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Macrocyclisation and functionalisation of unprotected peptides via divinyltriazine cysteine stapling

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1. General Experimental Procedures

All reagents and solvents were used as received unless otherwise stated. CH_2Cl_2, EtOAc, MeOH, MeCN and toluene were distilled from CaH_2. Tetrahydrofuran (THF) was dried over Na wire and distilled from a mixture of $LiAlH_4$ and CaH_2 with triphenylmethane as the indicator. Et_2O was distilled from a mixture of $LiAlH_4$ and CaH_2. Petroleum ether was distilled before use, with pet. ether 40–60 referring to the fraction between 40–60 °C. Peptide grade dimethylformamide (DMF) was purchased from Cambridge Reagents.

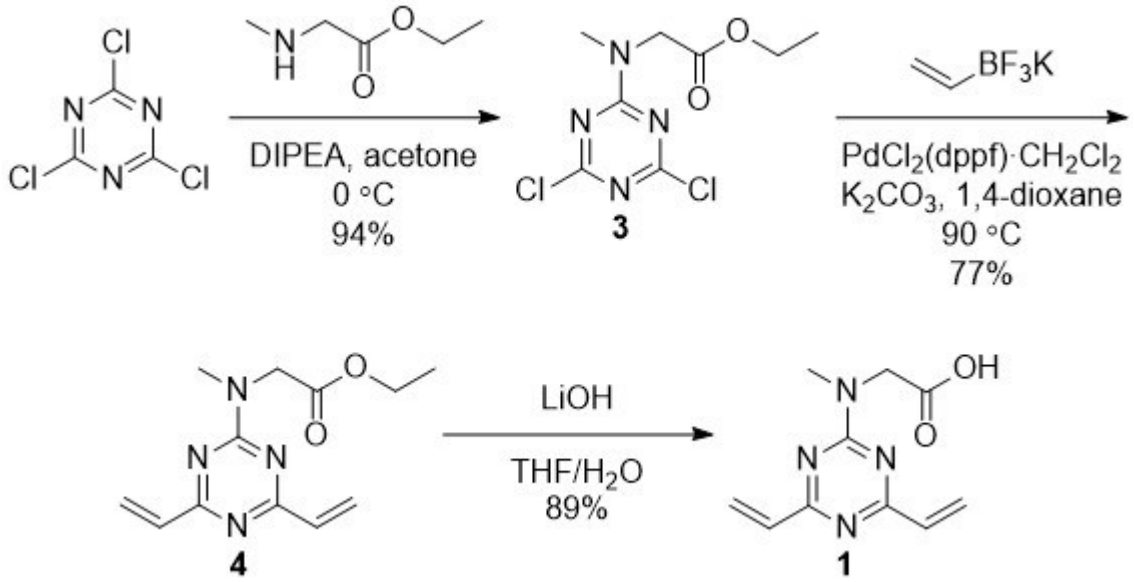
TLC analysis was performed on commercially prepared glass plates precoated with Merck silica gel F254. Visualisation was by the quenching of ultraviolet (UV) fluorescence (λmax = 254 nm) or by staining with potassium permanganate or ninhydrin. Retention factors (Rf) are quoted to the nearest 0.01. LCMS analysis was performed on a Waters ACQUITY H-Class UPLC with an ESCi MultiMode Ionisation Waters SQ Detector 2 spectrometer using MassLynx 4.1 software; LC system: solvent A: 2 mM NH_4OAc in H_2O/MeCN 95:5; solvent B: MeCN; solvent C: 2% HCO_2H; gradient: A/B/C, 90:5:5–0:95:5 over 1 min at a flow rate of 0.6 mL/min.

Flash column chromatography (FCC) was carried out using slurry-packed Merck 938 Keiselgel 60 SiO_2 (230–400 mesh) under a positive pressure of compressed air.

Infrared (IR) spectra were recorded neat on a Perkin-Elmer Spectrum One (FTIR) spectrometer with internal referencing. Selected absorption maxima (vmax) are reported in wavenumbers (cm^{-1}) with the following abbreviations: w, weak; m, medium; s, strong. Melting points (mp) were obtained on a Büchi B-545 melting point apparatus and are uncorrected.

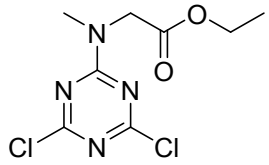
Analytical HPLC was run on an Agilent 1260 Infinity using a Supelcosil ABZ+PLUS column (150 mm × 4.6 mm, 3 μm) eluting with a linear gradient system (solvent A: 0.05% (v/v) TFA in H2O, solvent B: 0.05% (v/v) TFA in MeCN) over 15 minutes at a flow rate of 1 mL/min. Semi-preparative HPLC was run on an Agilent 1260 Infinity using a Supelcosil ABZ+PLUS column (250 mm × 21.2 mm, 5 μm) eluting with a linear gradient system (solvent A: 0.1% (v/v) TFA in H_2O, solvent B: 0.05% (v/v) TFA in MeCN) over 20 minutes at a flow rate of 20 mL/min. HPLC was monitored by UV absorbance at 220 and 254 nm. Retention times (R_t) are reported to the nearest 0.01 min.

2. Synthetic Procedures



SI Scheme 1: Synthesis of divinyltriazine linker **1** from commercially available cyanuric chloride.

ethyl *N*-(4,6-dichloro-1,3,5-triazin-2-yl)-*N*-methylglycinate (3)

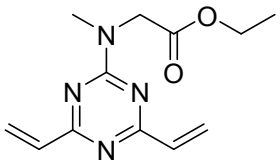


To a solution of cyanuric chloride (500 mg, 2.71 mmol) and sarcosine ethyl ester hydrochloride (416 mg, 2.71 mmol) in acetone (10.0 mL) at 0 ^{0}C was added dropwise *N*,*N*-diisopropylethylamine (0.944 mL, 5.42 mmol). After addition, the reaction mixture was kept stirring at 0 °C for 2 hr. Then the mixture was poured into ice-water, extracted with CH_2Cl_2. The organic layer was dried over anhydrous Na_2SO_4, filtrated and concentrated *in vacuo*. The residue was purified by column chromatography (0-20% EtOAc/petroleum ether 40-60) to provide the title compound **3** as a white solid (673 mg, 94%).

R_f (25% EtOAc/petroleum ether 40-60) = 0.38; **mp**: 76-77 °C; **δ_H*** (400 MHz, $CDCl_3$): 4.36 (s, 2H), 4.24 (q, 2H, *J* = 7.1 Hz), 3.27 (s, 3H), 1.30 (t, 3H, *J* = 7.1 Hz); **δ_C*** (101 MHz, CDCl3): 170.5, 170.0, 169.0, 165.6, 61.8, 50.8, 36.5, 14.2; **IR** ν_{max} (neat)/cm^{-1}: 2993w (C-H), 1734s (C=O), 1580s, 1547s, 1478s, 1418w, 1406w, 1379w, 1353w, 1325m, 1213s, 1171s, 1146m, 1115w, 1045m, 1020w; **HRMS** (ESI) calcd for $[C_8H_{11}Cl_2N_4O_2]^+$: 265.0259, found: 265.0254.

*Extra peaks are due to the presence of rotamers.

ethyl *N*-(4,6-divinyl-1,3,5-triazin-2-yl)-*N*-methylglycinate (4)

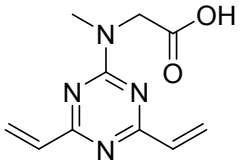


A mixture of **3** (331 mg, 1.25 mmol), potassium vinyltrifluoroborate (504 mg, 3.75 mmol) and K_2CO_3 (1.04 g, 7.50 mmol) in 1,4-dioxane (15 mL) and H_2O (1.5 mL) was degassed with nitrogen for 5 min at ambient temperature. $PdCl_2(dppf)\cdot CH_2Cl_2$ (102 mg, 0.13 mmol) was added at ambient temperature, then the reaction mixture was heated to 90 °C for 6 hr. The reaction mixture was cooled to ambient temperature and filtered through a celite bed under vacuum and washed with EtOAc. The filtrate was then diluted with EtOAc and washed with H_2O. The organic layer was washed with a saturated aqueous solution of NaCl, dried over anhydrous Na_2SO_4, filtered and concentrated *in vacuo*. The residue was purified by column chromatography (silica gel, gradient elution: 0:100 EtOAc:pet ether to 20:80 EtOAc:pet ether) to provide the title compound **4** as a pale-yellow oil (240 mg, 77%). This oil was employed immediately for the next step.

R_f (25% EtOAc/petroleum ether 40-60) = 0.53 **δ_H*** (500 MHz, d_6-DMSO, rt): 6.72-6.48 (m, 4H), 5.87-5.80 (m, 2H), 4.42 (s, 2H), 4.12 (q, 2H, *J* = 7.1 Hz), 3.22 (s, 3H), 1.18 (t, 3H, *J* = 7.1 Hz); **δ_C*** (126 MHz, d_6-DMSO, rt): 170.0, 169.5, 169.3, 165.1, 135.9, 135.8, 126.8, 126.7, 60.5, 50.2, 35.4, 14.1; **IR** ν_{max} (film)/cm^{-1}: 2982w (C-H), 1744m (C=O), 1639w, 1538s, 1509s, 1422w, 1405m, 1374w, 1357w, 1337w, 1281w, 1243w, 1192m, 1082w, 1050w, 1029w; **HRMS** (ESI) calcd for $[C_{12}H_{17}N_4O_2]^+$: 249.1346, found: 249.1336.

*Extra peaks are due to the presence of rotamers.

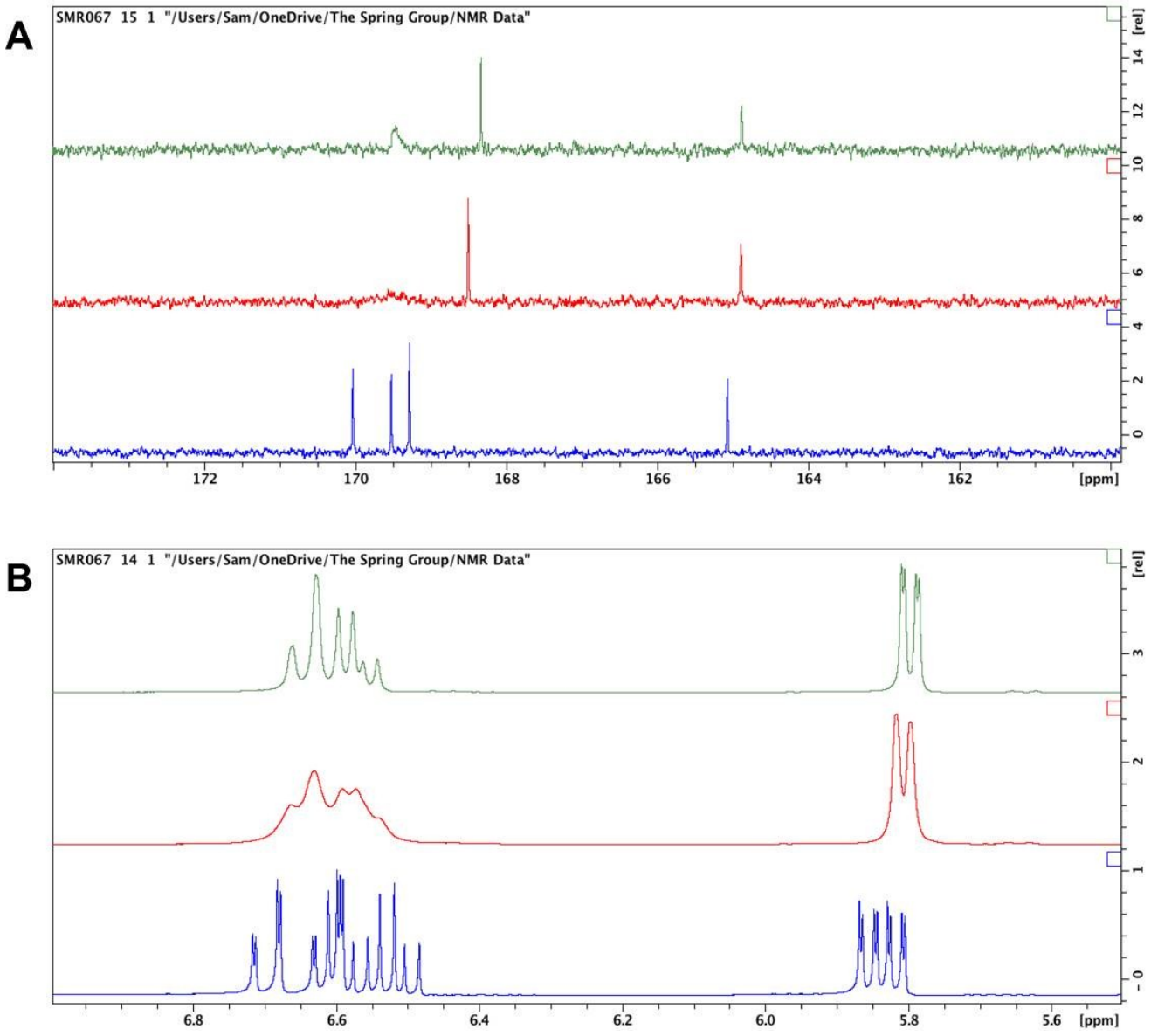
***N*-(4,6-divinyl-1,3,5-triazin-2-yl)-*N*-methylglycine (1)**



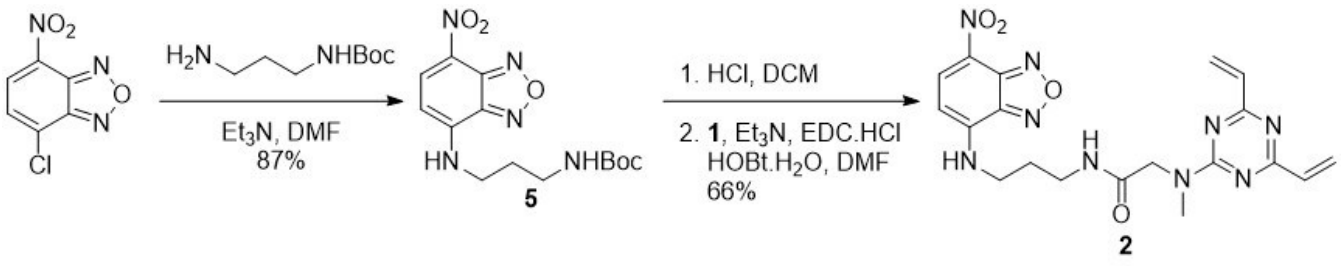
To a solution of **4** (224 mg, 0.90 mmol) in THF (6.0 mL) and H_2O (6.0 mL) at 0 °C was added $LiOH\cdot H_2O$ (38 mg, 0.90 mmol). The mixture was stirred at room temperature for 18 hr, and then diluted with H_2O, washed with Et_2O. The aqueous layer was neutralized with 1 N HCl aq. (0.90 mL, 0.90 mmol) and extracted with CH_2Cl_2. Then the organic layer was dried over anhydrous Na_2SO_4, filtered and concentrated *in vacuo*. The residue was triturated with pet ether to provide the title compound **1** as a white solid (178 mg, 89%).

