Electronic Supplementary Material (ESI) for Chemical Science.

Serine Promoted Synthesis of Peptide Thioester-Precursor on Solid Support for Native Chemical Ligation

Hader E. Elashal, Yonnette E. Sim, Monika Raj*

Department of Chemistry, Seton Hall University, 400 S Orange Ave, South Orange, NJ 07079

*e-mail: monika.raj@shu.edu

Supporting Information

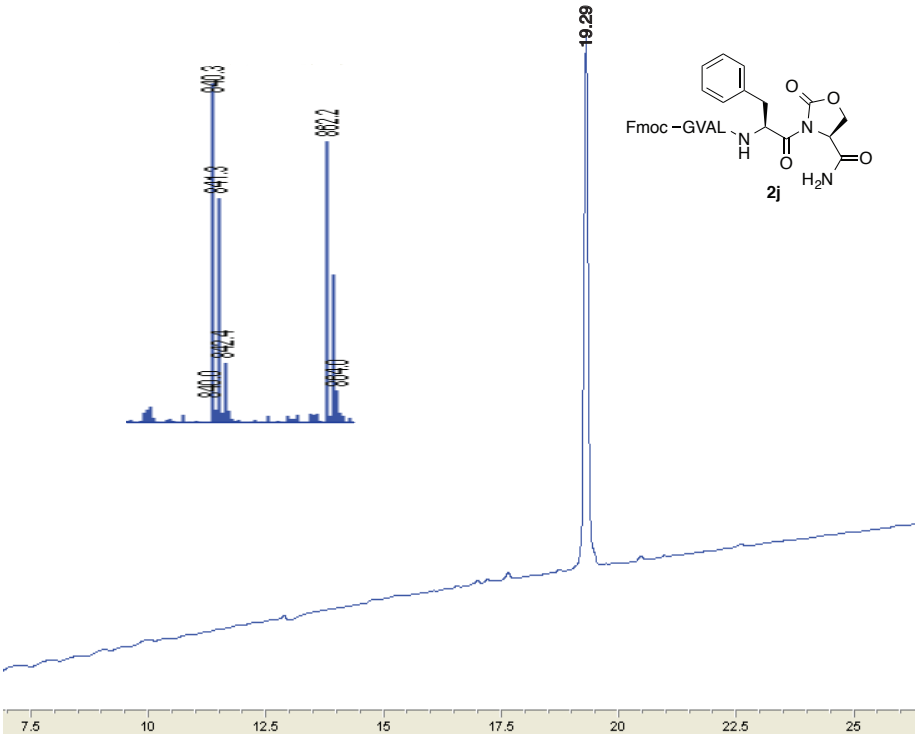
Table of Contents

Table S1: Substrate scope of serine cyclization on Fmoc-Gly-**Xaa**-**Ser**-Phe-Ala-Gly.[a]

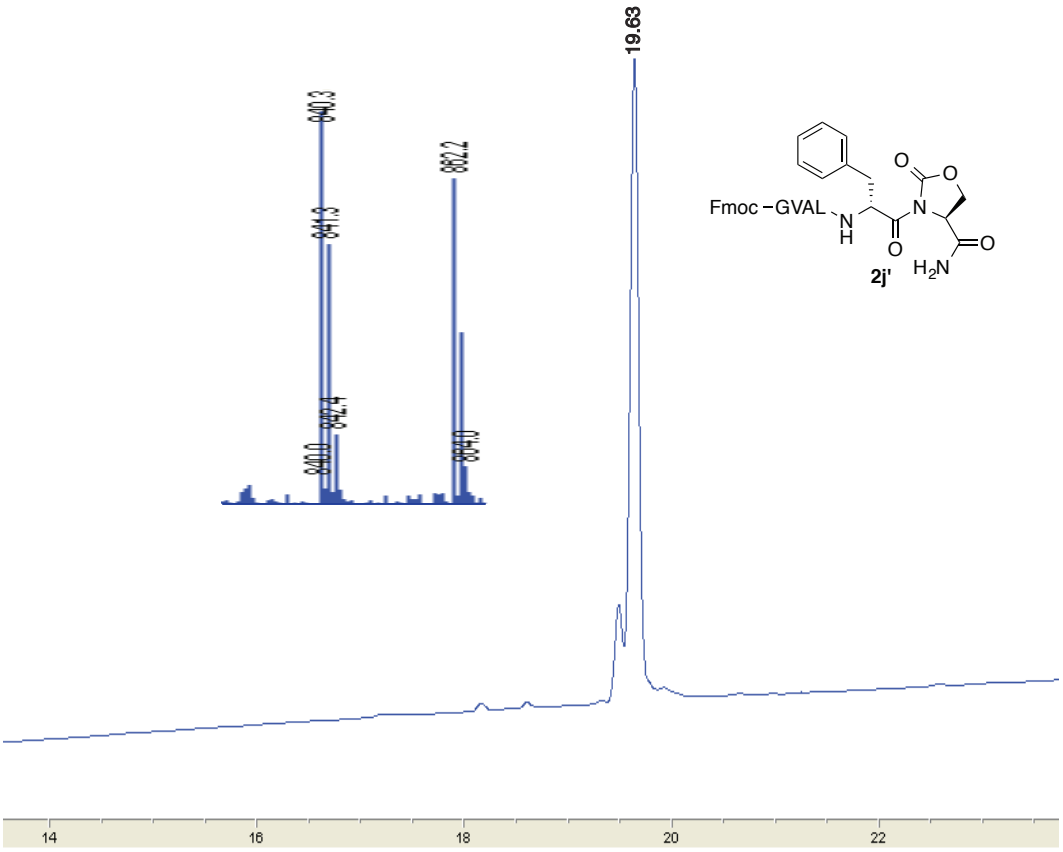
Substrate	Xaa	Conversion (%)[b]
1a'	Ala	>99
1b'	Gly	>99
1c'	Met	>99
1d'	His	>99
1e'	Tyr	>99
1f'	Trp	>99
1g'	Val	>99

[a]Reaction conditions: Peptide **1'** (25 mg, 0.7 mm/g) on solid support was reacted with DSC (10 equiv.), DIEA (10 equiv.), and a crystal of DMAP in DMF (3 mL) at room temperature for 17 h. [b]Conversion to cyclic urethane moiety **2'** was calculated from the absorbance at 220 nm using HPLC.

a)


19.29
840.3
841.3
842.4
840.0
882.2
884.0
Fmoc – GVAL
2j
7.5
10
12.5
15
17.5
20
22.5
25

b)


19.63
840.3
841.3
842.4
840.0
882.2
884.0
Fmoc – GVAL
2j'
14
16
18
20
22

c)

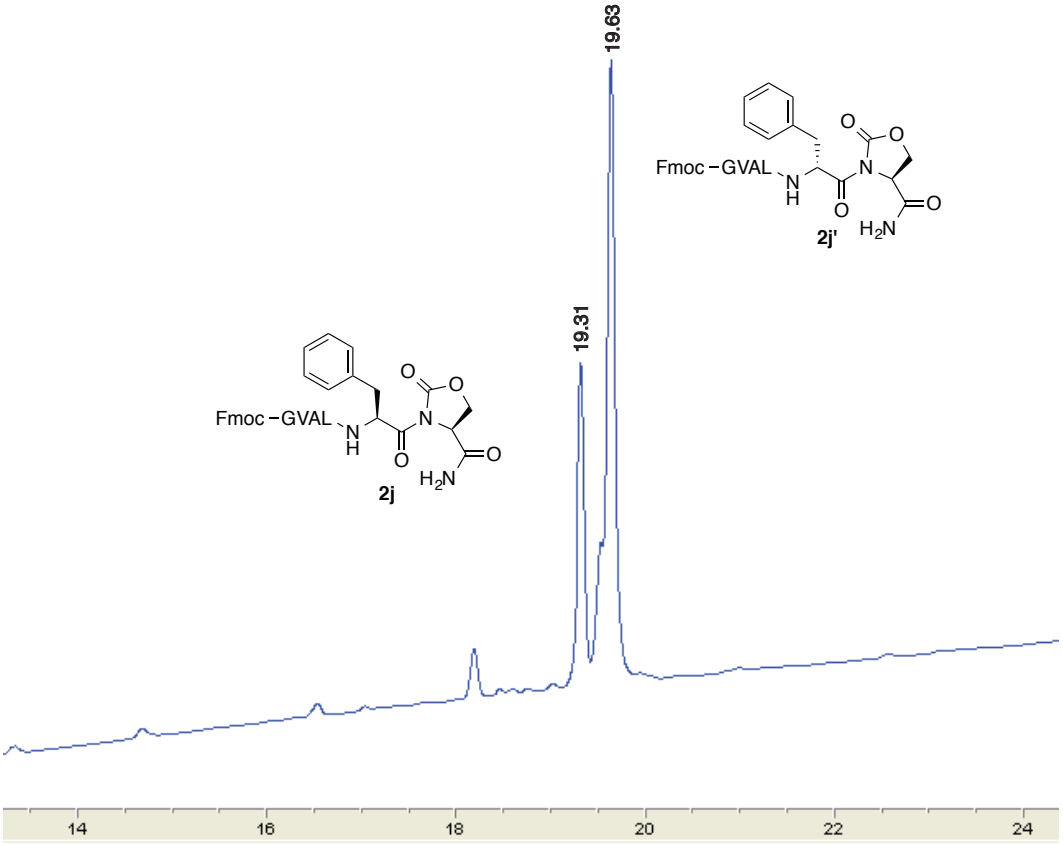
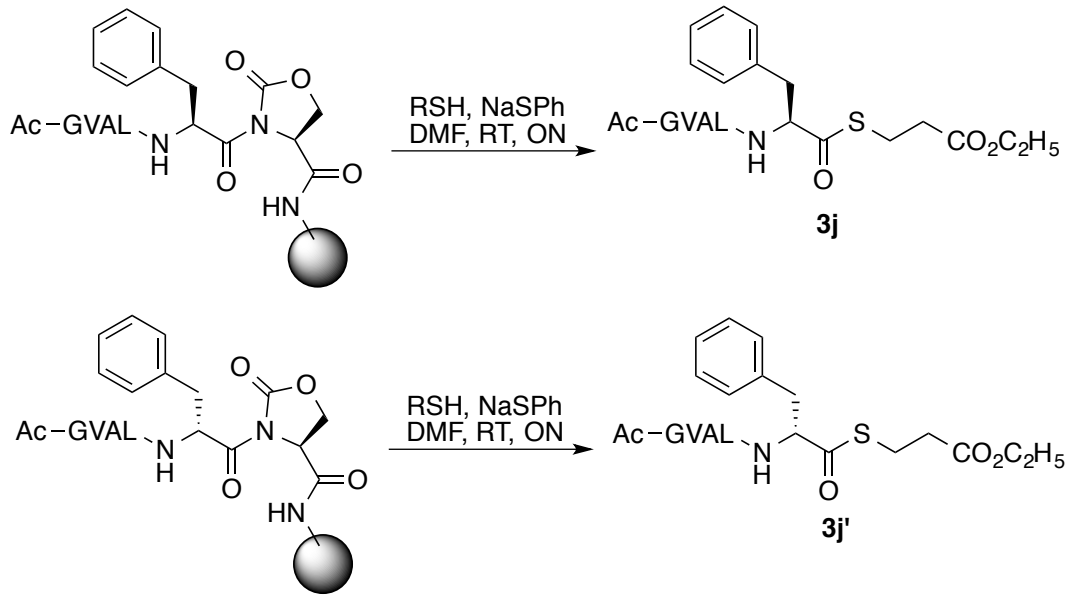
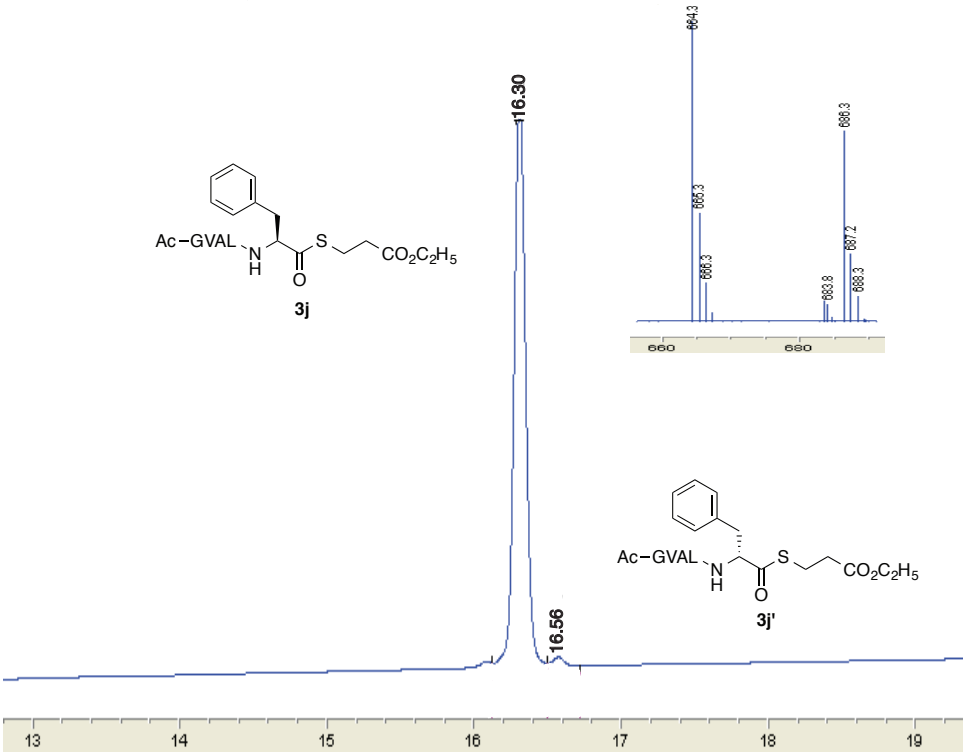


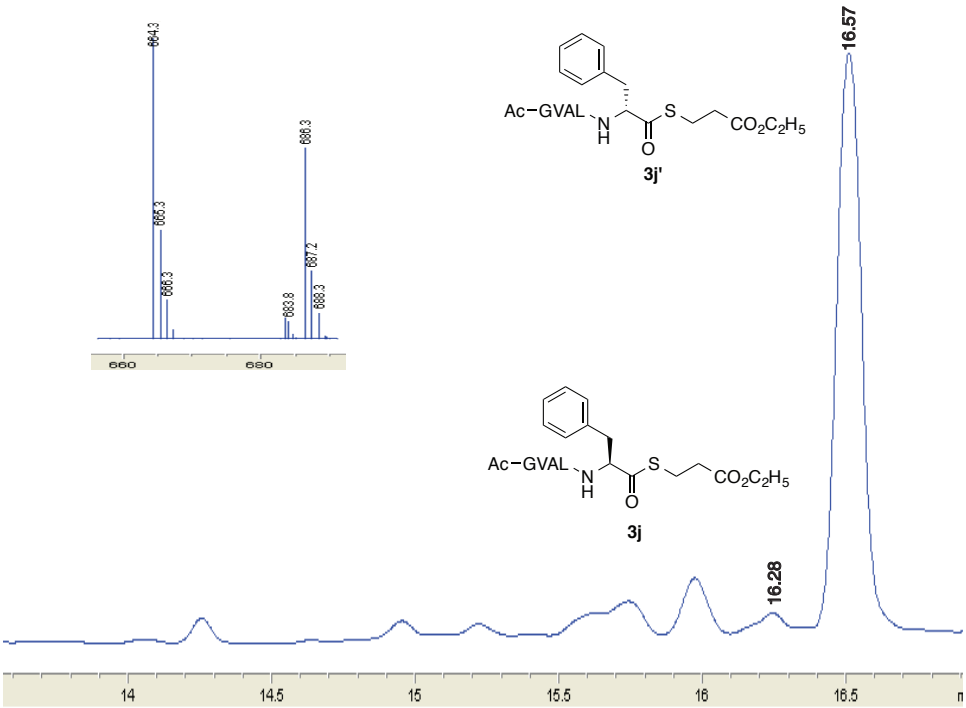
Figure S1. Investigation of potential epimerization during the formation of cyclic urethane moiety Fmoc-GVALF(*L*)-*Oxd* **2j** and epimer Fmoc-GVALF(*D*)-*Oxd* **2j'** (a) purified activated peptide Fmoc-GVALF(*L*)-*Oxd* **2j** (b) purified activated peptide diastereoisomer Fmoc-GVALF(*D*)-*Oxd* **2j'** and (c) mixture containing both diastereoisomers of activated peptides **2j** and **2j'**, demonstrating lack of detectable epimerization during serine activation.



a)


16.30
16.56
Ac–GVAL
N
H
S
O
CO2C2H5
3j
3j'
664.3
666.3
686.3
687.2
688.3
683.8
660
680
13
14
15
16
17
18
19

b)


16.57
16.28
Ac–GVAL
N
H
S
O
CO2C2H5
3j'
3j
664.3
666.3
686.3
687.2
688.3
683.8
660
680
14
14.5
15
15.5
16
16.5

c)

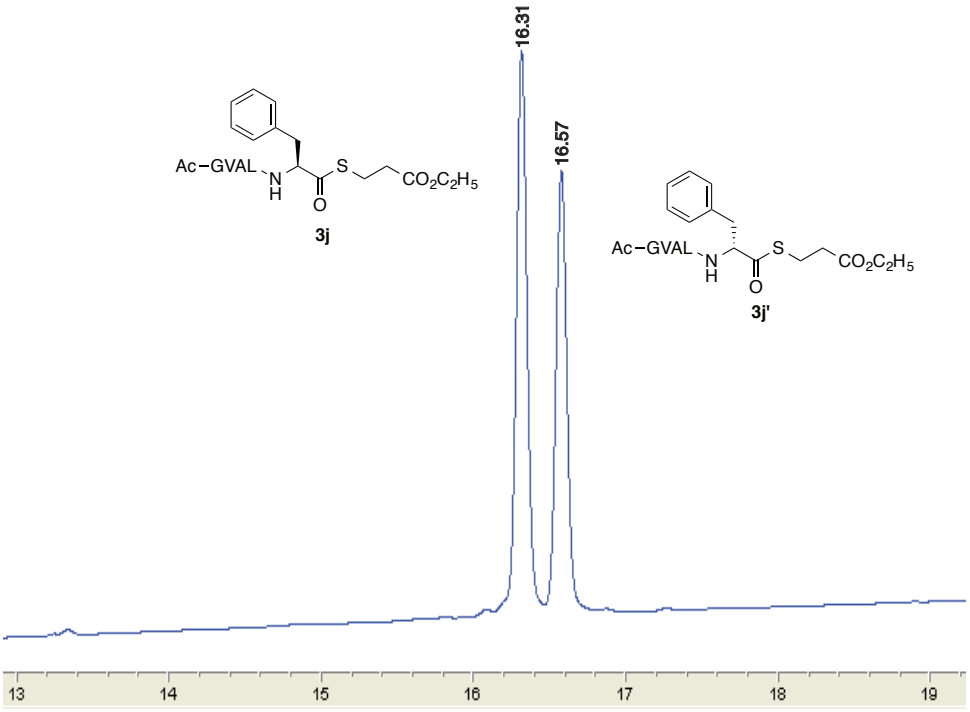


***Figure S2*.** Investigation of potential epimerization during the thioesterification process by the formation of peptide thioester Ac-GVALF(*L*)-COSR **3j** and epimer Ac-GVALF(*D*)-COSR **3j'** (a) purified peptide thioester Ac-GVALF(*L*)-$COS(CH_2)_2OH$ **3j** (b) purified peptide thioester diastereoisomer Ac-GVALF(*D*)-$COS(CH_2)_2OH$ **3j'** demonstrating less than 1% of epimerization and (c) mixture containing both diastereoisomers of peptide thioesters **3j** and **3j'**. RSH = $SH\text{-}(CH_2)_2\text{-}COOC_2H_5$

Methods

I. General. All commercial materials (Aldrich, Fluka, Nova) were used without further purification. All solvents were reagent grade or HPLC grade (Fisher). Anhydrous THF, diethyl ether, CH_2Cl_2, and DMF were obtained from a dry solvent system (passed through column of alumina) and used without further drying. All reactions were performed under air in round bottom flask. Yields refer to chromatographically pure compounds; % yield were obtained by comparison of HPLC peak areas of products and starting material. HPLC was used to monitor reaction progress.

Materials. Fmoc-amino acids were obtained from Nova Biochem is under (EMD Millipore Corporation)(Billerica, Massachusetts) and CreoSalus (Louisville, Kentucky). Rink amide resin was obtained from ChemPep Inc (Wellington, Florida). N,N,N′,N′-Tetramethyl-O-(1H-benzotriazol-1-yl)uronium hexafluorophosphate (HBTU) was obtained from CreoSalus (Louisville, Kentucky). N,N′-Disuccinimidyl carbonate (DSC) was obtained from Nova Biochem, under (EMD Millipore Corporation) (Billerica, Massachusetts). 4-Dimethylaminopyridine (DMAP): Merck KGaA (Darmstadt, Germany). N,N-Dimethylformamide (DMF): Macron Fine Chemicals (Center Valley, Pennsylvania). Dichloromethane (DCM), acetonitrile, N,N- Diisopropylethylamine (DIEA), N,N'-diisopropylcarbodiimide (DIC), Triethylsilane (TES), were purchased from (EMD Millipore Corporation)(Billerica, Massachusetts). Piperidine was purchased from Alfa Aesar (Ward Hill, Massachusetts). Trifluoroacetic acid (TFA) was purchased from VWR 100 Matsonford Road Radnor, PA. Diethyl Ether, 4-mercaptophenylacetic acid (MPAA), sodium thiophenolate (NaSPh), and Tris(2-carboxyethyl)phosphine hydrochloride TCEP.HCl : Sigma Aldrich (St. Louis, Missouri). Water was purified using a Millipore MilliQ water purification system.

NMR:
Proton NMR spectra were recorded on a 600 MHz spectrometer and carbon NMR spectra on a 151 MHz, spectrometer at ambient temperature. All NMR chemical shifts (δ) are referenced in ppm relative to residual solvent or internal tetramethylsilane. ^{1}H NMR chemical shifts referenced to residual DMSO-d_5 at 2.50 ppm, and ^{13}C NMR chemical shifts referenced to DMSO-d_6 at 39.52 ppm. Carbon NMR spectra are proton decoupled. NMR spectral data are reported as chemical shift (multiplicity, coupling constants (*J*), integration). Multiplicity is reported as follows: singlet (s), broad singlet (bs), doublet (d), doublet of doubles (dd), doublet of triplet (td), triplet (t) and multiplet (m). Coupling constant (*J*) in Hertz (Hz).

HPLC

Semi-Preparative HPLC:

Preparative HPLC chromatography (HPLC) was performed on Beckman Coulter equipped with System Gold 168 detector and 125P solvent module HPLC with C-18 reversed-phase column. All separations involved a mobile phase of 0.1% FA (v/v) in water (solvent A) and 0.1% FA (v/v) in acetonitrile (solvent B). Semi-preparative HPLC method using a linear gradient of 0–80% acetonitrile in 0.1% aqueous FA over 30 min at room temperature with a flow rate of 3.0

mL min^{-1}. The eluent was monitored by absorbance at 220 nm unless otherwise noted.

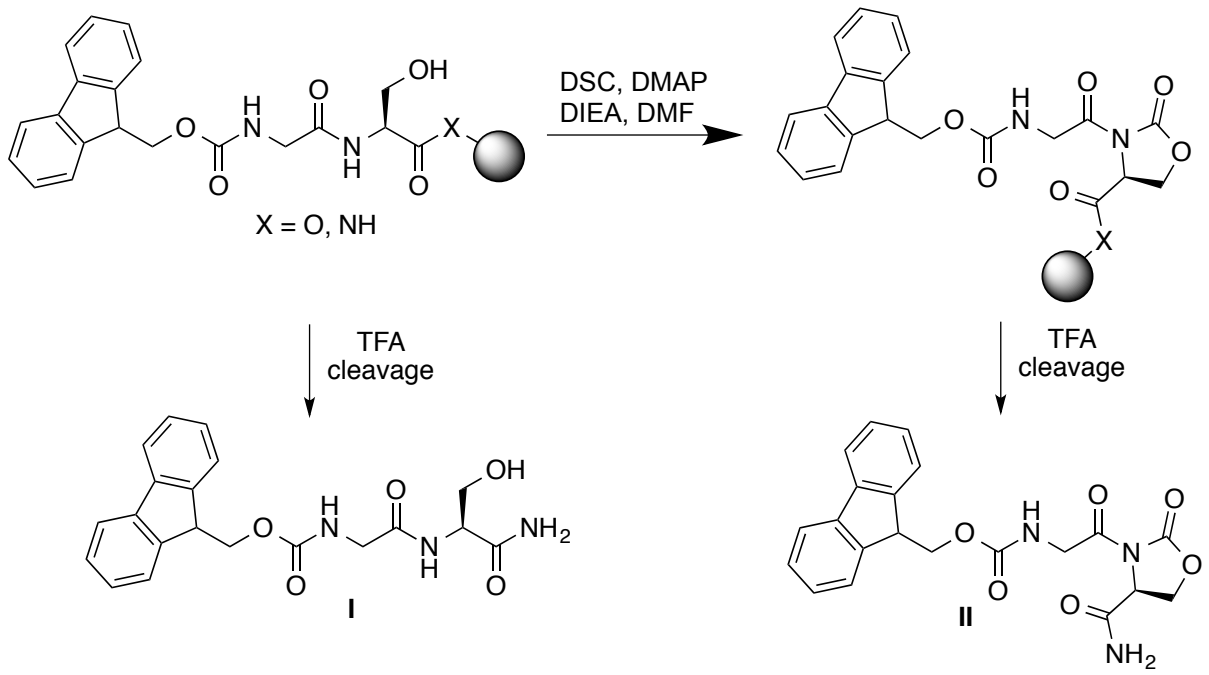
Analytical HPLC:

Analytical HPLC chromatography (HPLC) was performed on an Agilent 1100 series HPLC equipped with a 4.6 mm C-18 reversed-phase column. All separations involved mobile phase of 0.1% FA (v/v) in water (solvent A) and 0.1% FA (v/v) in acetonitrile (solvent B). Peptide compositions were evaluated by analytical reverse phase HPLC using a gradient of 0.1% FA in acetonitrile versus 0.1% FA in water. Analytical HPLC method using a linear gradient of 0–80% 0.1% FA (v/v) acetonitrile in 0.1% aqueous FA over 30 min at room temperature with a flow rate of 1.0 mL min^{-1}. The eluent was monitored by absorbance at 220 nm unless otherwise noted.

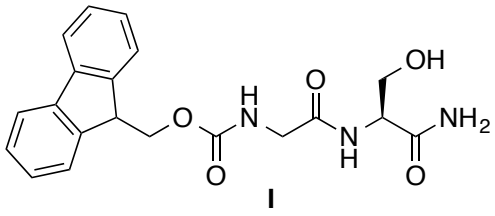
LCMS:
Mass spectrometry was performed using ultra high performance liquid chromatography-mass spectrometry using the Agilent 1100 Series LCMSD VL MS Spectrometer.

Fmoc Solid-Phase Peptide Synthesis.[1] Peptides were synthesized manually on a 0.25 mm scale using Rink amide resin. Fmoc–group was deprotected using 20% piperidine–DMF for 20 min to obtain a deprotected peptide-resin. Fmoc-protected amino acids (1.25 mm) were sequentially coupled on the resin using a HBTU (1.25 mm) and DIEA (1.25 mm) for 2 h at room temperature. Peptides were synthesized using standard protocols.[1] The peptide was cleaved from the resin using a cocktail of 95:2.5:2.5, trifluoroacetic acid:triisopropyl silane:water for 2 h. The resin was removed by filtration and the resulting solution was concentrated. The oily residue was triturated with diethyl ether to obtain a white suspension. The resulting solid was purified by HPLC.

(II) Serine Cyclization and NMR Spectra[2]:

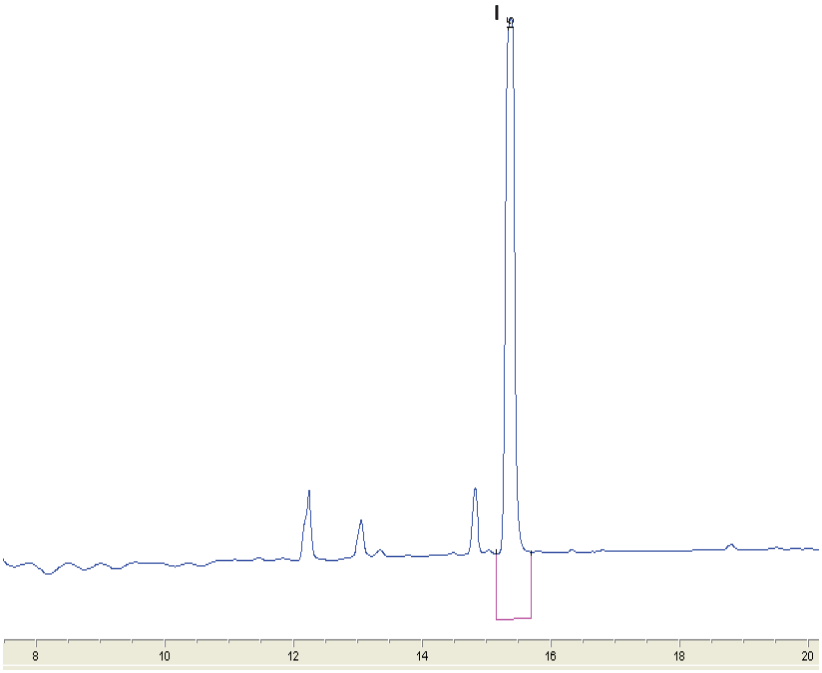


General procedure for the activation of serine to cyclic urethane moiety on solid support: To a peptide on the solid support (25-100 mg, 0.69 mm/g) was added a solution of DSC (10 equiv.), DIEA (10 equiv.) and catalytic amount of DMAP in dimethylformamide (DMF). The resin was left on the shaker for 17 h. The solution was drained and resin was washed with DMF followed by cleavage using a cocktail of 95:2.5:2.5, trifluoroacetic acid:triisopropyl silane:water for 2 h. The resin was removed by filtration and the resulting solution was concentrated. The oily residue was triturated with diethyl ether to obtain a white suspension. The resulting solid was purified by HPLC and analyzed by MS and NMR.



