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Supplementary information

A combinatorial approach toward smart libraries of discontinuous epitopes of HIV gp120 on a TAC synthetic scaffold

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1. General considerations

Unless stated otherwise, chemicals were obtained from commercial sources and used without further purification. Peptide grade DiPEA, CH_2Cl_2, NMP, TFA and HPLC grade solvents were purchased from Biosolve B.V. (Valkenswaard, The Netherlands). Fmoc-protected amino acids were purchased from GL Biochem Ltd. (Shanghai, China). Sidechain protecting groups for amino acids were as follows: Ser(tBu), Asp(OtBu), Glu(OtBu), Thr(tBu), Asn(Trt), Trp(Boc), Gln(Trt), Lys(Boc), Arg(Pbf).

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TentaGel S RAM resin functionalized with a modified Rink linker (particle size 90µm, capacity 0.20-0.27 mmol/g) was purchased from Rapp Polymere GmbH (Tübingen, Germany). 2-Chlorotrityl chloride resin (100-200 mesh, 1% DVB, 1.0-1.6 mmol/g) was purchased from Iris Biotech GmbH and was used for synthesis of the cyclic peptides.

Solid phase peptide synthesis was carried out in plastic syringes with a polyethylene frit (20 µm) obtained from Screening Devices B.V. The resin loading was determined by measuring the UV absorbance of the piperidine-dibenzofulvene adduct (λ_{max} 300 nm).[1]

All components used in gp120-capture ELISA experiments were purchased from ImmunoDiagnostics, Inc. (Woburn, MA).

Reactions were performed at room temperature. Solution phase reactions were monitored by TLC analysis and Rf-values were determined on Merck pre-coated silica gel 60 F-254 (0.25 mm) plates. Spots were visualized with UV-light, and by heating plates dipped in ninhydrine, Cl_2/N,N,N,N'-tetramethyl-4,4'-diaminodiphenylmethane (TDM)[2] or a KMnO4 solution. Column chromatography was performed using Silica-P Flash silica gel (60 Å, particle size 40-63 µm; Silicycle). Microwave reactions were performed in a Biotage Initiator (300W) reactor in sealed vessels suitable for reaction volumes of 0.5-2 mL.

1H NMR experiments were conducted on a 300 MHz Varian G-300 spectrometer, and chemical shifts are given in ppm (δ) relative to TMS (0.00 ppm). 13C NMR spectra were recorded at 75 MHz at a Varian G-300 spectrometer and chemical shifts are given in ppm (δ) relative to $CDCl_3$ (77 ppm).

Analytical HPLC was accomplished on a Shimadzu- 10Avp (Class VP) system using UV-detector operating at 214 and 254 nm. The mobile phase was 0,1% trifluoroacetic acid (TFA) in CH_3CN/H_2O 5:95 (buffer A) and 0,1% trifluoroacetic acid (TFA) in CH_3CN/H_2O 95:5 (buffer B). For analysis of protected (cyclic) peptides the mobile phase was 0,1% trifluoroacetic acid (TFA) in CH_3CN/H_2O 20:80 (buffer A) and 0,1% trifluoroacetic acid (TFA) in CH_3CN/iPrOH/H_2O 50:45:5 (buffer B). A Phenomenex Gemini C18 column (110

Å, 5 µm, 250×4.60 mm) was used at a flow rate of 1 mL min^{-1} using a standard protocol: 100% buffer A for 1 min, then a linear gradient of buffer B (0-100% in 30 min). Purification of the peptide-containing compounds was performed on a Prep LCMS-QP8000α HPLC system (Shimadzu) using a Phenomenex Gemini C18 column (10 µm, 110 Å, 250×20 mm) at a flow rate of 12.5 mL min^{-1} using a standard protocol: 100% buffer A for 5 min followed by a linear gradient of buffer B (0-55% in 100 min) using the same buffers as described for analytical HPLC.

