Electronic Supplementary Material (ESI) for Chemical Communications.

SUPPORTING INFORMATION

Bioinspired peptide stapling generates stable enzyme inhibitors

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List of abbreviations

ACN	Acetonitrile
APT	Attached proton test
COSY	Correlation spectroscopy
Dab	L-2,4-diaminobutyric acid
DCC	*N*,*N*′-dicyclohexylcarbodiimide
DCM	Dichloromethane
DIPEA	*N*,*N*-Diisopropylethylamine
DMF	Dimethylformamide
EDT	1,2-Ethanedithiol
EIC	Extracted-ion chromatogram
HBTU	3-[Bis(dimethylamino)methyliumyl]-3*H*-benzotriazol-1-oxide hexafluorophosphate
HEPES	2-[4-(2-Hydroxyethyl)piperazin-1-yl]ethane-1-sulfonic acid
HMBC	Heteronuclear multiple bond correlation
HOBt	*N*-Hydroxybenzotriazole
HPLC	High-performance liquid chromatography
HRMS	High-resolution mass spectrometry
HSQC	Heteronuclear single quantum coherence
LC-MS	Liquid chromatography-mass spectrometry
LRMS	Low-resolution mass spectrometry
MeOH	Methanol
TCEP	Tris(2-carboxyethyl)phosphine
TFA	Trifluoroacetic acid
TIPS	Triisopropylsilane

Instrumentation and materials used for chemical synthesis

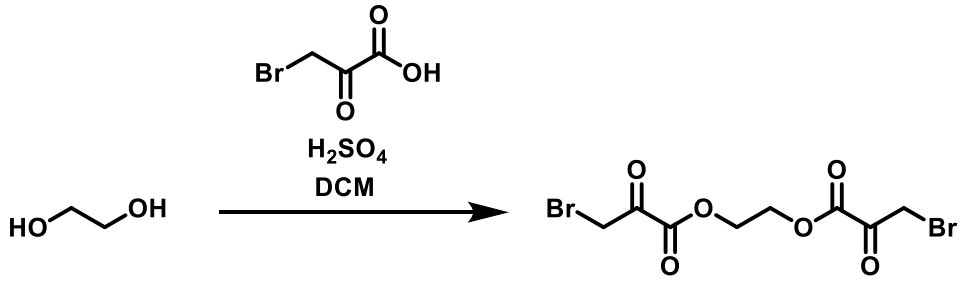
Small molecule NMR spectra were recorded on a Bruker Avance III 400 MHz equipped with a 5 mm Bruker probe head (PA BBO 400S1 BBF-H-D-05 Z SP). NMR spectra of compound **5** were recorded on a Bruker Avance III 800 MHz equipped with a 5 mm Bruker probe head (CP TCI 800S6 H-C/N-D-05 Z). All measurements were performed at 25 °C. Spectra were processed using MestReNova. Chemical shifts for all NMR spectra are reported in parts per million (ppm) and were referenced by their residual solvent peaks. Coupling constants (*J*) are recorded in Hz and significant multiplicities are reported as singlet (s), doublet (d), triplet (t), multiplet (m), doublet of doublets (dd), doublet of triplets (dt), triplet of doublets (td), apparent singlet (app s), and apparent doublet (app d). Deuterated solvents were purchased from Cambridge Isotope Laboratories (USA). Low-resolution electrospray ionisation mass spectrometry analysis was performed on a Waters LCT Premier orthogonal acceleration time-of-flight mass spectrometer. High-resolution electrospray ionisation mass analysis was performed on a Thermo Scientific Orbitrap Elite mass spectrometer. Low-resolution electron ionisation mass spectrometry analysis was performed on an Agilent 6890/5973 operating at 70 eV. High-resolution electron ionisation mass spectrometry analysis was performed on a Waters AutoSpec Primer operating at 70 eV. Liquid chromatography-mass spectrometry analysis was performed on an Agilent HPLC/MS (1260/6120) equipped with a reverse-phase column (Poroshell 120 EC-C18 3.0 x 50 mm, 2.7 μm) held at 30 °C. A flow rate of 0.3 ml/min was utilised and elution was monitored by UV absorbance (254 and 214 nm). Acquisition and analysis were performed using LC/MSD Chemstation. Presentation of chromatograms was done using OriginPro. Analytical thin-layer chromatography analysis was performed on pre-coated silica gel aluminium-backed plates (Merck silica gel 60 F_{254}), using visualisation under UV light (254 nm). Synthesised compounds were purified using a Biotage Isolera One automated flash chromatography system equipped with Biotage SNAP Ultra silica gel cartridges. Elution was monitored by UV absorbance (254 and 280 nm). Peptides were purified using preparative HPLC using a Waters 600 controller equipped with a reverse-phase column (SymmetryPrep C18, 100 Å, 7 μm, 19 x 150 mm), autosampler (717 plus), diode array detector (2996), and a Waters Fraction Collector III. A flow rate of 10 ml/min was utilised and elution was monitored by UV absorbance (254 and 200 nm). Acquisition and analysis were performed using Waters Empower 2 software. Fmoc solid-phase peptide synthesis was performed manually using filtered polypropylene syringes purchased from Torviq (USA). Rink amide resin (capacity 0.67 mmol/g) was purchased from Auspep (Australia). Fmoc-protected amino

acids were purchased from GL Biochem (China) and included Fmoc-Arg(Pbf)-OH, Fmoc-Asn(Trt)-OH, Fmoc-Asp(OtBu)-OH, Fmoc-Cys(Trt)-OH, Fmoc-Gln(Trt)-OH, Fmoc-Gly-OH, Fmoc-Ile-OH, Fmoc-Leu-OH, Fmoc-Lys(Boc)-OH, Fmoc-Pro-OH, Fmoc-Ser(tBu)-OH, and Fmoc-Tyr(tBu)-OH. All other reagents were purchased from either AK Scientific (USA) or Sigma Aldrich (USA) and were used without further purification.

Analytical LC-MS method

Solvent A: 5% ACN:water (20 mM ammonium acetate pH 5); Solvent B: 90% ACN:water (20 mM ammonium acetate pH 5); Gradient: 0% B for 1 min, 0 – 100% B over 10 min, 100% B for 3 min; Flow rate: 0.3 ml/min.

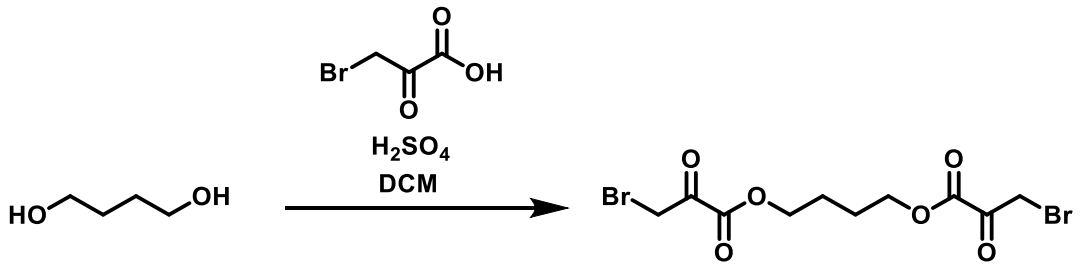
Synthesis of ethane-1,2-diyl bis(3-bromo-2-oxopropanoate) (a)



Scheme S1. Procedure for the synthesis of **a**.

A 100 ml round bottom flask was charged with ethylene glycol (45 µl, 0.80 mmol), bromopyruvic acid (540 mg, 3.2 mmol), and H_2SO_4 (10 µl) in DCM (50 ml). The mixture was refluxed under nitrogen for 2 days before being washed with saturated $NaHCO_3$, water (x3), brine, dried over $MgSO_4$, and filtered. The solvent was removed under reduced pressure and the residue was recrystallised (DCM:*n*-hexane) to yield the title compound (24.3 mg, 8%) as white crystals. **^{1}H NMR** (400 MHz, CD_3CN) δ = 4.54 (s, 4H), 4.53 (s, 4H) ppm; **^{13}C NMR** (100 MHz, CD_3CN) δ = 185.4, 159.1, 64.7, 34.3 ppm; **LRMS** (ESI^-) *m/z*: 356.8 ([M($^{79}Br_2$)-H]$^-$, 1), 358.8 ([M($^{81}Br^{79}Br$)-H]$^-$, 2), 360.9 ([M($^{81}Br_2$)-H]$^-$, 1); **HRMS** (ESI^-) *m/z*: [M-H]$^-$ Calcd for $C_8H_8{}^{79}Br_2O_6$ 356.8609, Found 356.8617; Calcd for $C_8H_8{}^{81}Br^{79}BrO_6$ 358.8589, Found 358.8578; Calcd for $C_8H_8{}^{81}Br_2O_6$ 360.8568, Found 360.8560; **TLC** R_f: 0.58 (5% MeOH:DCM).

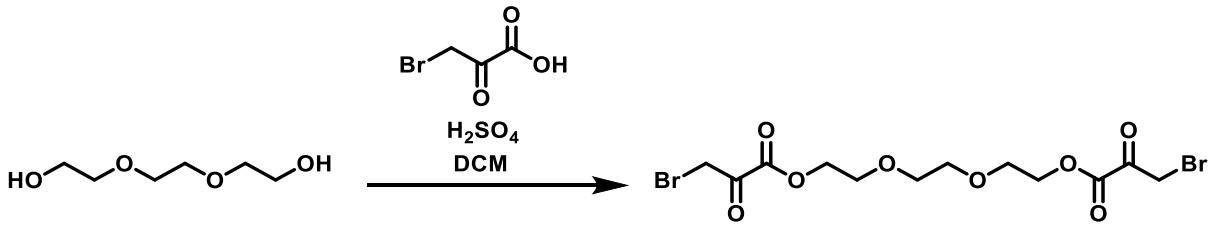
Synthesis of butane-1,4-diyl bis(3-bromo-2-oxopropanoate) (b)



Scheme S2. Procedure for the synthesis of **b**.

A 50 ml round bottom flask was charged with 1,4-butanediol (25 µl, 0.28 mmol), bromopyruvic acid (190 mg, 1.1 mmol), and H_2SO_4 (10 µl) in DCM (25 ml). The resulting mixture was stirred under nitrogen for 24 h before being purified using a 10 g silica gel cartridge (0 – 5% MeOH:DCM). The fractions containing the product were washed with water (x3), brine, dried over $MgSO_4$, and filtered. The solvent was removed under reduced pressure to yield the title compound (51.0 mg, 46%) as a yellow oil. **^{1}H NMR** (400 MHz, $CDCl_3$) δ = 4.40 – 4.33 (m, 4H), 4.31 (s, 4H), 1.93 – 1.85 (m, 4H) ppm; **^{13}C NMR** (100 MHz, $CDCl_3$) δ = 184.5, 159.4, 66.3, 30.7, 25.0 ppm; **LRMS** (ESI^-) *m/z*: 384.8 ([M($^{79}Br_2$)-H]$^-$, 1), 386.8 ([M($^{81}Br^{79}Br$)-H]$^-$, 2), 388.7 ([M($^{81}Br_2$)-H]$^-$, 1); **HRMS** (ESI^-) *m/z*: [M-H]$^-$ Calcd for $C_{10}H_{12}{}^{79}Br_2O_6$ 384.8922, Found 384.8920; Calcd for $C_{10}H_{12}{}^{81}Br^{79}BrO_6$ 386.8902, Found 386.8898; Calcd for $C_{10}H_{12}{}^{81}Br_2O_6$ 388.8881, Found 388.8878; **TLC** R_f: 0.45 (5% MeOH:DCM).

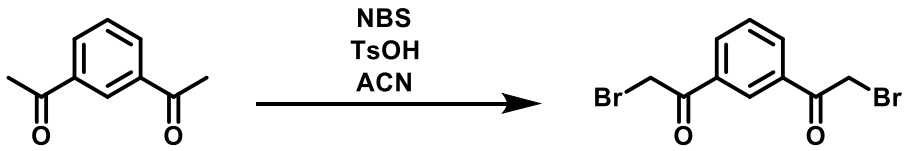
Synthesis of (ethane-1,2-diylbis(oxy))bis(ethane-2,1-diyl) bis(3-bromo-2-oxopropanoate) (c)



Scheme S3. Procedure for the synthesis of **c**.

A 50 ml round bottom flask was charged with triethylene glycol (36 µl, 0.27 mmol), bromopyruvic acid (180 mg, 1.1 mmol), and H_2SO_4 (10 µl) in DCM (25 ml). The resulting mixture was refluxed under nitrogen for 24 h before being purified using a 10 g silica gel cartridge (0 – 3% MeOH:DCM). The fractions containing the product were washed with water (x3), brine, dried over $MgSO_4$, and filtered. The solvent was removed under reduced pressure to yield the title compound (18.7 mg, 16%) as a yellow oil. **^{1}H NMR** (400 MHz, $CDCl_3$) δ = 4.47 – 4.44 (m, 4H), 4.35 (s, 4H), 3.81 – 3.78 (m, 4H), 3.67 (s, 4H) ppm; **^{13}C NMR** (100 MHz, $CDCl_3$) δ = 184.4, 159.3, 70.8, 68.6, 66.0, 31.0 ppm; **LRMS** (ESI^-) *m/z*: 445.0 ([M($^{79}Br_2$)-H]$^-$, 1), 446.9 ([M($^{81}Br^{79}Br$)-H]$^-$, 2), 448.9 ([M($^{81}Br_2$)-H]$^-$, 1); **HRMS** (ESI^-) *m/z*: [M-H]$^-$ Calcd for $C_{12}H_{16}{}^{79}Br_2O_8$ 444.9134, Found 444.9135; Calcd for $C_{12}H_{16}{}^{81}Br^{79}BrO_8$ 446.9113, Found 446.9104; Calcd for $C_{12}H_{16}{}^{81}Br_2O_8$ 448.9093, Found 448.9088; **TLC** R_f: 0.55 (5% MeOH:DCM).

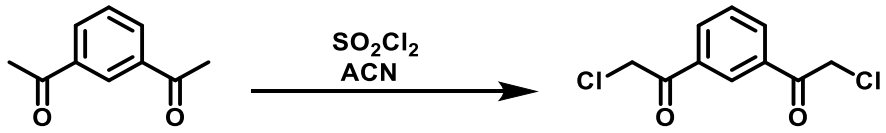
Synthesis of 1,1'-(1,3-phenylene)bis(2-bromoethan-1-one) (e)



Scheme S4. Procedure for the synthesis of **e**.

A 50 ml round bottom flask was charged with 1,3-diacetylbenzene (500 mg, 3.1 mmol) and *p*-toluenesulfonic acid monohydrate (1.5 g, 7.9 mmol) in dry acetonitrile (25 ml). The mixture was stirred under nitrogen at 0 °C for 15 min before freshly recrystallised *N*-bromosuccinimide (1.1 g, 6.2 mmol) was slowly added. Stirring continued for 1 h at 0 °C, followed by room temperature for 4 days. The solvent was removed under reduced pressure and the residue was taken up in DCM, washed with water (x3), brine, dried over Na_2SO_4, and filtered. The solvent was removed under reduced pressure and the crude product was purified using a 10 g silica gel cartridge (0 – 100% toluene:*n*-hexane) to yield the title compound as a white powder (228 mg, 23%). **^{1}H NMR** (400 MHz, $CDCl_3$) δ = 8.58 (t, *J* = 1.6 Hz, 1H), 8.23 (dd, *J* = 7.8, 1.8 Hz, 2H), 7.67 (t, *J* = 7.8 Hz, 1H), 4.48 (s, 4H) ppm; **^{13}C NMR** (100 MHz, $CDCl_3$) δ = 190.5, 134.7, 134.0, 129.8, 129.4, 30.5 ppm; **LRMS** (EI^+) *m/z* (%): 321.9 ($[M(^{81}Br_2)]^{+\bullet}$, 1), 319.9 ($[M(^{81}Br^{79}Br)]^{+\bullet}$, 2), 317.9 ($[M(^{79}Br_2)]^{+\bullet}$, 1), 228.0 ($[M(^{81}Br)\text{-}CH_2Br]^{+\bullet}$, 98), 225.0 ($[M(^{79}Br)\text{-}CH_2Br]^{+\bullet}$, 100); **HRMS** ($EI^+$) *m/z*: $[M]^{+\bullet}$ Calcd for $C_{10}H_8{}^{79}Br_2O_2$ 317.8886, Found 317.8885; Calcd for $C_{10}H_8{}^{81}Br^{79}BrO_2$ 319.8865, Found 319.8852; Calcd for $C_{10}H_8{}^{81}Br_2O_2$ 321.8845, Found 321.8836; **TLC** R_f: 0.48 (DCM).

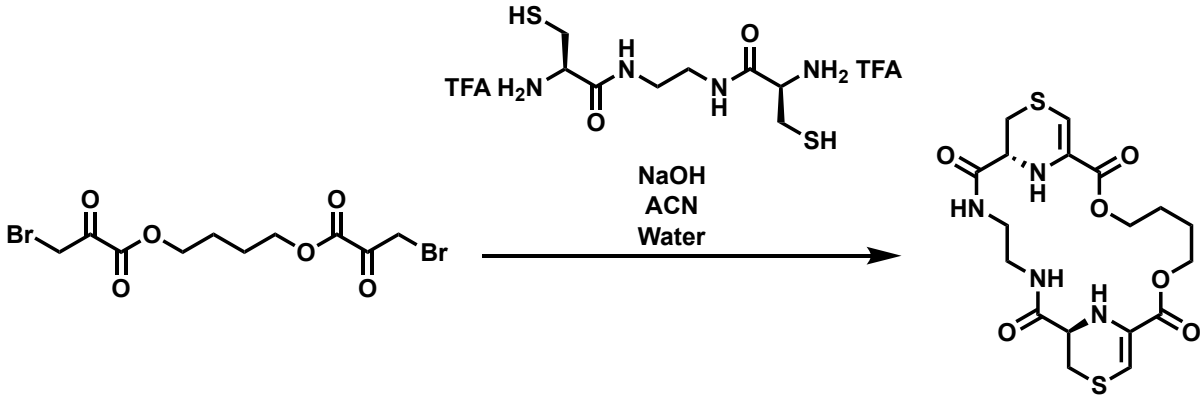
Synthesis of 1,1'-(1,3-phenylene)bis(2-chloroethan-1-one) (f)



Scheme S5. Procedure for the synthesis of **f**.

A 50 ml round bottom flask was charged with 1,3-diacetylbenzene (300 mg, 1.9 mmol) and dry acetonitrile (25 ml). The resulting mixture was stirred under nitrogen at 0 °C for 10 min before the dropwise addition of sulfuryl chloride (300 µl, 3.7 mmol). The mixture was stirred at 0 °C for 30 min and then room temperature for 5 days. The solvent was removed under reduced pressure and the crude product was purified using a 25 g silica gel cartridge (0 – 48% DCM:*n*-hexane) to yield the title compound (41.4 mg, 10%) as a white powder. **^{1}H NMR** (400 MHz, $CDCl_3$) δ = 8.51 (t, *J* = 1.8 Hz, 1H), 8.20 (dd, *J* = 7.8, 1.8 Hz, 2H), 7.67 (t, *J* = 7.8 Hz, 1H), 4.73 (s, 4H) ppm; **^{13}C NMR** (100 MHz, $CDCl_3$) δ = 190.4, 134.9, 133.6, 129.8, 128.6, 45.8 ppm; **LRMS** (EI^+) *m/z* (%): 234.0 ($[M(^{37}Cl_2)]^{+\bullet}$, 1), 232.0 ($[M(^{37}Cl^{35}Cl)]^{+\bullet}$, 2), 230.0 ($[M(^{35}Cl_2)]^{+\bullet}$, 4), 183.1 ($[M(^{37}Cl)\text{-}CH_2Cl]^{+\bullet}$, 35), 181.1 ($[M(^{35}Cl)\text{-}CH_2Cl]^{+\bullet}$, 100); **HRMS** ($EI^+$) *m/z*: $[M]^{+\bullet}$ Calcd for $C_{10}H_8{}^{35}Cl_2O_2$ 229.9896, Found 229.9888; Calcd for $C_{10}H_8{}^{37}Cl^{35}ClO_2$ 231.9866, Found 231.9856; Calcd for $C_{10}H_8{}^{37}Cl_2O_2$ 233.9837, Found 233.9835; **TLC** R_f: 0.52 (DCM).

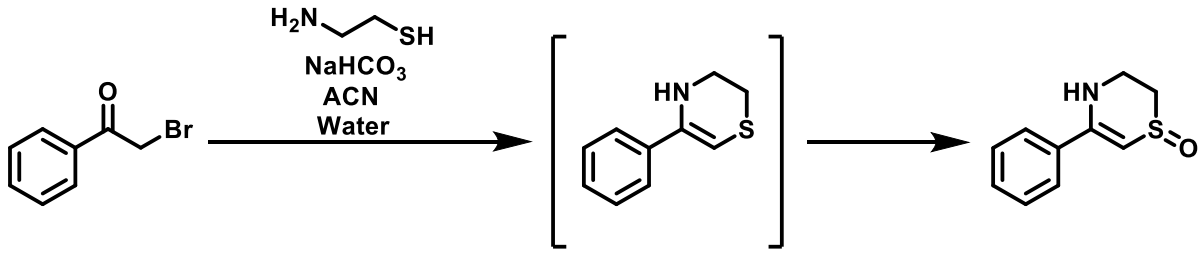
Synthesis of compound 5



Scheme S6. Procedure for the synthesis of **5**.

A 10 ml round bottom flask was charged with (2*R*,2'*R*)-*N*,*N*'-(ethane-1,2-diyl)bis(2-amino-3-mercaptopropanamide) bis(trifluoroacetic acid)[1] (42 mg, 85 μmol) in 50% ACN:water (5 ml). The pH was adjusted to 10 with a diluted NaOH solution. A 1 ml solution of **b** (30 mg, 77 μmol) in ACN was added dropwise to the mixture. After 24 h, the solvent was removed under reduced pressure and the crude product was purified by preparative HPLC (5 – 90% MeOH:water + 0.1% formic acid) to yield the title compound (11.2 mg, 26%) as a white powder. **^{1}H NMR** (800 MHz, 50% CD_3CN:D_2O pD 7) δ = 6.09 (s, 2H), 4.39 (t, *J* = 3.3 Hz, 2H), 4.38 – 4.34 (m, 2H), 4.14 – 4.09 (m, 2H), 3.40 (d, *J* = 11.7 Hz, 2H), 3.18 (app d, *J* = 12.6, 2.1 Hz, 2H), 3.09 (d, *J* = 11.7 Hz, 2H), 2.35 (dd, *J* = 12.5, 3.5 Hz, 2H), 1.81 (app s, 4H) ppm; **^{13}C NMR** (200 MHz, 50% CD_3CN:D_2O pD 7) δ = 172.9, 162.9, 127.9, 101.0, 65.0, 55.4, 38.7, 25.0, 24.1 ppm; **LRMS** (ESI^+) *m/z*: 457.1 $[M+H]^+$; **HRMS** (ESI^+) *m/z*: $[M+H]^+$ Calcd for $C_{18}H_{24}N_4O_6S_2$ 457.1216, Found 457.1215; **TLC** R_f: 0.32 (5% MeOH:DCM).

Synthesis of 5-phenyl-3,4-dihydro-2*H*-1,4-thiazine 1-oxide (6)



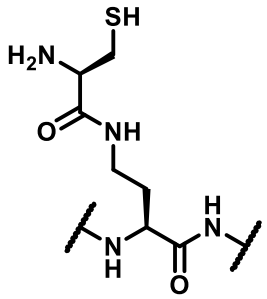
Scheme S7. Procedure for the synthesis and proposed structure of **6**.

