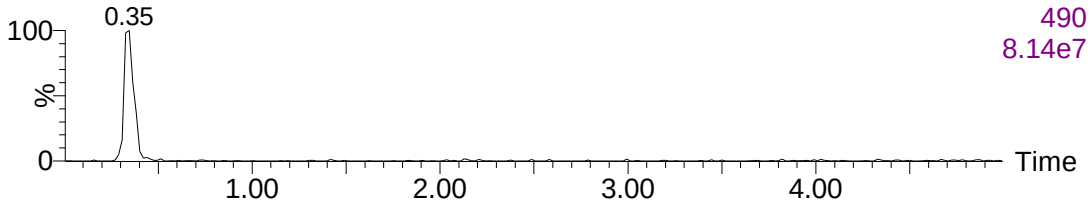

100
%
0
1.32
1.00
2.00
3.00
4.00
Time
403
7.69e8

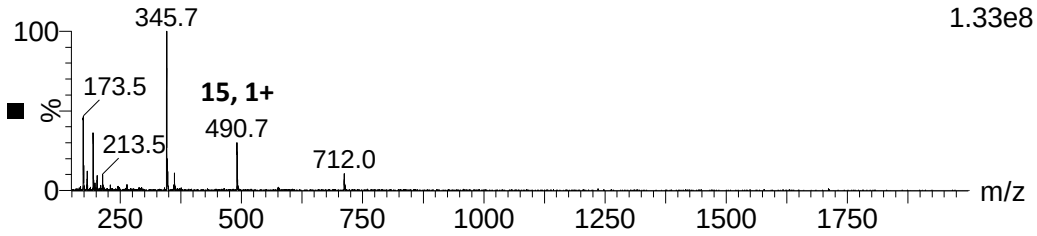

14, 1+
403.2
404.6
405.7
406.7
274.6
100
%
0
250
500
750
1000
1250
1500
1750
m/z
1.34e8

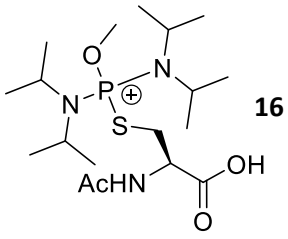

N
N
N
N
P
N
N
S
AcHN
OH
O

15

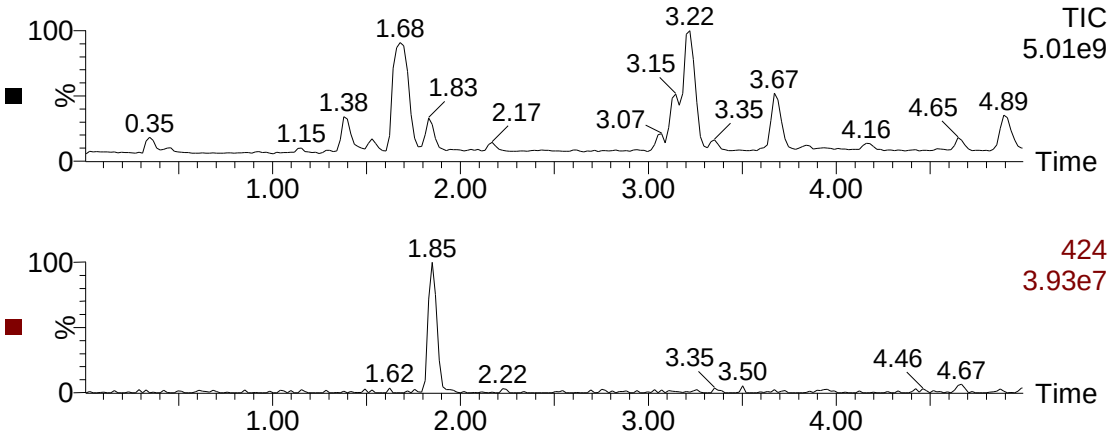

100
%
0
0.35
0.50
1.62
TIC
4.91e9
Time
1.00
2.00
3.00
4.00

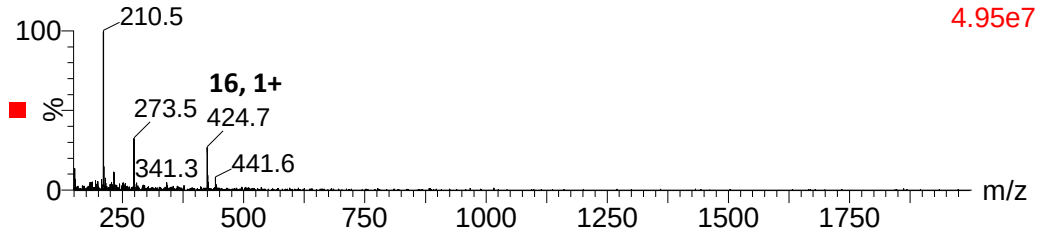

100
%
0
0.35
490
8.14e7
Time
1.00
2.00
3.00
4.00


100
%
0
345.7
173.5
213.5
15, 1+
490.7
712.0
1.33e8
m/z
250
500
750
1000
1250
1500
1750

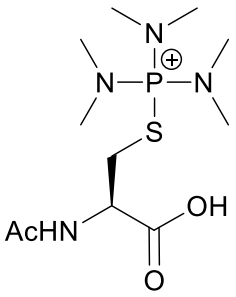

O
N
N
P
S
AcHN
OH
O

16

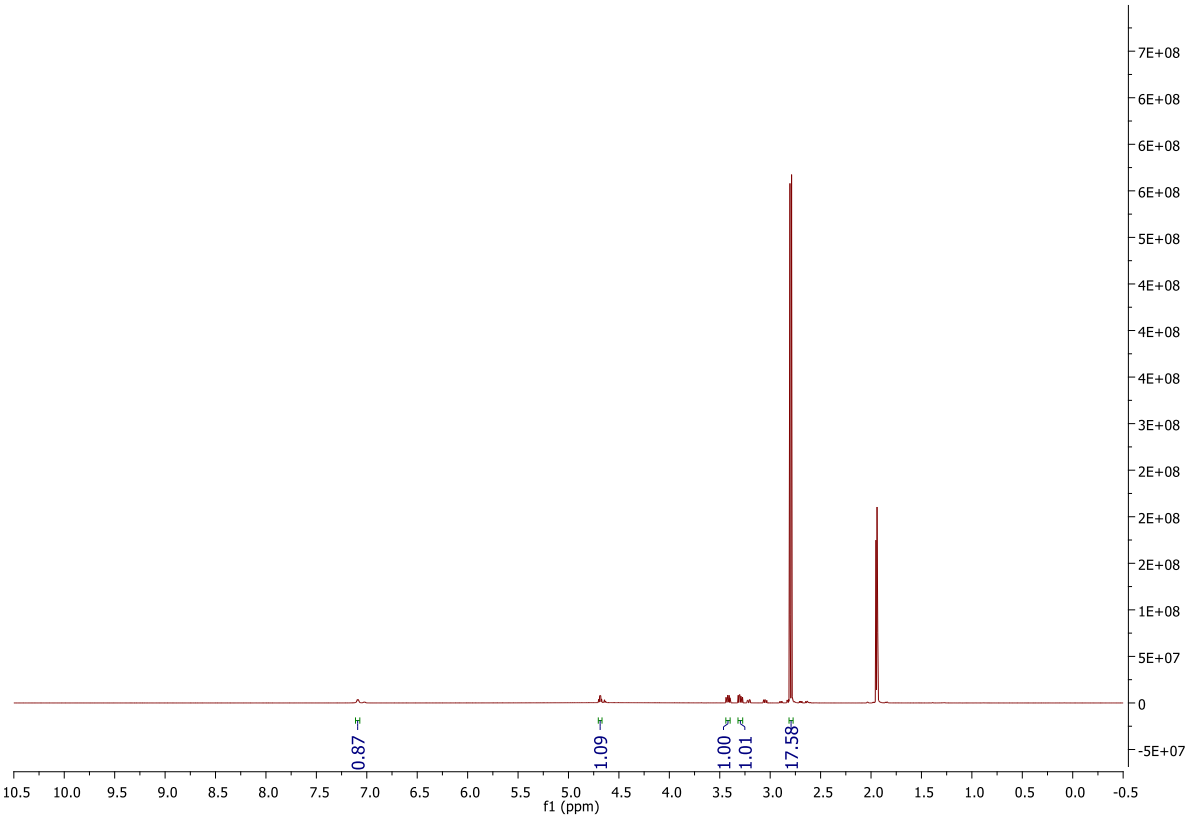

TIC
5.01e9
100
%
0
0.35
1.15
1.38
1.68
1.83
2.17
3.07
3.15
3.22
3.35
3.67
4.16
4.65
4.89
Time
1.00
2.00
3.00
4.00
424
3.93e7
1.62
1.85
2.22
3.35
3.50
4.46
4.67

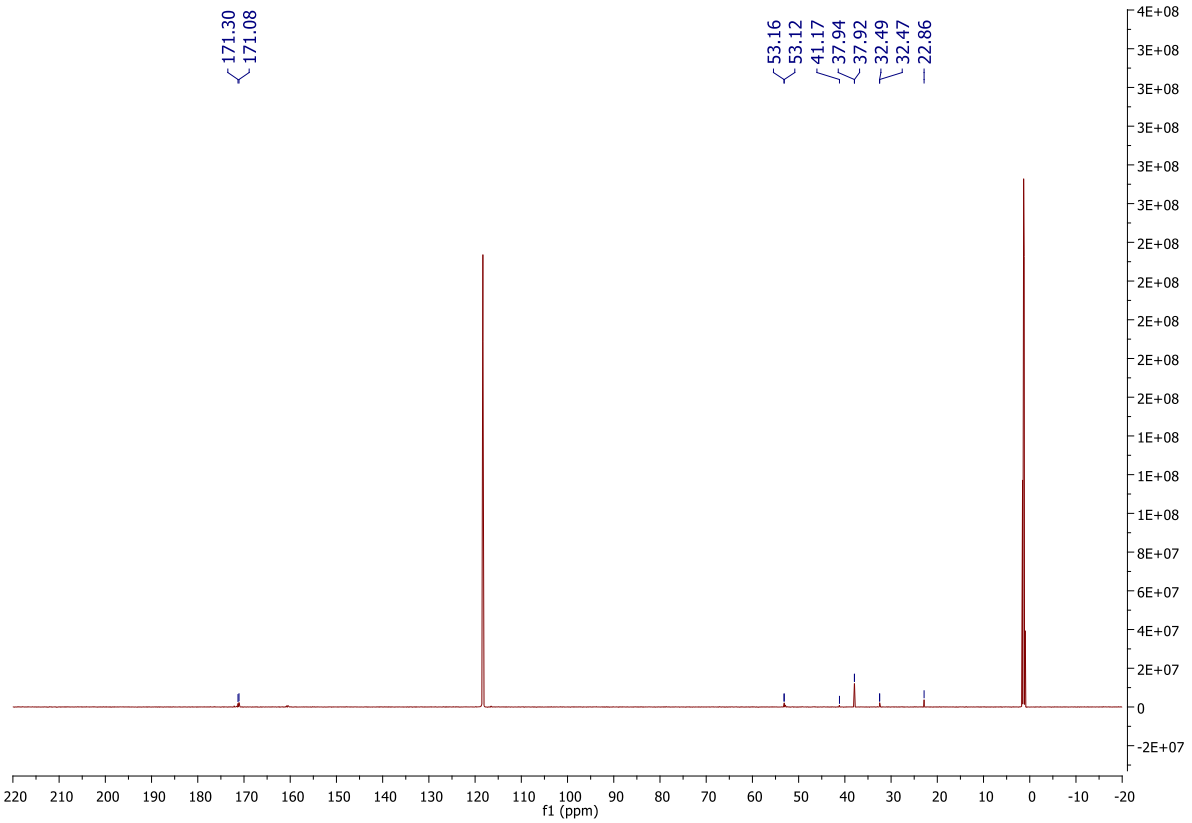

4.95e7
100
%
0
210.5
16, 1+
273.5
424.7
341.3
441.6
m/z
250
500
750
1000
1250
1500
1750

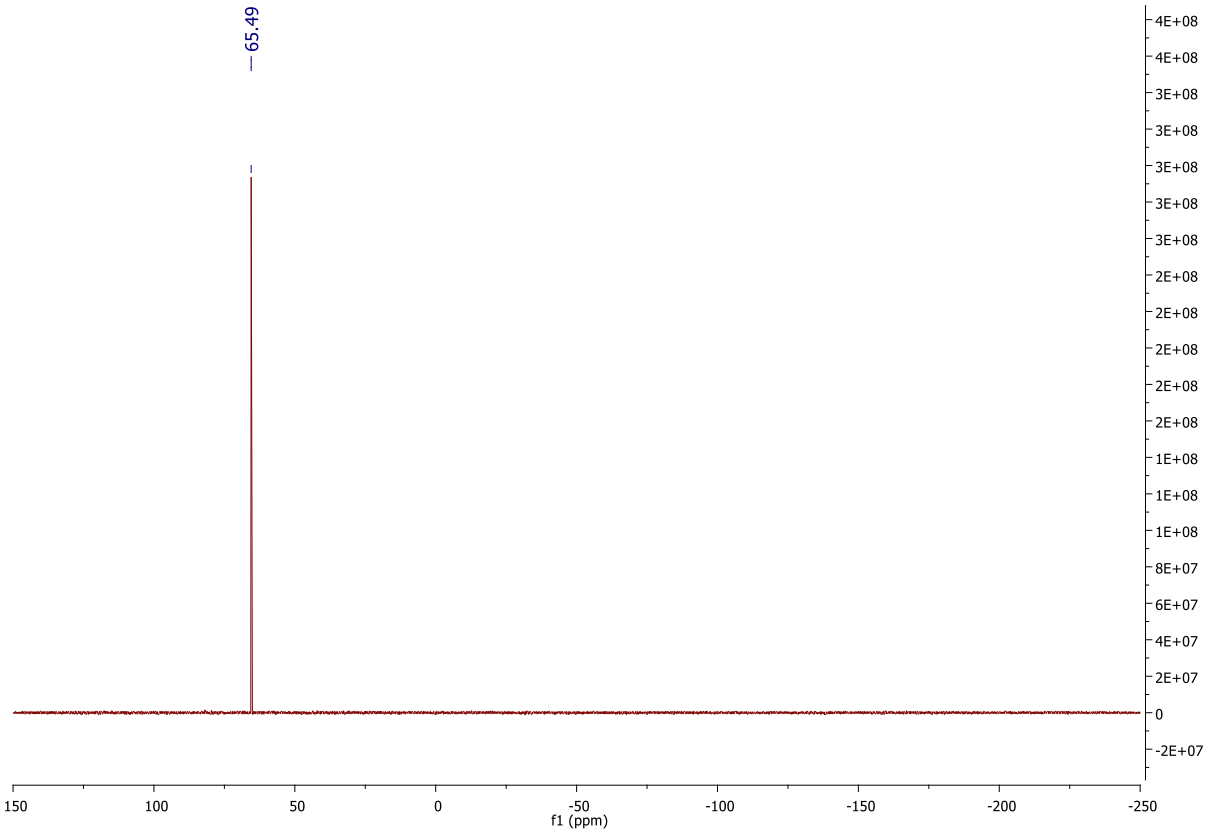
*Preparation and isolation of thiophosphonium **12***



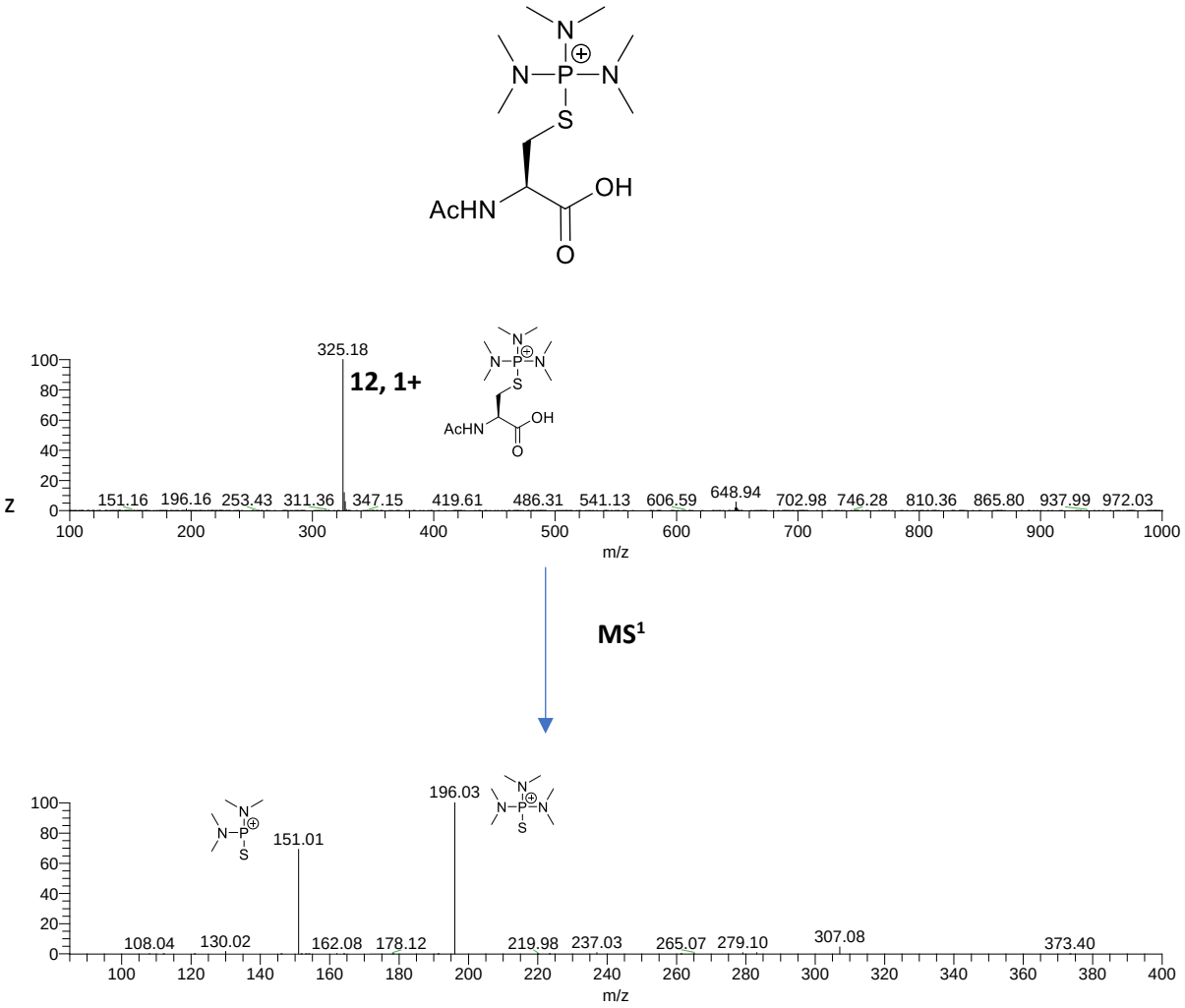
To a solution of disulfide **4** (75 mg, 0.24 mmol) in MeCN (2 mL) was added H_2O (2mL). HMPT **1** (194 µL, 1.19 mmol, 5 equiv.) was then added, the reaction mixed thoroughly, and allowed to incubate at 21 °C for 1 h. Solvent was partially removed *in vacuo*, and the resulting 0.5 mL solution was acidified with 1.5 mL of 1M HCl. The resulting solution was then filtered to remove precipitates, and half of the resulting solution was purified *via* semi-preparative HPLC. Fractions containing the desired product were then lyophilise, yielding thiophosphonium **12** as a colourless gum (40 mg, 0.13 mmol 52%). ^{1}H NMR (700 MHz, CD_3CN) δ 7.09 (d, *J* = 6.9 Hz, 1H), 4.70-4.67 (m, 1H), 3.42-3.40 (m, 1H), 3.32-3.28 (m, 1H). 2.80 (d, *J* = 11.3 Hz, 18 H); ^{13}C NMR (175 MHz, CD_3CN) δ 171.3 (C), 171.1 (C), 53.1 (CH), 41.2 (CH), 37.9 (CH_3), 32.5 (CH), 22.9 (CH_3); ^{31}P NMR (284 MHz, CD_3CN) δ 65.5 ($P(NMe_2)_3$); IR (thin film) 3273, 2914, 1657, 1167, 987; LRMS (ESI) 325 (100, $[M]^+$); HRMS (ESI) calcd for $C_{11}H_{26}N_4O_3PS$ $[M]^+$ 325.1463; observed 325.1454.


