Electronic Supplementary Information

Specific N-terminal attachment of TMTHSI-containing click linkers to native peptides and proteins for strain-promoted azide alkyne cycloaddition

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Contents

Methods and materials

Reagents and starting materials were obtained from commercial suppliers unless otherwise noted. ^{1}H-NMR spectra were recorded on a Bruker Avance-400 ultrashield NMR spectrometer, using $CDCl_3$ or DMSO-d_6 as solvent and are reported in ppm using TMS (0.00 ppm) as an internal standard.

For synthesis of compounds TMTHSI-2PCA and TMTHSI-glycol-2PCA:

UPLC-MS method A: Instrument: Agilent 1260 Infinity, 1260 G1312B Bin. Pump, 1260 G1367E WPS, 1260 TCC G1316A Column Comp. 1260 G1315C DAD (210-320 nm, 210 nm), PDA (210-320 nm), G6130B MSD ESI pos/neg (mass range 100-800), Column: EVO C18 (100x4.6mm 2.6µm), Flow: 0.8 ml/min; Column Temp: 40°C, Eluent A: 10 mM ammonium bicarbonate in water (pH 9.5), Eluent B: Acetonitrile, Gradient: t=0 min 5% B, t=8.0 min 98% B, t=10 min 98% B, Postrun: 2 min.

LC-MS method B: Instrument: Agilent 1260 Infinity II, 1260 G7112B Bin. Pump, 1260 G7167A Multisampler, 1260 MCT G7116A Column Comp. 1260 G7115A DAD (210, 220 and 210-320nm), PDA (210-320nm), G6130B MSD (ESI pos/neg) mass range 90-1500, Column: XSelect CSH C18 (30x2.1mm 3.5µ), Flow: 1 ml/min, Column temp: 25°C, Eluent A: 10mM Ammoniumbicarbonate in water (pH 9.5), Eluent B: Acetonitrile, Gradient: t=0 min 5% B, t=1.6 min 98% B, t=3 min 98% B, Postrun: 1.2 min.

UPLC-MS method C: Instrument: Waters I-Class UPLC, Binary Solvent Manager (BSM), Sample Manager-FTN (SM-FTN) and Sample Organizer (SO), Column Manager (CM-A), PDA 210-320nm and 220 nm, QDa ESI 100-800 (pos) 100-800 (neg), Column: Acquity HSS T3 C18 (100x2.1mm 1.7µm) Flow: 0.45 ml/min; Column temp: 60°C, Eluent A: 0.1% formic acid in water, Eluent B: 0.1% formic acid in acetonitrile, Gradient: t=0 min 5% B, t=0.5 min 5% B, t=5 min 98% B, t=6 min 98% B, Postrun: 1 min.

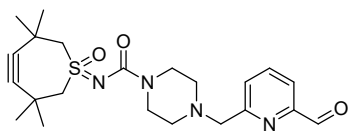
Basic prep MPLC method: Instrument type: Reveleris™ prep MPLC; column: Waters XSelect CSH C18 (145x25 mm, 10µ); Flow: 40 mL/min; Column temp: room temperature; Eluent A: 10 mM ammoniumbicarbonate in water pH = 9.0); Eluent B: 99% acetonitrile + 1% 10 mM ammoniumbicarbonate in water; Gradient: t=0 min 5% B, t=1 min 5% B, t=2 min 30% B, t=17 min 70% B, t=18 min 100% B, t=23 min 100% B; Detection UV: 230, 254, 280 nm.

For Evasin functionalization, click to CDP-azide and purification:

Preparative and semi-preparative HPLC were performed on a Waters DeltaPrep Preparative Chromatography System with EmPower 2 software. Either a C18 22 mm x 250 mm (C18 preparative) column, C18 10 mm x 250 mm (C18 semi-preparative) column, or C4 10 mm x 250 mm (C4 semi-preparative; VYDAC) column was used. The preparative system was used for peptide amounts up to 50 mg in combination with a flow of 20 ml/min, whereas the semi-preparative systems were used for a maximum of 20 mg of peptide in combination with a 12 ml/min flow. Absorption was measured at a wavelength of 214 nm.
Buffer A: 0.1% TFA in H_2O and buffer B: 0.1% TFA in a 9/1 (v/v) mixture of ACN in H_2O.

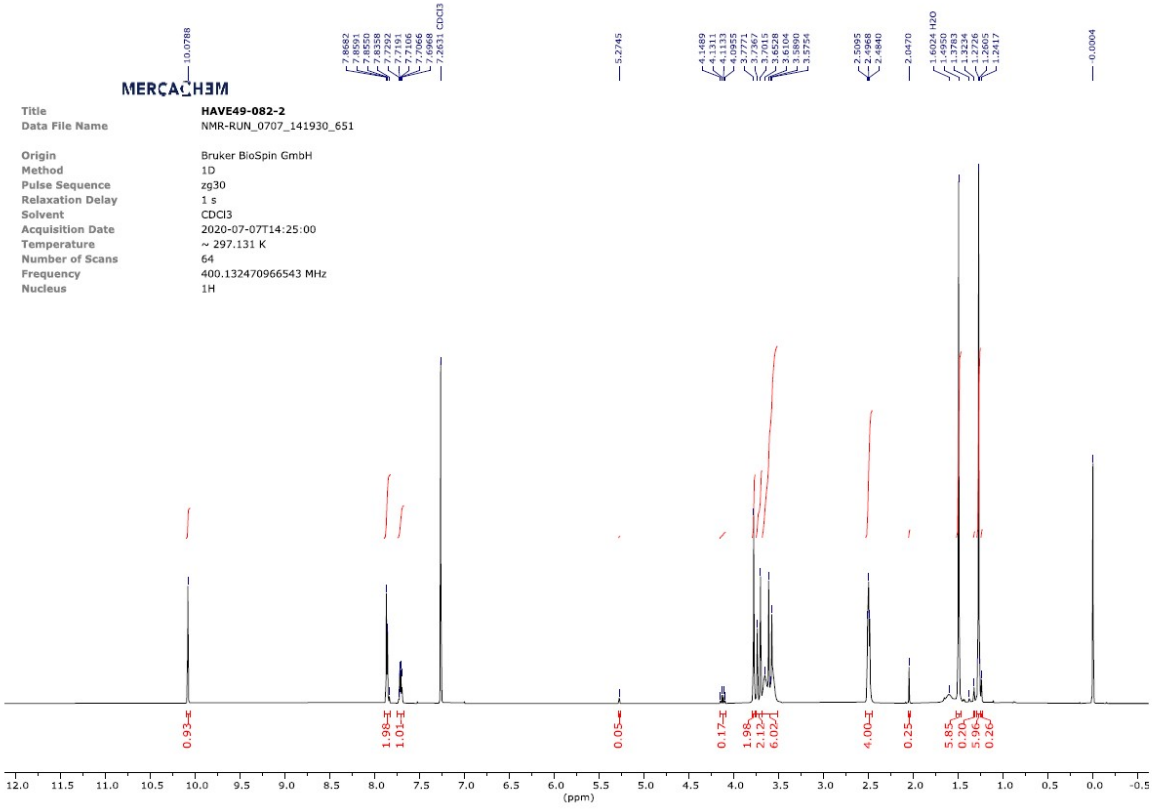
Ultra-high performance liquid chromatography (UPLC) electron-spray ionization (ESI)-MS was performed on a Waters UHPLC XEVO-G2QTOF (XEVO) system. Absorption was measured at a wavelength of 220 nm. Deconvoluted molecular masses of the polypeptides were retrieved based on experimental mass to charge ratios (m/z) using the MaxEnt3 feature within the MassLynx software.
Buffer A: 0.1% FA in H_2O, buffer B: 0.09 % FA in ACN/H_2O 9/1 (v/v). Column: ACQUITY UPLC Peptide BEH C18 column, 130 A, 1.7 um, 2.1 mm x 50 mm.

S1: Synthesis of TMTHSI-2PCA 3



TMTHSI-2PCA (**3**)

A suspension of TMTHSI-OSu **1** (118 mg, 0.346 mmol) and DIPEA (301 µl, 1.73 mmol) in DCM (2.0 ml) was added to a suspension of 6-(1-piperazinylmethyl)-2-pyridinecarboxaldehyde bistosylate salt **2** (190 mg, 0.346 mmol) in ACN (2.0 ml). The mixture was stirred at room temperature for 20 hrs. The mixture was quenched through addition of aq. $NaHCO_3$ solution and extracted with DCM (3x 10 ml). The combined organic extracts were passed over a phase separator. The combined organic filtrates were dried over Na_2SO_4 and concentrated *in vacuo*. A yellow residue was obtained which was purified by flash column chromatography with isocratic elution using EtOAc to afford an off-white solid residue. The residue was triturated from Et_2O, washed with pentane and air-dried to afford 76 mg (51% yield, 0.18 mmol) of title compound **3** as an off-white solid residue. ^{1}H NMR (400 MHz, $CDCl_3$) δ 10.08 (s, 1H), 7.89 – 7.82 (m, 2H), 7.74 – 7.67 (m, 1H), 3.78 (s, 2H), 3.72 (d, *J* = 14.1 Hz, 2H), 3.68 – 3.51 (m, 6H), 2.50 (t, *J* = 5.1 Hz, 4H), 1.50 (s,6H), 1.27 (s, 6H). UPLC-MS (method A): t_r= 5.03 min, purity: 94.7%, M/z $[M+H]^+$ 431.