I

HPLC Trace: Retention time = 15.37, Purity: >95% (HPLC analysis at 220 nm)



HRMS: *m/z* 384.155, (calcd $[M+H]^+$ = 384.148)

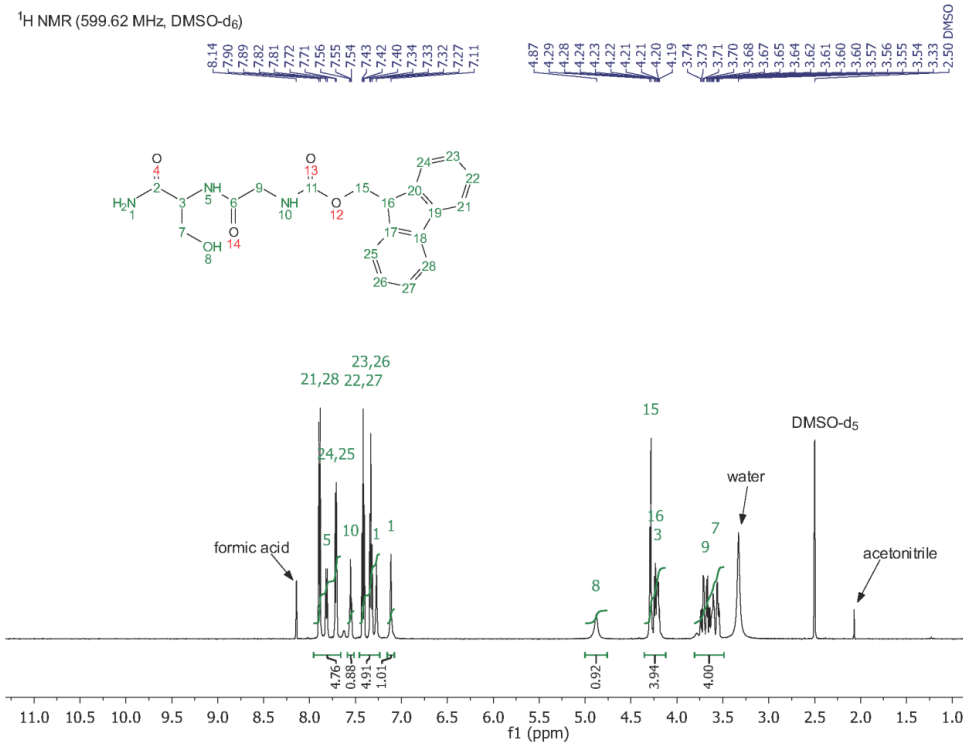
NMR

^{1}H NMR chemical shifts referenced to residual DMSO-d_6 at 2.50 ppm, and ^{13}C NMR chemical shifts referenced to DMSO-d_6 at 39.52 ppm.

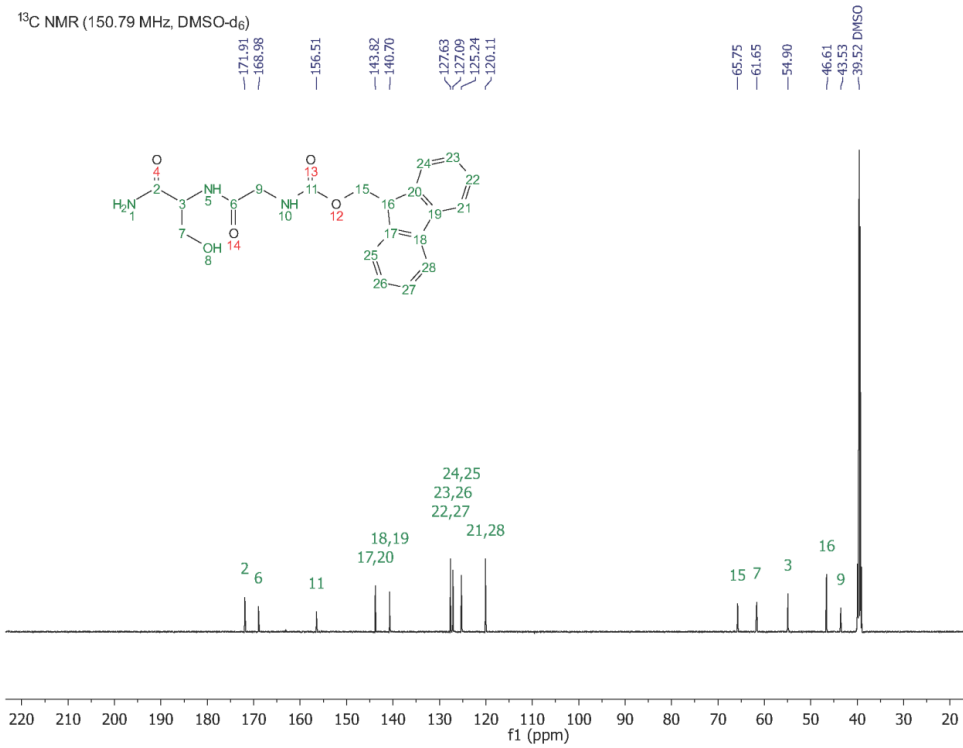
^{1}H NMR: (600 MHz, DMSO-d_6) δ 7.89 (d, J = 7.5 Hz, 2H), 7.81 (d, J = 8.0 Hz, 1H), 7.71 (d, J = 7.5 Hz, 2H), 7.55 (t, J = 6.0 Hz, 1H), 7.42 (t, J = 7.4 Hz, 2H), 7.33 (t, J = 7.4 Hz, 2H), 7.27 (s, 1H), 7.11 (s, 1H), 4.87 (br s, 1H), 4.29 (d, J = 6.8 Hz, 2H), 4.26 – 4.17 (om, 2H), 3.72 (dd, J = 16.8, 6.0 Hz, 1H), 3.66 (dd, J = 16.8, 6.0 Hz, 1H), 3.61 (dd, J = 10.5, 5.4 Hz, 1H), 3.55 (dd, J = 10.5, 4.8 Hz, 1H).

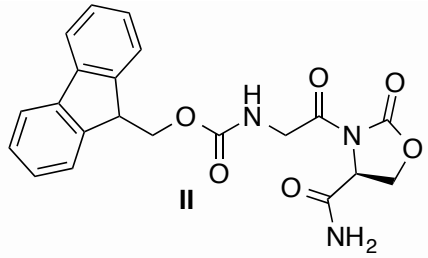
^{13}C NMR: (151 MHz, DMSO-d_6) δ 171.9, 169.0, 156.5, 143.8, 140.7, 127.6, 127.1, 125.2, 120.1, 65.8, 61.7, 54.9, 46.6, 43.5.

^{1}H NMR (599.62 MHz, DMSO-d$_6$)

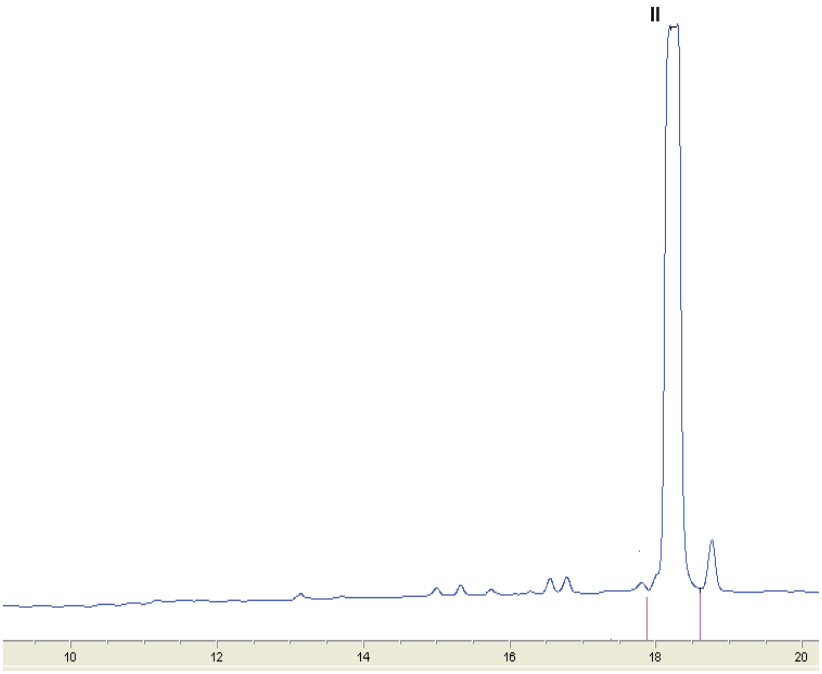


^{13}C NMR (150.79 MHz, DMSO-d$_6$)





HPLC Trace: Retention time = 18.3 min, Purity: >95% (HPLC analysis at 220 nm)



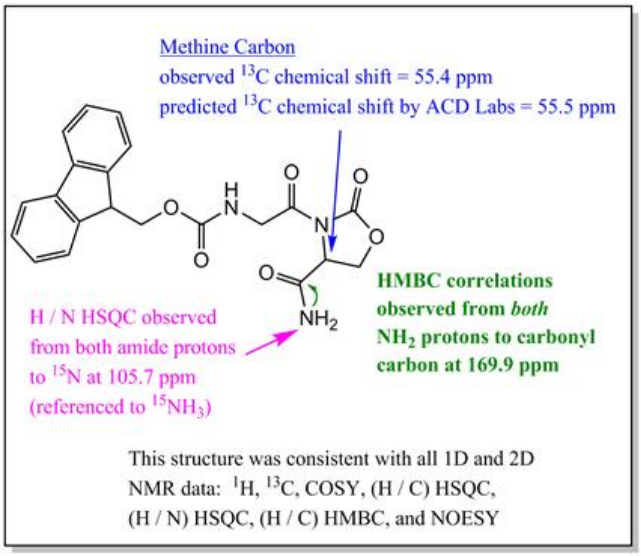
HRMS: *m/z* 432.116, (calcd $[M+Na]^+$ = 432.121)

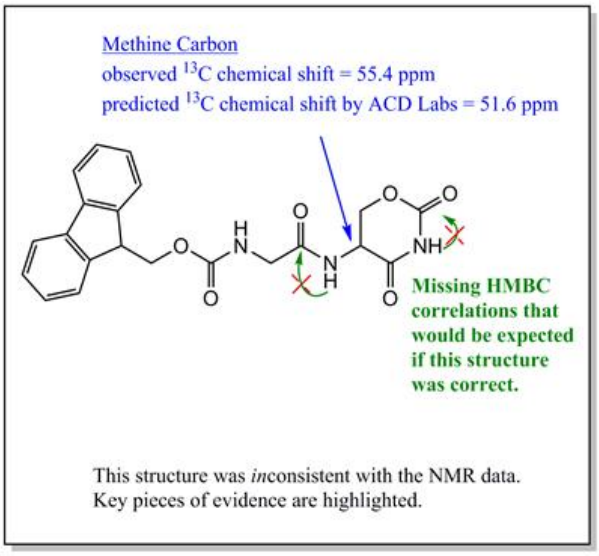
NMR

^{1}H NMR chemical shifts referenced to residual DMSO-d_6 at 2.50 ppm, and ^{13}C NMR chemical shifts referenced to DMSO-d_6 at 39.52 ppm.

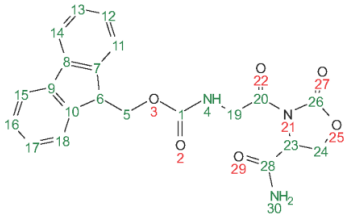
^{1}H NMR (600 MHz, DMSO-d_6) δ 7.90 (d, J = 7.5 Hz, 2H), 7.75 (s, 1H), 7.72 (d, J = 7.4 Hz, 2H), 7.64 (t, J = 6.0 Hz, 1H), 7.42 (t, J = 7.5 Hz, 2H), 7.39 (s, 1H), 7.33 (t, J = 7.4 Hz, 2H), 4.73 (dd, J = 9.1, 3.1 Hz, 1H), 4.60 (t, J = 9.0 Hz, 1H), 4.36 – 4.15 (m, 6H).

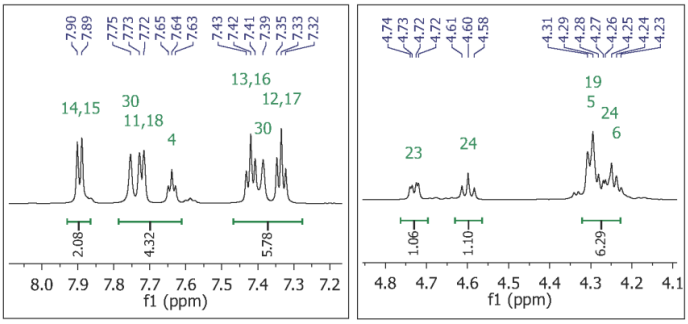
^{13}C NMR (151 MHz, DMSO-d_6) δ 169.9, 169.1, 156.4, 153.5, 143.8, 140.7, 127.6, 127.1, 125.2, 120.1, 66.8, 65.8, 55.4, 46.6, 44.1.

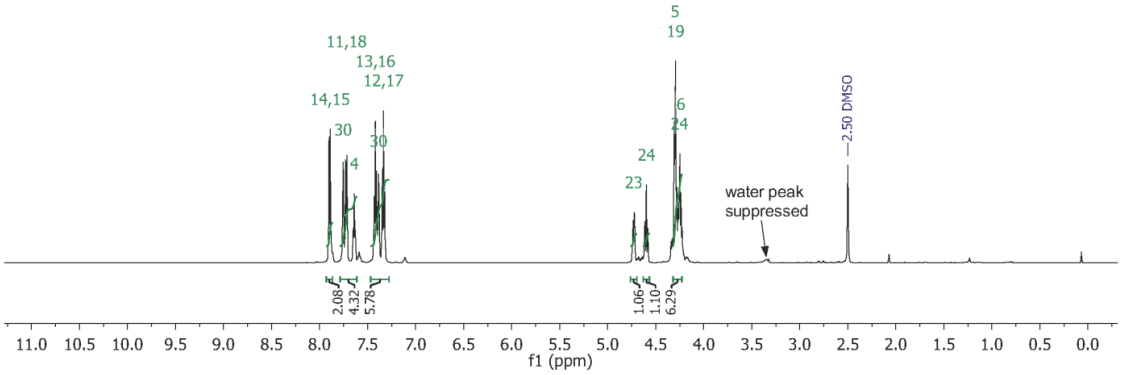


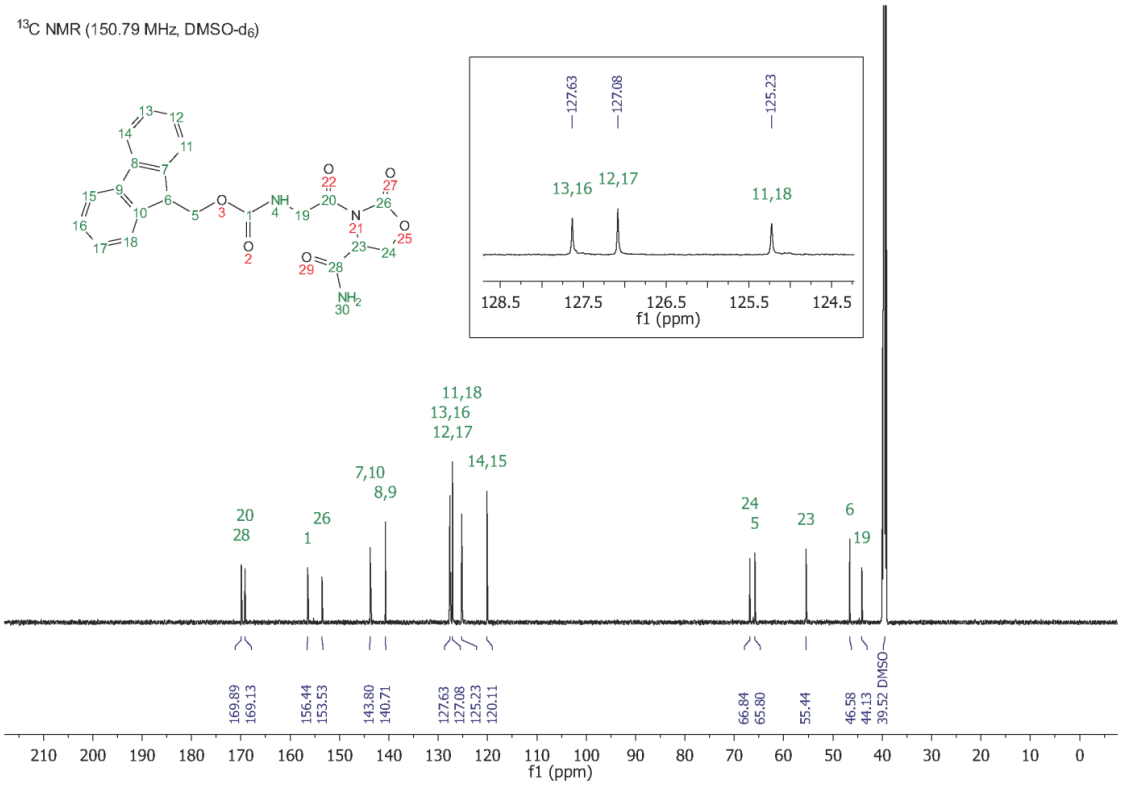


^{1}H NMR (599.62 MHz, DMSO-d_6)
presaturation used for water suppression

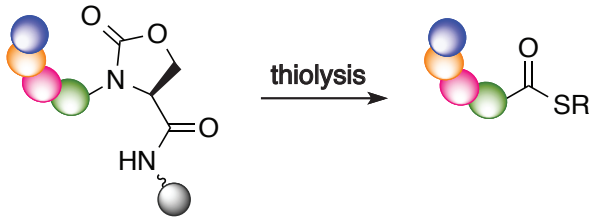







13C NMR (150.79 MHz, DMSO-d6)
127.63
127.08
125.23
13,16
12,17
11,18
128.5
127.5
126.5
125.5
124.5
f1 (ppm)
11,18
13,16
12,17
14,15
7,10
8,9
20
28
26
1
24
5
23
6
19
169.89
169.13
156.44
153.53
143.80
140.71
127.63
127.08
125.23
120.11
66.84
65.80
55.44
46.58
44.13
39.52 DMSO
210
200
190
180
170
160
150
140
130
120
110
100
90
80
70
60
50
40
30
20
10
0
f1 (ppm)

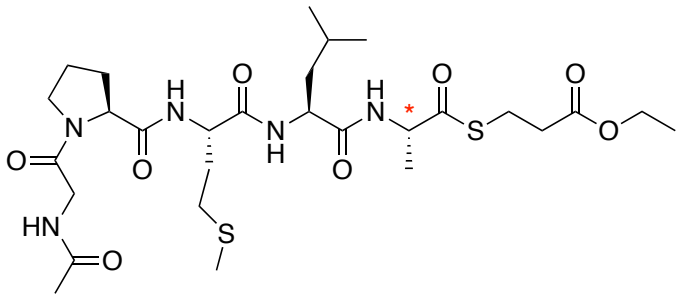
III. Synthesis of peptide thioesters from activated serine on the solid support.



General procedure for the synthesis of peptide thioesters from solid support: To an activated peptide as cyclic urethane moiety on the solid support (25 mg – 0.7 mm/g), thiol (100 μL), catalytic amount of sodium thiolate (0.5 equiv.) and 1 mL of DMF was added and resin was left on shaker at room temperature for 20 h. The resin was filtered which is followed by the removal of solvent under high vacuum. The resulting peptide thioester was analyzed by MS and HPLC. HPLC: 0.1% FA (v/v) in water (solvent A): 0.1% FA (v/v) acetonitrile (solvent B); gradient 0-80 %, 0.1% FA (v/v) acetonitrile in 25 min, flow rate = 1.0 mL/min, detection wavelength 220 nm.

NMR and MS of Peptide Thioesters

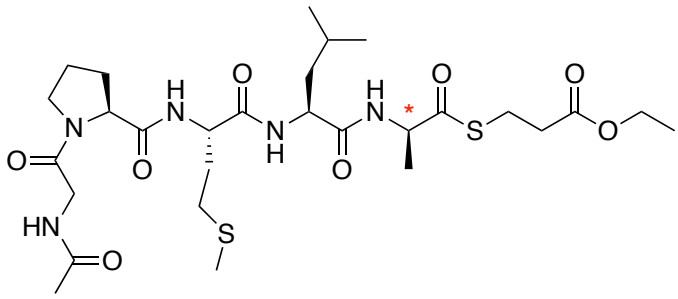
NMR spectra were recorded on a Bruker 600 MHz AVANCE III HD spectrometer with a triple resonance TXI probe at ambient temperature. Each sample was fully dissolved in 150 μL DMSO-d_6 and transferred to a 3 mm NMR tube. Data are reported as follows: ^{1}H NMR chemical shifts in parts per million (δ, ppm) referenced to residual DMSO-d_6 at 2.50 ppm and ^{13}C NMR chemical shifts in parts per million (δ, ppm) referenced to DMSO-d_6 at 39.52 ppm. Multiplicity abbreviations are s = singlet, d = doublet, t = triplet, q = quartet, om = overlapped multiplet, and proton-proton coupling constants (*J*) were measured in Hz. HRMS data were recorded on an Agilent 6520 Q-ToF mass spectrometer using positive polarity electrospray ionization (+ESI). IUPAC nomenclature generated by ACD Labs, version 12.5.



Ac-Gly-Pro-Met-Leu-Ala(*L*)-COS$(CH_2)_2CO_2C_2H_5$- 3G

***N*-acetylglycyl-*N*-[(5*S*,8*S*,11*S*)-11-methyl-8-(2-methylpropyl)-6,9,12,16-tetraoxo-17-oxa-2,13-dithia-7,10-diazanonadecan-5-yl]-L-prolinamide**: ^{1}H NMR (600 MHz, DMSO-d_6) δ 8.40 (d, *J* = 7.5 Hz, 1H), 8.05 (d, *J* = 7.8 Hz, 1H), 8.00 (t, *J* = 6.0 Hz, 1H), 7.75 (d, *J* = 8.2 Hz, 1H), 4.42-4.24 (om, 4H), 4.06 (q, *J* = 7.0 Hz, 2H), 3.94-3.82 (om, 2H), 3.58-3.45 (om, 2H), 2.96 (t, *J* = 6.9 Hz, 2H), 2.54 (t, *J* = 6.9 Hz, 2H), 2.48-2.38 (om, 2H), 2.06-2.03 (om, 1H), 2.02 (s, 3H), 1.96-1.87 (om, 4H), 1.86 (s, 3H), 1.83-1.75 (om, 1H), 1.59-1.41 (om, 3H), 1.25 (d, *J* = 7.2 Hz,

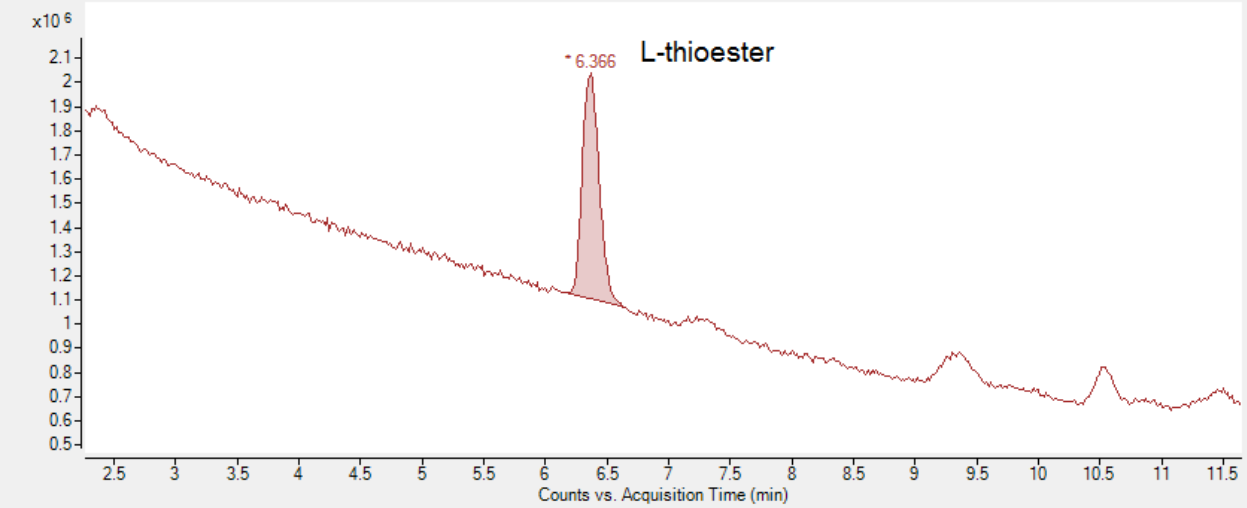
3H), 1.18 (t, *J* = 7.1 Hz, 3H), 0.88 (d, *J* = 6.8 Hz, 3H), 0.84 (d, *J* = 6.4 Hz, 3H). ^{13}C NMR (151 MHz, DMSO-d_6) δ 201.4, 172.0, 171.8, 171.1, 170.8, 169.5, 167.5, 60.2, 59.7, 54.8, 52.2, 51.0, 46.1, 41.4, 40.6, 33.7, 31.5, 29.6, 29.1, 24.3, 24.2, 23.2, 22.9, 22.4, 21.6, 17.2, 14.6, 14.1. HRMS (+ESI) calc. for $C_{28}H_{48}N_5O_8S_2^+$: 646.2939, found 646.2946.