A 50 ml round bottom flask was charged with phenacyl bromide (200 mg, 1.0 mmol), cysteamine (93 mg, 1.2 mmol), and sodium bicarbonate (250 mg, 3.0 mmol) in 50% ACN:water (25 ml). After 18 h, the mixture was concentrated under reduced pressure, extracted into DCM, washed with water (x3), brine, dried over Na_2SO_4, and filtered. The crude product was purified by preparative HPLC (5 – 90% MeOH:water + 0.1% formic acid) to yield the title compound (12.9 mg, 7%) as a yellow powder. **^{1}H NMR** (400 MHz, 8% D_2O:CD_3CN) δ = 7.52 (d, *J* = 7.9 Hz, 1H), 7.49 – 7.44 (m, 1H), 7.44 – 7.37 (m, 2H), 5.39 (s, 1H), 3.74 (dt, *J* = 13.9, 3.4 Hz, 1H), 3.58 (td, *J* = 14.0, 2.3 Hz, 1H), 2.91 (dt, *J* = 13.9, 2.7 Hz, 1H), 2.38 (td, *J* = 14.0, 3.7 Hz, 1H) ppm. **^{13}C NMR** (100 MHz, 8% D_2O:CD_3CN) δ = 151.24, 137.22, 130.81, 129.39, 127.19, 88.89 (t, *J* = 26.8 Hz), 42.30, 31.63 ppm: **^{13}C NMR** (100 MHz, 8% H_2O:CD_3CN) δ = 151.39, 137.33, 130.82, 129.40, 127.20, 88.29, 42.36, 31.77 ppm. **LRMS** (ESI^+) *m/z*: 194.2 $[M+H]^+$; **HRMS** (ESI^+) *m/z*: $[M+H]^+$ Calcd for $C_{10}H_{11}NOS$ 194.0640, Found 194.0636; **TLC** R_f: 0.34 (5% MeOH:DCM).

General procedure for solid-phase synthesis of 1 – 4 and 7

In a 10 ml filtered polypropylene syringe, Rink amide resin (Auspep 0.67 mmol/g) was swelled in DCM for 30 min. The resin was then washed with DMF (x3) before Fmoc deprotection with 25% piperidine in DMF for 10 min (x2). The resin was then washed with DMF (x3), DCM (x3), and DMF (x3). The resin was treated with a solution of *N*-Fmoc-protected amino acid (3 Eq), HBTU (3 Eq), HOBt (3 Eq), and DIPEA (4 Eq) in DMF (1.5 ml). The mixture was agitated for 1 h before being washed with DMF (x3), DCM (x3), and DMF (x3). The Fmoc deprotection, washing, and coupling steps were repeated until the desired peptide sequence had been formed. After a final Fmoc deprotection step, the resin was washed with DMF (x3), DCM (x3), diethyl ether (x3), and dried under reduced pressure for 2 h. The peptide was cleaved from the resin using a 1.5 ml mixture of TFA (91%), TIPS (3%), EDT (3%), and water (3%). The mixture was agitated for 2 h before being poured into ice-cold diethyl ether (40 ml). The precipitate was centrifuged and washed with cold diethyl ether (x3). The diethyl ether was decanted, and the crude peptide was dried under reduced pressure for 2 h. The product was purified by preparative HPLC using a gradient eluent system (5 – 90% MeOH:water + 0.1% TFA) over 20 min. The fractions containing the product were combined and lyophilised to yield the product. All purified peptides were stored at -20 °C. The sequences of all synthesised peptides are listed in Table S1. Identity was confirmed by high-resolution mass spectrometry (Table S2).

Table S1. List of the peptide sequences. The unnatural amino acid, Dys, is described in previous work.[2]



Dys

Peptide	Sequence
1	H-Cys-Tyr-Ile-Gln-Asn-Dys-Pro-Leu-Gly-NH_2
2	H-Dys-Ser-Lys-Arg-Dys-NH_2
3	Ac-Dys-Asp-Asp-Asp-Dys-NH_2
4	Ac-Dys-Asn-Gln-Dys-NH_2
7	H-Dys-Lys-Arg-Lys-Dys-NH_2

Table S2. HRMS results for all synthesised peptides.

Peptide	Molecular formula	HRMS calculated	HRMS found
1	$C_{47}H_{76}N_{14}O_{13}S_2$	1109.5236 $[M+H]^+$	1109.5244 $[M+H]^+$
2[a]	$C_{29}H_{58}N_{14}O_8S_2$	795.4082 $[M+H]^+$	793.3907 $[M+H]^+$
3	$C_{28}H_{46}N_{10}O_{14}S_2$	811.2715 $[M+H]^+$	811.2728 $[M+H]^+$
4	$C_{25}H_{45}N_{11}O_9S_2$	708.2921 $[M+H]^+$	708.2921 $[M+H]^+$
7[a]	$C_{32}H_{63}N_{15}O_7S_2$	834.4555 $[M+H]^+$	834.4542 $[M+H]^+$
1a	$C_{55}H_{78}N_{14}O_{17}S_2$	1293.5008 $[M+Na]^+$	1293.5007 $[M+Na]^+$
2a	$C_{37}H_{60}N_{14}O_{12}S_2$	957.4035 $[M+H]^+$	957.4059 $[M+H]^+$
3a	$C_{36}H_{48}N_{10}O_{18}S_2$	995.2487 $[M+Na]^+$	995.2495 $[M+Na]^+$
4a	$C_{33}H_{47}N_{11}O_{13}S_2$	892.2694 $[M+Na]^+$	892.2710 $[M+Na]^+$
1b	$C_{57}H_{82}N_{14}O_{17}S_2$	1321.5321 $[M+Na]^+$	1321.5339 $[M+Na]^+$
2b	$C_{39}H_{64}N_{14}O_{12}S_2$	985.4348 $[M+H]^+$	985.4354 $[M+H]^+$
3b	$C_{38}H_{52}N_{10}O_{18}S_2$	1023.2800 $[M+Na]^+$	1023.2804 $[M+Na]^+$
4b	$C_{35}H_{51}N_{11}O_{13}S_2$	920.3007 $[M+Na]^+$	920.3024 $[M+Na]^+$
1c	$C_{59}H_{86}N_{14}O_{19}S_2$	1381.5533 $[M+Na]^+$	1381.5537 $[M+Na]^+$
2c	$C_{41}H_{68}N_{14}O_{14}S_2$	1045.4559 $[M+H]^+$	1045.4589 $[M+H]^+$
3c	$C_{40}H_{56}N_{10}O_{20}S_2$	1083.3011 $[M+Na]^+$	1083.3024 $[M+Na]^+$
4c	$C_{37}H_{55}N_{11}O_{15}S_2$	980.3218 $[M+Na]^+$	980.3237 $[M+Na]^+$
1d	$C_{51}H_{74}N_{14}O_{13}S_2$	1177.4899 $[M+Na]^+$	1177.4919 $[M+Na]^+$
2d	$C_{33}H_{56}N_{14}O_8S_2$	841.3925 $[M+H]^+$	841.3934 $[M+H]^+$
3d	$C_{32}H_{44}N_{10}O_{14}S_2$	879.2378 $[M+Na]^+$	879.2382 $[M+Na]^+$
4d	$C_{29}H_{43}N_{11}O_9S_2$	776.2584 $[M+Na]^+$	776.2599 $[M+Na]^+$
1e	$C_{57}H_{78}N_{14}O_{13}S_2$	1253.5212 $[M+Na]^+$	1253.5215 $[M+Na]^+$
2e	$C_{39}H_{60}N_{14}O_8S_2$	917.4238 $[M+H]^+$	917.4245 $[M+H]^+$
3e	$C_{38}H_{48}N_{10}O_{14}S_2$	955.2691 $[M+Na]^+$	955.2689 $[M+Na]^+$
4e	$C_{35}H_{47}N_{11}O_9S_2$	852.2897 $[M+Na]^+$	852.2908 $[M+Na]^+$
1f	$C_{57}H_{78}N_{14}O_{13}S_2$	1253.5212 $[M+Na]^+$	1253.5217 $[M+Na]^+$
2f	$C_{39}H_{60}N_{14}O_8S_2$	917.4238 $[M+H]^+$	917.4250 $[M+H]^+$
3f	$C_{38}H_{48}N_{10}O_{14}S_2$	955.2691 $[M+Na]^+$	955.2694 $[M+Na]^+$
4f	$C_{35}H_{47}N_{11}O_9S_2$	852.2897 $[M+Na]^+$	852.2910 $[M+Na]^+$
7a	$C_{40}H_{67}N_{15}O_{11}S_2$	998.4664 $[M+H]^+$	998.4664 $[M+H]^+$
7b	$C_{42}H_{71}N_{15}O_{11}S_2$	1026.4977 $[M+H]^+$	1026.4978 $[M+H]^+$
7c	$C_{44}H_{75}N_{15}O_{13}S_2$	1086.5188 $[M+H]^+$	1086.5187 $[M+H]^+$

[a] HRMS results of the cyclic disulfide reported.

General procedure for the synthesis and calculation of abundance of 1a – 4f in solution

10 µl of peptide (**1** – **4**) (10 mM in water) was added to 150 µl of buffer (10 mM HEPES pH 7.5, 4 mM TCEP, 75% ACN). After 2 min, 15 µl of linker (**a** – **f**) (10 mM in ACN) was added. The solutions were incubated for 1 h before analysis by LC-MS. Stability was confirmed by remeasuring after 24 h. Abundance of product was determined by integrating peaks of the EICs of the cyclised peptide, major side products, and remaining linear/cyclic disulfide peptide starting material. Identity was confirmed by high-resolution mass spectrometry (Table S2).

Determination of stapling kinetics of 1b and degradation of 1e

The stapling kinetics of **1b** and degradation of **1e** were determined by LC-MS (EIC) using the same reaction procedure as described above. Samples were taken at different time points (Figure S1) and analysed by LC-MS (EIC) using the following method:

Solvent A: 5% ACN:water (20 mM ammonium acetate pH 5); solvent B: 90% ACN:water (20 mM ammonium acetate pH 5); Gradient: 0 – 100% B over 3 min, 100% B for 1.5 min; Flow rate: 0.6 ml/min.

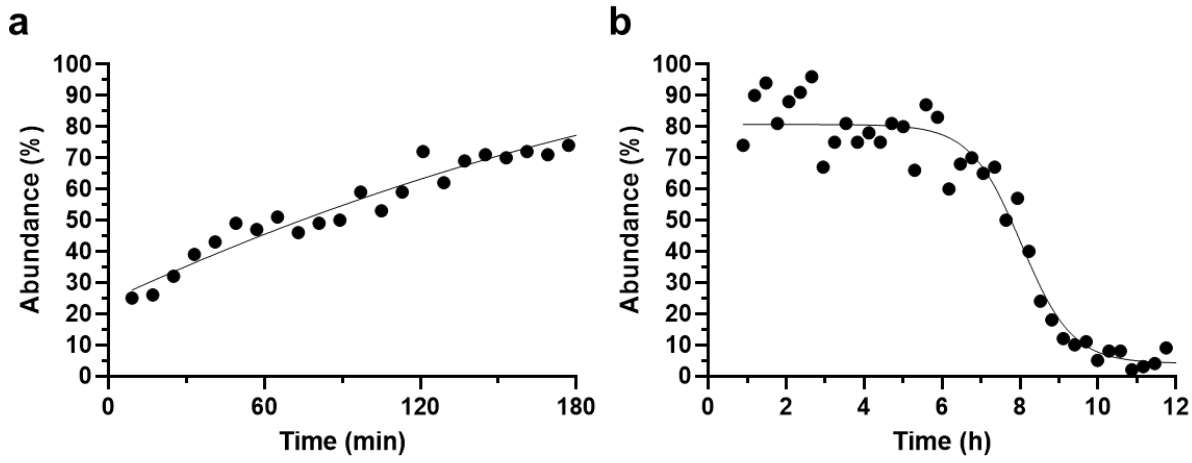


Figure S1. (a) Stapling kinetics of **1b** from 10 – 180 min. (b) Degradation of **1e** from 1 – 12 h.

Synthesis and isolation of 1b

300 µl of **1** (10 mM in 50% ACN:water) was added to 4.5 ml of buffer (10 mM HEPES pH 7.5, 4 mM TCEP, 75% ACN). After 2 min, 450 µl of **b** (10 mM in ACN) was added. The mixture was incubated for 24 h and reaction completion was confirmed by LC-MS. The stapled peptide was purified by preparative HPLC using a gradient eluent system (5 – 90% MeOH:water + 0.1% formic acid) over 20 min. Identity was confirmed by high-resolution mass spectrometry (Table S2).

Synthesis and isolation of 7a, 7b, and 7c

200 µl of **7** (10 mM in water) was added to 3 ml of buffer (10 mM HEPES pH 7.5, 4 mM TCEP, 75% ACN). After 2 min, 300 µl of linker (**a** – **c**) (10 mM in ACN) was added, the mixture was incubated for 16 h and reaction completion was confirmed by LC-MS. The stapled peptides were purified by preparative HPLC using a gradient eluent system (5 – 90% MeOH:water + 0.1% formic acid) over 20 min. Identity was confirmed by high-resolution mass spectrometry (Table S2).

Zika virus protease NS2B-NS3 assay

The unlinked ZIKV protease NS2B-NS3 construct bZiPro was expressed and purified as published, using Addgene plasmid #86846.[3] Experiments were performed in duplicate. The assay was conducted in 96-well plates (black U-bottom, Greiner Bio-One) in a total volume of 100 µl using 10 mM Tris pH 8.5, 1 mM CHAPS, 20% glycerol. All measurements were performed in duplicate. Stock solutions of reduced **7** were prepared in buffer (10 mM Tris pH 8, 1 mM TCEP) and used immediately. Varying concentrations of the purified peptides **7**, **7a**, **7b**, and **7c** were incubated with ZiPro (0.3 nM) for 10 minutes. The enzymatic reaction was initiated by addition of the substrate Bz-Nle-Lys-Lys-Arg-AMC (Mimotopes) at a final concentration of 5 µM. Fluorescence was monitored by a fluorophotometer (Tecan Infinite M Plex plate reader) for 5 min at 460 nm using 360 nm excitation. Initial velocities were derived from the linear range, and 100% enzyme activity was defined as the initial velocity of blank wells containing no peptide. Percent inhibition was calculated as a fraction of 100% enzyme activity. The IC_{50} values were calculated in GraphPad Prism 9.

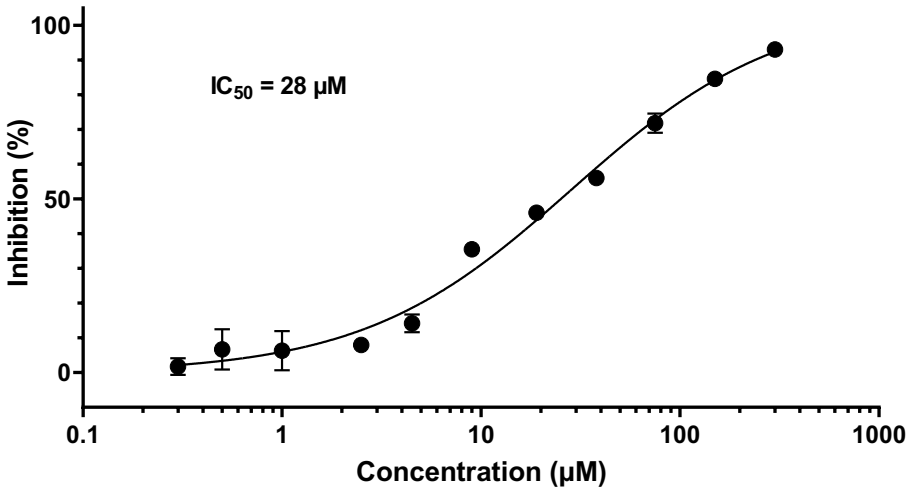


Figure S2. Dose-response curve and IC_{50} of peptide **7**.

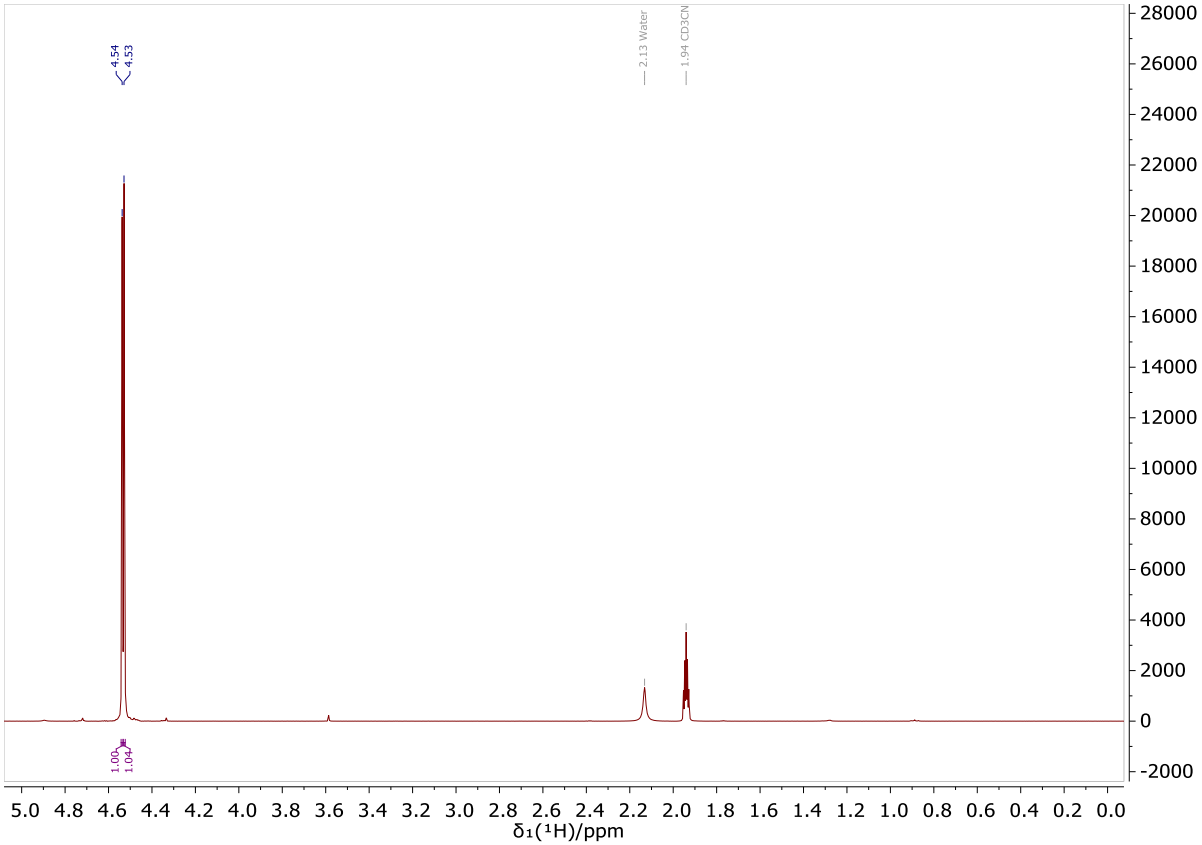


Figure S3. ^{1}H-NMR spectrum (400 MHz, CD_3CN) of compound **a**.

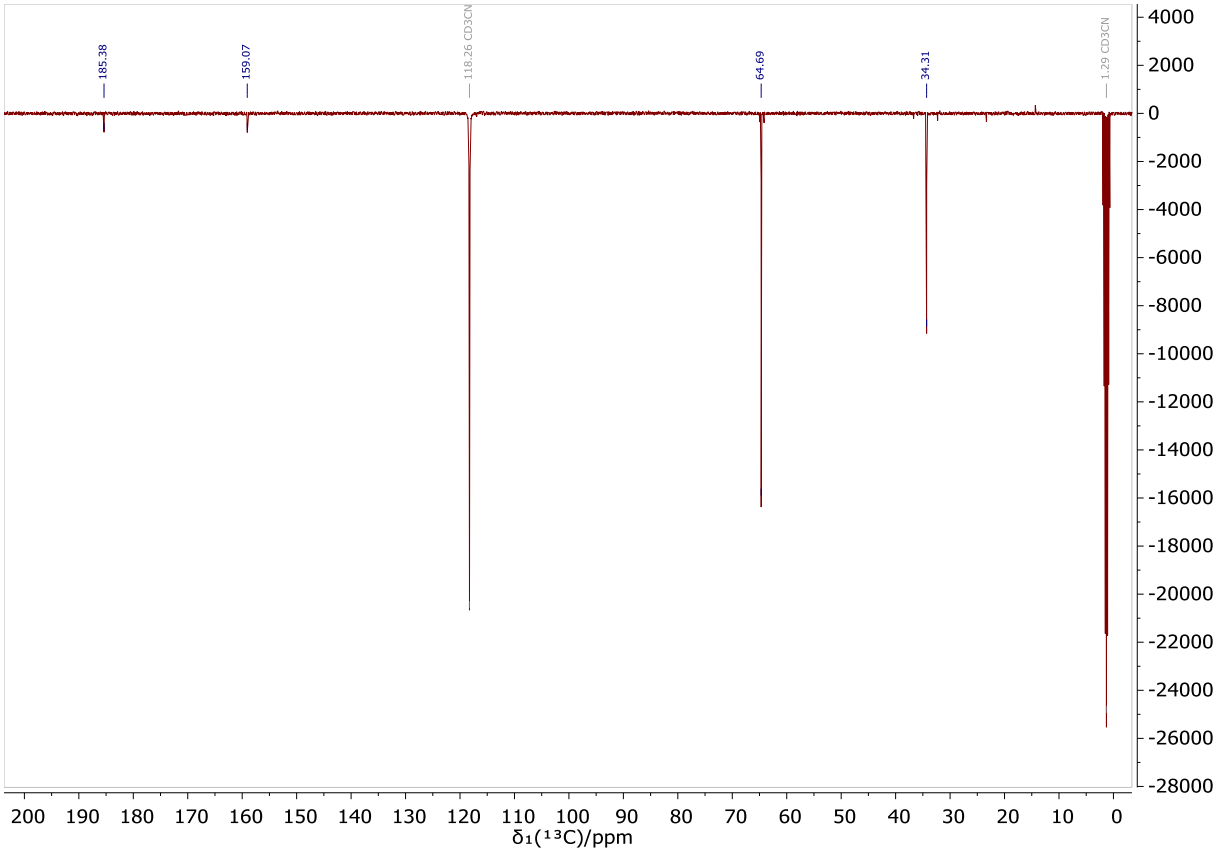


Figure S4. ^{13}C-NMR APT spectrum (100 MHz, CD_3CN) of compound **a**.

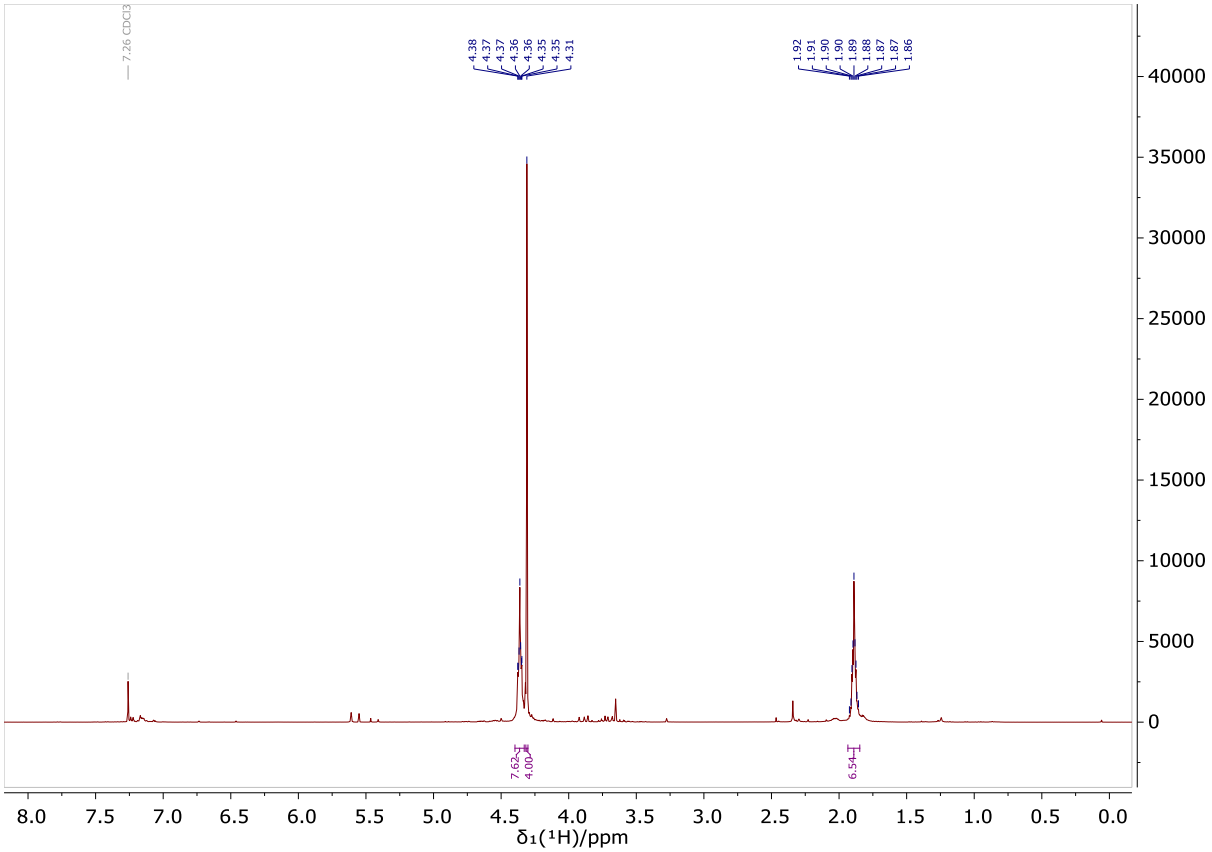


Figure S5. ^{1}H-NMR spectrum (400 MHz, $CDCl_3$) of compound **b**.