0.87
1.09
1.00
1.01
17.58
f1 (ppm)

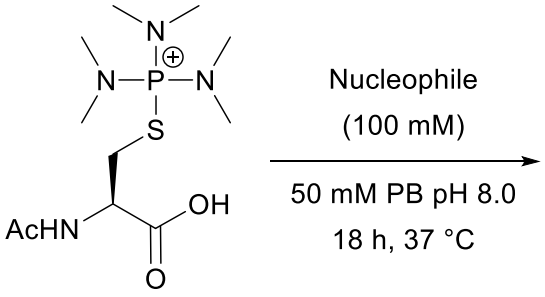

171.30
171.08
53.16
53.12
41.17
37.94
37.92
32.49
32.47
22.86
f1 (ppm)


65.49
4E+08
4E+08
3E+08
3E+08
3E+08
3E+08
3E+08
3E+08
2E+08
2E+08
2E+08
2E+08
2E+08
2E+08
1E+08
1E+08
1E+08
8E+07
6E+07
4E+07
2E+07
0
-2E+07
150
100
50
0
-50
-100
-150
-200
-250
f1 (ppm)

MSMS analysis of 12



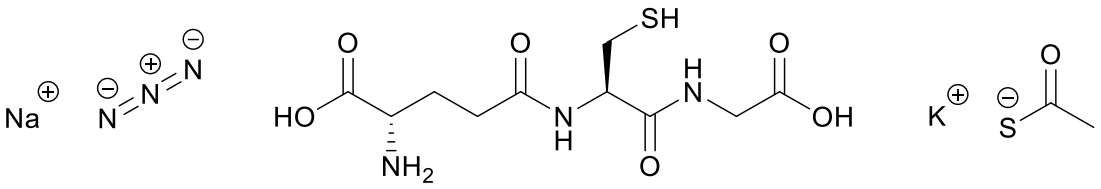
Stability study of thiophosphonium **12**



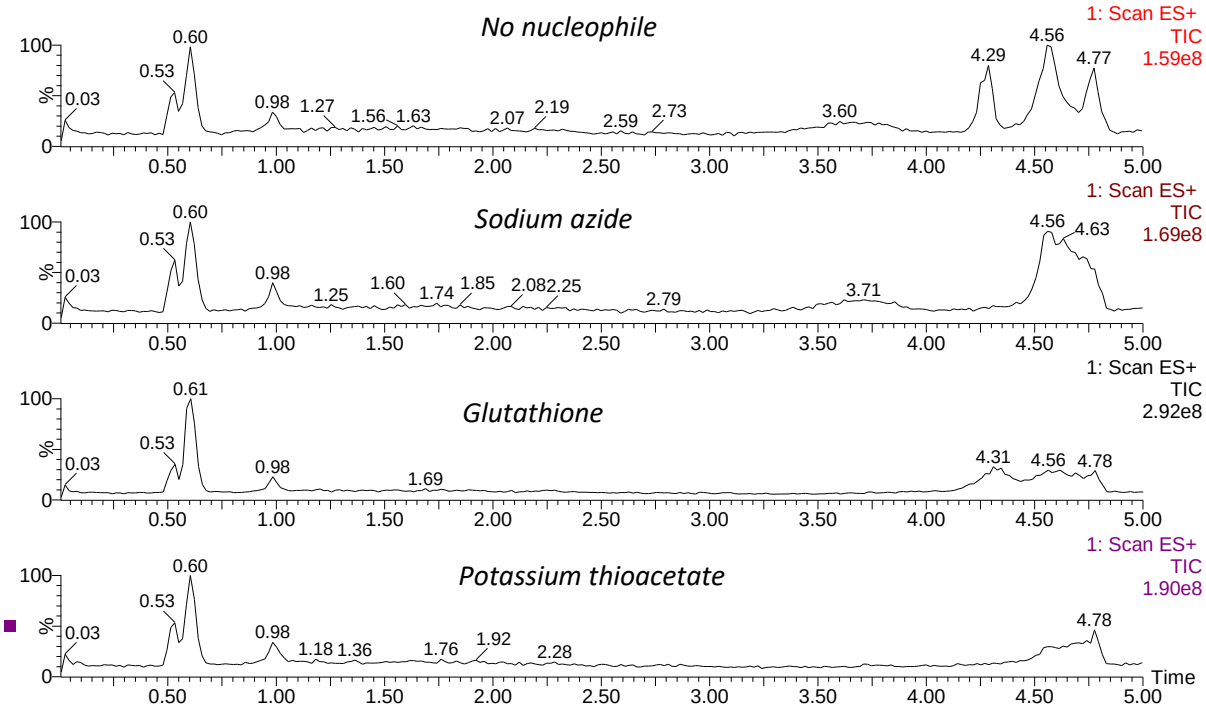
1 mM

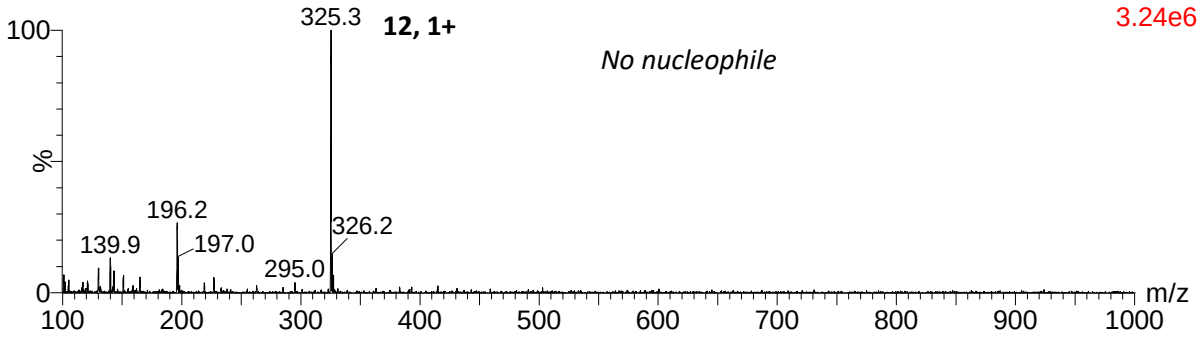
A 5 µL aliquot of a 20 mM thiophosphonium **12** stock in H_2O was charged with 70 µL of H_2O and 25 µL of 0.2 M PB pH 8.0 buffer. The solution was then charged with 5 µL of a 200 mM nucleophile stock solution in H_2O. The solution was mixed thoroughly and allowed to incubate at 37 °C for 18 h. Decomposition of/nucleophilic addition to thiophosphonium **12** was assessed by LCMS analysis.

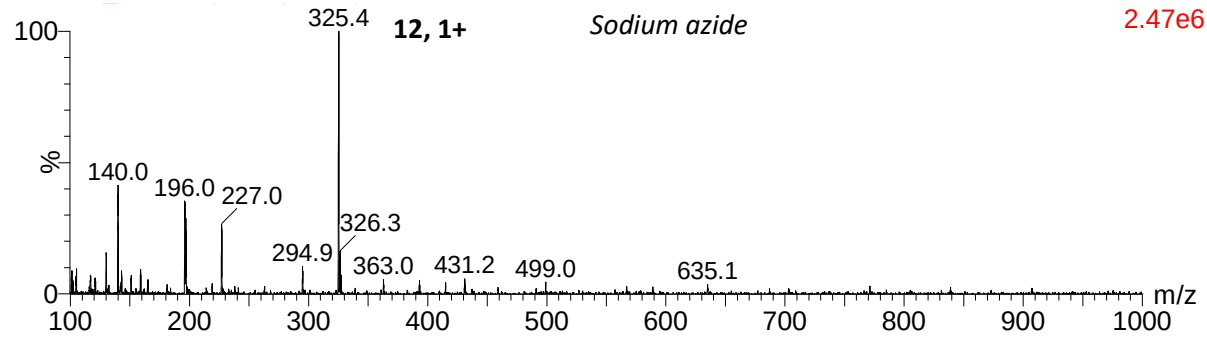
Nucleophiles:

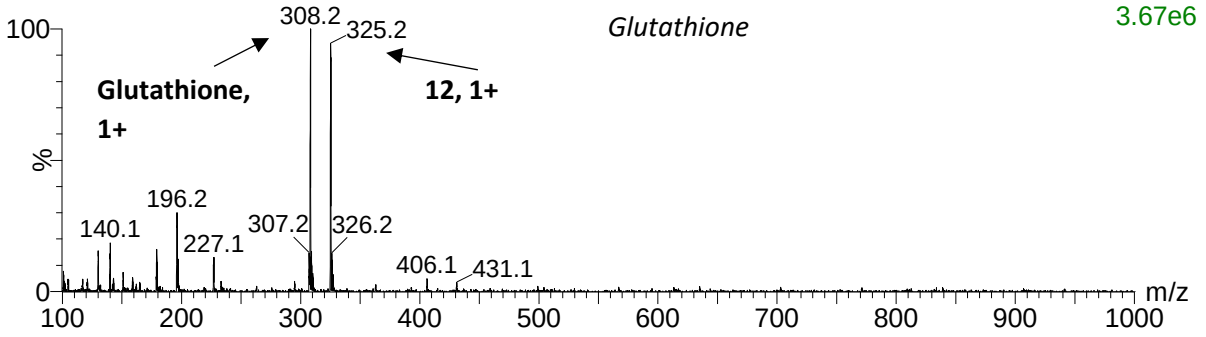


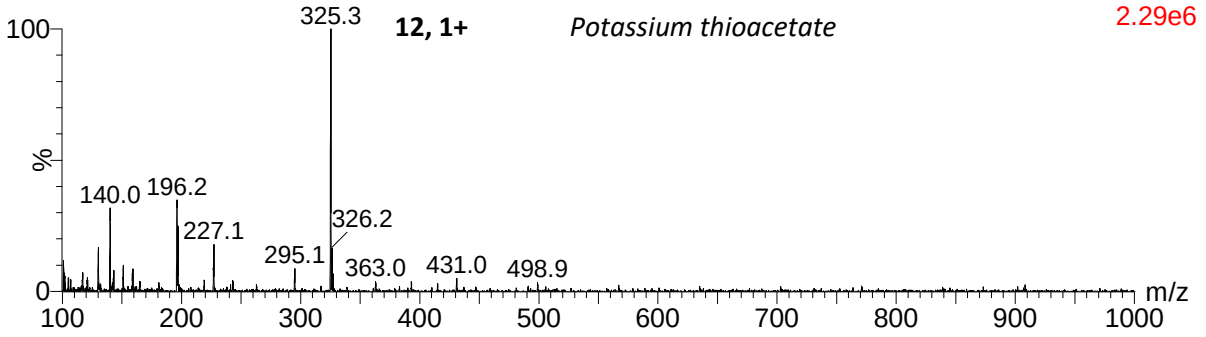
Total ion counts

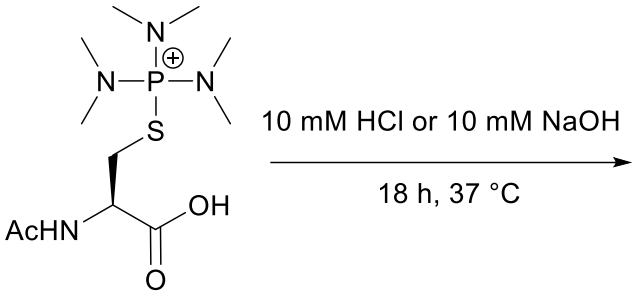



325.3
12, 1+
3.24e6
No nucleophile
100
%
0
196.2
139.9
197.0
295.0
326.2
m/z
100
200
300
400
500
600
700
800
900
1000


325.4
12, 1+
Sodium azide
2.47e6
100
%
0
140.0
196.0
227.0
326.3
294.9
363.0
431.2
499.0
635.1
m/z
100
200
300
400
500
600
700
800
900
1000


308.2
325.2
Glutathione
3.67e6
Glutathione,
1+
12, 1+
100
%
0
196.2
140.1
227.1
307.2
326.2
406.1
431.1
m/z
100
200
300
400
500
600
700
800
900
1000

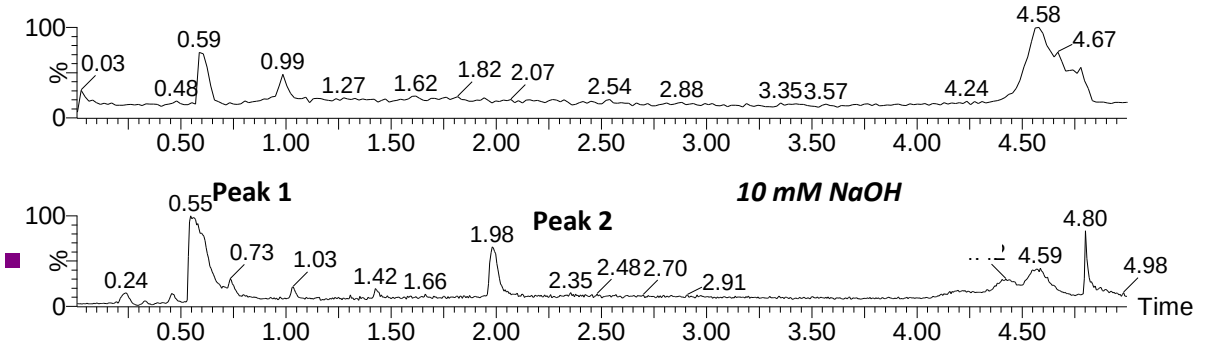

325.3
12, 1+
Potassium thioacetate
2.29e6
100
%
0
140.0
196.2
227.1
295.1
326.2
363.0
431.0
498.9
m/z
100
200
300
400
500
600
700
800
900
1000

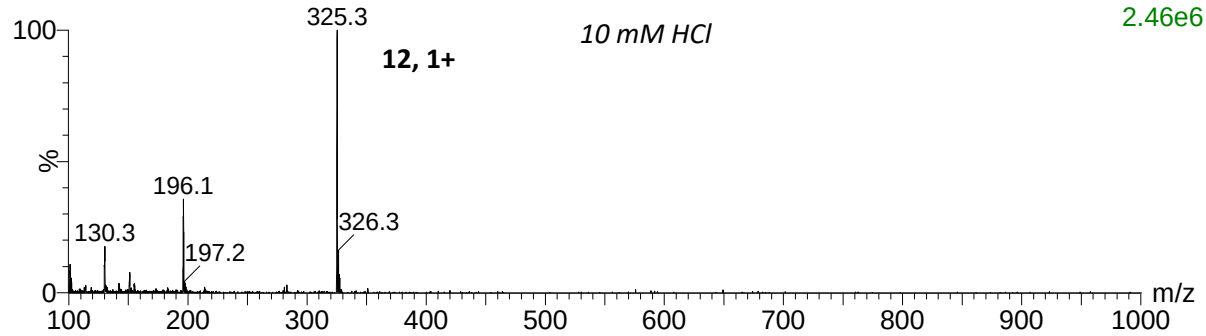


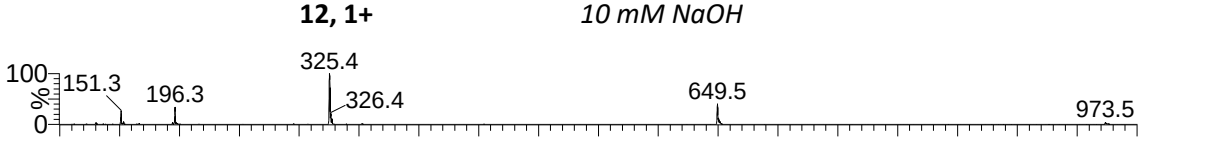
1 mM

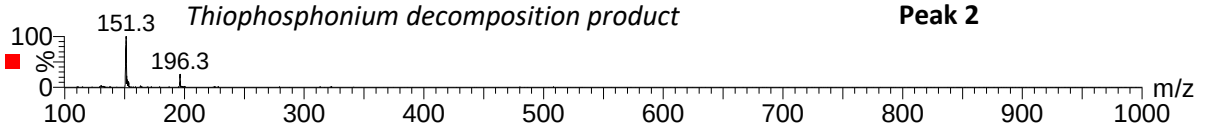
A 5 µL aliquot of a 20 mM thiophosphonium **12** stock in H_2O was charged with 45 µL of H_2O. The solution was then charged with 50 µL of either 20 mM HCl or 20 mM NaOH. The solution was mixed thoroughly and allowed to incubate at 37 °C for 18 h. Decomposition of thiophosphonium **12** was assessed by LCMS analysis.

Total ion counts





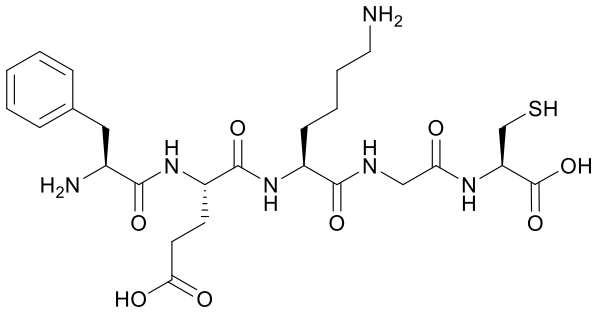


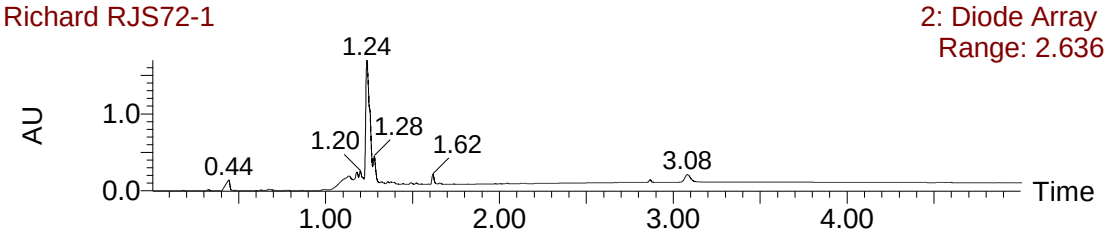


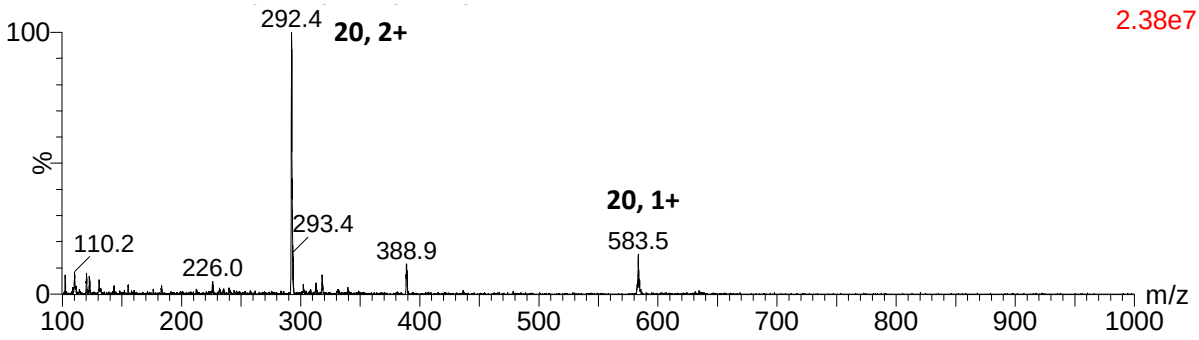
Peptides

FEKGC **20**

Synthesised using 200 mg resin (0.19 mmol g^{-1}. Yield = 18 mg (82% yield)

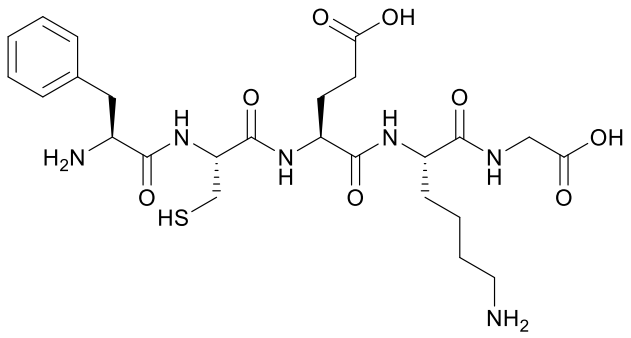


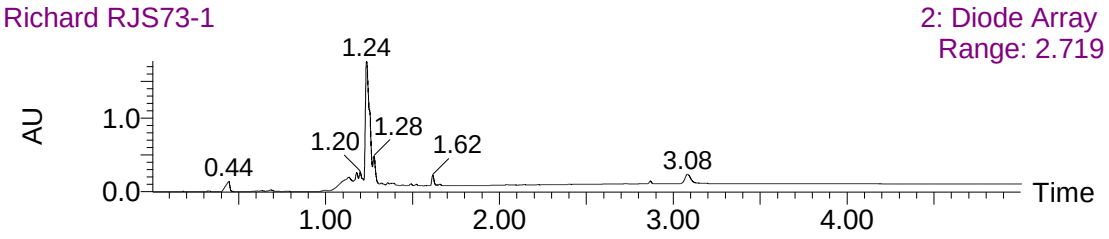


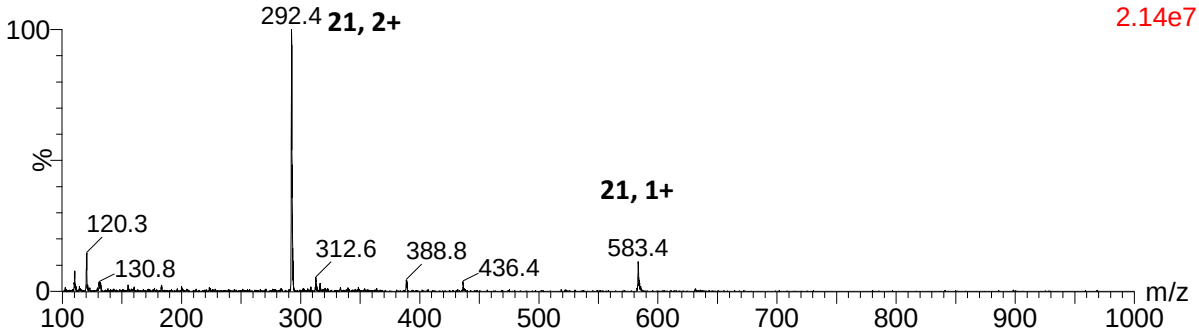


FCEKG **21**

Synthesised using 200 mg resin (0.19 mmol g^{-1}. Yield = 15 mg (68% yield)