R_f (20% MeOH/$CHCl_3$) = 0.37; **mp**: >300 °C (decomposed); **δ_H*** (400 MHz, d_6-DMSO): 12.77 (br s, 1H), 6.72-6.49 (m, 4H), 5.87-5.81 (m, 2H), 4.35 (2H, s, NCH_2CO_2), 2.20 (3H, s, NMe); **δ_C*** (101 MHz, d_6-DMSO): 170.8, 170.1, 169.6, 165.2, 136.0, 136.0, 126.9, 126.9, 50.1, 35.5; **IR** ν_{max} (neat)/cm^{-1}: 2494w, 1714m (C=O), 1640w, 1544s, 1504s, 1433w, 1407m, 1353w, 1293w, 1261w, 1223m, 1200m, 1171w, 1217w, 1099, 1069w, 1030w; **HRMS** (ESI) calcd for $[C_{10}H_{13}N_4O_2]^+$: 221.1033, found: 221.1023.

The NMR spectra of the purified triazine-based linkers **3**, **4** and **1**, suggested the presence of rotameric forms as a result of restricted rotation about the C-N bond outside of the triazine ring. To test this hypothesis, carbon and proton NMR spectra for 2 were recorded at room temperature, 80 °C and 120 °C. These spectra displayed a coalescence of the signals at elevated temperatures (SI Fig X).

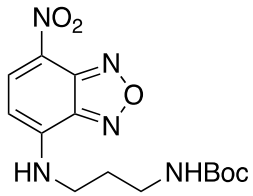


SI Figure 1: A) Expanded ^{13}C NMR spectra of **3** at room temperature (blue), 80 °C (red) and 120 °C (green). **B**) Expanded ^{1}H NMR spectra of **3** at room temperature (blue), 80 °C (red) and 120 °C (green).



SI Scheme 2: Synthesis of fluorescently functionalised divinyltriazine linker **2** through the use of a nitrobenzofuran (NBD) fluorescent tag.

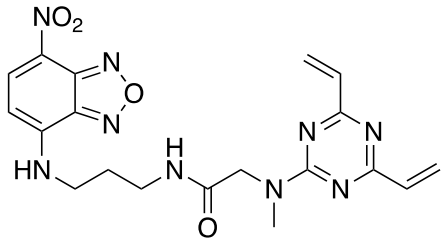
***tert*-butyl (3-((7-nitrobenzo[*c*][1,2,5]oxadiazol-4-yl)amino)propyl)carbamate (5)**



To a solution of 4-chloro-7-nitrobenzofurazan (200 mg, 1.00 mmol) in DMF (6 mL) was added triethylamine (0.140 mL, 1.00 mmol) and *N*-Boc-1,3-diaminopropane (209 mg, 1.20 mmol) and the mixture stirred at rt for 4 h. Upon completion, the reaction mixture was poured into a saturated aqueous solution of NH_4Cl and extracted with EtOAc (4 × 30 mL). The combined organic phases were washed with H_2O and brine, dried ($MgSO_4$) and concentrated *in vacuo* to yield **5** (295 mg, 0.870 mmol, 87%) as brown oil.

R_f 0.21 (SiO_2; 50% EtOAc/PE); **δ_H** (400 MHz, $CDCl_3$) 8.43 (d, 1H, *J* = 8.8 Hz), 8.02 (DMF), 7.55 (br s, 1H), 6.16 (d, 1H, *J* = 8.8 Hz), 4.92 (s, 1H), 3.60 (br s, 2H), 3.34-3.29 (m, 2H), 2.97 (DMF), 2.88 (DMF), 1.92 (br s, 2H), 1.45 (s, 9H) **δ_C** (101 MHz, $CDCl_3$) 162.8 (DMF), 157.2, 144.4, 144.1, 136.8, 129.1, 128.3, 125.4, 123.3, 98.4, 80.2, 40.9, 37.4, 36.7 (DMF), 31.6 (DMF), 29.3, 28.5 **HRMS** (ESI) *m/z* found $[M+Na]^+$ 360.1281, $C_{14}H_{19}N_5O_5{}^{23}Na^+$ required 360.1278.

2-((4,6-divinyl-1,3,5-triazin-2-yl)(methyl)amino)-*N*-(3-((7-nitrobenzo[*c*][1,2,5]oxadiazol-4-yl)amino)propyl)acetamide (2)



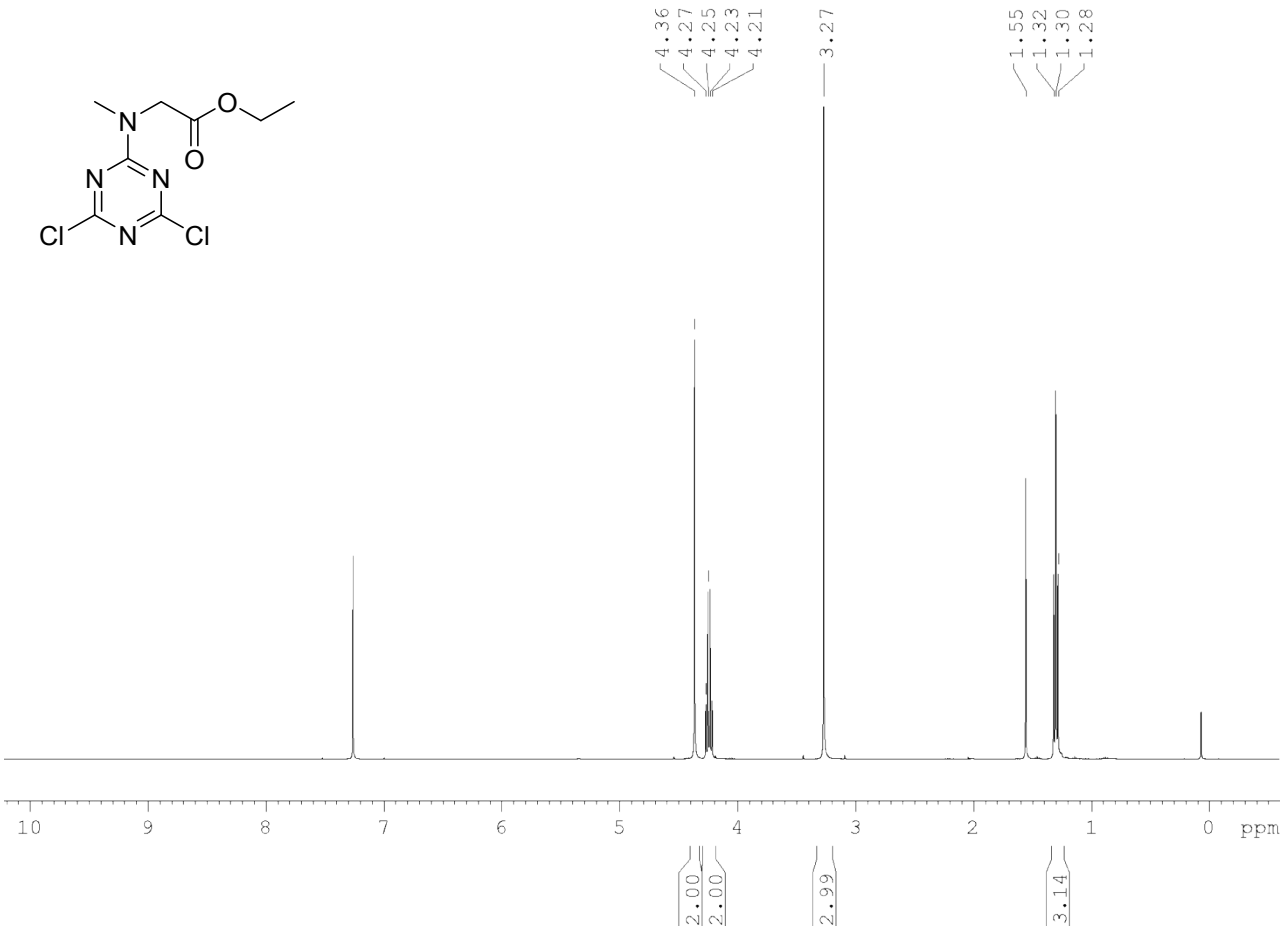
To a solution of **5** (270 mg, 0.800 mmol) in CH_2Cl_2 (1 mL) was added HCl (4M in 1,4-dioxne, 6 mL) and the mixture stirred at rt for 16 h. Upon completion, the reaction was concentrated *in vacuo* to yield the deprotected amine as a fine brown solid.

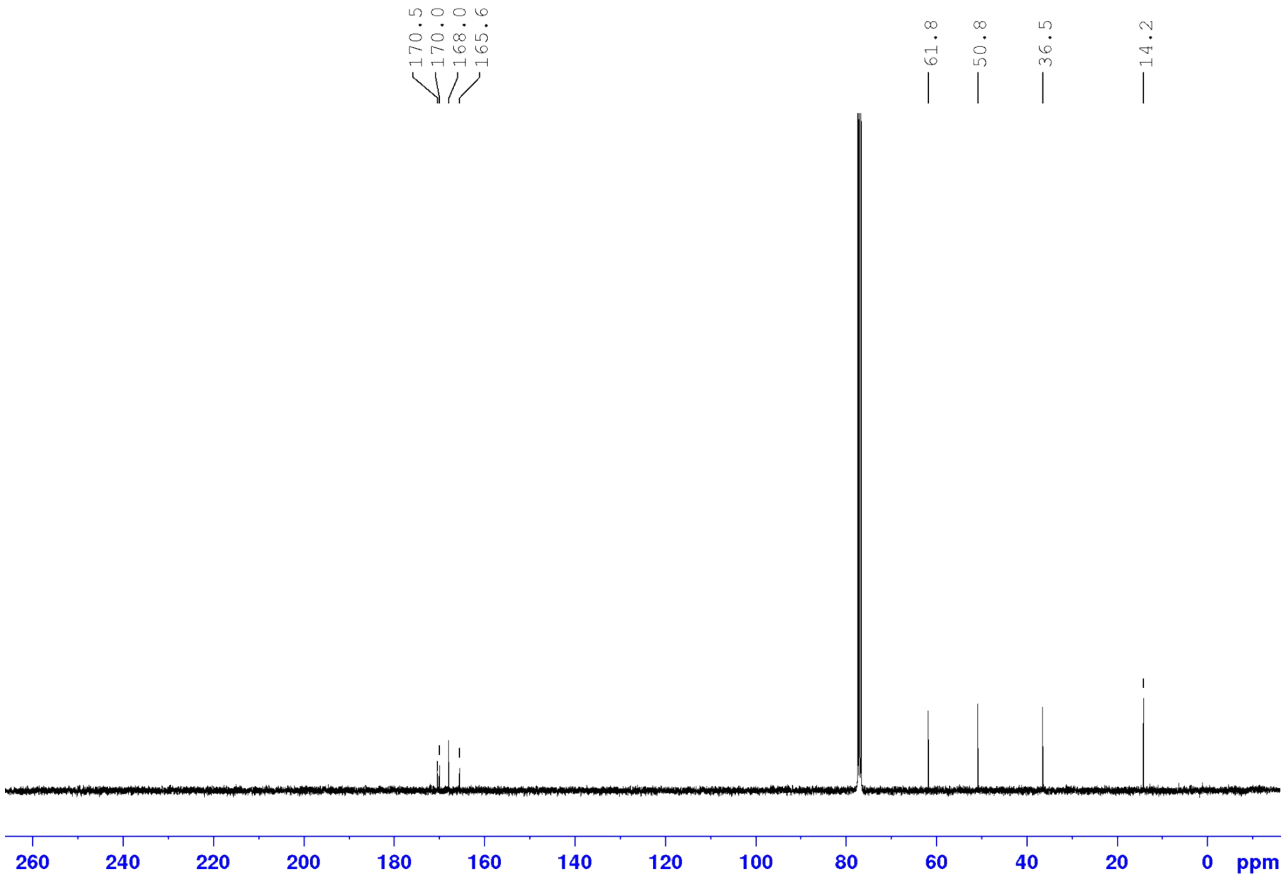
To a solution of divinyltriazine **1** (25.0 mg, 0.114 mmol) in DMF (1.5 mL) at 0 °C was slowly added triethylamine (15.9 µL, 0.114 mmol), followed by EDC·HCl (21.8 mg, 0.114 mmol), HOBt monohydrate (19.3 mg, 0.114 mmol) and deprotected amine (15.6 mg, 0.0570 mmol). The reaction was warmed to rt and stirred for 18 h at rt. Upon completion, the reaction mixture was poured into a 0.5 M aqueous HCl solution and extracted with CH_2Cl_2 (4 × 20 mL). The combined organic phases were washed with saturated Na_2CO_3 (2 × 30 mL), brine (2 × 30 mL), dried ($MgSO_4$) and concentrated *in vacuo* to yield **2** (16.4 mg, 0.0370 mmol, 66% over two steps) as an orange solid.

R_f 0.3 (SiO_2; EtOAc); **δ_H** (400 MHz, DMSO-d_6) 9.45 (s, 1H), 8.47 (d, 1H, *J* = 8.6 Hz), 8.08 (s, 1H), 6.71-6.44 (m, 4H), 6.31 (d, 1H, *J* = 8.6 Hz), 5.86-5.74 (m, 2H), 4.25 (s, 2H), 3.46-3.42 (m, 2H), 3.23-3.19 (m, 5H), 1.82 (t, 2H, *J* = 6.7 Hz). **δ_C** (101 MHz, DMSO-d_6) 169.8, 169.5, 168.3, 165.2, 145.1, 144.4, 144.1, 137.9, 136.0, 135.9, 126.7, 126.6, 120.7, 99.1, 51.6, 41.0, 36.2, 35.7, 27.7. **HRMS** (ESI) *m/z* found $[M+H]^+$ 440.1792, $C_{19}H_{22}N_9O_4^+$ required 440.1789.