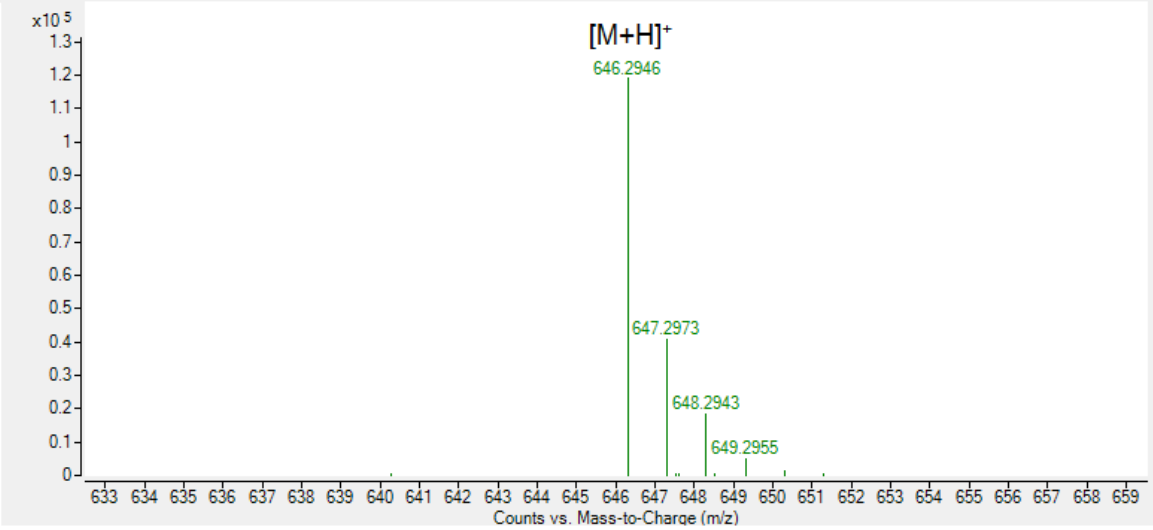


Ac-Gly-Pro-Met-Leu-Ala(*D*)-COS$(CH_2)_2CO_2C_2H_5$- 3G

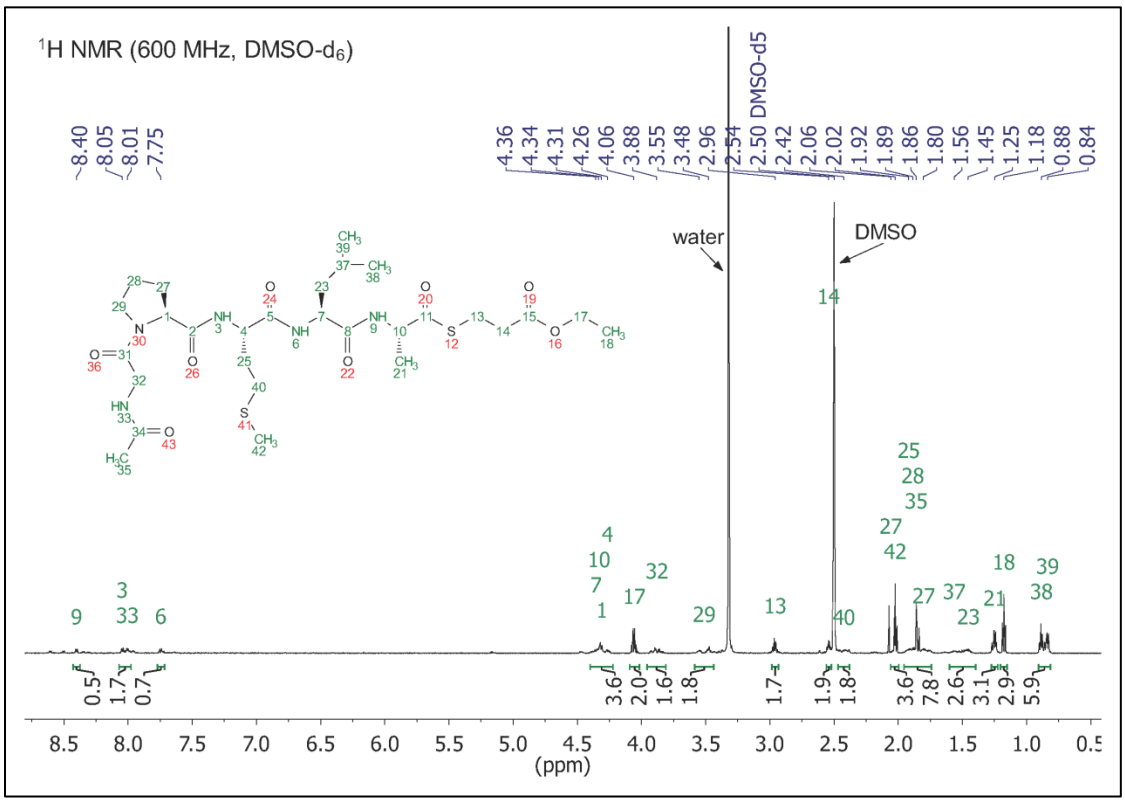
***N*-acetylglycyl-*N*-[(5*S*,8*S*,11*R*)-11-methyl-8-(2-methylpropyl)-6,9,12,16-tetraoxo-17-oxa-2,13-dithia-7,10-diazanonadecan-5-yl]-L-prolinamide**: ^{1}H NMR (600 MHz, DMSO-d_6) δ 8.50 (d, *J* = 7.1 Hz, 1H), 8.03 (d, *J* = 8.0 Hz, 1H), 8.00 (t, *J* = 5.5 Hz, 1H), 7.73 (d, *J* = 8.2 Hz, 1H), 4.39-4.24 (om, 4H), 4.06 (q, *J* = 7.2 Hz, 2H), 3.91 (dd, *J* = 16.9, 5.5 Hz, 1H), 3.84 (dd, *J* = 17.0, 5.3 Hz, 1H), 3.57-3.43 (om, 2H), 2.97 (t, *J* = 6.9 Hz, 2H), 2.54 (t, *J* = 7.0 Hz, 2H), 2.48-2.37 (om, 2H), 2.03 (s, 3H), 1.96-1.87 (om, 3H), 1.85 (s, 3H), 1.82-1.74 (om, 2H), 1.67-1.60 (m, 1H), 1.57-1.43 (om, 3H), 1.26 (d, *J* = 7.3 Hz, 3H), 1.18 (t, *J* = 7.2 Hz, 3H), 0.89 (d, *J* = 6.7 Hz, 3H), 0.86 (d, *J* = 6.5 Hz, 3H). ^{13}C NMR (151 MHz, DMSO-d_6) δ 201.6, 172.0, 171.7, 171.1, 170.8, 169.4, 167.4, 60.2, 59.6, 54.8, 51.9, 50.7, 46.0, 41.4, 40.4, 33.7, 31.6, 29.6, 29.1, 24.3, 24.1, 23.2, 23.1, 22.4, 21.5, 17.2, 14.7, 14.1. HRMS (+ESI) calc. for $C_{28}H_{48}N_5O_8S_2^+$: 646.2939, found 646.2932.

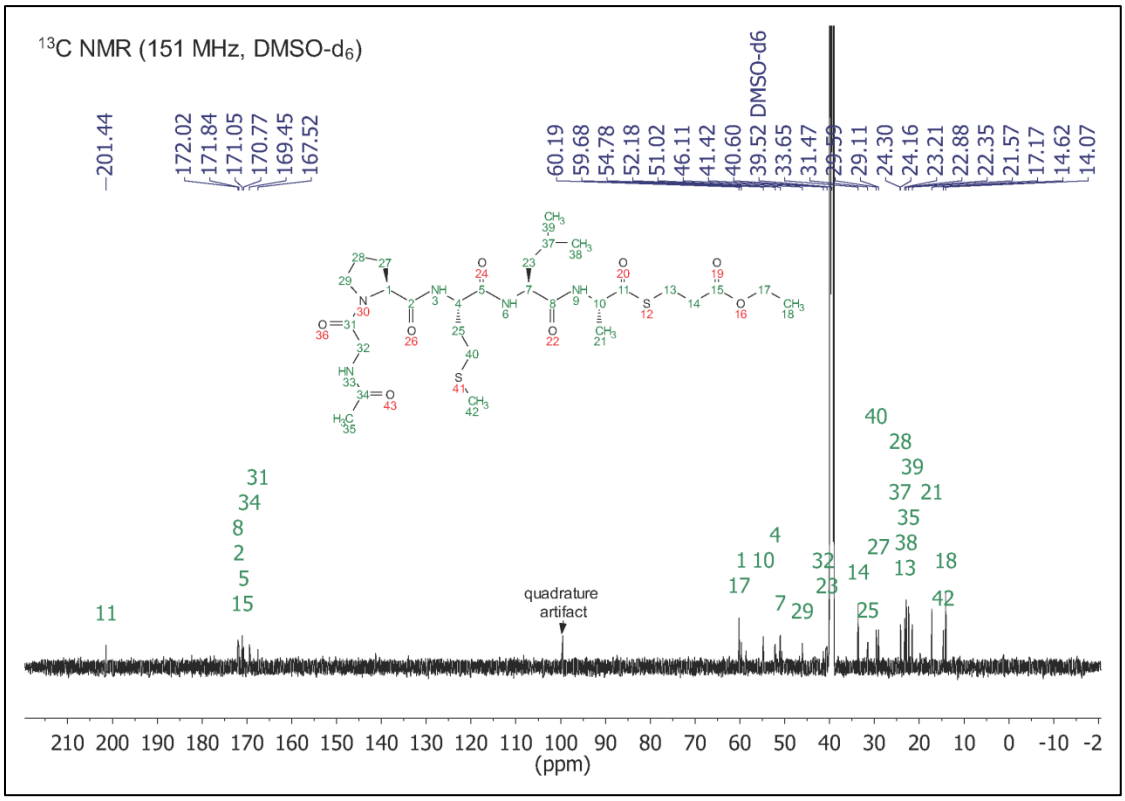
HRMS Spectra* Ac-Gly-Pro-Met-Leu-Ala(*L*)-COS$(CH_2)_2CO_2C_2H_5$-3G



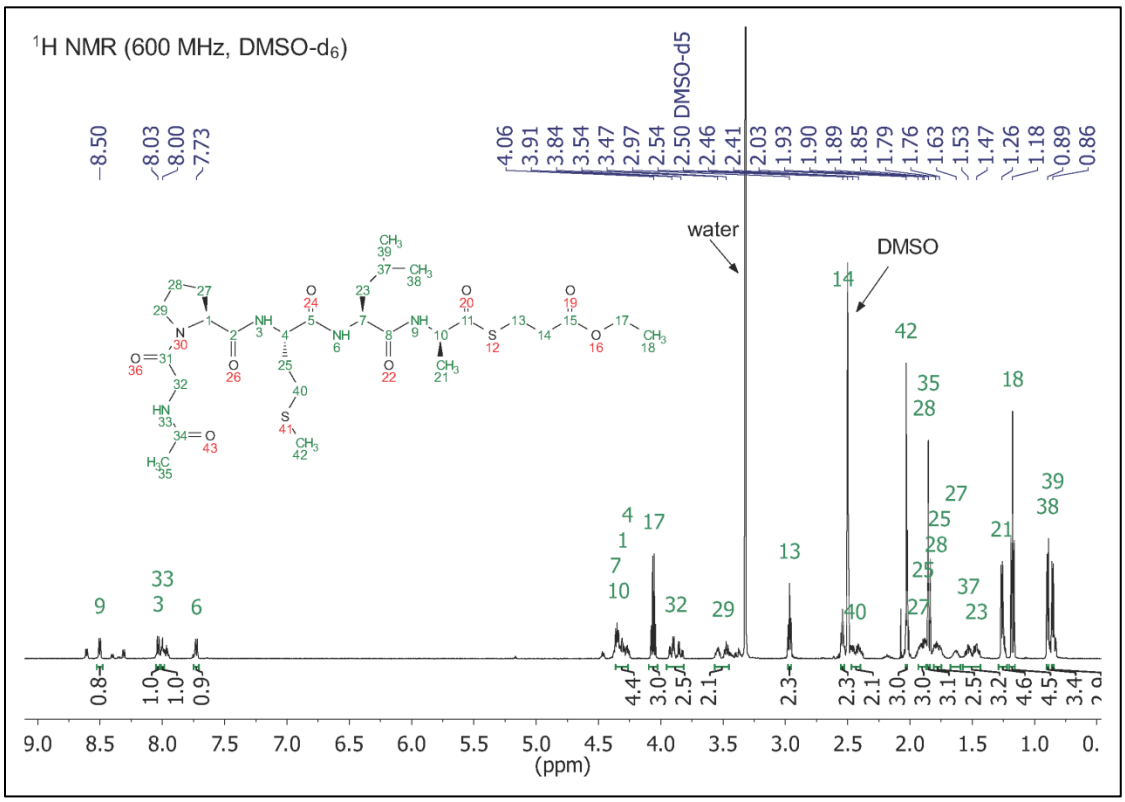


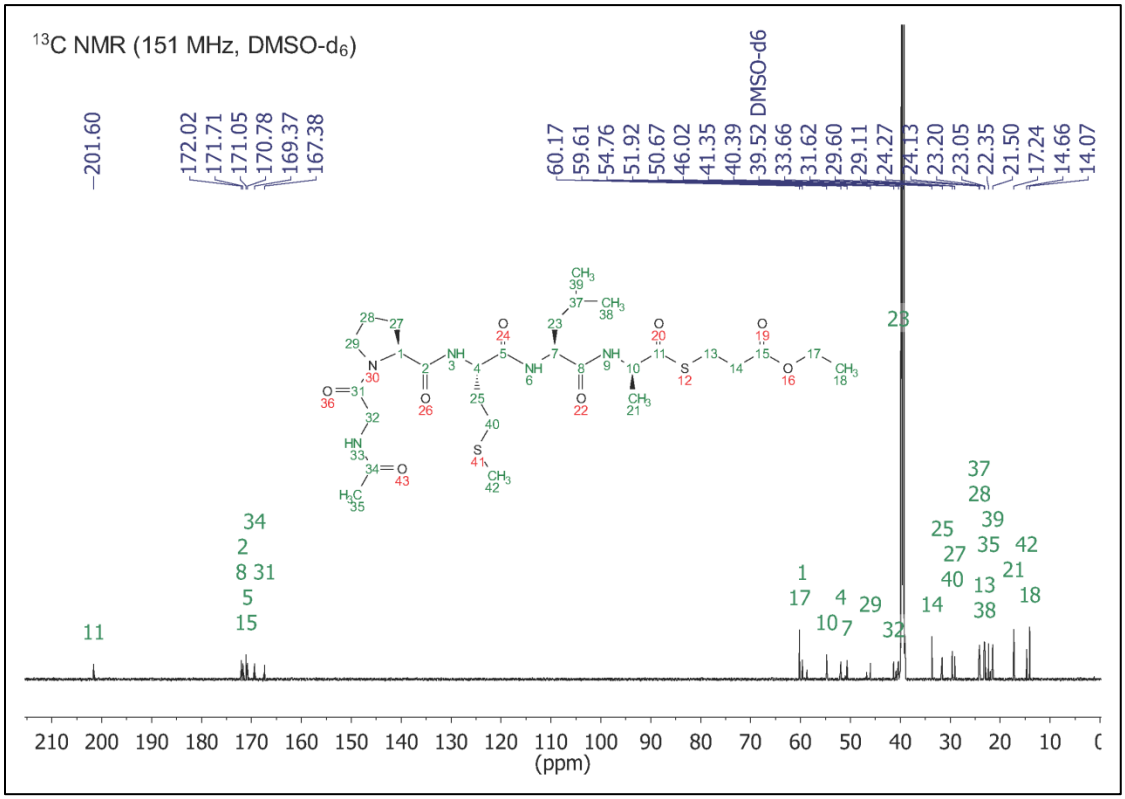
NMR Spectra for Ac-Gly-Pro-Met-Leu-Ala(*L*)-COS(CH_2)$_2$$CO_2$$C_2$$H_5$-3G



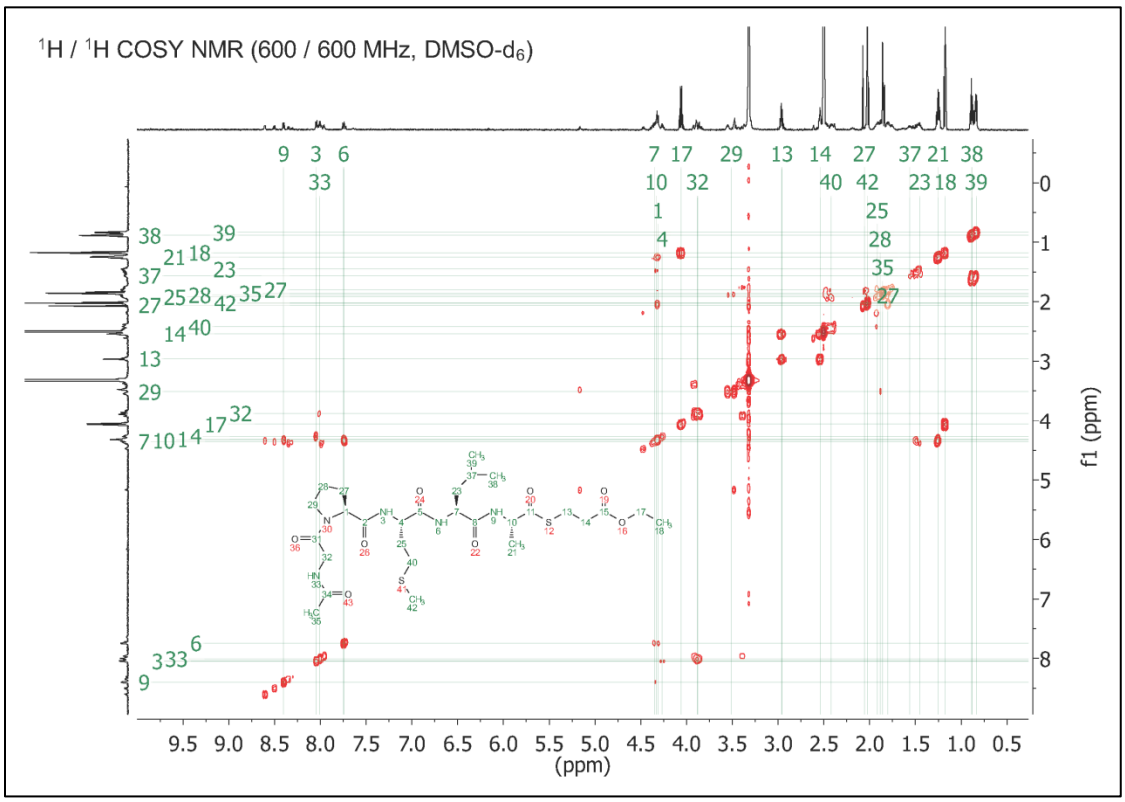

13C NMR (151 MHz, DMSO-d6)
201.44
172.02
171.84
171.05
170.77
169.45
167.52
60.19
59.68
54.78
52.18
51.02
46.11
41.42
40.60
39.52 DMSO-d6
33.65
31.47
29.59
29.11
24.30
24.16
23.21
22.88
22.35
21.57
17.17
14.62
14.07
quadrature artifact
210 200 190 180 170 160 150 140 130 120 110 100 90 80 70 60 50 40 30 20 10 0 -10 -2
(ppm)

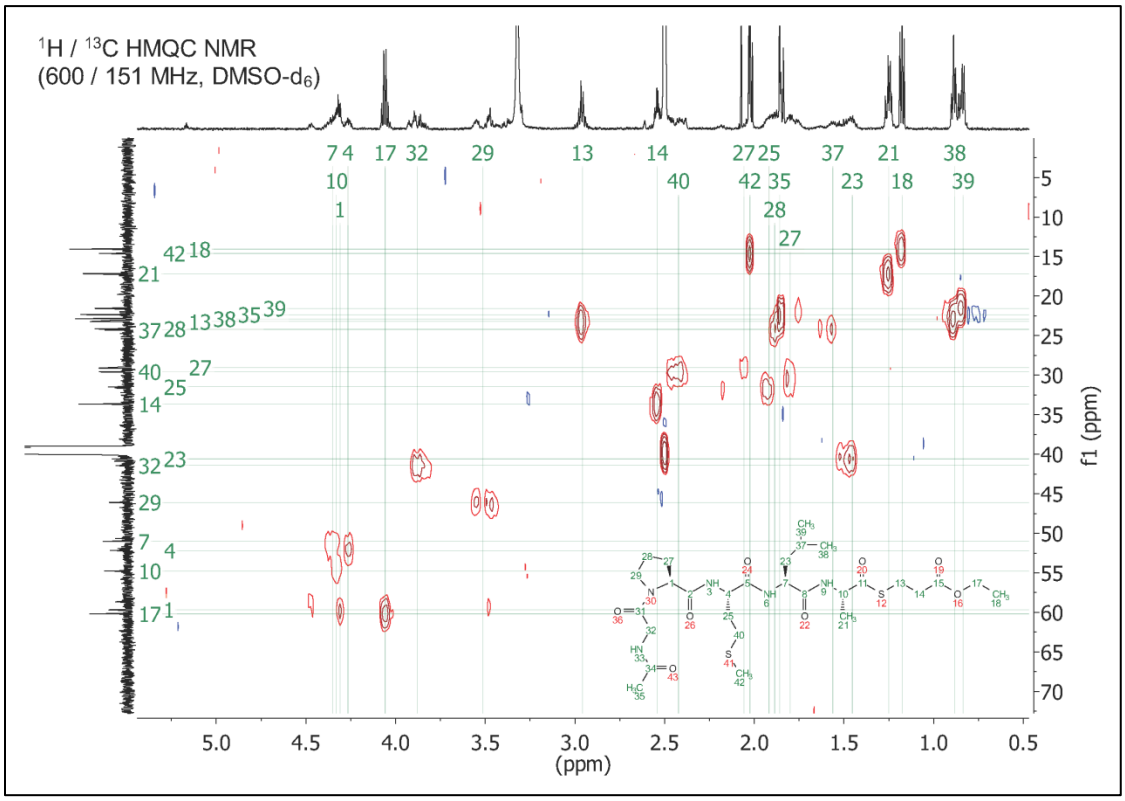
NMR Spectra for Ac-Gly-Pro-Met-Leu-Ala(*D*)-COS$(CH_2)_2CO_2C_2H_5$-3G

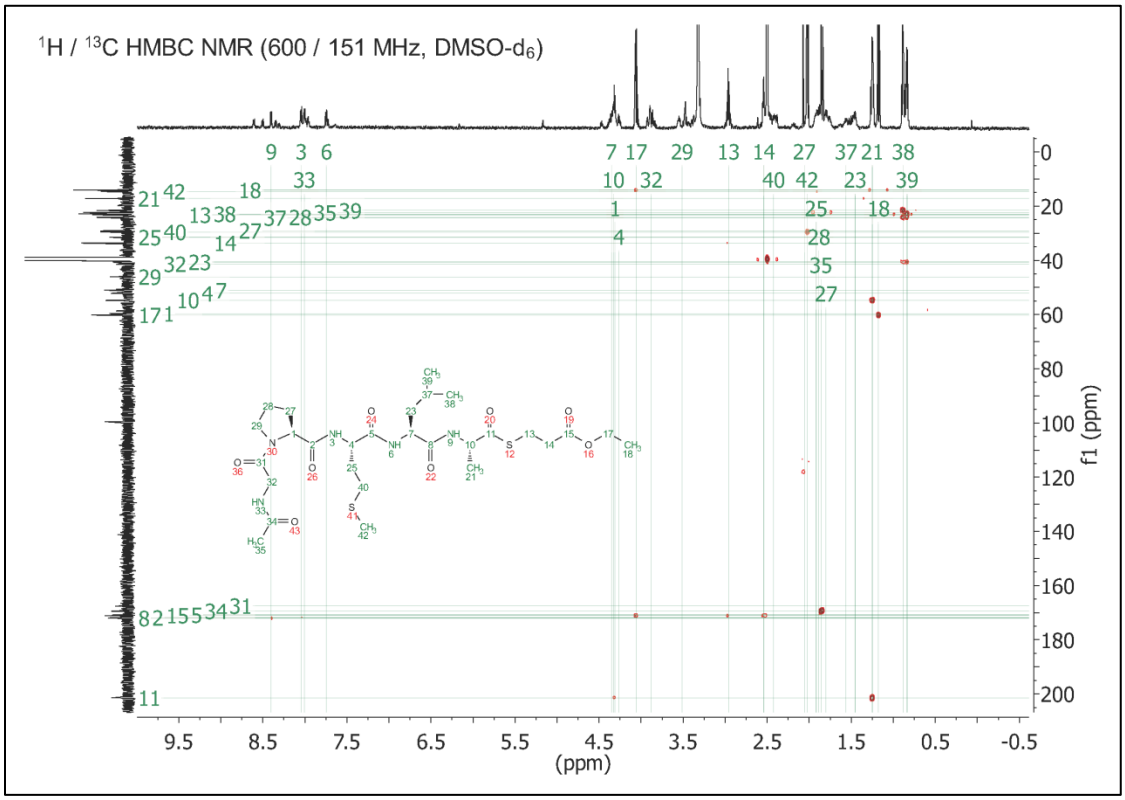


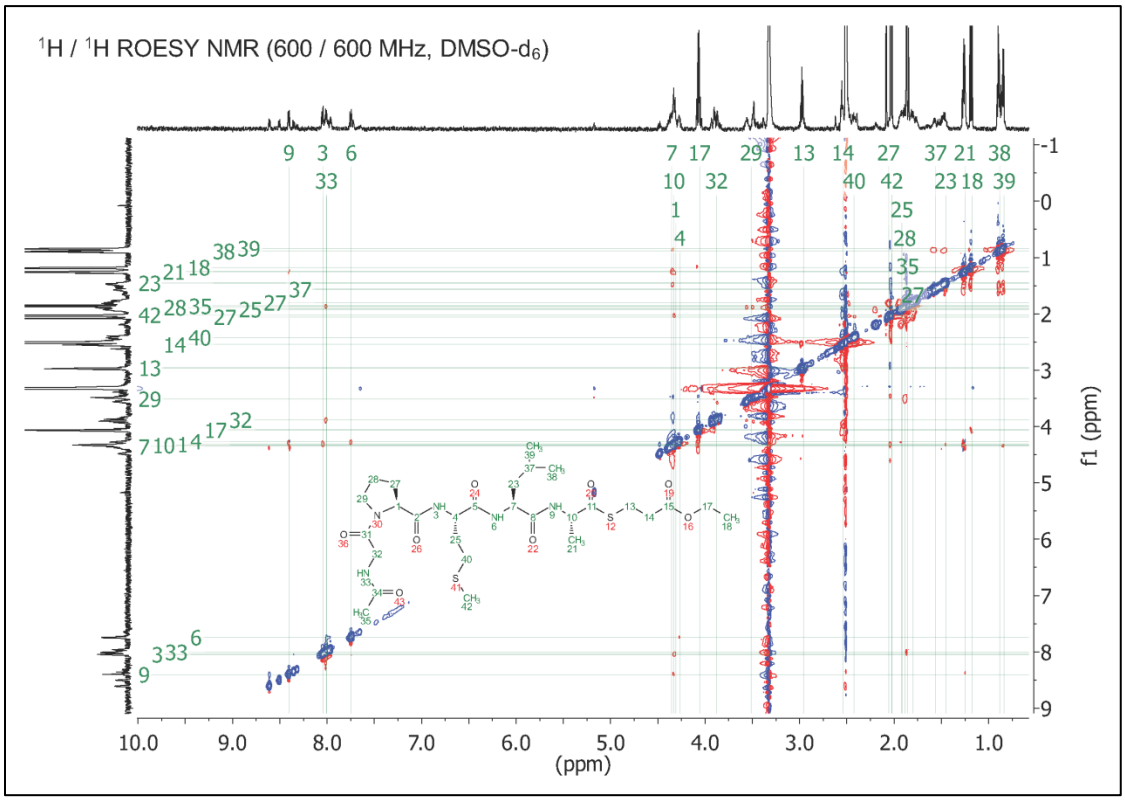

13C NMR (151 MHz, DMSO-d6)
DMSO-d6
201.60
172.02
171.71
171.05
170.78
169.37
167.38
60.17
59.61
54.76
51.92
50.67
46.02
41.35
40.39
39.52
33.66
31.62
29.60
29.11
24.27
24.13
23.20
23.05
22.35
21.50
17.24
14.66
14.07
(ppm)

2D NMR Spectra for Ac-Gly-Pro-Met-Leu-Ala(*L*)-COS$(CH_2)_2CO_2C_2H_5$-3G

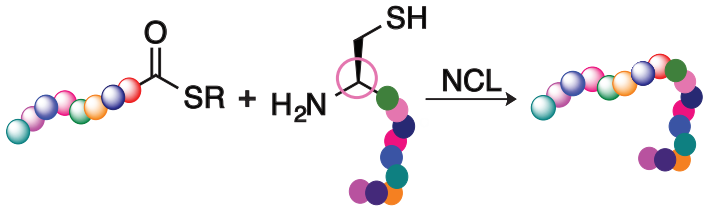



^{1}H / ^{13}C HMQC NMR
(600 / 151 MHz, DMSO-d$_6$)
f1 (ppm)
(ppm)


^{1}H / ^{13}C HMBC NMR (600 / 151 MHz, DMSO-d$_6$)
f1 (ppm)
(ppm)



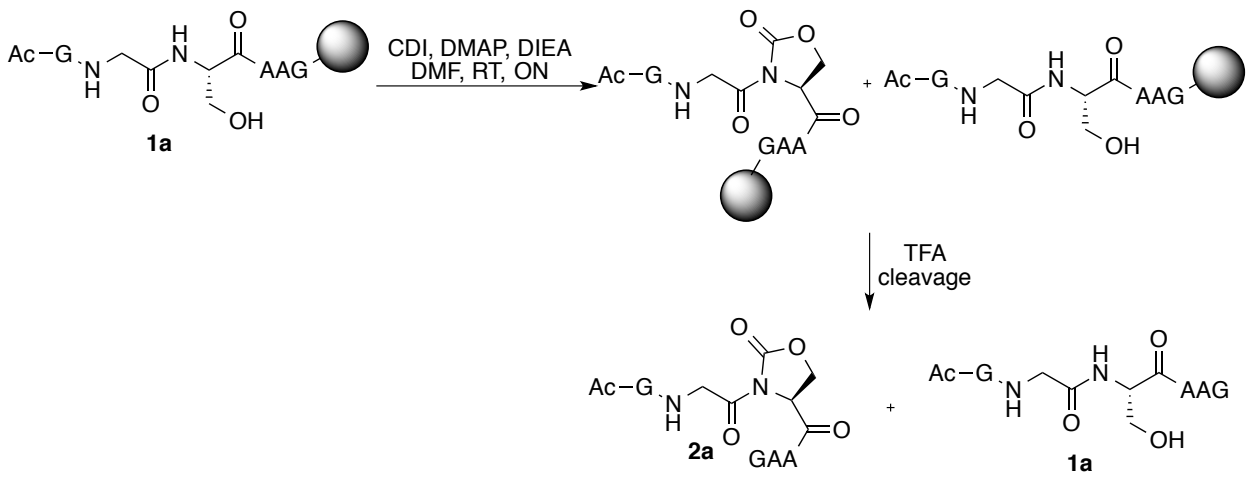
IV. Native Chemical Ligation of peptide thioesters obtained from activated serine on the solid support.