Electrospray ionization mass spectrometry (ESI-MS) was performed on a Shimadzu LCMS-QP8000 single quadrupole bench-top mass spectrometer operating in a positive ionization mode or a Thermo- Finnigan LCQ Deca XP Max ion trap mass spectrometer. Analytical LC-MS was preformed on Thermo- Finnigan LCQ Deca XP Max. MALDI-TOF-MS spectra were recorded on a Kratos Analytical (Shimadzu) AXIMA CFR mass spectrometer using α-cyano-4-hydroxycinnamic acid (CHCA) or sinapic acid as a matrix and human ACTH (18-39) or bovine insulin oxidized B chain as references. High resolution electrospray ionization (ESI) mass spectra were measured on a Micromass LCT mass spectrometer calibrated with CsI. All reported mass values are monoisotopic.

The microtiterplate reader used in the ELISA experiments was a BioTek µQuant (Beun de Ronde, Abcoude, The Netherlands). Software used for data analysis was the Full Mode-KC4 version 3.4.

2. Experimental section

Peptide synthesis

2-Chlorotrityl chloride resin (100-200 mesh, 1% DVB, 1.0-1.6 mmol/g, Iris Biotech GMBH) was used for synthesis of the cyclic peptides. Peptides were synthesized using conventional Fmoc/tBu chemistry.[1] Solvents used for synthesis were dried on molsieves (4Å), 10 mL solvent per gram of resin was used.

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Immobilization of the first monomer: Resin (1 g, 1.0-1.6 mmol) was swelled in CH_2Cl_2 (3 × 2 min). To the resin, a solution of 2 mmol Fmoc-amino acid and 5 mmol DiPEA dissolved in CH_2Cl_2 was added and the mixture was shaken during 1 hour at room temperature. After that, the reaction vessel was drained and the resin was washed with DMF (2 × 2 min), followed by treatment (2 × 10 min) with 10 mL of a mixture of CH_2Cl_2/MeOH/DiPEA (80:15:5 (v/v/v)) to quench any remaining reactive 2-chlorotrityl chloride. The resin was washed again with DMF (3× 2 min) and CH_2Cl_2 (3 × 2 min) and dried *in vacuo*. The loading efficiency was determined by photometric quantification of the absorbance of the dibenzofulvene-piperidine adduct at 300 nm.

Linear peptide synthesis: The resin was washed with NMP (3 × 10 mL per gram of resin, 2 min). The *N*-terminal Fmoc protecting group was removed using 20% piperidine in NMP (3 × 10 mL per gram of resin, 8 min) followed by washing steps with NMP (3 × 10 mL, 2 min), CH_2Cl_2 (3 × 10 mL, 2 min) and NMP (3 × 10 mL, 2 min). The amino acid coupling mixtures were prepared by dissolving 4 equivalents of amino acid, 4 equivalents of HOBt and HBTU and 8 equivalents of DiPEA in NMP and coupled during 60 min. Fmoc-azidolysine was coupled overnight using 2 equivalents of amino acid, 2 equivalents of the coupling reagents HBTU and HOBt and 4 equivalents of DiPEA. The resin was washed with NMP (3 × 10 mL, 2 min) and CH_2Cl_2 (3×10mL, 2 min) after every coupling step. The coupling steps and deprotection steps were monitored using the Kaiser test or bromophenol blue test in case of secondary amines.[3]

After coupling of the final Fmoc-amino acid, acetylation was carried out to protect unreacted residues, and prevent them from participation in the cyclization reaction. The resin was treated with 10 mL capping solution containing Ac_2O (4.72 mL, 42.7 mmol), DiPEA (2.18 mL, 22.8 mmol) and HOBt (0.23 g, 1.7 mmol) in NMP (100 mL) for 2 × 15 min, followed by washing steps with NMP (3 × 10 mL, 2 min), CH_2Cl_2 (3 × 10 mL, 2 min) and NMP (3 × 10 mL, 2 min). Finally, the *N*-terminal Fmoc-group was removed using the conditions mentioned earlier, to obtain the linear sidechain-protected azidopeptide **2**.