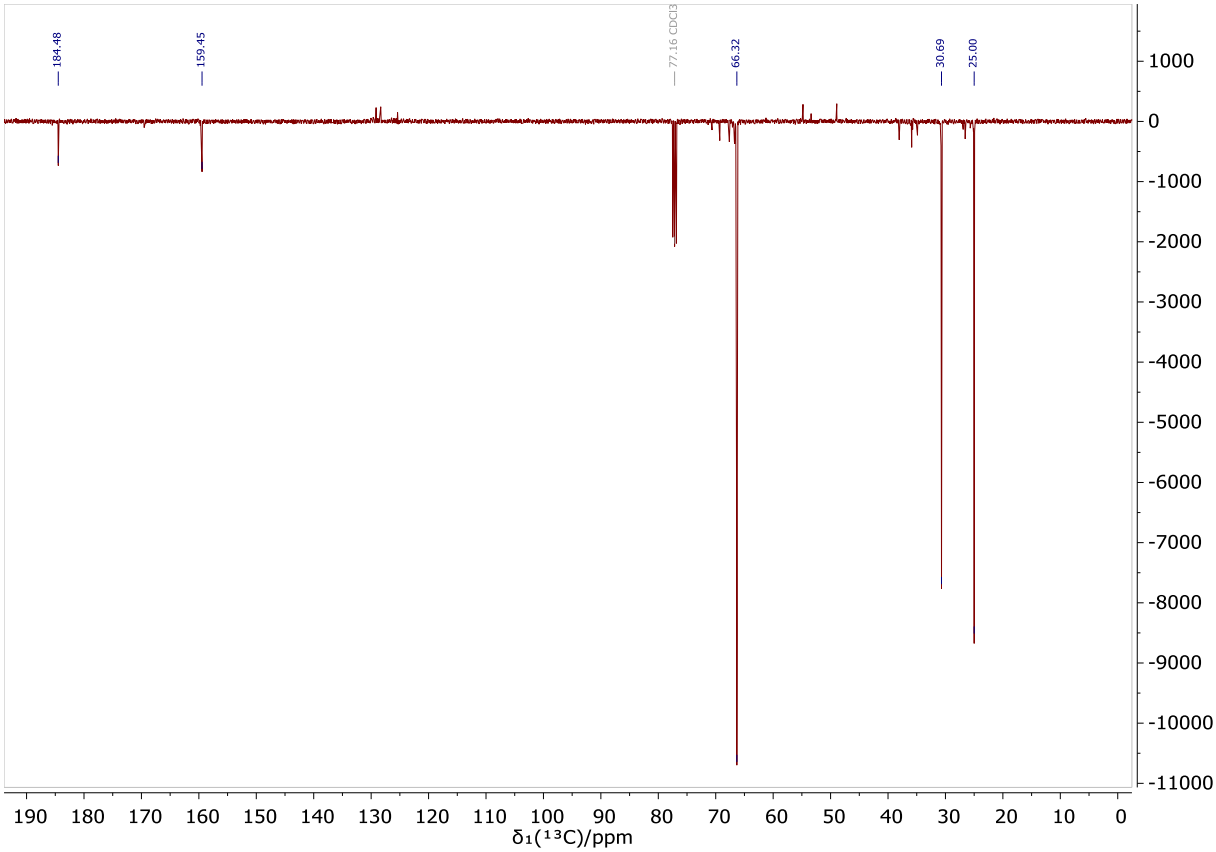


Figure S6. ^{13}C-NMR APT spectrum (100 MHz, $CDCl_3$) of compound **b**.

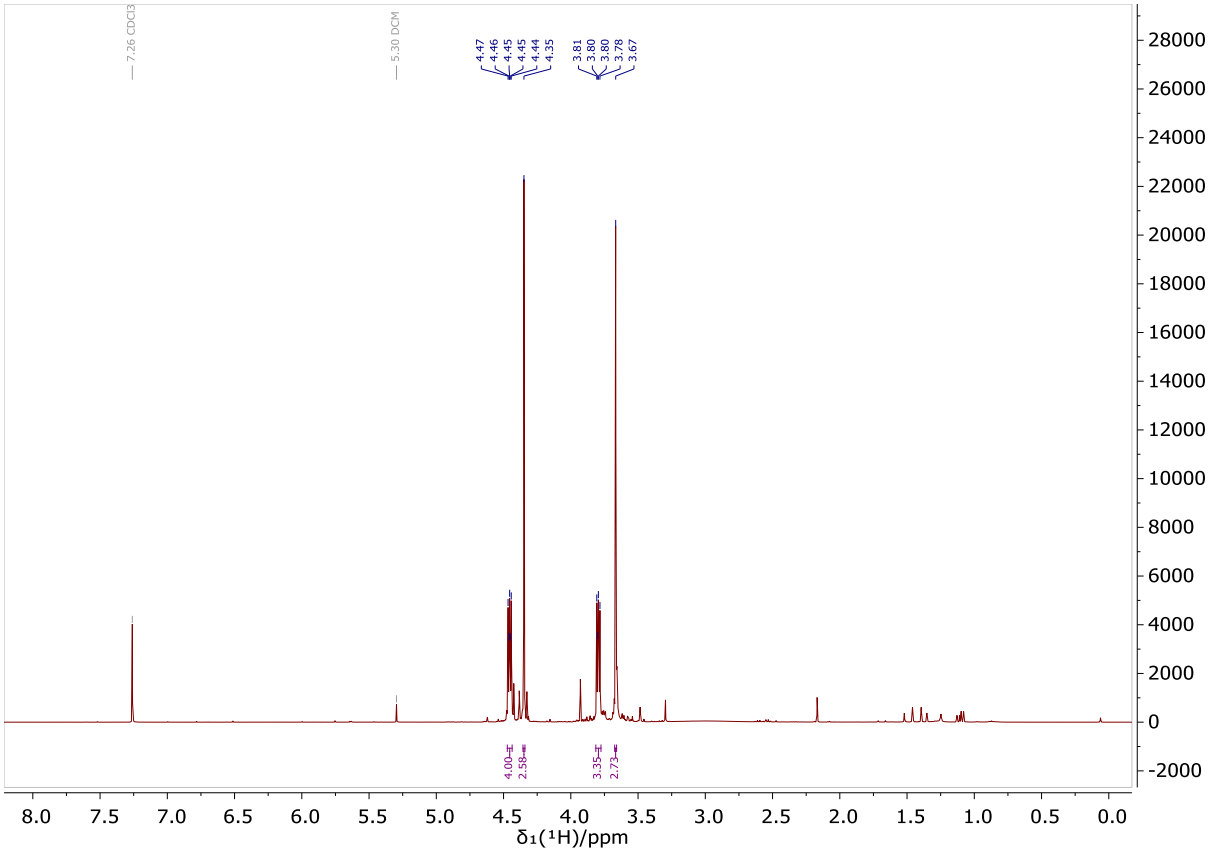


Figure S7. ^{1}H-NMR spectrum (400 MHz, $CDCl_3$) of compound **c**.

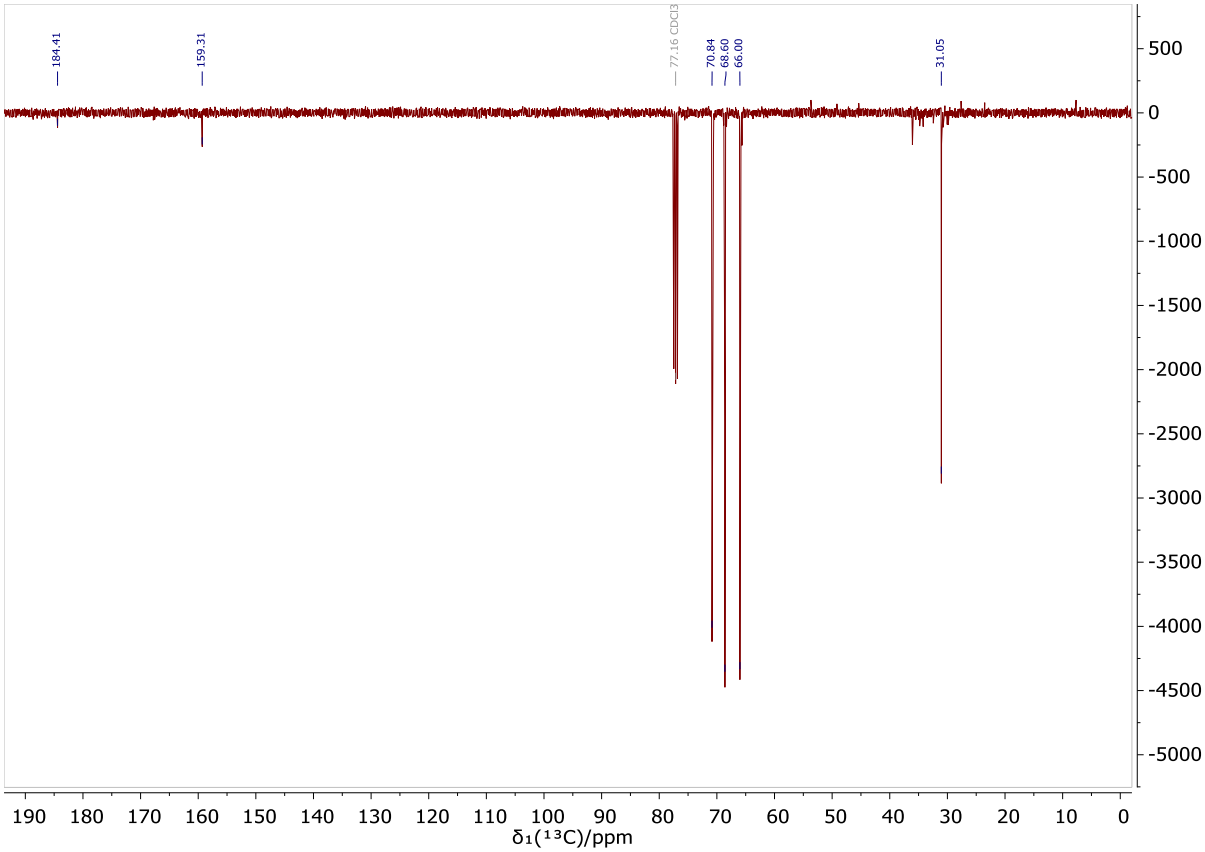


Figure S8. ^{13}C-NMR APT spectrum (100 MHz, $CDCl_3$) of compound **c**.

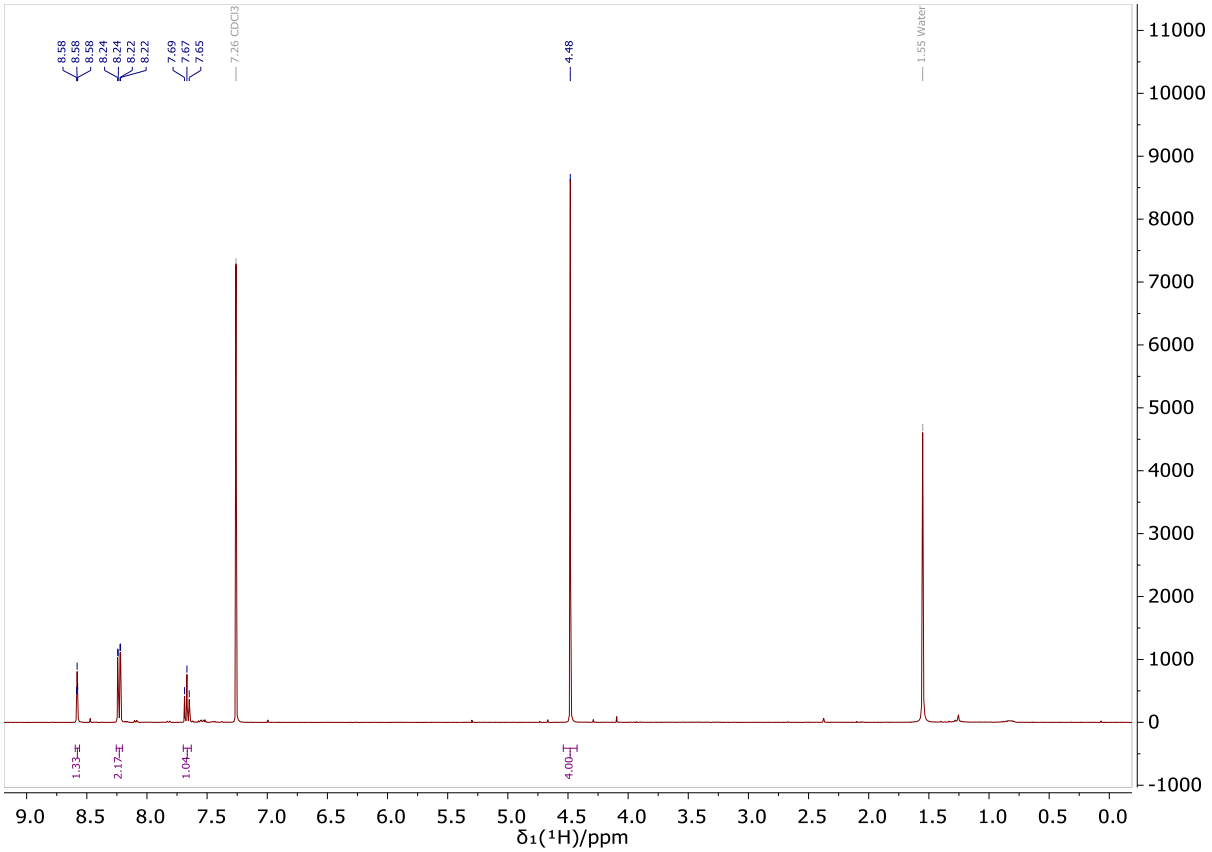


Figure S9. ^{1}H-NMR spectrum (400 MHz, $CDCl_3$) of compound **e**.

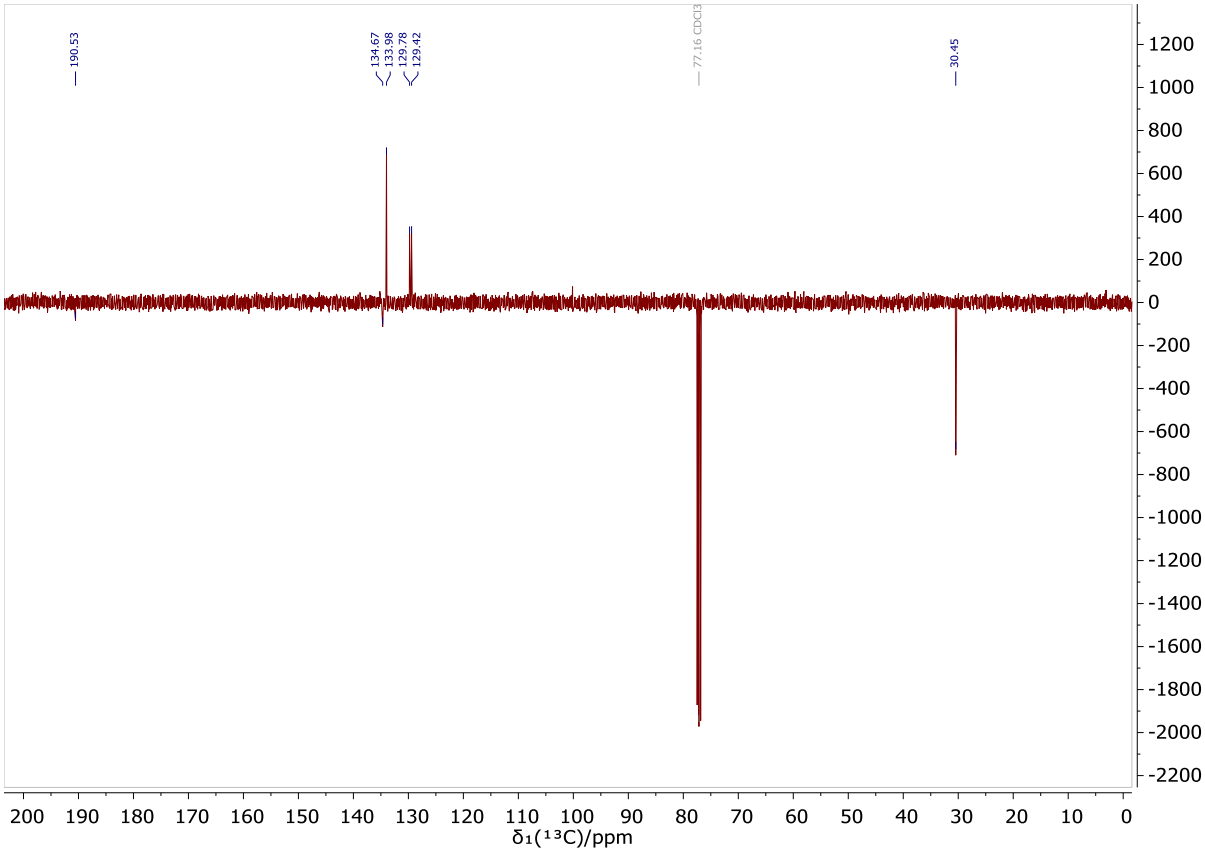


Figure S10. ^{13}C-NMR APT spectrum (100 MHz, $CDCl_3$) of compound **e**.

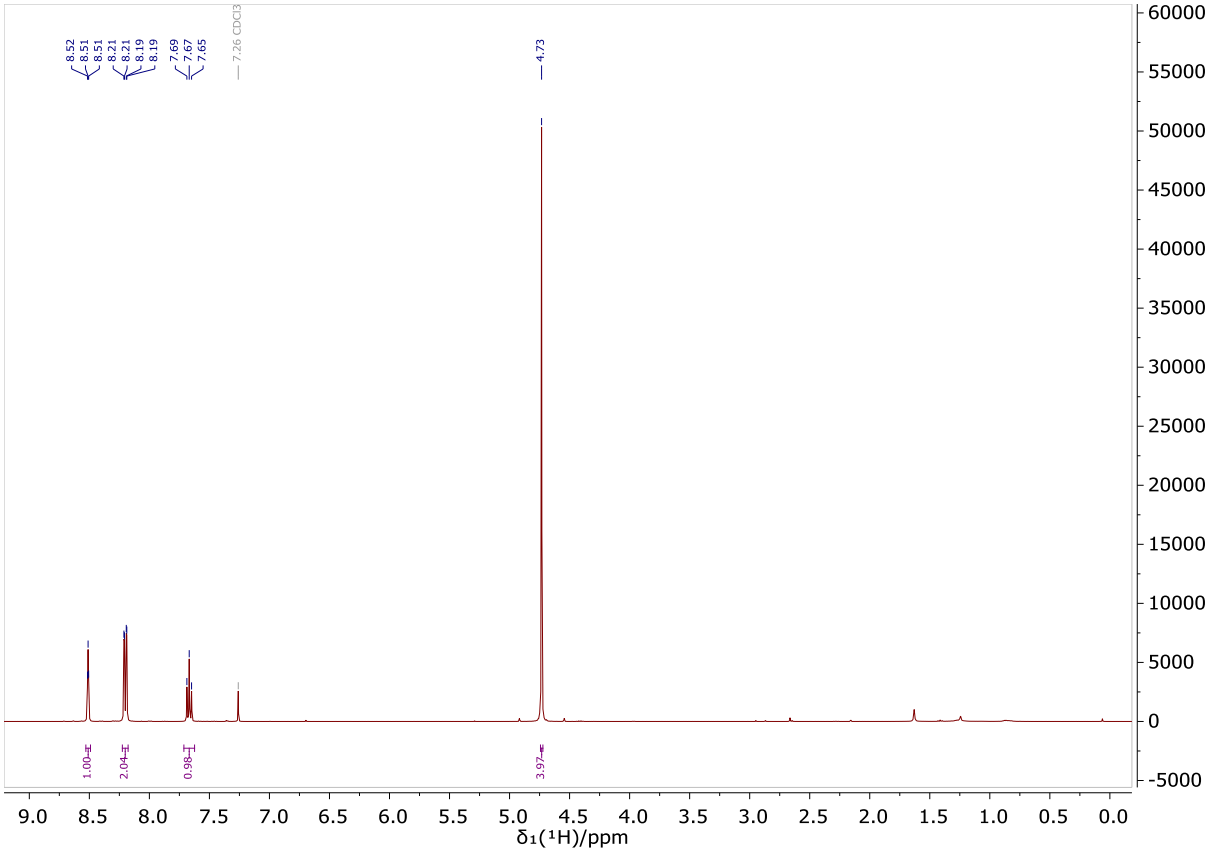


Figure S11. ^{1}H-NMR spectrum (400 MHz, $CDCl_3$) of compound **f**.

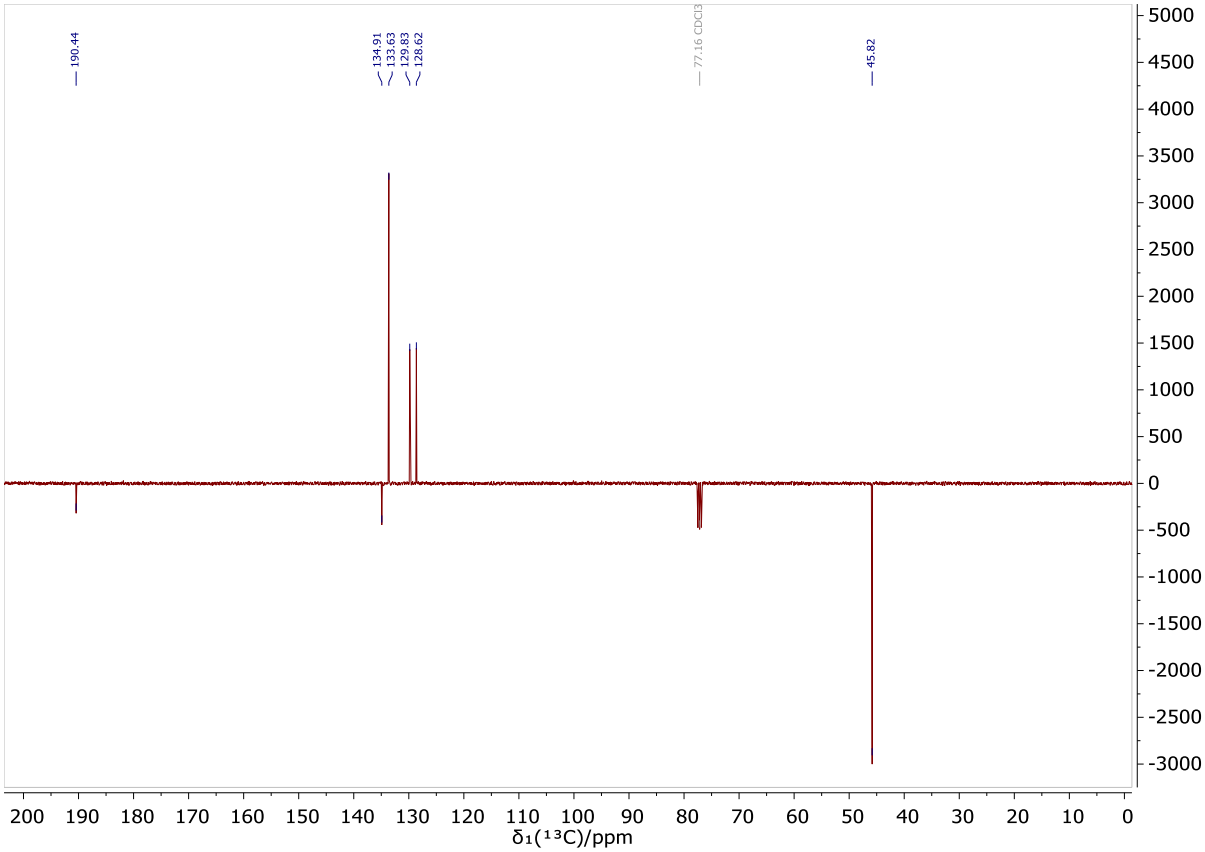


Figure S12. ^{13}C-NMR APT spectrum (100 MHz, $CDCl_3$) of compound **f**.