General procedure for the native chemical ligation for synthesis of large peptides:

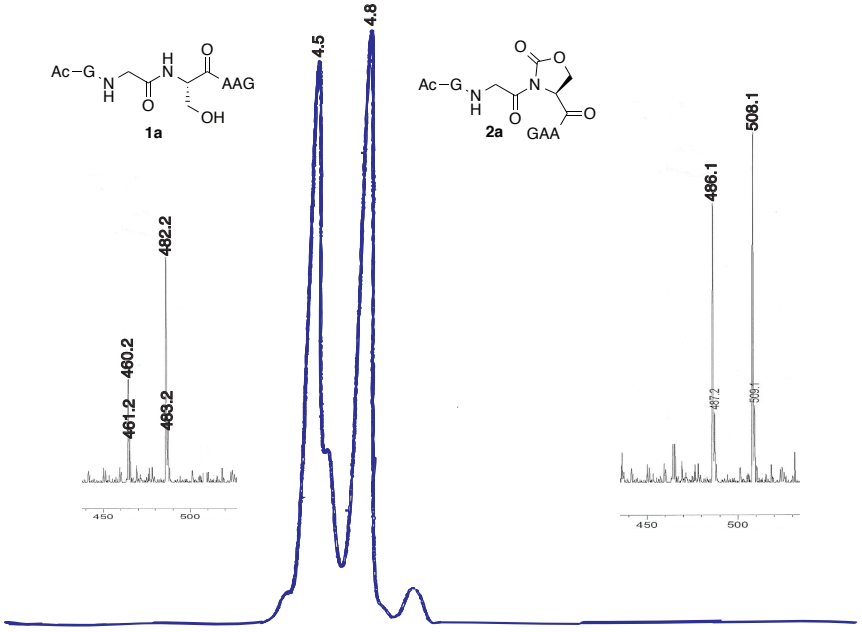
900 µL of ligation buffer pH 7.1 (0.2 M sodium phosphate buffer and 6 M guanidine hydrochloride) was added to 1.0 mg of the peptide thioester **3G/3h** (final concentration 1.3 mM) and 1.3 mg of peptide CRAFS (final concentration 2 mM) under argon. The mixture was treated with MPAA (3.8 mg, 20 mM) and TCEP.HCl (5.5 mg, 17 mM) and pH of solution was adjusted to 7.0 by using 2N NaOH solution. The ligation was carried out at 37°C and monitored by RP HPLC. After completion of reaction, TCEP.HCl (30 mM in water) was added into the reaction mixture and it was left for stirring at room temperature for 1 h followed by purification of ligated peptide using RP-HPLC.

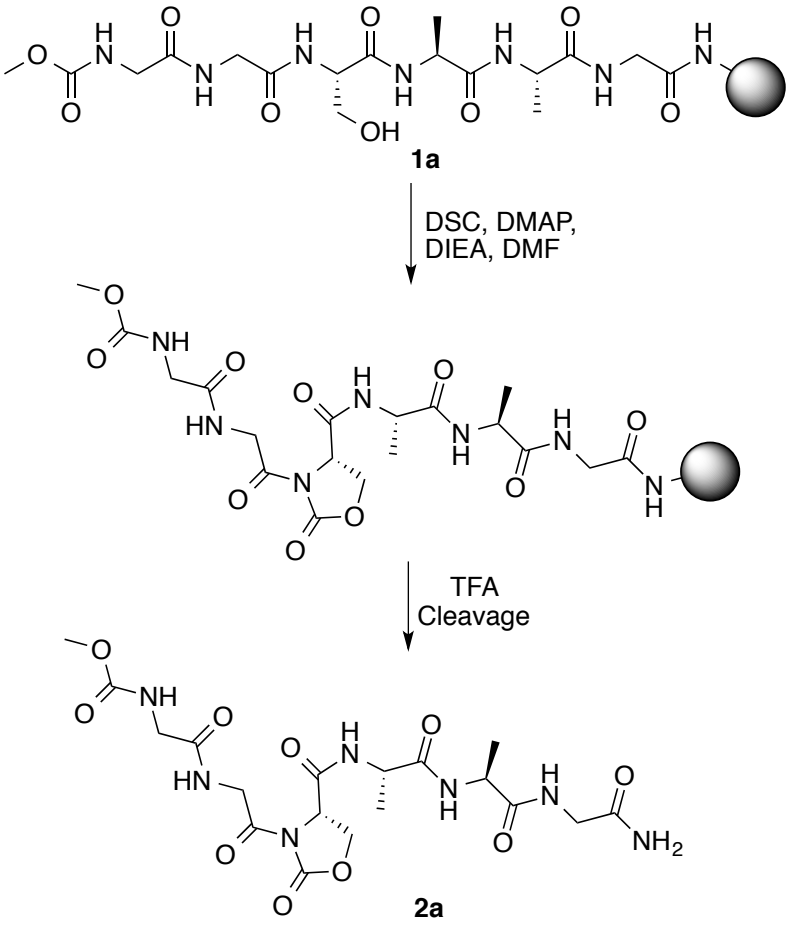
V. Peptide Characterization and HPLC Traces



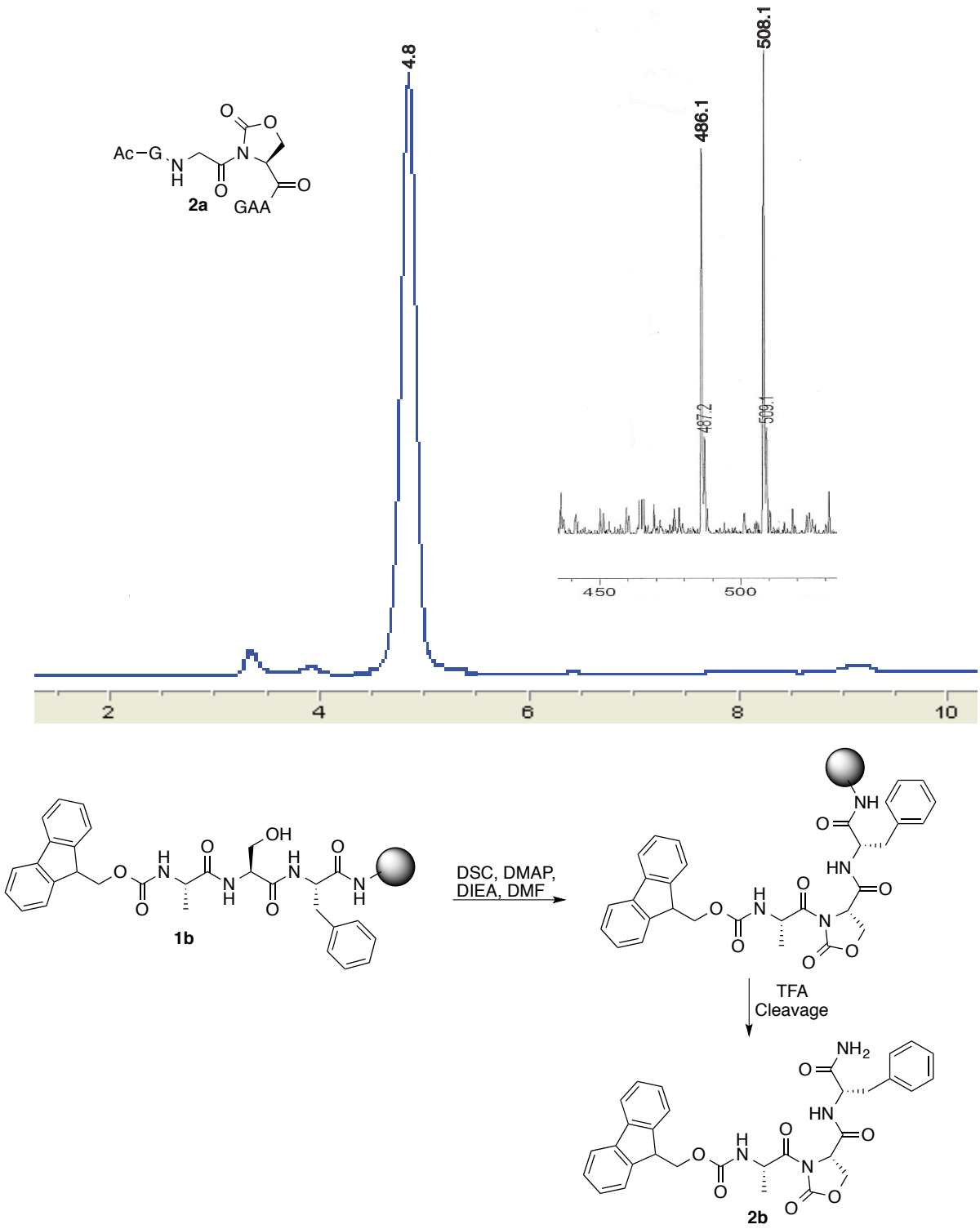
Ac-Gly-Gly-Ser-Ala-Ala-Gly-NH$_2$ (1a). LCMS: *m/z* 460.2 (calcd $[M+H]^+$ = 460.5), *m/z* 482.2 (calcd $[M+Na]^+$ = 482.5). Purity: >95% (HPLC analysis at 220 nm). Retention time: 4.5 min.

Ac-Gly-Gly-Oxd-Ala-Ala-Gly-NH$_2$ (2a). LCMS: *m/z* 486.1 (calcd $[M+H]^+$ = 486.4), *m/z* 508.1 (calcd $[M+Na]^+$ = 508.4). Purity: >95% (HPLC analysis at 220 nm). Retention time: 4.8 min.

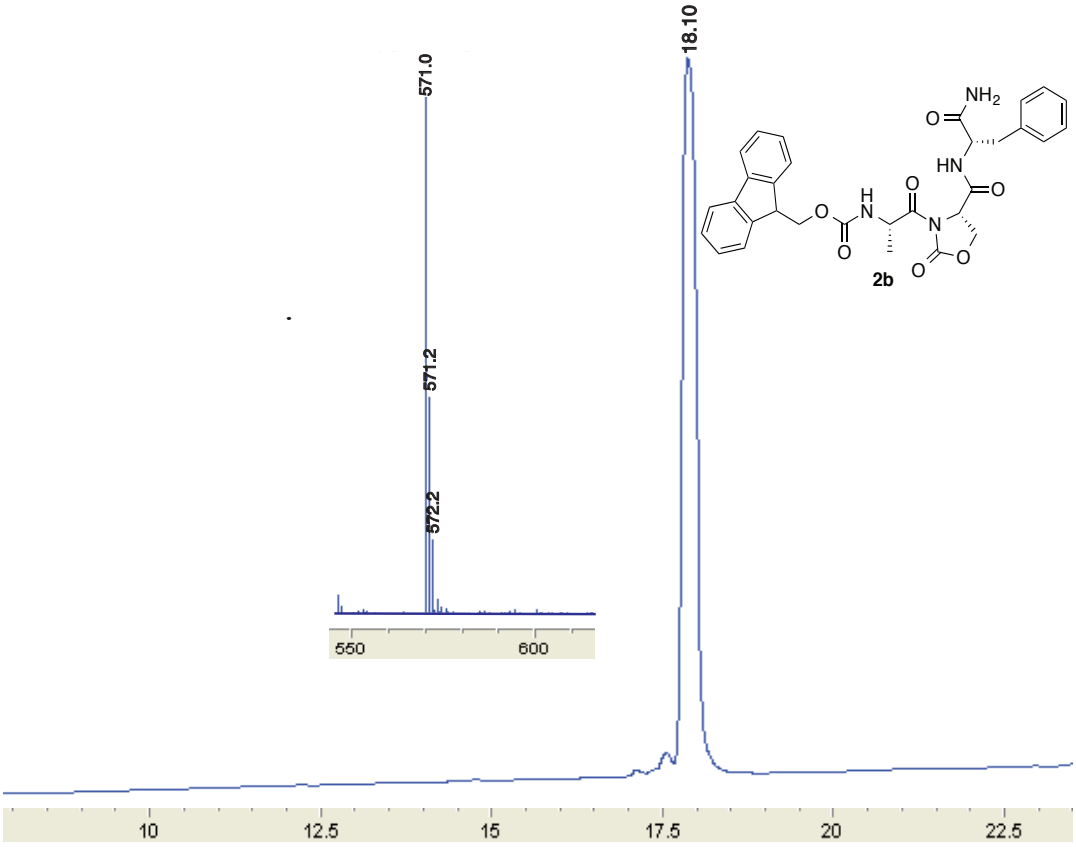


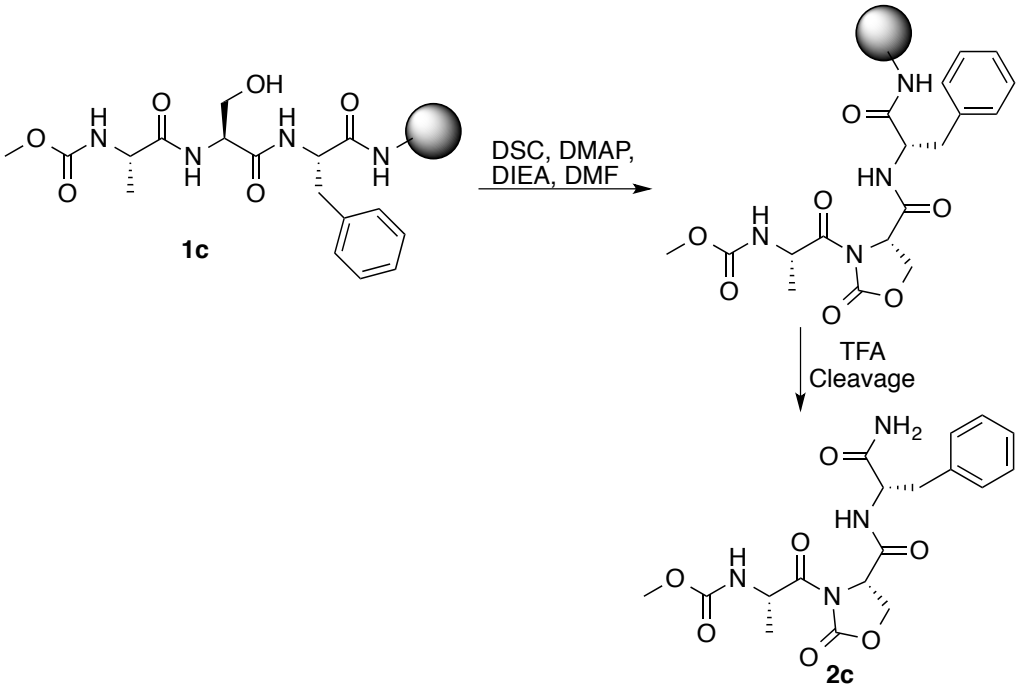


Ac-Gly-Gly-Oxd-Ala-Ala-Gly-NH_2 (2a). LCMS: *m/z* 486.1 (calcd $[M+H]^+$ = 486.4), *m/z* 508.1 (calcd $[M+Na]^+$ = 508.4). Purity: >95% (HPLC analysis at 220 nm). Retention time: 4.8 min

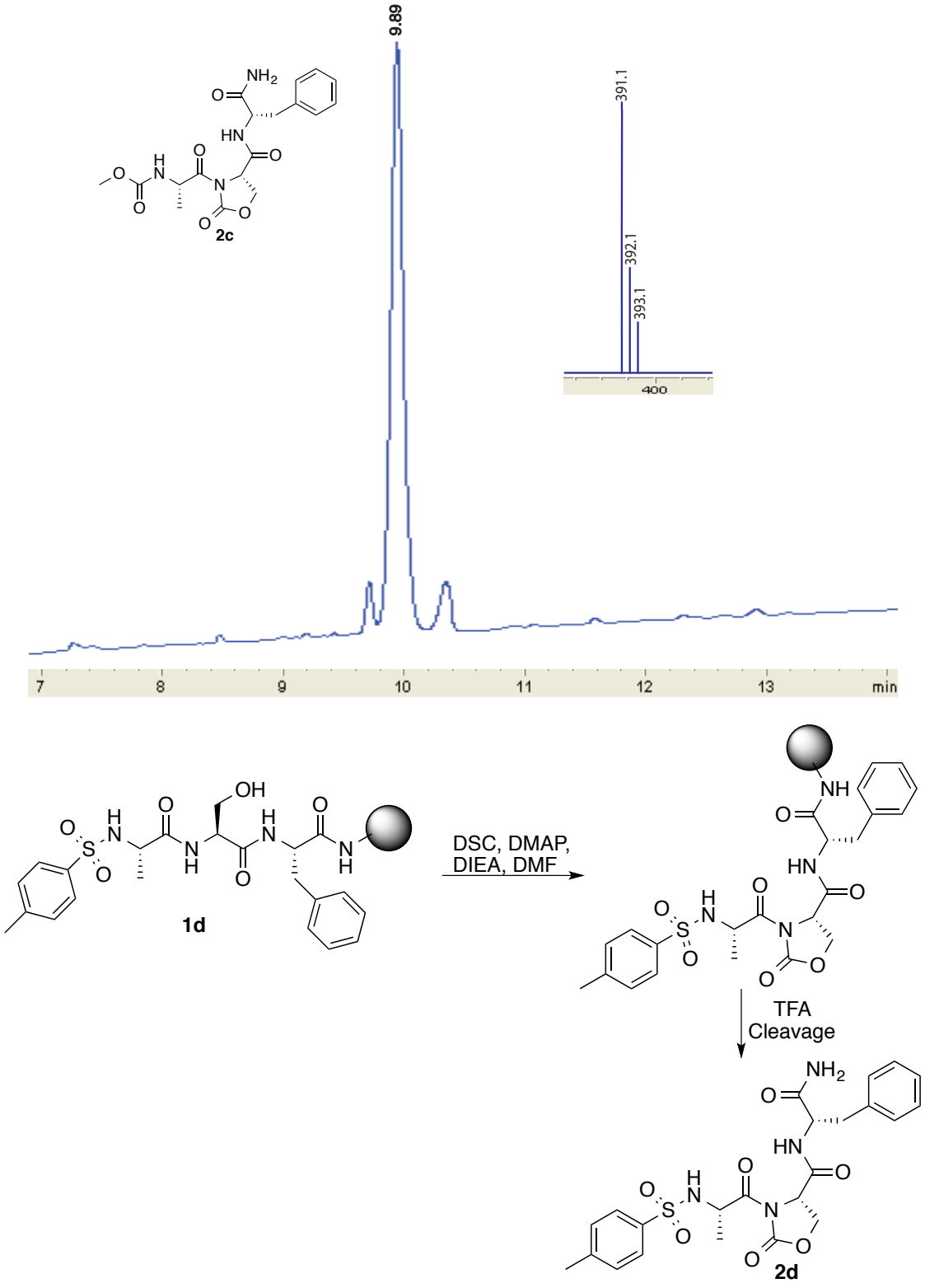


Fmoc-Ala-Oxd-Phe-NH$_2$ (2b). LCMS: *m/z* 571.0 (calcd $[M+H]^+$ = 571.3). Purity: >95% (HPLC analysis at 220 nm). Retention time: 18.1 min

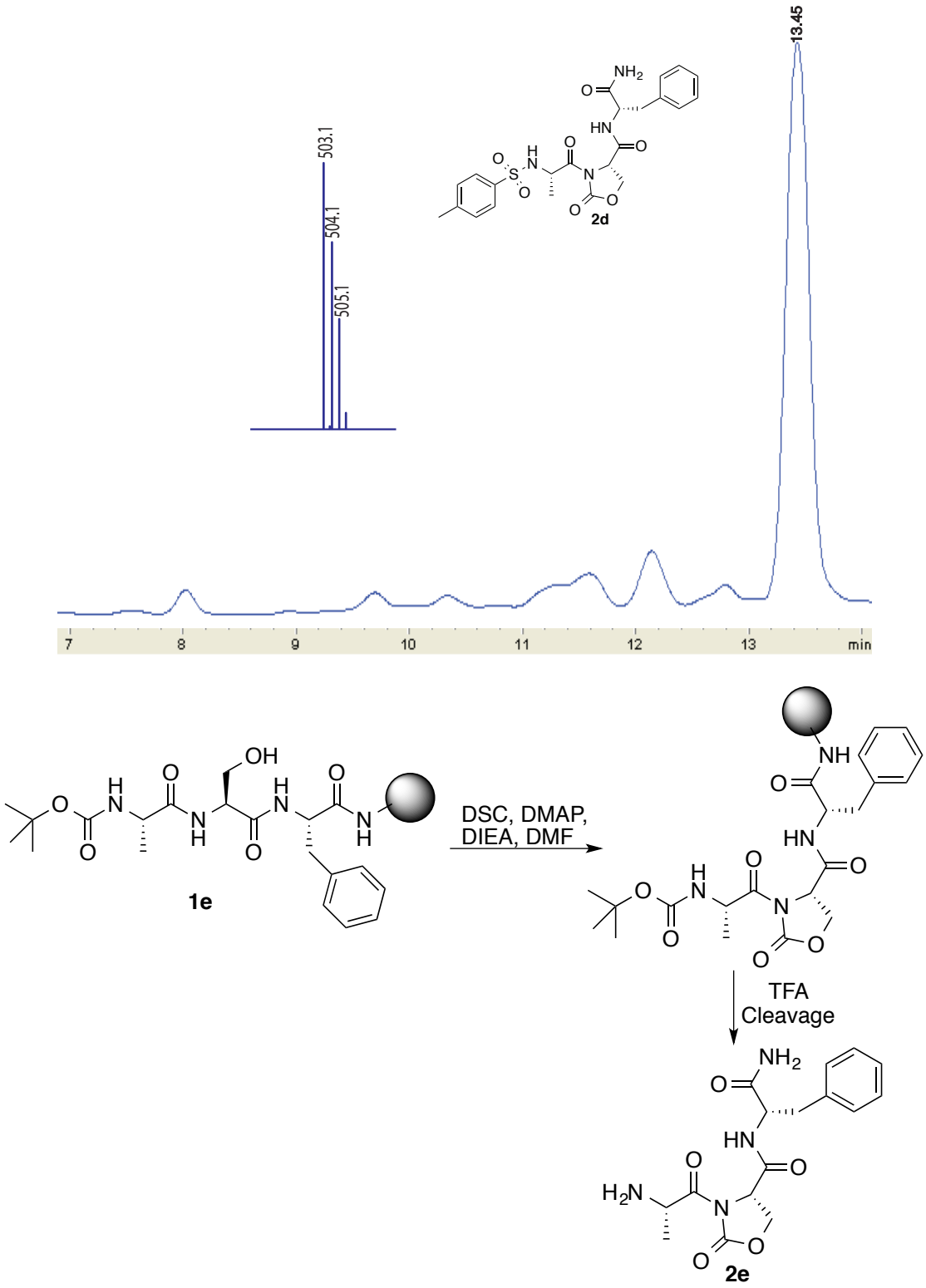




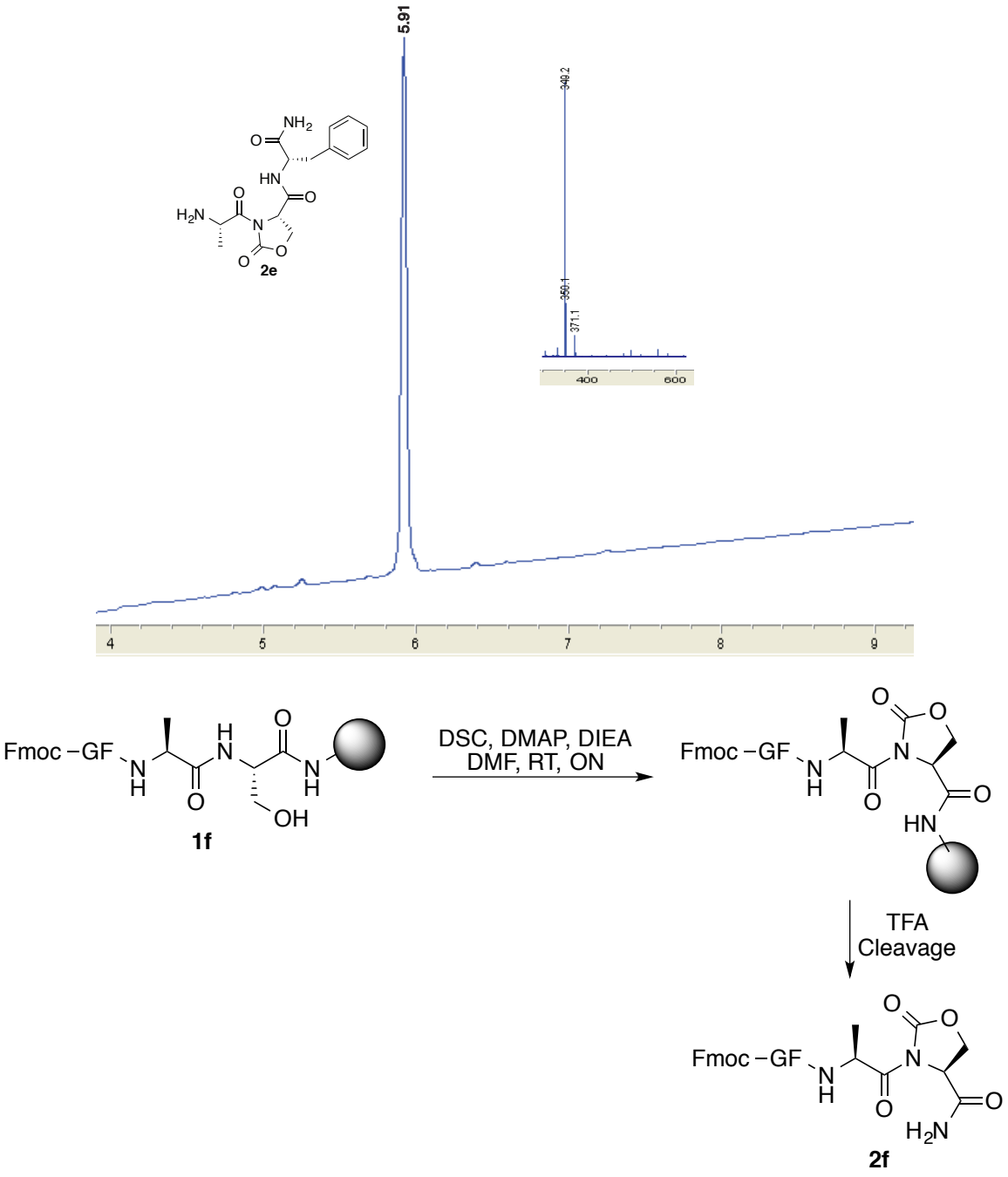
Ac-Ala-Oxd-Phe-NH$_2$ (2c). LCMS: *m/z* 391.1 (calcd $[M+H]^+$ = 391.5). Purity: >95% (HPLC analysis at 220 nm). Retention time: 9.89 min



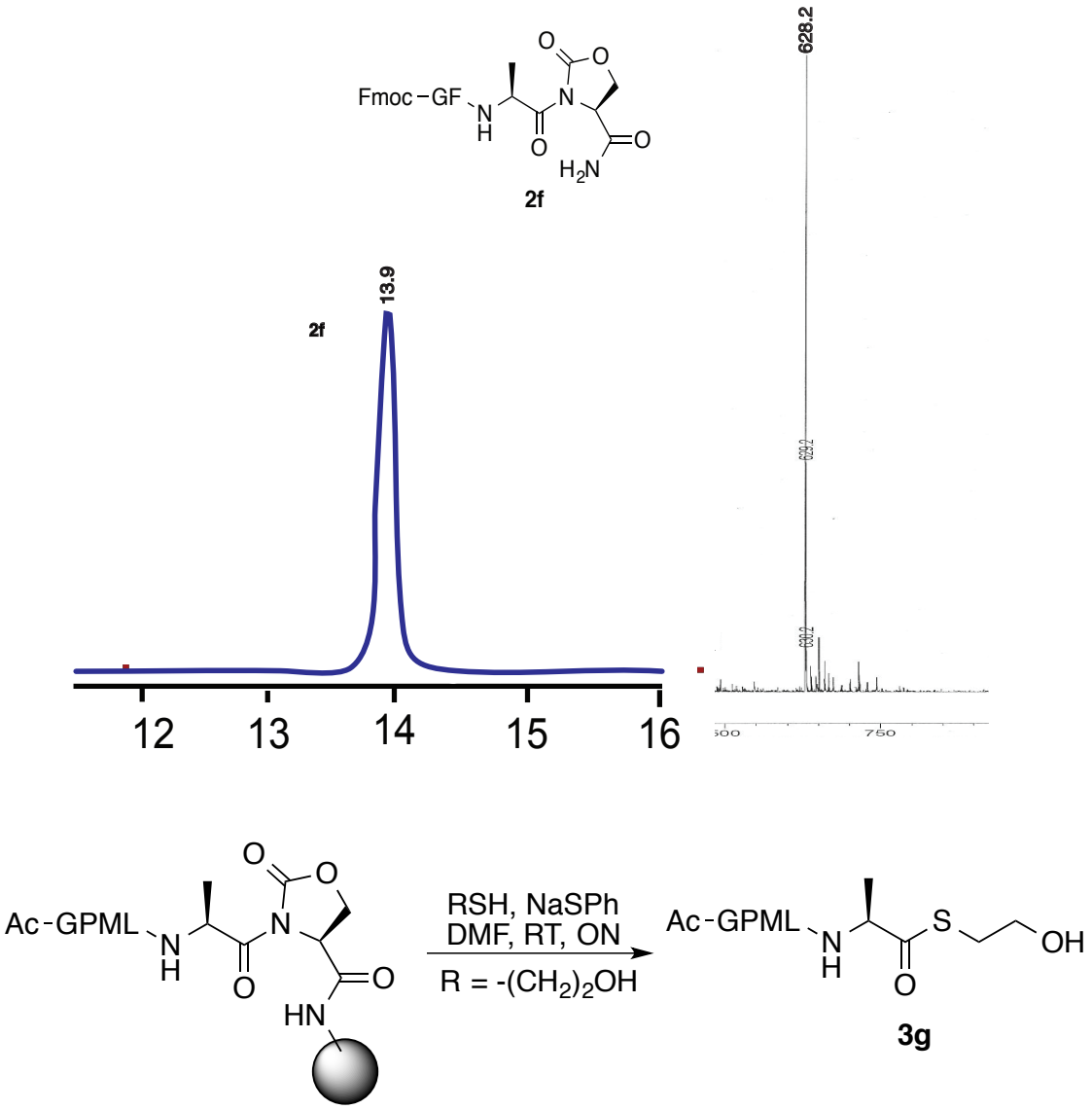
Ts-Ala-Oxd-Phe-NH$_2$ (2d). LCMS: *m/z* 503.1 (calcd $[M+H]^+$ = 503.2). Purity: >95% (HPLC analysis at 220 nm). Retention time: 13.45 min