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Cleavage of peptide from solid support and cyclization: The sidechain-protected linear azidopeptide **2** was cleaved from the resin using a solution of TFE in CH_2Cl_2 (2:8 (v/v)), 10 mL for each gram of resin, 1 hour reaction time. The liquid was filtered from the resin beads and concentrated *in vacuo*. The residue **3** was dissolved in CH_2Cl_2 or DMF, depending on the solubility of the peptide **3**, with a final peptide concentration of 1mg/mL. To this solution 1.2 equivalents of BOP and 2.4 equivalents of DiPEA were added and the reaction mixture was stirred at room temperature. Progress of the cyclization reaction was monitored with analytical HPLC. Depending on the sequence and the solvent, reaction times varied from 16 hours to three days. When full conversion was observed with analytical HPLC, the reaction mixture was concentrated *in vacuo* and the protected cyclic peptide was dissolved in EtOAc. The organic phase was washed with a 1N $KHSO_4$ solution, water, 5% $NaHCO_3$ solution and brine, dried over Na_2SO_4 and concentrated *in vacuo*.

Side-chain deprotection: The purified sidechain-protected cyclic peptides *(vide infra)* were treated with a solution (10 mL per 0.25 mmol) of TFA/TIS/H_2O (95:2.5:2.5 (v/v/v)). The reaction mixture was stirred during 3 hours, followed by precipitation of the peptides from MTBE/hexane 1:1 (v/v) at -20°C. The precipitates were dissolved in $^tBuOH/H_2O$ 1:1 (v/v) and lyophilized. The purity of the peptides **4a-c** was analyzed with analytical HPLC and the peptides were characterized with mass spectrometry.

Purification of cyclic peptides 4a-c

Compound 4a, loop 1: K(N_3)LTRDGGNG

The crude protected cyclic peptide was purified with column chromatography in CH_2Cl_2/EtOH 95:5. The product was obtained as a yellow oil. R_f: 0.67 (CH_2Cl_2/EtOH 9:1). Synthesis on 0.5 mmol scale. Yield after deprotection of sidechains: 106 mg (23%).

$[M+H]^+$ monoisotopic calculated for $C_{36}H_{60}N_{16}O_{13}$: 925.4599, $[M+2H]^{2+}$calculated: 463.2336, HRMS, $[M+2H]^{2+}$ found: 463.2316, HPLC: Rt= 13.84 min, purity 94.5%.

Compound 4b, loop 2: INMWQEVGKAK(N_3)G

The crude protected cyclic peptide was purified with column chromatography in CH_2Cl_2/MeOH 95:5. The product was obtained as a yellow oil. R_f: 0.59 (CH_2Cl_2/MeOH 9:1). Synthesis on 0.25 mmol scale. Yield after deprotection of sidechains: 210 mg (61%). HPLC analysis shows some small impurities, therefore the product is purified another time with preparative HPLC. Pure product was obtained as a white powder. Yield: 40 mg (12%).

$[M+H]^+$ monoisotopic calculated for $C_{60}H_{93}N_{19}O_{16}S$: 1368.6841, $[M+2H]^{2+}$calculated: 684.8457, HRMS $[M+2H]^{2+}$ found: 684.8446, HPLC: Rt= 17.51 min, purity= 95.3%

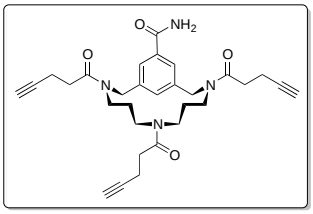
Compound 4c, loop 3: SGGDPEIVTK(N_3)G

The crude protected cyclic peptide was purified with column chromatography using a gradient of CH_2Cl_2/MeOH 97:3 to 9:1. The product was obtained as colorless oil. R_f: 0.61 (CH_2Cl_2/MeOH 9:1)

Synthesis on 0.25 mmol scale. Yield after deprotection of sidechains: 56 mg (21%).