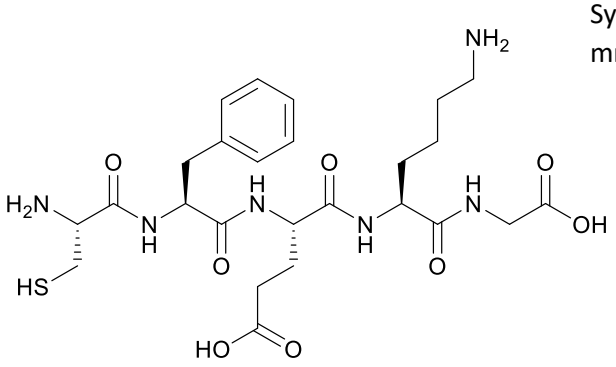


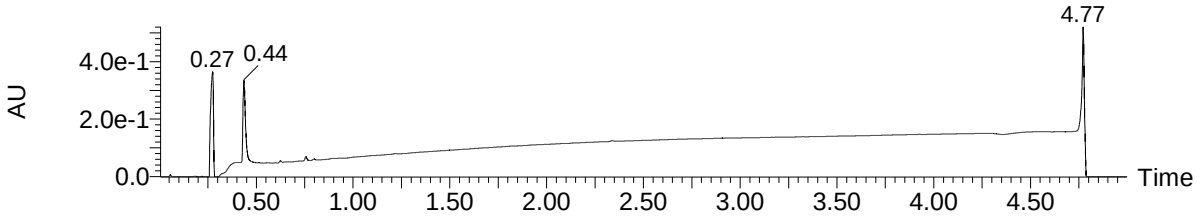


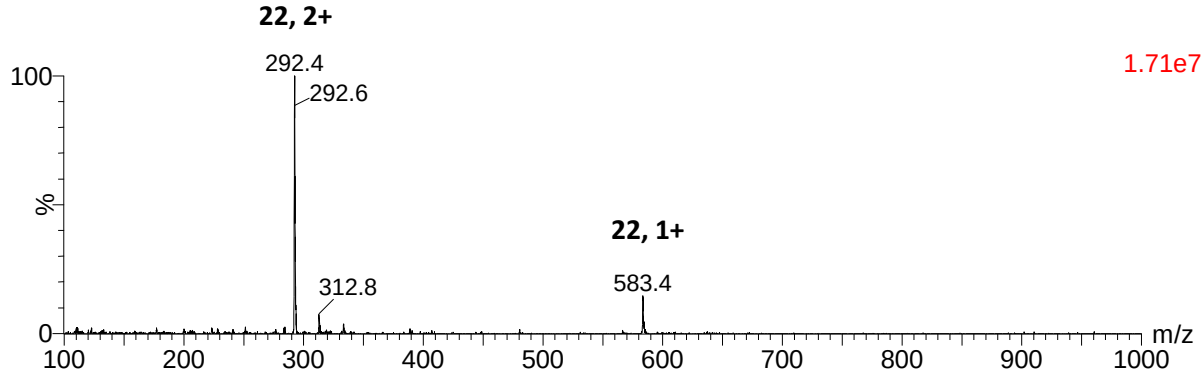


CFEKG **22**

Synthesised using 200 mg resin (0.19 mmol g^{-1}. Yield = 20 mg (90% yield)

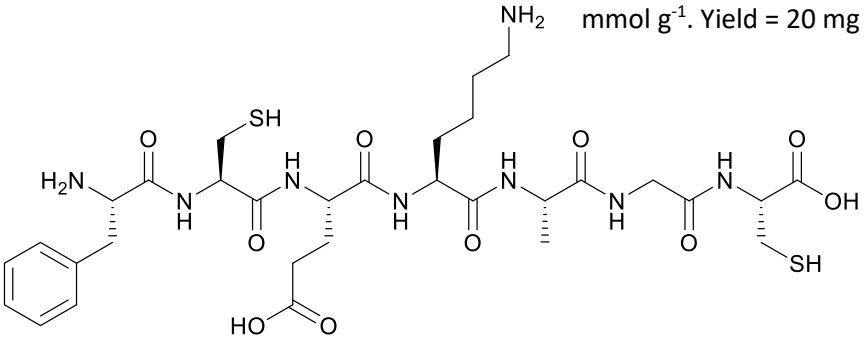


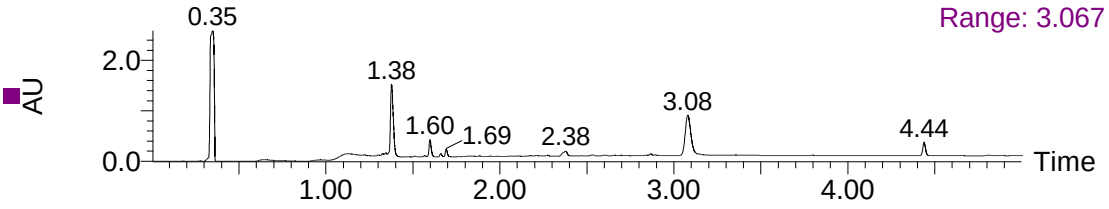


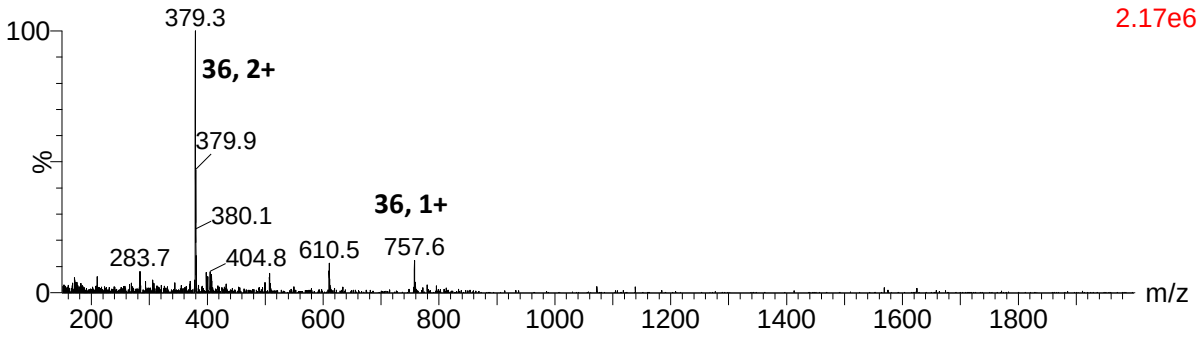


FCEKAGC **36**

Synthesised using 175 mg resin (0.21 mmol g^{-1}. Yield = 20 mg (72% yield).



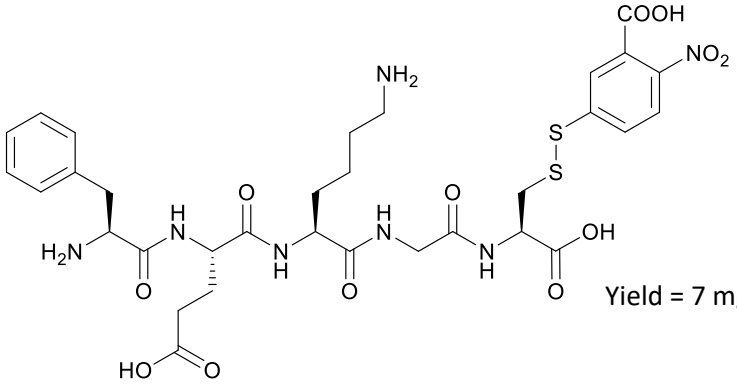




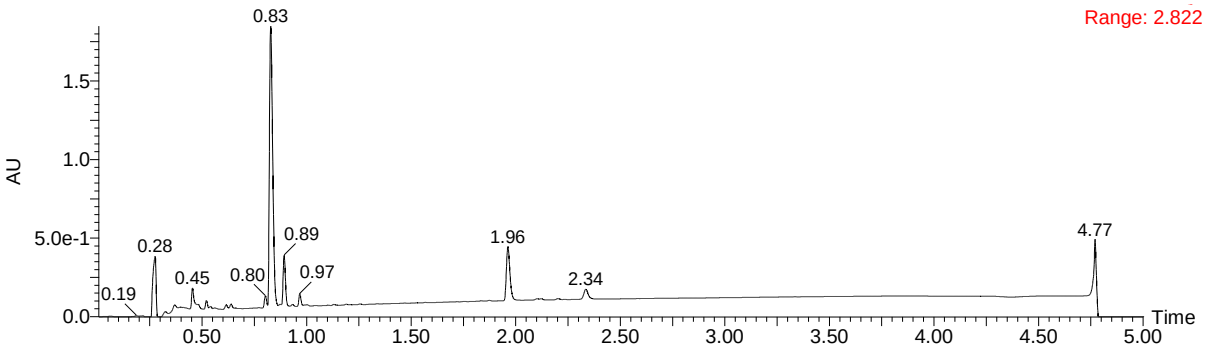
Formation of disulfide-containing peptides 24, 25 and 26

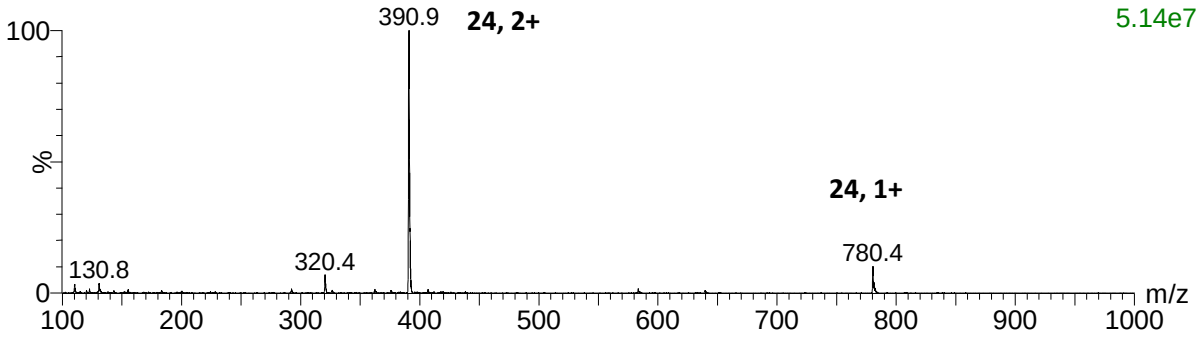
To a solution of cysteine containing peptides **20, 21** or **22** (10 mg) in 0.1 M PB pH 8.0 (900 µL) was added Ellman's reagent **23** (12.6 mg) in DMF (100 µL). The reaction was mixed thoroughly, and allowed to incubate at 21 °C for 1 h. Samples were purified using a solid phase extraction cartridge, and subsequently lyophilised to give a white powder.

Peptide 24

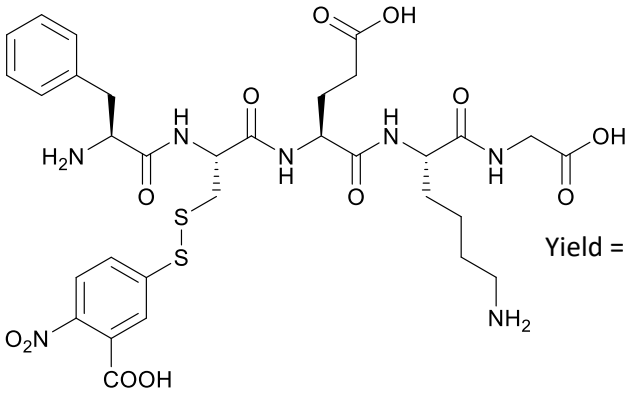


Yield = 7 mg (54% yield).

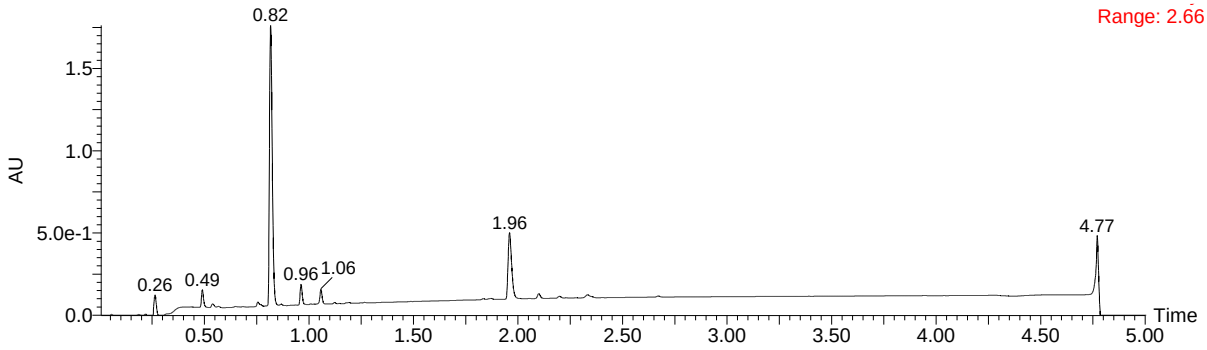


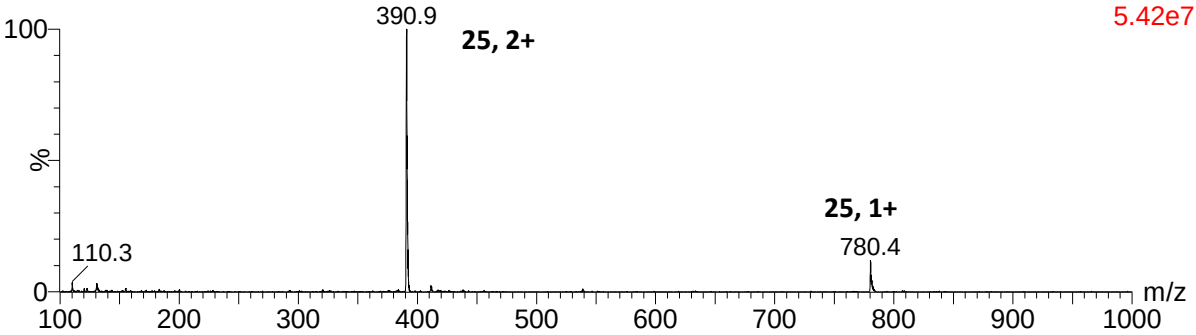


Peptide 25

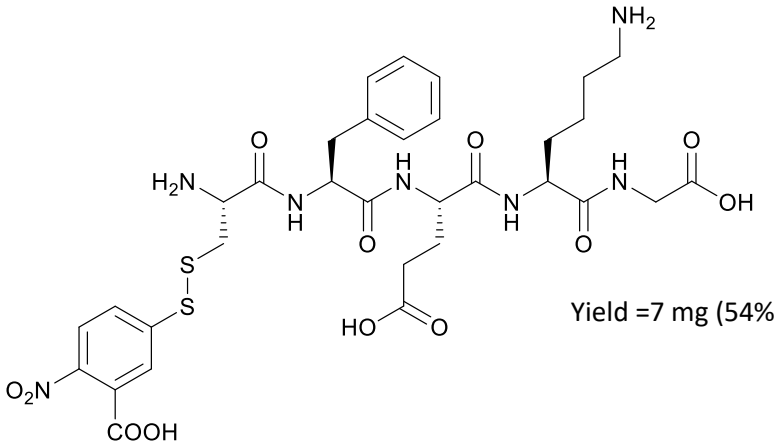


Yield = 5 mg (38% yield).

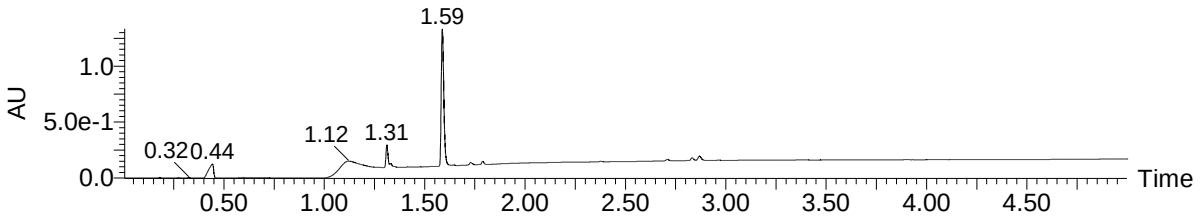


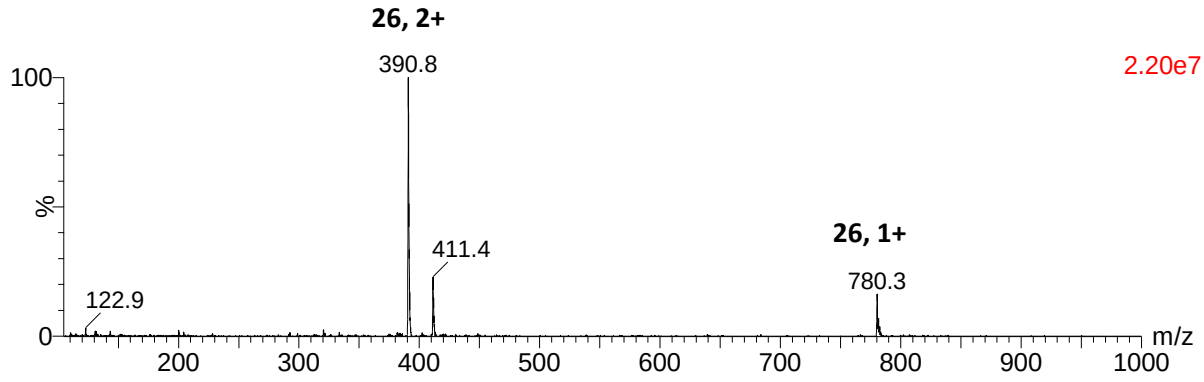


Peptide 26

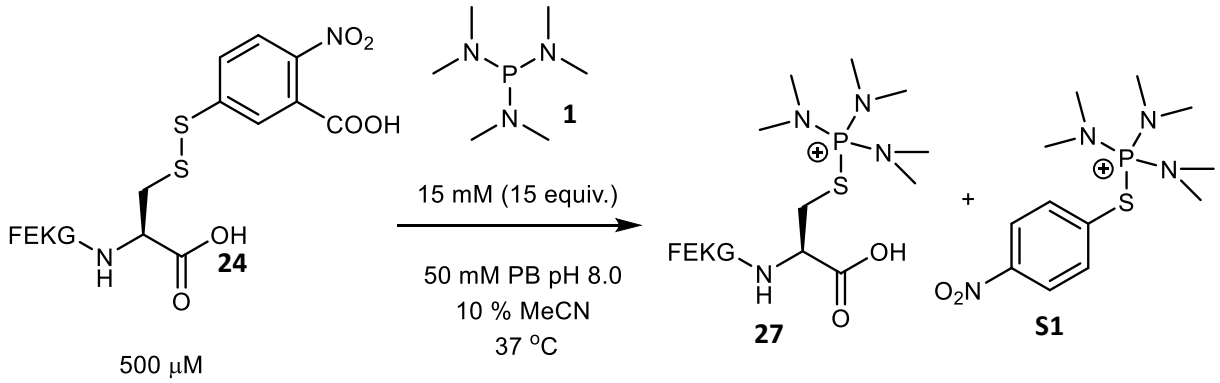


Yield =7 mg (54% yield).