Ala-Oxd-Phe-NH$_2$ (2e). LCMS: *m/z* 349.2 (calcd $[M+H]^+$ = 349.5). Purity: >95% (HPLC analysis at 220 nm). Retention time: 5.91 min

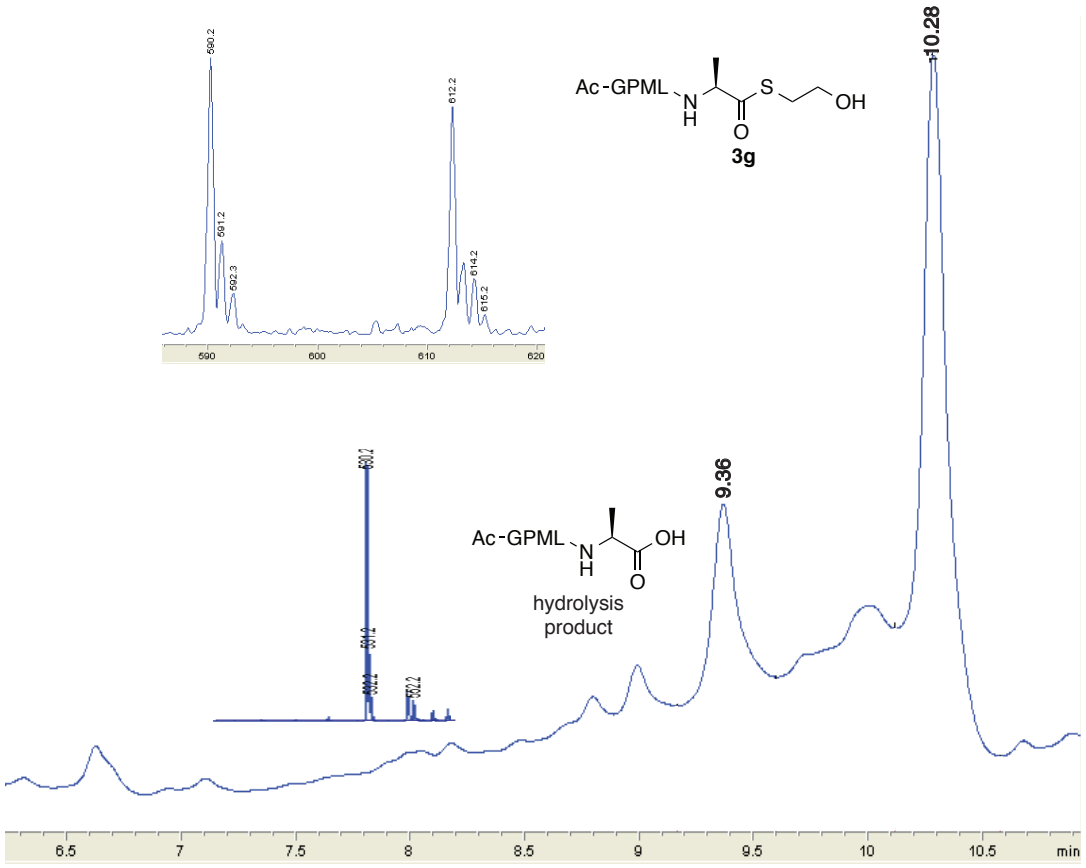


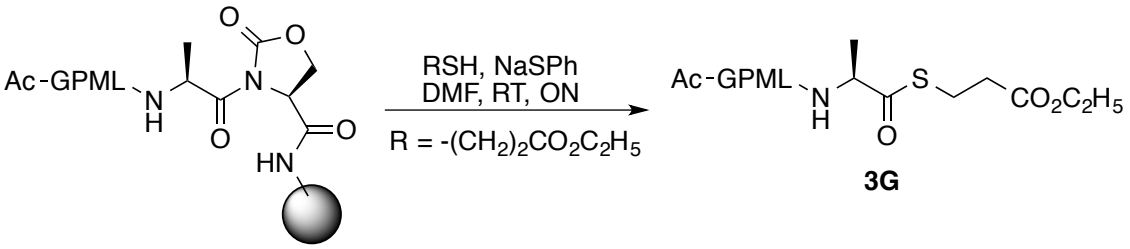
Fmoc-Gly-Phe-Ala-Oxd-NH$_2$ (2f). LCMS: *m/z* 628.2 (calcd $[M+H]^+$ = 628.5). Purity: >95% (HPLC analysis at 220 nm). Retention time: 13.9 min



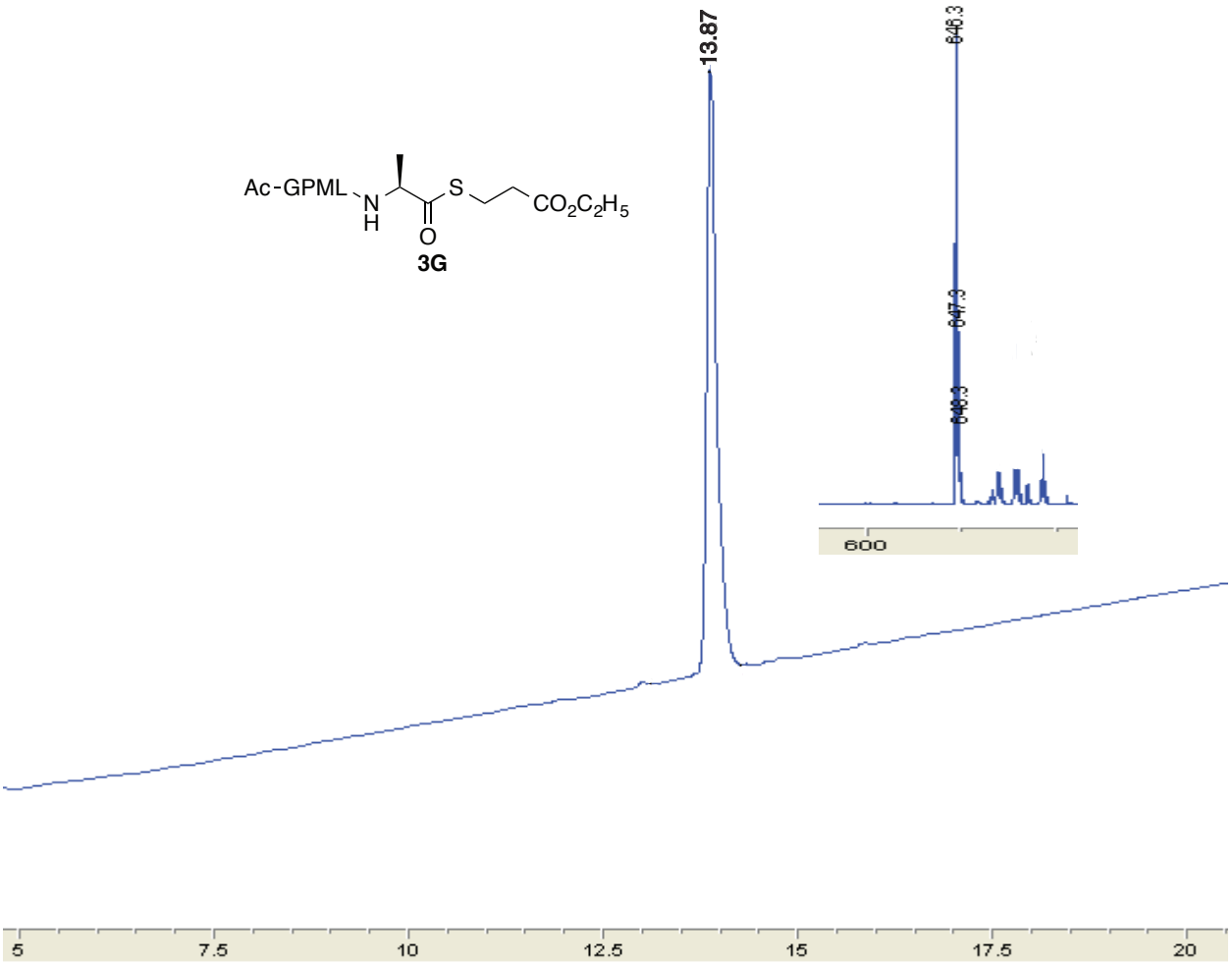
Ac-Gly-Pro-Met-Leu-Ala-COS$(CH_2)_2$OH (3g). LCMS: *m/z* 590.2 (calcd $[M+H]^+$ = 590.6), *m/z* 612.2 (calcd $[M+ Na]^+$ = 612.6). Purity: >95% (HPLC analysis at 220 nm). Retention time:10.28 min

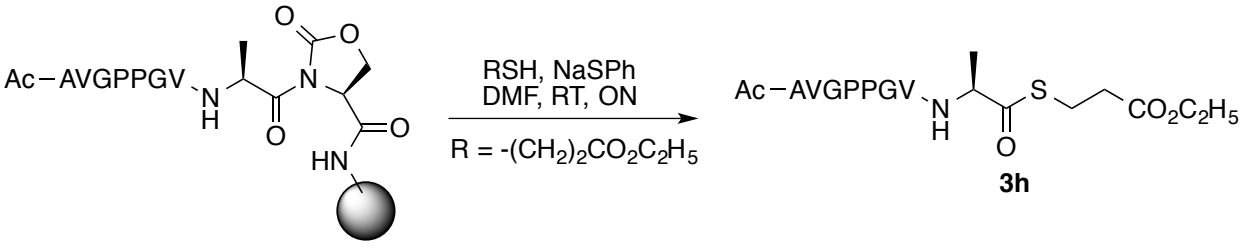
Ac-Gly-Pro-Met-Leu-Ala-OH. LCMS: *m/z* 530.2 (calcd $[M+H]^+$ = 530.5), *m/z* 552.2 (calcd $[M+Na]^+$ = 552.5). Purity: >95% (HPLC analysis at 220 nm). Retention time: 9.36 min



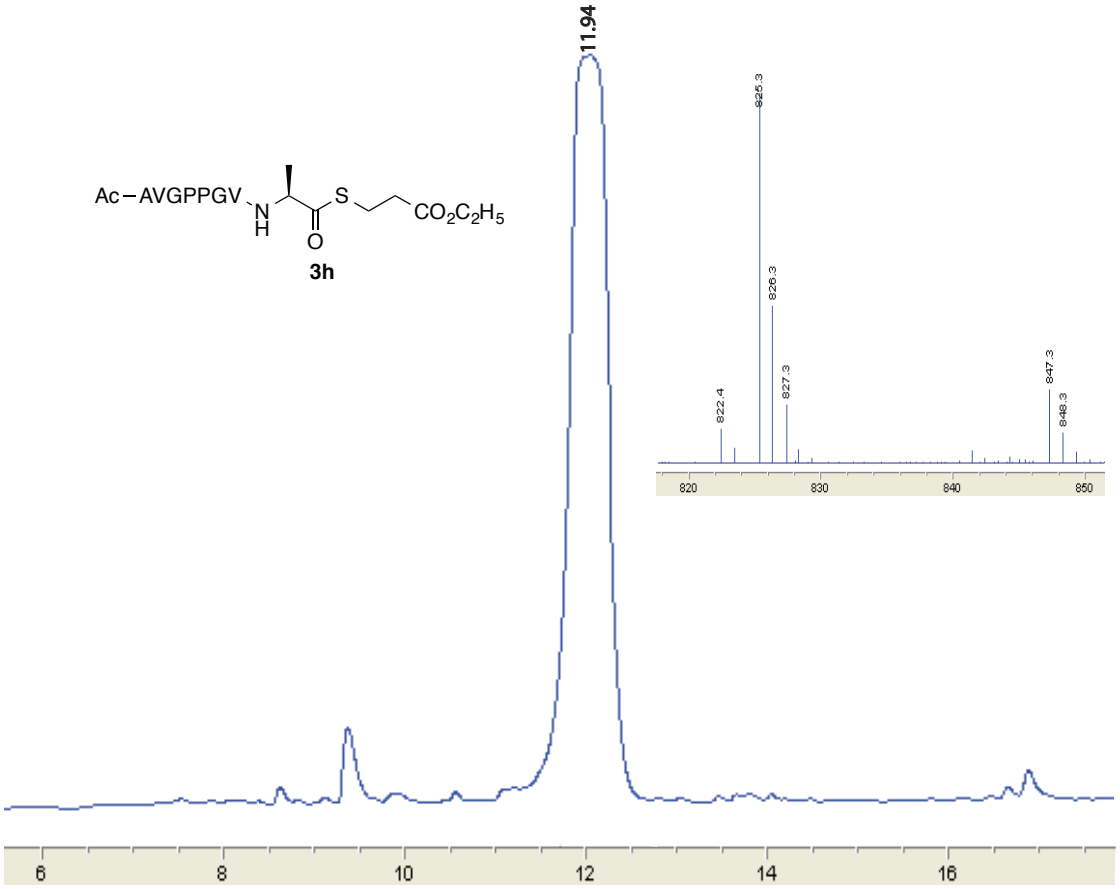


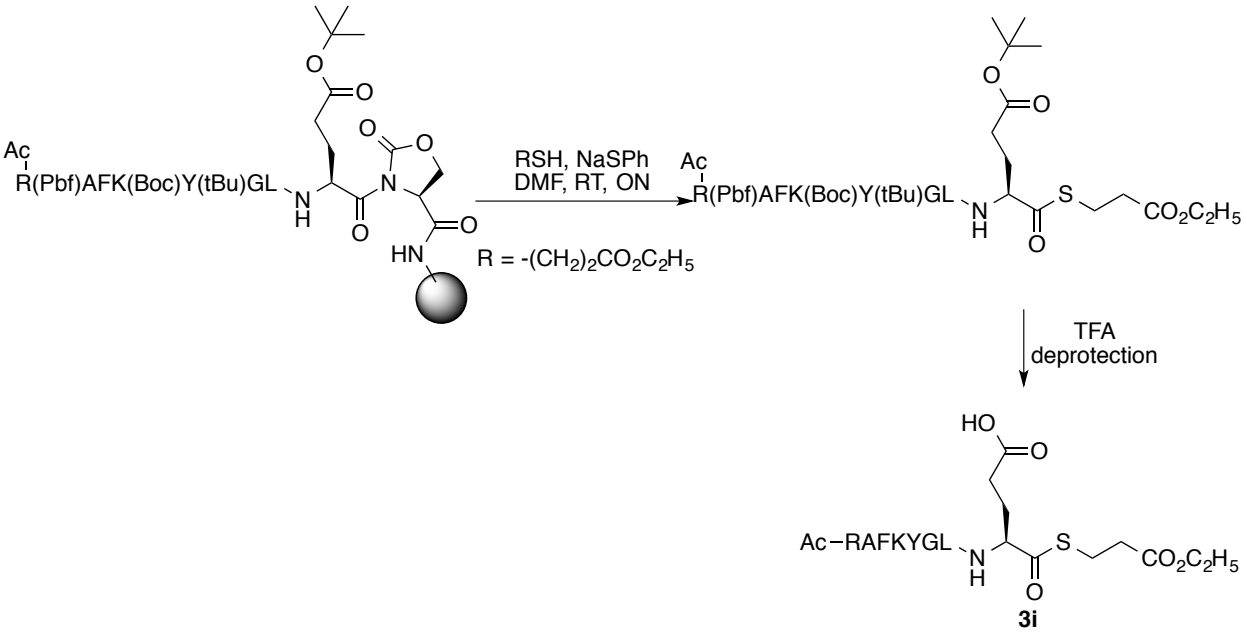
Ac-Gly-Pro-Met-Leu-Ala-COS$(CH_2)_2CO_2C_2H_5$ (3G). LCMS: *m/z* 646.3 (calcd $[M+H]^+$ = 646.7). Purity: >95% (HPLC analysis at 220 nm). Retention time: 13.87 min





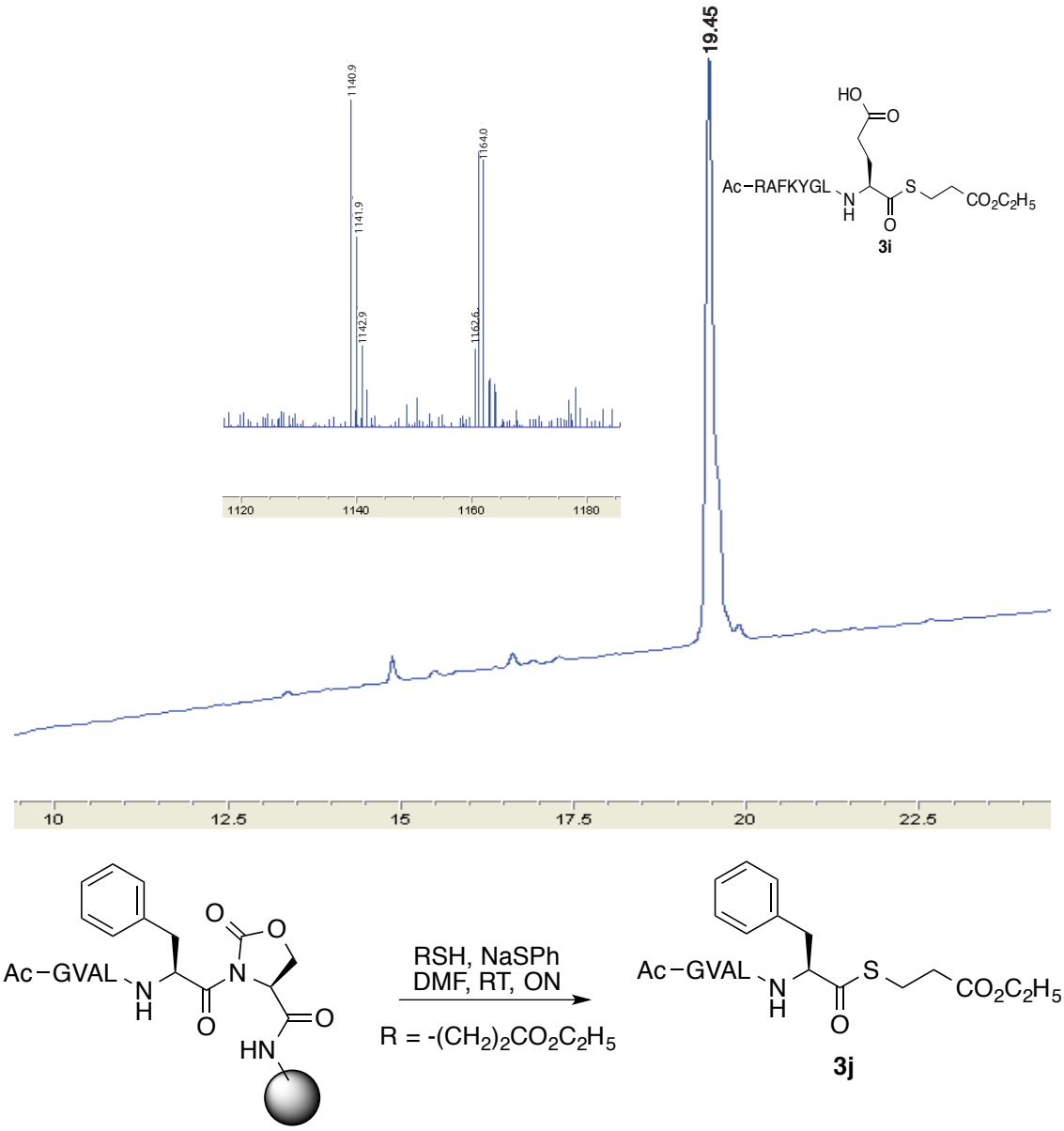
Ac-Ala-Val-Gly-Pro-Pro-Gly-Val-Ala-COS$(CH_2)_2CO_2C_2H_5$ (3h). LCMS: *m/z* 825.3 (calcd $[M+H]^+$ = 825.7), *m/z* 847.3 (calcd $[M+Na]^+$ = 847.7). Purity: >95% (HPLC analysis at 220 nm). Retention time: 11.94 min



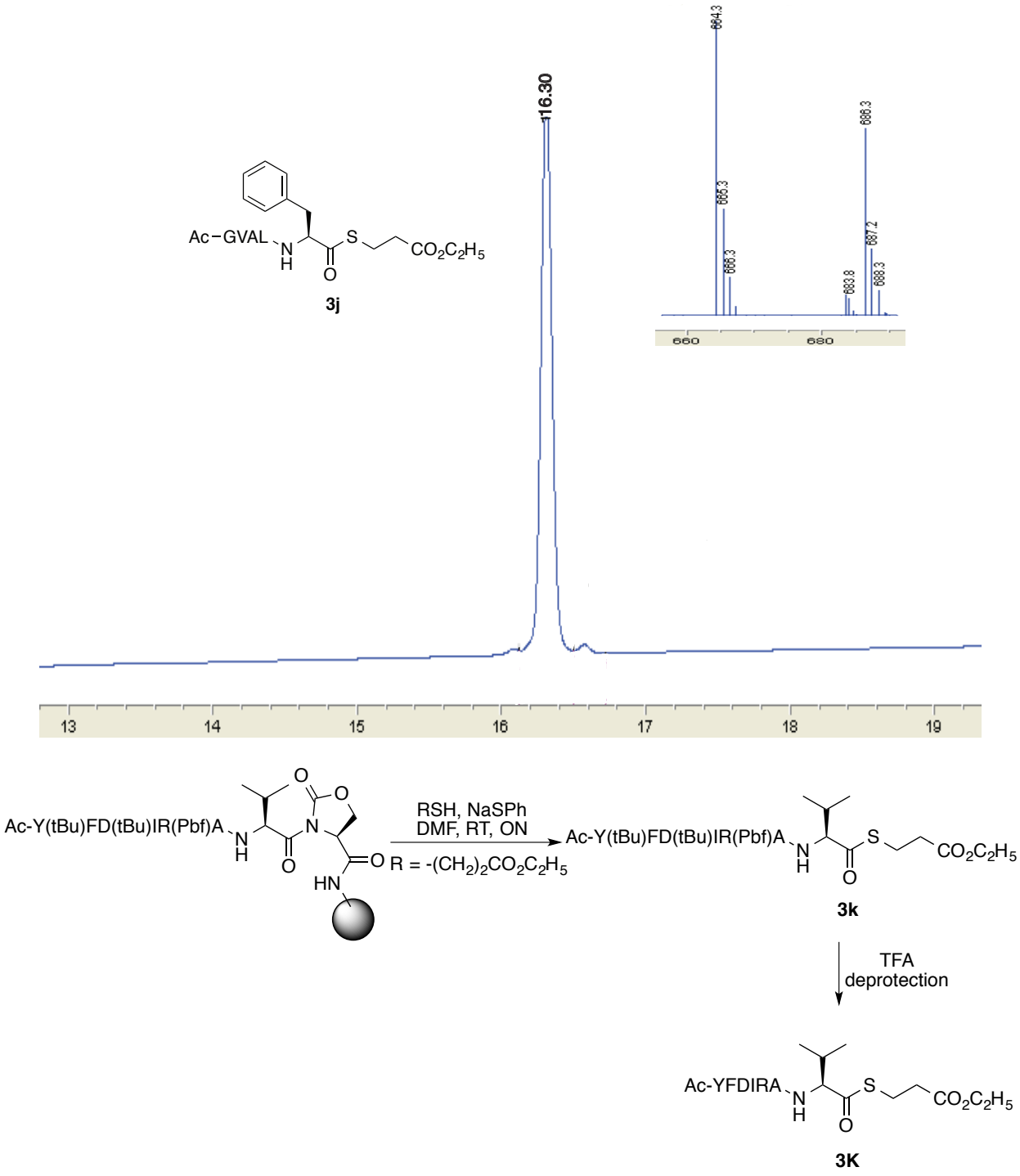


Ac-Arg-Ala-Phe-Lys-Tyr-Gly-Leu-Glu-COS$(CH_2)_2CO_2C_2H_5$ (3i). LCMS: *m/z* 1140.9 (calcd

[*M*+H]$^+$ = 1140.5), *m/z* 1163.9 (calcd [*M*+Na]$^+$ = 1163.5). Purity: >95% (HPLC analysis at 220 nm). Retention time: 19.45 min.

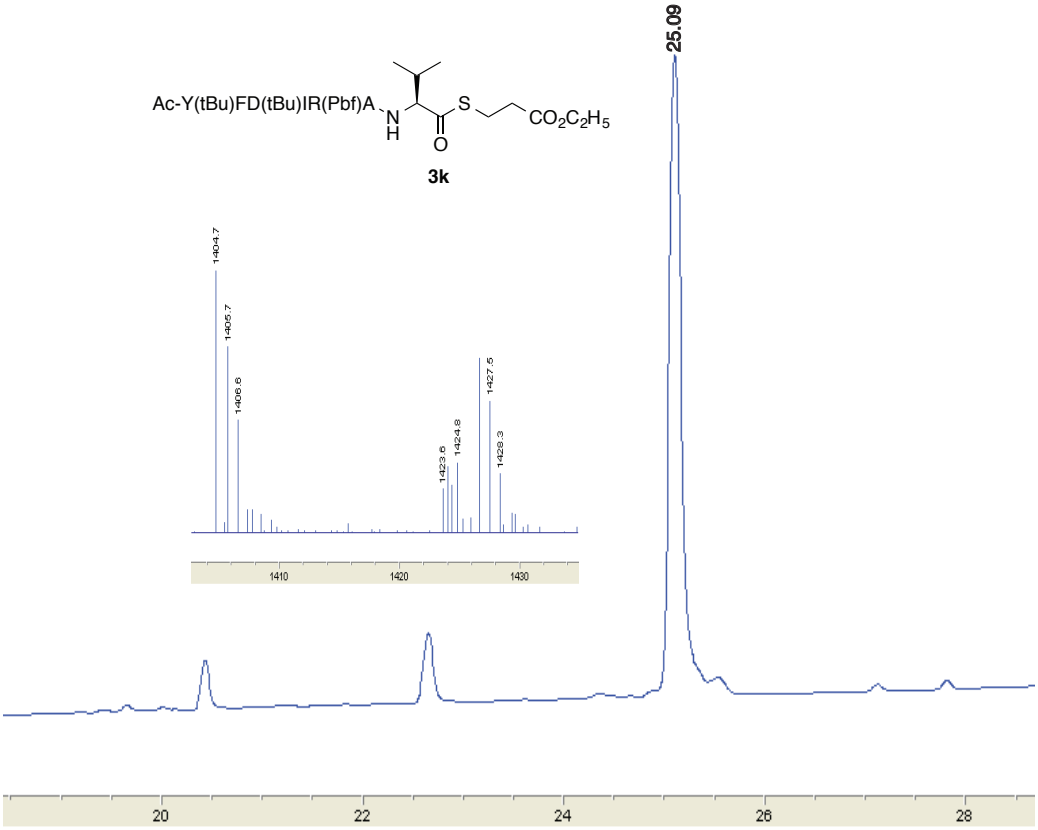


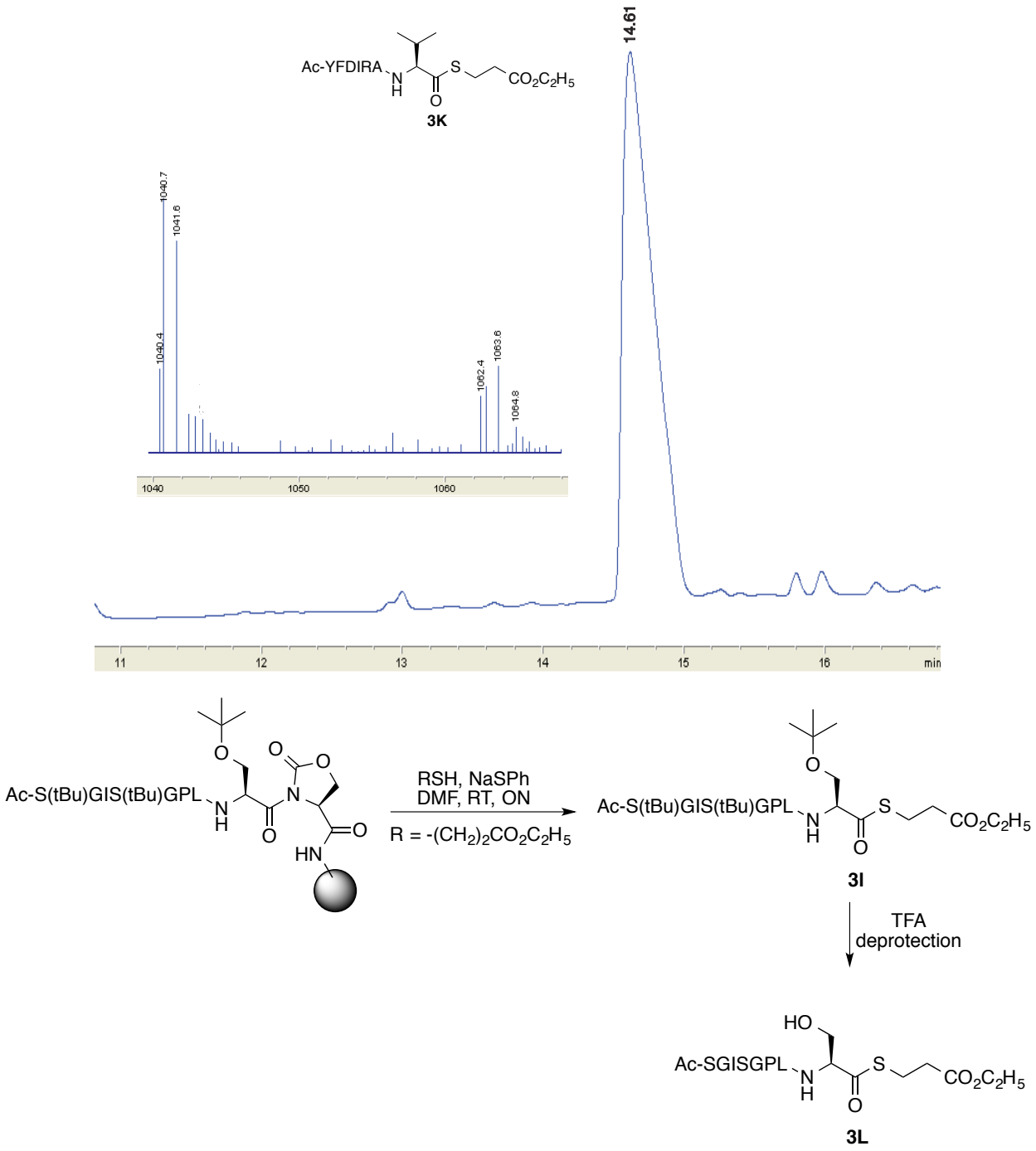
Ac-Gly-Val-Ala-Leu-Phe-COS(CH$_2$)$_2$CO$_2$C$_2$H$_5$ (3j). LCMS: *m/z* 664.3 (calcd [*M*+H]$^+$ = 664.6), *m/z* 686.3 (calcd [*M*+Na]$^+$ = 686.6). Purity: >95% (HPLC analysis at 220 nm). Retention time: 16.30 min.