$[M+H]^+$ monoisotopic calculated for $C_{44}H_{70}N_{14}O_{17}$: 1067.5116, $[M+2H]^{2+}$calculated: 534.2594, HRMS $[M+2H]^{2+}$ found: 534.2652, HPLC: Rt= 15.52 min, purity= 100%

Compound 5: *N*,*N*,*N*-tris(pent-4-ynoic)-triazacyclophane scaffold



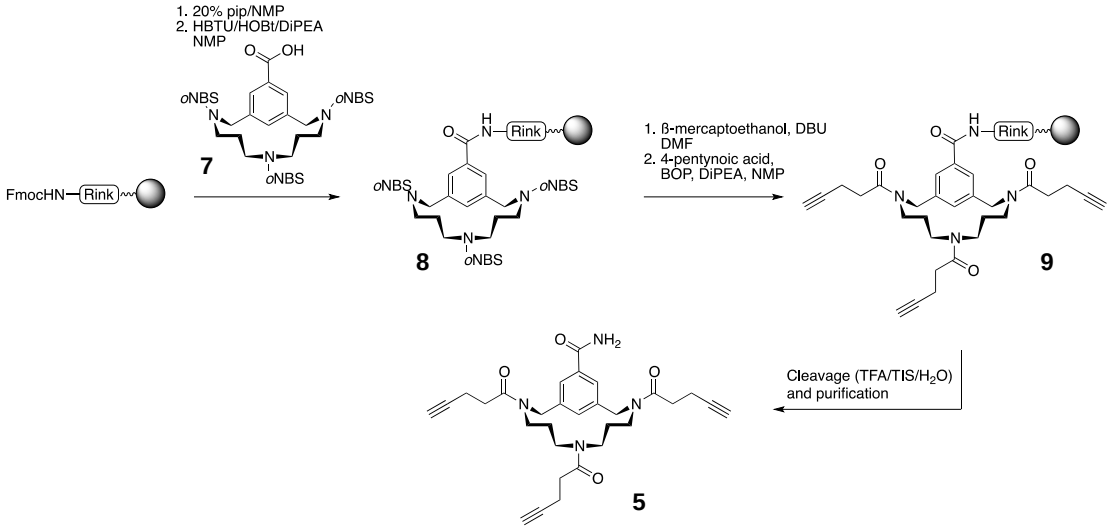
Tentagel S RAM resin (4 g, 0.24 mmol/g) was swelled in NMP (3 × 10 mL per gram of resin, 2 min). For all subsequent reactions also 10 mL solvent per gram of resin was used. The *N*-terminal Fmoc protecting group was removed using 20% piperidine in NMP (3 × 8 min) followed by washing steps with NMP (3 × 2 min), CH_2Cl_2 (3 × 2 min) and NMP (3 × 2 min). *N*,*N*,*N*-tris(2-nitrophenylsulfonamido)-triazacyclophane scaffold **7**[4] (1.60 g, 1.92 mmol, 2 eq), 2 equivalents of HOBt and HBTU and 4 equivalents of DiPEA were dissolved

in NMP, added to the resin and coupled overnight. After coupling, the resin **8** was washed with NMP (3 × 2 min), CH_2Cl_2 (3 × 2 min) and DMF (3 × 2 min) followed by treatment with 15 eq DBU and 30 eq β-mercaptoethanol in DMF for. After 30 minutes, the solution was drained and the procedure was repeated one more time. The resin was washed with DMF (3 × 2 min), CH_2Cl_2 (3 × 2 min) and NMP (3 × 2 min), followed by coupling of 4-pentynoic acid (3 eq per amine, 847.6 mg, 8.64 mmol) using 3 equivalents of HOBt and HBTU and 6 equivalents of DiPEA in NMP. The mixture was allowed to react for two hours followed by the standard washing procedure. Resin **9** (2.17 g, 0.5 mmol) was treated with 40 mL TFA/TIS/H_2O (95:2.5:2.5 (v/v/v)) during 2.5 hours, after which the mixture was concentrated *in vacuo* and applied to a silica column eluting with CH_2Cl_2/MeOH (95:5). After solvent removal the product was obtained as a white nanocrystalline foam. (131 mg, 51%). A schematic overview of the synthesis of compound **5** is shown in scheme 1.