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Eluent A	10mM ammoniumbicarbonate in water (pH=9.5)
Eluent B	100% acetonitrile
Gradient	t=0 min 5% B, t=8 min 98% B, t=10 min 98% B
Posttime	2 min
Detection PDA	210-320nm, 260nm
MS	SQD2
Detection MS	ESI (scan)
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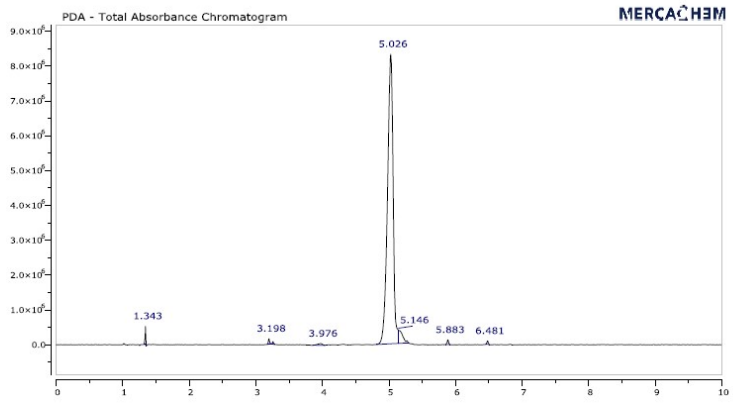
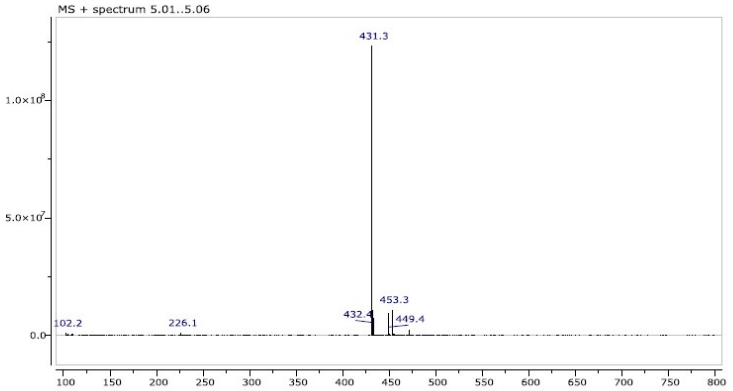
Mass range	100-800 (neg)
Time	10 min
Cone	15 V; Scan time: 0.4 s

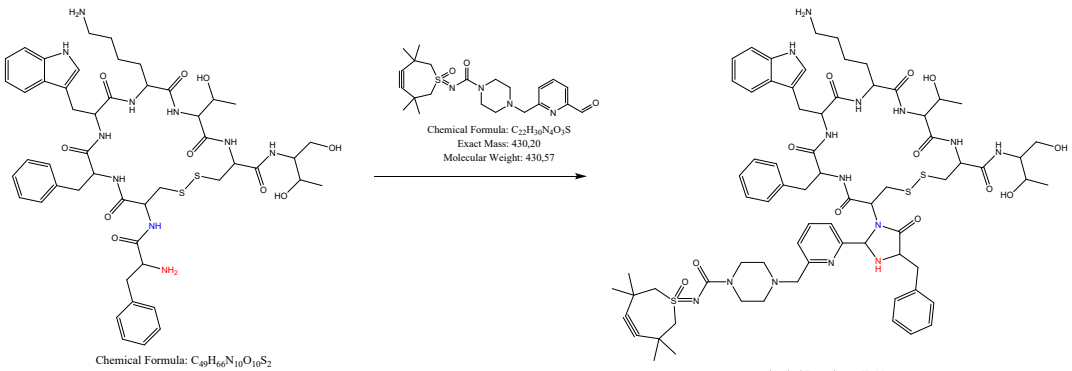


Integrals spectrum PDA - Total Absorbance Chromatogram

rt (min)	height	area	area (%)
1.34	537015	11436115	0.56
3.20	158964	7167560	0.35
3.26	73839	2832587	0.14
3.98	48333	8603080	0.42
5.03	8318109	1950363883	94.73
5.15	378069	65058835	3.16
5.88	136701	8207956	0.40
6.48	100306	5270772	0.26



S2: TMTHSI-2PCA conjugation to octreotide/ LTX315



In a 2 mL HPLC vial with stirring bar, 0.5 mg of octreotide (0.49 nmol) or LTX315 (0.35 nmol) was dissolved in 1mL of 100 mM sodium phosphate buffer with pH adjusted to 7.5. In parallel, 3 mg of TMTHSI-2PCA (6.97 nmol) was dissolved in 0.5 mL of ACN. The TMTHSI-2PCA solution was added to the peptide solution, with a final solvent of 2:1 (buffer: ACN). The reaction mixture was stirred overnight after which LC-MS was used to analyze the reaction mixture.

LC-MS method:

LC:

UPLC column:	Acquity UPLC Peptide CSH 1.7µm; 2.1 x 100 mm
Eluent A:	0.1% TFA in MilliQ water
Eluent B:	0.1% TFA in ACN
Needle flush solvent:	MilliQ water / ACN (10:90 v/v)
Sample tray Temperature:	20 °C
Column Temperature:	40 °C
Flow:	0.5 mL/min
Injection volume:	2.0 µL
UV Detection:	210 nm
Resolution	1.2 nm
Sampling rate	10 point/sec
Run time:	8 minutes

Time (min)	Flow rate (ml/min)	%A	%B	Curve
Initial	0.500	98.0	2.0	-
0.50	0.500	98.0	2.0	6
5.00	0.500	60.0	40.0	6
5.01	0.500	0.0	100.0	6
6.00	0.500	0.0	100.0	6
6.01	0.500	98.0	2.0	6
8.00	0.500	98.0	2.0	6

MS:

Capillary	3.00 kV
Cone	70.00 V
R_F	2.50 V
Extract	3.00 V
Source temperature	150 °C
Desolvation temperature	400 °C
Cone gas flow	50 L/Hr
Desolvation gas flow	650 L/Hr
SIR	ES+
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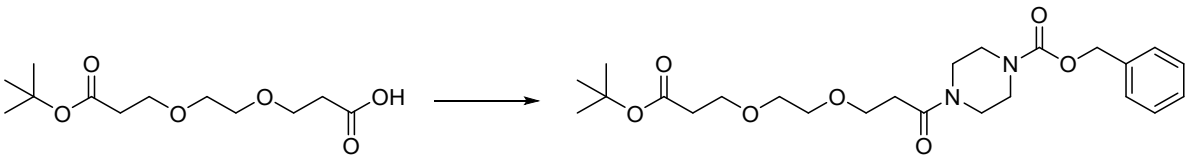
MS Scan	ES+
Mass range	400 to 1400