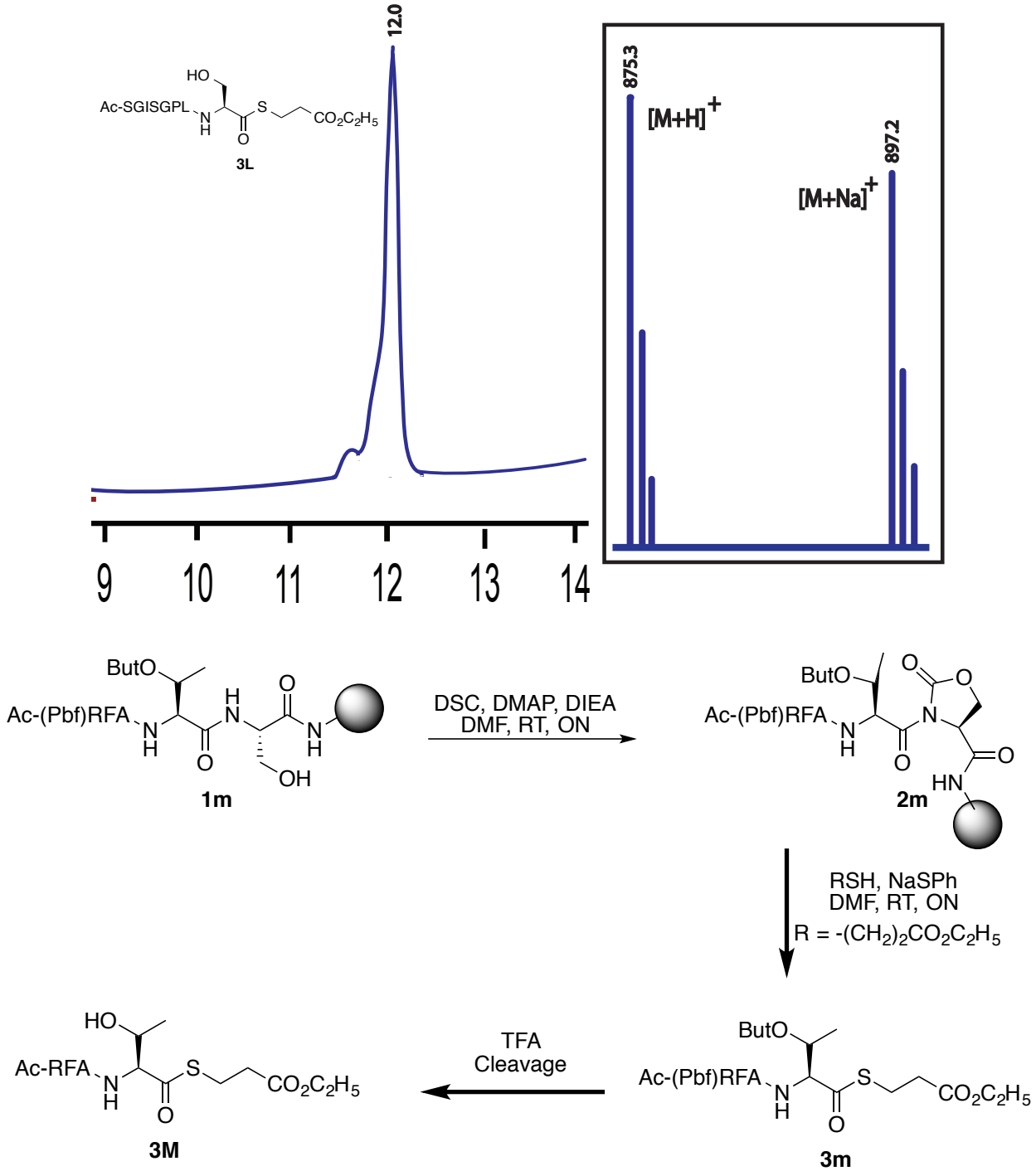
Ac-Tyr(tBu)-Phe-Asp(tBu)-Ile-Arg(Pbf)-Ala-Val-COS$(CH_2)_2CO_2C_2H_5$ (3k). LCMS: *m/z* 1404.7 (calcd $[M+H]^+$ = 1404.3), *m/z* 1426.5 (calcd $[M+Na]^+$ = 1426.3). Purity: >95% (HPLC analysis at 220 nm). Retention time: 25.09 min.

Ac-Tyr-Phe-Asp-Ile-Arg-Ala-Val-COS$(CH_2)_2CO_2C_2H_5$ (3K). LCMS: *m/z* 1040.7 (calcd $[M+H]^+$ = 1040.8), *m/z* 1063.6 (calcd $[M+Na]^+$ = 1063.8). Purity: >95% (HPLC analysis at 220 nm). Retention time: 14.61 min.

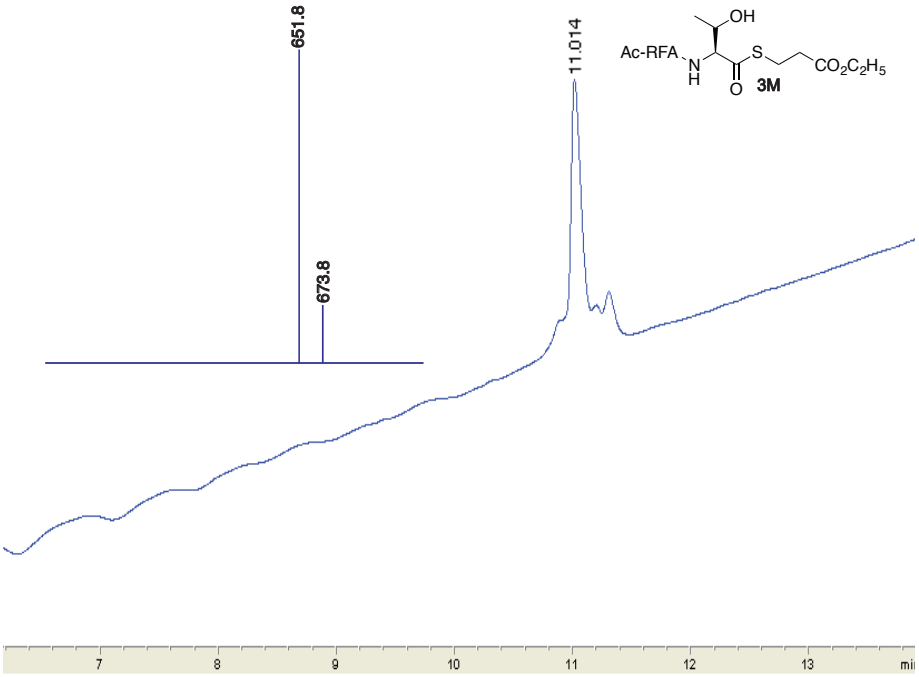


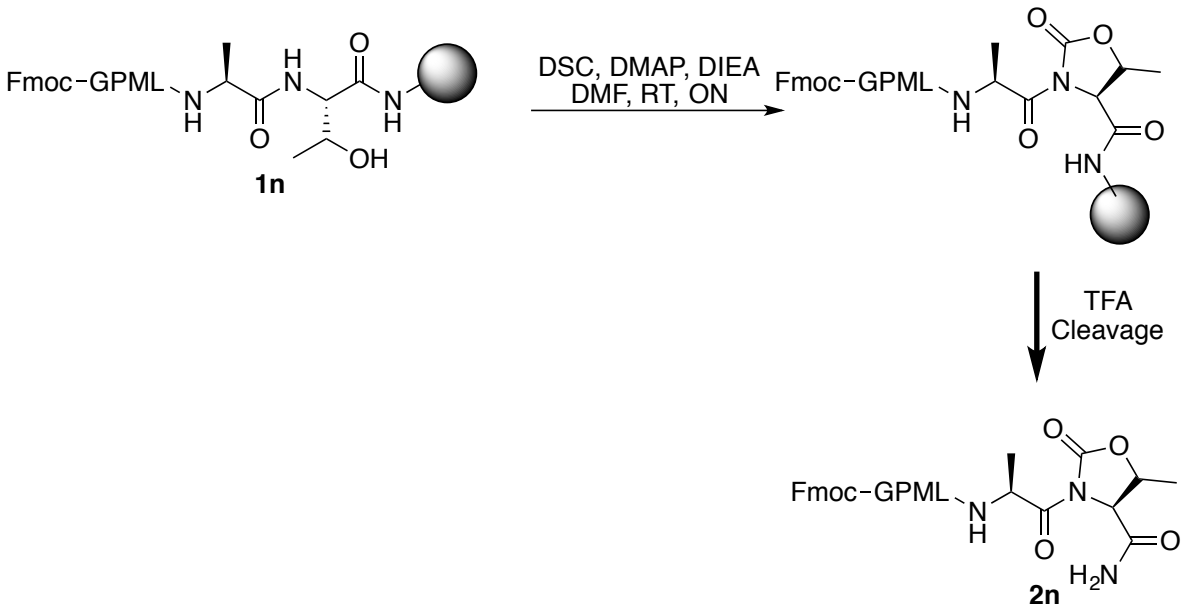


Ac-Ser-Gly-Ile-Ser-Gly-Pro-Leu-Ser-COS$(CH_2)_2CO_2C_2H_5$ (3L). LCMS: *m/z* 875.3 (calcd $[M+H]^+$ = 875.7), *m/z* 897.2 (calcd $[M+Na]^+$ = 897.7). Purity: >95% (HPLC analysis at 220 nm). Retention time: 12.0 min.

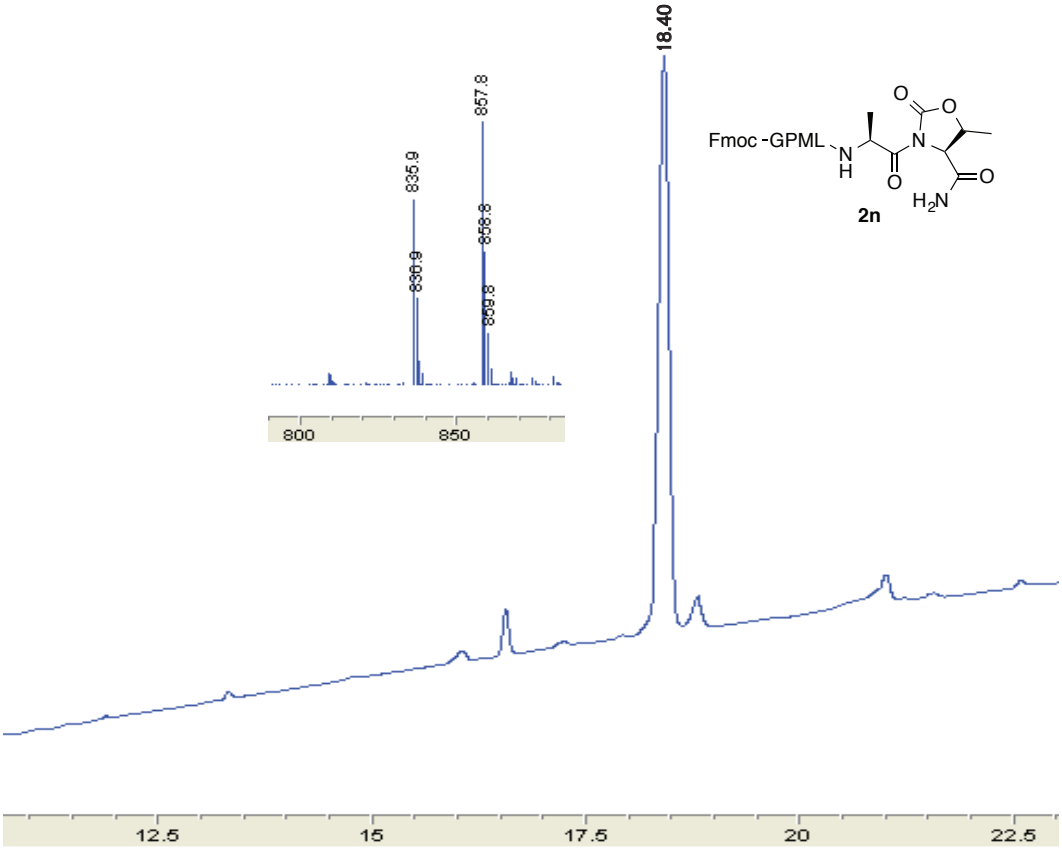


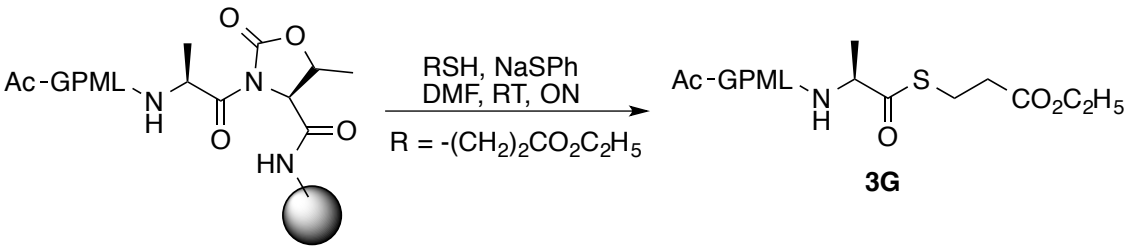
Ac-Arg-Phe-Ala-Thr-COS$(CH_2)_2CO_2C_2H_5$ (3M). LCMS: *m/z* 651.8 (calcd $[M+H]^+$ = 651.5), *m/z* 673.8 (calcd $[M+Na]^+$ = 673.5). Purity: >95% (HPLC analysis at 220 nm). Retention time: 11.01 min. Isolated yield = 50 %


651.8
673.8
11.014
OH
Ac-RFA
N
H
S
O
3M
$CO_2C_2H_5$
7
8
9
10
11
12
13
mi

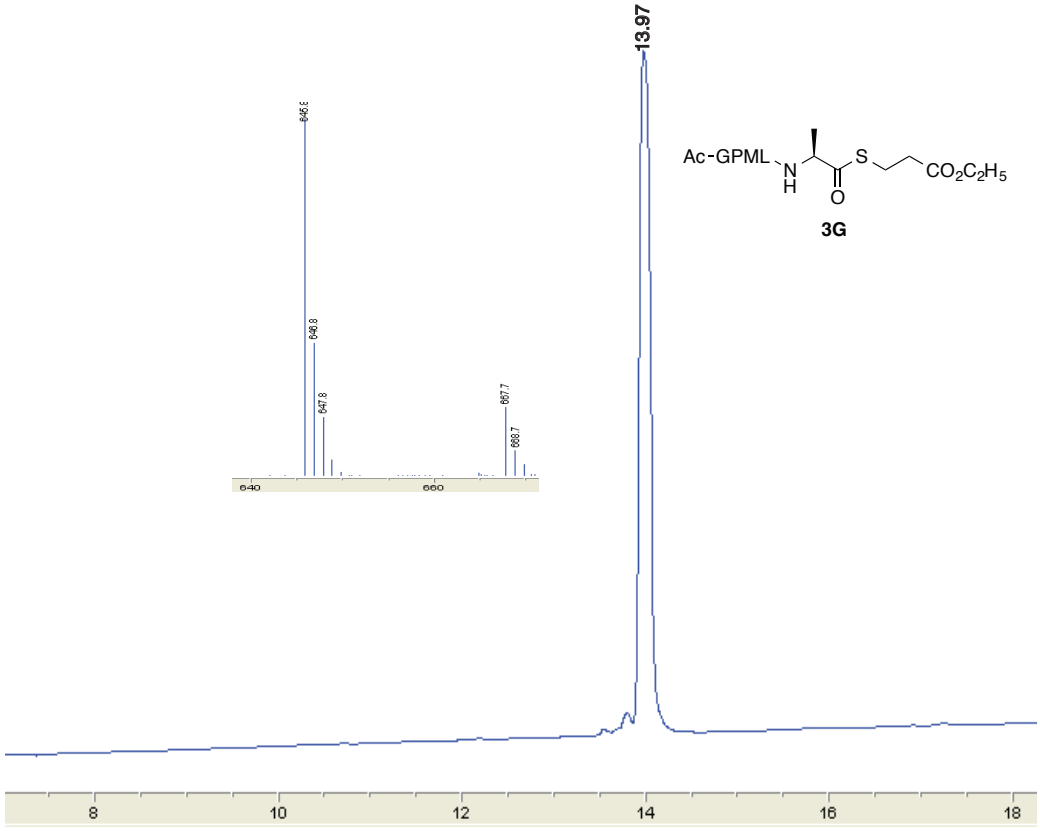


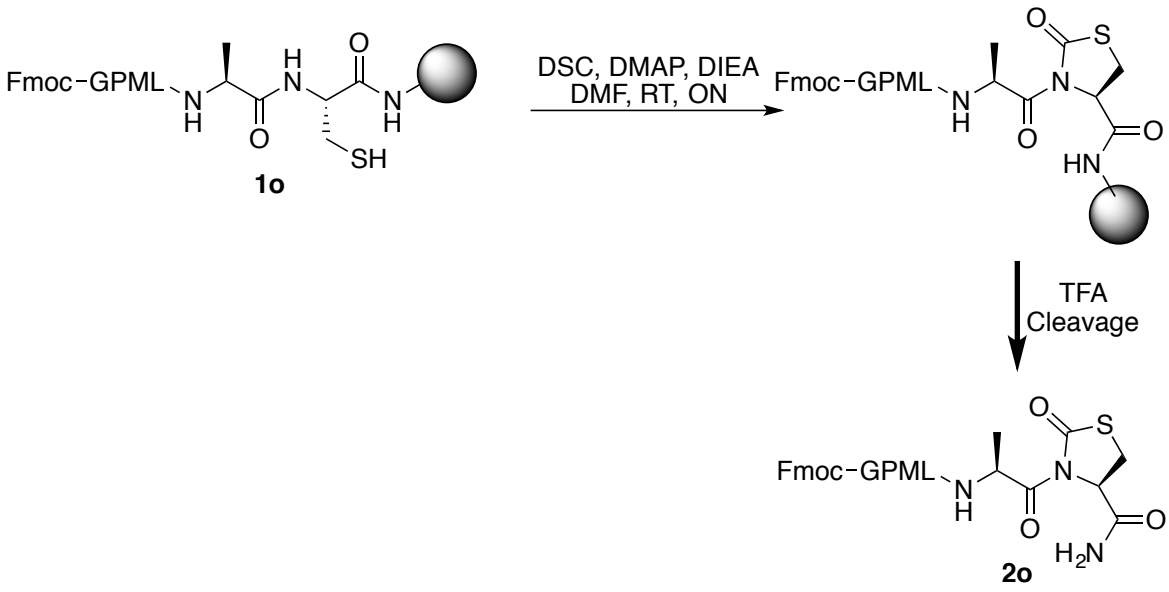
Fmoc-Gly-Pro-Met-Leu-Ala-Oxd^{Me} (2n). LCMS: *m/z* 835.9 (calcd $[M+H]^+$ = 835.7), *m/z* 857.8 (calcd $[M+Na]^+$ = 857.7). Purity: >95% (HPLC analysis at 220 nm). Retention time: 18.4 min.



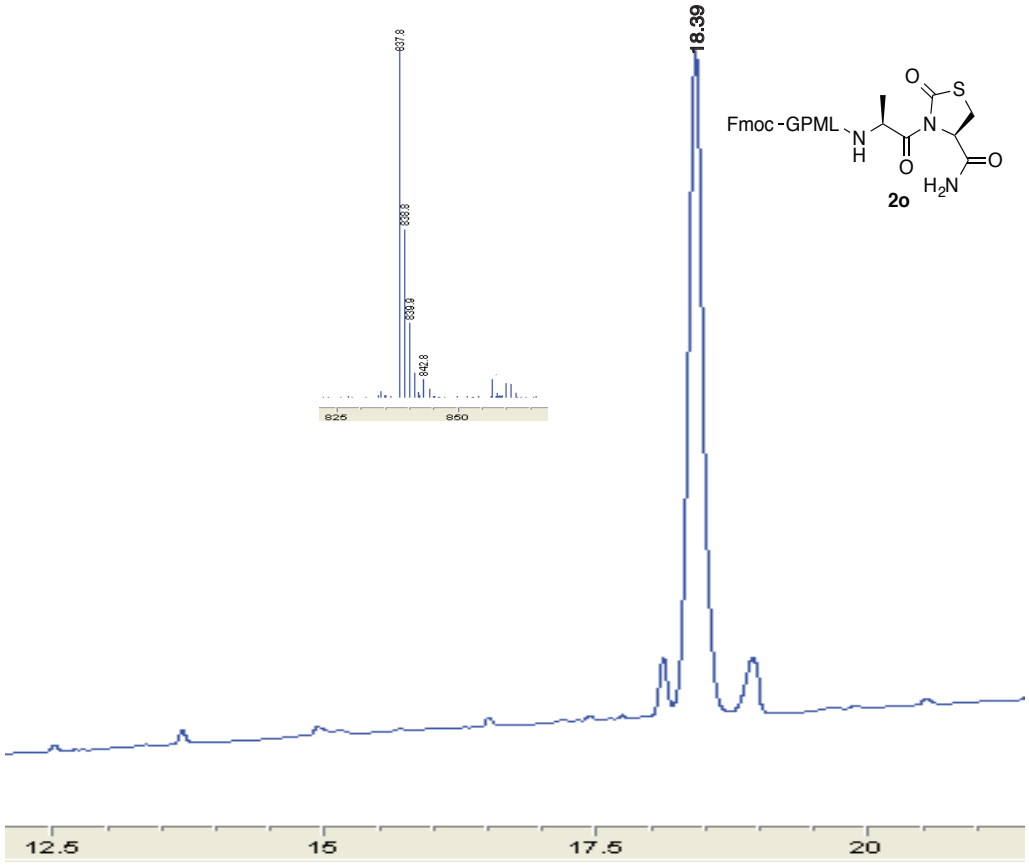


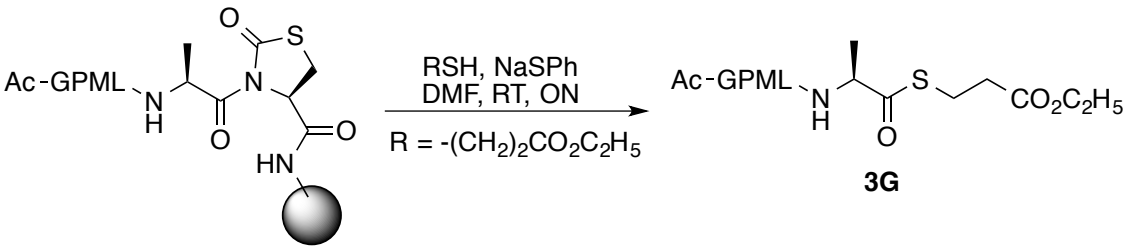
Ac-Gly-Pro-Met-Leu-Ala-COS$(CH_2)_2CO_2C_2H_5$ (3G). LCMS: *m/z* 645.8 (calcd $[M+H]^+$ = 645.5), *m/z* 667.7 (calcd $[M+Na]^+$ = 667.5). Purity: >95% (HPLC analysis at 220 nm). Retention time: 13.97 min.



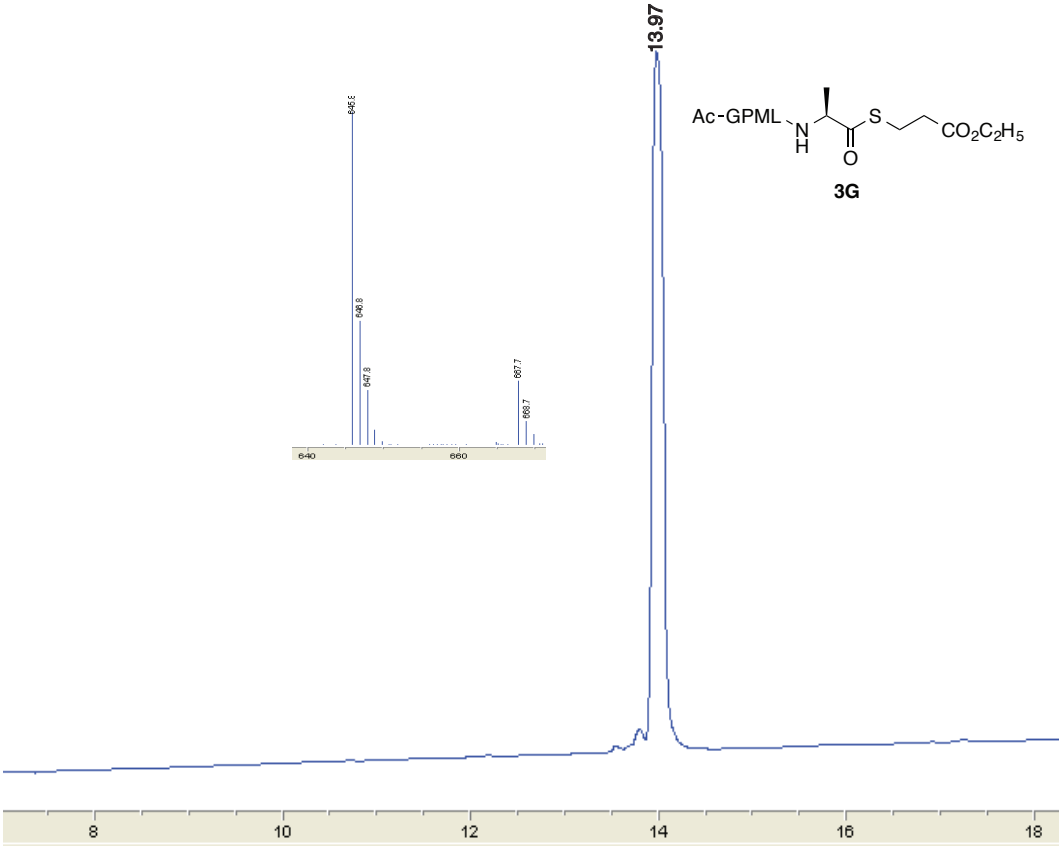


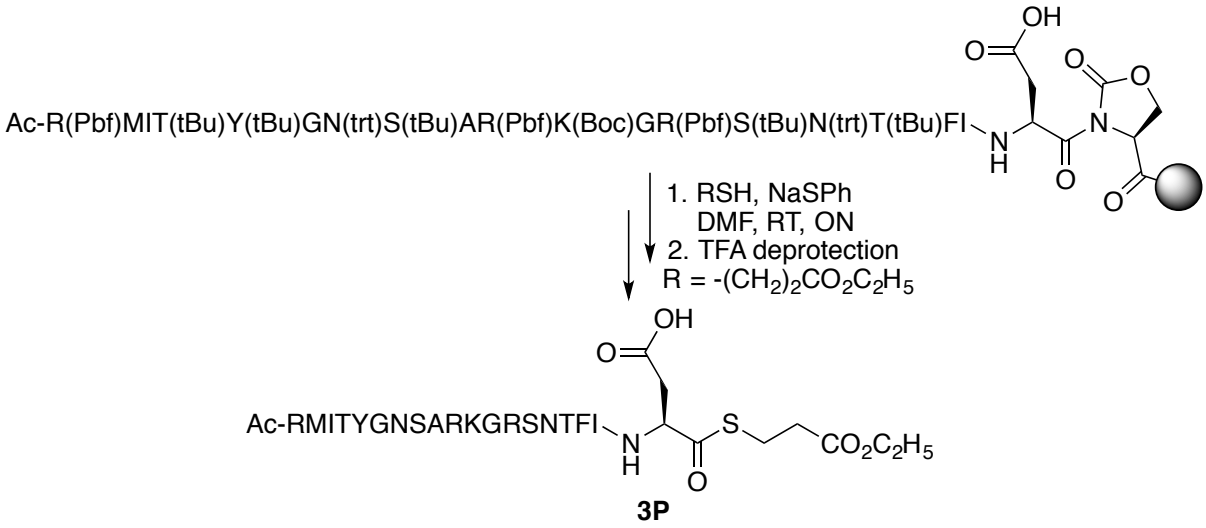
Fmoc-Gly-Pro-Met-Leu-Ala-Thz (2o). LCMS: *m/z* 837.8 (calcd $[M+H]^+$ = 837.7), *m/z* 859.8 (calcd $[M+Na]^+$ = 859.7). Purity: >95% (HPLC analysis at 220 nm). Retention time: 18.39 min.