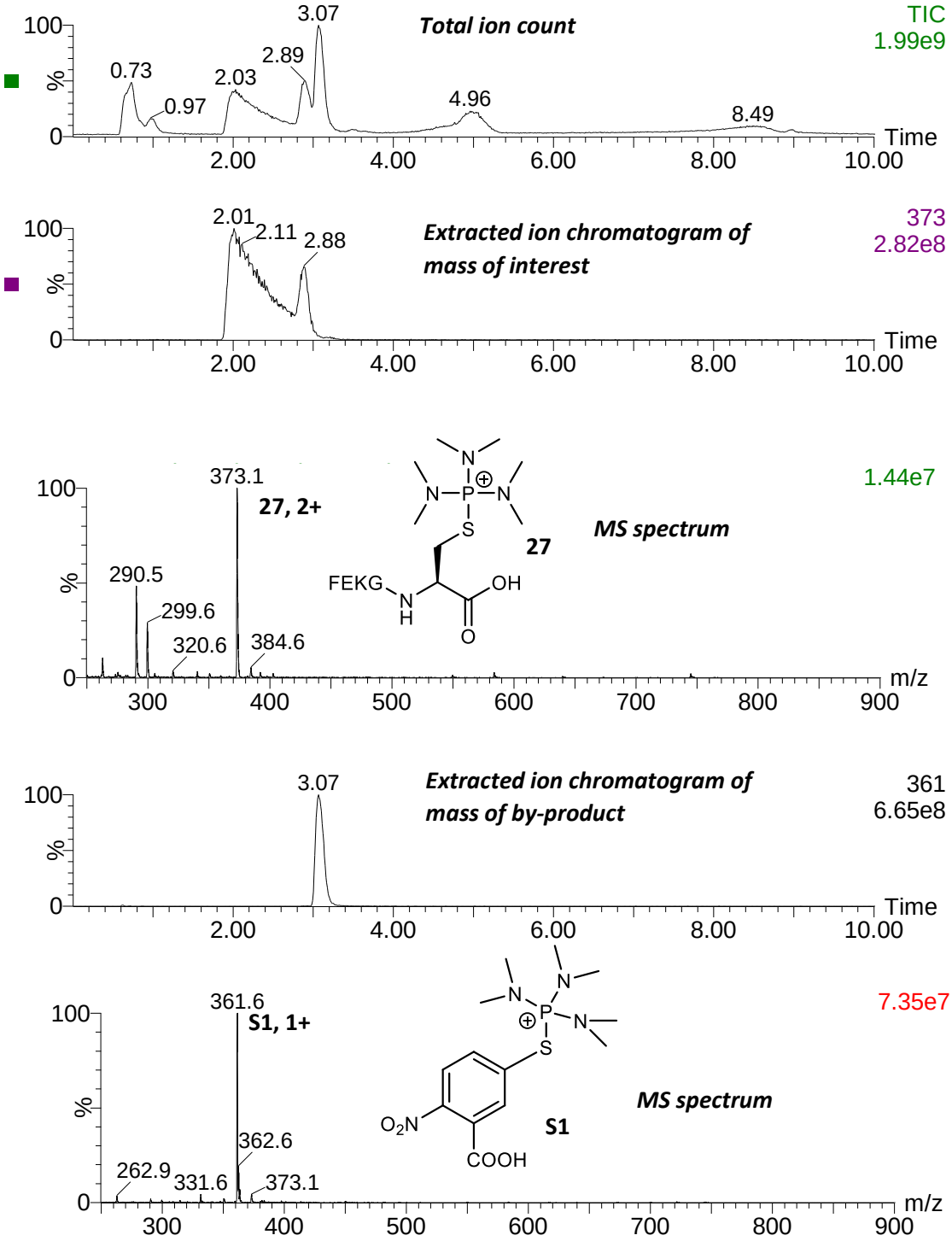


Reaction of peptide disulfide 24 with HMPT 1

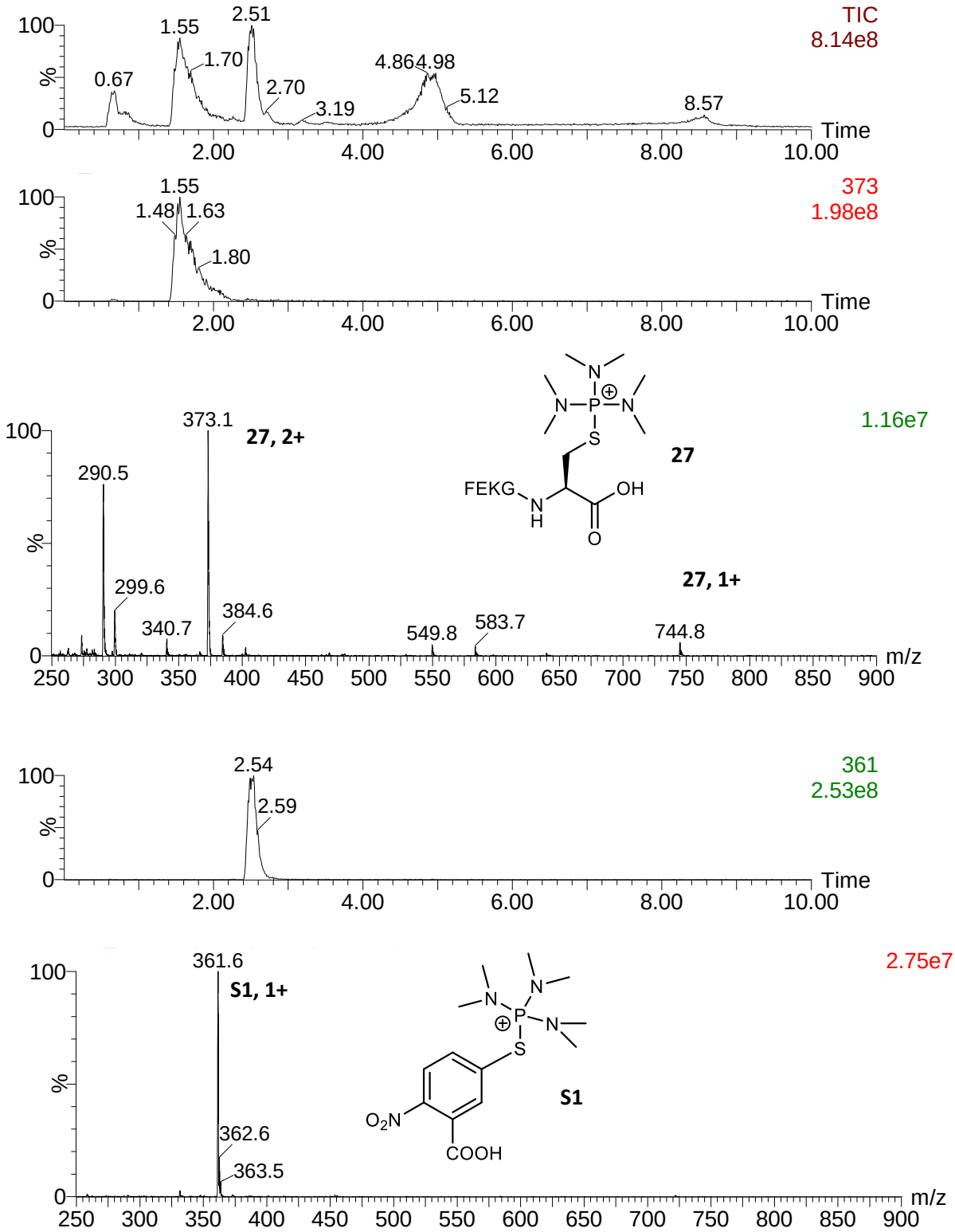


A 10 µL aliquot of a 2.5 mM disulfide **24** stock in H_2O was charged with 25 µL of 0.1 M PB pH 8.0 buffer and 10 µL H_2O. The solution was then charged with 5 µL of a 150 mM HMPT **1** stock solution in MeCN. The solution was mixed thoroughly, and allowed to incubate at 37 °C. Formation of thiophosphonium **27** (along with observation of Sar thiophosphonium species **S1**) was assessed by LCMS analysis.

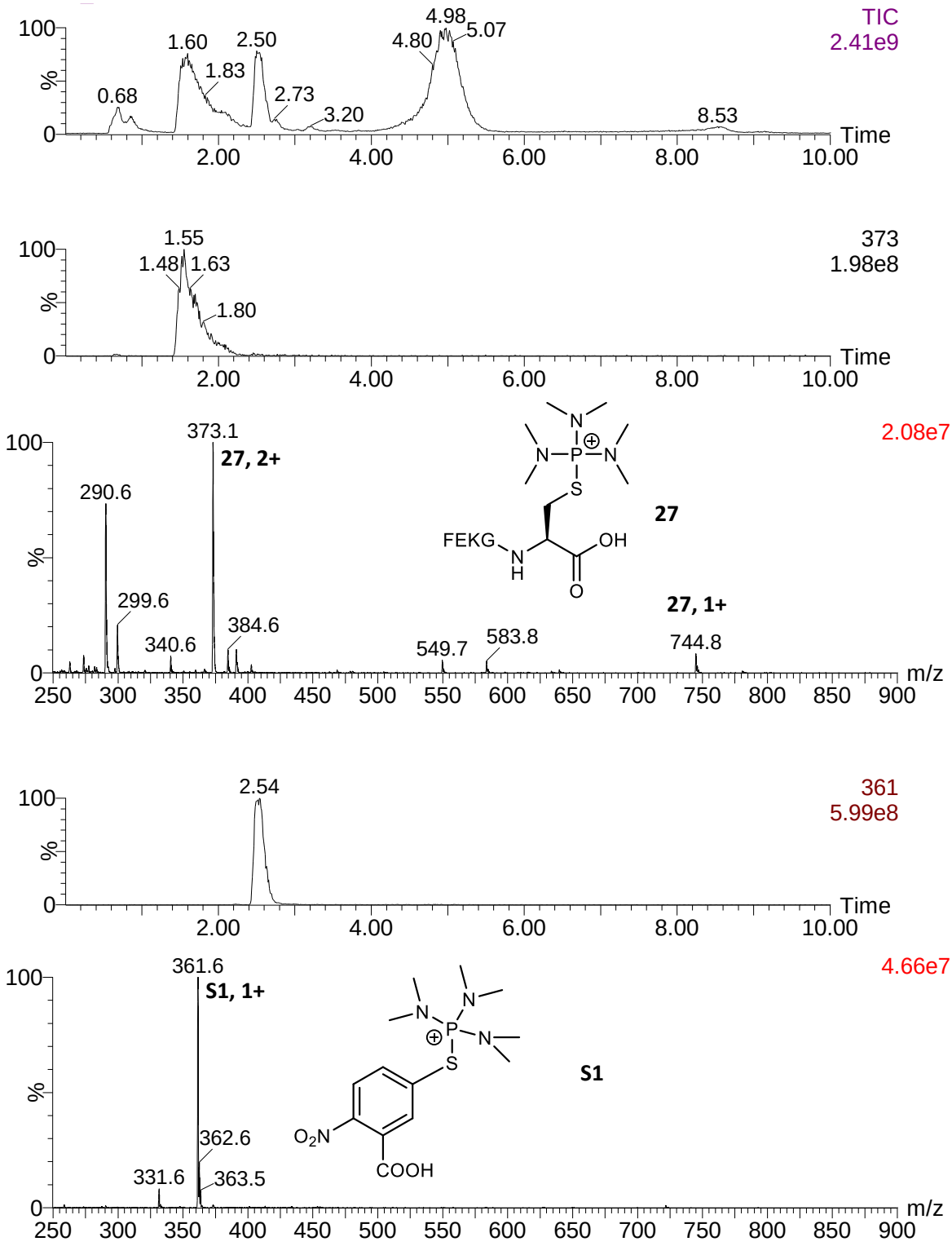
5 min


Total ion count
TIC
1.99e9
3.07
2.89
0.73
2.03
0.97
4.96
8.49
Time
Extracted ion chromatogram of mass of interest
373
2.82e8
2.01
2.11
2.88
MS spectrum
1.44e7
373.1
27, 2+
290.5
299.6
320.6
384.6
27
FEKG
OH
m/z
Extracted ion chromatogram of mass of by-product
361
6.65e8
MS spectrum
7.35e7
361.6
S1, 1+
362.6
262.9
331.6
373.1
S1
COOH
m/z

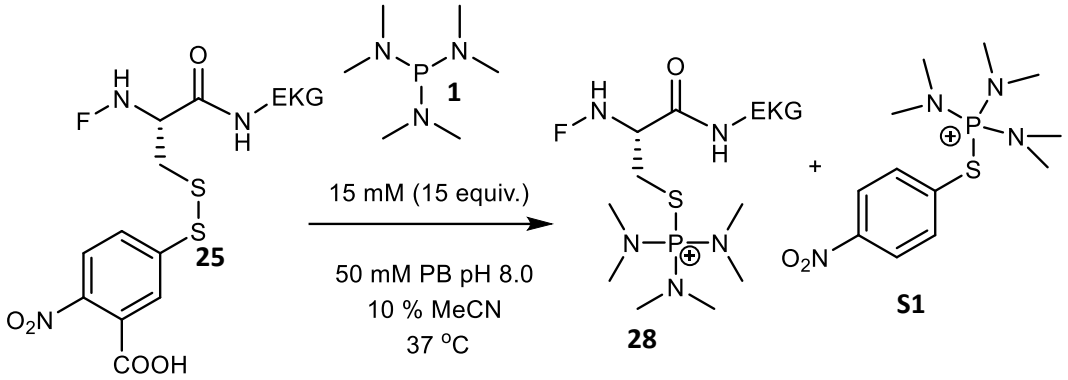
1h


TIC
8.14e8
0.67
1.55
1.70
2.51
2.70
3.19
4.86
4.98
5.12
8.57
Time
373
1.98e8
1.48
1.55
1.63
1.80
Time
1.16e7
373.1
27, 2+
290.5
299.6
340.7
384.6
549.8
583.7
744.8
m/z
27
27, 1+
FEKG
OH
361
2.53e8
2.54
2.59
Time
2.75e7
361.6
S1, 1+
362.6
363.5
m/z
S1
COOH

18h

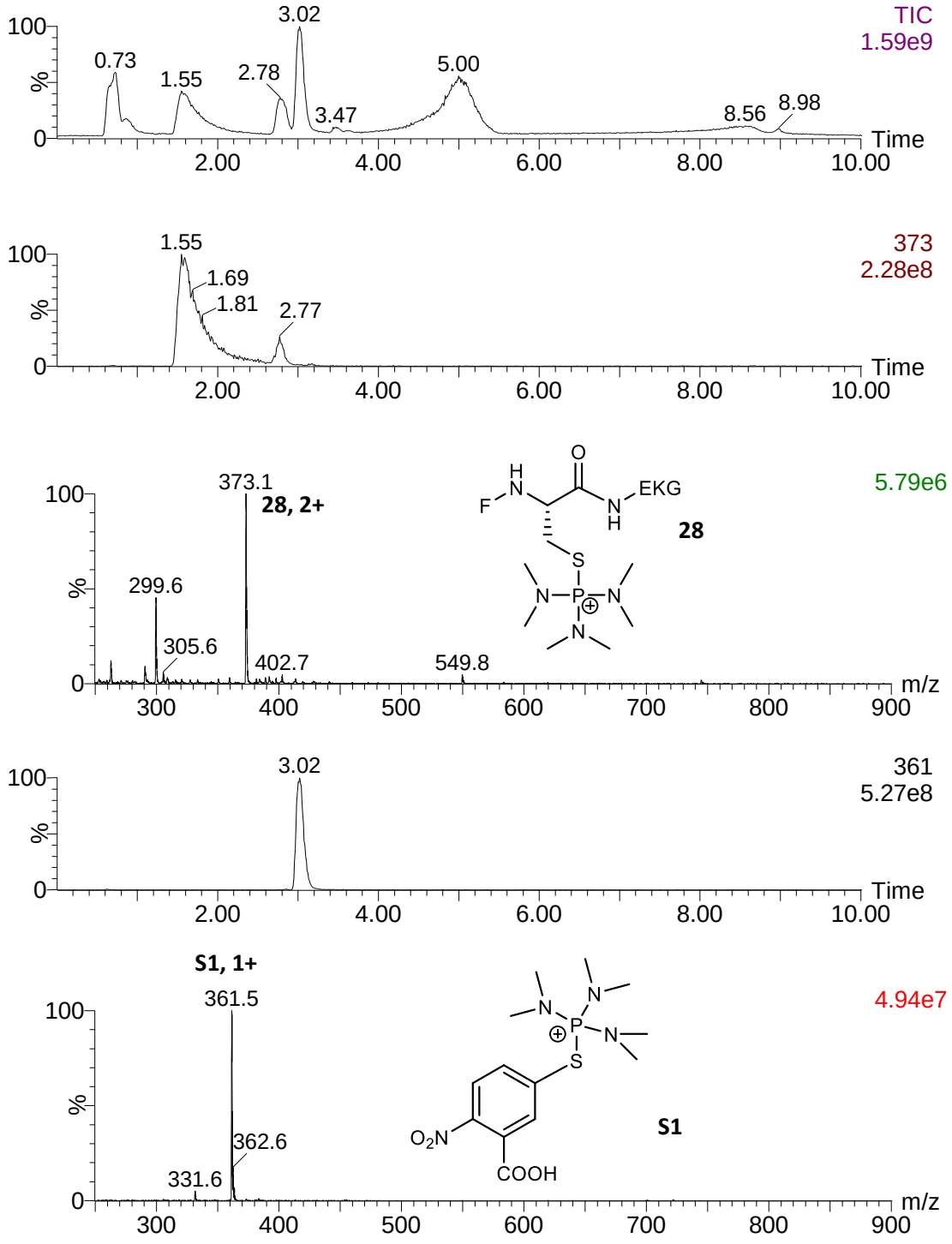

TIC
2.41e9
0.68
1.60
1.83
2.50
2.73
3.20
4.80
4.98
5.07
8.53
Time
373
1.98e8
1.48
1.55
1.63
1.80
2.08e7
373.1
27, 2+
290.6
299.6
340.6
384.6
549.7
583.8
744.8
27
27, 1+
FEKG
OH
361
5.99e8
2.54
4.66e7
361.6
S1, 1+
362.6
331.6
363.5
S1
COOH
m/z

Reaction of peptide disulfide 25 with HMPT 1

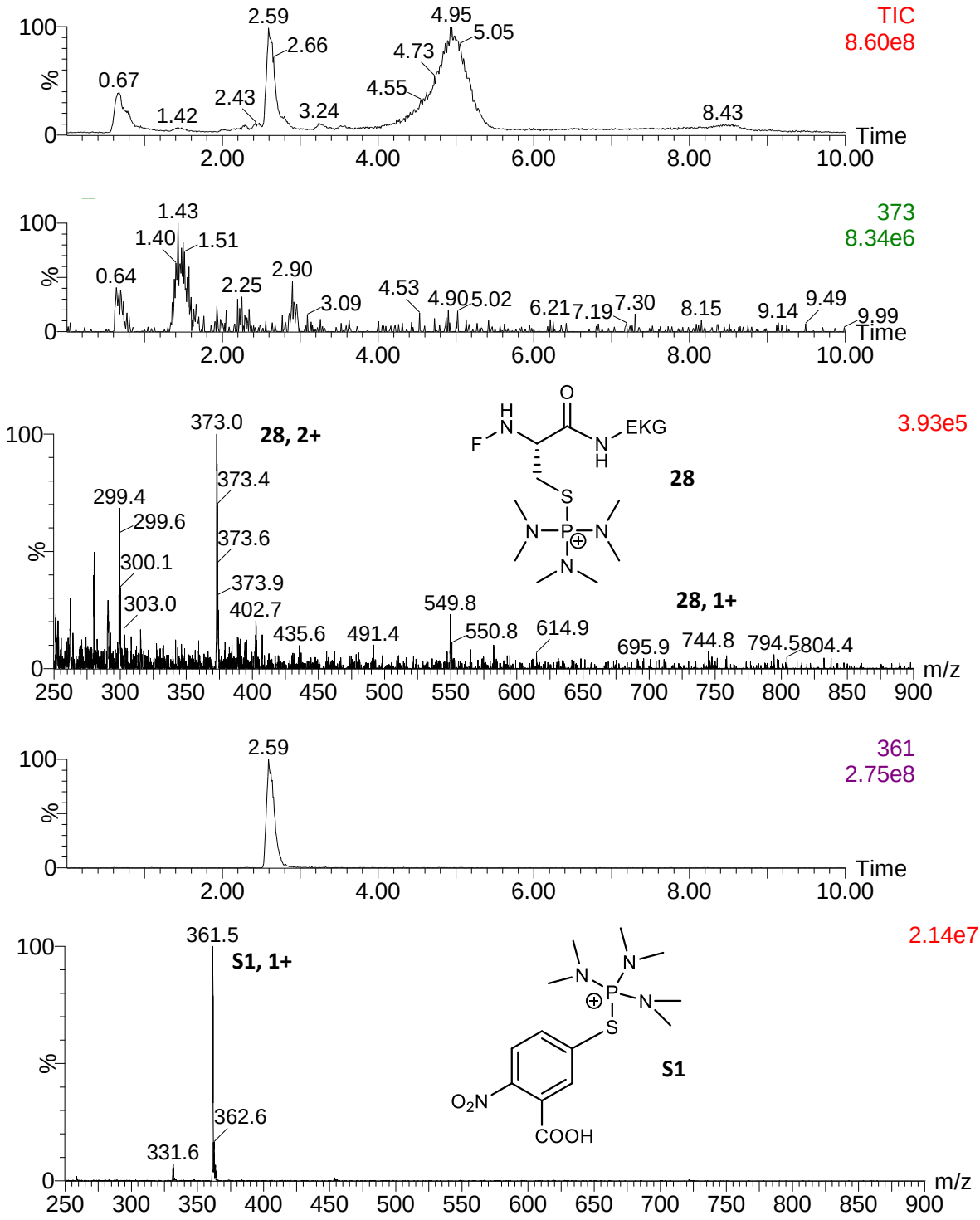


A 10 µL aliquot of a 2.5 mM disulfide **25** stock in H_2O was charged with 25 µL of 0.1 M PB pH 8.0 buffer and 10 µL H_2O. The solution was then charged with 5 µL of a 150 mM HMPT **1** stock solution in MeCN. The solution was mixed thoroughly, and allowed to incubate at 37 °C. Formation of thiophosphonium **28** was appraised by LCMS analysis at various time points.