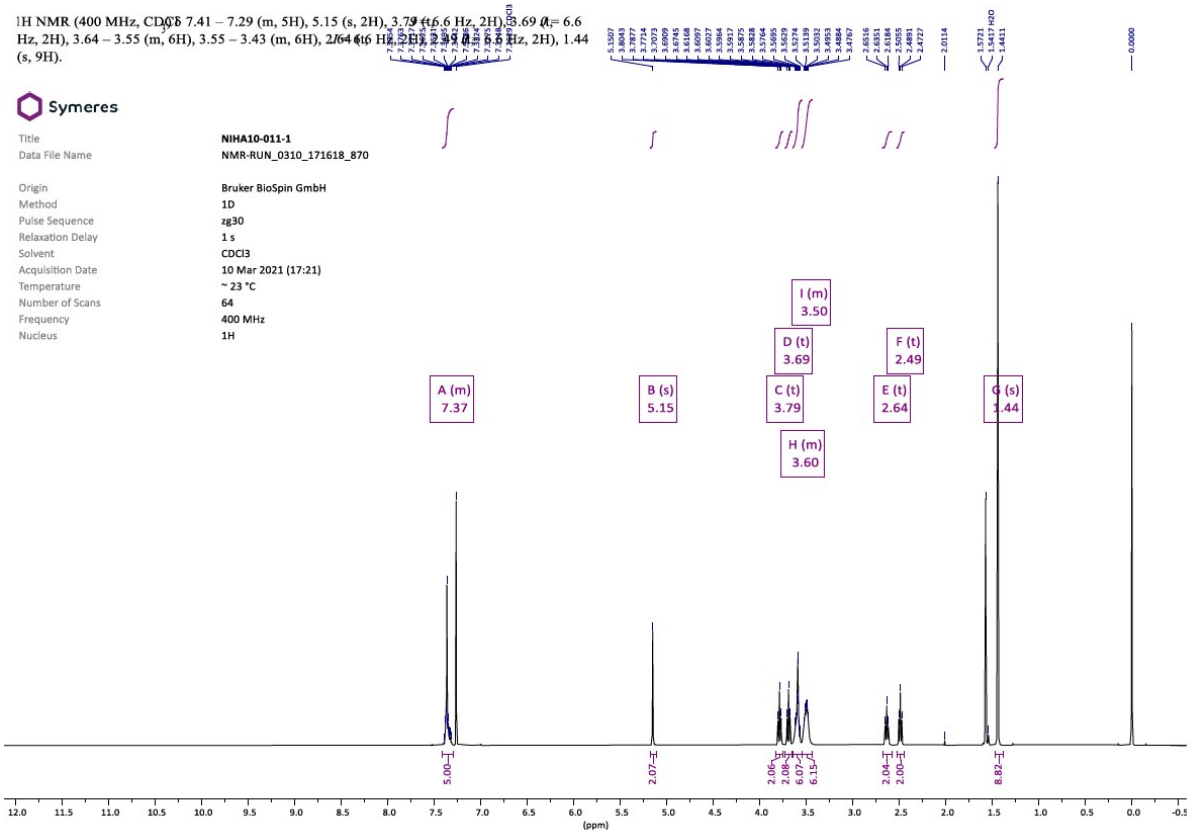
S3: Synthesis of TMTHSI-glycol-2PCA 16

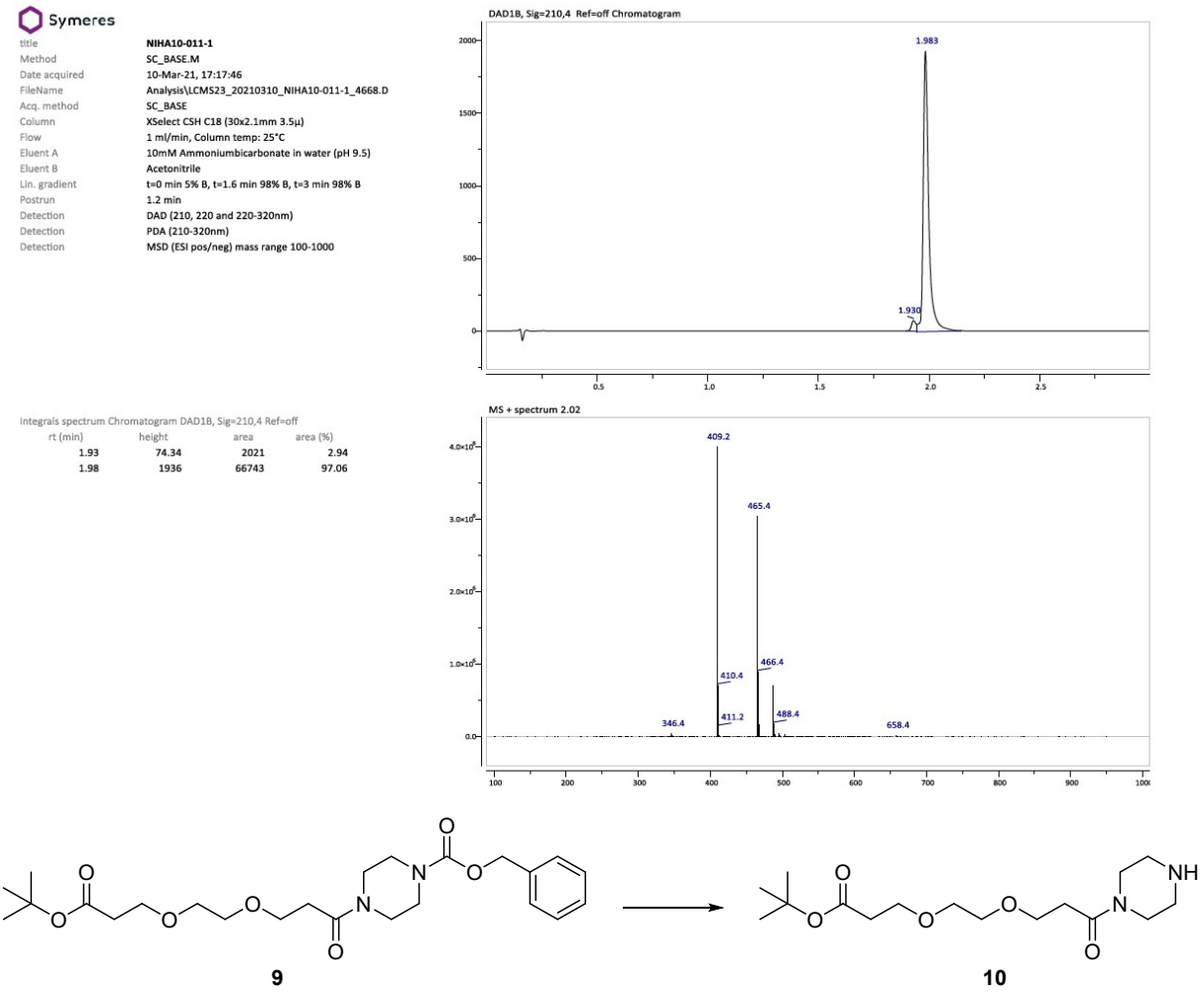


8 **9**

benzyl 4-(3-(2-(3-(tert-butoxy)-3-oxopropoxy)ethoxy)propanoyl)piperazine-1-carboxylate (**9**)

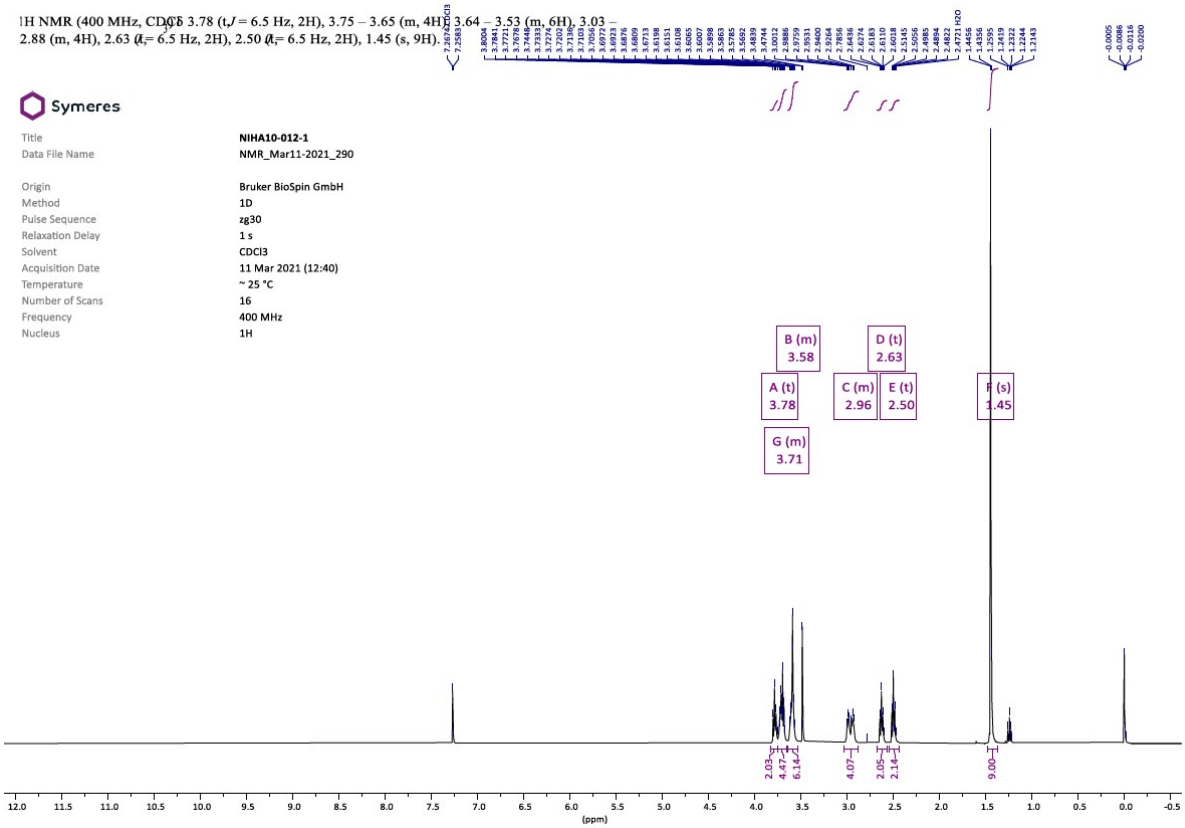
To an oven-dried vial was added anhydrous DMF (1.27 mL), HATU (159 mg, 0.419 mmol), and carboxylic acid-PEG2-*t*-butyl ester **8** (100 mg, 0.381 mmol) which were mixed at room temperature under argon atmosphere. DIPEA (100 µL, 0.572 mmol) was added, and after 10 min 1-Cbz-piperazine (88 µL, 0.457 mmol) was added. The yellow solution was stirred at room temperature for 2 hours. The reaction mixture was diluted with DMSO (2.0 mL) and injected into a basic prep-MPLC system. All fractions containing product were isolated to obtain 161 mg (91%, 0.347 mmol) of title compound **9** as a tan oil. ^{1}H NMR (400 MHz, $CDCl_3$) δ 7.41 – 7.29 (m, 5H), 5.15 (s, 2H), 3.79 (t, *J* = 6.6 Hz, 2H), 3.69 (t, *J* = 6.6 Hz, 2H), 3.64 – 3.55 (m, 6H), 3.55 – 3.43 (m, 6H), 2.64 (t, *J* = 6.6 Hz, 2H), 2.49 (t, *J* = 6.6 Hz, 2H) 1.44 (s, 9H); LC-MS (method B): t_r= 1.98 min, purity: 97.1%, M/z $[M+H]^+$ 465, $[M-t\text{Bu}+H]^+$ 409.