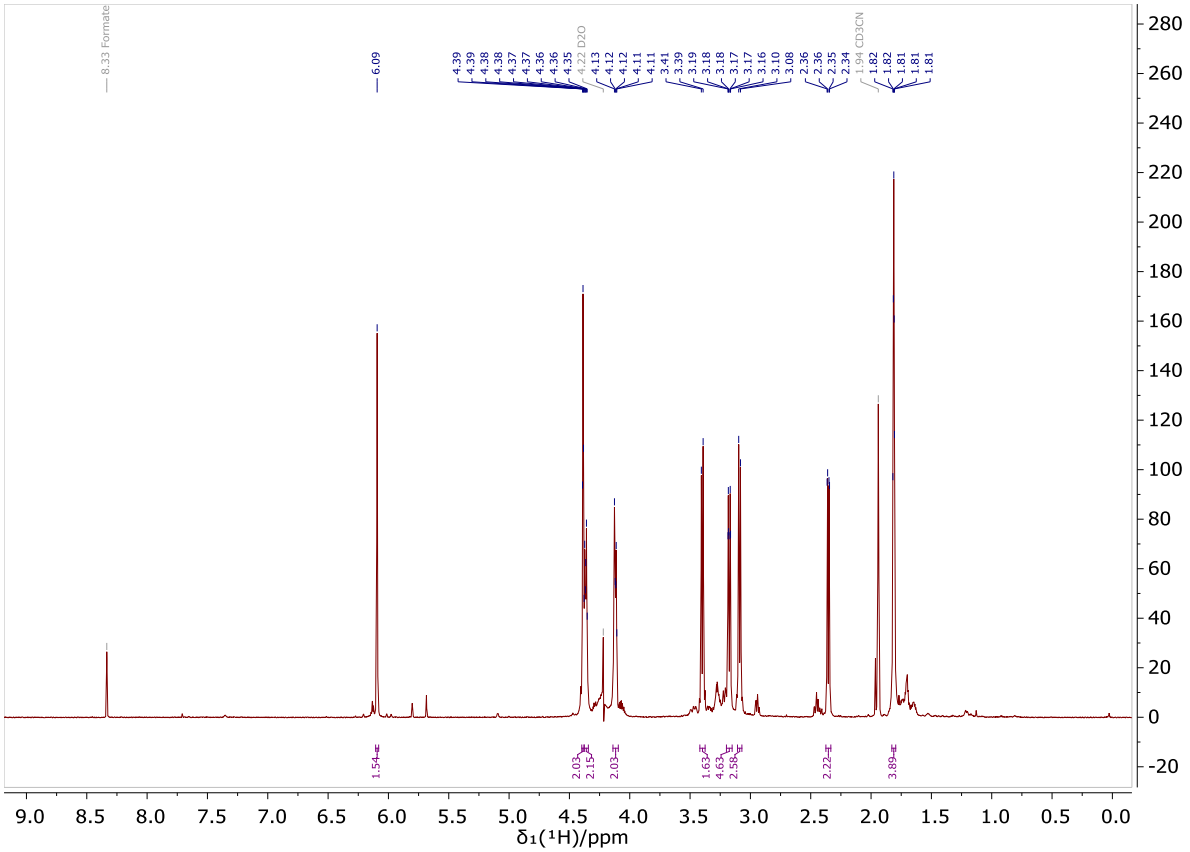


Figure S13. Water suppressed ^{1}H-NMR spectrum (800 MHz, 50% CD_3CN:D_2O pD 7) of compound **5**.

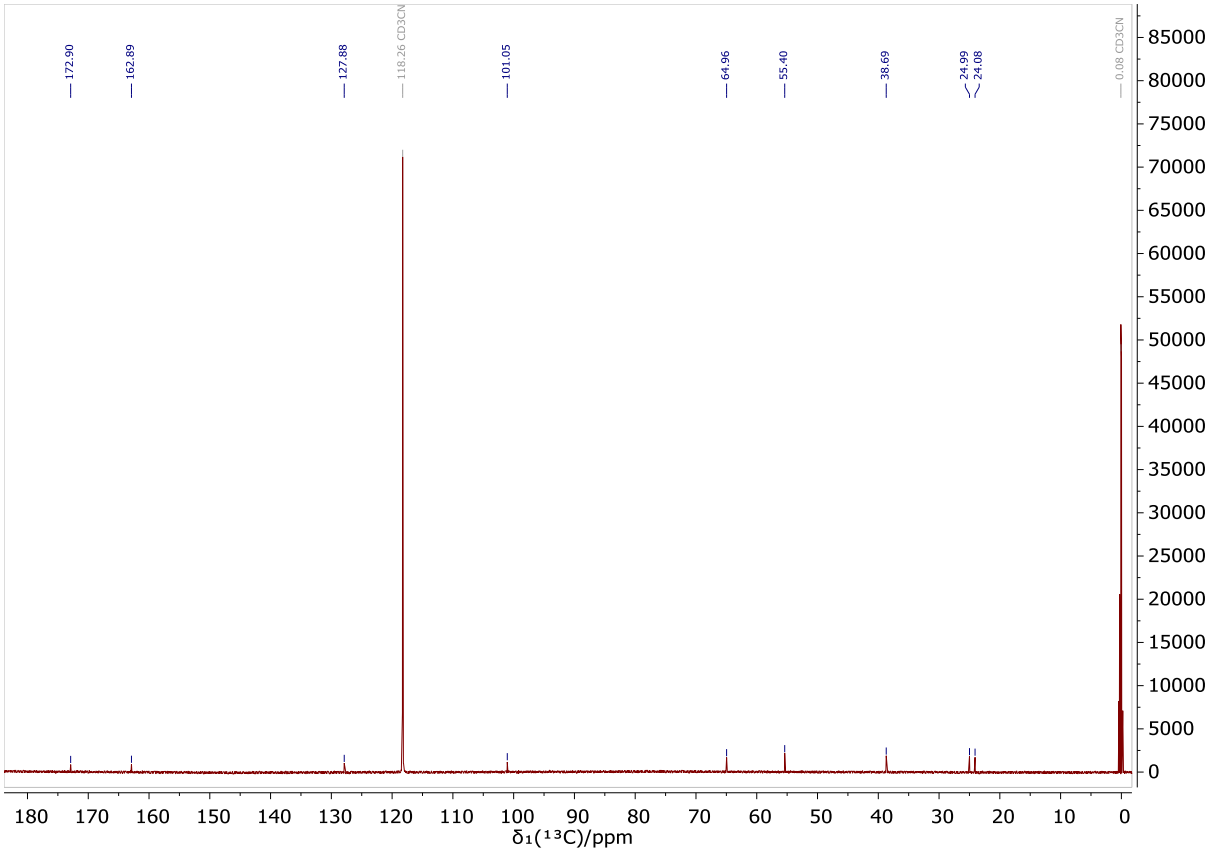


Figure S14. ^{13}C-NMR spectrum (200 MHz, 50% CD_3CN:D_2O pD 7) of compound **5**.

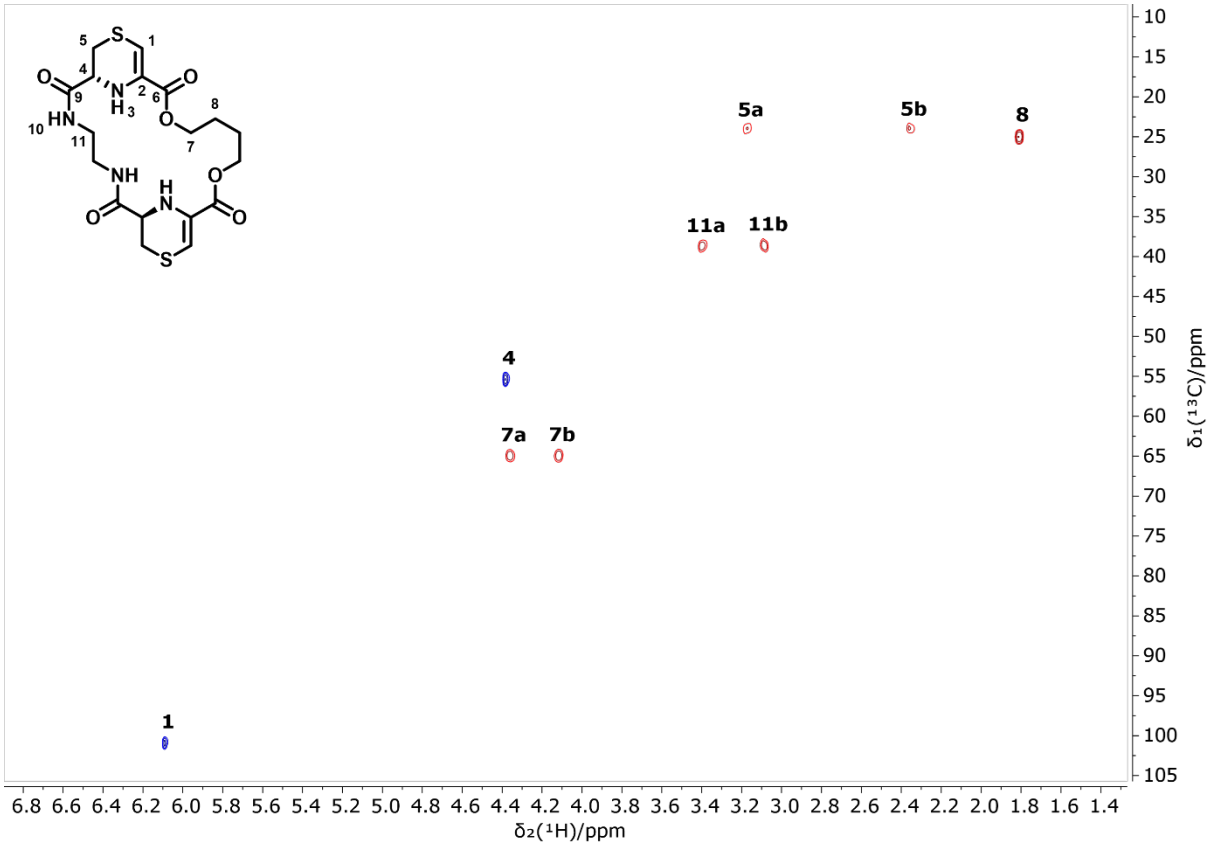


Figure S15. [^{13}C,^{1}H]-HSQC-NMR spectrum (800/200 MHz, 50% CD_3CN:D_2O pD 7) of compound **5**. Peak assignments are indicated.

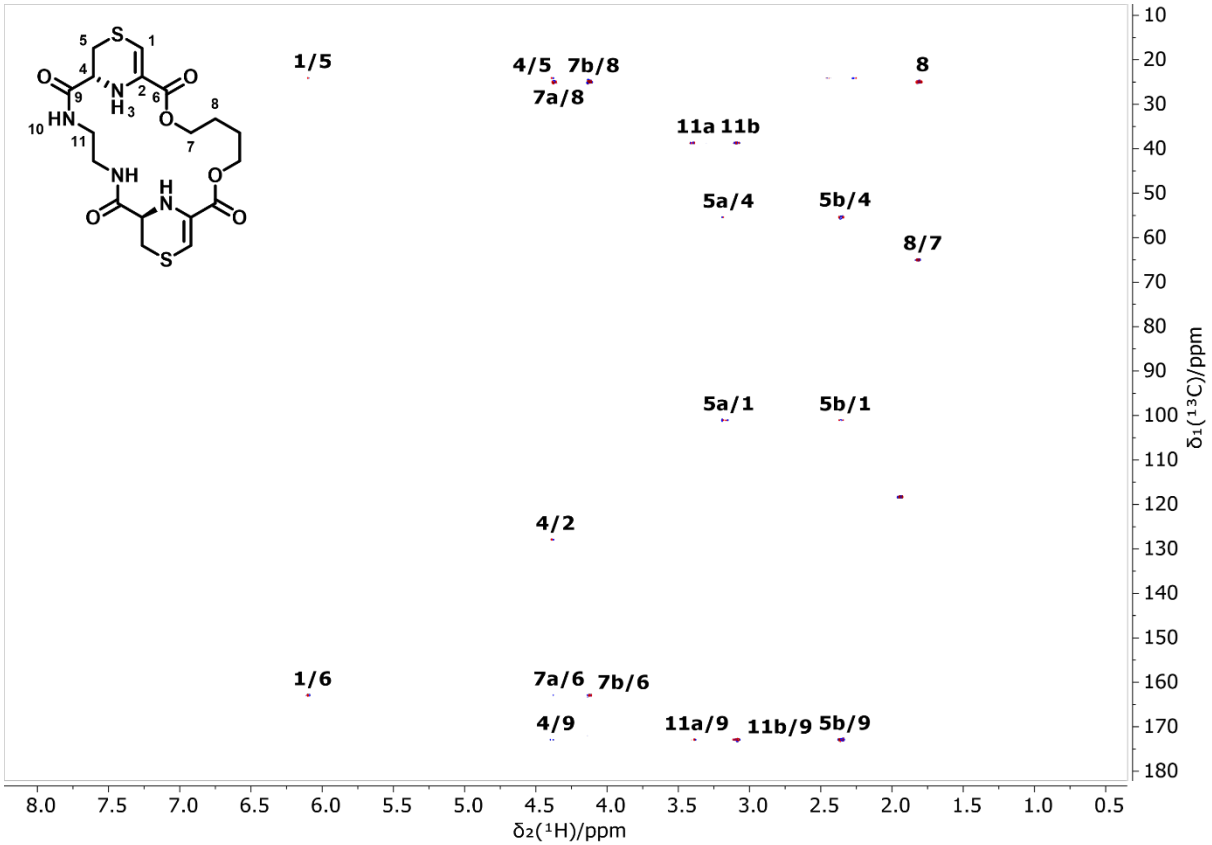


Figure S16. [^{13}C,^{1}H]-HMBC-NMR spectrum (800/200 MHz, 50% CD_3CN:D_2O pD 7) of compound **5**. Couplings are indicated.

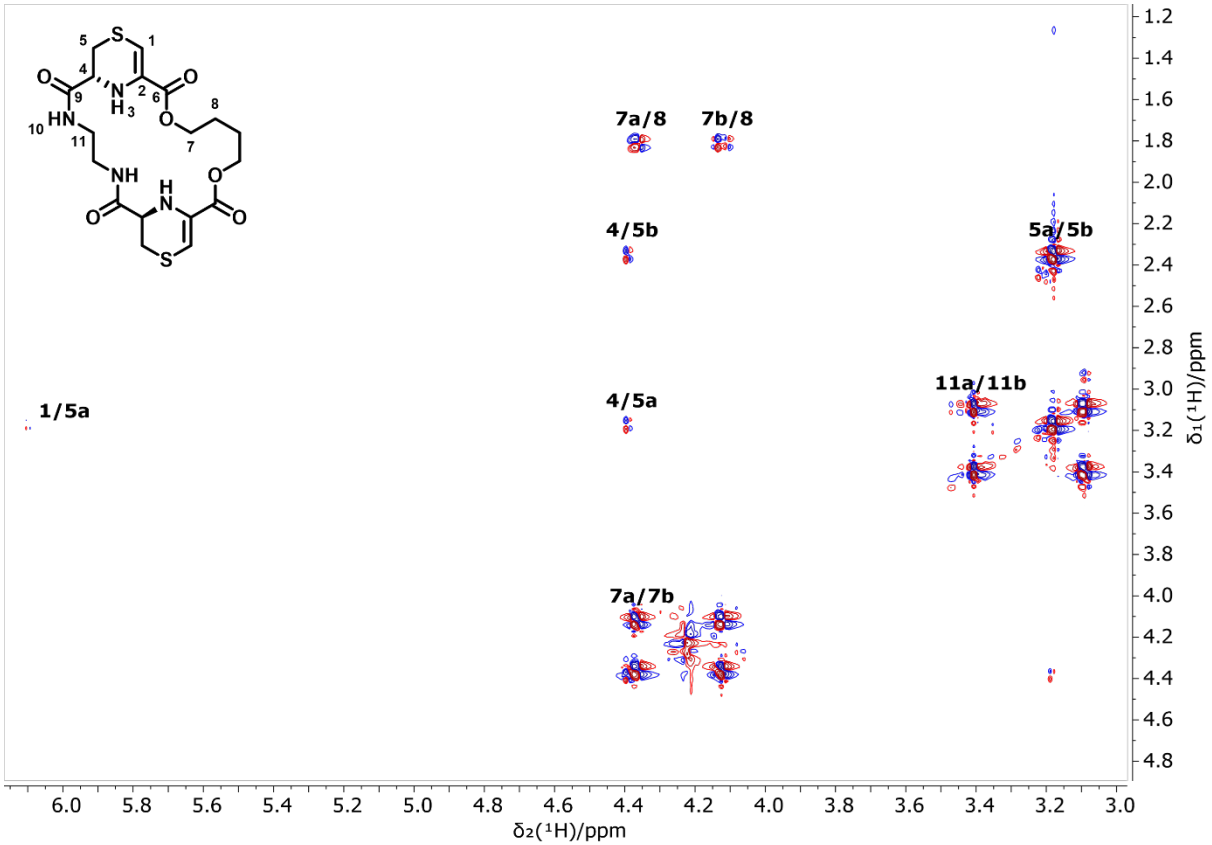


Figure S17. ^{1}H-COSY-NMR spectrum (800 MHz, 50% CD_3CN:D_2O pD 7) of compound **5**. Couplings are indicated.

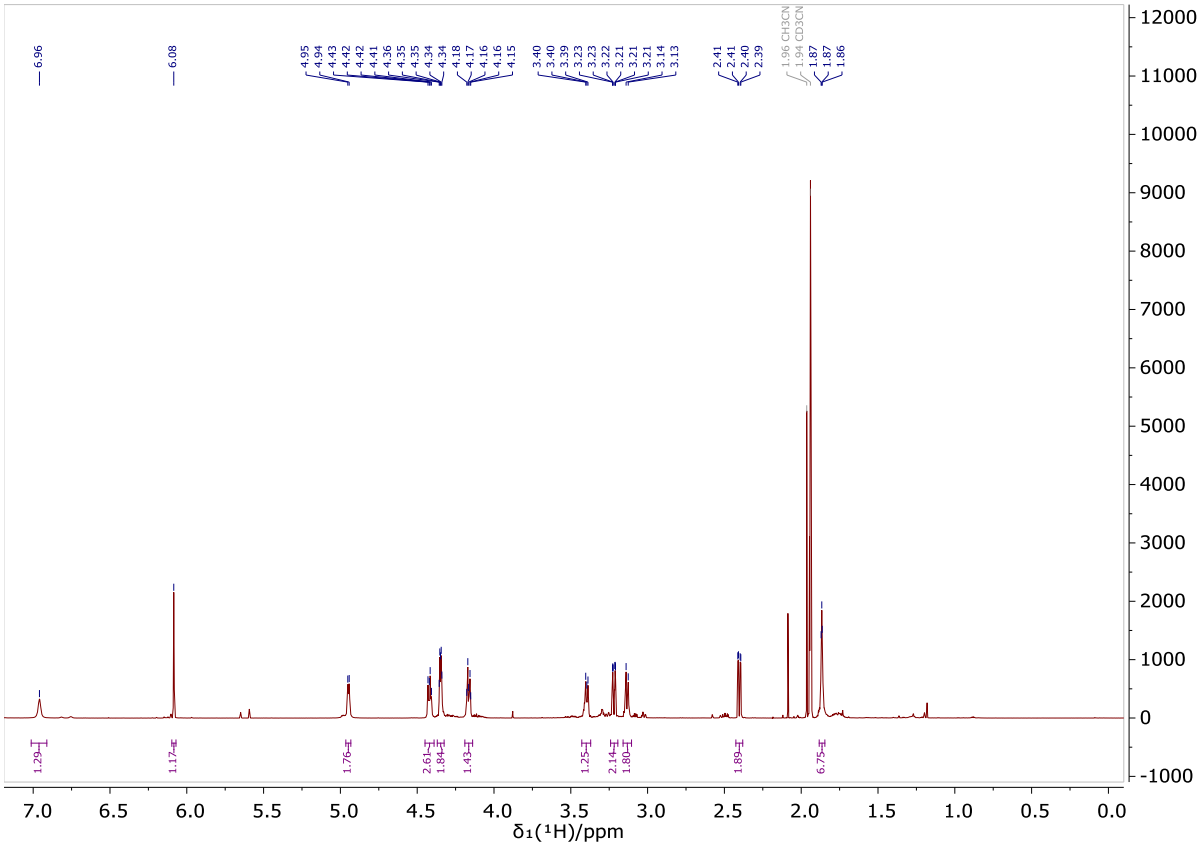


Figure S18. ^{1}H-NMR spectrum (800 MHz, CD_3CN) of compound **5**.

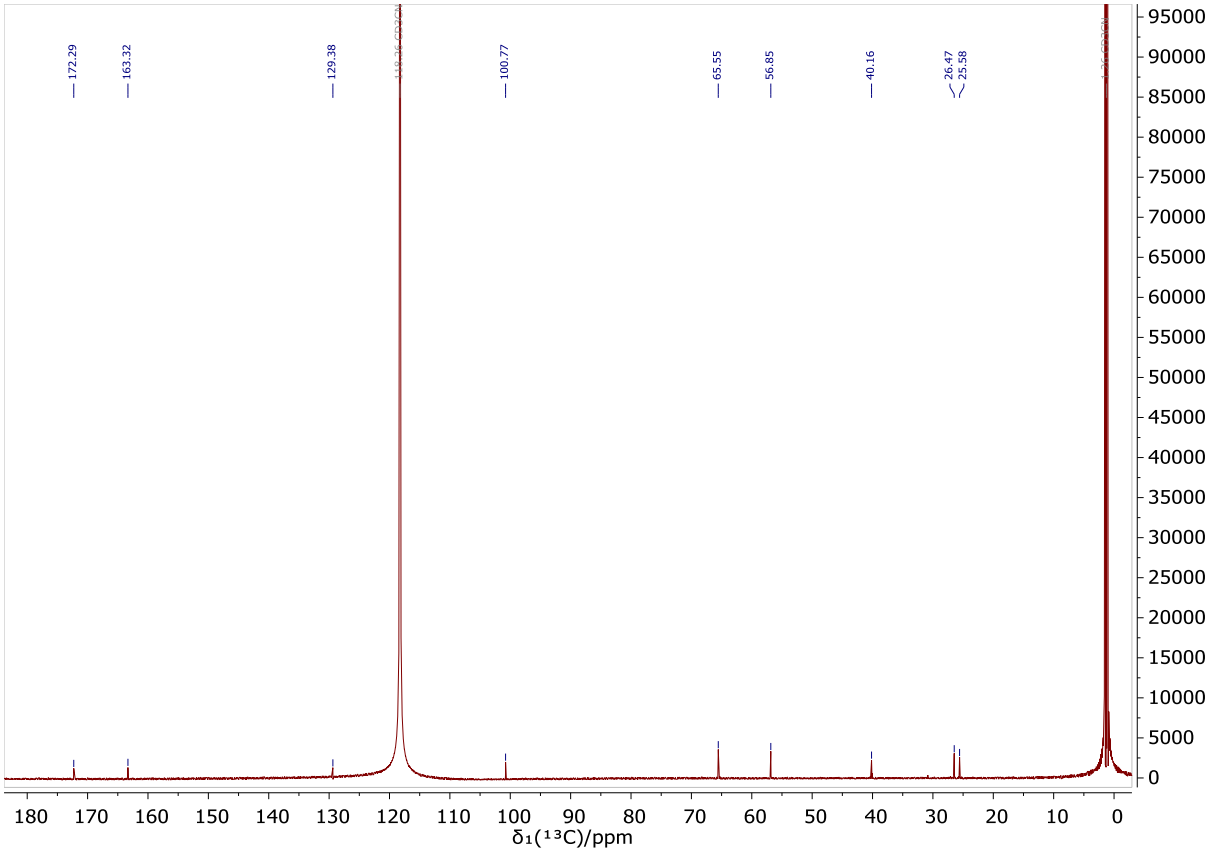


Figure S19. ^{13}C-NMR spectrum (200 MHz, CD_3CN) of compound **5**.