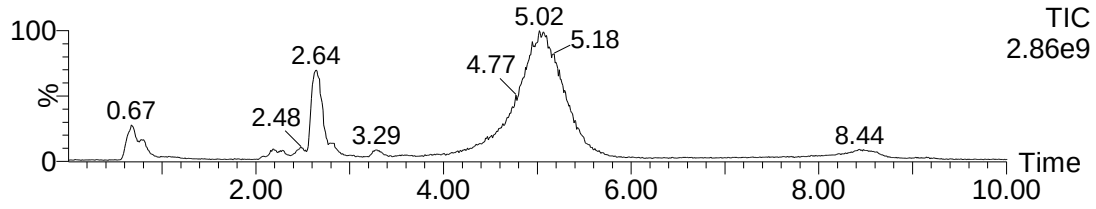
5 min


TIC
1.59e9
3.02
0.73
1.55
2.78
3.47
5.00
8.56
8.98
Time
373
2.28e8
1.55
1.69
1.81
2.77
373.1
28, 2+
299.6
305.6
402.7
549.8
28
5.79e6
361
5.27e8
S1, 1+
361.5
362.6
331.6
S1
4.94e7
m/z

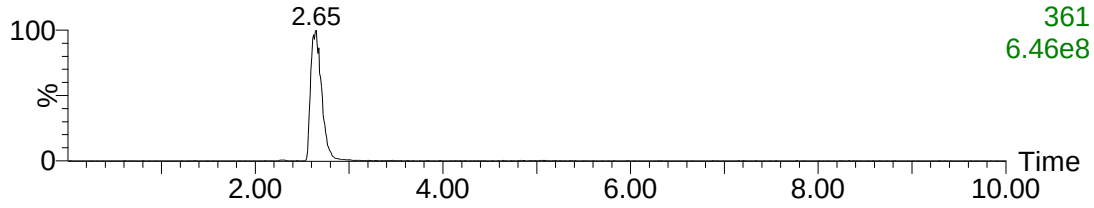
1 h

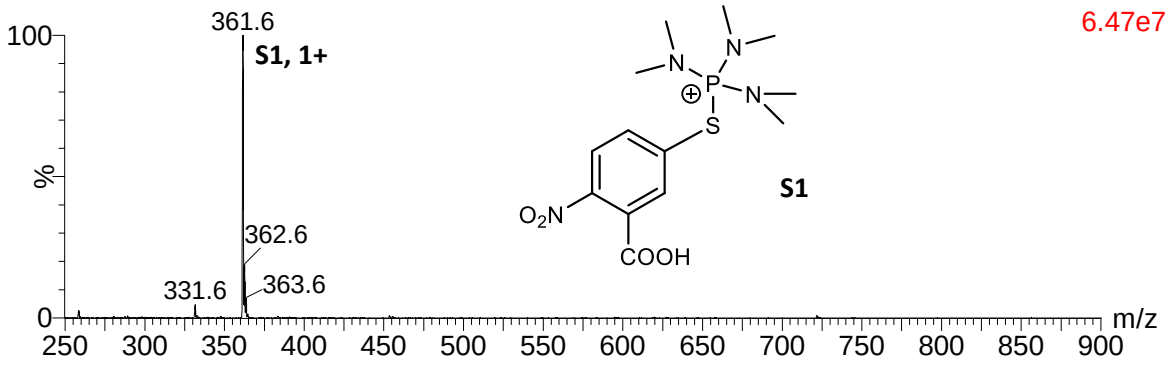

TIC
8.60e8
373
8.34e6
3.93e5
28, 2+
28
28, 1+
361
2.75e8
2.14e7
S1, 1+
S1
Time
m/z

18h

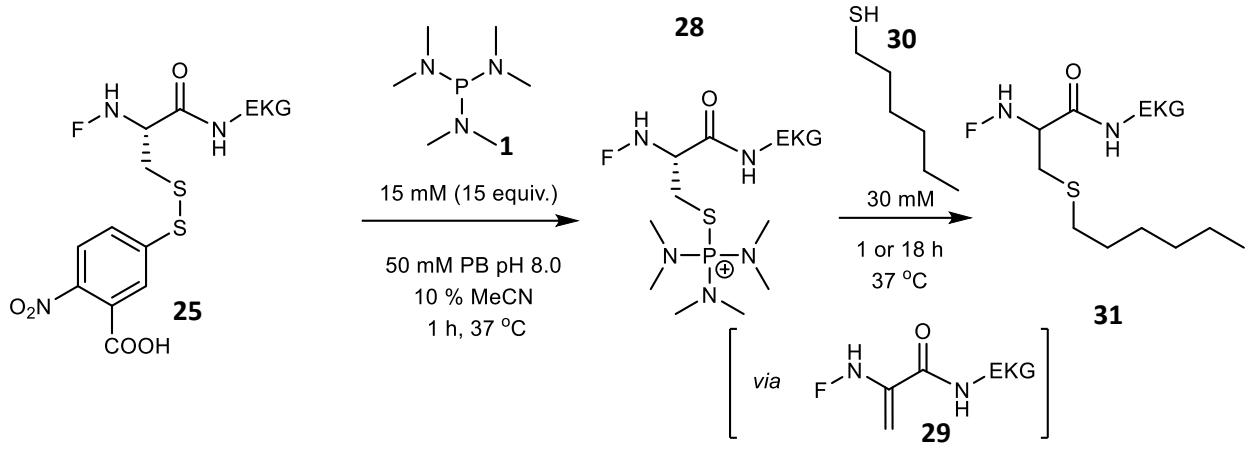


Thiophosphonium not detected



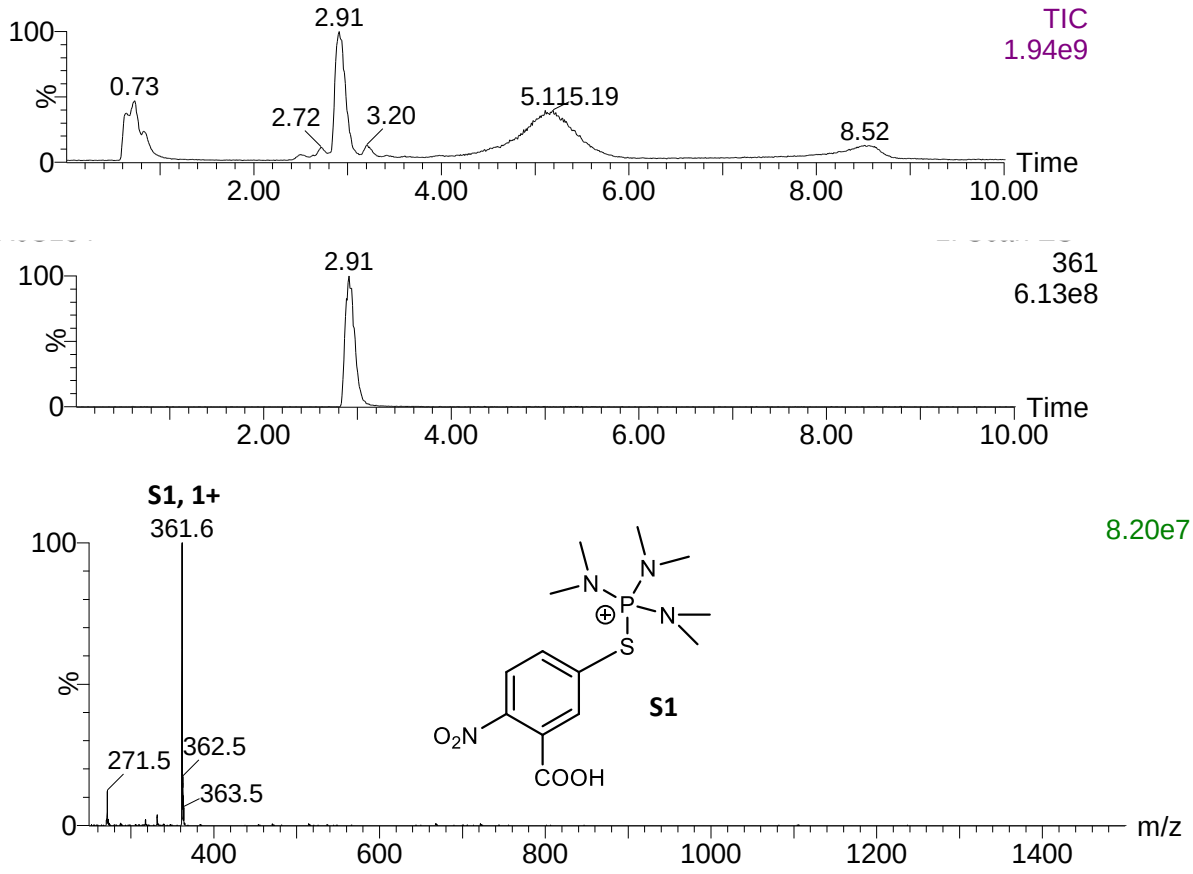


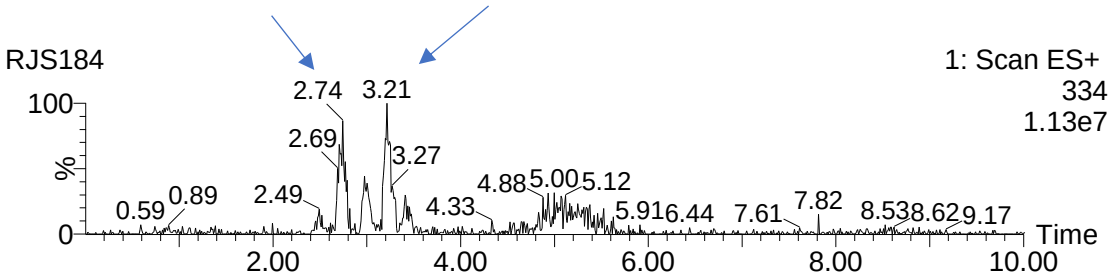
Reaction of peptide 25 with HMPT 1 followed by *n*-hexanethiol 27 addition

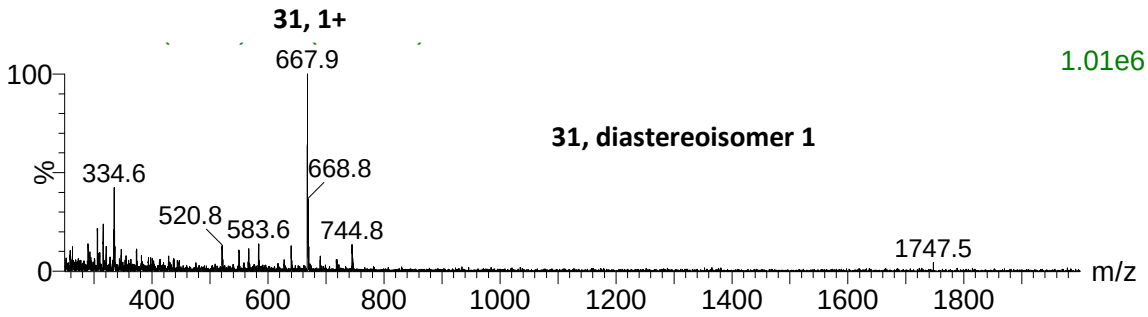


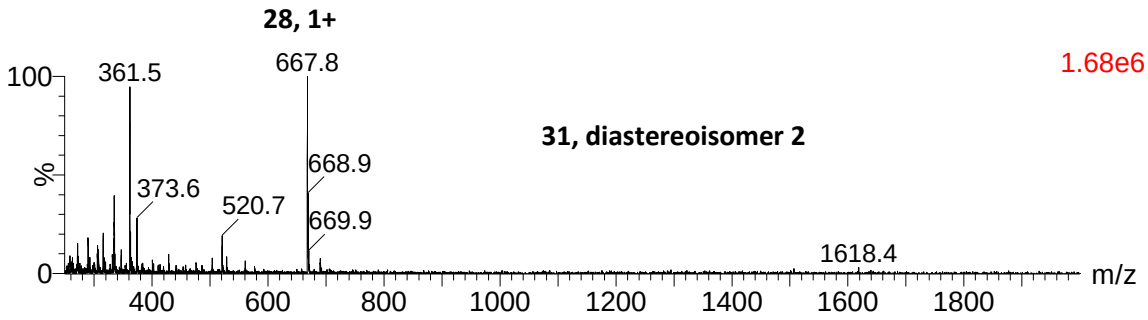
A 10 µL aliquot of a 2.5 mM disulfide **25** stock in H_2O was charged with 25 µL of 0.1 M PB pH 8.0 buffer and 10 µL H_2O. The solution was then charged with 5 µL of a 150 mM HMPT **1** stock solution in MeCN. The solution was mixed thoroughly, and allowed to incubate at 37 °C for 1 h. After this time, the reaction mixture was charged with 50 µL of a 60 mM *n*-hexanethiol **30** stock solution in H_2O:MeCN (1:1). Formation of thiol-capped peptide **31** was appraised by LCMS analysis at various time points. *N.B. Two peaks at different retention times for peptide **31** were observed – we hypothesise that these corresponds to the two different diastereoisomers that result from thiol attack at either face of the Dha side chain.*[1]

1h

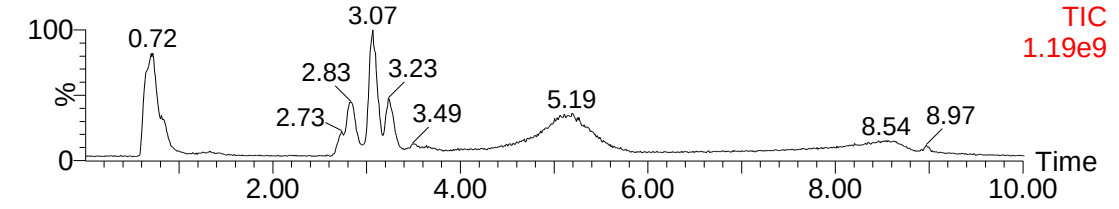


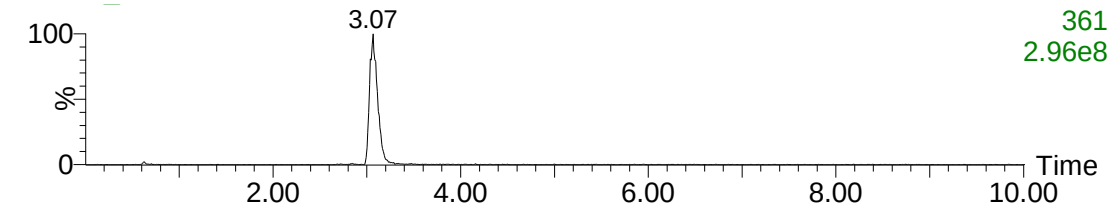

RJS184
1: Scan ES+
334
1.13e7
100
%
0
2.74
3.21
2.69
3.27
0.59
0.89
2.49
4.33
4.88
5.00
5.12
5.91
6.44
7.61
7.82
8.53
8.62
9.17
Time
2.00
4.00
6.00
8.00
10.00

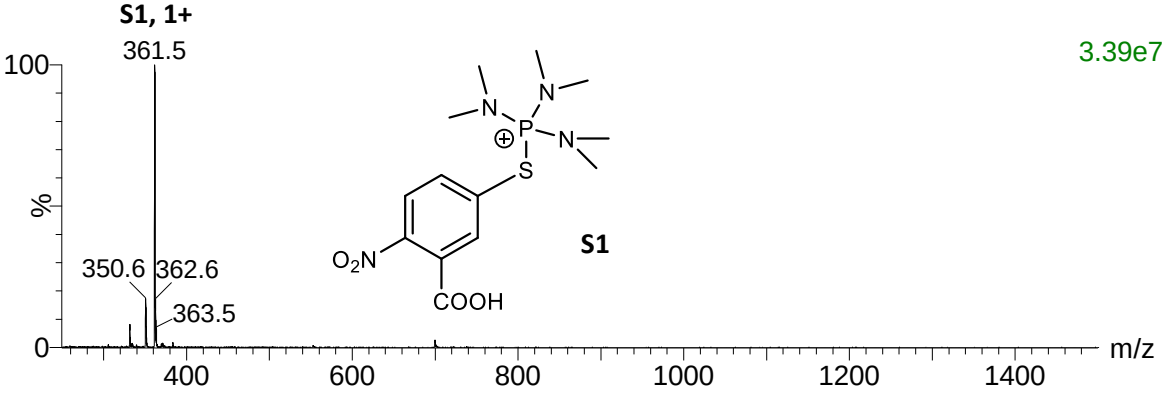

31, 1+
667.9
1.01e6
31, diastereoisomer 1
100
%
0
334.6
668.8
520.8
583.6
744.8
1747.5
m/z
400
600
800
1000
1200
1400
1600
1800

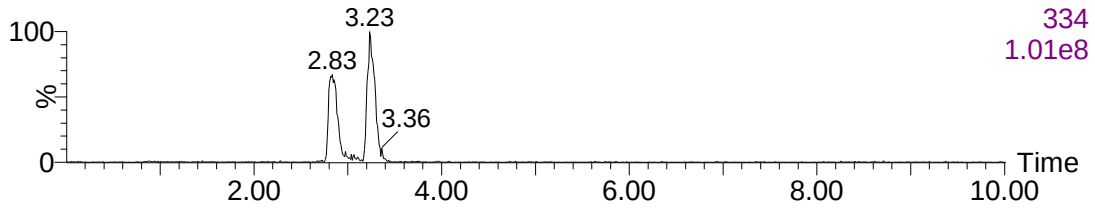

28, 1+
667.8
1.68e6
31, diastereoisomer 2
100
%
0
361.5
373.6
520.7
668.9
669.9
1618.4
m/z
400
600
800
1000
1200
1400
1600
1800

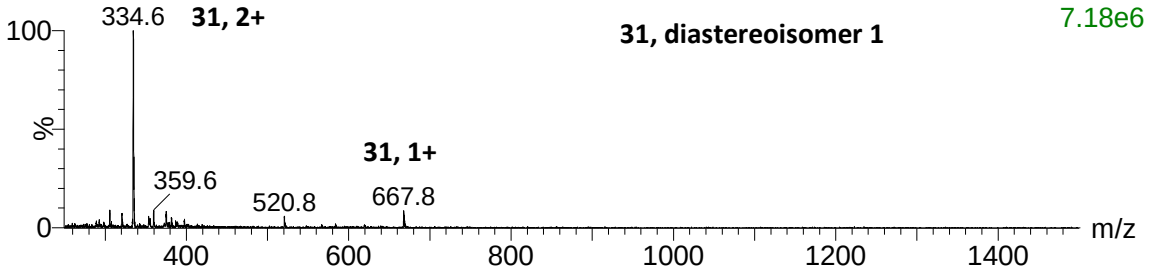
18h

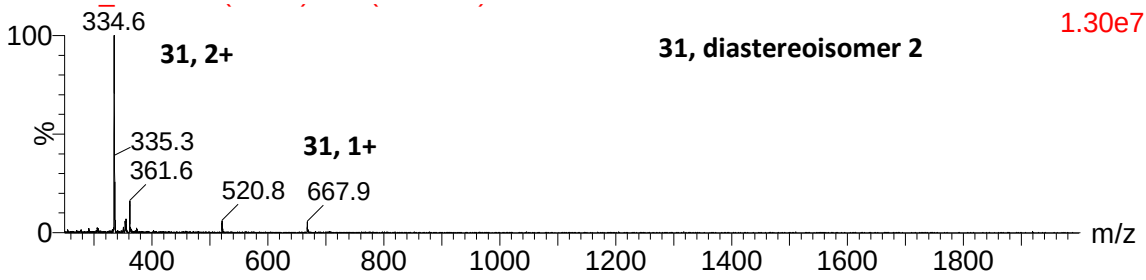

TIC
1.19e9
100
%
0
0.72
3.07
2.83
3.23
2.73
3.49
5.19
8.54
8.97
Time
2.00
4.00
6.00
8.00
10.00


361
2.96e8
100
%
0
3.07
Time
2.00
4.00
6.00
8.00
10.00

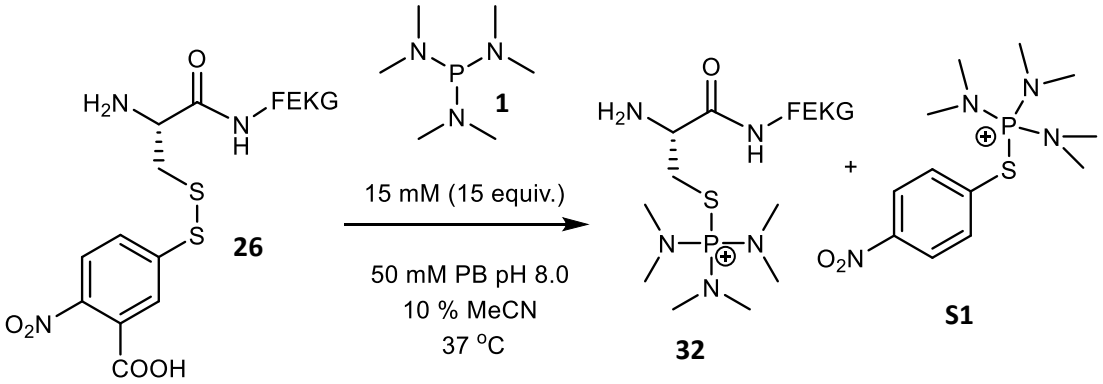

S1, 1+
361.5
3.39e7
100
%
0
350.6
362.6
363.5
O2N
COOH
S1
m/z
400
600
800
1000
1200
1400


100
%
0
3.23
2.83
3.36
334
1.01e8
Time
2.00
4.00
6.00
8.00
10.00


100
%
0
334.6
31, 2+
31, diastereoisomer 1
7.18e6
31, 1+
359.6
520.8
667.8
m/z
400
600
800
1000
1200
1400