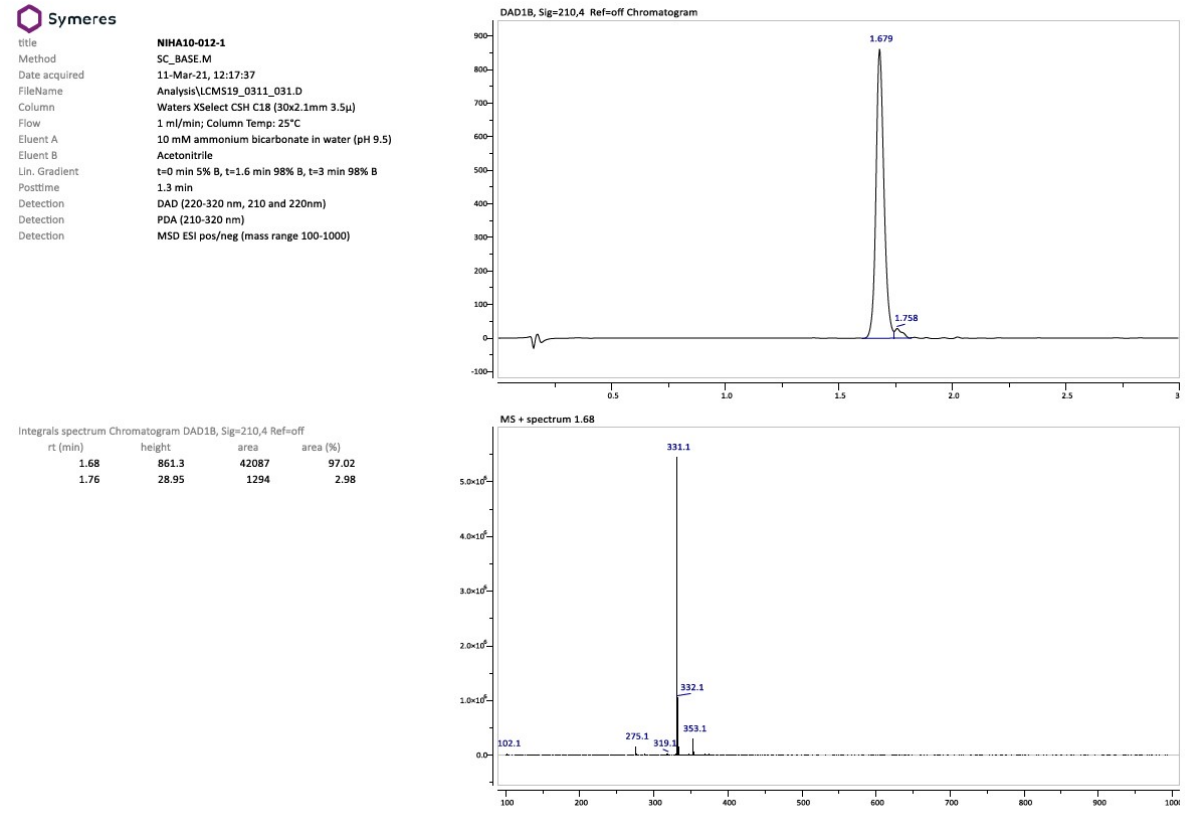




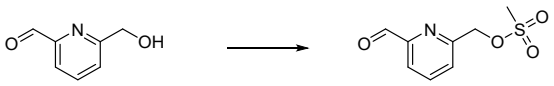
Tert-butyl 3-(2-(3-oxo-3-(piperazin-1-yl)propoxy)ethoxy)propanoate (**10**)

Cbz-protected compound **9** (160 mg, 0.344 mmol) was dissolved in EtOH (3.44 mL) after which 10% Pd/C (37 mg, 0.034 mmol) was added. A hydrogen atmosphere was created above the mixture and a hydrogen balloon was attached to the flask. The mixture was stirred vigorously at room temperature for 2 hours. The reaction mixture was diluted using EtOH (5 mL) and taken up in a syringe followed by filtration through using a 45 µm syringe filter. After completion of the filtration, the filter was washed using EtOH (10 mL). The colorless filtrate was concentrated *in vacuo* to yield 128 mg (>99% yield) of **10** as a colorless film. ^{1}H NMR (400 MHz, $CDCl_3$) δ 3.78 (t, *J* = 6.5 Hz, 2H), 3.75 – 3.65 (m, 4H), 3.64 – 3.53 (m, 6H), 3.03 – 2.88 (m, 4H), 2.63 (t, *J* = 6.5 Hz, 2H), 2.50 (t, *J* = 6.5 Hz, 2H) 1.45 (s, 9H); LC-MS (method B): t_r= 1.68 min, purity: 97.0%, M/z $[M+H]^+$ 331.





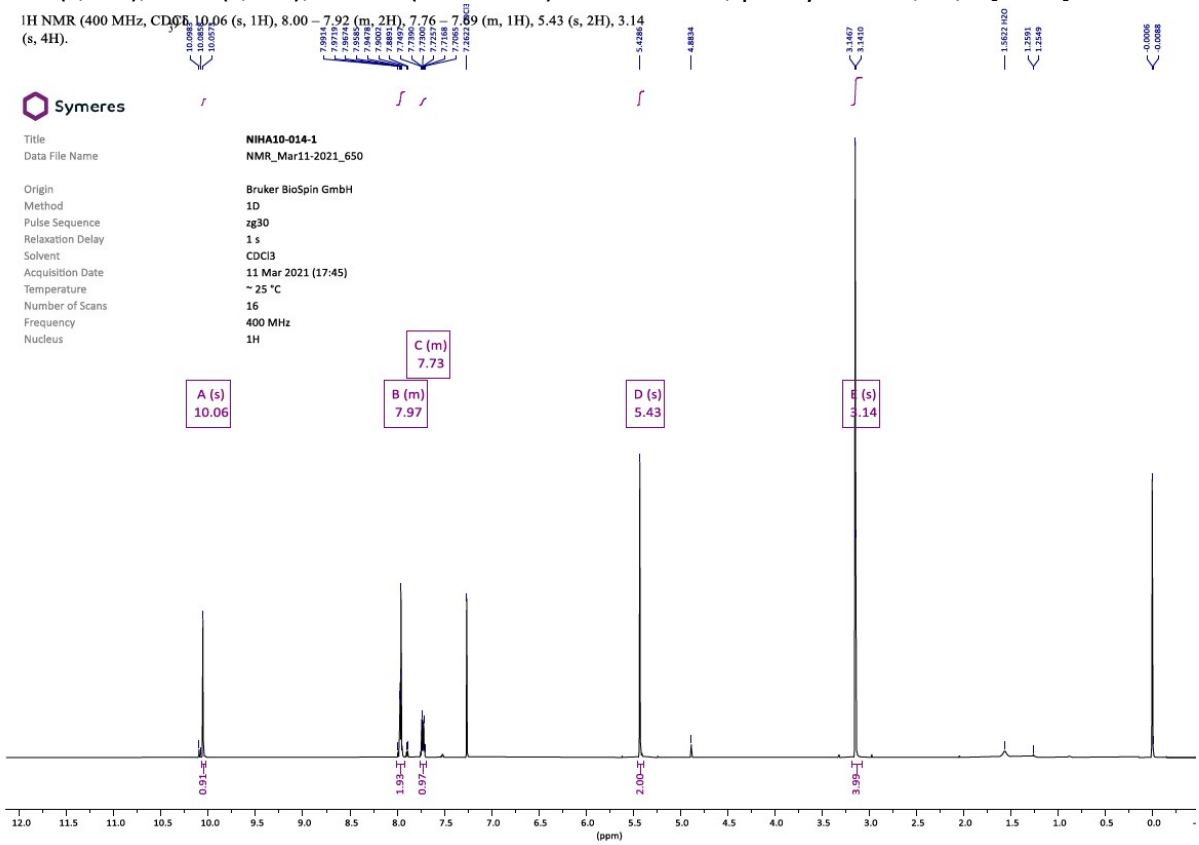
Synthesis of **12** from **11** was only performed when **12** was not commercially available. For the batch used for subsequent synthesis, this was not required.



12 13

(6-formylpyridin-2-yl)methyl methanesulfonate (**13**)

6-(Hydroxymethyl)picolinaldehyde **12** (900 mg, 6.56 mmol) and TEA (2.74 mL, 19.7 mmol) were dissolved in DCM (50 mL) and cooled to 0 °C under an argon atmosphere. mesyl-Cl (0.563 mL, 7.22 mmol) was added dropwise and the mixture was stirred for 1 hour at room temperature. The mixture was concentrated *in vacuo* and purified using flash column chromatography eluting with a gradient from heptane:EtOAc 9:1 to 4:6. All fractions containing product were isolated to yield 526 mg (37%, 2.44 mmol) of title compound **13** as a tan oil. ^{1}H NMR (400 MHz, $CDCl_3$) δ 10.06 (s, 1H), 8.00 – 7.92 (m, 2H), 7.76 – 7.69 (m, 1H), 5.44 (s, 2H), 3.14 (s, 4H); LC-MS (method B): t_r= 1.26 min, purity: 94.5%, M/z $[M+H]^+$ 216.



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Eluent B	Acetonitrile
Lin. gradient	t=0 min 5% B, t=1.6 min 98% B, t=3 min 98% B
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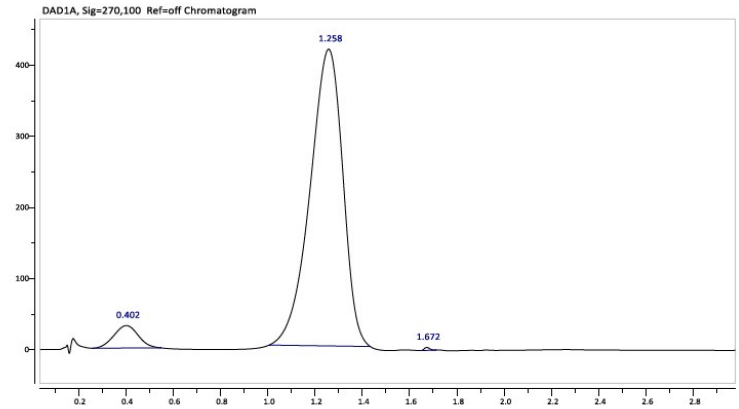
Detection	PDA (210-320nm)
Detection	MSD (ESI pos/neg) mass range 100-1000