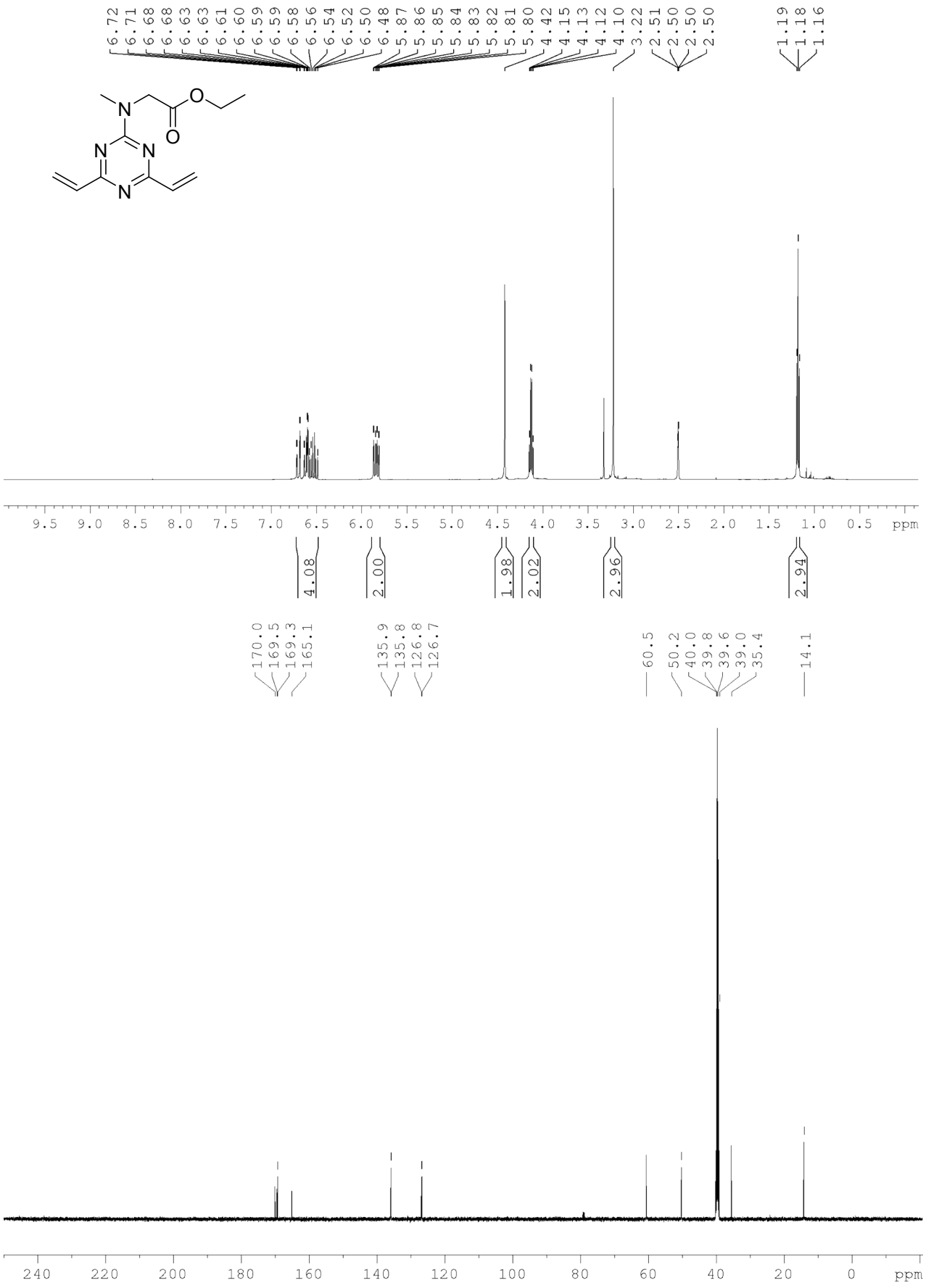
3. NMR spectra

ethyl *N*-(4,6-dichloro-1,3,5-triazin-2-yl)-*N*-methylglycinate (3)

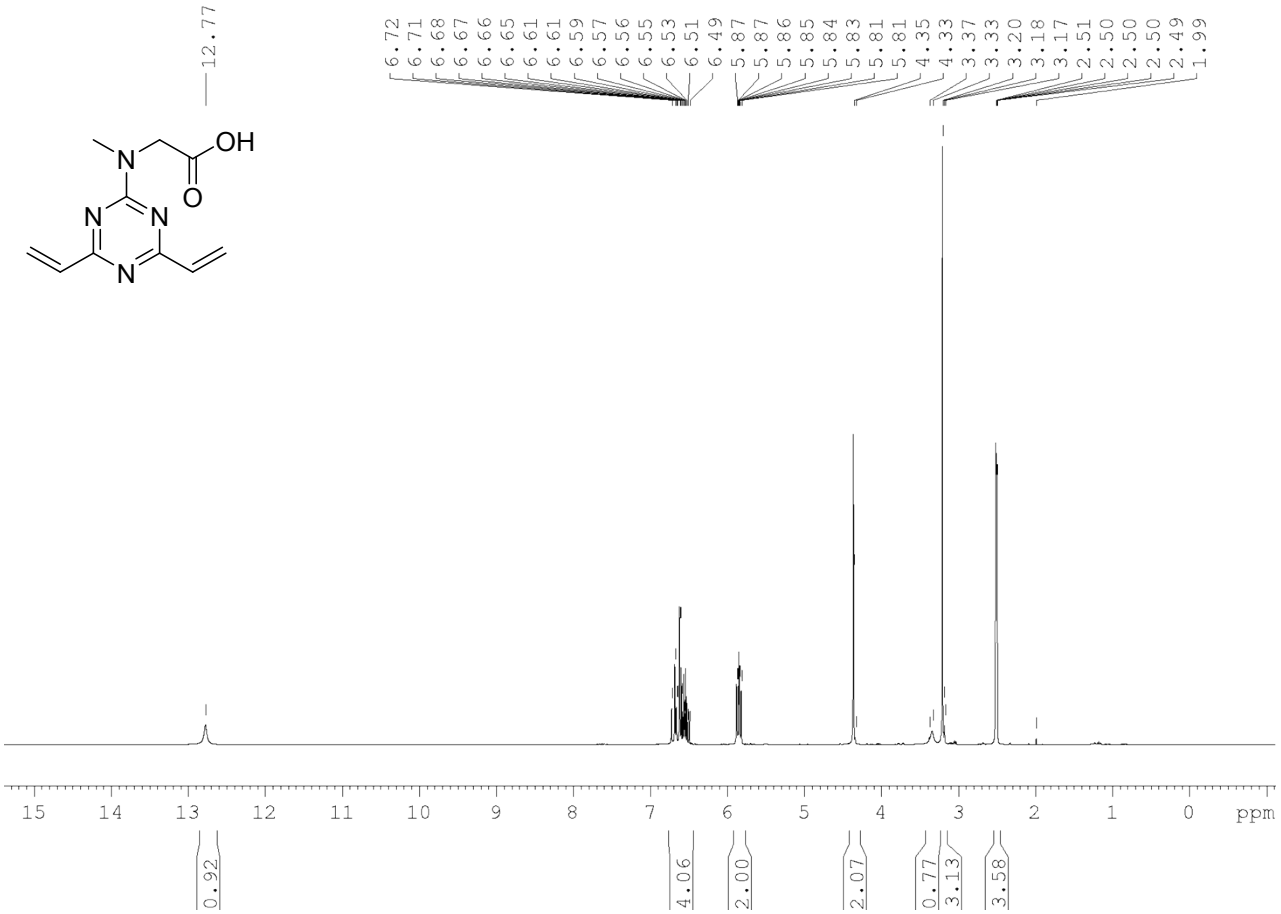


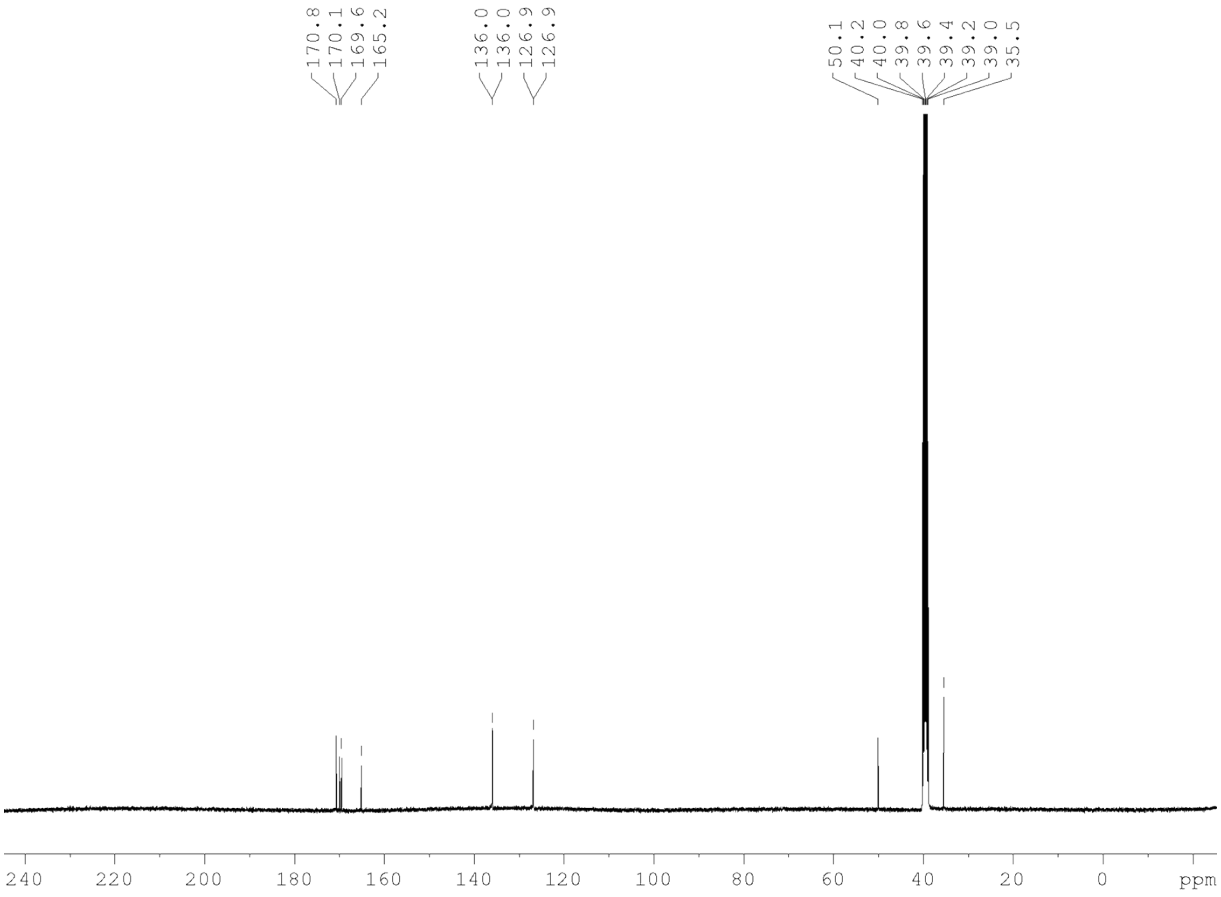


ethyl *N*-(4,6-divinyl-1,3,5-triazin-2-yl)-*N*-methylglycinate (4)

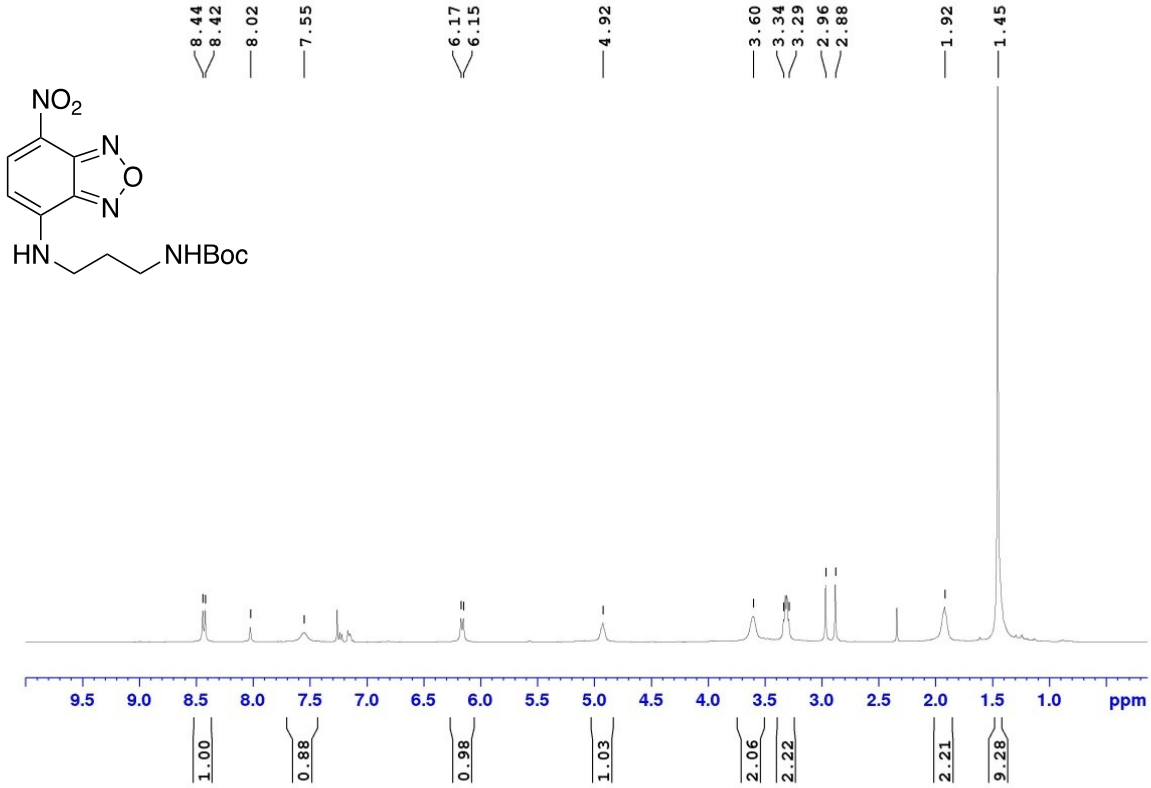

6.72 6.71 6.68 6.68 6.63 6.63 6.61 6.60 6.59 6.59 6.58 6.56 6.54 6.52 6.50 6.48 5.87 5.86 5.85 5.84 5.83 5.82 5.81 5.80 4.42 4.15 4.13 4.12 4.10 3.22 2.51 2.50 2.50 2.50 1.19 1.18 1.16
N
O
O
N
N
N
9.5 9.0 8.5 8.0 7.5 7.0 6.5 6.0 5.5 5.0 4.5 4.0 3.5 3.0 2.5 2.0 1.5 1.0 0.5 ppm
4.08 2.00 1.98 2.02 2.96 2.94
170.0 169.5 169.3 165.1 135.9 135.8 126.8 126.7 60.5 50.2 40.0 39.8 39.6 39.0 35.4 14.1
240 220 200 180 160 140 120 100 80 60 40 20 0 ppm