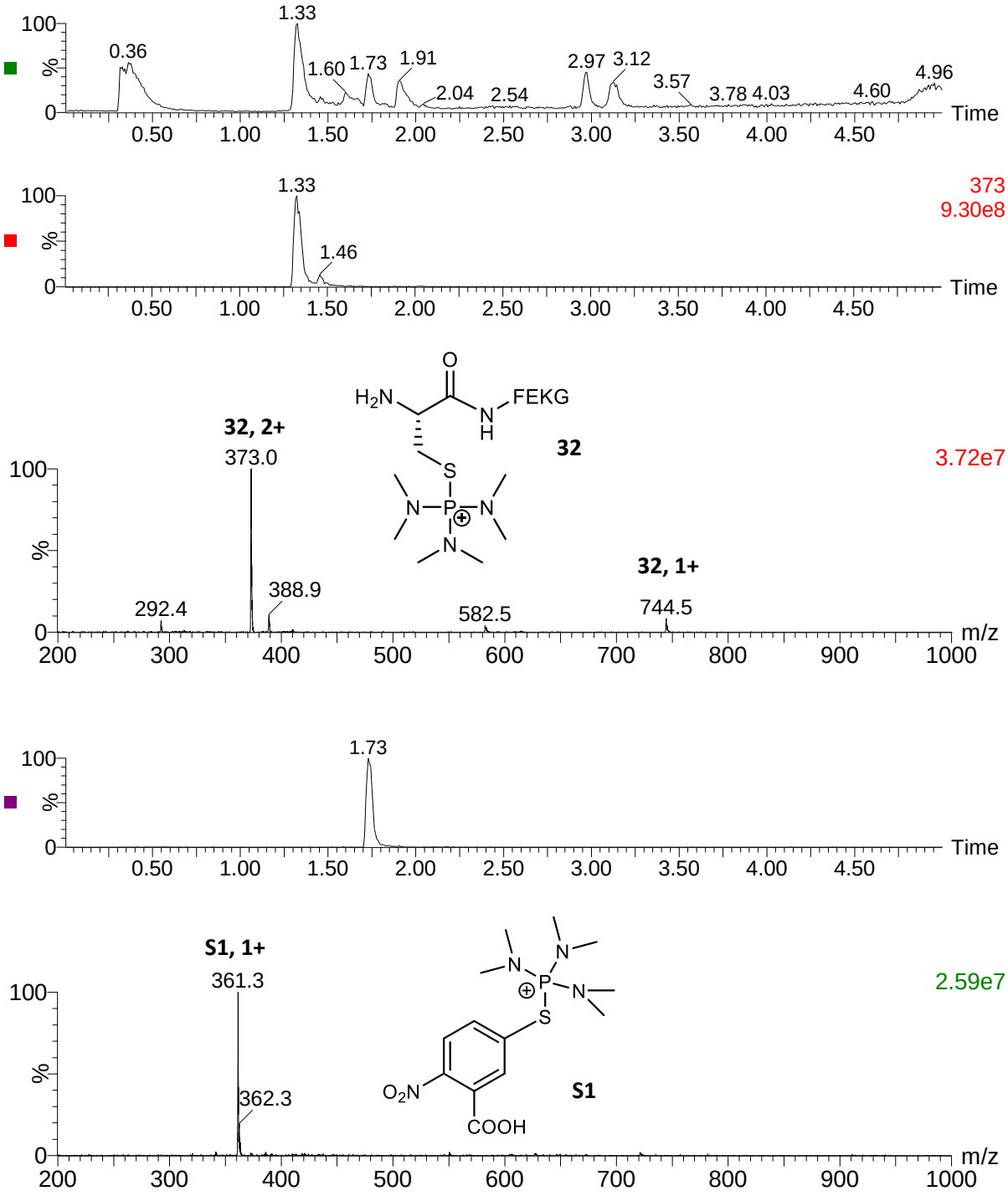

100
%
0
334.6
31, 2+
31, diastereoisomer 2
1.30e7
335.3
361.6
31, 1+
520.8
667.9
m/z
400
600
800
1000
1200
1400
1600
1800

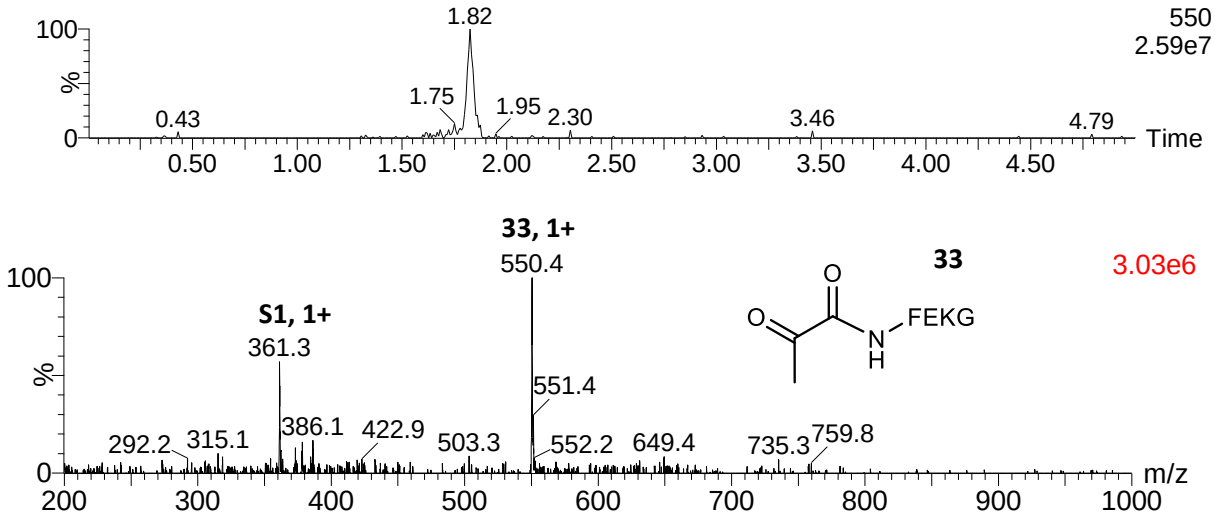
Reaction of peptide 26 with HMPT 1



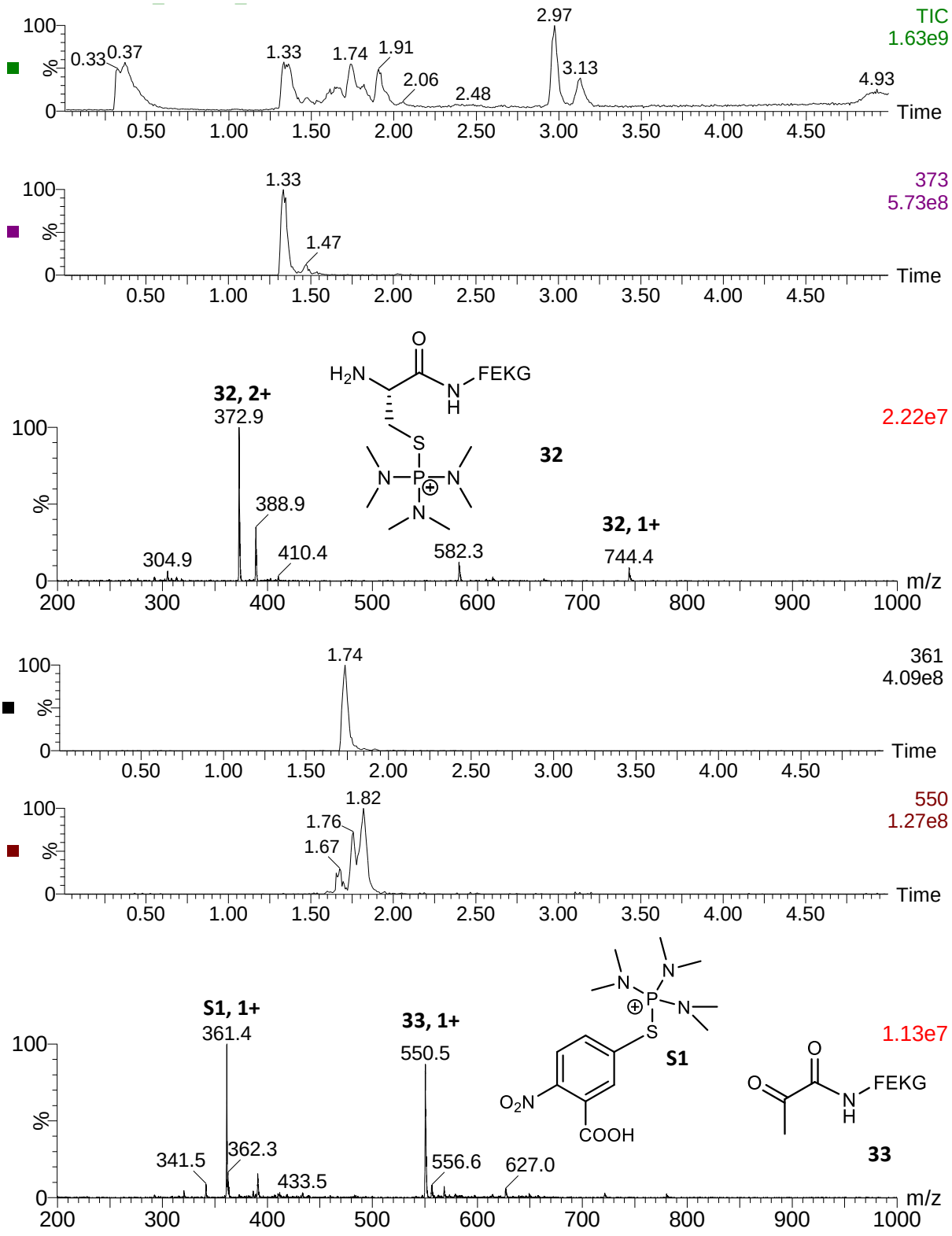
A 10 µL aliquot of a 2.5 mM disulfide **26** stock in H_2O was charged with 25 µL of 0.1 M PB pH 8.0 buffer and 10 µL H_2O. The solution was then charged with 5 µL of a 150 mM HMPT **1** stock solution in MeCN. The solution was mixed thoroughly, and allowed to incubate at 37 °C. Formation of thiophosphonium **32** was appraised by LCMS analysis at various time points.

1 h

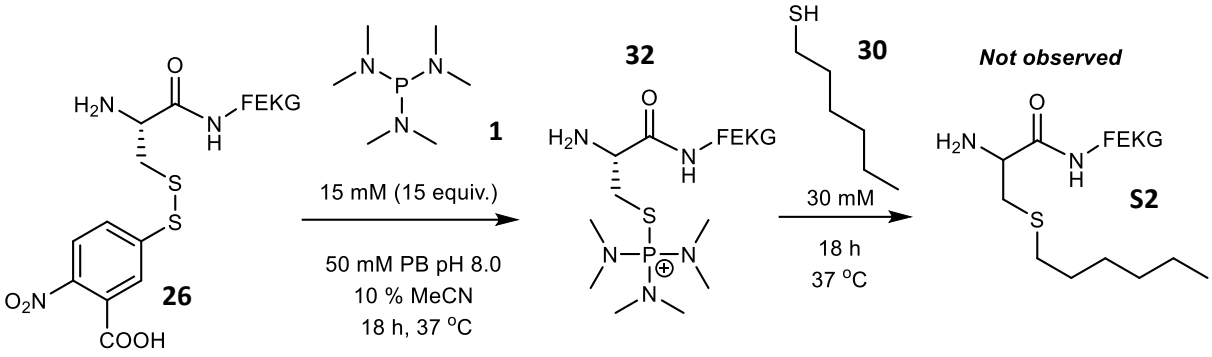

1.33
0.36
1.60
1.73
1.91
2.04
2.54
2.97
3.12
3.57
3.78
4.03
4.60
4.96
Time
373
9.30e8
1.46
32, 2+
373.0
32
3.72e7
388.9
292.4
582.5
32, 1+
744.5
m/z
1.73
S1, 1+
361.3
2.59e7
362.3
S1
COOH


100
%
0
1.82
1.75
1.95
2.30
0.43
3.46
4.79
Time
0.50
1.00
1.50
2.00
2.50
3.00
3.50
4.00
4.50
550
2.59e7
33, 1+
550.4
33
3.03e6
S1, 1+
361.3
551.4
386.1
422.9
292.2
315.1
503.3
552.2
649.4
735.3
759.8
O
O
N
H
FEKG
m/z
200
300
400
500
600
700
800
900
1000

18 h

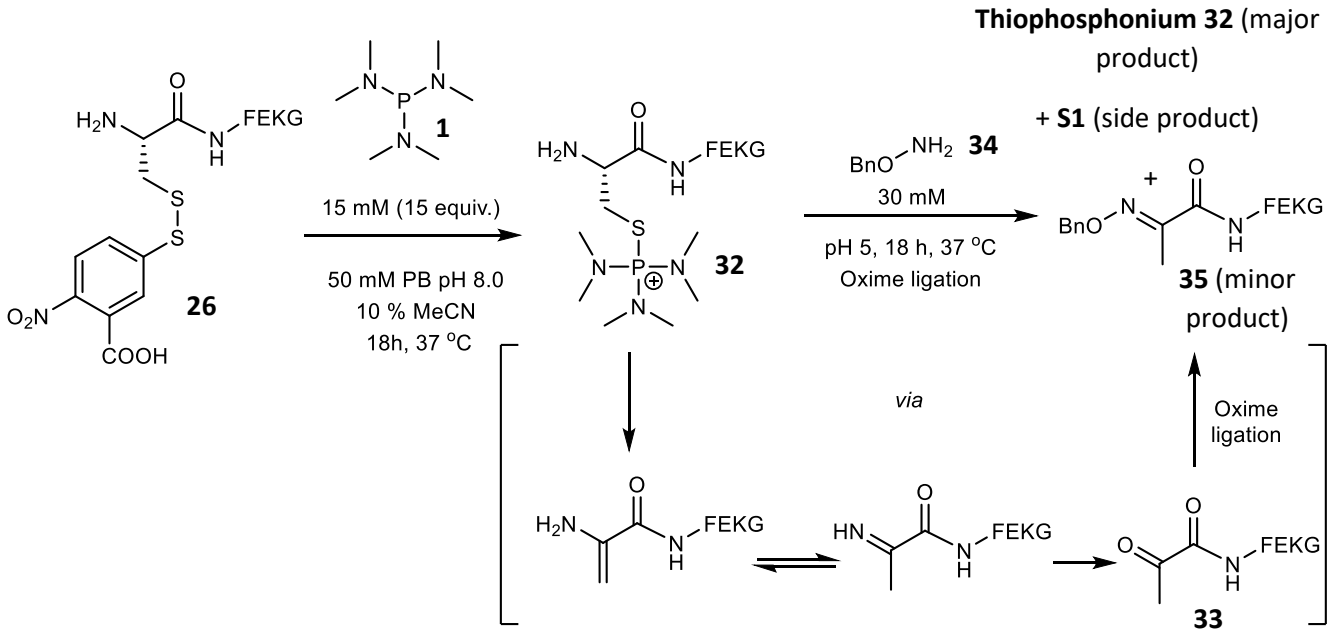

TIC
1.63e9
0.33
0.37
1.33
1.74
1.91
2.06
2.48
2.97
3.13
4.93
Time
373
5.73e8
1.33
1.47
32, 2+
372.9
388.9
304.9
410.4
582.3
32, 1+
744.4
32
2.22e7
m/z
361
4.09e8
1.74
550
1.27e8
1.82
1.76
1.67
S1, 1+
361.4
341.5
362.3
433.5
33, 1+
550.5
556.6
627.0
S1
33
1.13e7

Reaction of peptide 26 with HMPT 1 followed by *n*-hexanethiol 30 addition

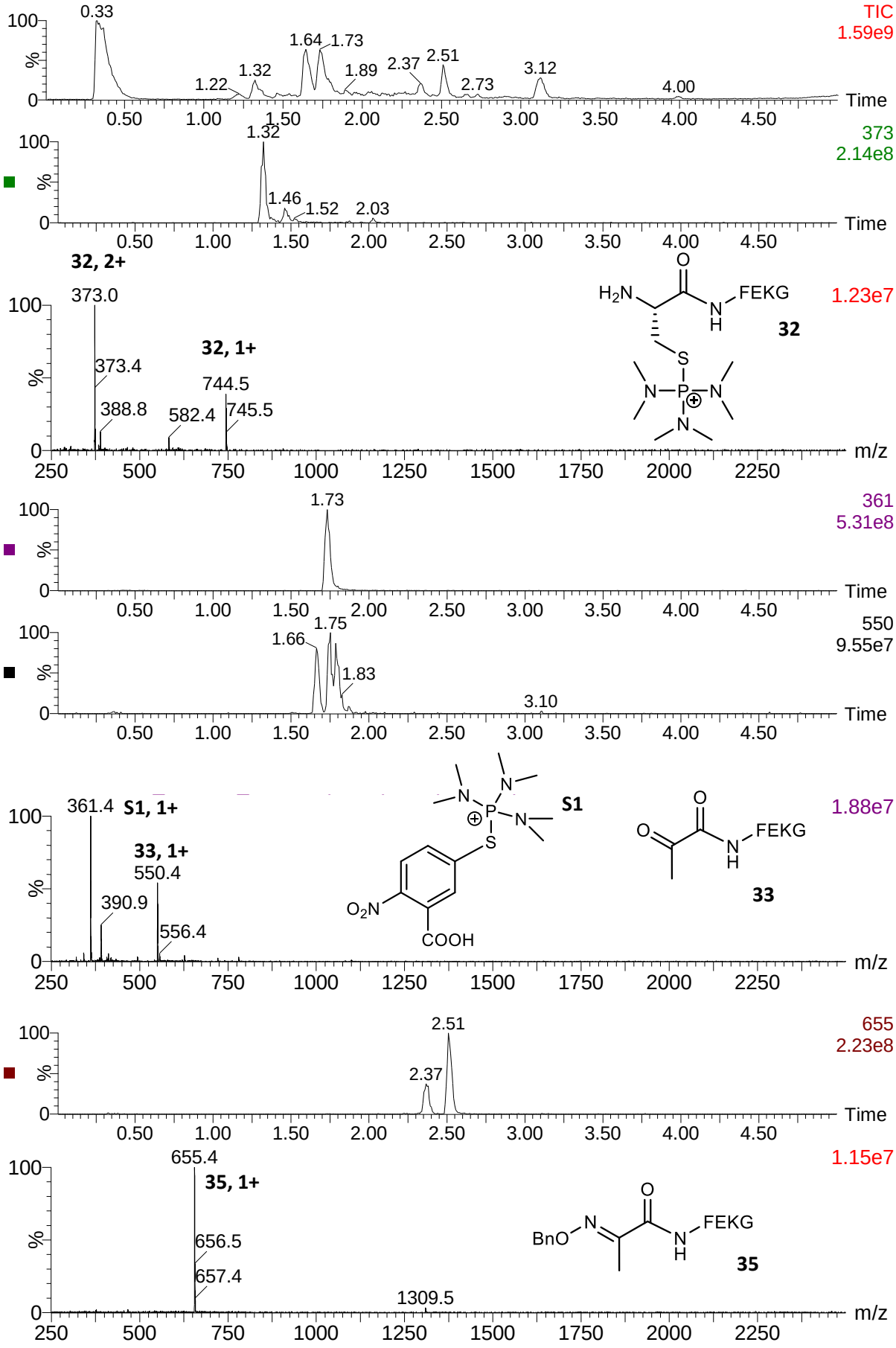


A 10 µL aliquot of a 2.5 mM disulfide **26** stock in H_2O was charged with 25 µL of 0.1 M PB pH 8.0 buffer and 10 µL H_2O. The solution was then charged with 5 µL of a 150 mM HMPT **1** stock solution in MeCN. The solution was mixed thoroughly, and allowed to incubate at 37 °C for 18 h. After this time, the reaction mixture was charged with 50 µL of a 60 mM *n*-hexanethiol **30** stock solution in H_2O:MeCN (1:1). No formation of a thiol-capped peptide **S2** was observed *via* LCMS.

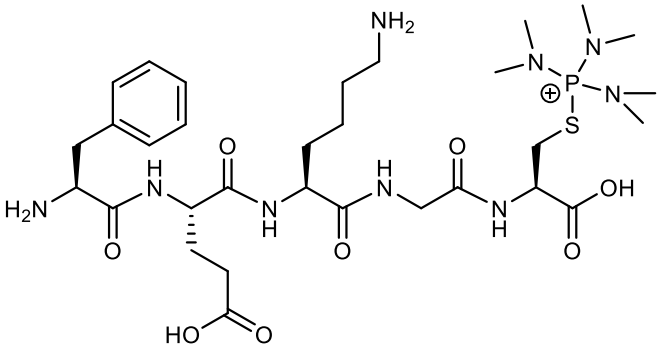
Reaction of peptide 26 with HMPT 1 followed by benzyloxyamine 34 addition



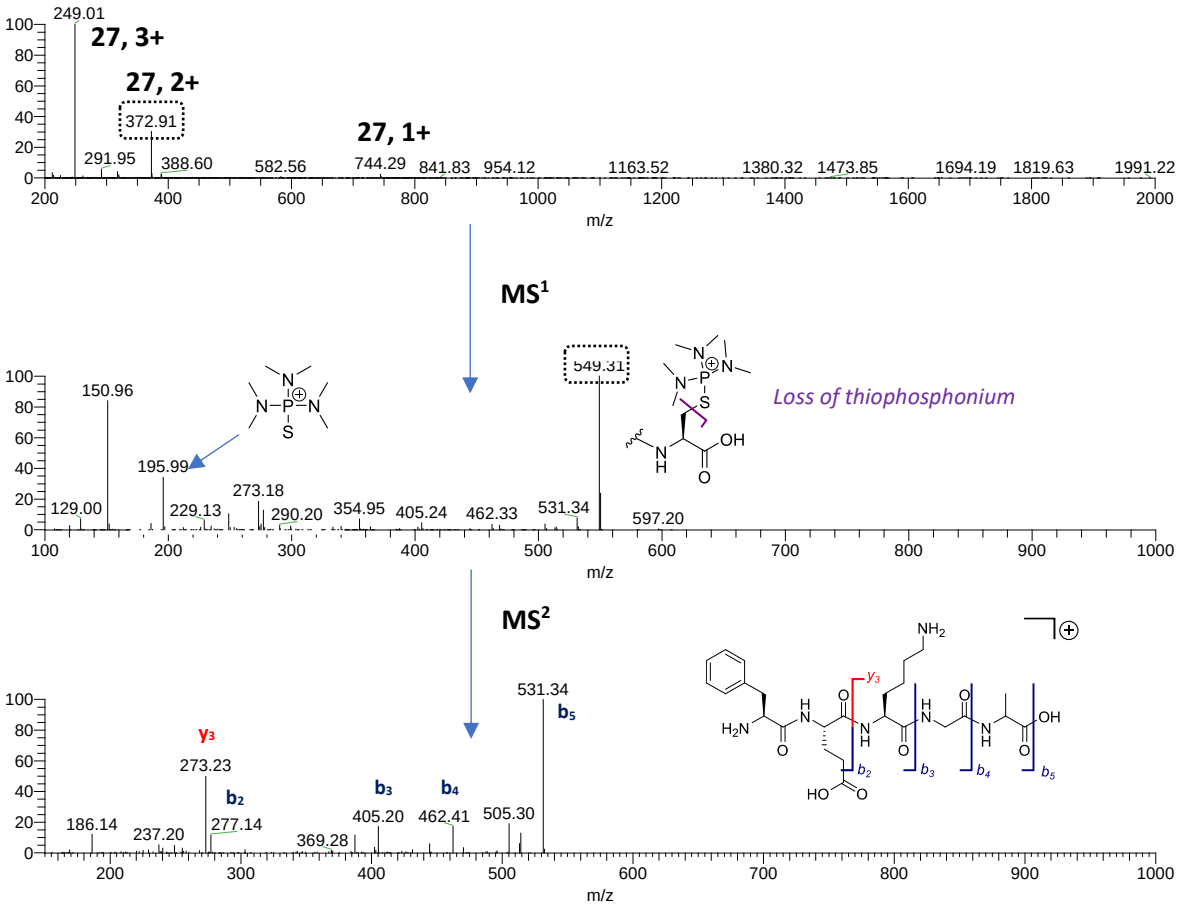
A 10 µL aliquot of a 2.5 mM disulfide **26** stock in H_2O was charged with 25 µL of 0.1 M PB pH 8.0 buffer and 10 µL H_2O. The solution was then charged with 5 µL of a 150 mM HMPT **1** stock solution in MeCN. The solution was mixed thoroughly, and allowed to incubate at 37 °C for 18 h. After this time, the reaction mixture was charged with 50 µL of a 60 mM benzyloxyamine **34** stock solution in 0.2 M NaOAc pH 5 buffer. Formation of minor product oxime peptide **35** was appraised by LCMS analysis.