Integrals spectrum Chromatogram DAD1A, Sig=270,100 Ref=off

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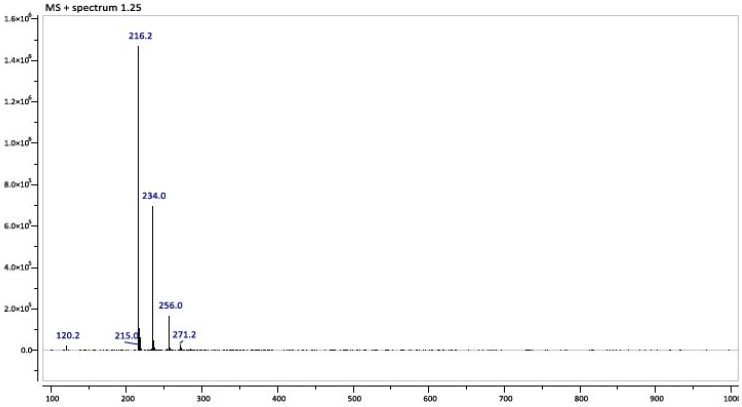
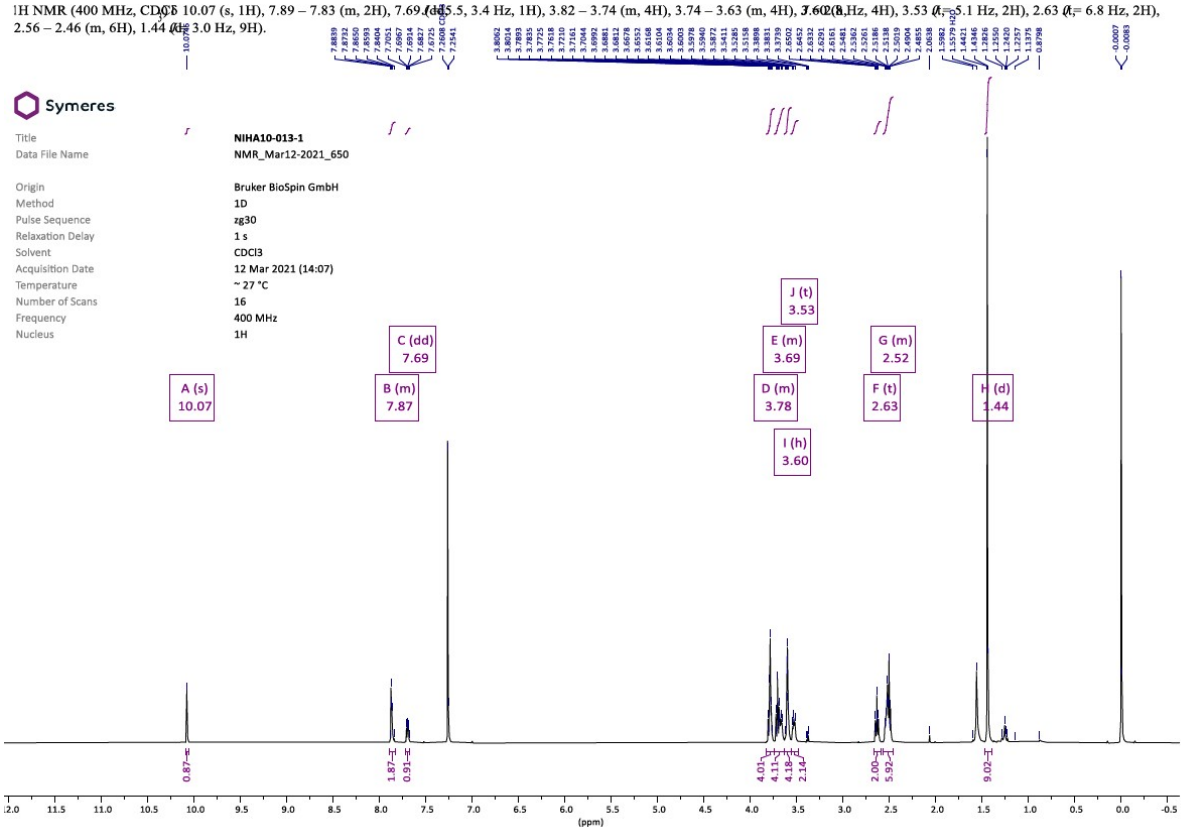
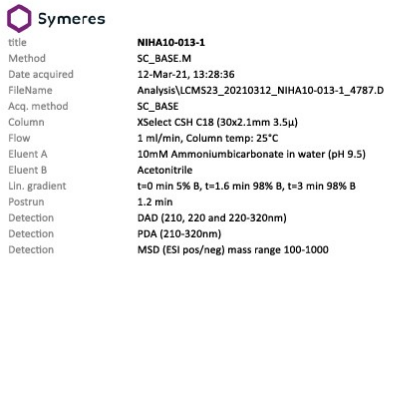


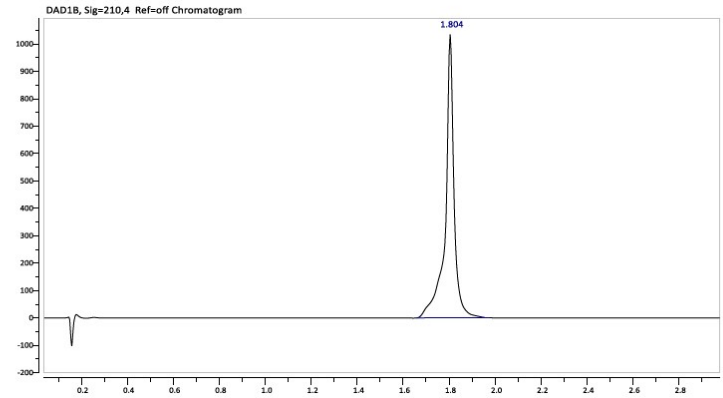
Tert-butyl 3-(2-(3-(4-((6-formylpyridin-2-yl)methyl)piperazin-1-yl)-3-oxopropoxy)ethoxy)propanoate (**14**)

Piperazine **10** (128 mg, 0.387 mmol) was dissolved in anhydrous ACN (1.94 mL) after which K_2CO_3 (118 mg, 0.852 mmol) and mesylate **13** (92 mg, 0.43 mmol) were added under argon atmosphere. The mixture was heated overnight to 60 °C. The reaction solution was taken up in a syringe and reaction vial was washed with ACN (2.0 mL) and taken up in the same syringe. The contents of the syringe were filtered using a 45 µm syringe filter and concentrated *in vacuo*. The residue was purified using basic prep MPLC. All fractions containing product were isolated to yield 123 mg (71%, 0.274 mmol) of title compound **14** as a colorless oil. ^{1}H NMR (400 MHz, $CDCl_3$) δ 10.07 (s, 1H), 7.89 – 7.83 (m, 2H), 7.69 (dd, *J* = 5.5, 3.4 Hz, 1H), 3.82 – 3.74 (m, 4H), 3.74 – 3.63 (m, 4H), 3.60 (h, *J* = 2.8 Hz, 4H), 3.53 (t, *J* = 5.1 Hz, 2H), 2.63 (t, *J* = 6.8 Hz, 2H), 2.56 – 2.46 (m, 6H), 1.44 (s, 9H); LC-MS (method B): t_r= 1.80 min, purity: >99%, M/z $[M+H]^+$ 450.

¹H NMR (400 MHz, CDCl3) δ 10.07 (s, 1H), 7.89 – 7.83 (m, 2H), 7.69 (dd, J = 5.5, 3.4 Hz, 1H), 3.82 – 3.74 (m, 4H), 3.74 – 3.63 (m, 4H), 3.60 (h, J = 6.8 Hz, 4H), 3.53 (t, J = 5.1 Hz, 2H), 2.63 (t, J = 6.8 Hz, 2H), 2.56 – 2.46 (m, 6H), 1.44 (d, J = 3.0 Hz, 9H).







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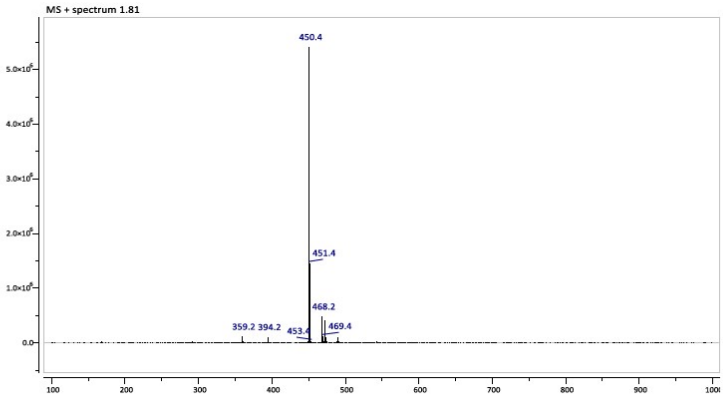

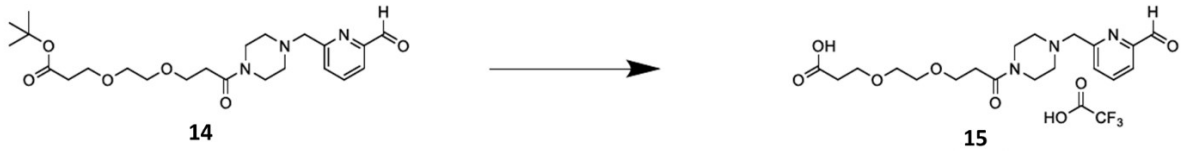

14 15

3-(2-(3-2PCA-3-oxopropoxy)ethoxy)propanoic acid TFA salt (**15**)

Tert-butyl ester **14** (119 mg, 0.265 mmol) was dissolved in DCM (1.32 mL) after which TFA (326 µL, 4.24 mmol) was added. The solution was stirred at room temperature for 6 hours. The mixture was concentrated *in vacuo* to obtain 134 mg (>99%, 0.264 mmol) of title compound **15** as a tan oil which was used in a followup reaction without further purification. LC-MS (method B): t_r= 1.22 min, purity: 92.8%, M/z $[M+H]^+$ 394.