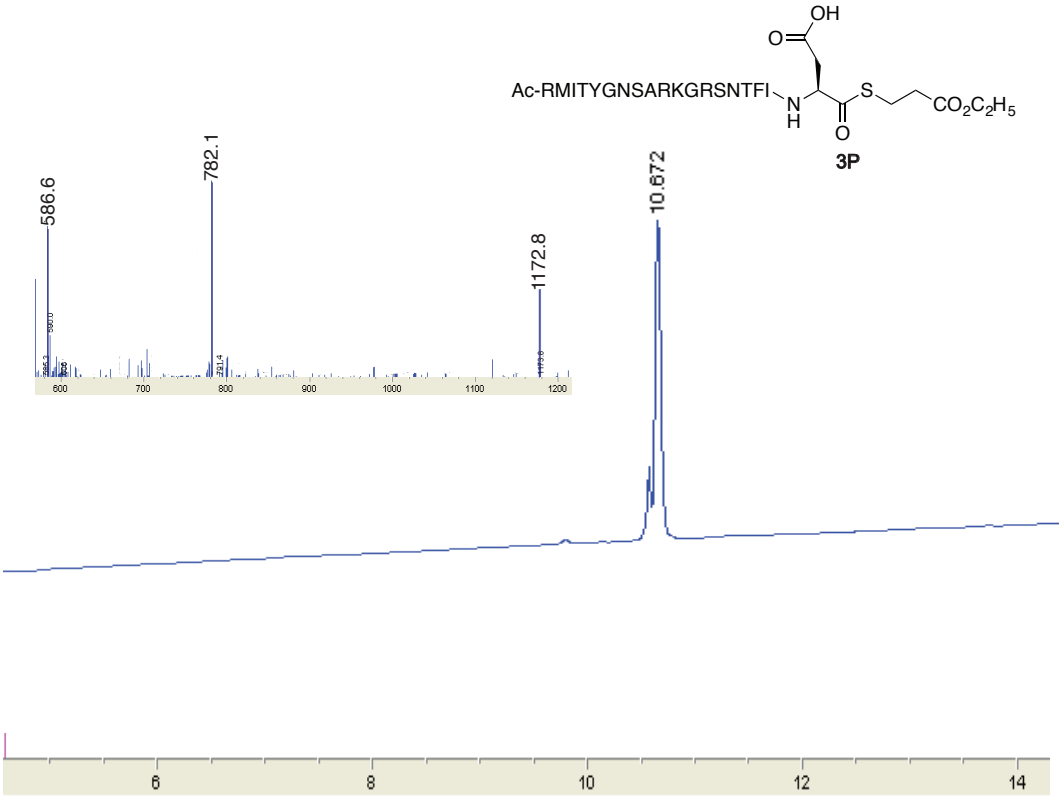


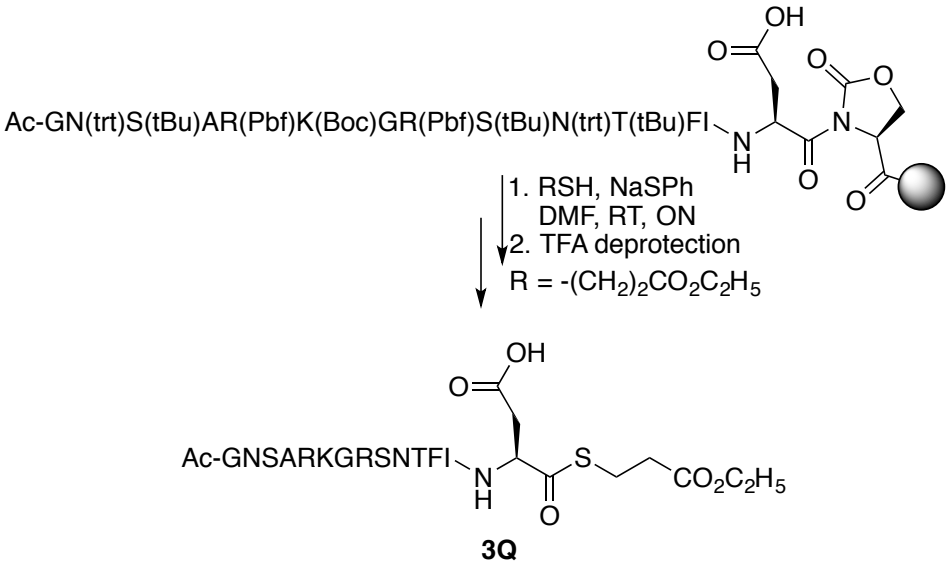
Ac-Gly-Pro-Met-Leu-Ala-COS$(CH_2)_2CO_2C_2H_5$ (3G). LCMS: *m/z* 645.8 (calcd $[M+H]^+$ = 645.5), *m/z* 667.7 (calcd $[M+Na]^+$ = 667.5). Purity: >95% (HPLC analysis at 220 nm). Retention time: 13.97 min.



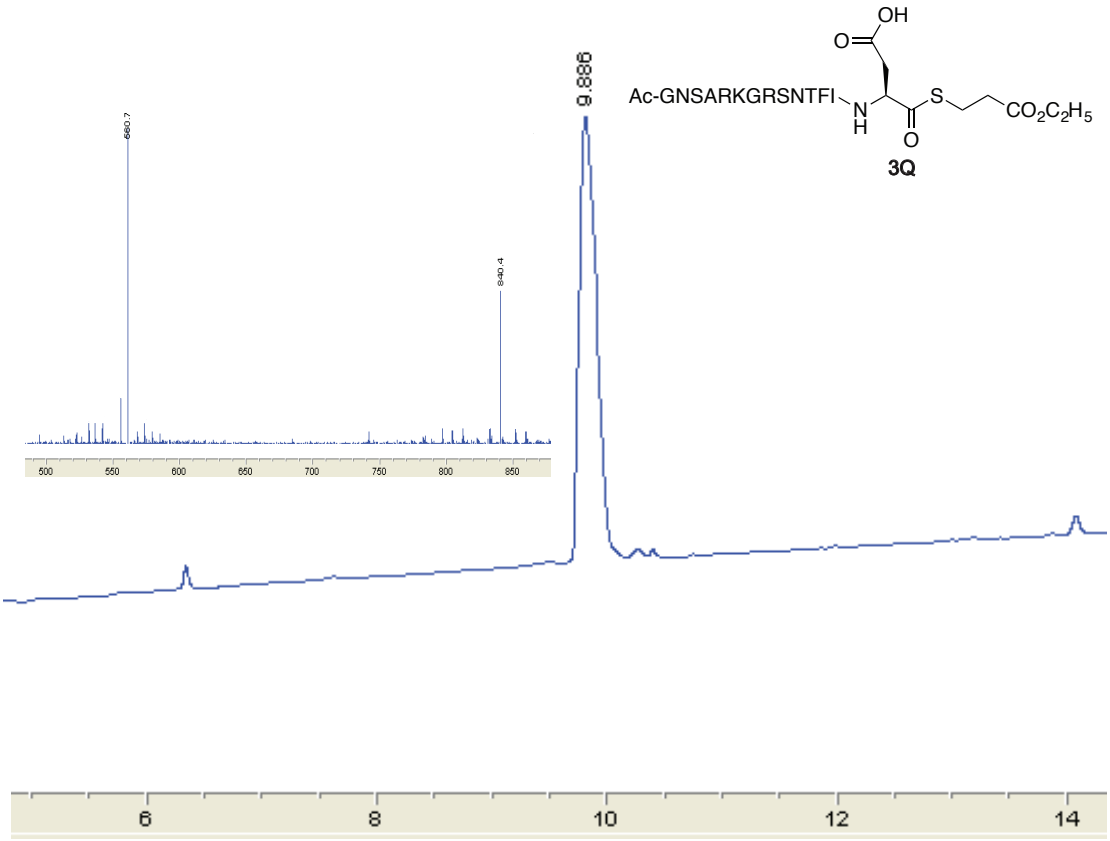


Ac-RMITYGNSARKGRSNTFID-COS$(CH_2)_2CO_2C_2H_5$ (3P). LCMS: *m/z* 1172.8 (calcd $[M+2H]^{2+}$ = 1172.5), *m/z* 782.1 (calcd $[M+3H]^{3+}$ = 782.0), *m/z* 586.6 (calcd $[M+4H]^{4+}$= 586.7). Purity: >95% (HPLC analysis at 220 nm). Retention time: 10.67 min. Isolated yield = 31%

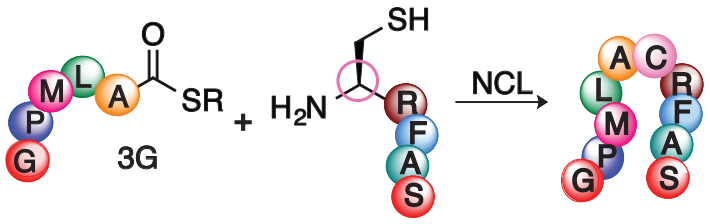




Ac-GNSARKGRSNTFID-COS$(CH_2)_2CO_2C_2H_5$ (3Q). LCMS: *m/z* 840.4 (calcd $[M+2H]^{2+}$ = 840.9), *m/z* 560.7 (calcd $[M+3H]^{3+}$ = 560.9). Purity: >95% (HPLC analysis at 220 nm). Retention time: 9.88 min. Isolated yield = 39 %

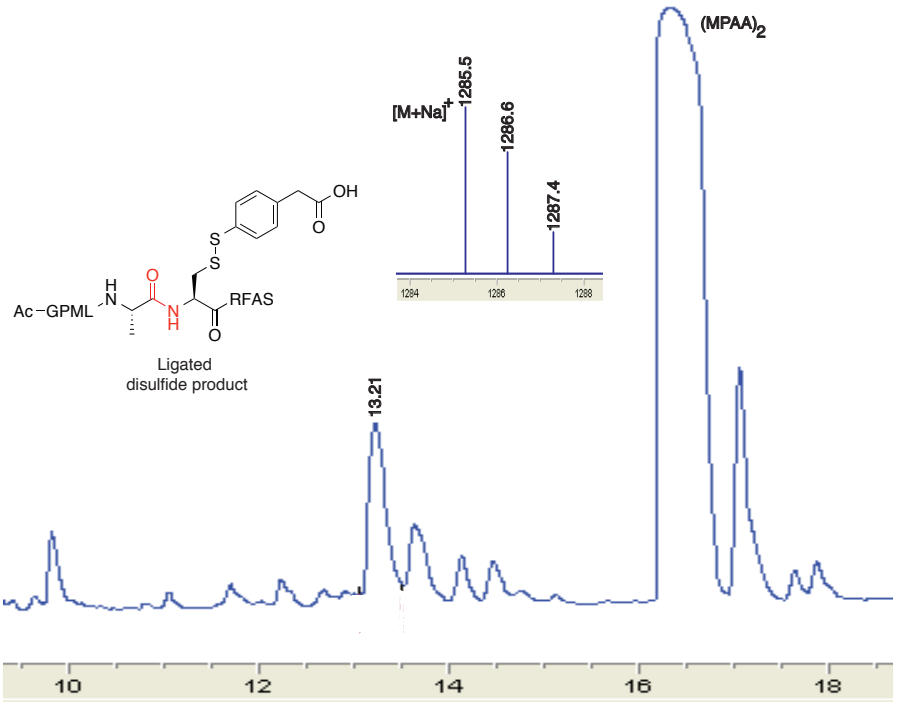


Native Chemical Ligation

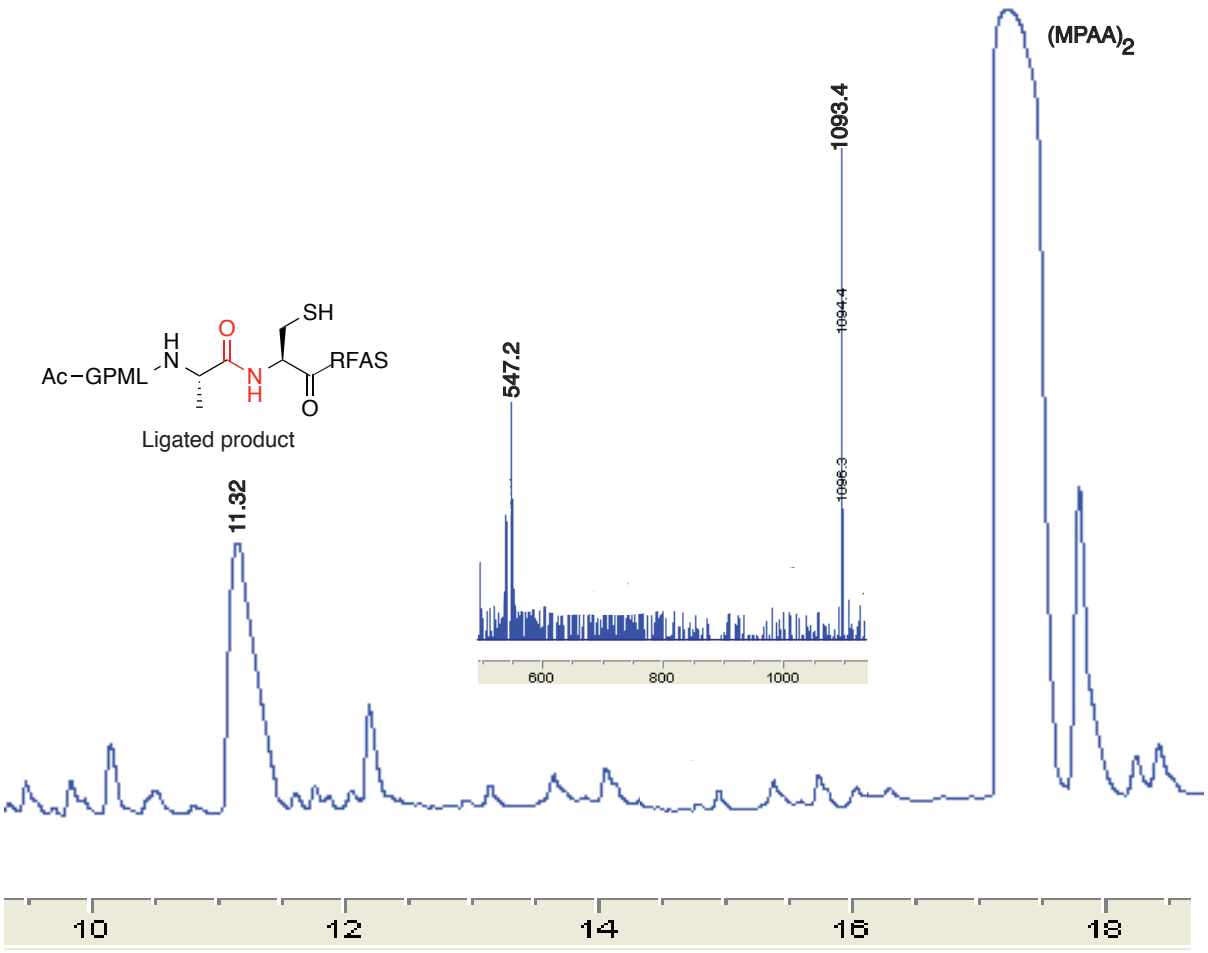


Ac-Gly-Pro-Met-Leu-Ala-Cys(MPAA)-Arg-Phe-Ala-Ser. LCMS: *m/z* 1285.5 (calcd $[M+Na]^+$ = 1285.8). Purity: >95% (HPLC analysis at 220 nm). Retention time: 13.2 min.

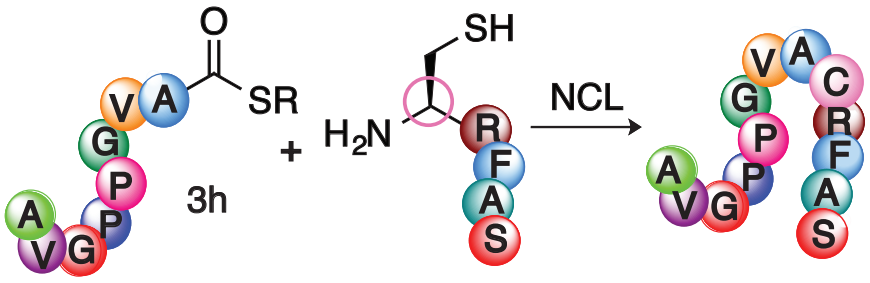
Ac-Gly-Pro-Met-Leu-Ala-Cys-Arg-Phe-Ala-Ser. LCMS: *m/z* 1093.4 (calcd $[M+H]^+$ = 1093.3), *m/z* 547.2 (calcd $[M+2]^{2+}$ = 547.6). Purity: >95% (HPLC analysis at 220 nm). Retention time: 11.32 min.



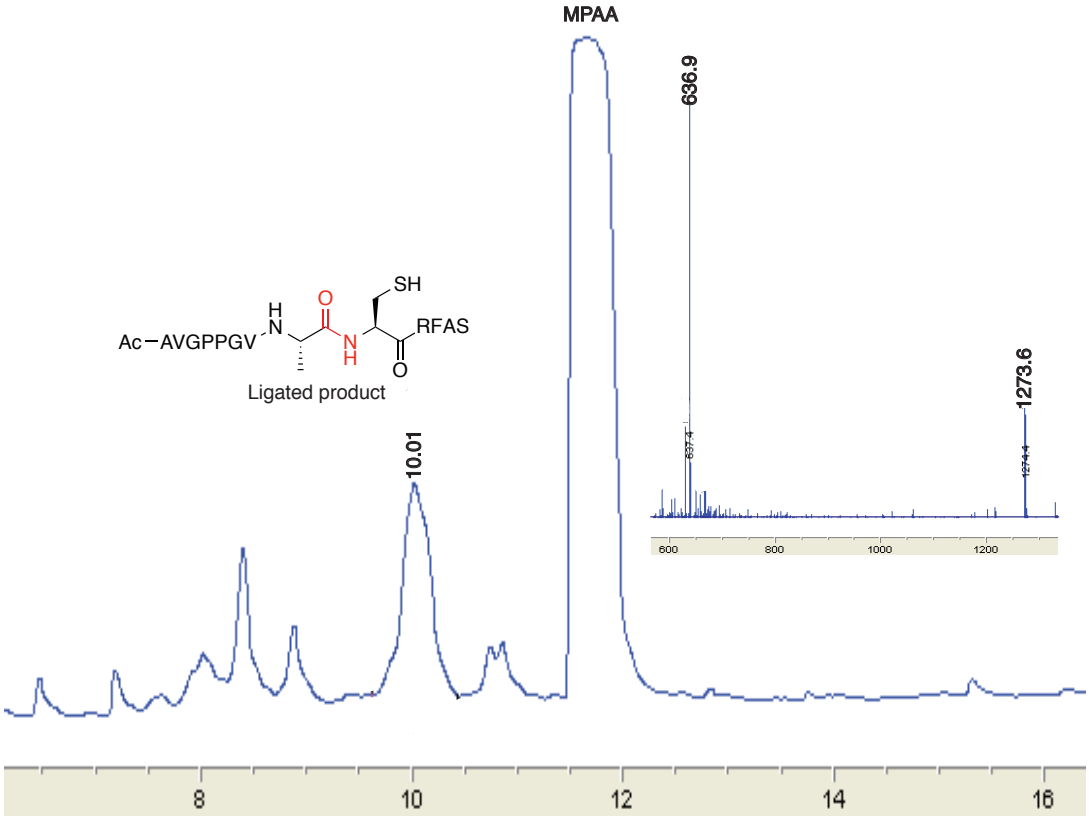
Native Chemical Ligation reaction before quenching it with TCEP.HCl



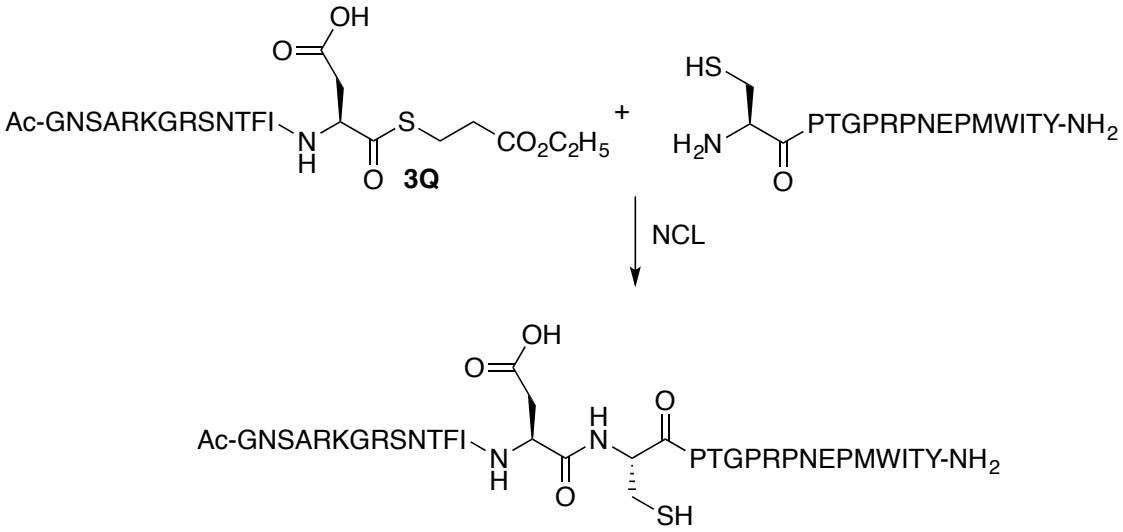
Native Chemical Ligation reaction after quenching it with TCEP.HCl



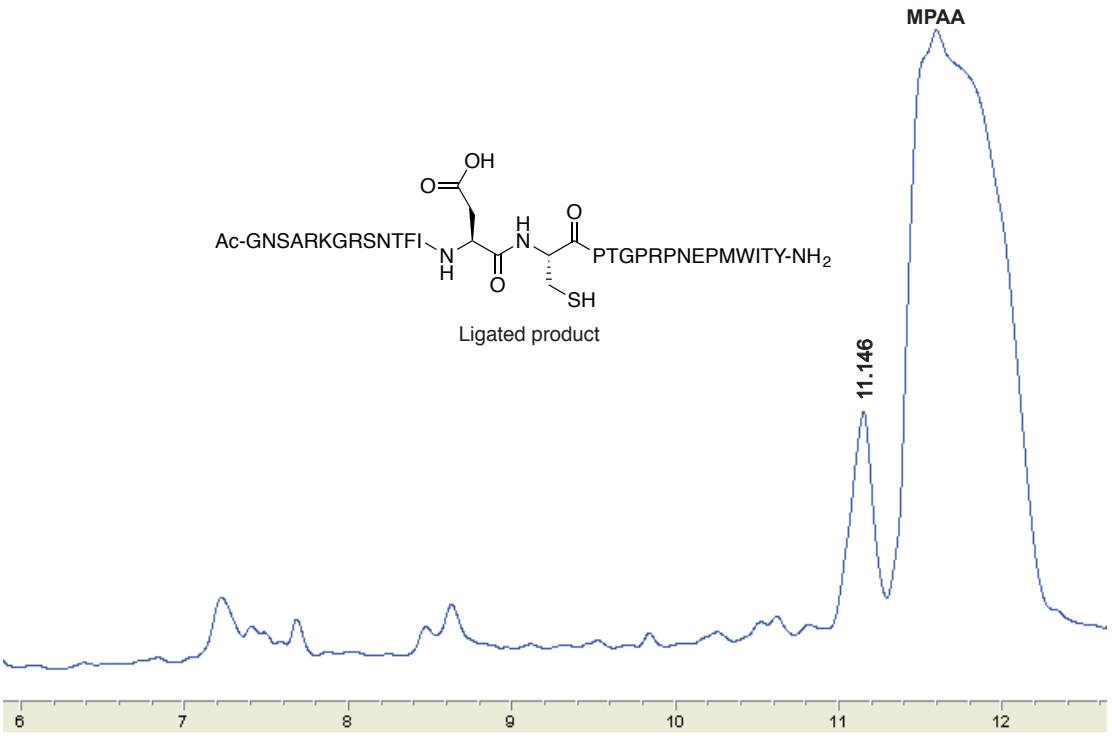
Ac-Ala-Val-Gly-Pro-Pro-Gly-Val-Ala-Cys-Arg-Phe-Ala-Ser. LCMS: *m/z* 1273.6 (calcd $[M+H]^+$ = 1273.4), *m/z* 636.9 (calcd $[M+2]^{2+}$ = 637.2). Purity: >95% (HPLC analysis at 220 nm). Retention time: 10.01 min.



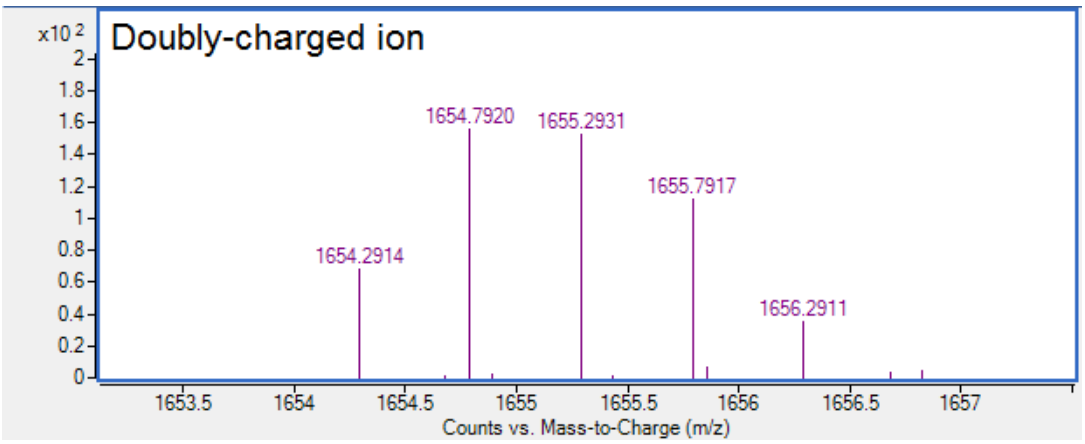
Native Chemical Ligation reaction after quenching it with TCEP.HCl

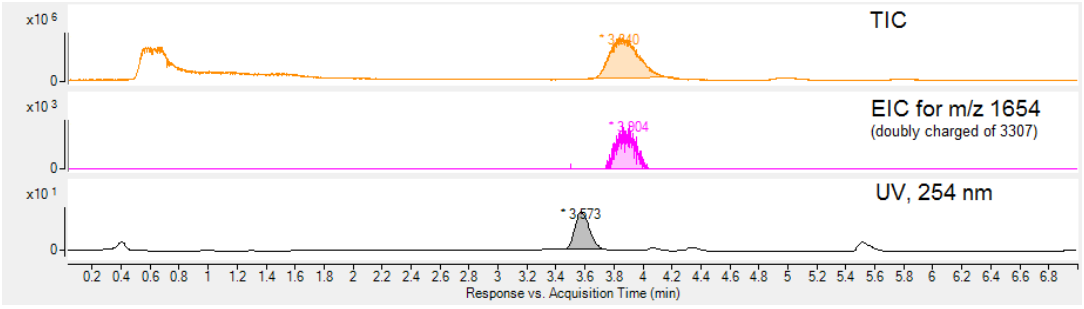


Ac-GNSARKGRSNTFIDCPTGPRPNEPMWITY-NH$_2$. LCMS: *m/z* 3308.6 (calcd $[M+H]^+$ = 3308.4), *m/z* 1654.8 (calcd $[M+2]^{2+}$ = 1654.7). Purity: >95% (HPLC analysis at 220 nm). Retention time: 11.14 min