***N*-(4,6-divinyl-1,3,5-triazin-2-yl)-*N*-methylglycine (1)**





***tert*-butyl (3-((7-nitrobenzo[*c*][1,2,5]oxadiazol-4-yl)amino)propyl)carbamate (5)**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6.17
6.15
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3.34
3.29
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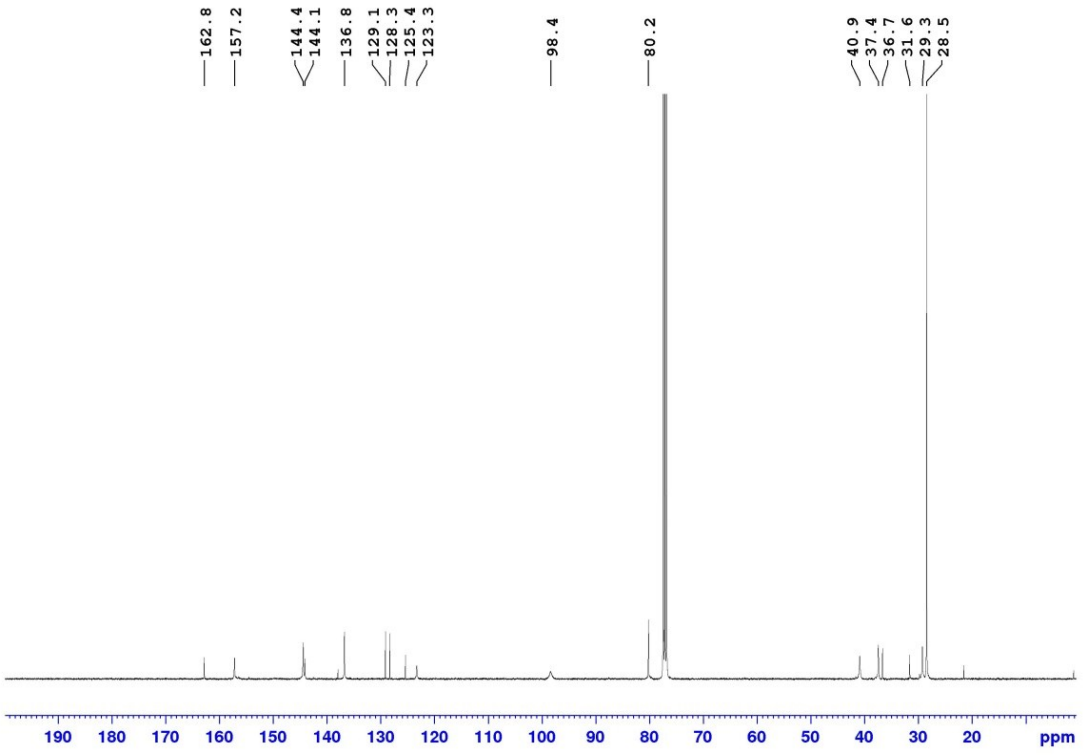
1.92
1.45
NO2
N
O
N
HN
NHBoc
9.5 9.0 8.5 8.0 7.5 7.0 6.5 6.0 5.5 5.0 4.5 4.0 3.5 3.0 2.5 2.0 1.5 1.0 ppm
1.00
0.88
0.98
1.03
2.06
2.22
2.21
9.28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157.2
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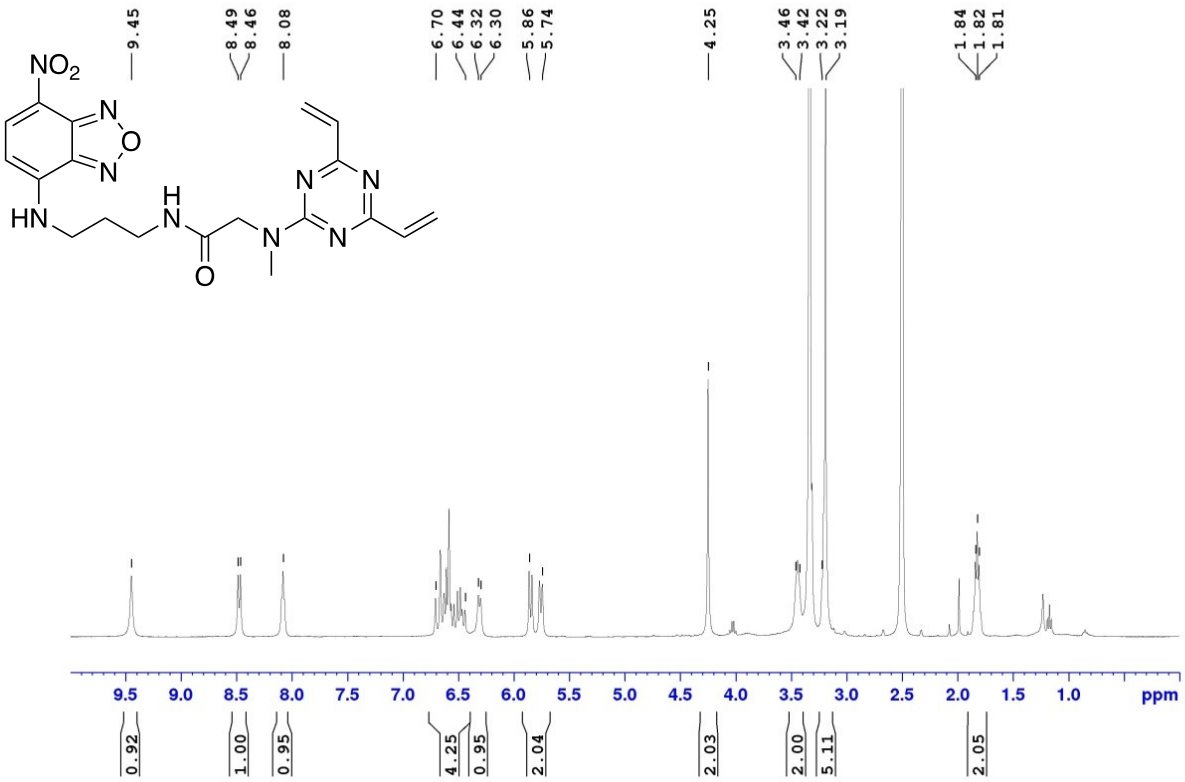
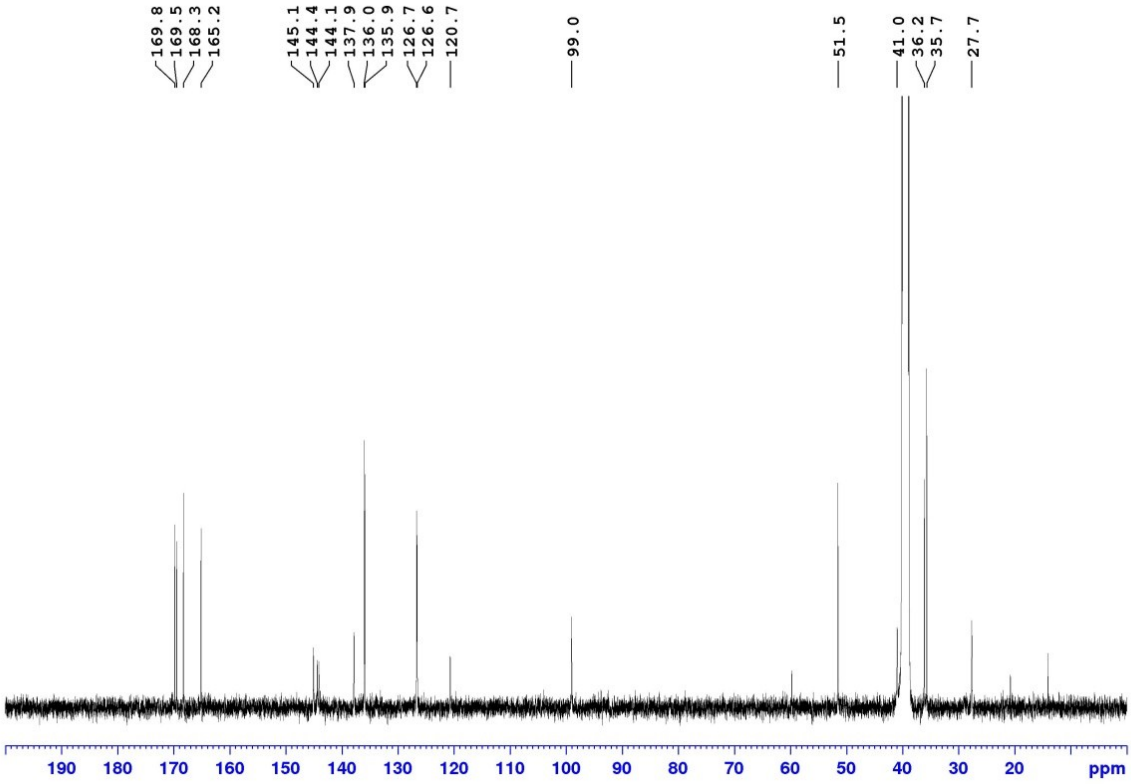
136.8
129.1
128.3
125.4
123.3
98.4
80.2
40.9
37.4
36.7
31.6
29.3
28.5
190 180 170 160 150 140 130 120 110 100 90 80 70 60 50 40 30 20 ppm

2-((4,6-divinyl-1,3,5-triazin-2-yl)(methyl)amino)-*N*-(3-((7-nitrobenzo[*c*][1,2,5]oxadiazol-4-yl)amino)propyl)acetamide (2)





4. Peptide synthesis

Peptide synthesis was carried out on solid-phase using Fmoc-protecting group strategy on a CEM LibertyBlue Automated Microwave Peptide Synthesizer using Rink Amide MBHA LL resin (0.33 mmol/g) (Merck Millipore). Fmoc-protected amino acids were made up as a solution of 0.2 M in DMF to give 5 equivalents relative to resin loading. Oxyma Pure was made up as a 1 M solution in DMF to give 5 equivalents relative to resin and *N*,*N*'-Diisopropylcarbodiimide as a 1 M solution in DMF to give 10 equivalents relative to the resin. All amino acid couplings were single coupled and heated to 90 °C for two mins, except for Fmoc-Arg(Pbf)-OH which was double coupled and heated to 75 °C for 300 seconds for each coupling. Fmoc deprotection was carried out using 20 % (v/v) piperidine in DMF and heated at 90 °C for 60 seconds.

General procedure for manual peptide synthesis

Peptide synthesis was carried out on solid-phase using Fmoc-protecting group strategy using Rink Amide MBHA LL resin (0.33 mmol/g) (Merck Millipore). The resin was swollen in anhydrous DMF for 30 minutes, then drained. Fmoc-deprotection was carried out twice with a solution of 20% v/v piperidine/DMF with vigorous shaking for 1 min, followed by sequential washes of the resin with DMF, MeOH and CH_2Cl_2. Amino acid coupling was carried out using Fmoc-protected amino acid (2 equiv.) dissolved in anhydrous DMF followed by addition of 1-[Bis(dimethylamino)methylene]-1H-1,2,3-triazolo[4,5-*b*]pyridinium 3-oxide hexafluorophosphate (HATU) (2 equiv.). The resulting solution was added to the prepared resin. *N*,*N*-diisopropylethylamine (DIPEA) (4 equiv.) was added and the reaction shaken vigorously for 3 min, then drained and washed sequentially with DMF, MeOH and CH_2Cl_2. Successful Fmoc-deprotection and amino acid coupling were confirmed by Kaiser test.

Cleavage of peptide from resin

Synthesised peptides were cleaved from the resin using a cleavage cocktail of TFA/TIPS/H_2O (95:2.5:2.5) for either 1 hour at 42 °C or 3 hours at room temperature. The resin was filtered, the filtrate collected and the TFA blown off with a steady stream of nitrogen. The peptide was precipitated and washed three times in cold diethyl ether, then spun down to a pellet before the diethyl ether was removed and the peptide dried under a steady stream of nitrogen.

Peptide purification was carried out using a semi-preparative HPLC on an Agilent 1260 Infinity using a Supelcosil ABZ+PLUS column (250 mm × 21.2 mm, 5 µm) eluting with a linear gradient system (solvent A: 0.1% (v/v) TFA in water, solvent B: 0.05% (v/v) TFA in acetonitrile) over 20 minutes at a flow rate of 20 mL/min. Analytical HPLC was run on an Agilent 1260 Infinity using a Supelcosil ABZ+PLUS column (150 mm × 4.6 mm, 3 µm) eluting with a linear gradient system (solvent A: 0.05% (v/v) TFA in water, solvent B: 0.05% (v/v) TFA in acetonitrile) over 15 minutes at a flow rate of 1 mL/min. HPLC was monitored by UV absorbance at 220 and 254 nm.

General method for peptide stapling

The peptide was dissolved in 50 mM sodium phosphate buffer (pH 8), followed by addition of divinyltriazne linker dissolved in acetonitrile, to make a final peptide concentration of 2 mg/mL. The reaction was allowed to stir at room temperature for 1 hour before the reaction mixture either lyophilised or directly purified by reverse phase preparative HPLC.