TIC
1.59e9
0.33
1.22
1.32
1.64
1.73
1.89
2.37
2.51
2.73
3.12
4.00
Time
373
2.14e8
1.32
1.46
1.52
2.03
32, 2+
373.0
373.4
388.8
582.4
32, 1+
744.5
745.5
m/z
1.23e7
32
361
5.31e8
1.73
550
9.55e7
1.66
1.75
1.83
3.10
361.4
S1, 1+
33, 1+
550.4
390.9
556.4
S1
33
1.88e7
655
2.23e8
2.37
2.51
655.4
35, 1+
656.5
657.4
1309.5
35
1.15e7

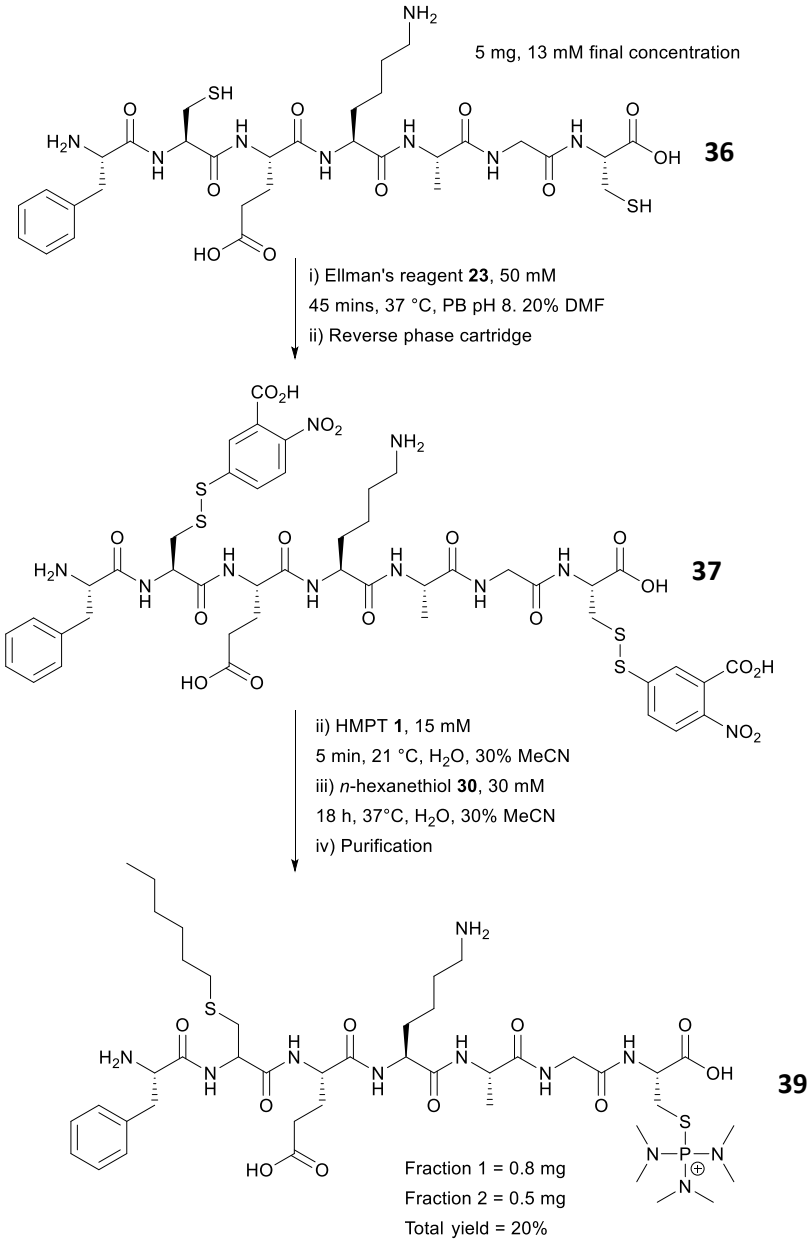
Peptide 27 MSMS data



Prior to MSMS analysis, a sample of peptide **27** (prepared as described previously) was passed through a solid phase extraction cartridge, washed with H_2O, eluted with 70% MeCN, and the resulting fraction was subsequently subjected to LCMS analysis, followed by MSMS analysis.

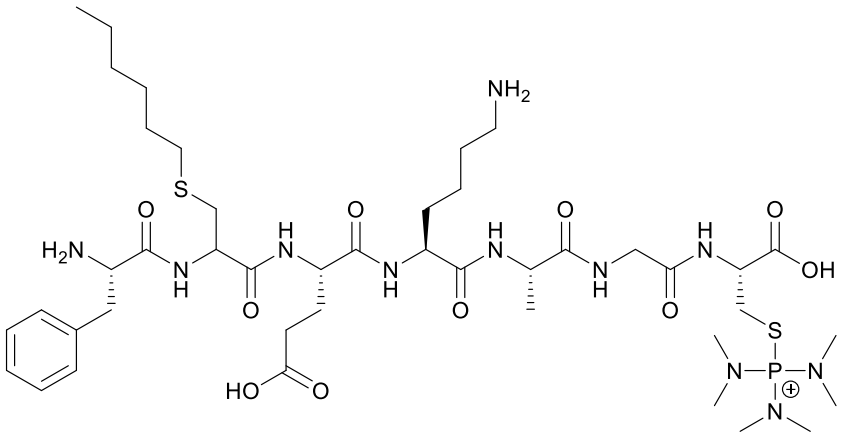


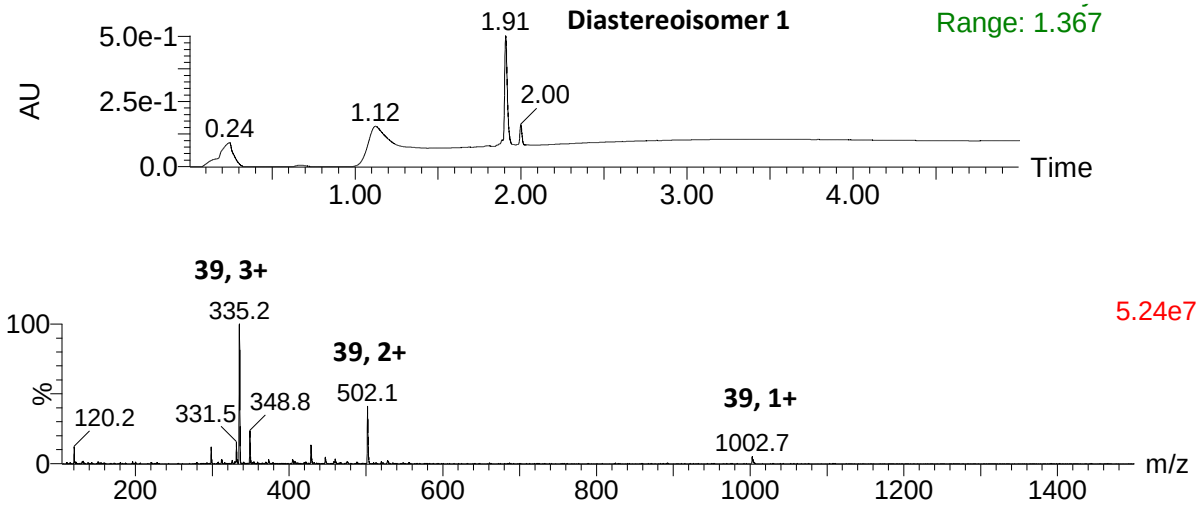
Preparation of dually modified peptide 39

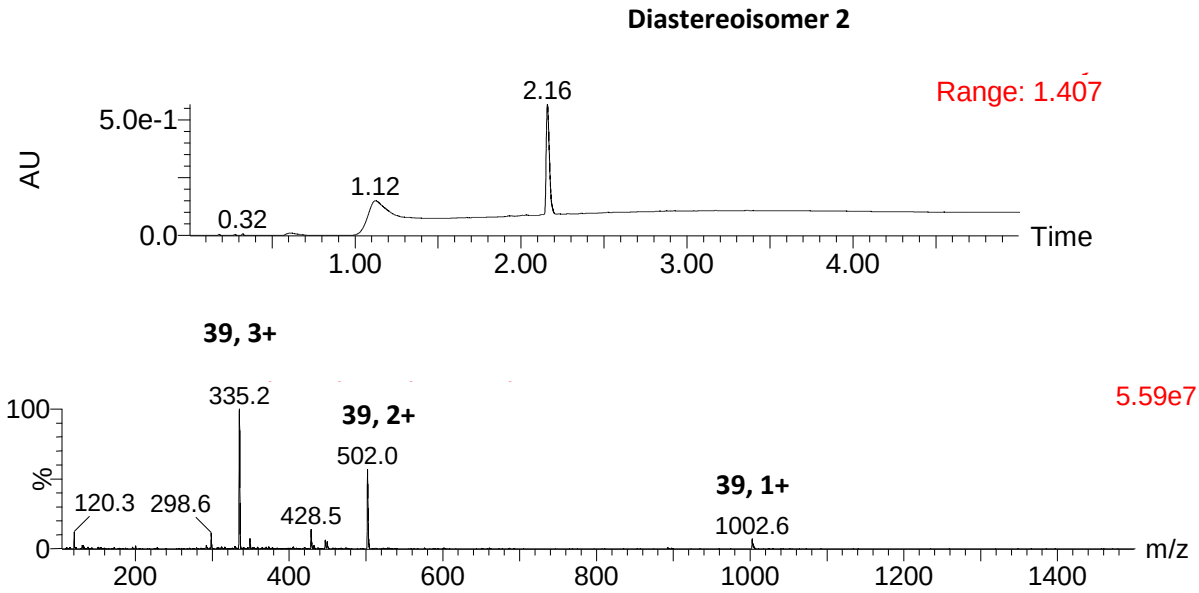


A 400 µL aliquot of a 16 mM FCEKGAC **36** stock (containing 5 mg total peptide content) in 0.2 M PB pH 8.0 was charged with 100 µL of a 250 mM Ellman's reagent **23** stock. The solution was mixed thoroughly and allowed to incubate at 37 °C for 45 min. The solution was then passed through a solid phase extraction cartridge. Fractions obtained from eluting with 10% MeCN, 30% MeCN and 50% MeCN, which contained the Ellman's capped peptide **37** were subsequently pooled. To the pooled fractions (3 mL total volume) was added HMPT **1** (8.2 µL, 15 mM final concentration). The solution was mixed thoroughly and allowed to sit at 21 °C for 5 min. The solution was then charged with *n*-hexanethiol **30** (12.8 µL, 30 mM final concentration) and allowed to incubate at 37 °C for 18 h. After this time, the reaction mixture was acidified with formic acid, desalted using a solid phase extraction cartridge, and subjected to semi-preparative HPLC. Two fractions containing the desired dually modified peptide **39** were obtained and subsequently lyophilised, both giving white powders (0.8 mg in Fraction 1 and 0.5 mg in Fraction 2). LCMS analysis and MSMS analysis were subsequently performed to confirm the presence of dually modified peptide **39**.

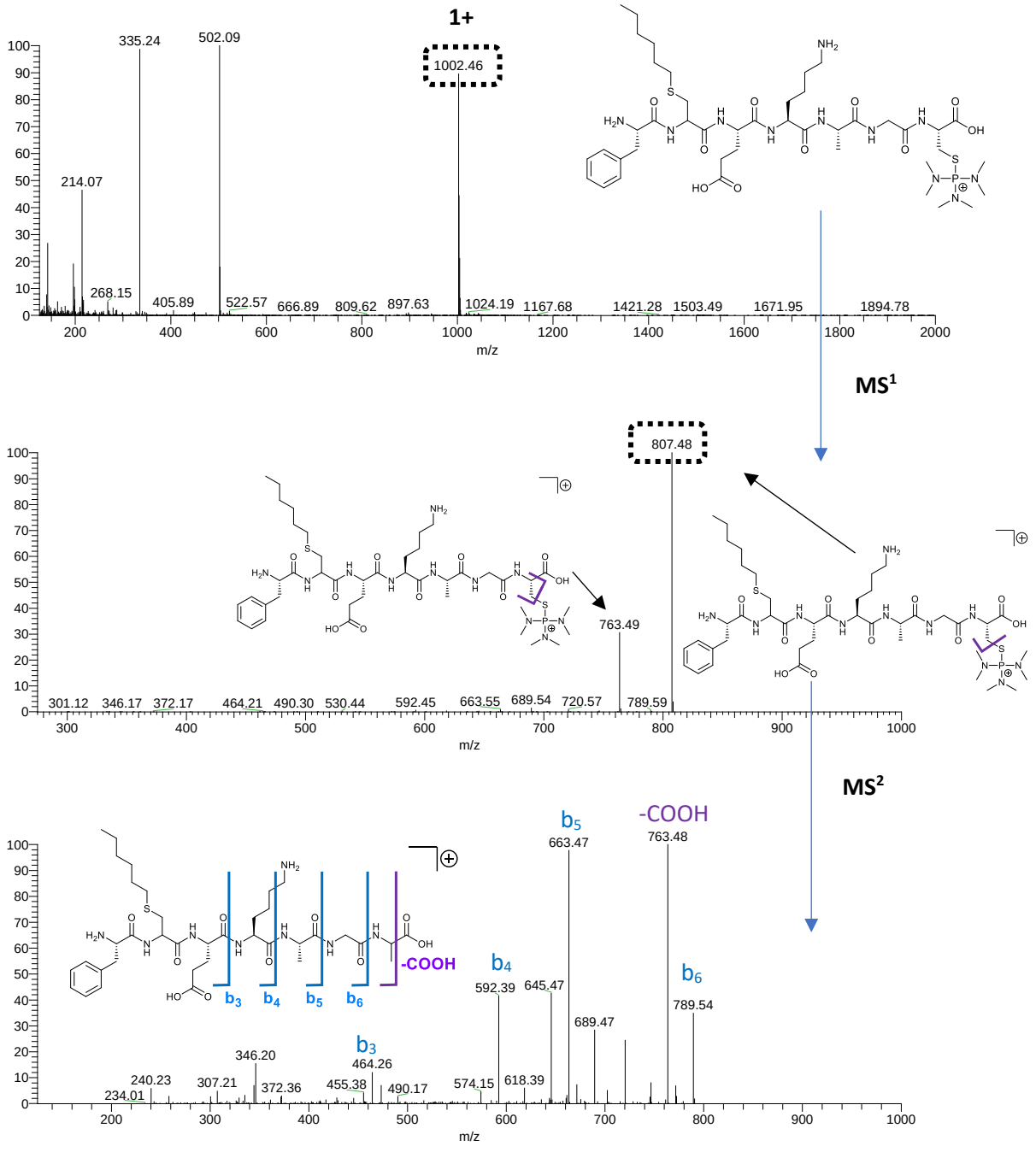
Peptide 39


NH2
S
O
H2N
N
H
O
HO
OH
S
N
P
N
N

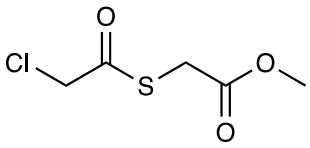

Diastereoisomer 1
Range: 1.367
1.91
2.00
1.12
0.24
5.0e-1
2.5e-1
0.0
AU
Time
1.00
2.00
3.00
4.00
39, 3+
335.2
39, 2+
502.1
39, 1+
1002.7
120.2
331.5
348.8
5.24e7
100
%
0
m/z
200
400
600
800
1000
1200
1400


Diastereoisomer 2
Range: 1.407
2.16
1.12
0.32
5.0e-1
0.0
AU
Time
1.00
2.00
3.00
4.00
39, 3+
335.2
39, 2+
502.0
39, 1+
1002.6
120.3
298.6
428.5
5.59e7
100
%
0
m/z
200
400
600
800
1000
1200
1400

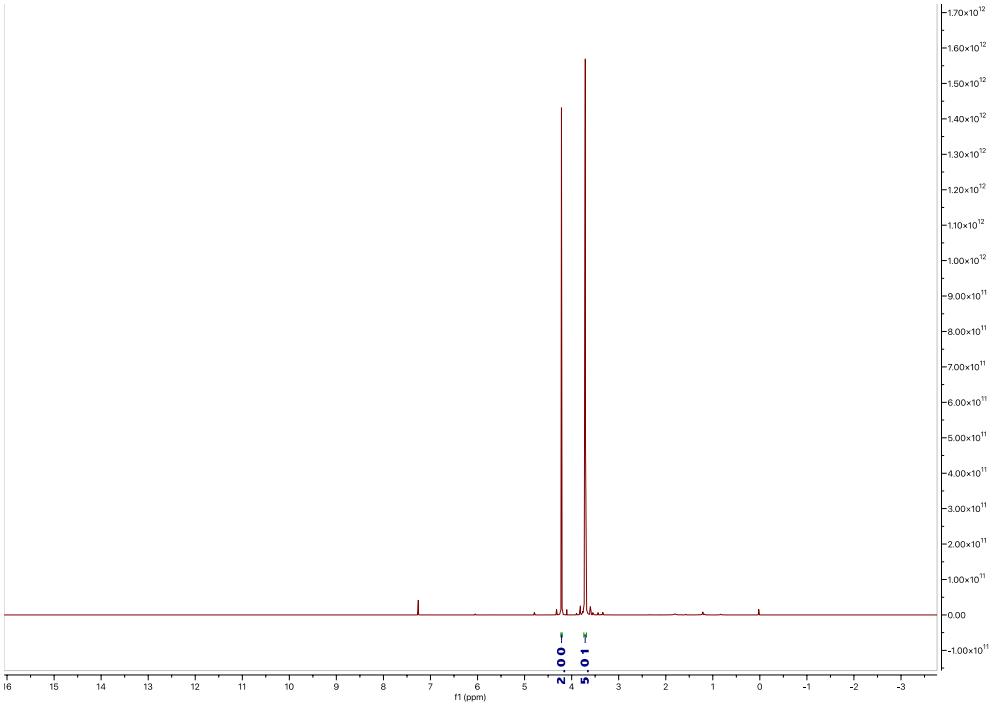
Dually modified peptide 39 MSMS data

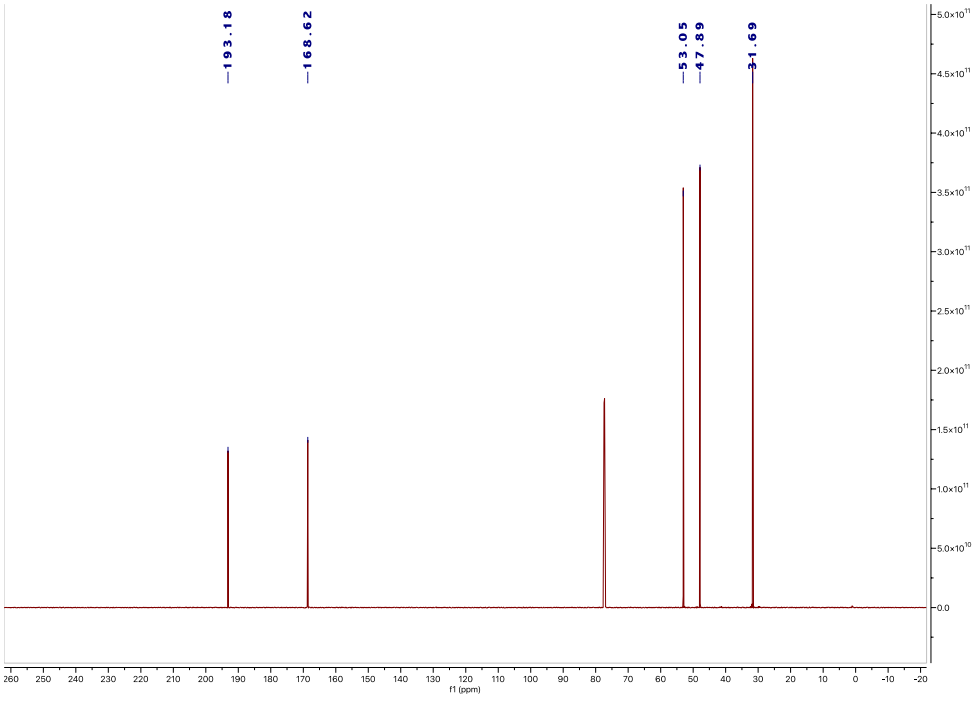


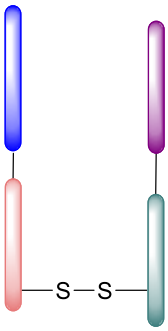
α-Chlorothioester S3



To a solution of methyl thioglycolate (0.280 mL, 3.14 mmol, 1.0 eq.) in DCM (2 mL) was added triethylamine (0.438 mL, 3.13 mmol, 1.0 eq.). The mixture was then added dropwise over 2 h time into a stirring solution of chloroacetyl chloride (0.750 ml, 9.43 mmol, 3.0 eq.) in DCM (10 mL). The resulting solution was allowed to stir under an argon atmosphere at 21 °C overnight. Purification was then achieved by flash chromatography (Cyclohexane:EtOAc, 0:100 -> 60:40), yielding the target compound **S3** (502 mg, 87% yield) as light-yellow oil.^{1}H NMR (700 MHz, $CDCl_3$) δ 4.21 (s, 2H), 3.71 (m, 5H); ^{13}C NMR (150 MHz, $CDCl_3$) δC 193.2 (C), 168.7 (C), 52.8 (CH_3), 47.6 (CH_2), 31.3 (CH_2); IR (oil) 3003, 2955, 1794, 1680; LRMS (ESI) 207.0 (100, [^{37}ClM + Na]$^+$, 90), 205.0 ([^{35}ClM + Na]$^+$, 100), 183.0 ([M+H]+, 55), 151.0 ([^{35}ClM – OCH_3], 40); HRMS (ESI) m/z calcd for $C_5H_7{}^{35}ClO_3S$ [M+H]$^+$ 182.9883, observed 182.9888.