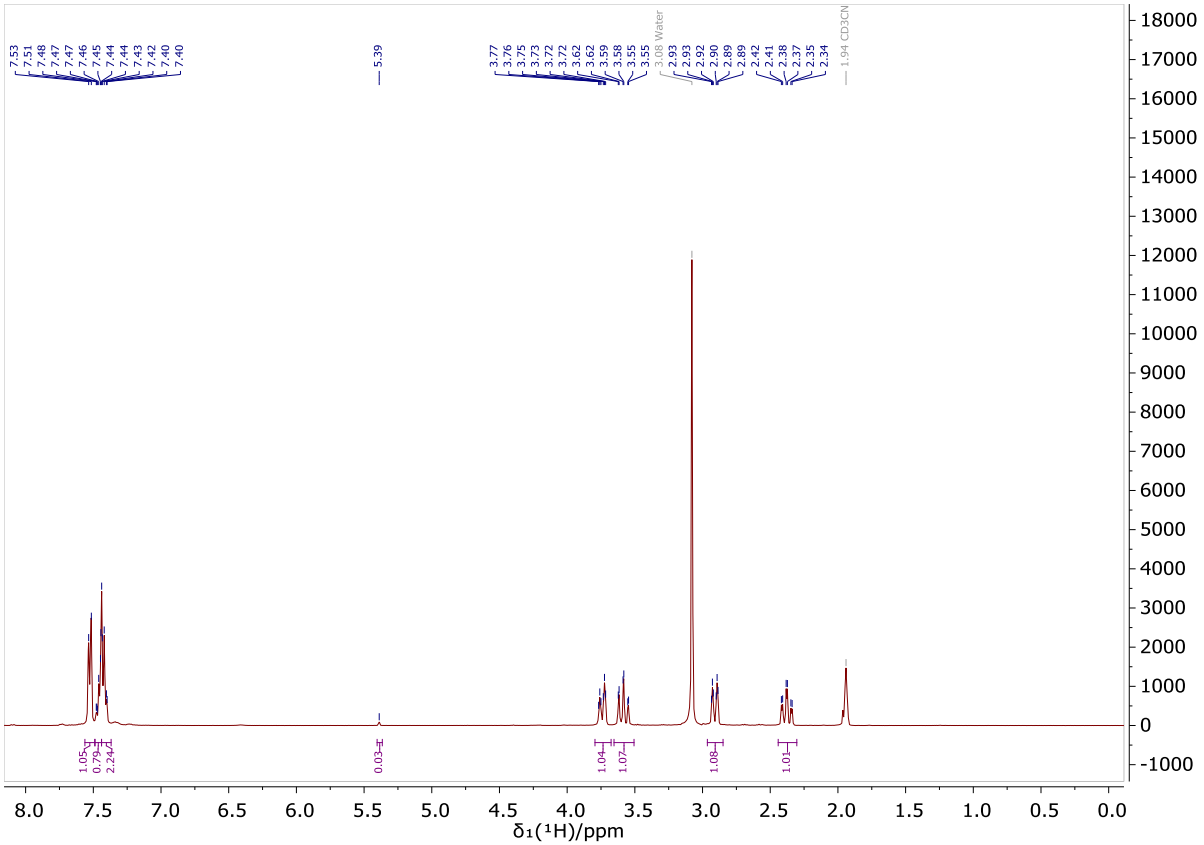


Figure S20. ^{1}H-NMR spectrum (400 MHz, 8% D_2O in CD_3CN) of compound **6**.

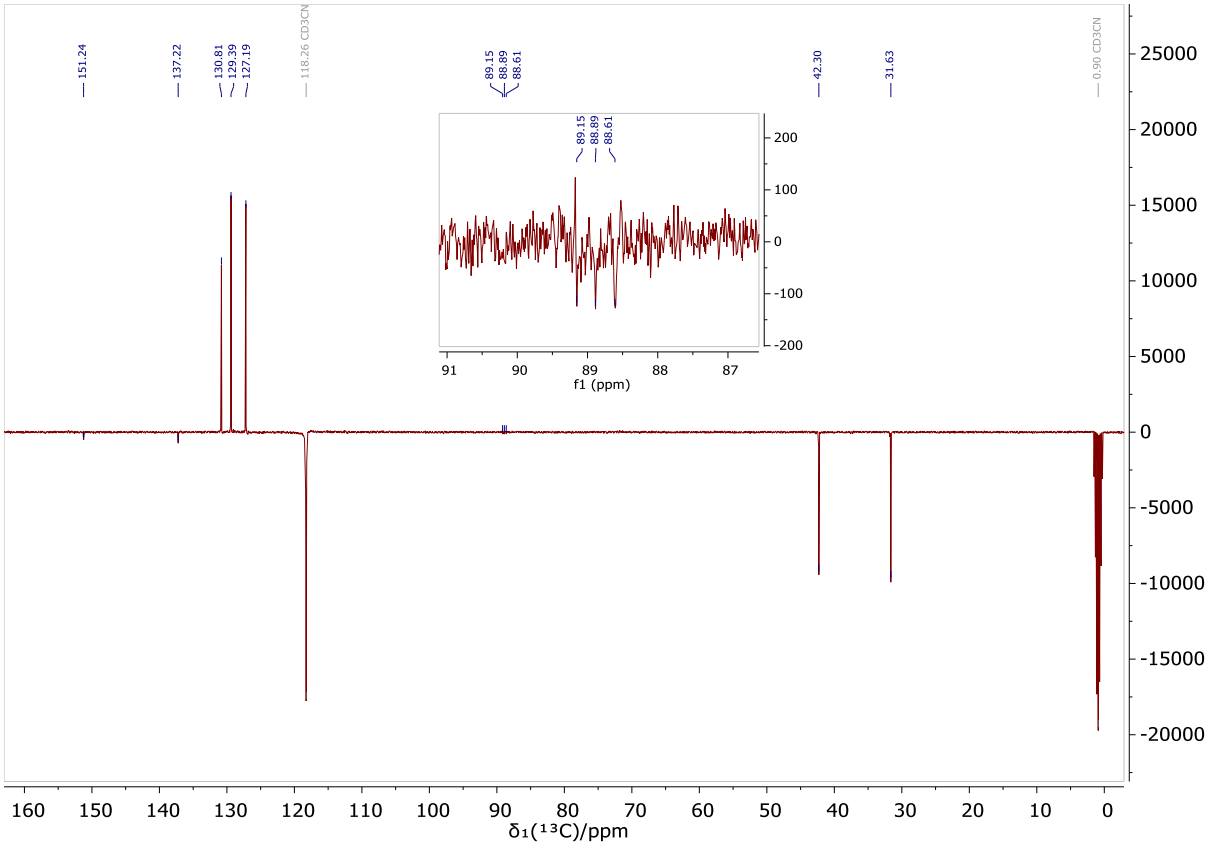


Figure S21. ^{13}C-NMR APT spectrum (100 MHz, 8% D_2O in CD_3CN) of compound **6**.

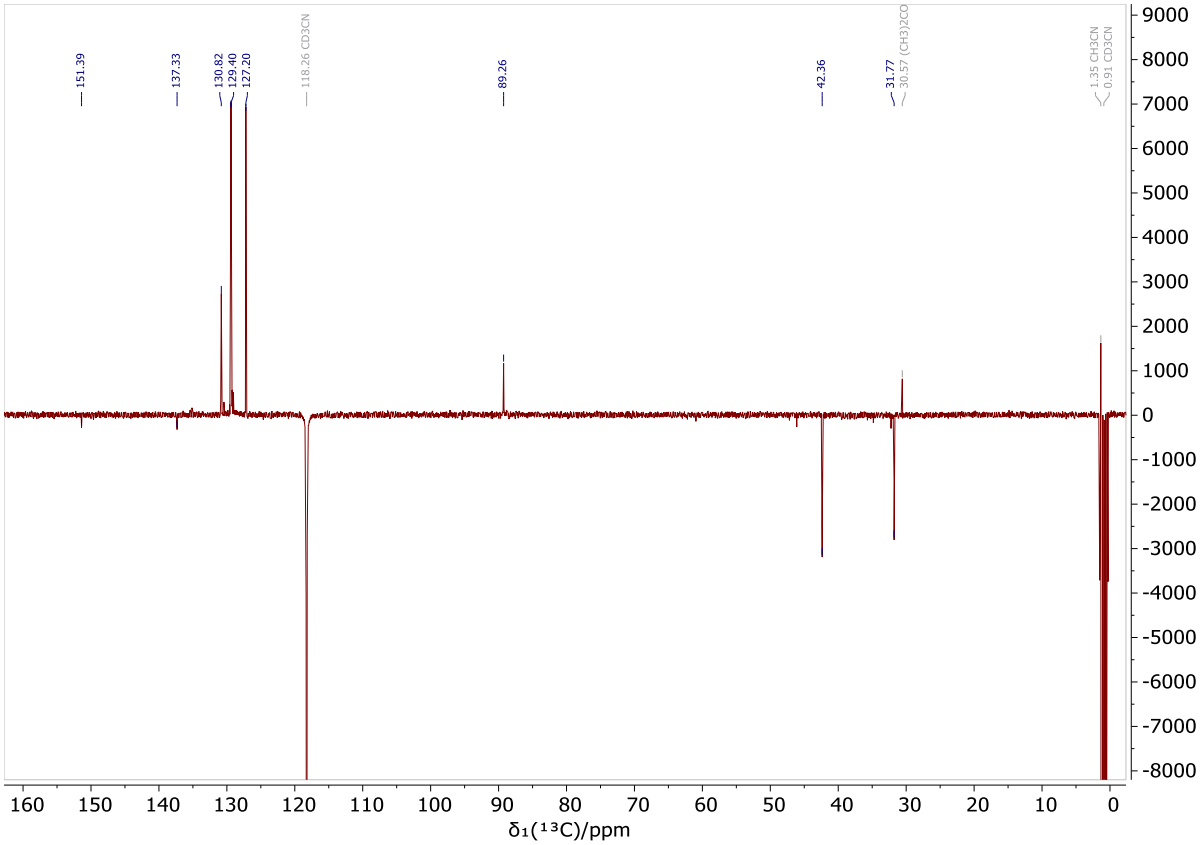


Figure S22. ^{13}C-NMR APT spectrum (100 MHz, 8% H_2O in CD_3CN) of compound **6**.

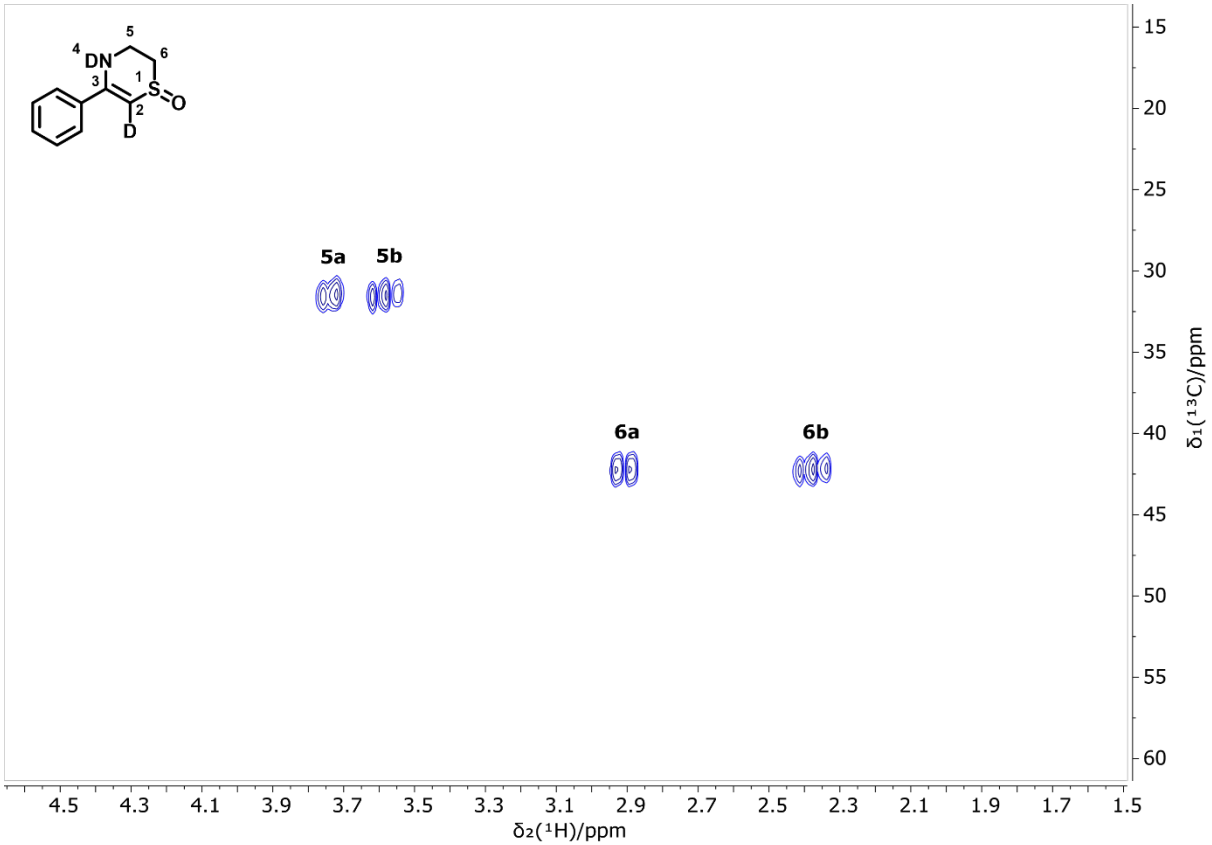


Figure S23. [^{13}C,^{1}H]-HSQC-NMR spectrum (400/100 MHz, 8% D_2O in CD_3CN) of compound **6**. Peak assignments are indicated.

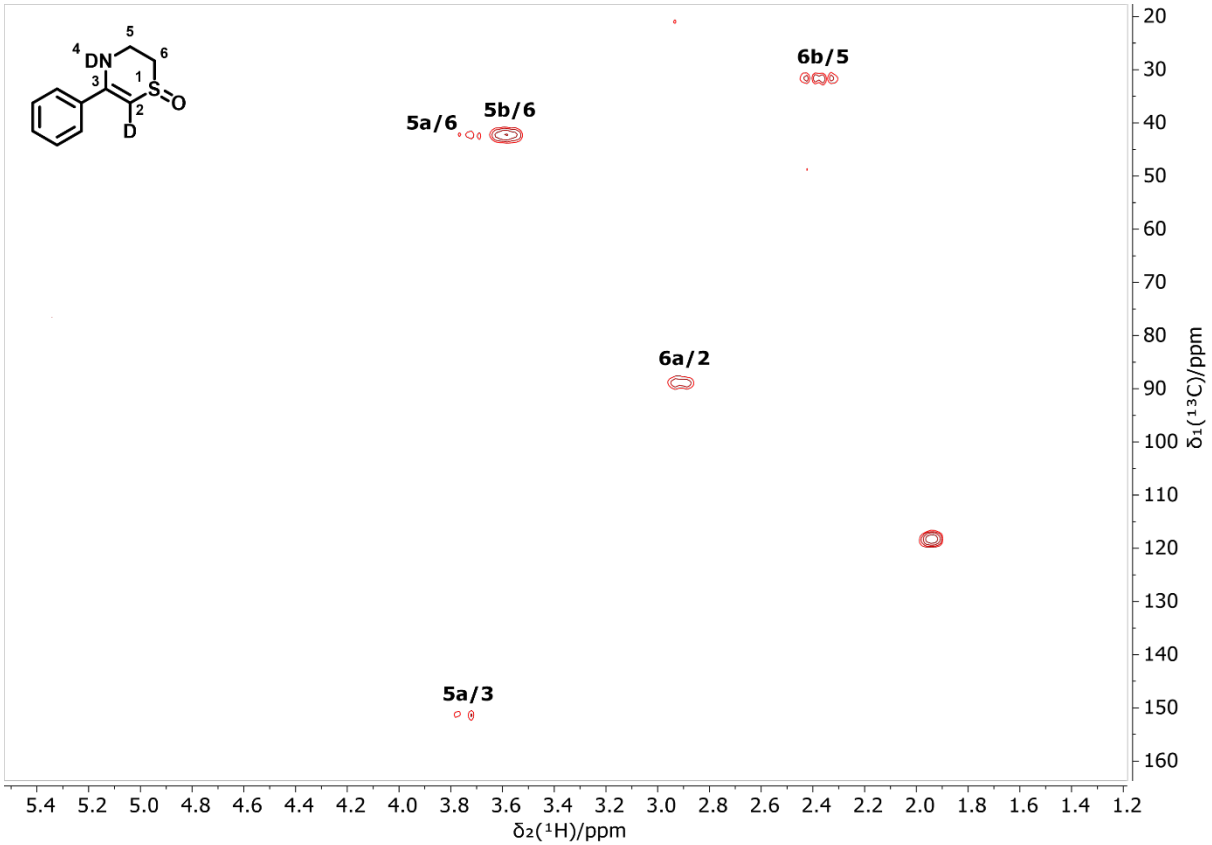


Figure S24. [^{13}C,^{1}H]-HMBC-NMR spectrum (400/100 MHz, 8% D_2O in CD_3CN) of compound **6**. Couplings are indicated.

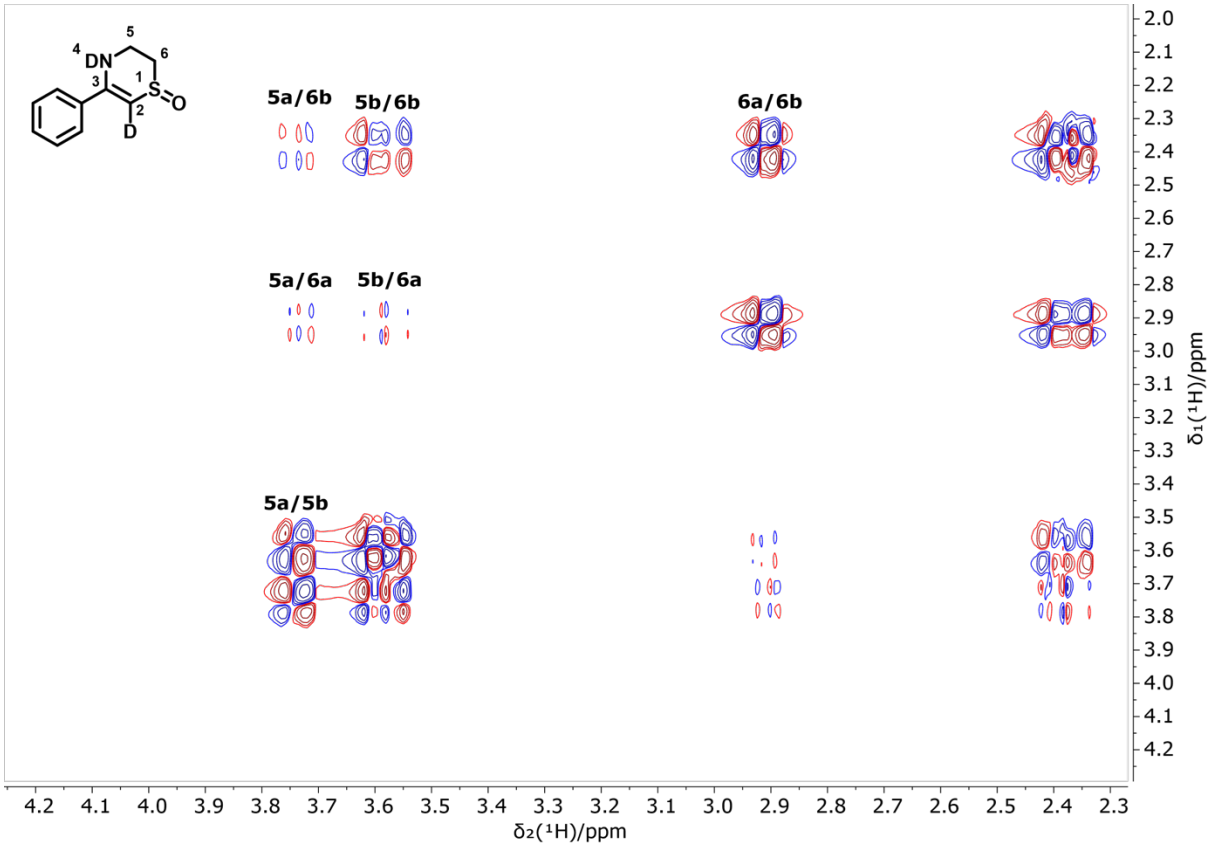
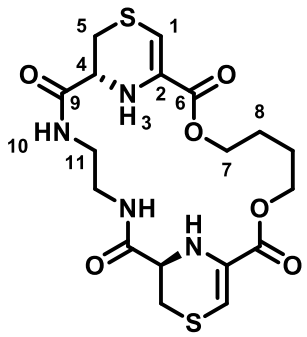


Figure S25. ^{1}H-COSY-NMR spectrum (400 MHz, 8% D_2O in CD_3CN) of compound **6**. Couplings are indicated.

Table S3. Chemical shifts of **5** in 100% CD_3CN and 50% CD_3CN:D_2O (pD 7) reported in ppm.



Atom	^1H (100%CD_3CN)	^{13}C (100% CD_3CN)	^1H (50% CD_3CN)	^{13}C (50% CD_3CN)
1	6.08	100.8	6.09	101.1
2	-	129.4	-	127.9
3	4.95	-	-	-
4	4.35	56.9	4.39	55.4
5	2.40, 3.22	25.6	2.35, 3.18	24.1
6	-	163.3	-	162.9
7	4.16, 4.42	65.6	4.12, 4.36	65.0
8	1.87	26.5	1.81	25.0
9	-	172.3	-	172.9
10	6.96	-	-	-
11	3.14, 3.40	40.2	3.09, 3.40	38.7

Chromatograms of all linear and cyclic peptides

All chromatograms reported below were acquired using the LC-MS method A. Compounds were identified by their corresponding *m*/*z* ratio and are labelled on each chromatogram.

Analytical chromatograms of purified peptides 1 – 4 and 7

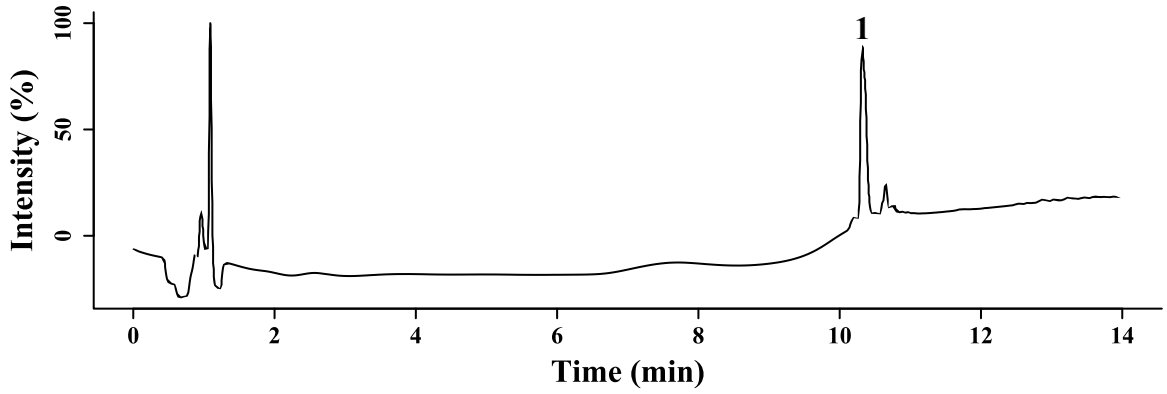


Figure S26. 214 nm chromatogram of purified **1** showing linear peptide (10.32 min).