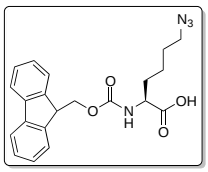
HRMS monoisotopic Calcd for $C_{30}H_{36}N_4O_4$ $[M+H]^+$, 517.2809, found 517.2785.

HPLC: Rt= 23.87 min, purity= 99.2%

Scheme 1: Synthesis of *N,N,N*-tris(pent-4-ynoic)-triazacyclophane scaffold 5



Fmoc-L-ε-azidolysine[5]



Fmoc-Lys(Boc)-OH (10 g, 21.32 mmol) was dissolved in 100 mL CH_2Cl_2. The solution was stirred during 1 hour after which a white suspension was obtained. The solvents were evaporated and the residue was dissolved in 100 mL water and 200 mL MeOH. $CuSO_4$ (53.2 mg, 0.21 mmol) was added and the pH of the solution was brought to 8.5 with K_2CO_3. Imidazole sulfonyl azide HCl salt (5.36 g, 25.59 mmol, synthesized according to the procedure by Stick *et al.*[5]) was added and the pH of the reaction mixture was maintained at 8.5 by addition of K_2CO_3. The clear blue solution was stirred overnight, after which MeOH was evaporated and the aqueous solution was acidified to pH 2 by addition of 2N HCl. The aqueous phase was extracted four times with 100 mL CH_2Cl_2 and the combined CH_2Cl_2 fractions were dried over Na_2SO_4 and concentrated *in vacuo*. The resulting yellow oil was purified with column chromatography in MeOH/acetone/AcOH 95:5:1 to CH_2Cl_2/acetone/AcOH 90:10:1 when the product started eluting. Pure product was obtained as a colorless oil that crystallized into a white solid. Yield: 7.91 g (94%). R_f: 0.35 (CH_2Cl_2/acetone/AcOH 90:10:1). ^{1}H-NMR (300 MHz, $CDCl_3$, TMS): δ 1.26-1.32 (m, 2H, Lys-$^{\delta}$CH_2), 1.46-1.49 (m, 1H, Lys-$^{\gamma}$CH_2), 1.58-1.65 (m, 1H, Lys-$^{\gamma}$CH_2), 1.65-1.80 (m, 1H, Lys-$^{\beta}$CH_2), 1.85-2.01 (m, 1H, Lys-$^{\beta}$CH_2), 3.26-3.30 (t, 2H, Lys-$^{\varepsilon}$CH_2), 4.20-4.24 (t, 1H, Lys-$^{\alpha}$CH), 4.42-4.44 (d, 2H, Fmoc-CH_2), 4.55 (br.s, 1H, Fmoc-CH), 5.26-5.29 (d, 1H, NH), 7.23-7.43 (m, 4H, Fmoc-^{AR}CH), 7.57-7.60 (d, 2H, Fmoc-^{AR}CH), 7.75-7.77 (d, 2H, Fmoc-^{AR}CH)

^{13}C-NMR (75 MHz, $CDCl_3$): δ 22.40, 28.32, 31.82, 47.11, 51.02, 53.48, 67.11, 119.99, 125.01, 127.06, 127.74, 141.30, 143.60, 156.06, 176.71

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General procedure for scaffold conjugation of cyclic peptides by optimized CuAAc.[6,7]

10-fold stock solutions of $CuSO_4$ (44.9 µmol, 11.2 mg in 1 mL H_2O) and sodium ascorbate (89.9 µmol, 17.8 mg in 1 mL H_2O) were prepared. TBTA[8,9] was dissolved in 100 µL DMF. Peptides were dissolved individually in the smallest possible amount (100-500 µL) of DMF (4Å).