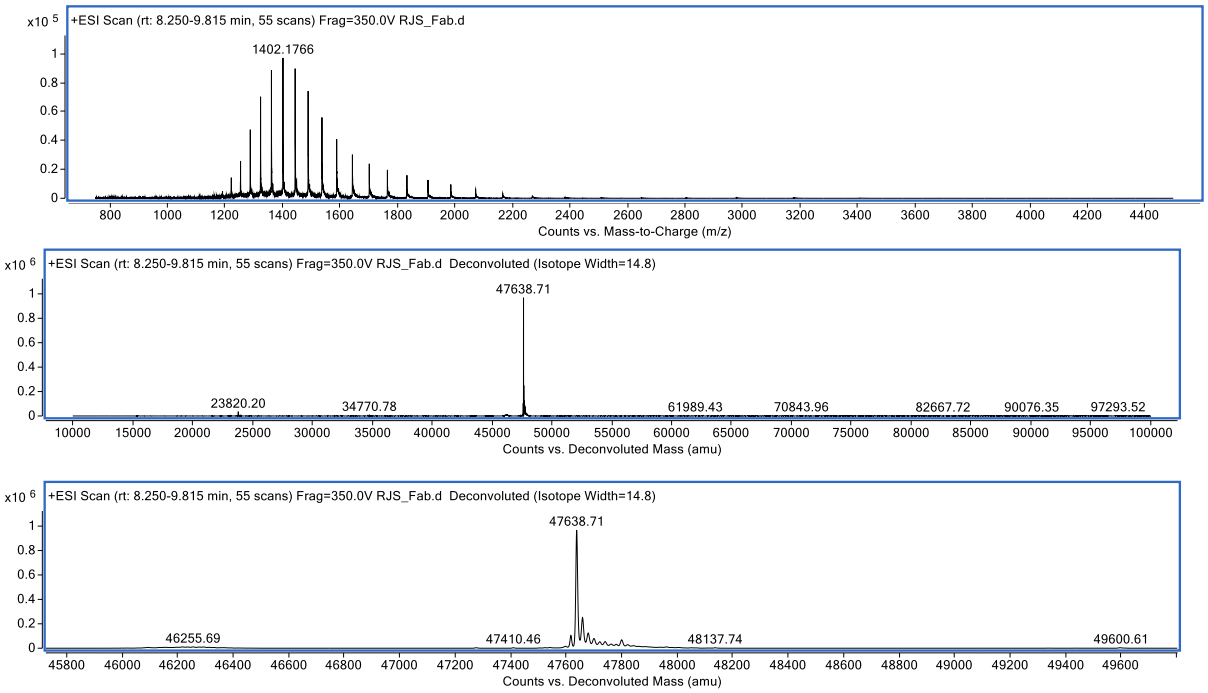



193.18
168.62
53.05
47.89
31.69
5.0×10^11
4.5×10^11
4.0×10^11
3.5×10^11
3.0×10^11
2.5×10^11
2.0×10^11
1.5×10^11
1.0×10^11
5.0×10^10
0.0
260 250 240 230 220 210 200 190 180 170 160 150 140 130 120 110 100 90 80 70 60 50 40 30 20 10 0 -10 -20
f1 (ppm)

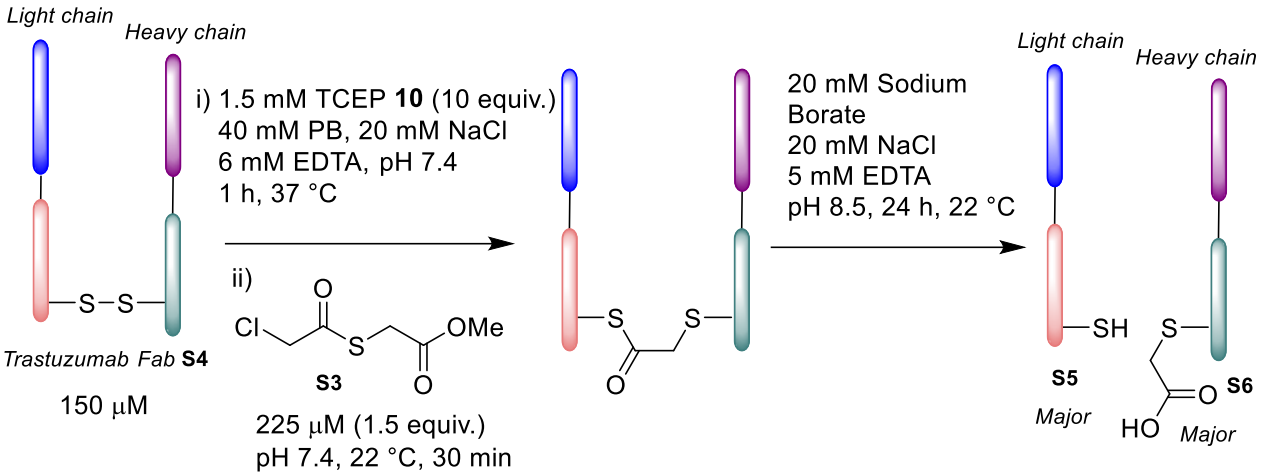


S4

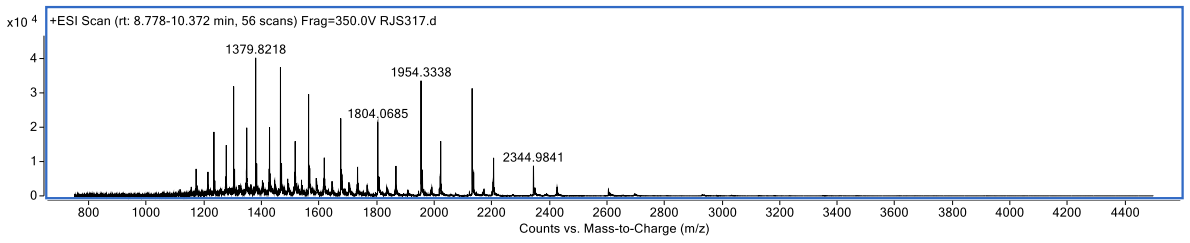
Trastuzumab Fab **S4** was obtained through pepsin/papain digestion of trastuzumab as described previously.[3] Concentration was determined photometrically using ε_{280} = 68,590 M^{-1} cm^{-1}. Calculated mass = 47638 Da, Found 47639 Da

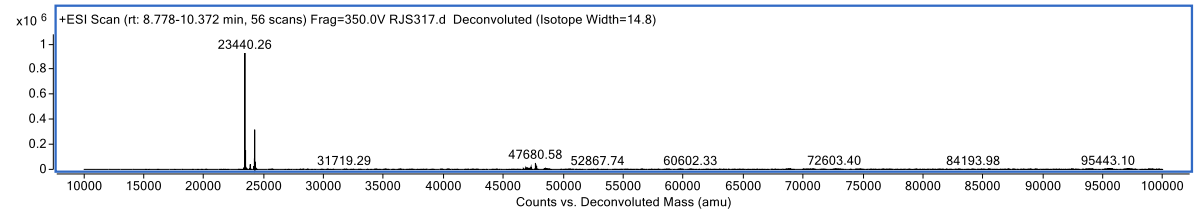


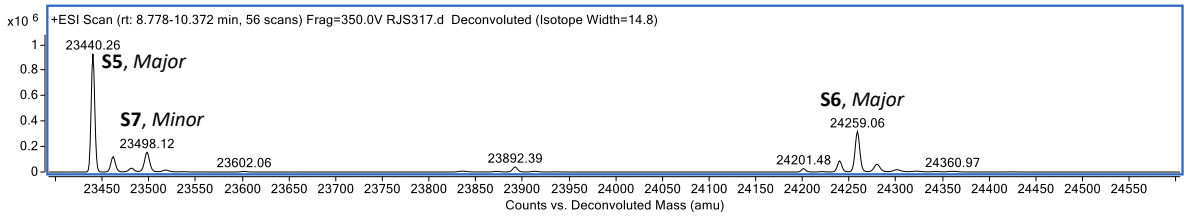
Free LC thiol, capped HC

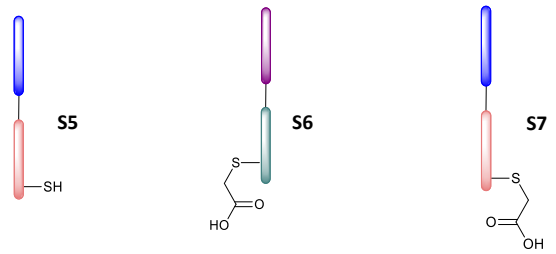


A 20 µL aliquot of a 150 µM Fab **S4** stock in buffer (40 mM PB, 20 mM NaCl, 6 mM EDTA, pH 7.4) was charged with 2 µL of a 15 mM tris(2-carboxyethyl)phospine (TCEP) **10** stock solution in H_2O. The solution was incubated at 37 °C for 1.5 h. The solution was then charged with 2 µL of a 225 mM stock solution of α-chlorothioester **S3** in DMF and incubated at 22 °C for 30 min. The solution was then buffer swapped (3 x Vivaspin, 10 kDa, Sigma-Aldrich) into degassed BBS buffer (20 mM Sodium Borate, 20 mM NaCl, 5 mM EDTA, pH 8.5) and left at 22 °C for 24 h, resulting in the bridged species being hydrolysed on the heavy chain and free thiol on the light chain.

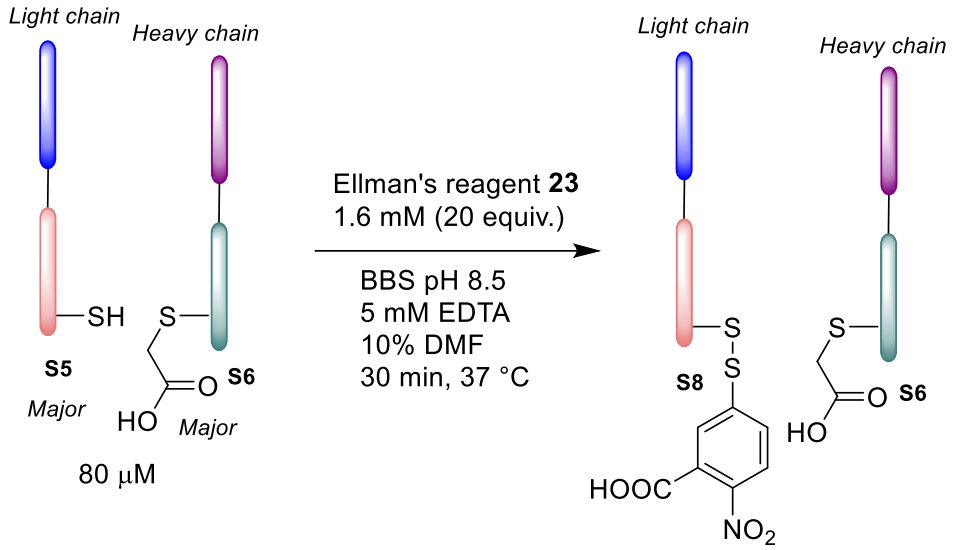




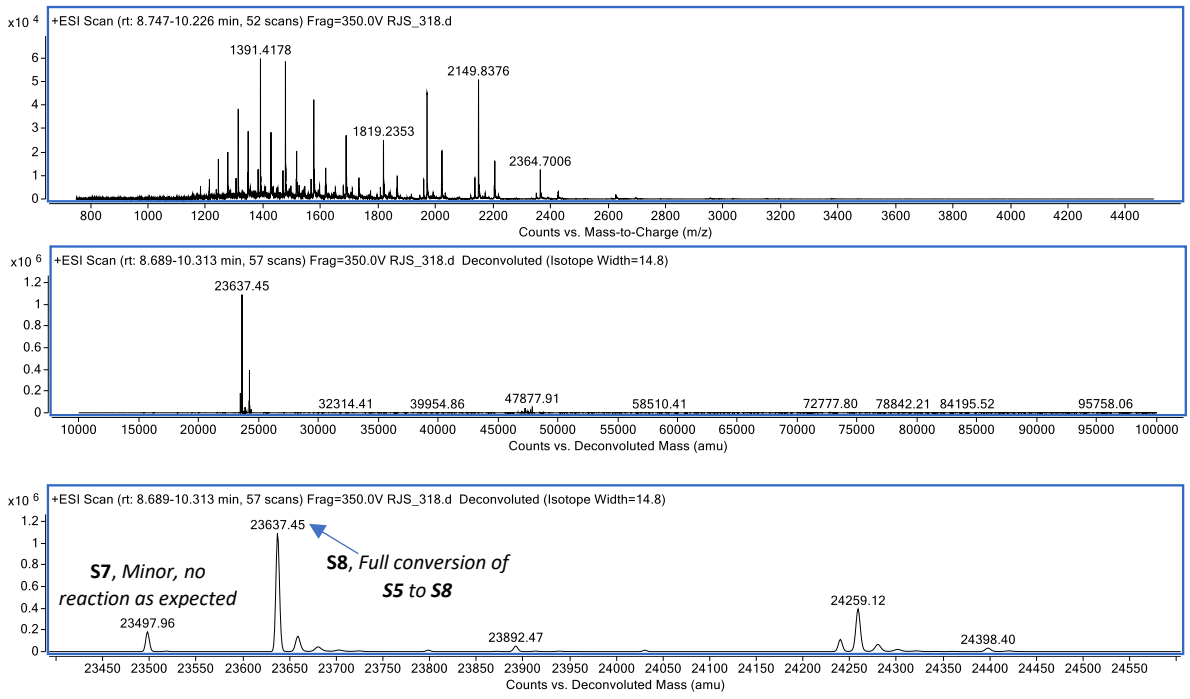




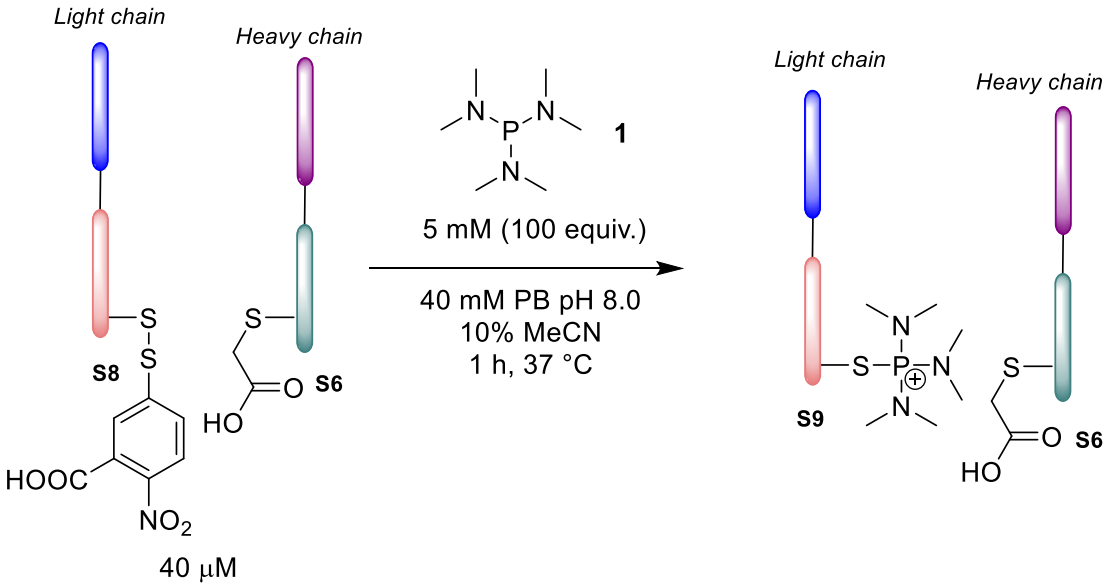
Ellmans LC, capped HC



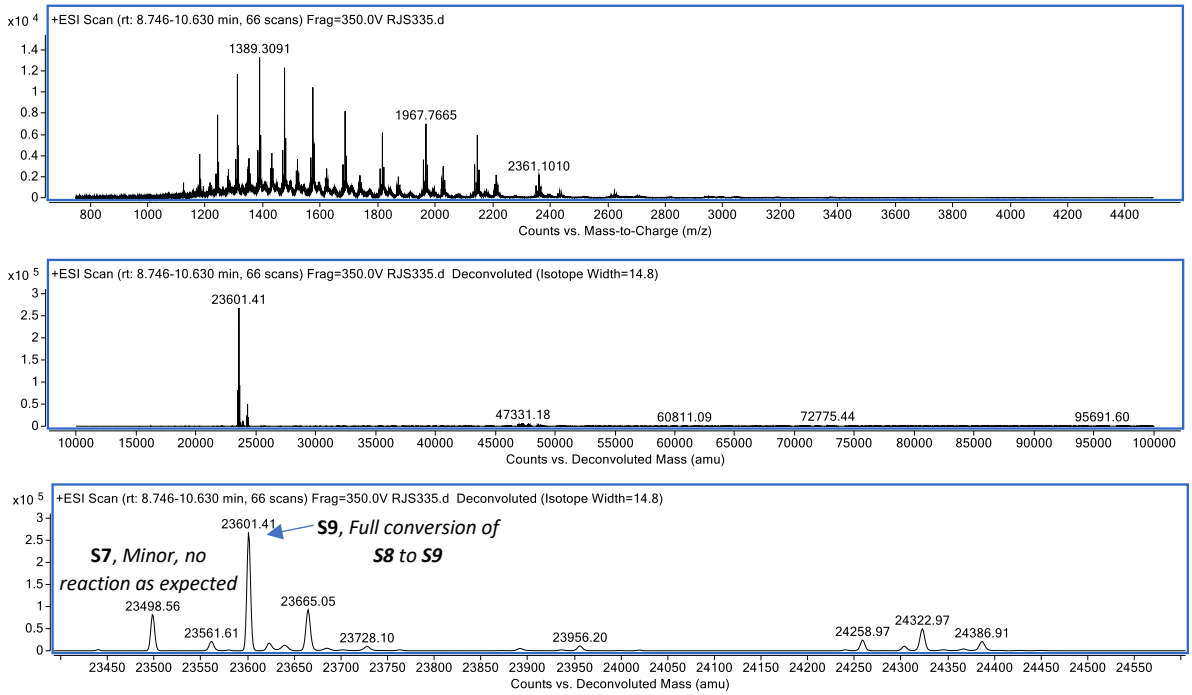
A 50 µL aliquot containing both light chain **S5** and heavy chain **S6**(20 µM) in BBS buffer (20 mM Sodium Borate, 20 mM NaCl, 5 mM EDTA, pH 8.5) prepared as described previously was charged with 5 µL of a 1.6 mM stock solution of Ellman's reagent **23** in DMF. The solution was then allowed to incubate at 37 °C for 30 min. After this, the sample was then desalted using a ZebaSpin 7 kDa (ThermoScientific™), eluting with H_2O.



Thiophosphonium LC, capped HC



A 20 µL aliquot containing both light chain **S8** and heavy chain **S6** (40 µM) in H_2O, prepared as described previously, was charged with 2.5 µL of 0.2 M PB pH 8.0 buffer. The solution was then charged with 2.5 µL of a 50 mM stock solution of HMPT **1** in MeCN. The solution was then allowed to incubate at 37 °C for 1 h. After this, the sample was then desalted using a ZebaSpin (ThermoScientific™), eluting with H_2O.



NB: Adducts of +64 observed on light chain and heavy chain

References

1 R. J. Chari, V. S. Goldmacher, J. M. Lambert, W. A. Blattler, *US Pat*., US5208020A, 1993

2 B. N. Naidu, M. E. Sorenson, T. P. Connolly and Y. Ueda, Michael Addition of Amines and Thiols to Dehydroalanine Amides: A Remarkable Rate Acceleration in Water, *J. Org. Chem*., 2003, **68**, 10098–10102.

3 M. T. W. Lee, A. Maruani, J. R. Baker, S. Caddick and V. Chudasama, Next-generation disulfide stapling: reduction and functional re-bridging all in one, *Chem. Sci*., 2016, **7**, 799–802.