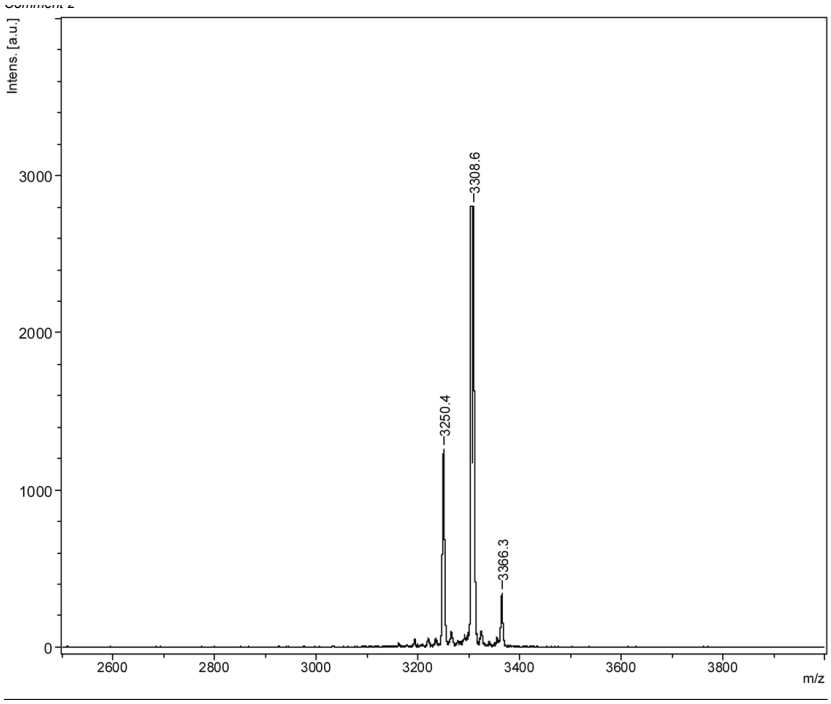


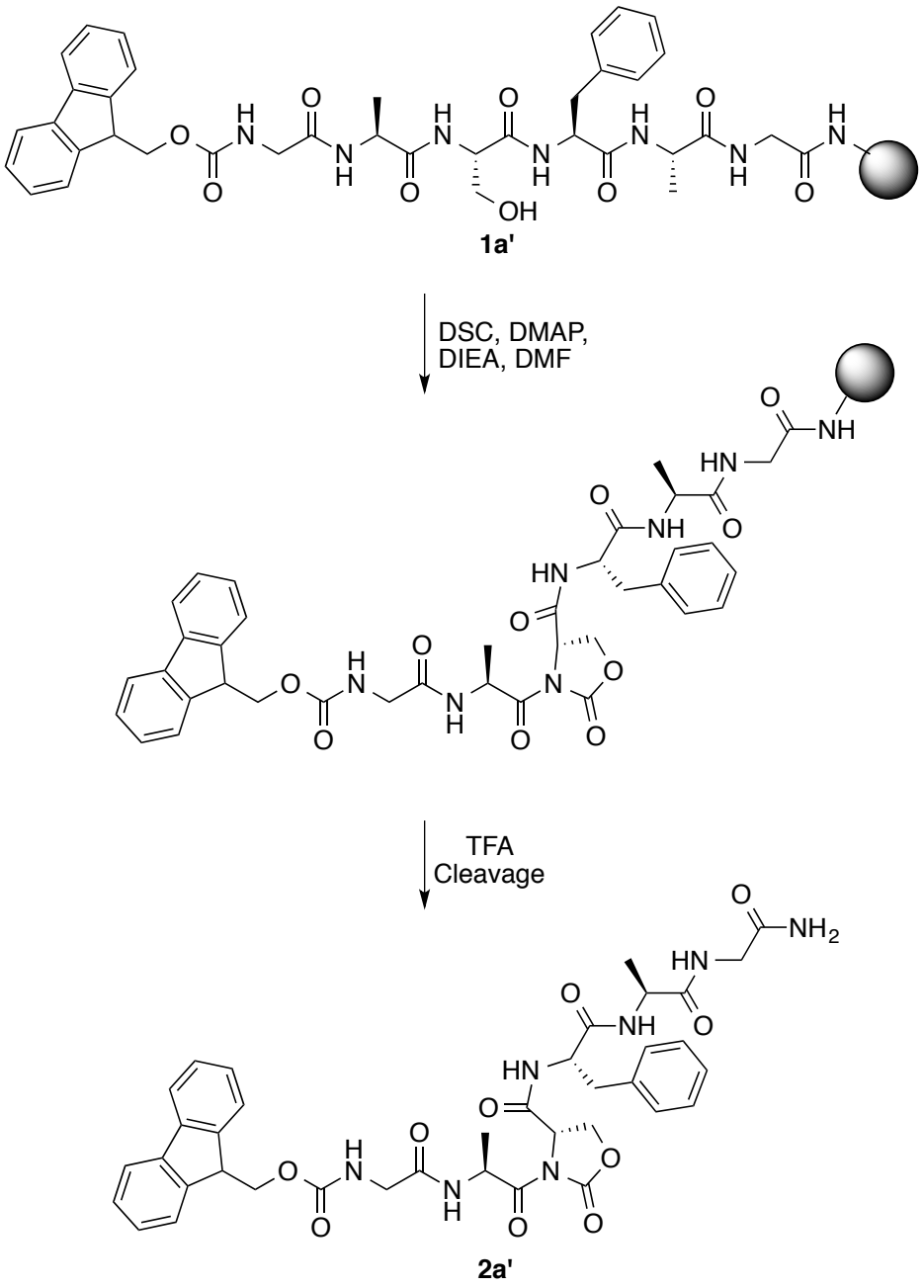
Native Chemical Ligation reaction after quenching it with TCEP.HCl





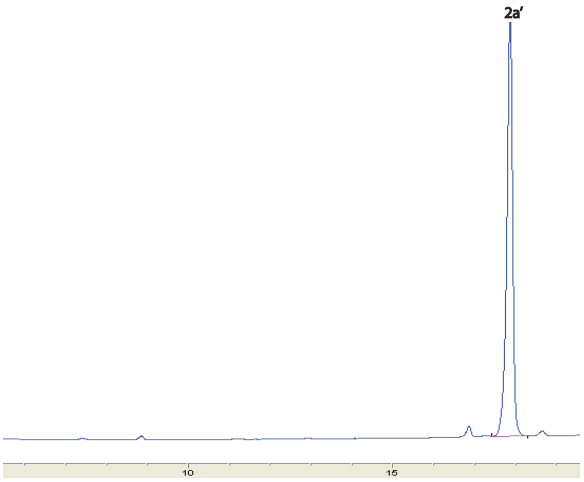
MALDI of ligated Product 29 amino acid long peptide *Ac-GNSARKGRSNTFIDCPTGPRPNEPMWITY-NH$_2$*

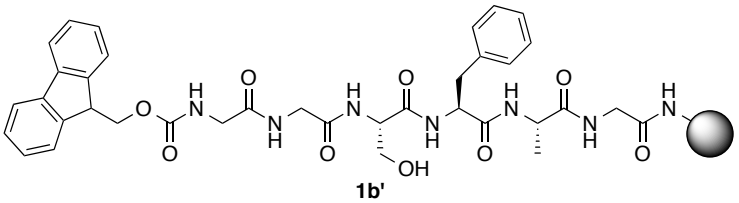




Fmoc-Gly-Ala-Oxd-Phe-Ala-Gly-NH_2 (2a'). LCMS: *m/z* 756.40 (calcd $[M+H]^+$ = 756.10), 778.5 (calcd $[M+Na]^+$ = 778.0). Purity: >95% (HPLC analysis at 254 nm). Retention time: 17.9 min.

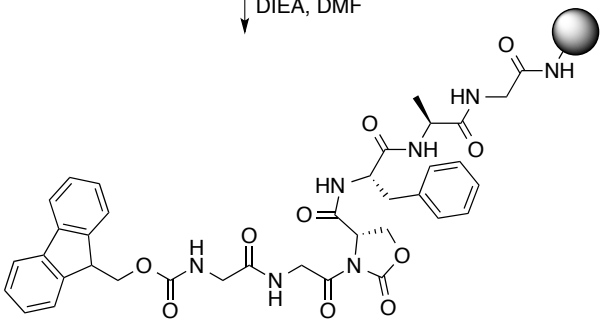
HPLC spectra of the cyclic urethane containing peptide **2a'**



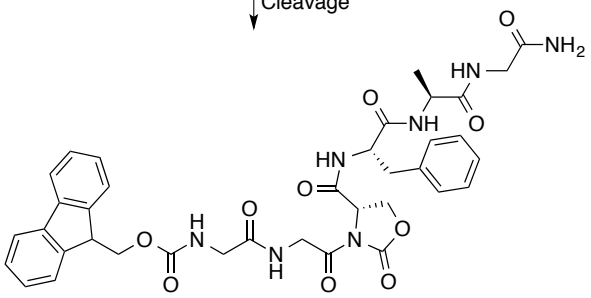


1b'

DSC, DMAP,
DIEA, DMF



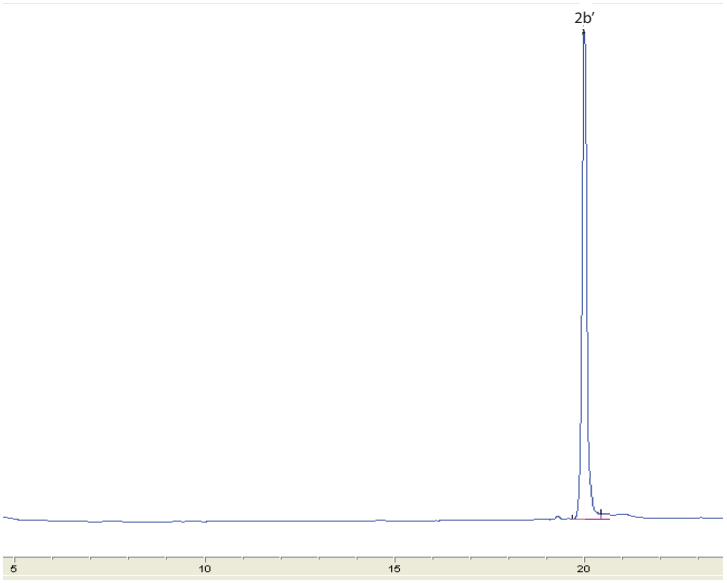
TFA
Cleavage

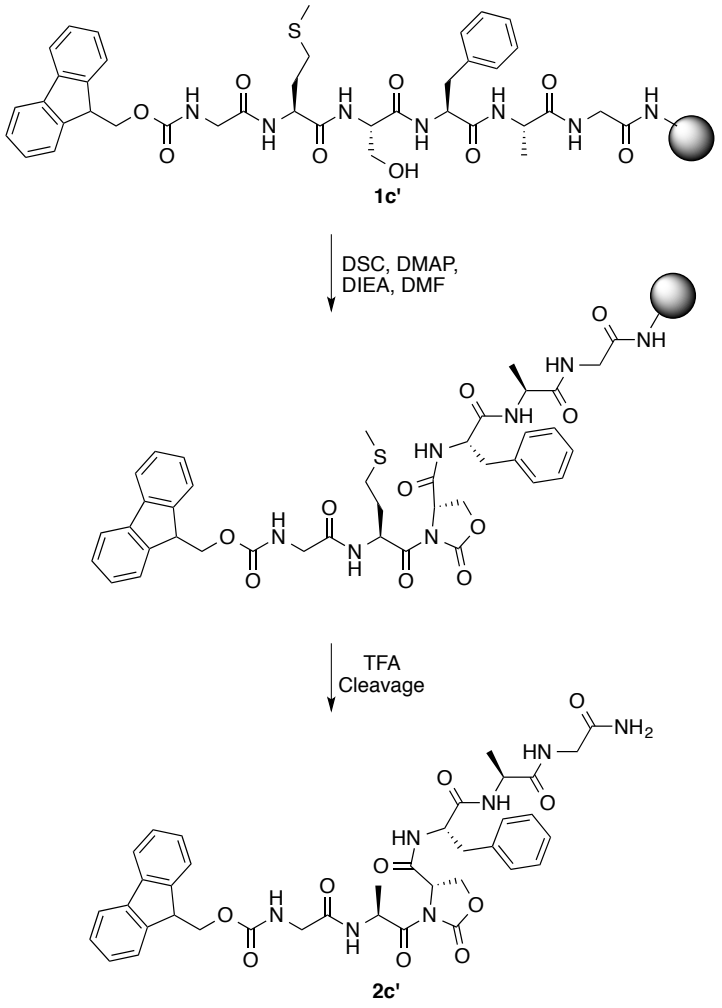


2b'

Fmoc-Gly-Gly-Oxd-Phe-Ala-Gly-NH_2 (2b'). LCMS: *m/z* 742.40 (calcd $[M+H]^+$ = 742.50), 371.9 (calcd $[(M+2]^{2+}$ = 371.75). Purity: >95% (HPLC analysis at 254 nm). Retention time: 20.1 min.

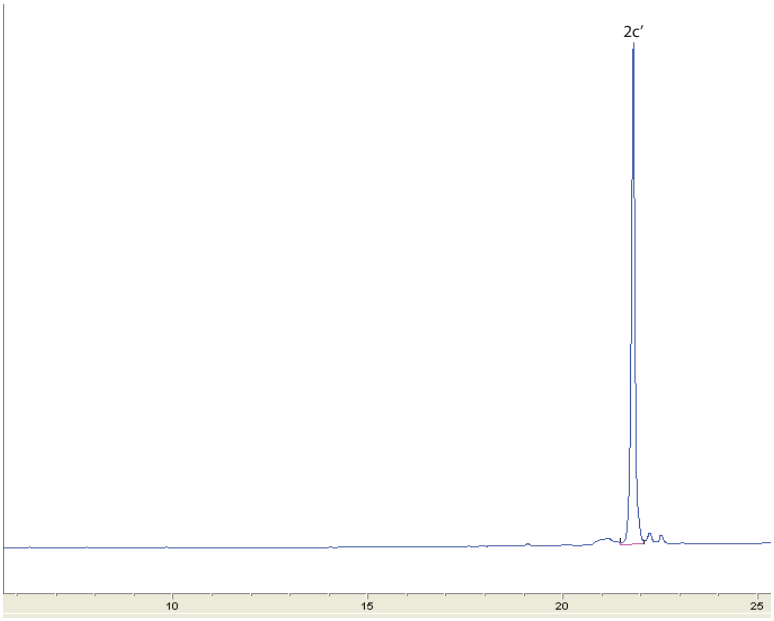
HPLC spectra of the cyclic urethane containing peptide **2b'**

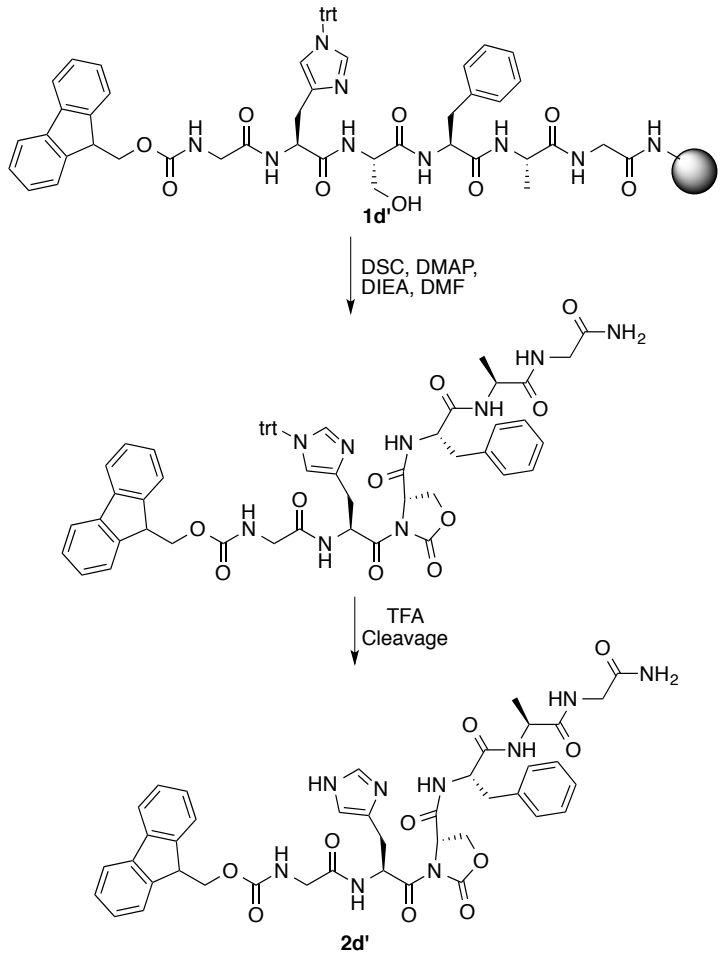




Fmoc-Gly-Met-Oxd-Phe-Ala-Gly-NH_2 (2c'). LCMS: *m/z* 816.0 (calcd $[M+H]^+$ = 816.20), 408.2 (calcd $(M+2)^{2+}$ = 408.8). Purity: >95% (HPLC analysis at 254 nm). Retention time: 21.8 min.

HPLC spectra of the cyclic urethane containing peptide **2c'**

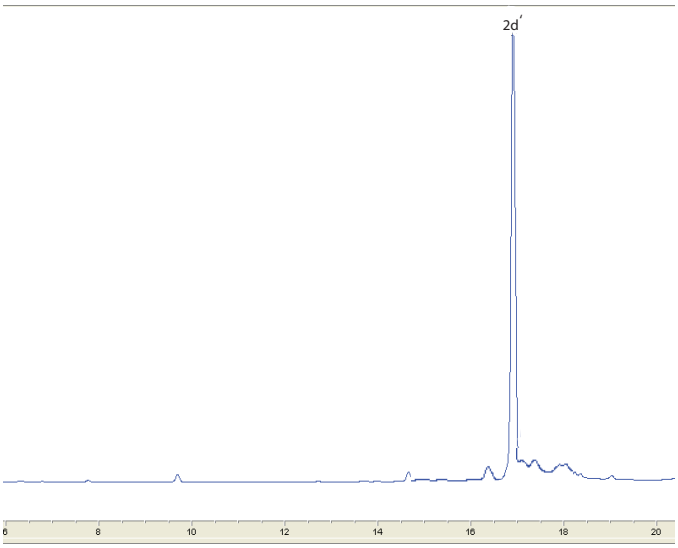


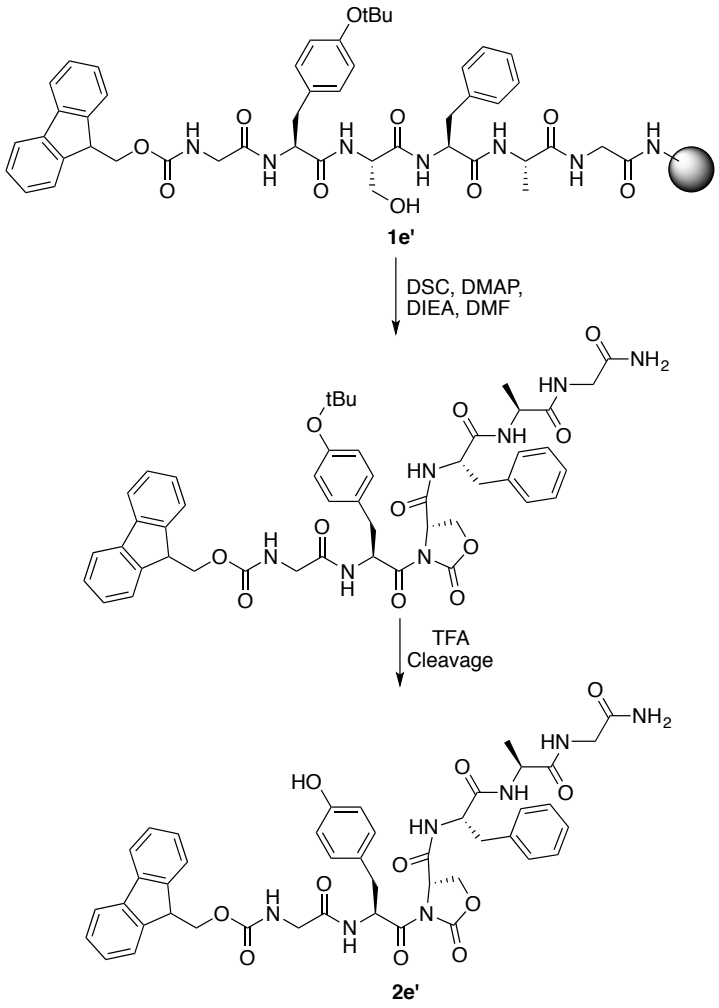


Fmoc-Gly-His-Oxd-Phe-Ala-Gly-NH$_2$ (2d'). LCMS: *m*/*z* 822.10 (calcd [*M*+H]$^+$ = 822.60),

411.2 (calcd $(M+2)^{2+}$ = 411.79). Purity: >95% (HPLC analysis at 254 nm). Retention time: 16.9 min.

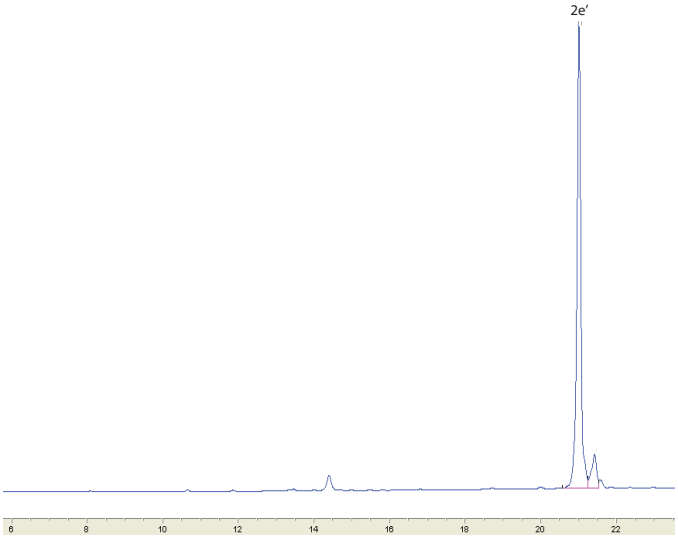
HPLC spectra of the cyclic urethane containing peptide **2d'**

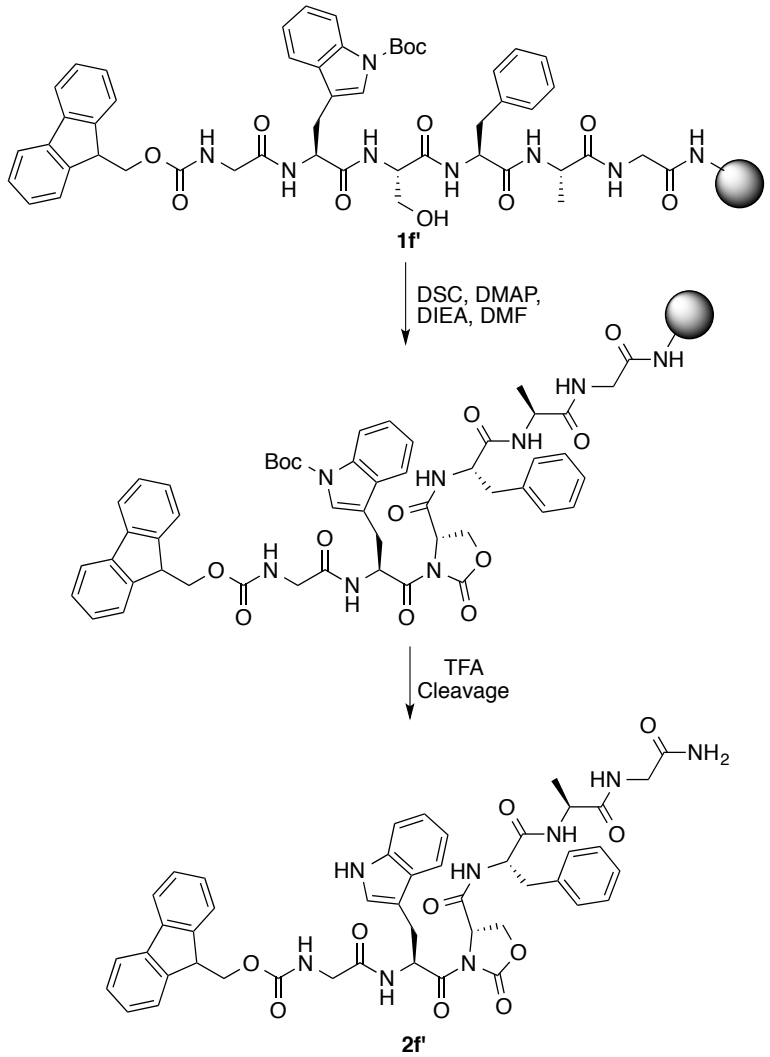




Fmoc-Gly-Tyr-Oxd-Phe-Ala-Gly-NH_2 (2e'). LCMS: *m/z* 848.20 (calcd $[M+H]^+$ = 848.62), 424.1 (calcd $[(M+2)^{2+}$ = 424.81). Purity: >95% (HPLC analysis at 254 nm). Retention time: 21.1 min.

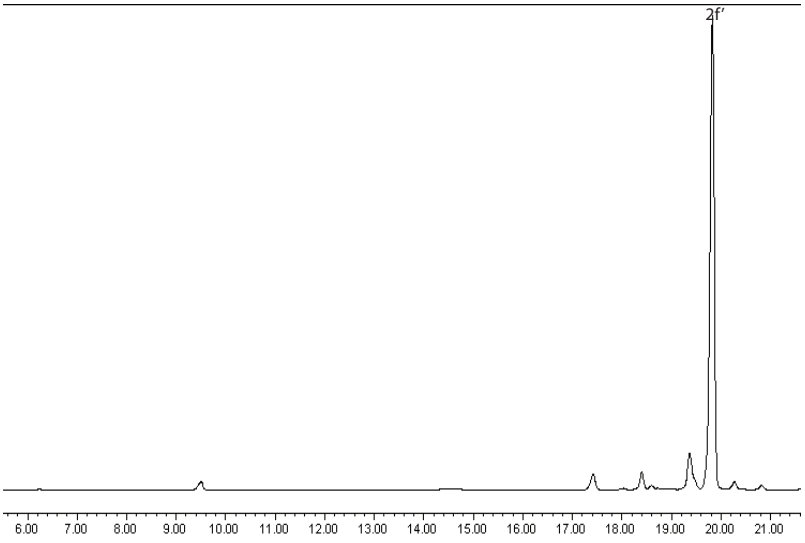
HPLC spectra of the cyclic urethane containing peptide **2e'**

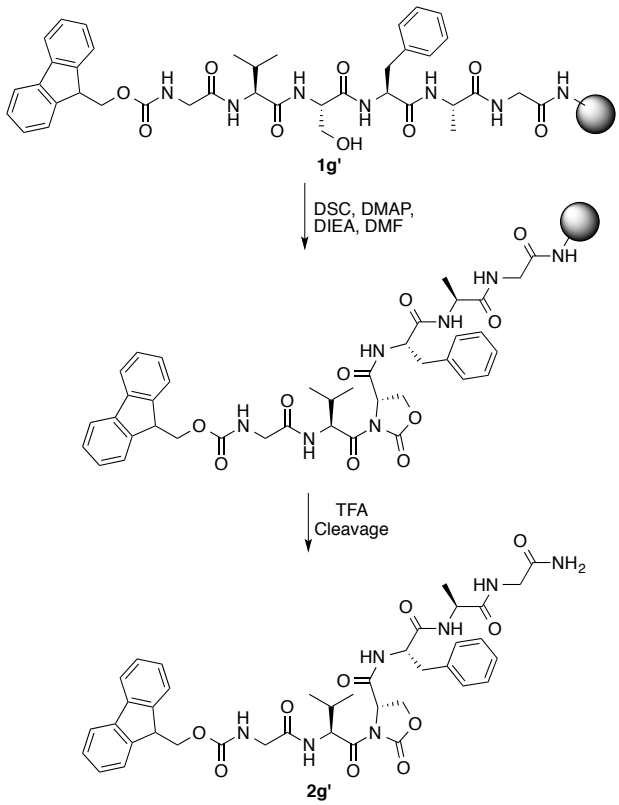

2e'
6
8
10
12
14
16
18
20
22


Boc
OH
1f'
DSC, DMAP,
DIEA, DMF
Boc
TFA
Cleavage
NH2
2f'

Fmoc-Gly-Trp-Oxd-Phe-Ala-Gly-NH_2 (2f'). LCMS: *m/z* 871.20 (calcd $[M+H]^+$ = 871.6), 436.0 (calcd $(M+2)^{2+}$ = 436.33). Purity: >95% (HPLC analysis at 254 nm). Retention time: 19.8 min.

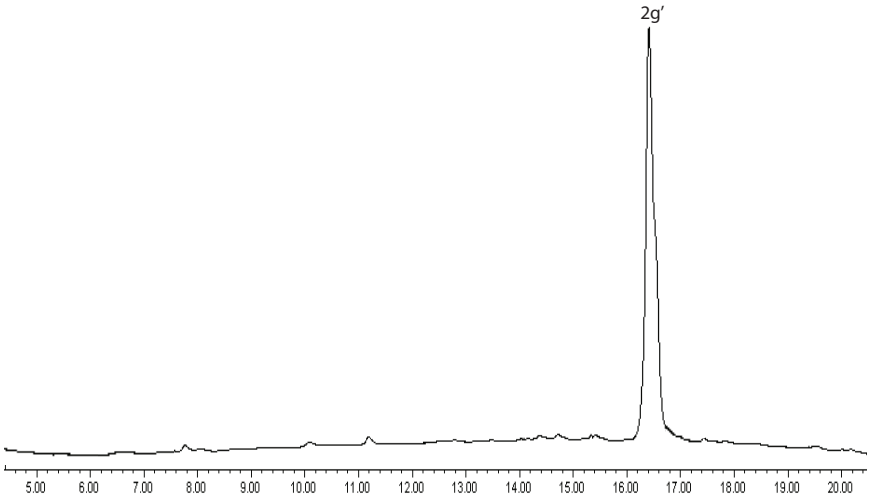
HPLC spectra of the cyclic urethane containing peptide **2f'**





Fmoc-Gly-Val-Oxd-Phe-Ala-Gly-NH_2 (2g'). LCMS: *m/z* 784.60 (calcd $[M+H]^+$ = 784.58), 392.5 (calcd $(M+2)^{2+}$ = 392.79). Purity: >95% (HPLC analysis at 254 nm). Retention time: 16.6 min.

HPLC spectra of the cyclic urethane containing peptide **2g'**



References:

(1) Chan, W. C.; White, P. D. *Fmoc solid phase peptide synthesis : a practical approach*; Oxford University Press: New York, 2000.

(2) H. E. Elashal and M. Raj, *Chem. Commun.*, 2016, **52**, 6304-6307.