Solutions of three peptides in DMF were prepared: loop 1 (compound **4a**, 7.49 µmol, 6.93 mg), loop 2 (compound **4b**, 7.49 µmol, 10.25 mg) and loop 3 (compound **4c**, 7.49 µmol, 7.99 mg). TAC scaffold **5** (7.49 µmol, 3.97 mg) was added to the microwave vessel and dissolved in 100 µL DMF (4Å). 100 µL of the $CuSO_4$ stock solution (4.49 µmol, 1.12 mg) and 100 µL of the sodium ascorbate stock solution (8.99 µmol, 1.78 mg) were added to the solution, followed by addition the TBTA solution (1.10 µmol, 0.60 mg). The peptide solutions were combined and added to the reaction mixture. Depending on the amount of DMF that was used to dissolve the peptides, H_2O was added to obtain a 3:2 DMF/H_2O ratio and 1-1.5 mL total volume. The microwave vessel was sealed and allowed to react in the microwave at 80°C during 25 minutes.

After reaction, a sample was taken from the reaction mixture for LC-MS analysis. Based on this analysis, the individual components were purified with preparative HPLC.

HIV-1 gp120 capture ELISA: Recombinant HIV-1IIIB gp120 protein (referred to as rgp120 hereafter unless otherwise noted) capture ELISA was performed according to the manufacturer's instructions (ImmunoDiagnostics, Inc., Woburn, MA). Firstly, various concentrations of free ligand rgp120 in sample buffer (up to 2 µg/ml, in a 2-fold dilution series) were added to a CD4-coated plate in the absence of test compounds. After a 60-min incubation at room temperature, the amounts of captured rgp120 were detected by

peroxidase-conjugated murine anti-gp120 MAb. We determined that optimal OD_{450}-values were obtained when an rgp120 concentration of 1 µg/ml was used.

When optimal concentrations were determined, competition experiments were performed. Test compounds diluted in sample buffer (50 µL 0,1% BSA in PBS) and 2% DMSO were added to the CD4-coated plate, immediately followed by addition of 50 µL 2 µg/ml rgp120 (final concentration 1 µg/mL). After 4 hours incubation at room temperature, the plate was washed with wash buffer (0,1% Tween 20 in PBS) followed by incubation with peroxidase-conjugated murine anti-gp120 MAb to detect the amounts of captured rgp120. Any unbound material was washed away using wash buffer, and plates were developed by adding 100 µL/well substrate solution (0,1 mg/mL TMB in 0.1 N NaOAc buffer pH 5.5, containing 0.003% H_2O_2). The reaction was stopped by adding 100 µL 4N sulfuric acid. Absorbances (ODs) were read at 450 nm using a microtiterplate reader. All assays were performed in duplicate and were independently repeated at least three times.