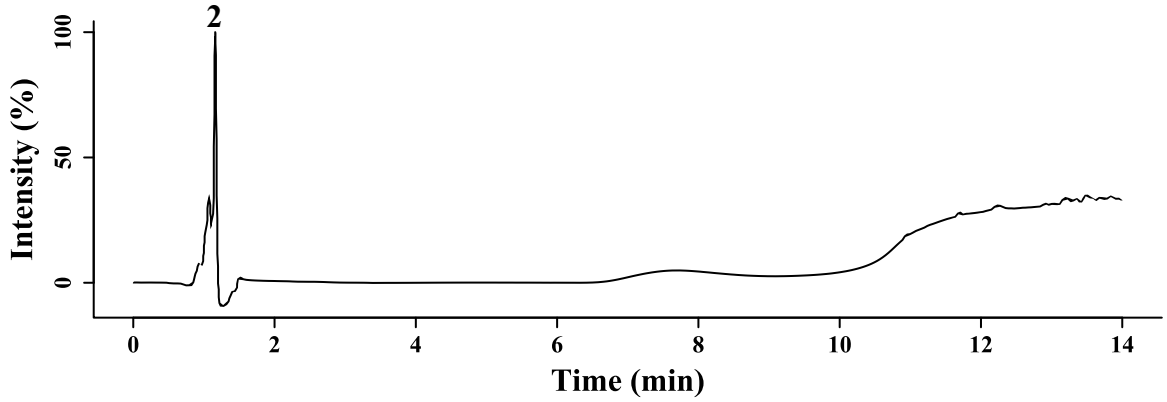


Figure S27. 214 nm chromatogram of purified **2** showing linear peptide (1.16 min).

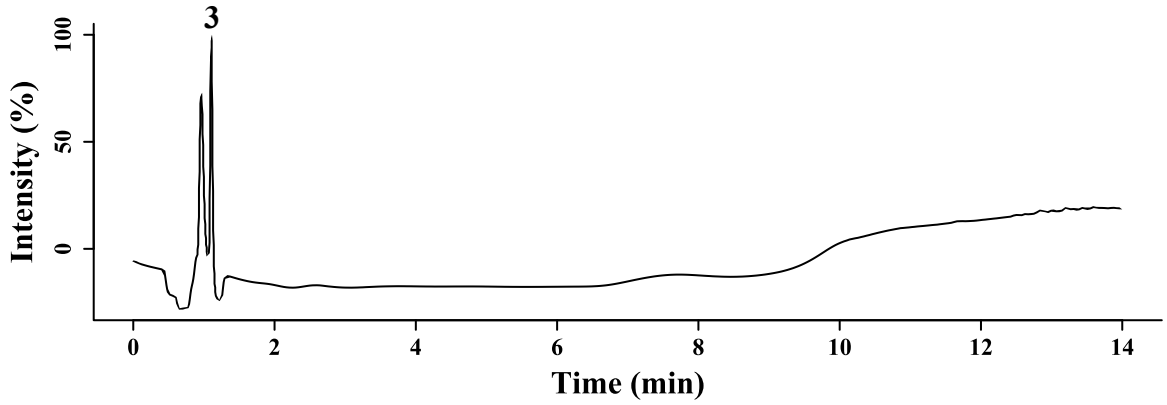


Figure S28. 214 nm chromatogram of purified **3** showing linear peptide (1.10 min).

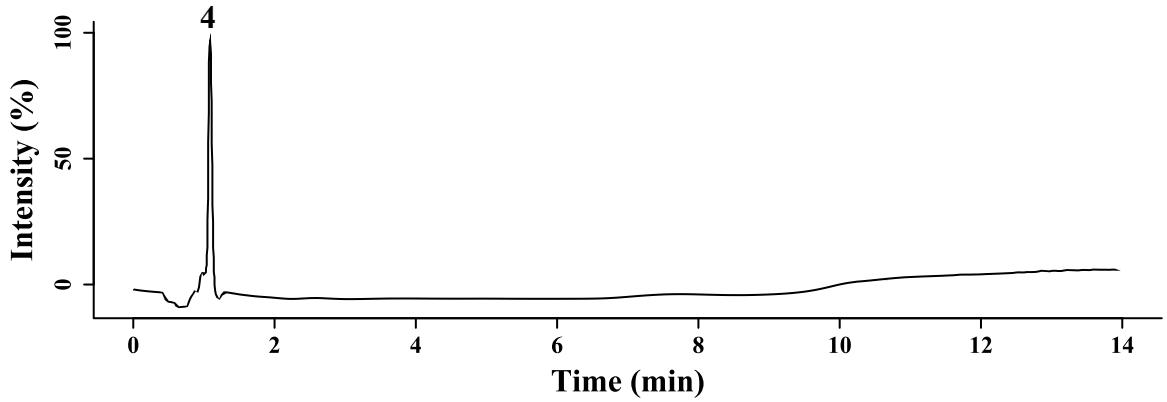


Figure S29. 214 nm chromatogram of purified **4** showing linear peptide (1.09 min).

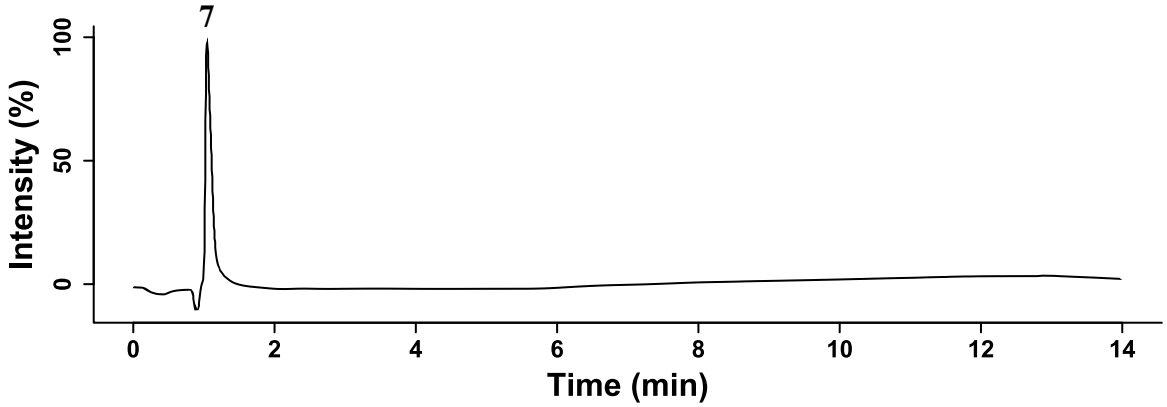


Figure S30. 214 nm chromatogram of purified **7** showing linear peptide (1.04 min).

Analytical chromatograms of purified stapled peptides

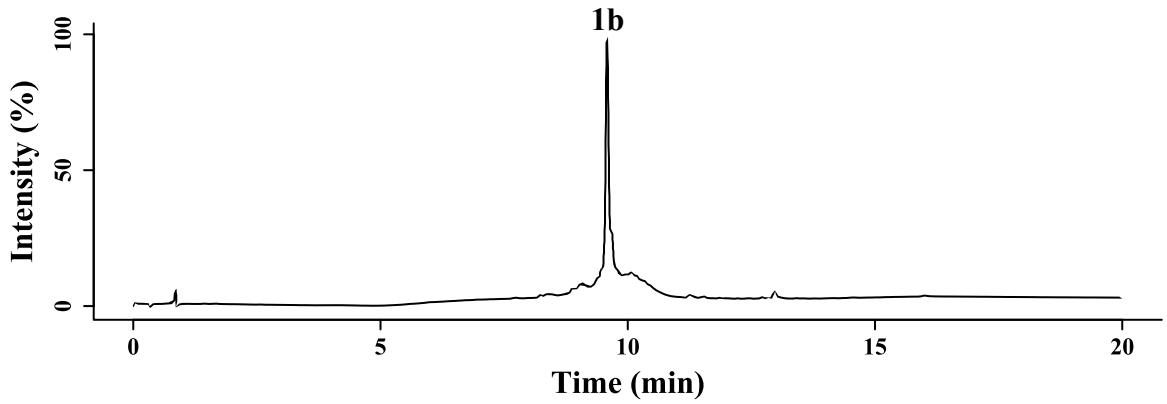


Figure S31. 254 nm chromatogram of purified **1b** showing **1b** (9.58 min).

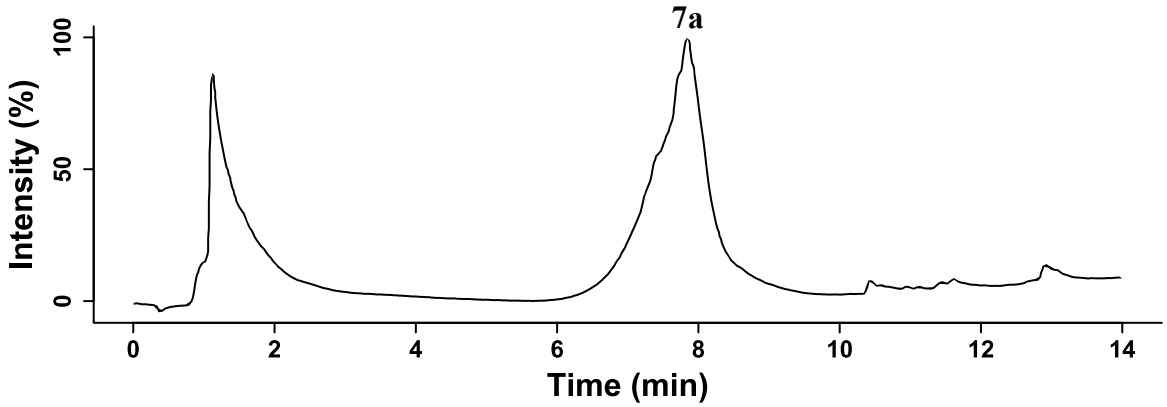


Figure S32. 254 nm chromatogram of purified **7a** showing **7a** (7.85 min).

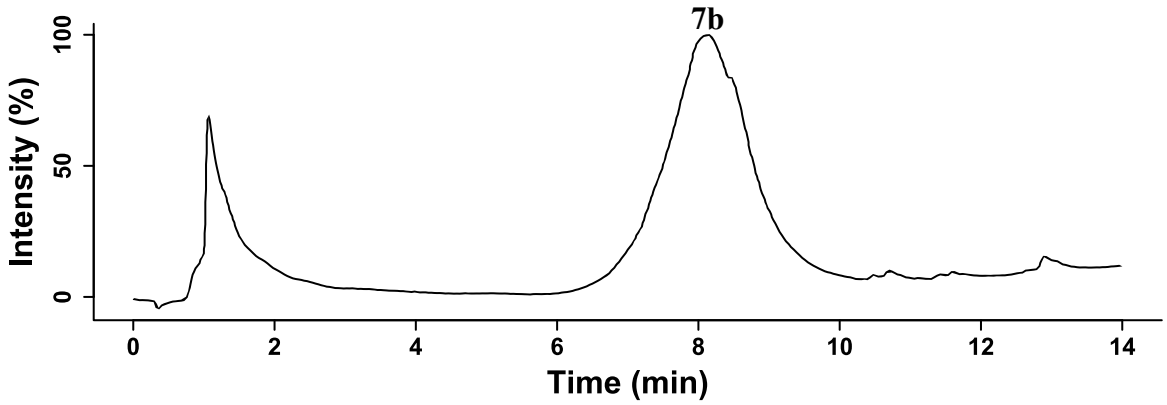


Figure S33. 254 nm chromatogram of purified **7b** showing **7b** (8.14 min).

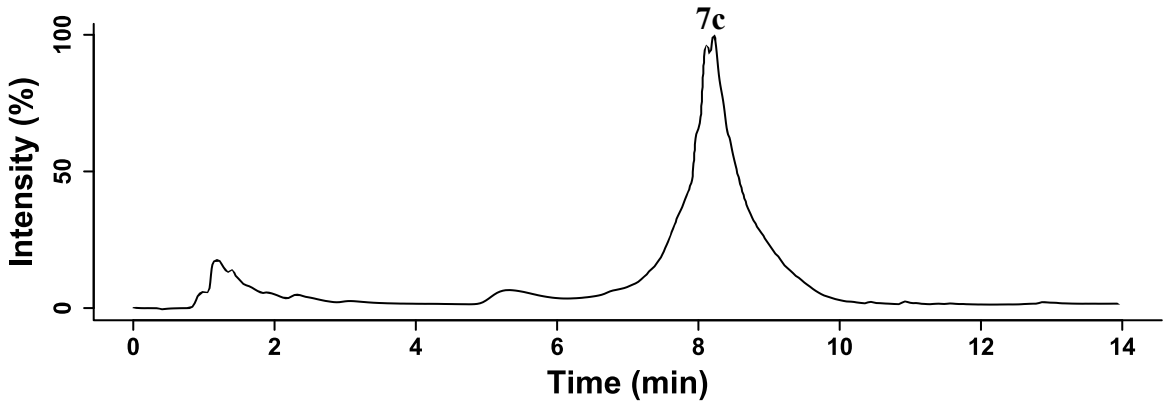


Figure S34. 254 nm chromatogram of purified **7c** showing **7c** (8.12 min).

Analytical chromatograms of *in situ* stapled peptides using linker a

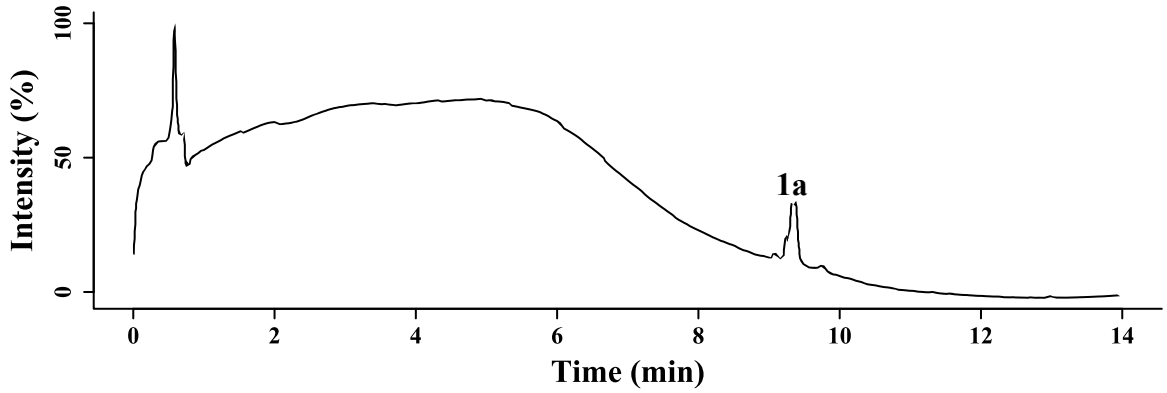


Figure S35. 254 nm chromatogram of crude **1a** after 1 h showing **1a** (9.38 min).

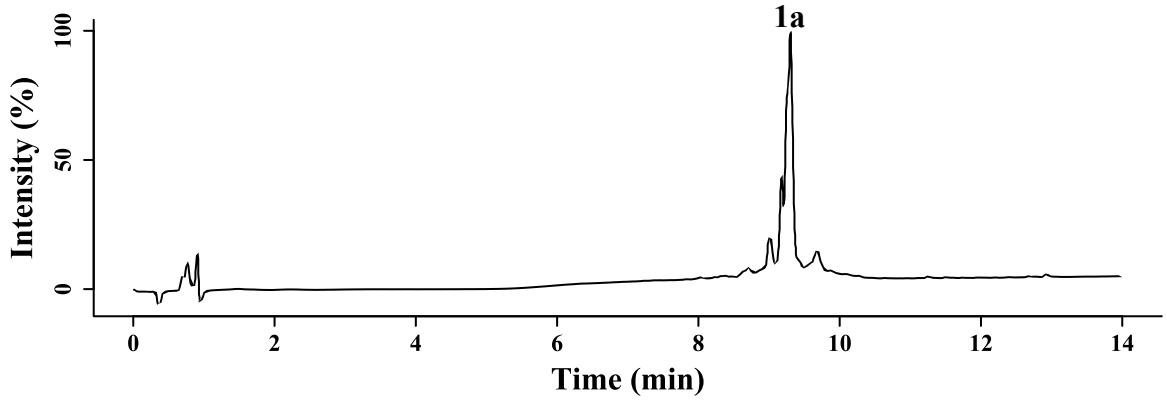


Figure S36. 254 nm chromatogram of crude **1a** after 24 h showing **1a** (9.30 min).

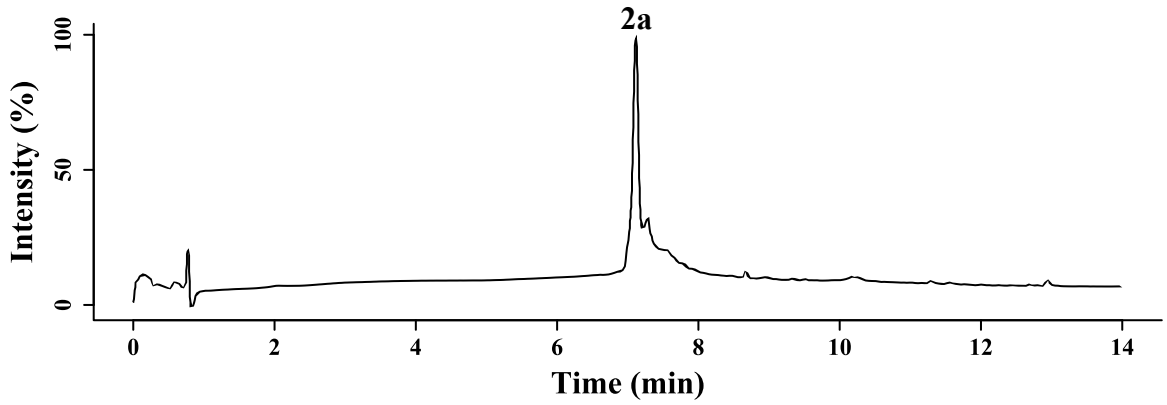


Figure S37. 254 nm chromatogram of crude **2a** after 1 h showing **2a** (7.12 min).

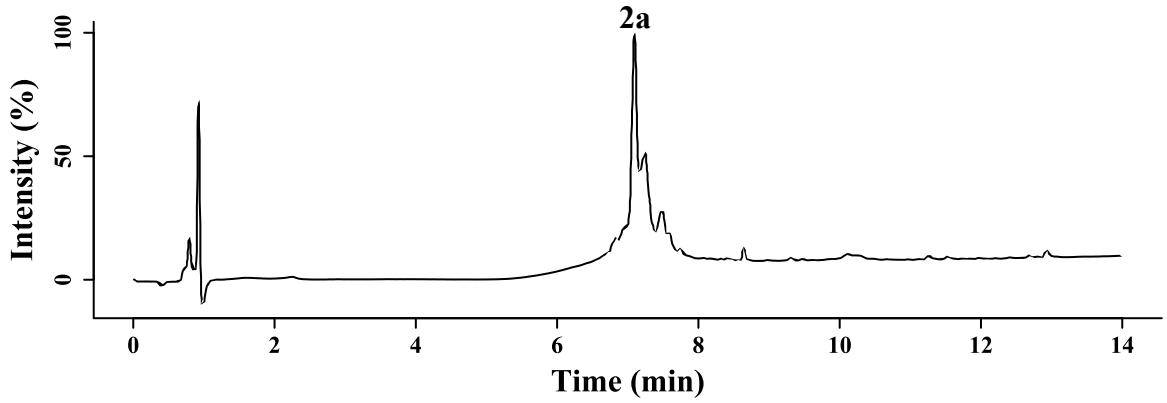


Figure S38. 254 nm chromatogram of crude **2a** after 24 h showing **2a** (7.10 min).

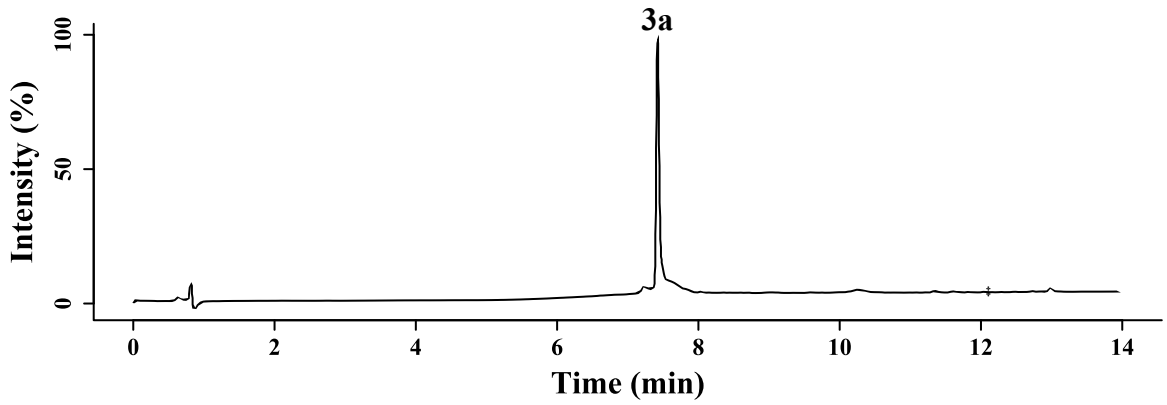


Figure S39. 254 nm chromatogram of crude **3a** after 1 h showing **3a** (7.42 min).

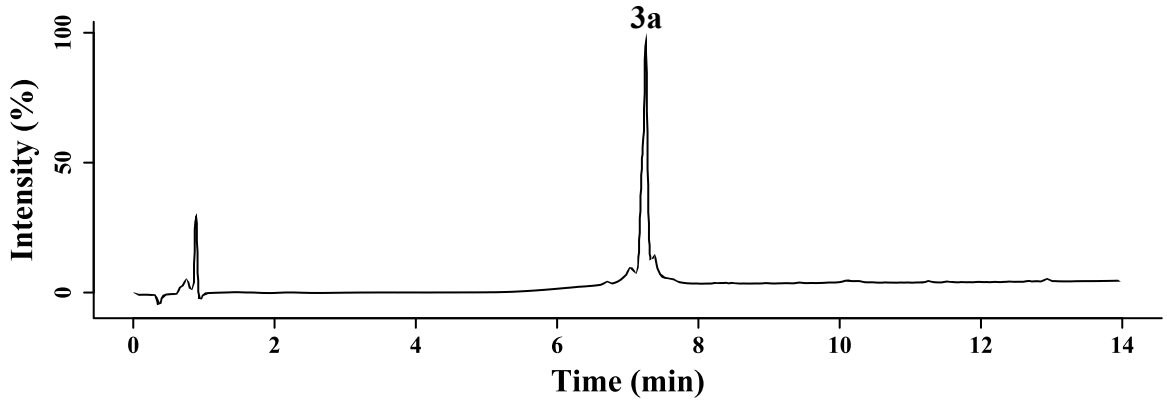


Figure S40. 254 nm chromatogram of crude **3a** after 24 h showing **3a** (7.25 min).

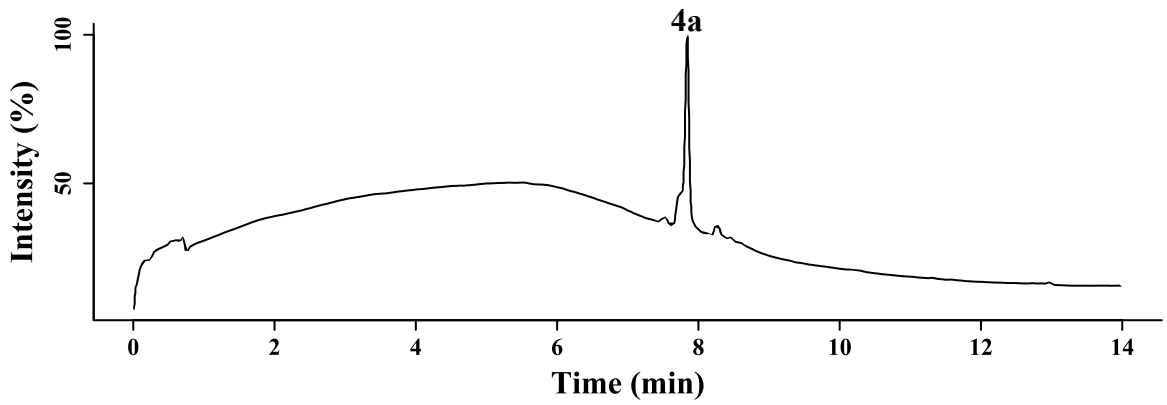


Figure S41. 254 nm chromatogram of crude **4a** after 1 h showing **4a** (7.84 min).