Symeres

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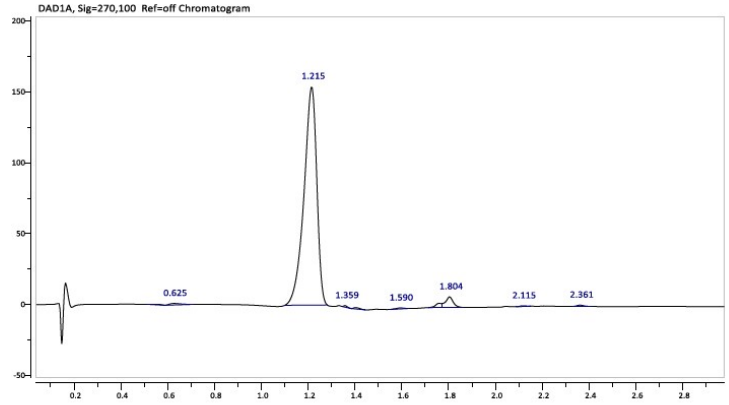
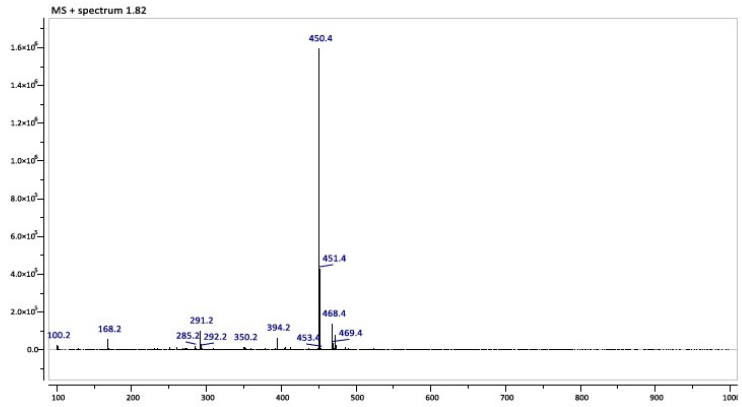
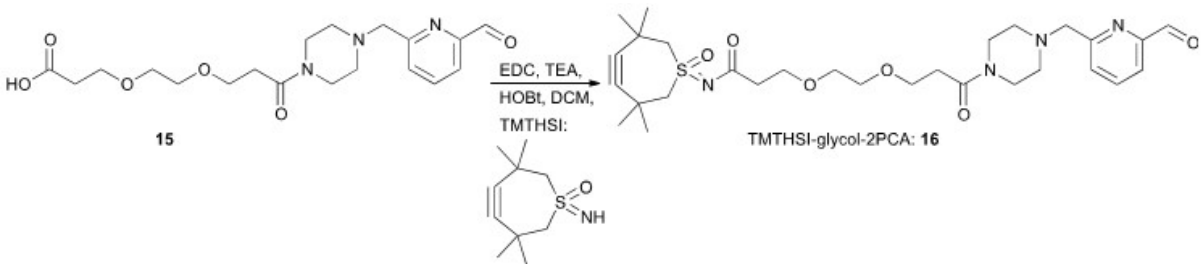
Eluent A	10mM Ammoniumbicarbonate in water (pH 9.5)
Eluent B	Acetonitrile
Lin. gradient	t=0 min 5% B, t=1.6 min 98% B, t=3 min 98% B
Postrun	1.2 min
Detection	DAD (210, 220 and 220-320nm)
Detection	PDA (210-320nm)
Detection	MSD (ESI pos/neg) mass range 100-1000



Integrals spectrum Chromatogram DAD1A, Sig=270,100 Ref=off

rt (min)	height	area	area (%)
0.56	0.4588	21.01	0.17
0.62	1.101	75.83	0.61
1.21	154.2	11695	94.47
1.36	1.110	22.40	0.18
1.40	0.9612	34.78	0.28
1.59	0.6975	34.37	0.28
1.76	2.950	101.9	0.82
1.80	7.542	344.5	2.78
2.12	0.4826	17.66	0.14
2.36	0.8841	32.56	0.26

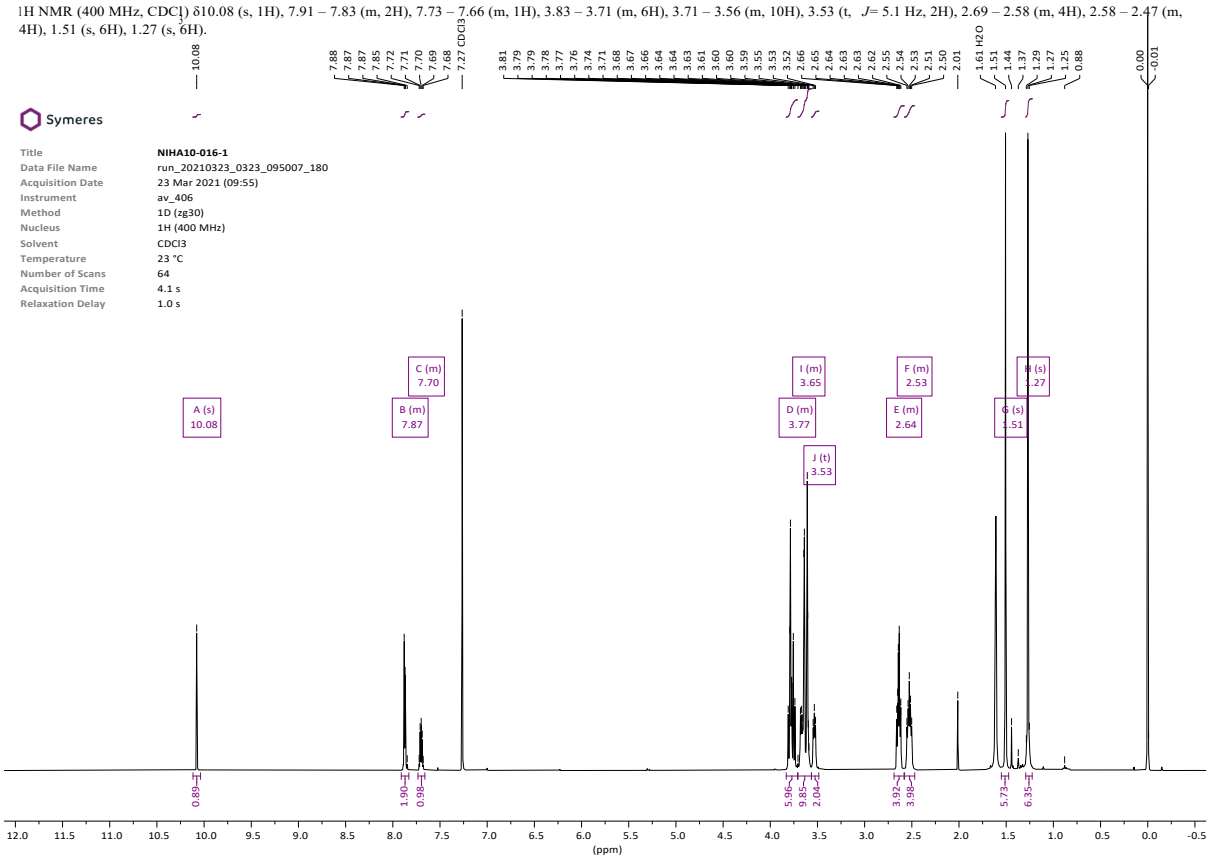




TMTHSI-glycol-2PCA (**16**)

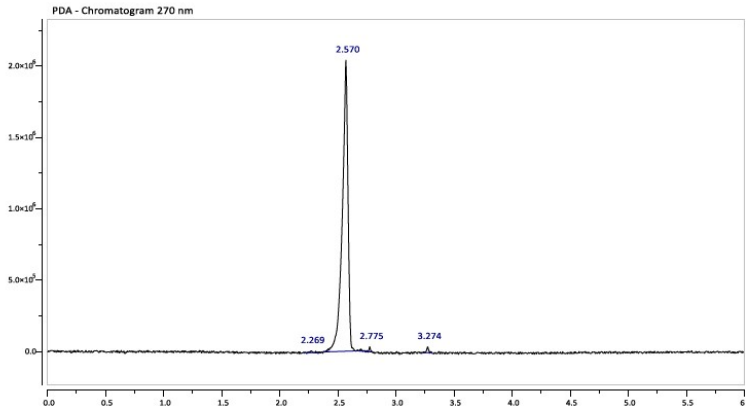
Carboxylic acid **15** (134 mg, 0.264 mmol) was suspended in anhydrous DCM (3.73 mL) and placed under an argon atmosphere. HOBt (52.6 mg, 0.343 mmol) and EDC (65.8 mg, 0.343 mmol) were added and the mixture was stirred for 10 min at room temperature. TMTHSI (68.4 mg, 0.343 mmol) dissolved in DCM (1.55 mL) was added dropwise followed by TEA (110 µL, 0.792 mmol). The solution was stirred at room temperature for 5 hours. After 1 hour additional EDC (65.8 mg, 0.343 mmol) and TEA (110 µL, 0.792 mmol) were added. The reaction mixture was concentrated *in vacuo* and injected in a basic prep-MPLC system.

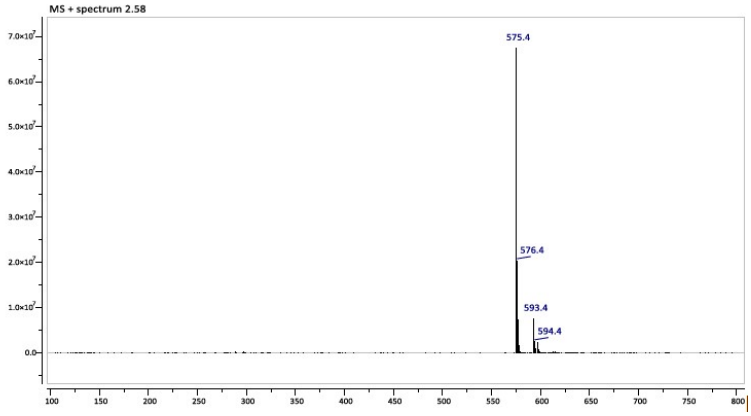
Fractions containing product were pooled and extracted using DCM (3x 20 mL). The combined organic phases were dried over Na_2SO_4, filtered and evaporated *in vacuo* to obtain 60 mg (40 %, 0.10 mmol) of compound **16** which was isolated as a colorless oil. ^{1}H NMR (400 MHz, $CDCl_3$) δ 10.08 (s, 1H), 7.91 – 7.83 (m, 2H), 7.73 – 7.66 (m, 1H), 3.83 – 3.71 (m, 6H), 3.71 – 3.56 (m, 10H), 3.53 (t, *J* = 5.1 Hz, 2H), 2.69 – 2.58 (m, 4H), 2.58 – 2.47 (m, 4H), 1.51 (s, 6H), 1.27 (s, 6H); UPLC-MS (Method C): t_r= 2.57 min, purity: 98.3%, M/z $[M+H]^+$ 575.