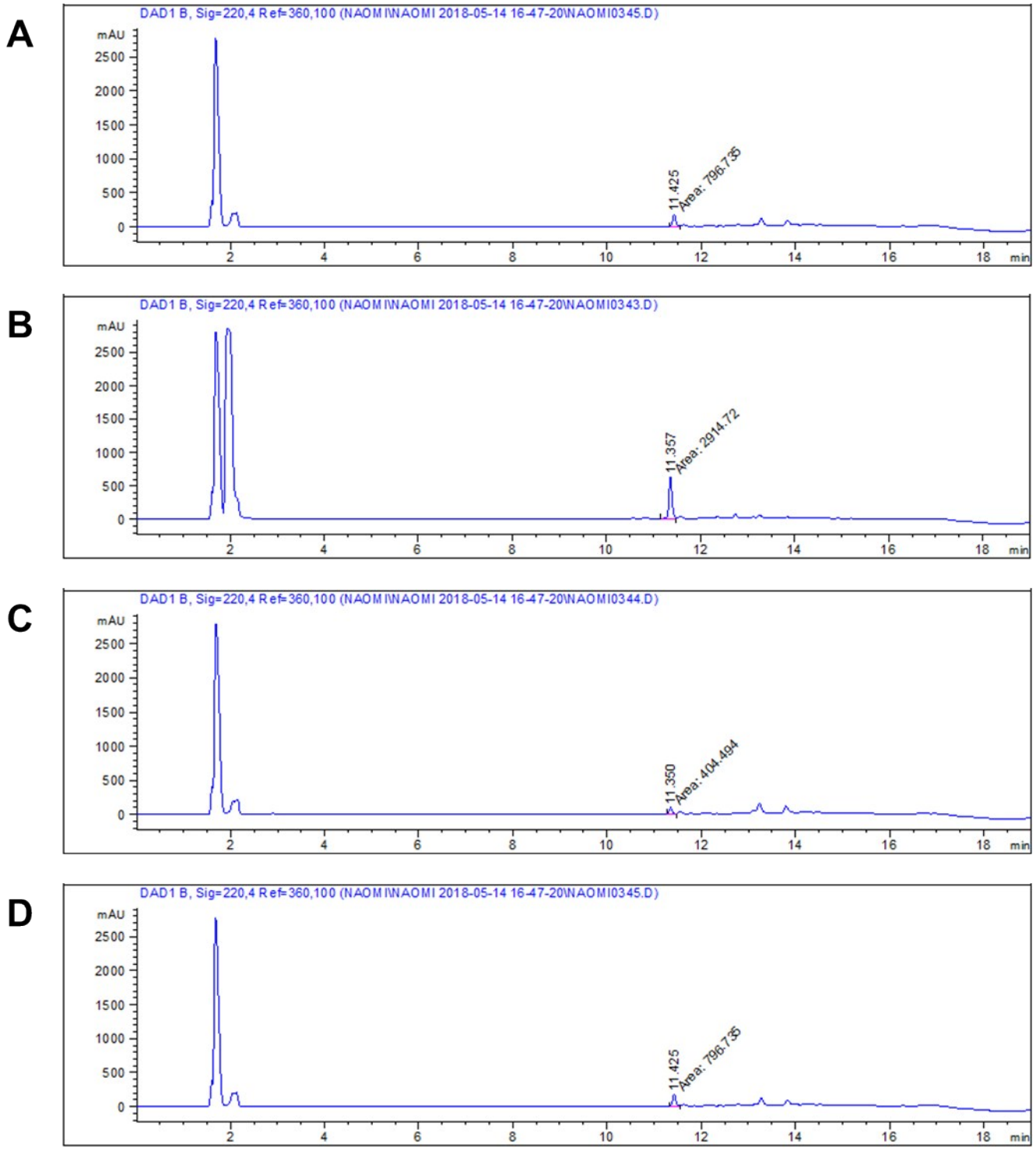
For the optimisation stapling reactions, 20 µL of the reaction solution was taken at specific time points and quenched with a solution of cysteine (100 µL, 33.5 $mgmL^{-1}$), followed by addition of MeCN:H_2O (200 µL, 1:1), before analysis by analytical HPLC.

Table S1. Additional conditions for the stapling reaction on peptide **P1-C** with divinyltriazine linker **4**.

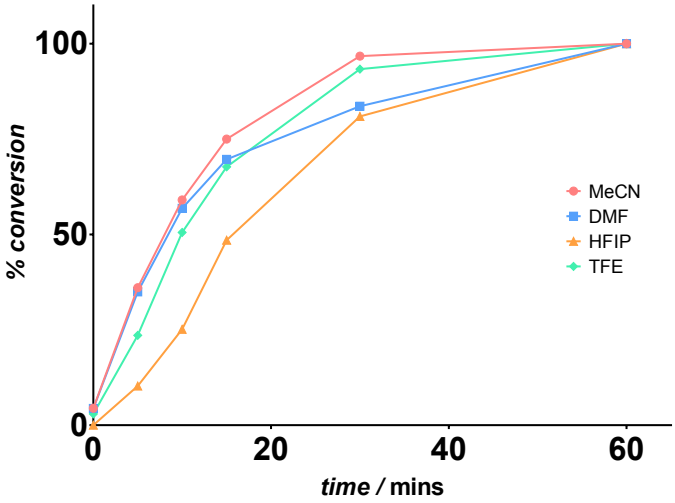
Entry	Aqueous solvent	Organic solvent	Solvent ratio (Aq:Org)	Peptide conc. (mg/mL)	% conversion after 60 mins
1	NaPi pH 8	MeCN	7:1	2	100
2	NaPi pH 8	DMF	7:1	2	100
3	NaPi pH 8	HFIP	7:1	2	100
4	NaPi pH 8	TFE	7:1	2	100
5	NaPi pH 8	MeCN	7:1	0.5	100
6	NaPi pH 8	MeCN	7:1	1	100
7	NaPi pH 8	MeCN	7:1	5	100
8	NaPi pH 8	MeCN	1:1	2	100
9	NaPi pH 8	MeCN	1:7	2	47
10	NaPi pH 8	MeCN	7:1	2[a]	100
11	MES pH 6	MeCN	7:1	2	37
12	Tris pH 9	MeCN	7:1	2	80
13	NaPi pH 8	DMF	7:1	2[a]	100
14	NaPi pH 8	HFIP	7:1	2[a]	100
15	NaPi pH 8	TFE	7:1	2[a]	100
16	NaPi pH 8	DMF	1:1	2	71
17	NaPi pH 8	DMF	1:7	2	2
18	NaPi pH 8	HFIP	1:1	2	81

19	NaP_i pH 8	HFIP	1:7	2	1
20	NaP_i pH 8	TFE	1:1	2	73
21	NaP_i pH 8	TFE	1:7	2	11

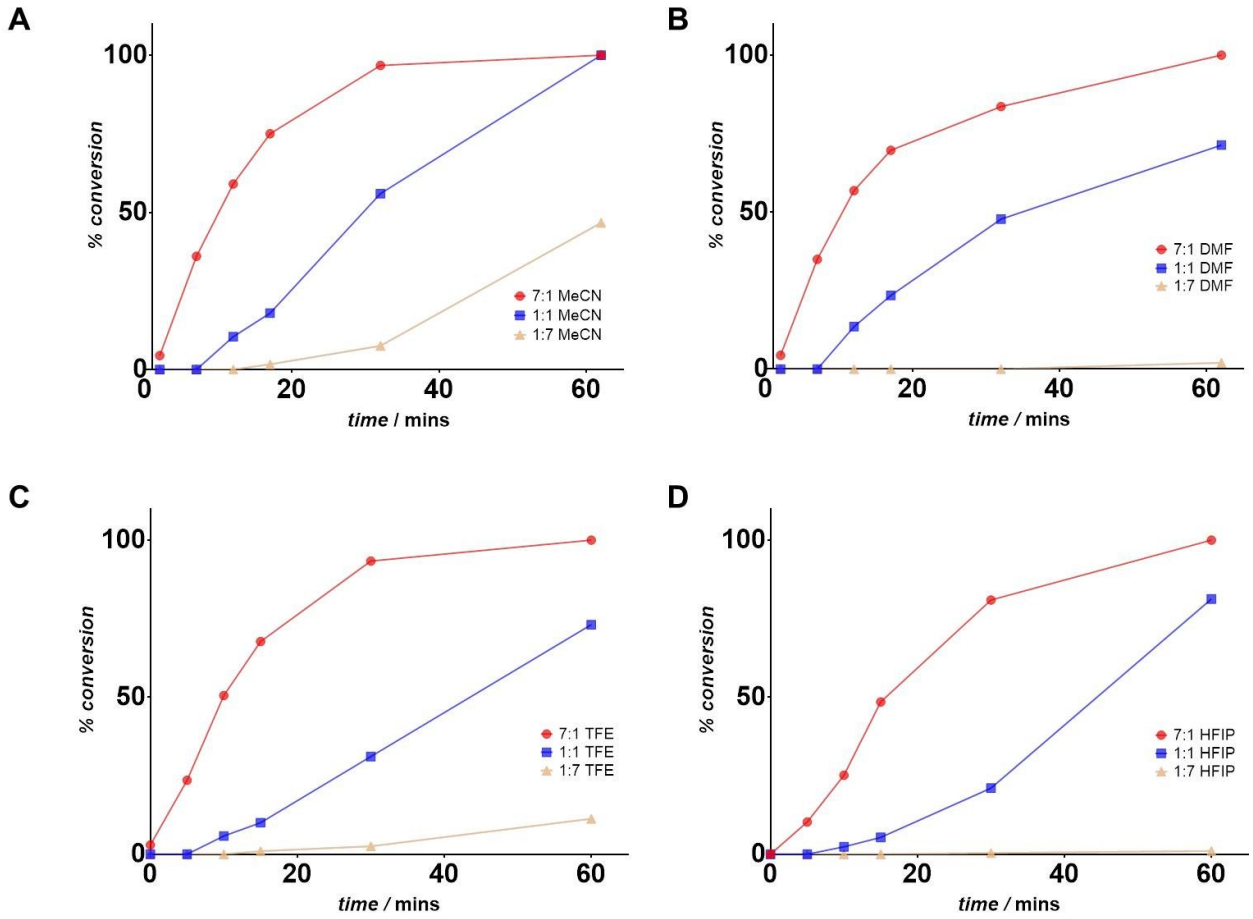
[a] In this case, crude linear peptide was used in the stapling reaction.



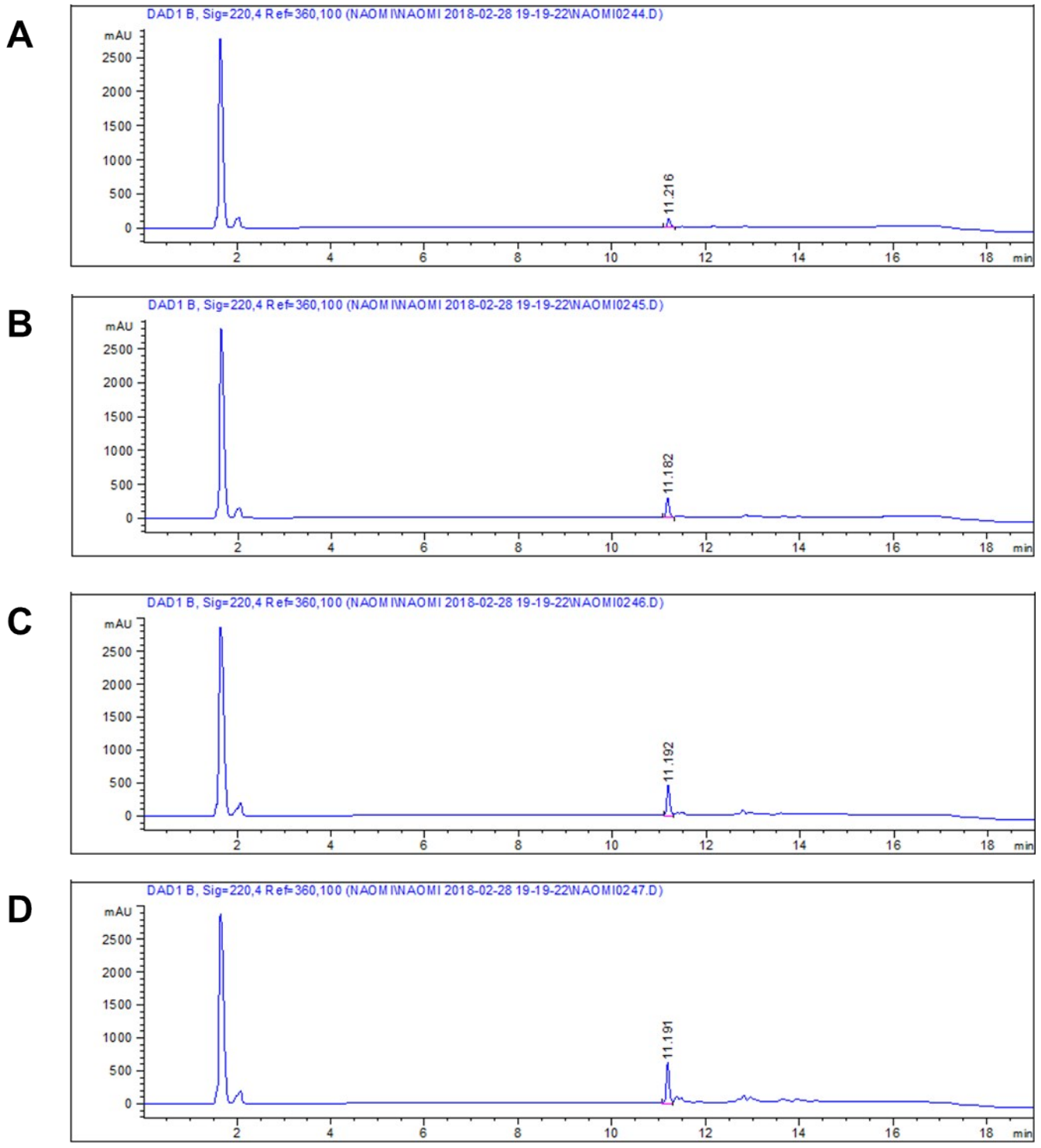
SI Figure 2: 60 min HPLC time point for stapling using different organic co-solvents, **A**) MeCN, **B**) DMF, **C**) HFIP and **D**) TFE.