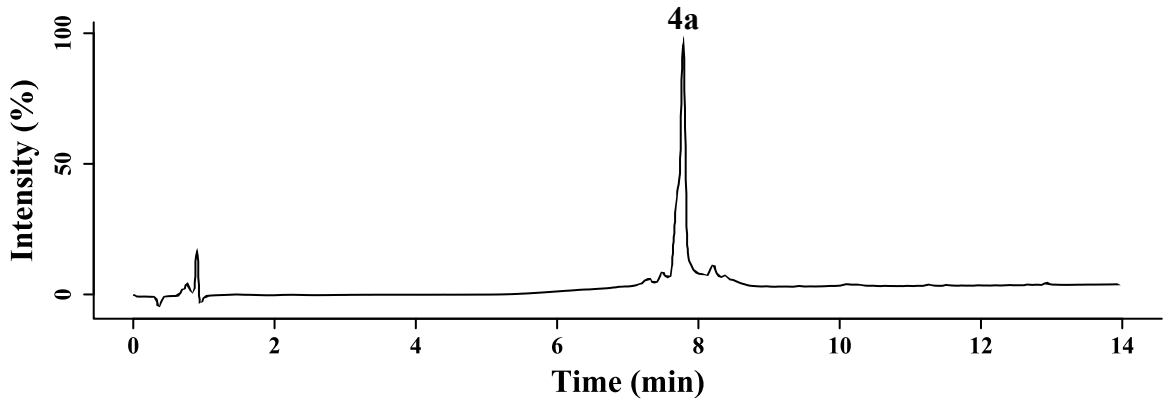


Figure S42. 254 nm chromatogram of crude **4a** after 24 h showing, **4a** (7.79 min).

Analytical chromatograms of *in situ* stapled peptides using linker b

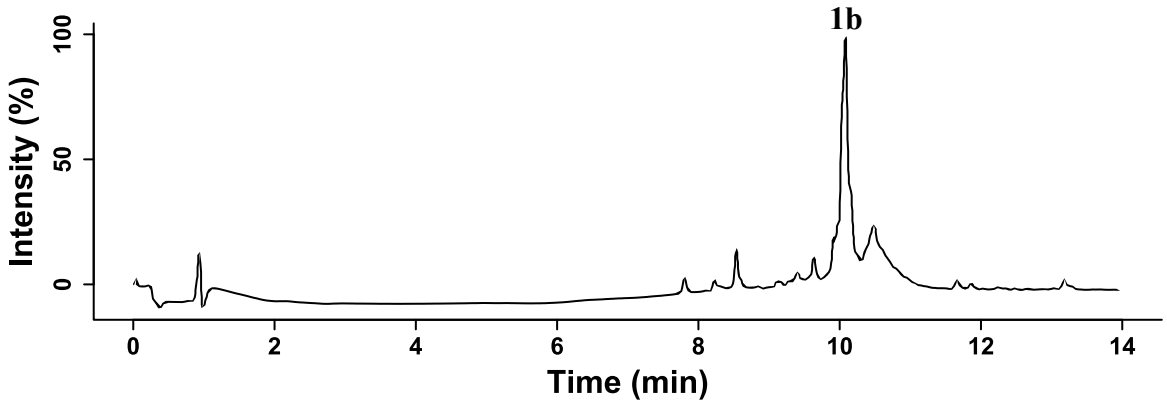


Figure S43. 254 nm chromatogram of crude **1b** after 1 h showing **1b** (10.08 min).

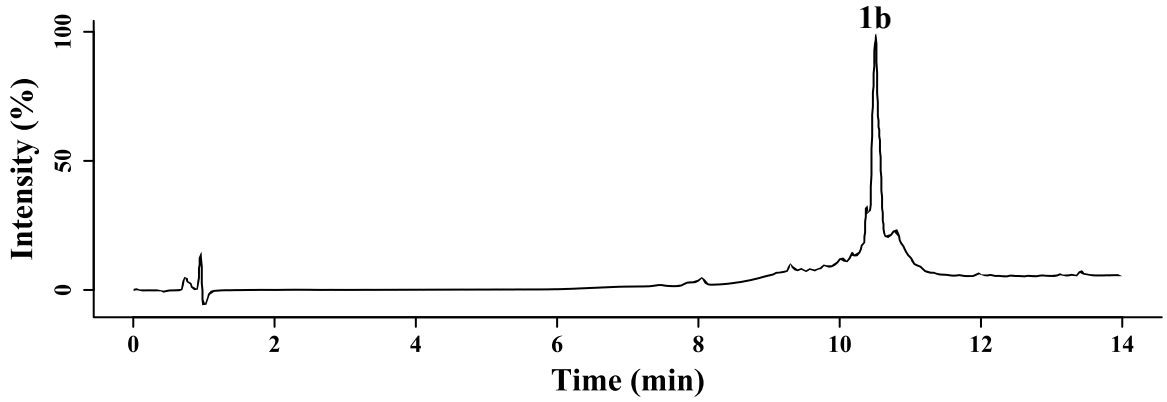


Figure S44. 254 nm chromatogram of crude **1b** after 24 h showing **1b** (10.51 min).

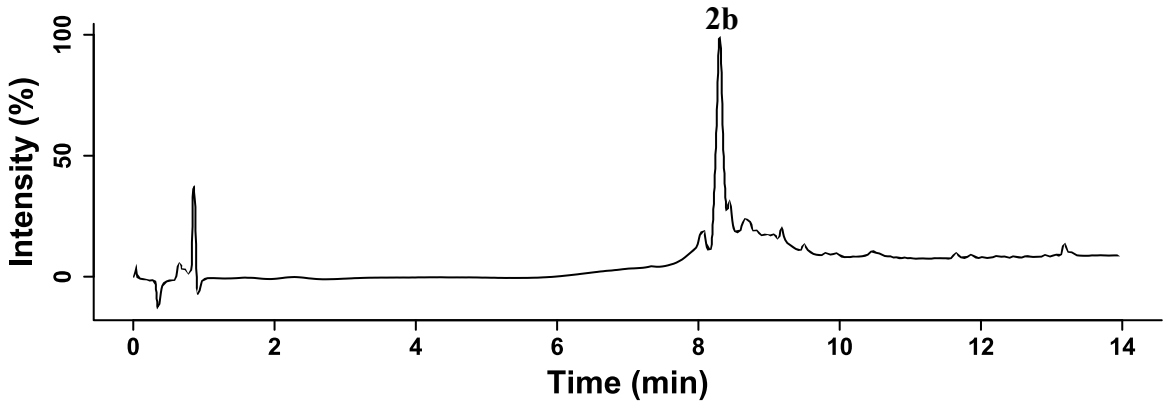


Figure S45. 254 nm chromatogram of crude **2b** after 1 h showing **2b** (8.30 min).

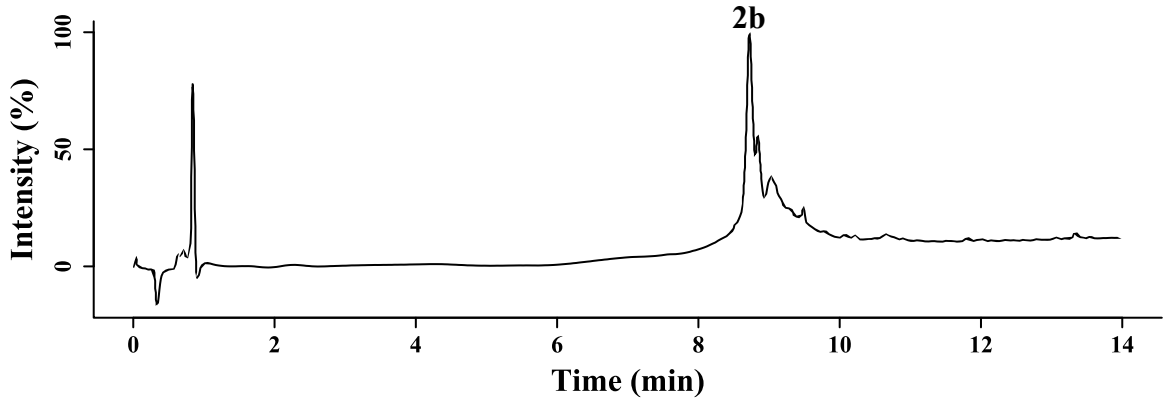


Figure S46. 254 nm chromatogram of crude **2b** after 24 h showing **2b** (8.72 min).

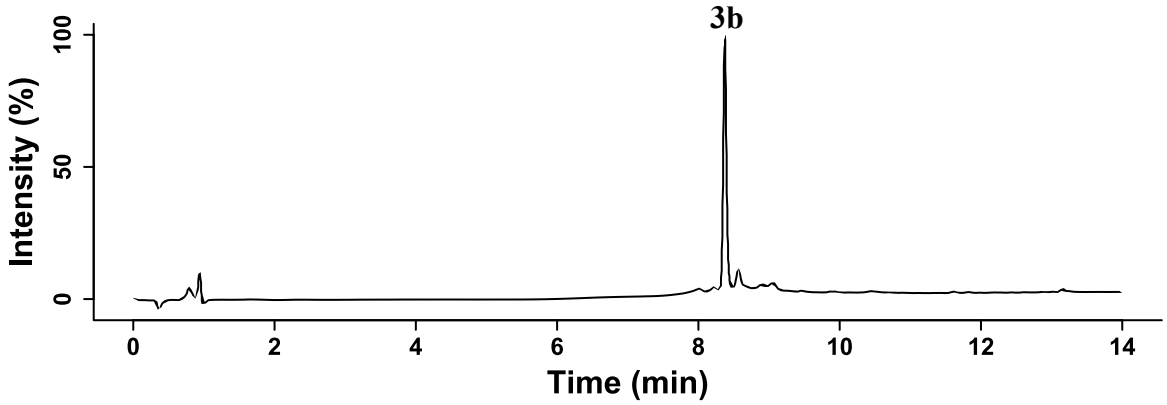


Figure S47. 254 nm chromatogram of crude **3b** after 1 h showing **3b** (8.38 min).

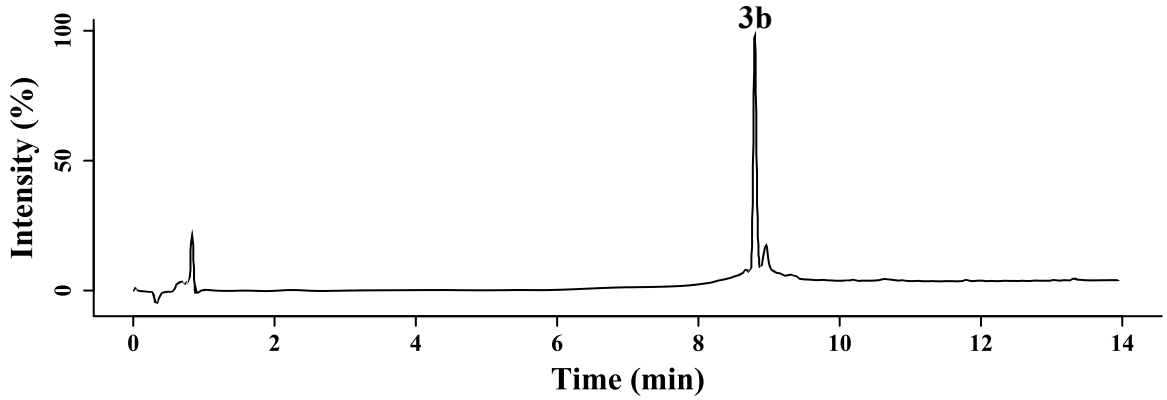


Figure S48. 254 nm chromatogram of crude **3b** after 24 h showing **3b** (8.80 min).

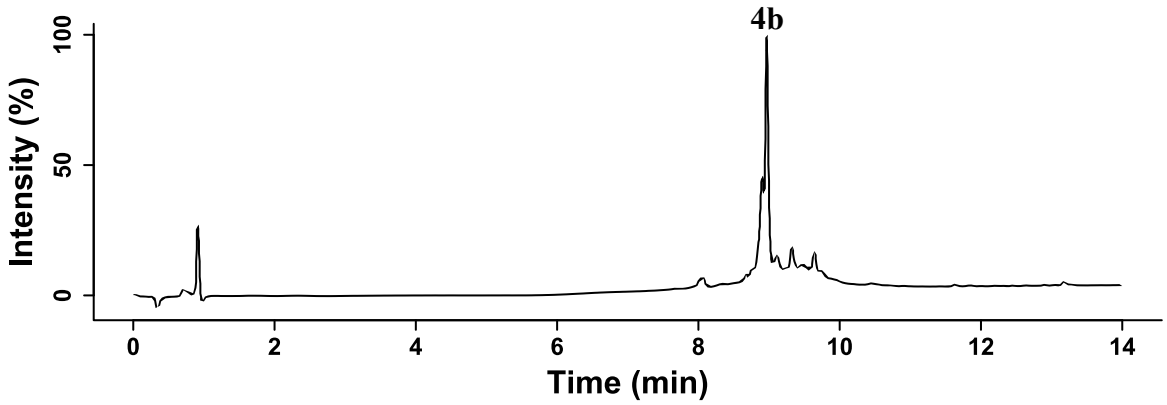


Figure S49. 254 nm chromatogram of crude **4b** after 1 h showing **4b** (8.97 min).

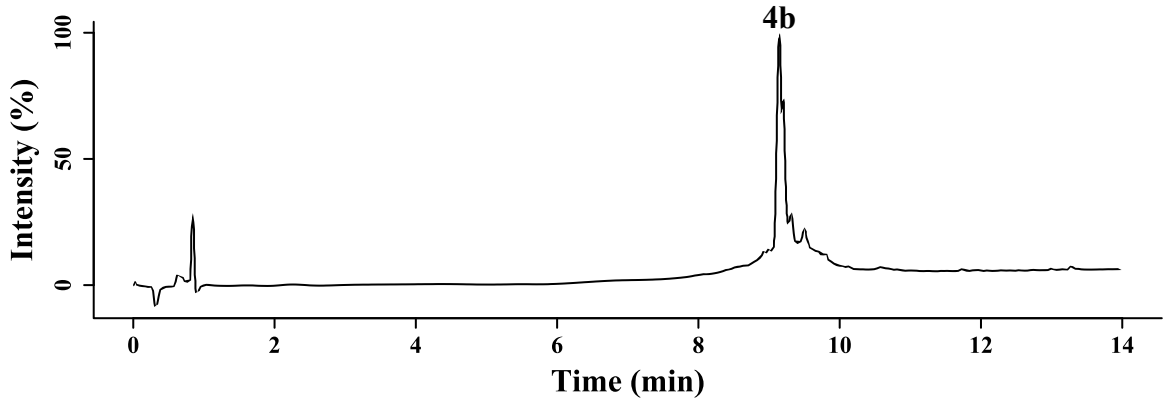


Figure S50. 254 nm chromatogram of crude **4b** after 24 h showing **4b** (9.20 min).

Analytical chromatograms of *in situ* stapled peptides using linker c

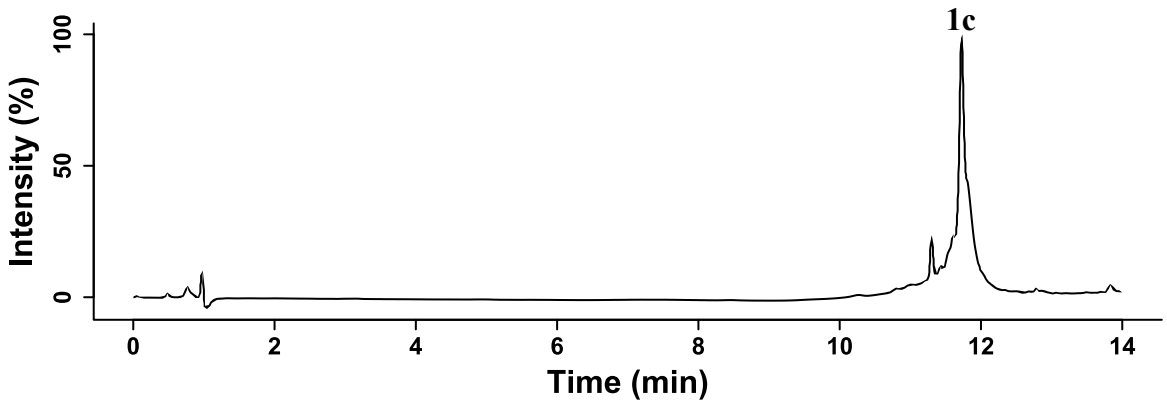


Figure S51. 254 nm chromatogram of crude **1c** after 1 h showing **1c** (11.72 min).

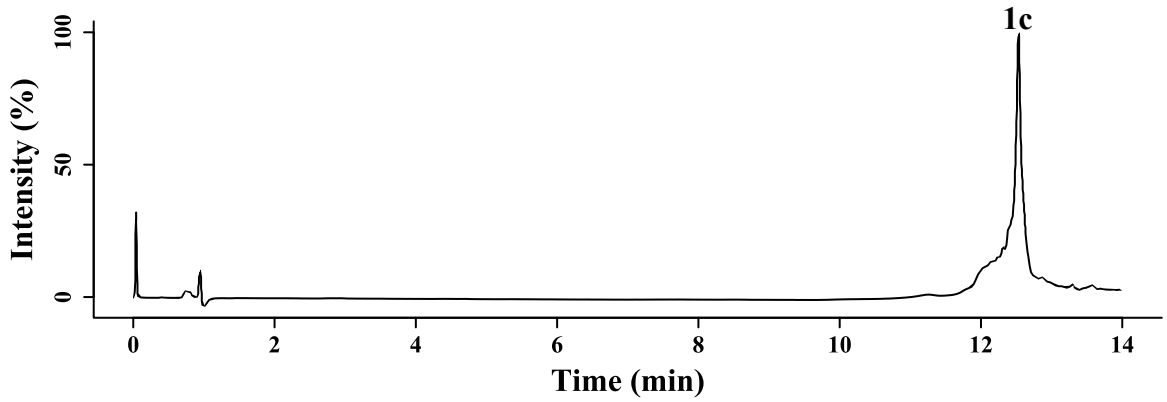


Figure S52. 254 nm chromatogram of crude **1c** after 24 h showing **1c** (12.53 min).

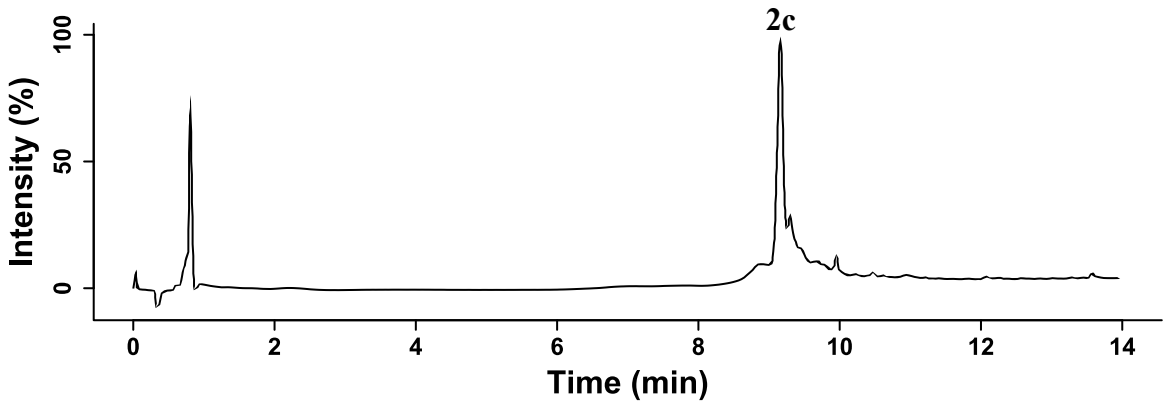


Figure S53. 254 nm chromatogram of crude **2c** after 1 h showing **2c** (9.16 min).

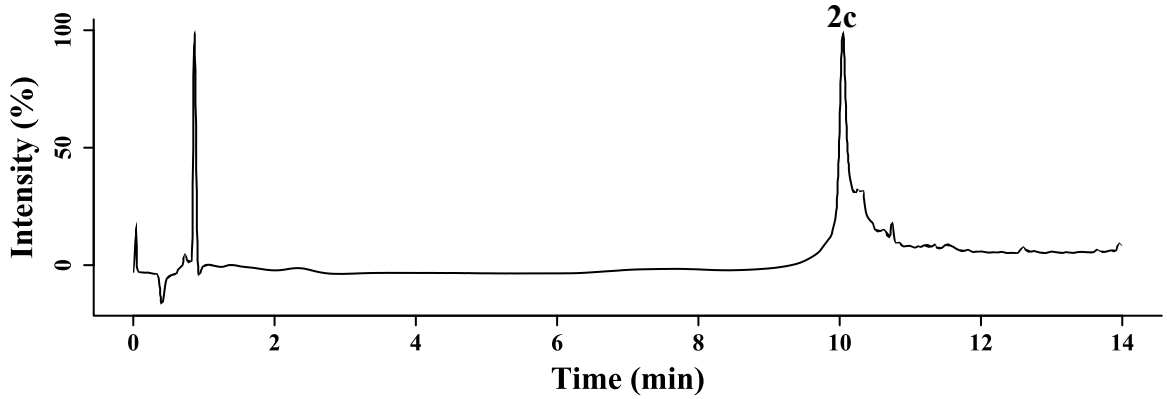


Figure S54. 254 nm chromatogram of crude **2c** after 24 h showing **2c** (10.05 min).

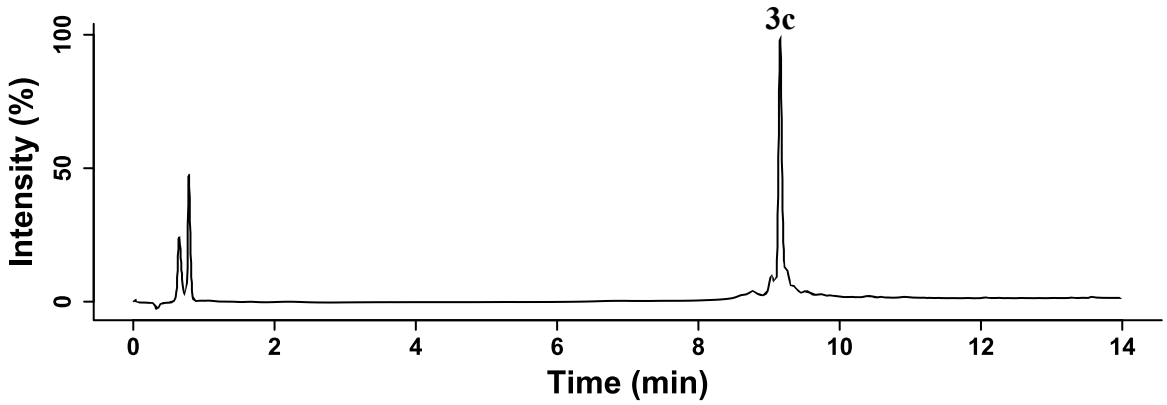


Figure S55. 254 nm chromatogram of crude **3c** after 1 h showing **3c** (9.16 min).