Symeres

title	**NIHA10-016-1**
Date acquired	**22-Mar-2021, 18:18:27**
FileName	**Analysis\LCMS20_210322_NIHA_011.raw**
UPLC	**Waters I-Class**
Acq. Method	**UPLC_T3_05-98_T60_6min**
Column	**Acquity HSS T3 C18 (100x2.1mm 1.7µm)**
Flow	**0.45 ml/min; Column temp: 60°C**
Eluent A	**0.1% formic acid in water**
Eluent B	**0.1% formic acid in acetonitrile**
Gradient	**t=0 min 5% B, t=0.5 min 5% B, t=5 min 98% B, t=6 min 98% B**
Posttime	**1 min**
Detection PDA	**210-320nm and 220 nm**
MS	**QDa**
Detection MS	**ESI (scan)**
Mass range	**105-800 (pos)**
Mass range	**105-800 (neg)**
Time	**6 min**
Cone	**15 V; Freq: 2 Hz**





Integrals spectrum PDA - Chromatogram 270 nm

rt (min)	height	area	area (%)
2.27	**13855**	**1197936**	**0.43**
2.57	**2039699**	**275422042**	**98.26**
2.70	**16456**	**711655**	**0.25**
2.78	**35882**	**1113000**	**0.40**
3.27	**41945**	**1862890**	**0.66**

S4: A2 peptide with TMTHSI-glycol-2PCA, synthesis and purification

In a HPLC vial with magnetic stirring bar, 1 mg of A2 (**17**, 0.4 nmole) was weighed and dissolved in 1 mL of 100 mM HEPES buffer pH 8.0. While stirring, 1.2 mg of TMTHSI-glycol-2PCA **16** sticky oil was measured by weighing a spatula before and after adding **16**. This spatula was placed in the HPLC vial, long enough for all oil stuck to the spatula to dissolve (taken out after approximately 1 hour). After overnight reaction, the reaction mixture was freezedried and redissolved in DMF. Hydroxylamine Wang resin was added (50 mg, loading: 1.5-2.5 mmol/g, Sigma-Aldrich) which resulted in decrease of linker present in solution (Figure 1, bottom)

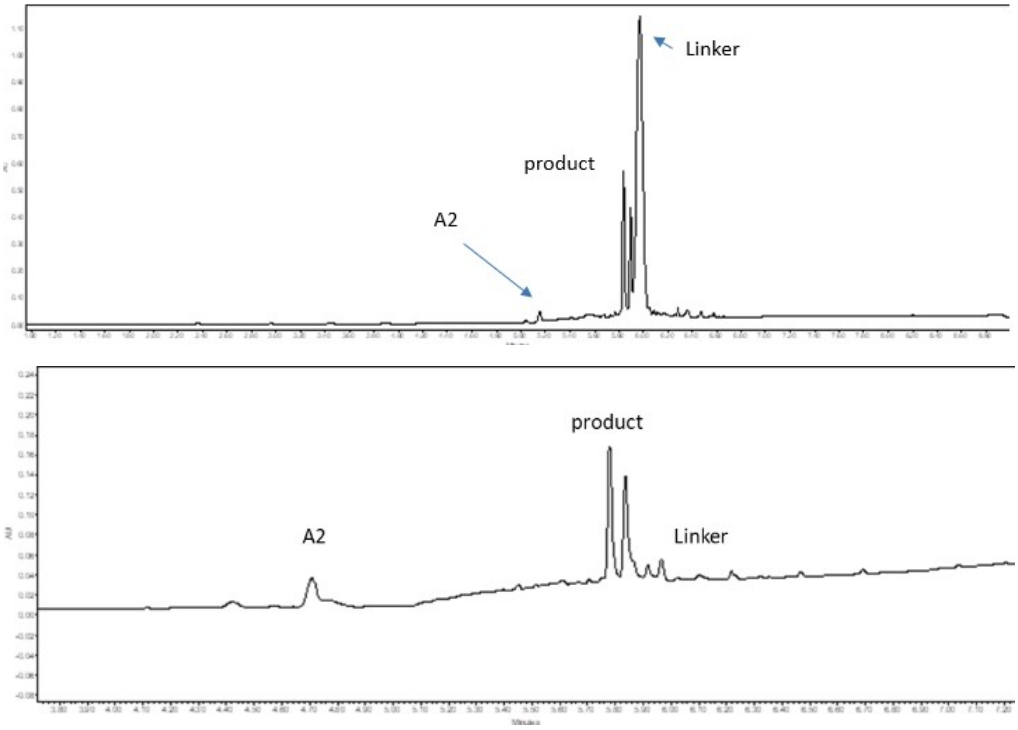


Figure 1: LC-trace of the reaction of A2 with TMTHSI-glycol-2PCA (top trace) after overnight reaction. The excess of linker is largely removed by purification using hydroxylamine beads (bottom trace)

S5: click reaction of **18** with azidoethanol

To 100 µL of **18** in a HPLC vial, 1 µL of azidoethanol was added. The vial was placed on a shaker for 5 minutes after which UPLC trace was again measured (Figure 2 below)

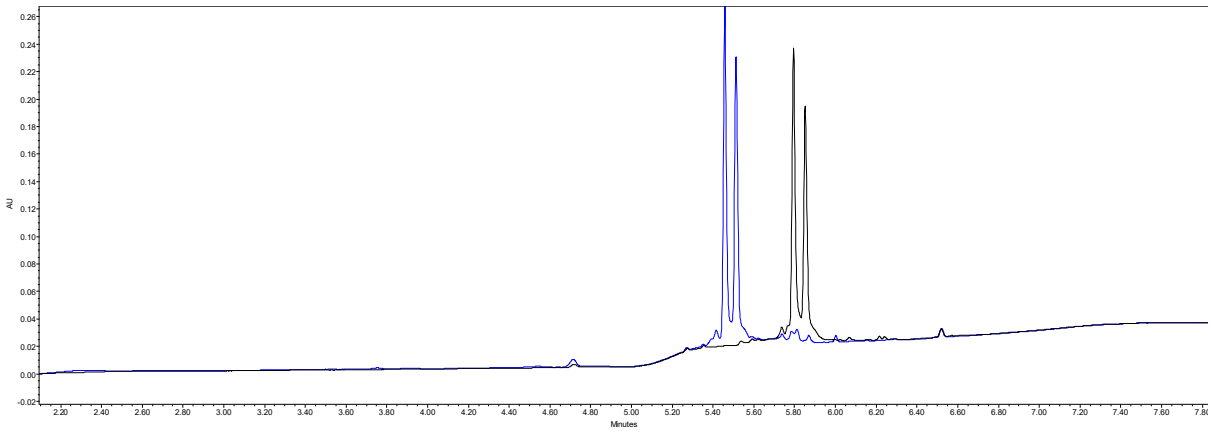


Figure 2: HPLC trace of the reaction of TMTMSI modified TP10-peptide with azido ethanol showing a shift of the product diastereomers. Black: before azidoethanol addition, Blue: 5 min after azidoethanol addition

S6: functionalization of 19 with 16 and subsequent purification

Oxidatively folded Evasin-3 **19** (3 mg, 0.4 nmol) was dissolved in 500 µl 100 mM phosphate buffer pH 7.4. TMTHSI-glycol-2PCA **16** (3 mg, 5.2 nmol) in 120 µl 60% ACN in H_2O was added and the reaction mixture was mixed at room temperature for 26 h. The reaction mixture was diluted by addition of 3.4 ml 0.1 M acetate buffer pH 4.0 containing 6 M Guanidine-HCl and filtered through a 0.2 µm filter before injection on a semi-prep-HPLC system for purification using the method described above. Fractions containing product were isolated to yield **20**.