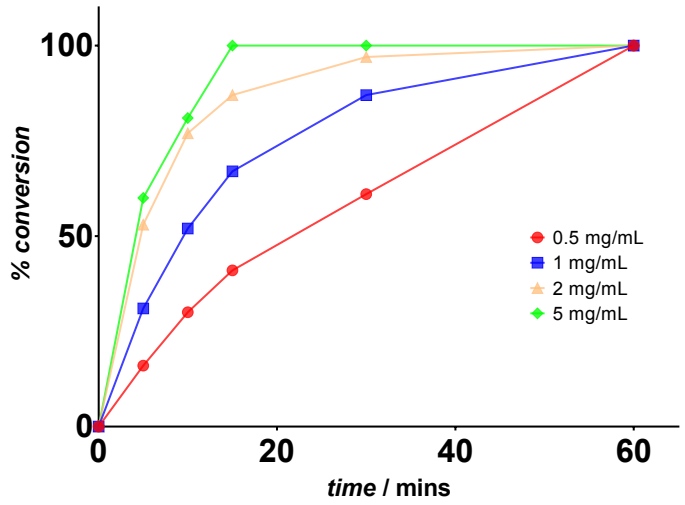
SI Figure 3: Stapling reaction of **P1-C** with divinyltriazine (DVT) linker **1** using different organic co-solvents MeCN (red), DMF (blue), HFIP (orange) and TFE (green). Percentage conversion was determined by analytical HPLC.



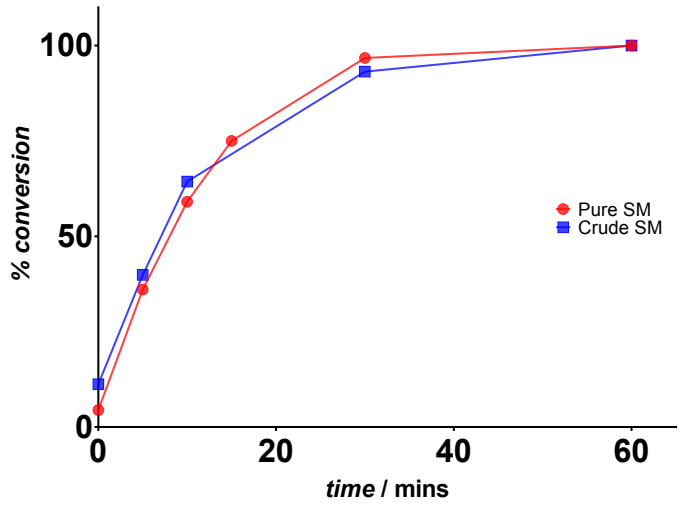
SI Figure 4: Study on the effect of different solvent ratios on the rates of stapling using **A**) MeCN, **B**) DMF, **C**) HFIP, and **D**) TFE as the organic co-solvents. Percentage conversion was determined by analytical HPLC.



SI Figure 5: 60 min HPLC time point for stapling using different peptide concentrations, **A**) 0.5 mg/mL, **B**) 1 mg/mL, **C**) 2 mg/mL and **D**) 5 mg/mL.



SI Figure 6: Stapling reaction of **P1-C** with divinyltriazine (DVT) linker **1** at different peptide concentrations 0.5 mg/mL (red), 1 mg/mL (blue), 2 mg/mL (orange) and 5 mg/mL (green). Percentage conversion was determined by analytical HPLC.



SI Figure 7: Stapling reaction of divinyltriazine (DVT) linker **1** using either crude (blue) or purified (red) linear peptide **P1-C**. Percentage conversion was determined by analytical HPLC.

5. Peptide Characterisation

Table S2. Characterisation of peptides synthesised.

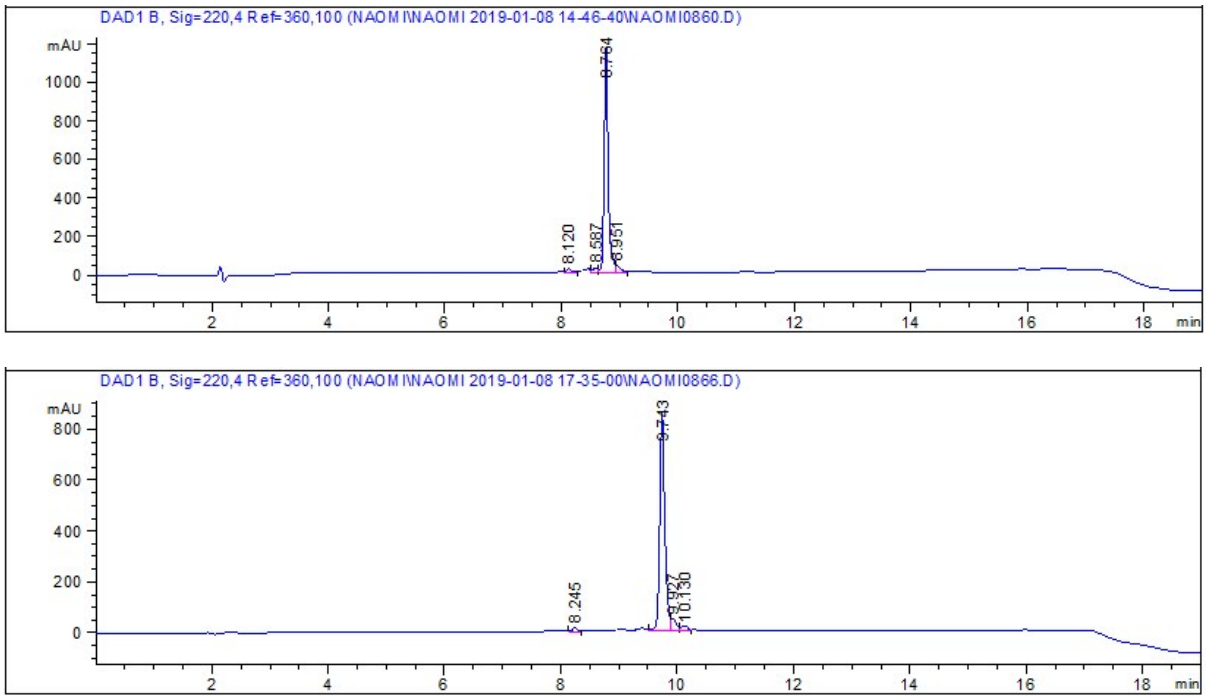
Peptide	Calculated MW $[M+H]^+$ (Da)	Found MW (Da)	R_t (min)	Isolated Yield (%)	Purity (%)
P1-WT	1660.8	1661.9 $[M+H]^+$	8.76[a] 9.74[b]	18	93
P1-C	1682.8	1682.6 $[M+H]^+$	9.92[a] 11.90[b]	47	96
P1-C-1	1902.9	1904.1 $[M+H]^+$	9.40[a] 11.04[b]	28	98
P1-S	1650.8	1651.9 $[M+H]^+$	9.18[a] 10.56[b]	34	86
P2-C	1009.5	1009.8 $[M+H]^+$	6.75[a] 4.63[b]	53	84
P2-C-1	1229.6	1230.7 $[M+H]^+$	7.44[a] 6.60[b]	38	89
P3-C	1105.4	1105.8 $[M+H]^+$	8.89[a] 8.34[b]	29	96
P3-C-1	1325.5	1326.8 $[M+H]^+$	8.44[a] 7.16[b]	23	92
P4-C	1639.7	1640.8 $[M+H]^+$	7.63[a] 6.75[b]	26	63
P4-C-1	1859.8	1860.7 $[M+H]^+$	7.17[a] 6.26[b]	28	88
P1-C-2	2121.9	1062.3 $[M+2H]^{2+}$	10.18[a] 12.55[b]	40	80

[a] 5-95% MeCN in water (+ 0.05% TFA), 15min gradient. [b] 20-60% MeCN in water (+ 0.05% TFA), 15min gradient.

Peptide Analytical HPLC Traces

All analytical HPLC traces for the peptides are reported for both 5-95% MeCN in water (+ 0.05% TFA), 15min gradient (top trace) and 20-60% MeCN in water (+ 0.05% TFA), 15min gradient (bottom trace).

P1-WT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8.587
8.951
min
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mAU
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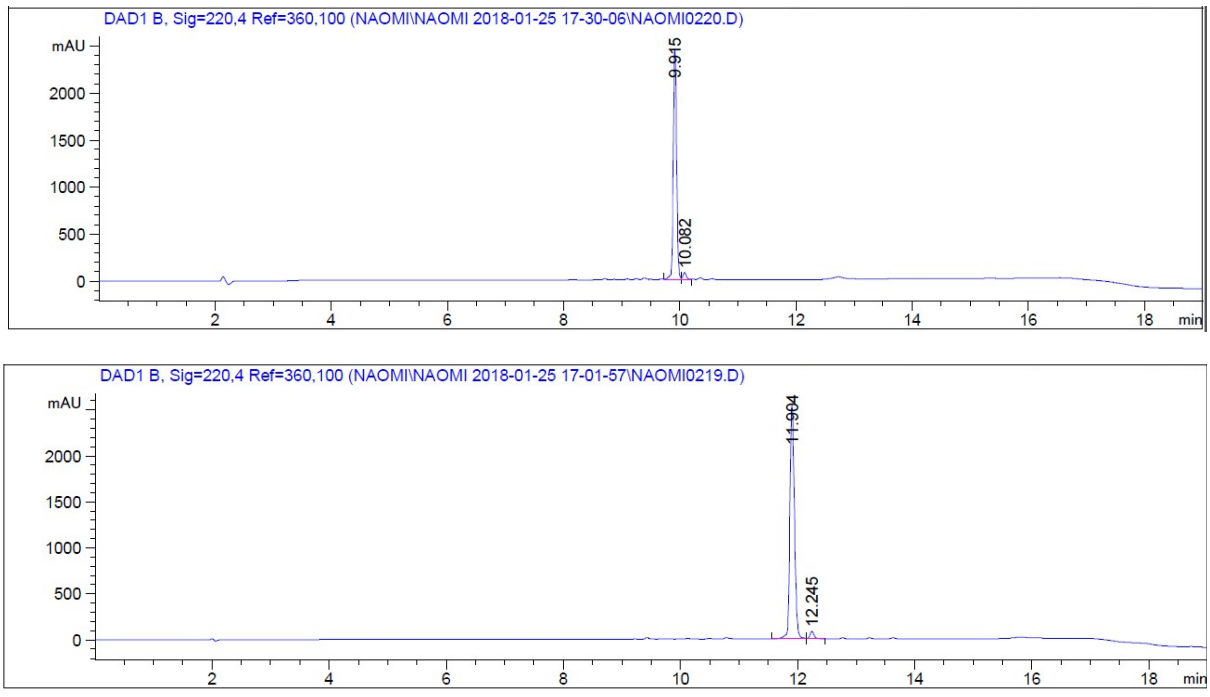
8.245
8.927
10.130
min

P1-C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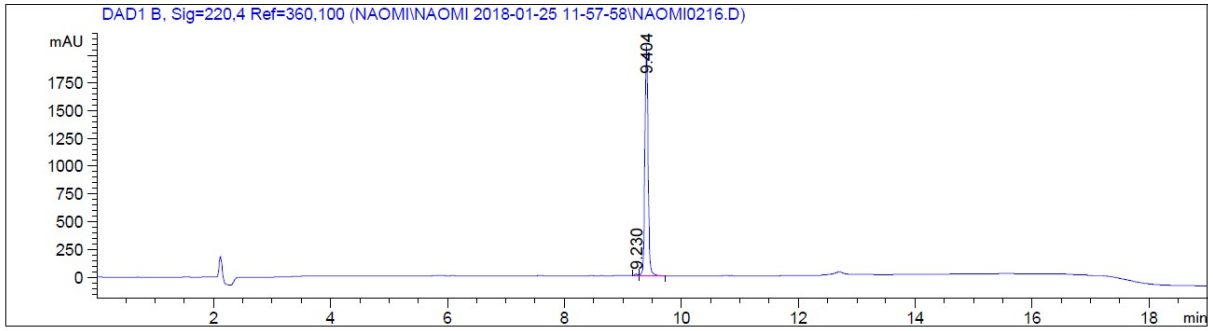
11.904
12.245
min

P1-C-1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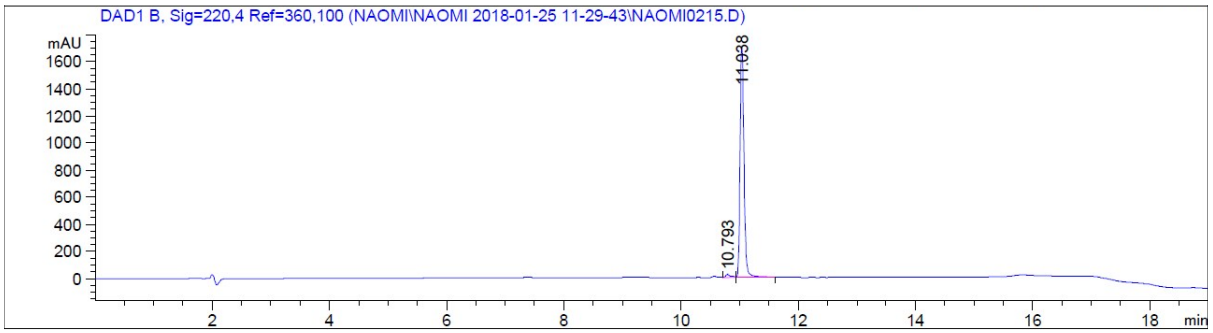
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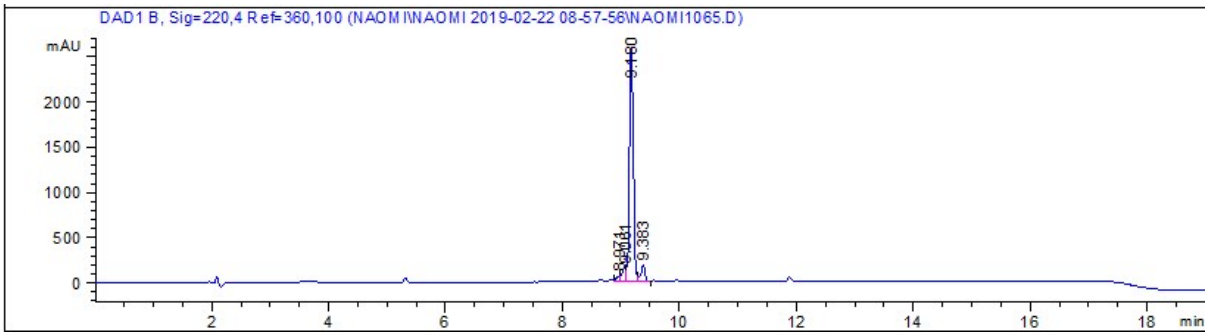
min