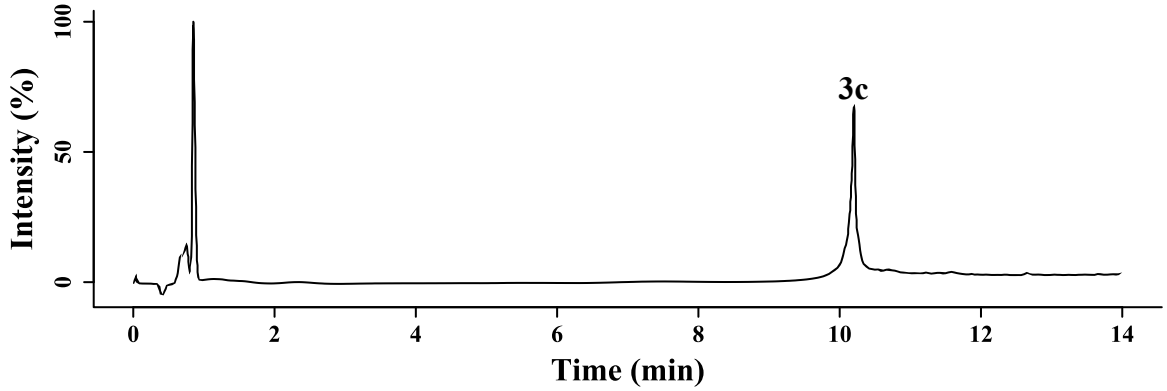


Figure S56. 254 nm chromatogram of crude **3c** after 24 h showing **3c** (10.20 min).

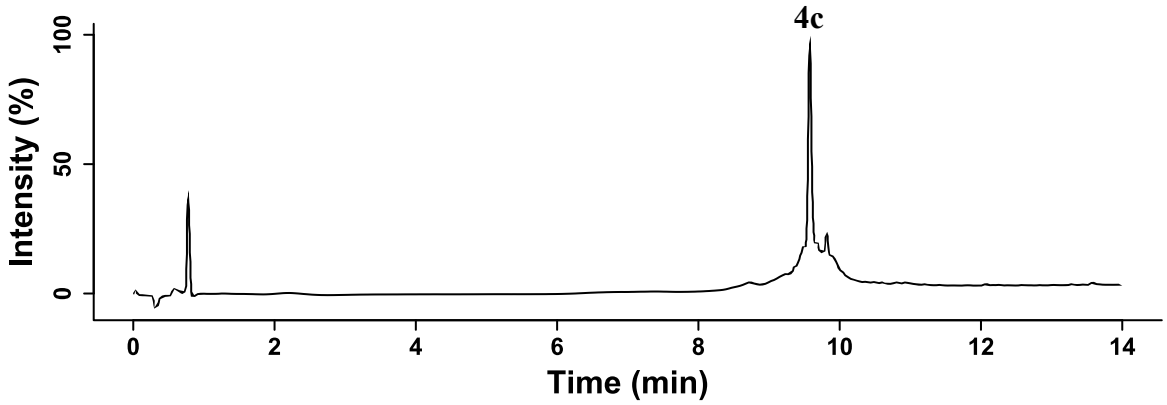


Figure S57. 254 nm chromatogram of crude **4c** after 1 h showing **4c** (9.58 min).

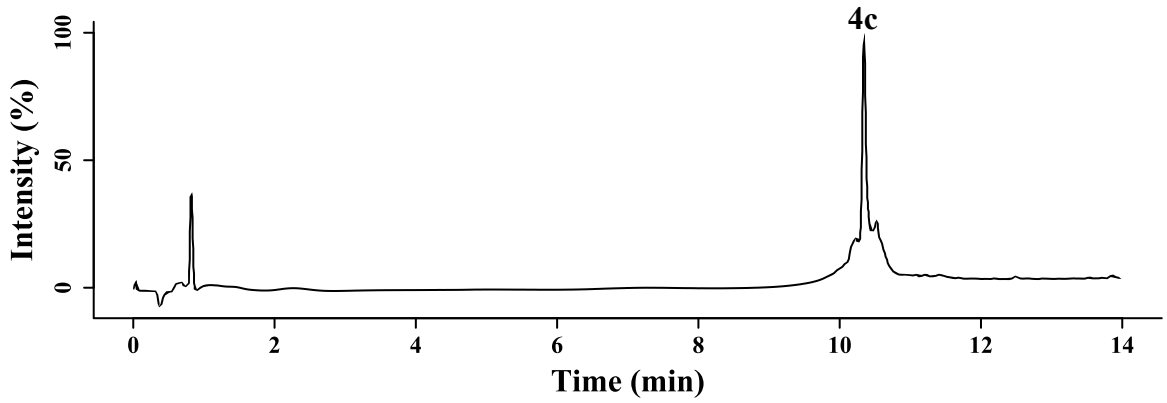


Figure S58. 254 nm chromatogram of crude **4c** after 24 h showing **4c** (10.34 min).

Analytical chromatograms of *in situ* stapled peptides using linker d

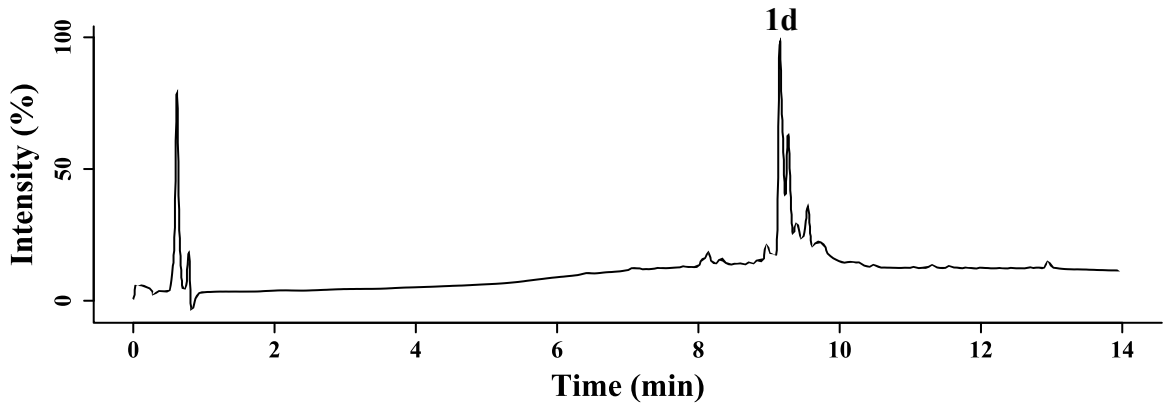


Figure S59. 254 nm chromatogram of crude **1d** after 1 h showing 1**d** (9.15 min).

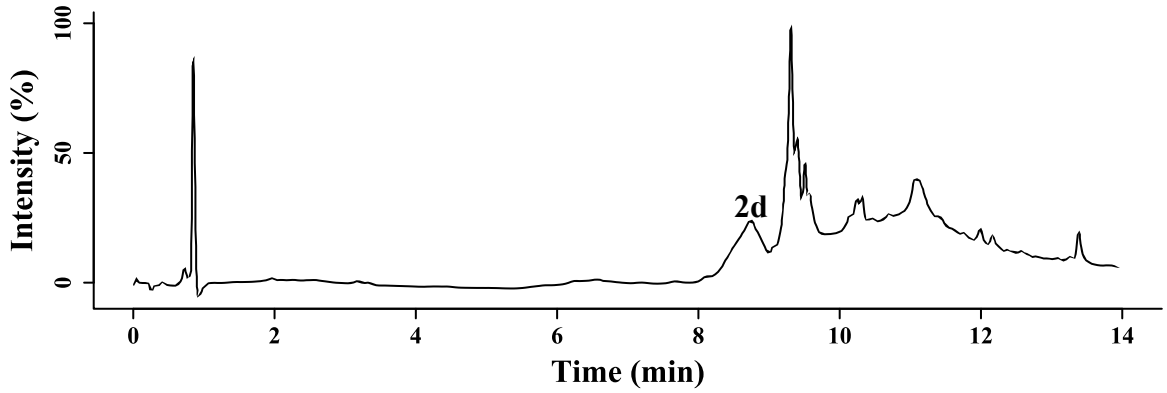


Figure S60. 254 nm chromatogram of crude **2d** after 1 h showing **2d** (8.74 min).

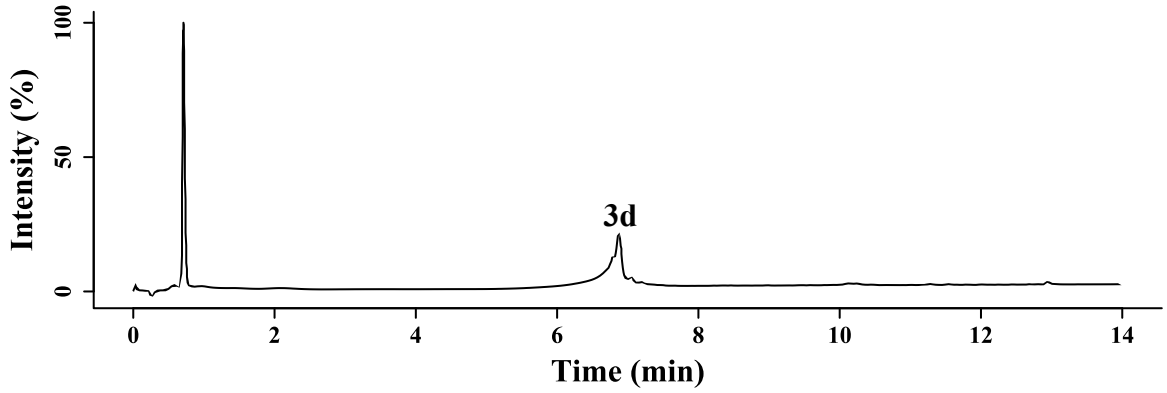


Figure S61. 254 nm chromatogram of crude **3d** after 1 h showing **3d** (6.88 min).

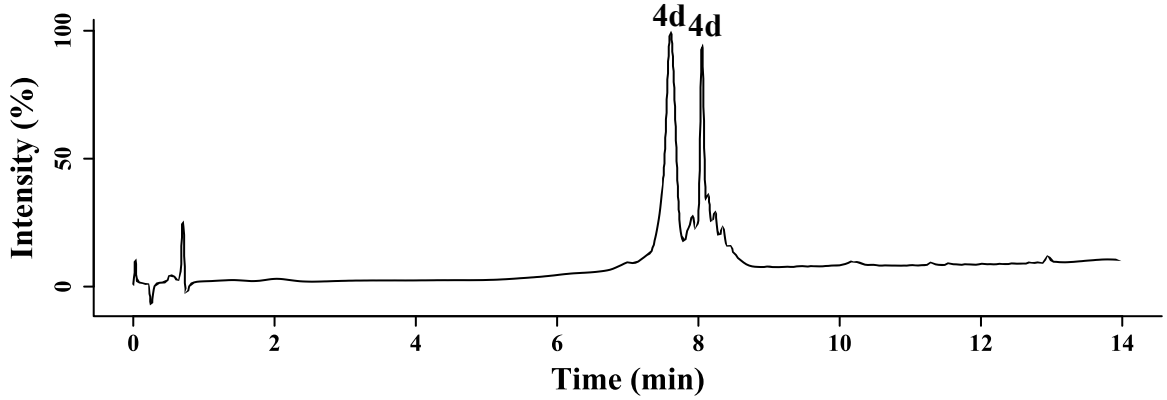


Figure S62. 254 nm chromatogram of crude **4d** after 1 h showing **4d** (7.61 and 8.05 min).

Analytical chromatograms of *in situ* stapled peptides using linker e

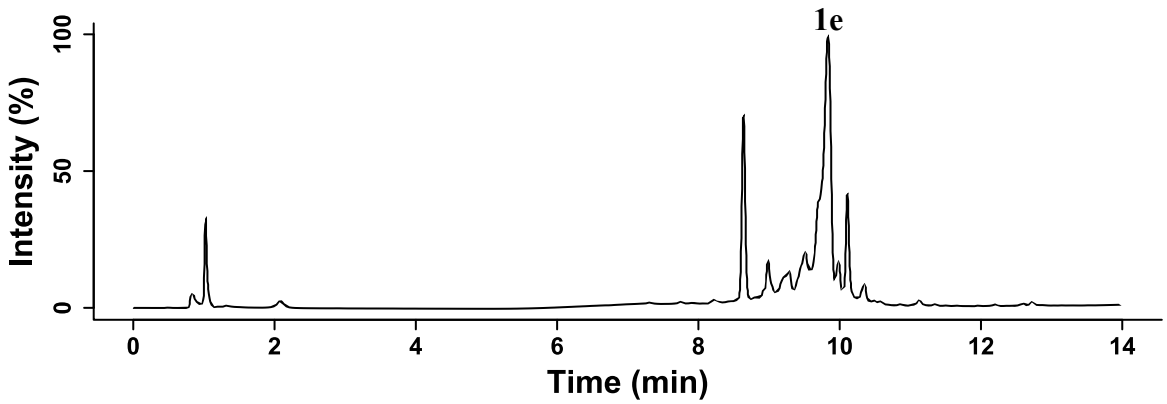


Figure S63. 254 nm chromatogram of crude **1e** after 1 h showing **1e** (9.84 min).

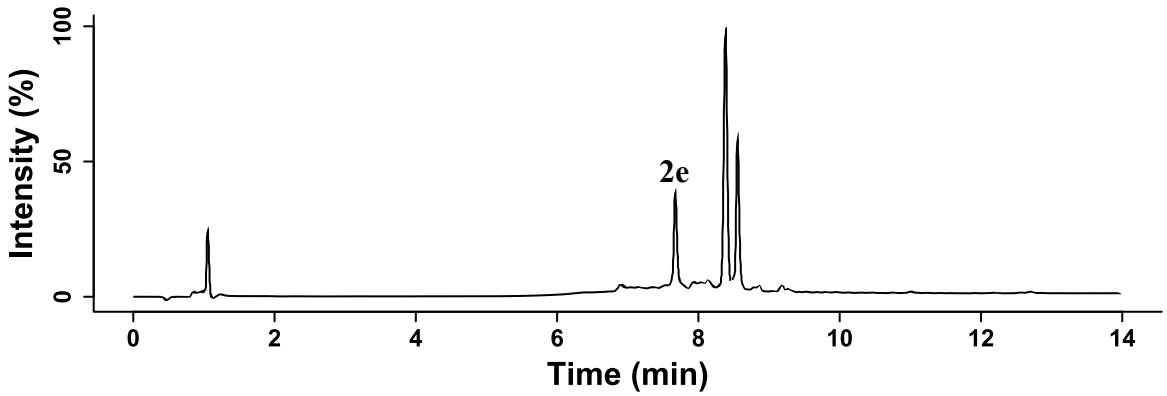


Figure S64. 254 nm chromatogram of crude **2e** after 1 h showing **2e** (7.67 min).

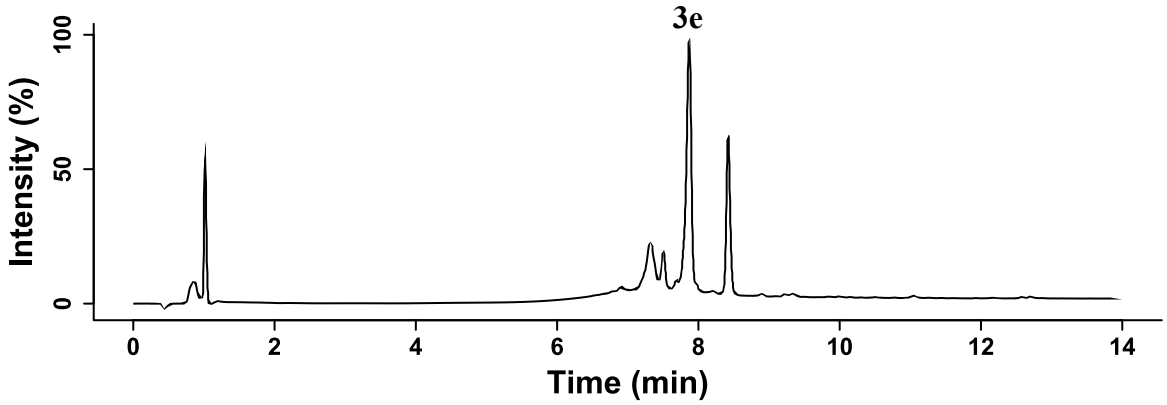


Figure S65. 254 nm chromatogram of crude **3e** after 1 h showing **3e** (7.87 min).

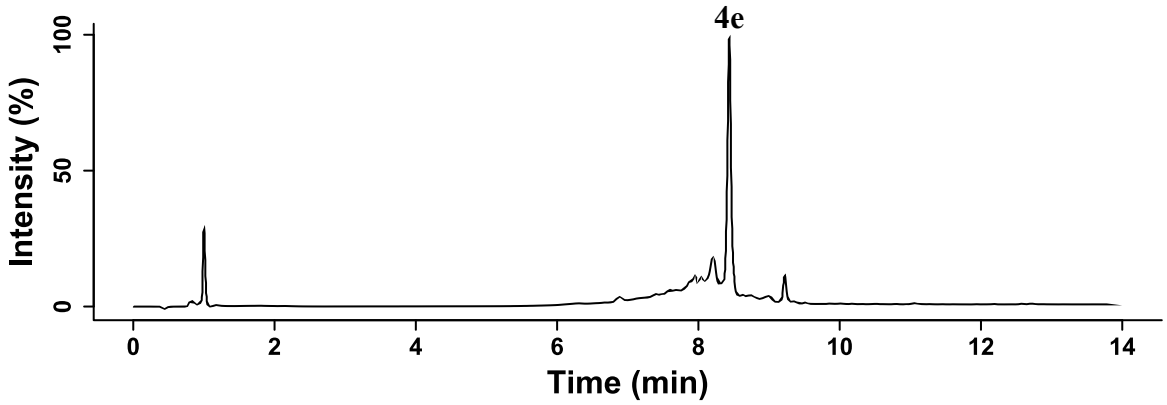


Figure S66. 254 nm chromatogram of crude **4e** after 1 h showing **4e** (8.44 min).

Analytical chromatograms of *in situ* stapled peptides using linker f

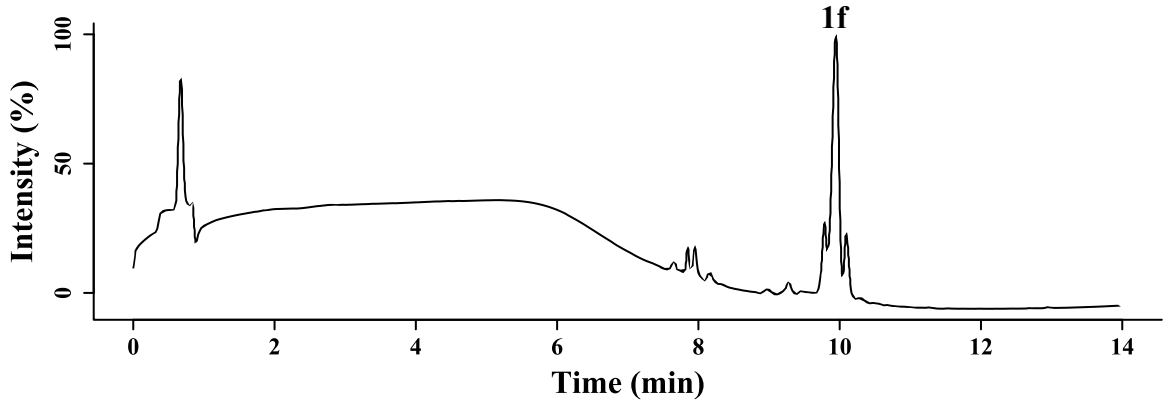


Figure S67. 254 nm chromatogram of crude **1f** after 1 h showing **1f** (9.95 min).

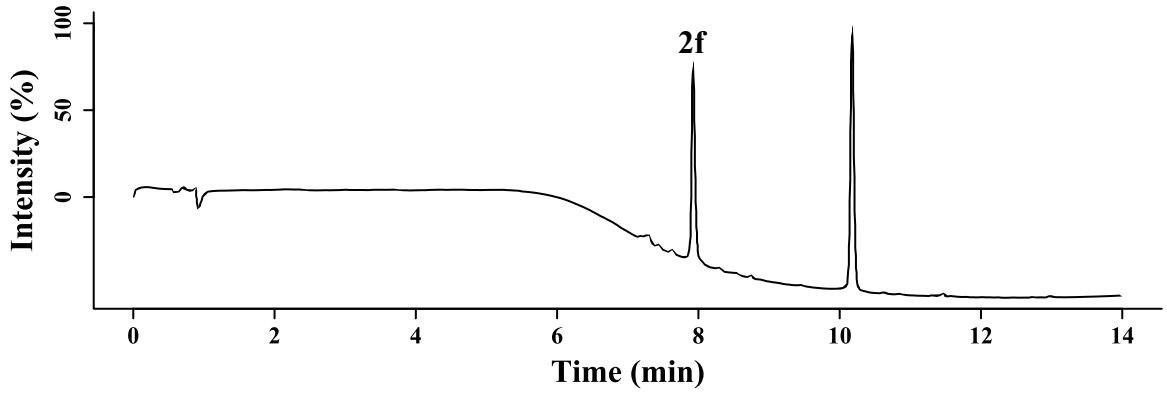


Figure S68. 254 nm chromatogram of crude **2f** after 1 h showing **2f** (7.92 min).

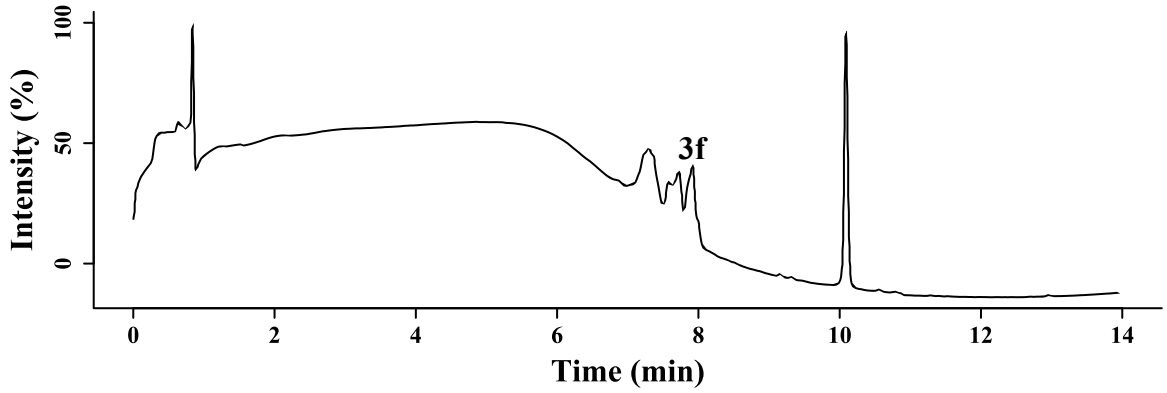


Figure S69. 254 nm chromatogram of crude **3f** after 1 h showing **3f** (7.92 min).

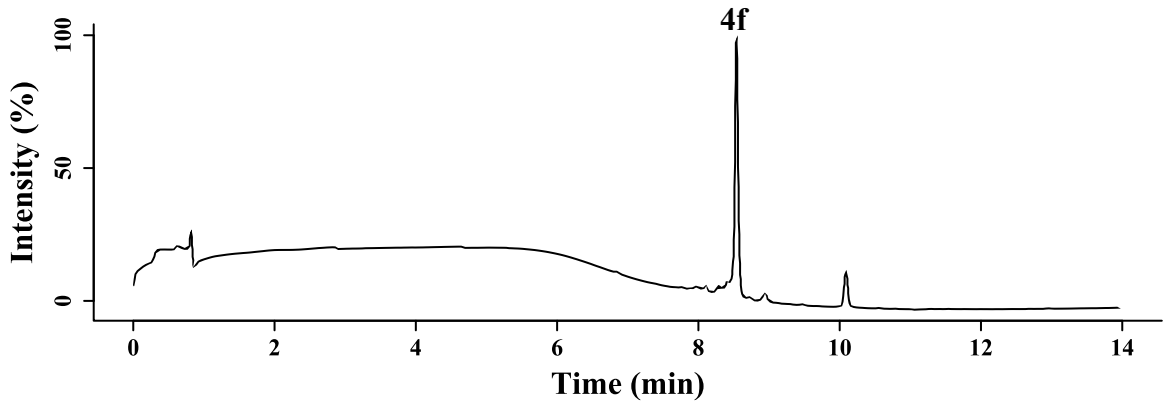


Figure S70. 254 nm chromatogram of crude **4f** after 1 h showing **4f** (8.54 min).

References

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2 Morewood R, Nitsche C. *Chem Sci*., 2020, **12**, 669-674.

3 Zhang Z, Li Y, Loh YR, Phoo WW, Hung AW, Kang C, Luo D. *Science* 2016, **354**, 1597-1600.