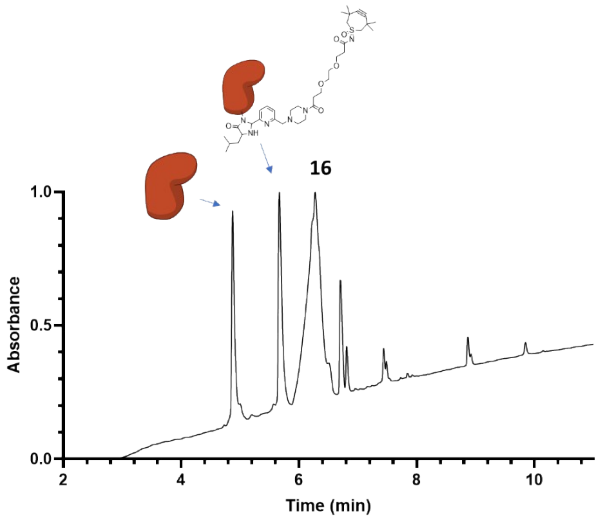


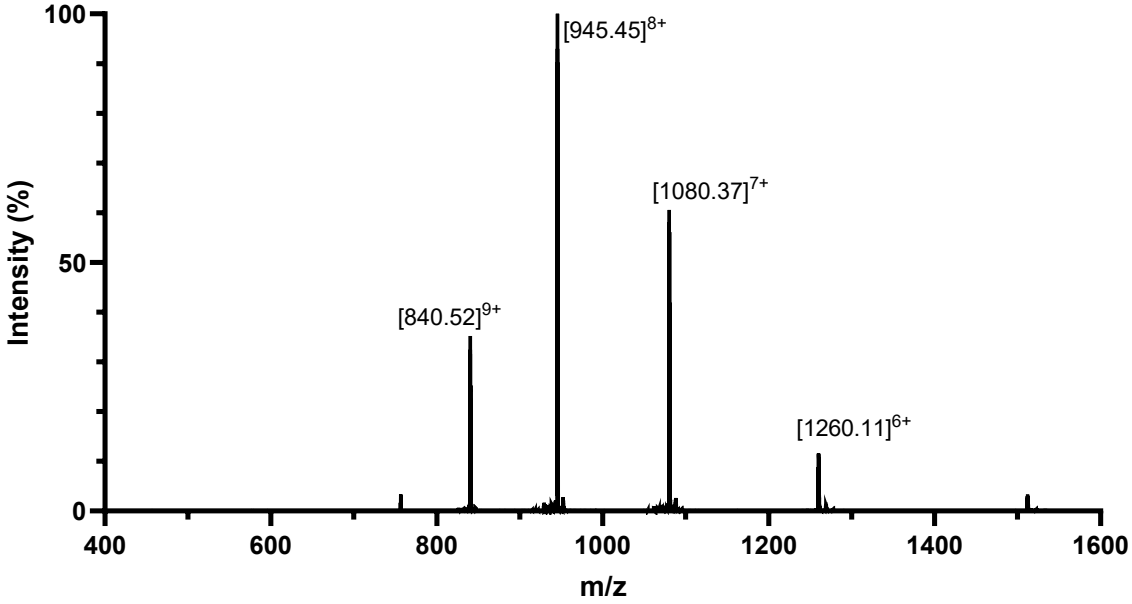
*Figure 3: HPLC trace of the reaction mixture of Evasin-3 **19** with TMTHSI-glycol 2PCA **16***

S7: click reaction of 20 with 21 and subsequent purification

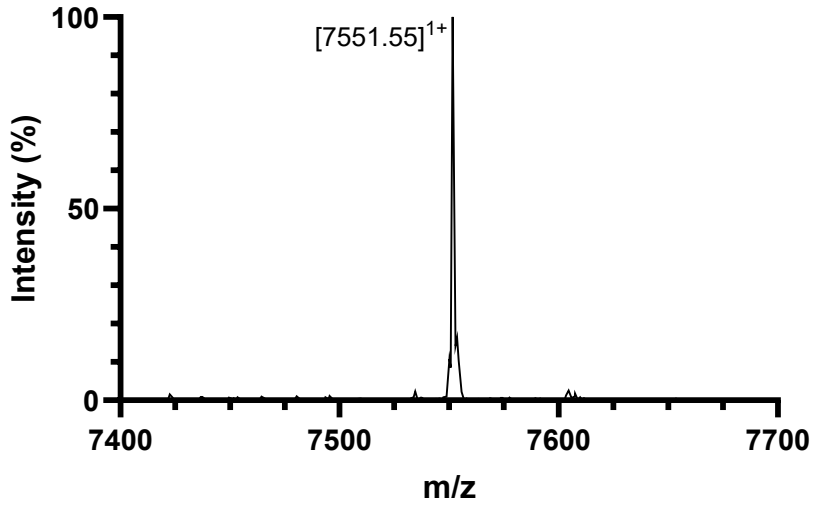
Evasin-3-TMTHSI **21** (500 µg, 0.066 nmole) was dissolved in 30 µl 67% ACN in H_2O. CDP-azide **20** (250 µg, 0.038 nmole) was dissolved in 50 µl 20% ACN in H_2O. **21** was added dropwise to **20** while stirred at room temperature and the reaction mixture was stirred for 3.5 h. Before injection on an analytical HPLC-system with the method described above, the

reaction mixture was diluted by addition of 80 µl H_2O. Fractions containing product were isolated to yield Evasin-3-CDP conjugate **22**.

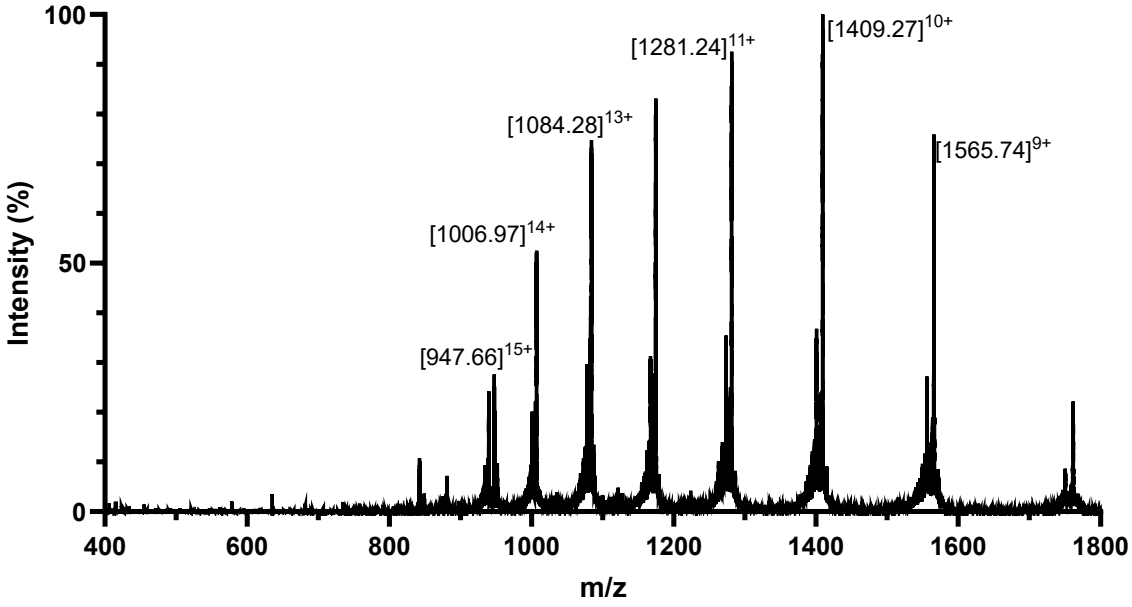
Mass spectra of **20** and **22**



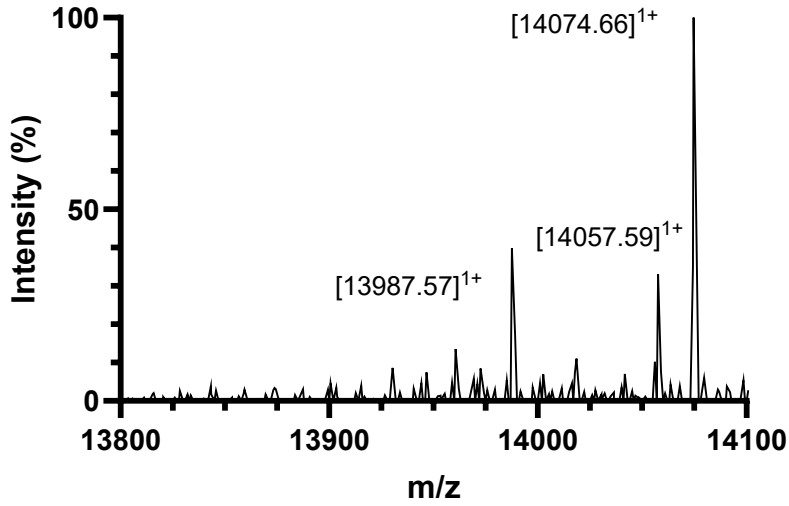
ESI-MS of **20**



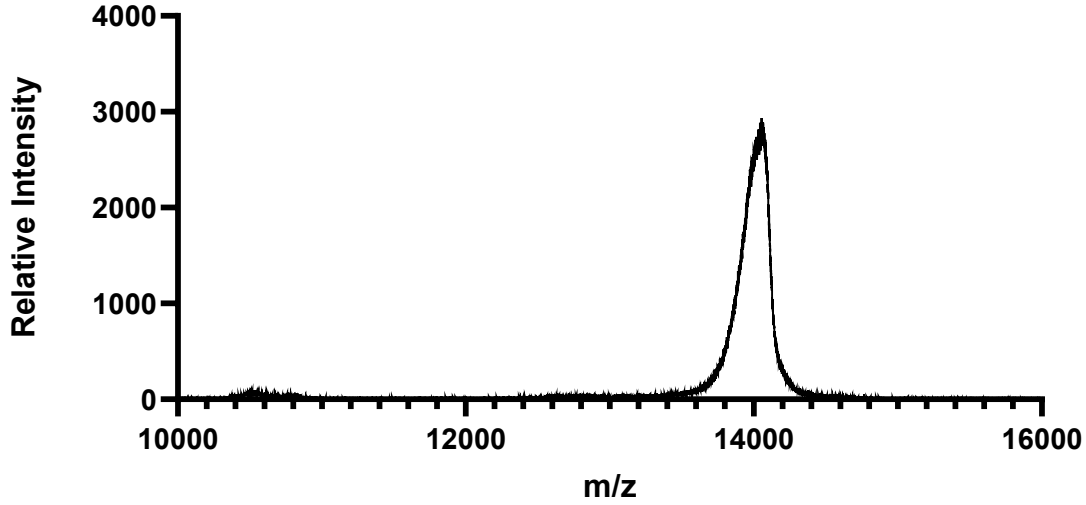
Deconvoluted MS of **20**



ESI-MS of **22**



Deconvoluted mass of **22**


Relative Intensity
4000
3000
2000
1000
0
10000
12000
14000
16000
m/z

MALDI of **22**