P1-S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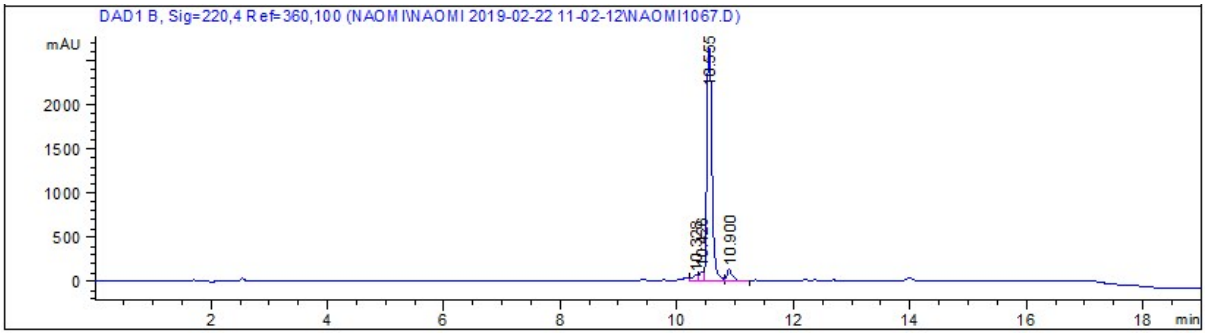
min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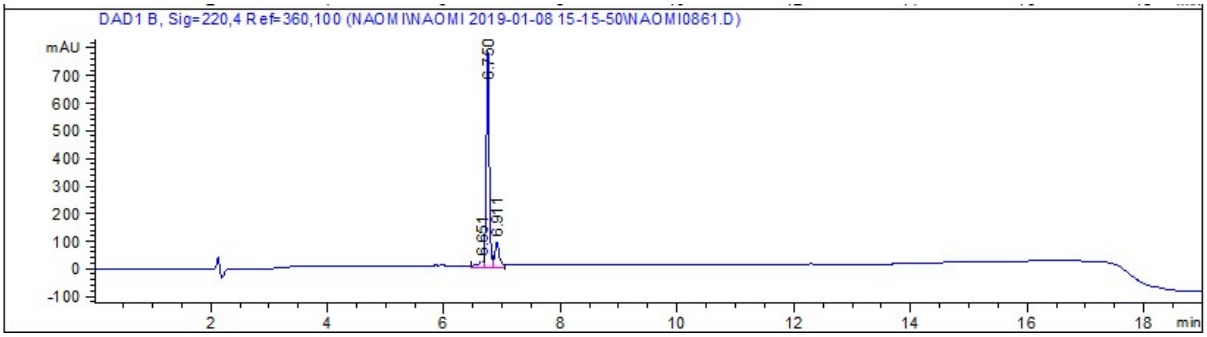
min

P2-C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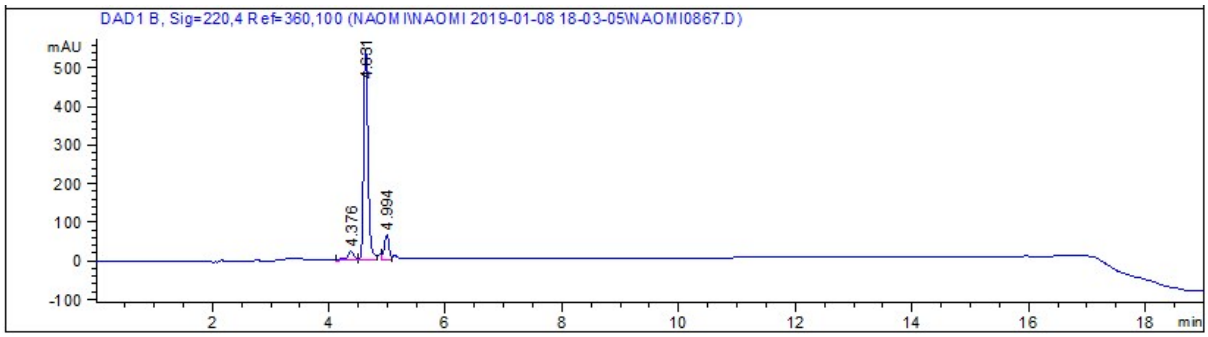
6.911
min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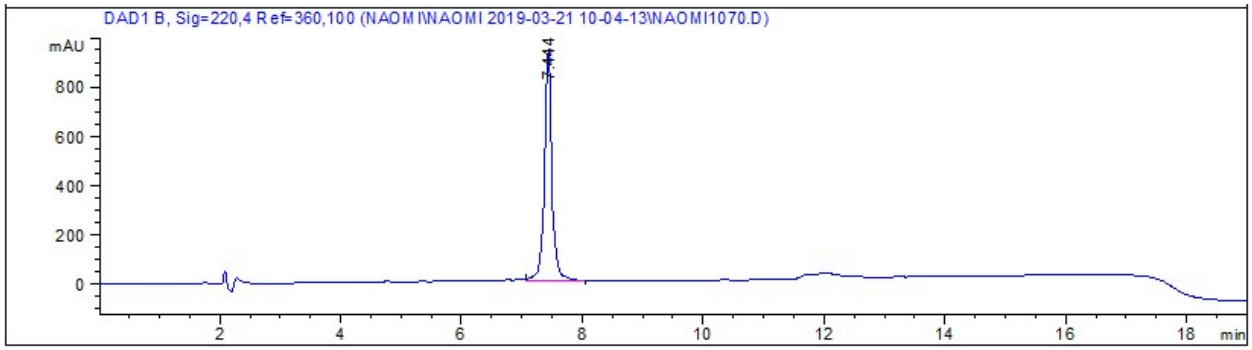
P2-C-1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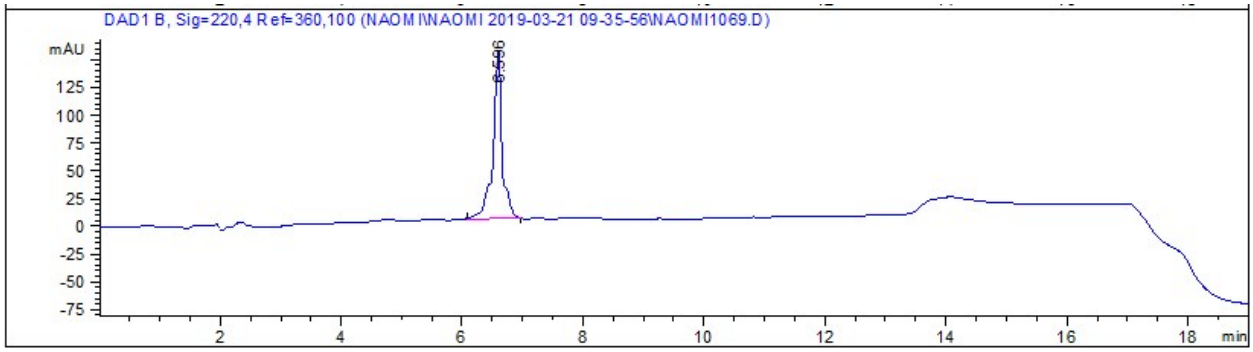
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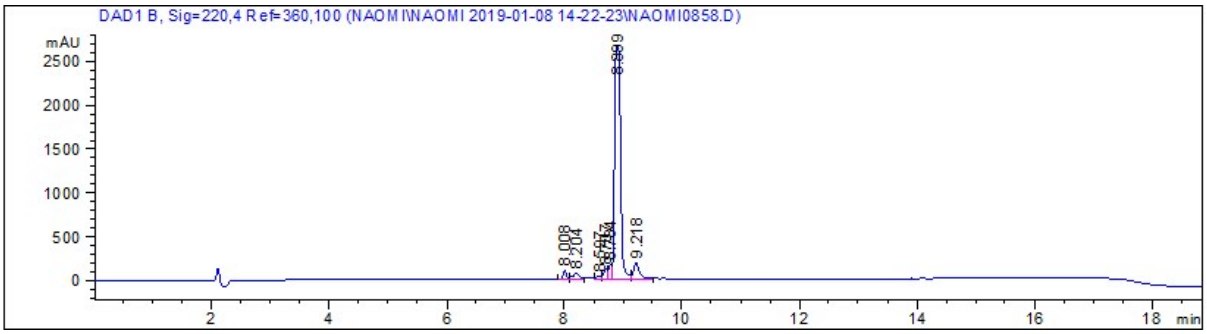
P3-C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1000
500
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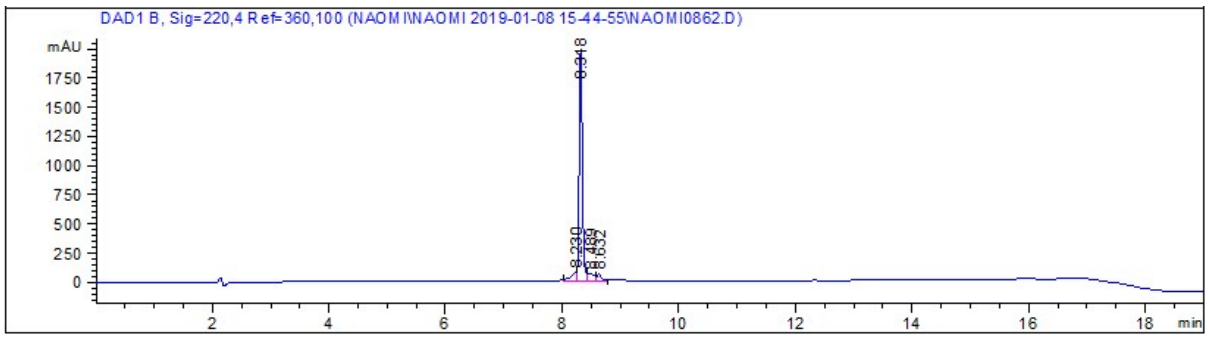
9.218
2
4
6
8
10
12
14
16
18
min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1000
750
500
250
0
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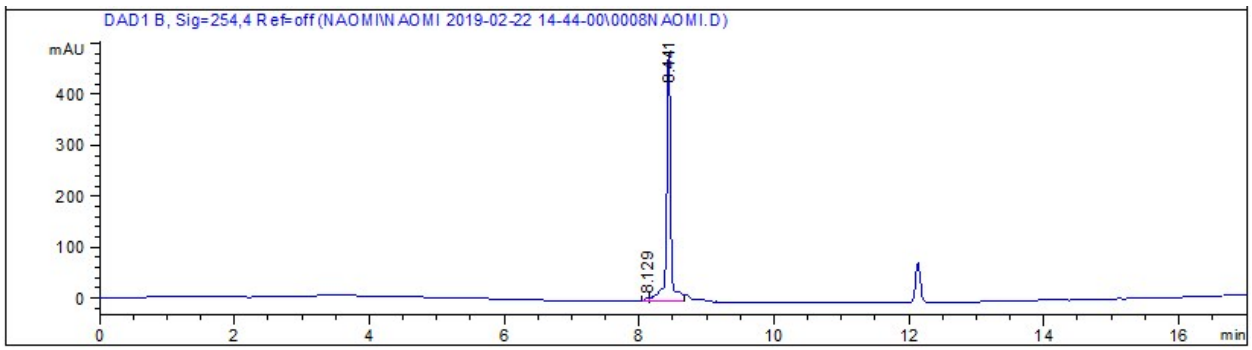
8.489
8.632
2
4
6
8
10
12
14
16
18
min

P3-C-1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4
6
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12
14
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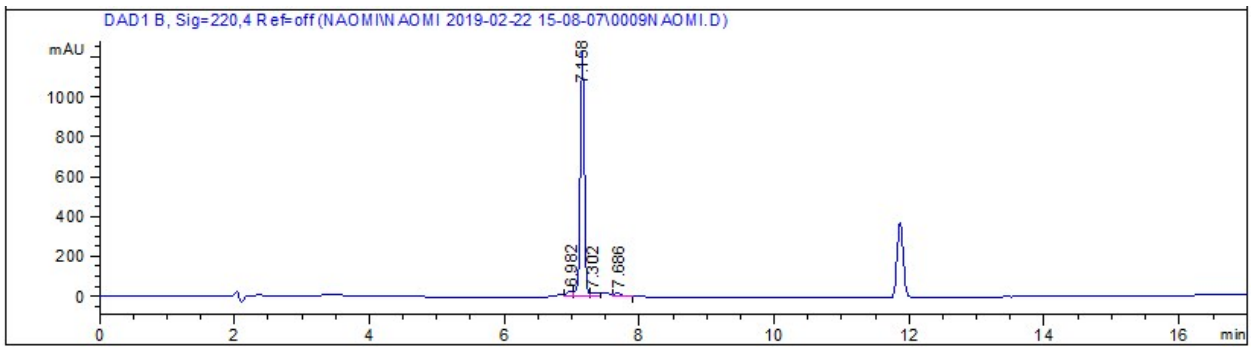
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7.302
7.686
0
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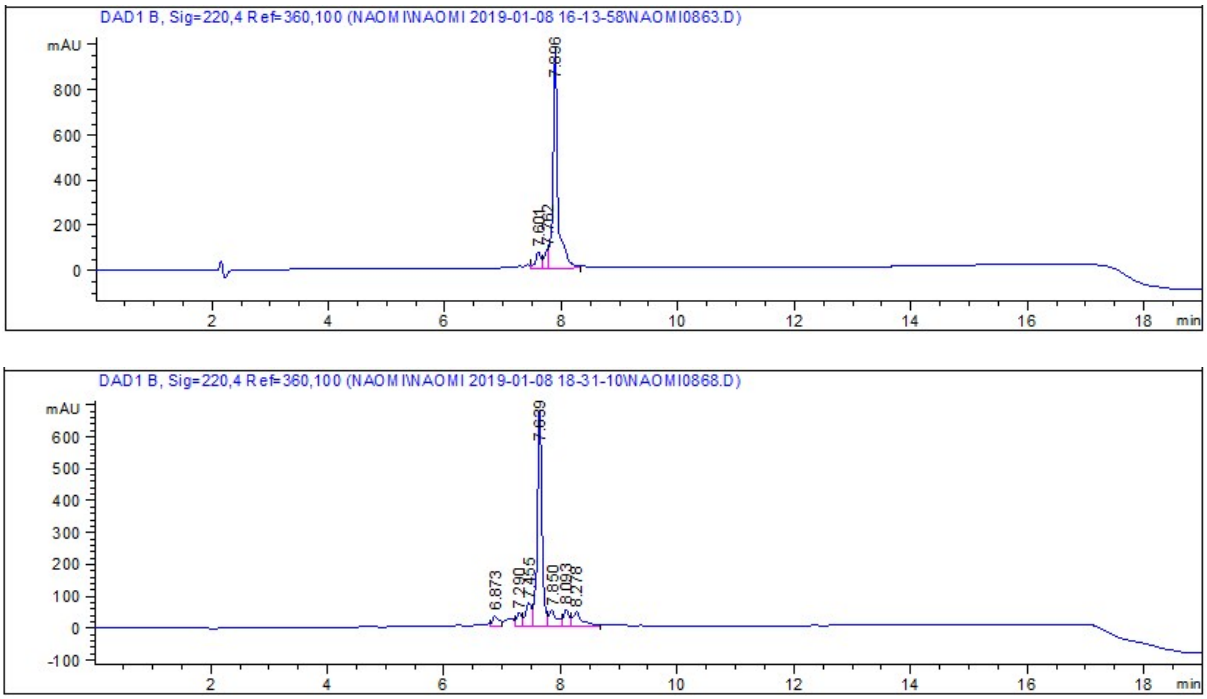
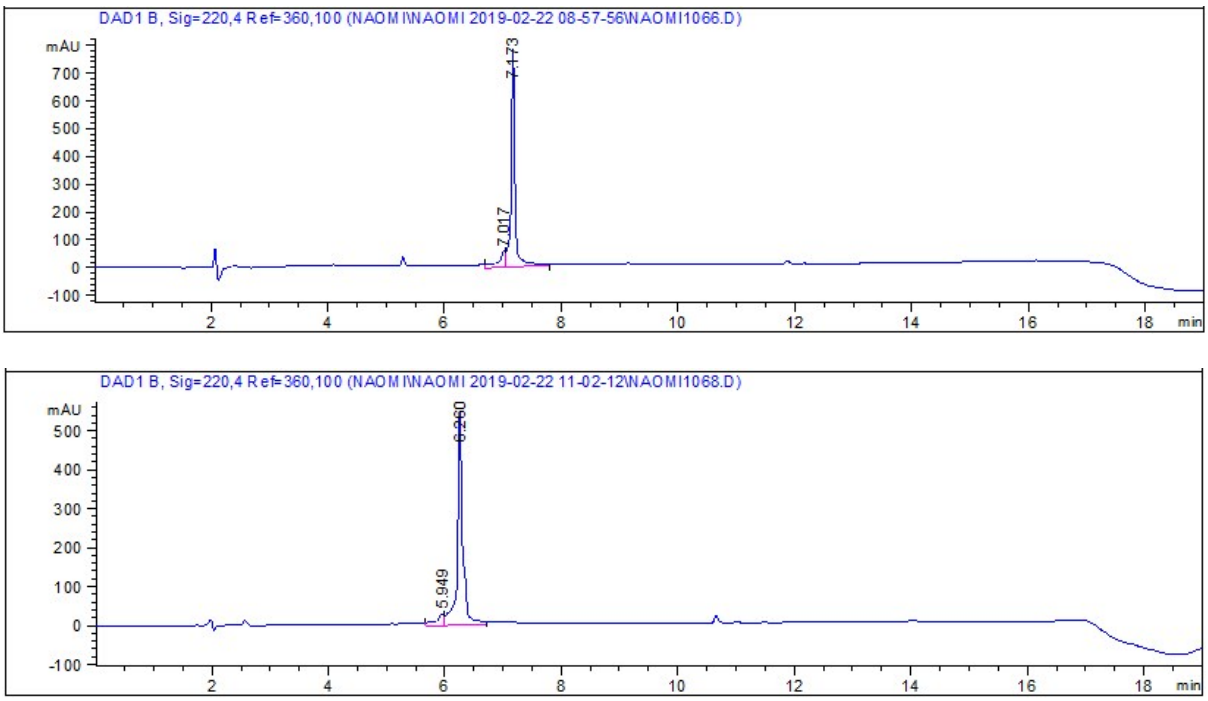
4
6
8
10
12
14
16
min

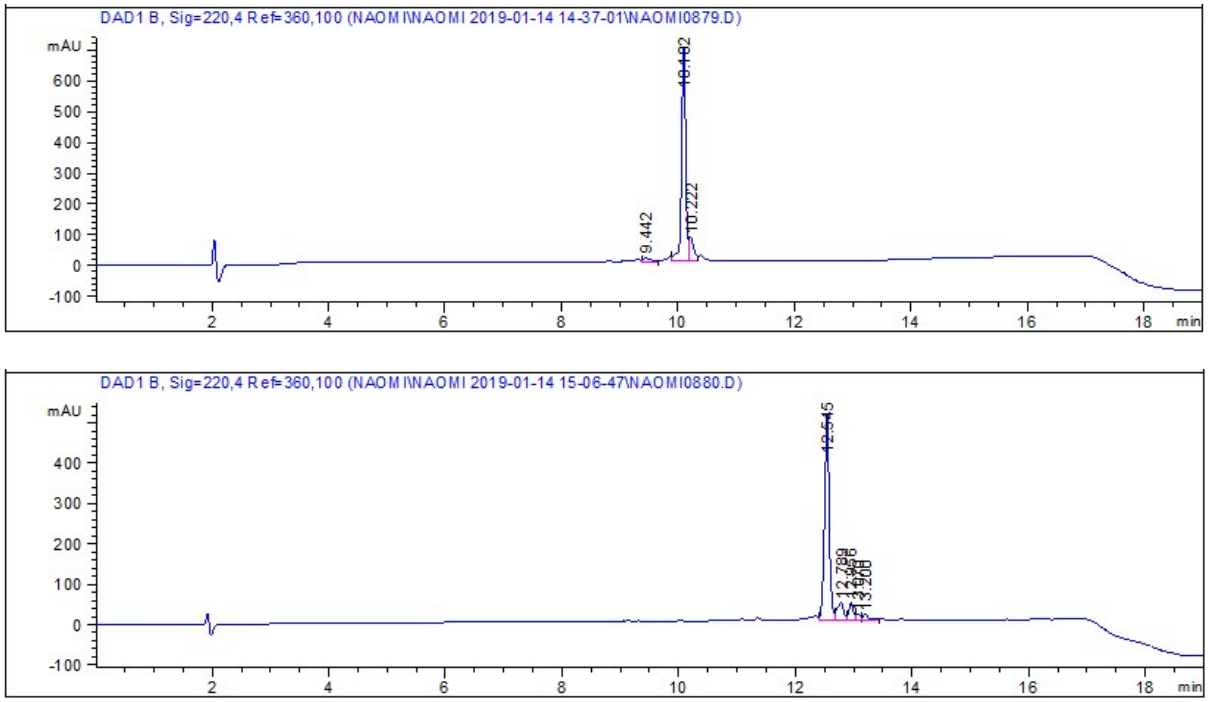
P4-C



P4-C-1



P1-C-2



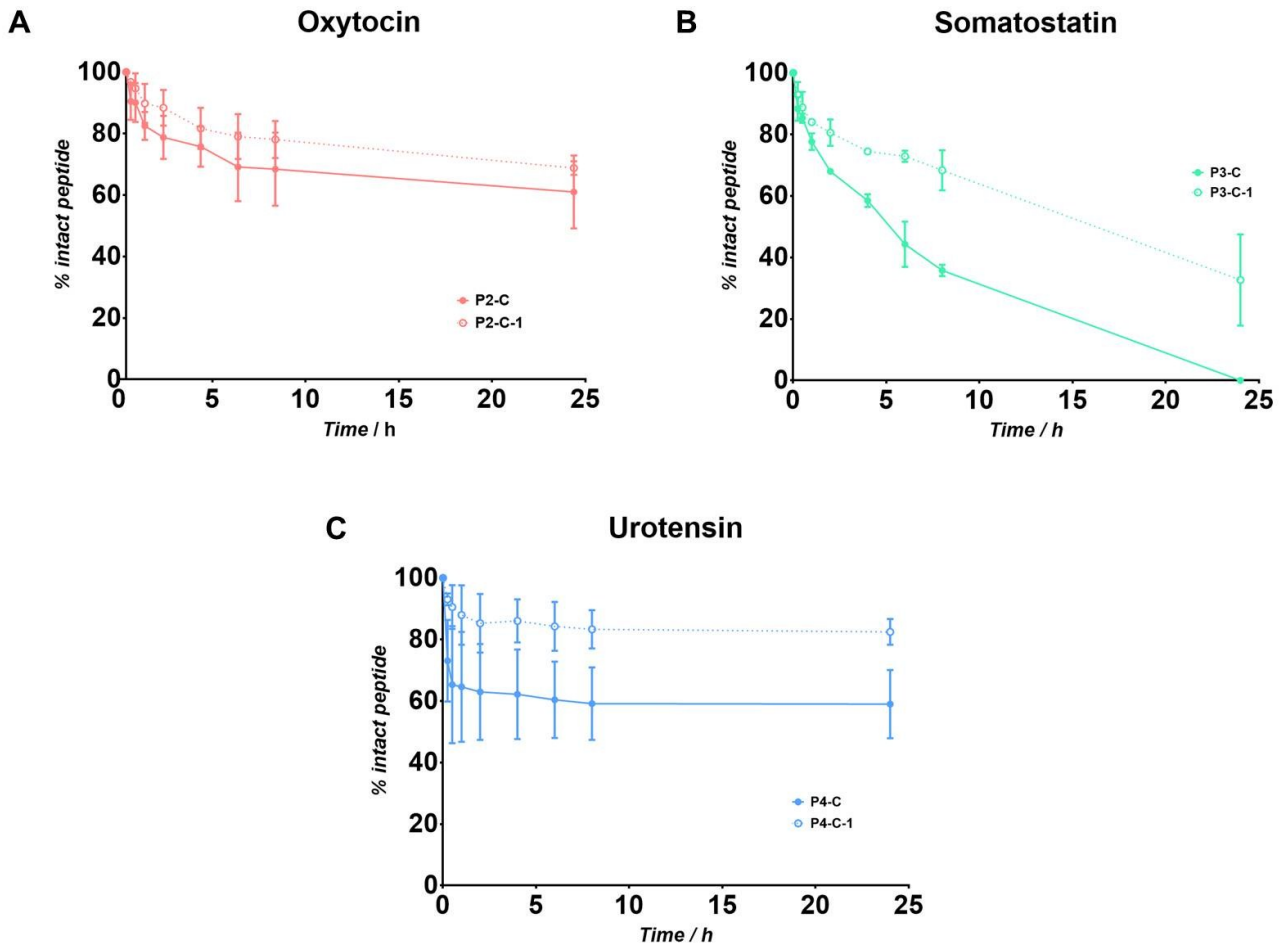
6. Circular Dichroism Spectroscopy

Circular dichroism spectra we obtained on an Aviv Model 410 CD spectrometer at 25 °C using a 1 mm path length, scanning between 250 and 195 nm at

Peptides were dissolved in 2:1 water/50 mM sodium phosphate, pH8. Helicity was calculated based on mean residue ellipticity at 222 nm.

7. Serum stability assay

Human serum (500 µL) and caffeine (10 µL, 15 mg/mL stock in water) as an internal standard, were left to equilibrate at 37 °C for 15 mins. Peptide (5 µL, 10 mM stock in DMSO) was added and incubated at 37 °C for 24 h, while taking 50 µL aliquots at various time points. Aliquots were immediately quenched with EtOH/DMSO (100 µL, 1:1, v/v), centrifuged at 13,400 rpm for 10 mins and the supernatant analysed by analytical HPLC, by comparing the peptide peak against the caffeine standard at 220 nm. TCEP was used to reduce disulfide bonds in linear peptides. Experiments were done in duplicate.



SI Figure 8: Serum stability assays on linear and stapled peptides of oxytocin, somatostatin and urotensin.

8. Competition fluorescence polarisation

Competition fluorescence polarisation was carried out using Mdm2 and a 5-TAMRA-labelled tracer peptide in a similar fashion as previously described by Lau *et al.* who also described the dissociation constant.[1] Stock solutions of peptides in DMSO (10 mM) were diluted in assay buffer (1 × PBS + 0.01% (v/v) Tween 20 + 3% (v/v) DMSO) to a top concentration of 10 µM, then 2/3-fold serial dilutions were made to give a 20-point titration. A stock solution of FP tracer (1 mM) was diluted in assay buffer to a concentration of 200 nM (final assay concentration of 50 nM). Mdm2 was diluted in assay buffer to a concentration of 380 nM (final assay concentration of 95 nM). Dilutions of peptides (20 µL), FP tracer (10 µL) and MDM2 (10 µL) were added to a 384-well plate (OptiPlate™ -384 F, Perkin Elmer) and incubated at room temperature for 1h. Titrations were conducted twice independently, in triplicate. Fluorescence polarisation was measured using a BMG ClarioStar plate reader using an excitation filter at 540 nm and emission filter at 590 nm, with a 20 nm bandwidth. Graphs were plotted using GraphPad Prism 7.03 and analysed using the following equation.

$$r = r_0 + (r_b + r_0) \times \frac{2\sqrt{(d^2 - 3e)}\cos(\theta/3) - d}{3K_{d1} + 2\sqrt{(d^2 - 3e)}\cos(\theta/3) - d}$$

$$d = K_{d1} + K_{d2} + [L]_{st} + [L]_t - [P]_t$$

$$e = K_{d1}([L]_t - [P]_t) + K_{d2}([L]_{st} - [P]_t) + K_{d1}K_{d2}$$

$$\theta = \cos^{-1}\left(\frac{-2d^3 + 9de - 27f}{2\sqrt{(d^2 - 3e)^3}}\right)$$

$$f = -K_{d1}K_{d2}[P]_t$$

Where, r is anisotropy measured, r_0 is anisotropy of free peptide, r_b is anisotropy of Mdm2:5-TAMRA peptide complex, K_{d1} is apparent dissociation constant of 5-TAMRA peptide to Mdm2, K_{d2} is dissociation constant of non-labelled ligand to Mdm2, $[P]_t$ is Mdm2 concentration, $[L]_t$ is non-labelled ligand concentration and $[L]_{st}$ is 5-TAMRA peptide concentration.

9. References

1. Y. H. Lau, P. de Andrade, S.-T. Quah, M. Rossmann, L. Laraia, N. Sköld, T. J. Sum, P. J. E. Rowling, T. L. Joseph, C. Verma, et al., *Chem. Sci.* **2014**, *5*, 1804–1809.