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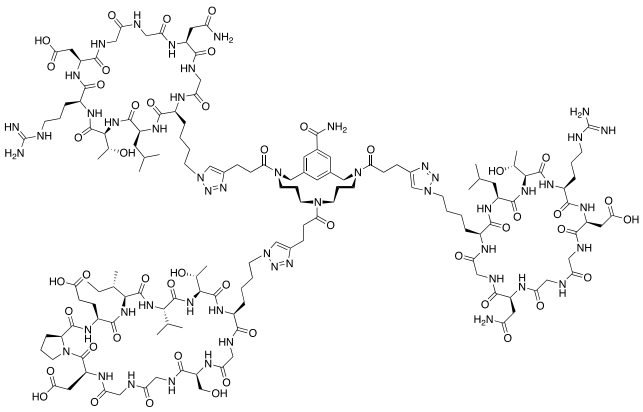
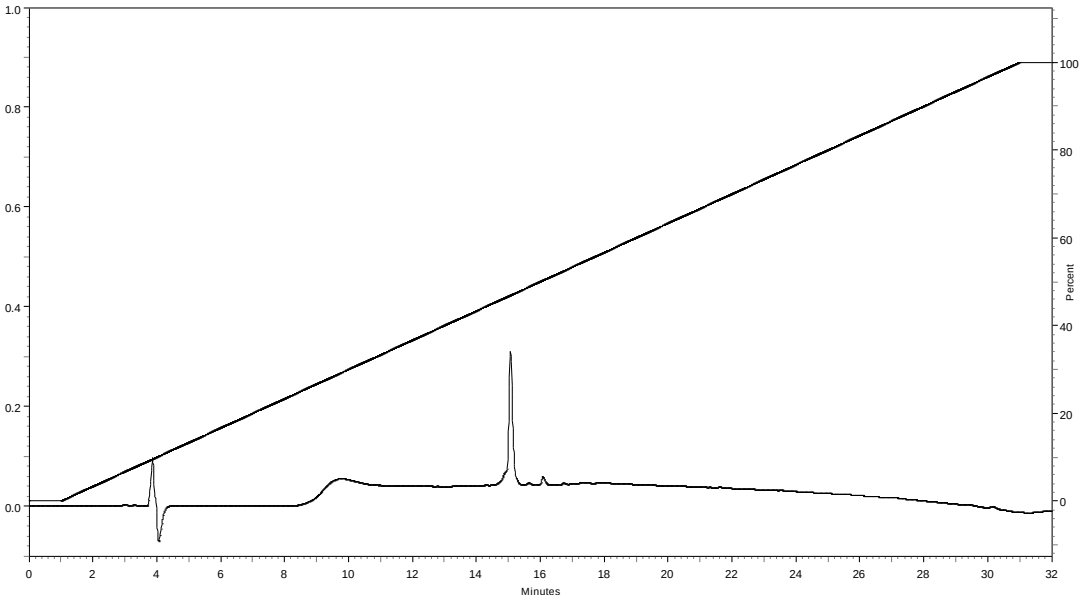
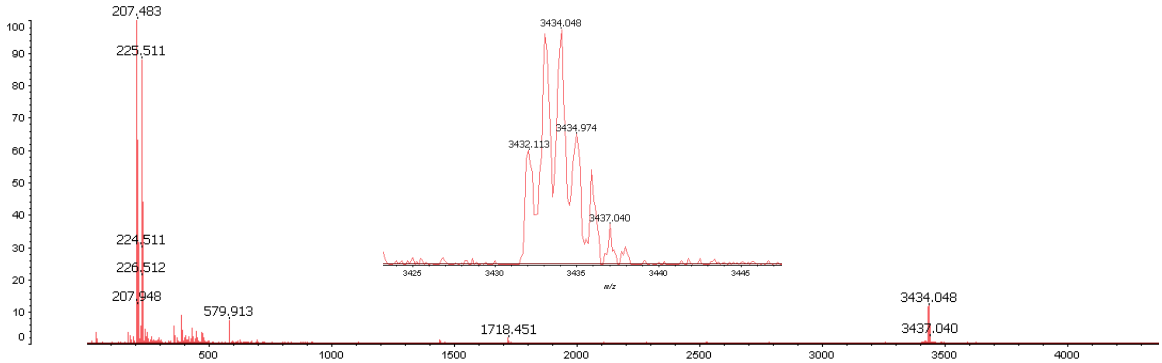
3. Analytical data of purified discontinuous epitope mimics

Scaffolded loops 1-1-3
$C_{146}H_{226}N_{50}O_{47}$
$[M+H]^+$ monoisotopic calculated: 3432.6904
Found: 3432.113





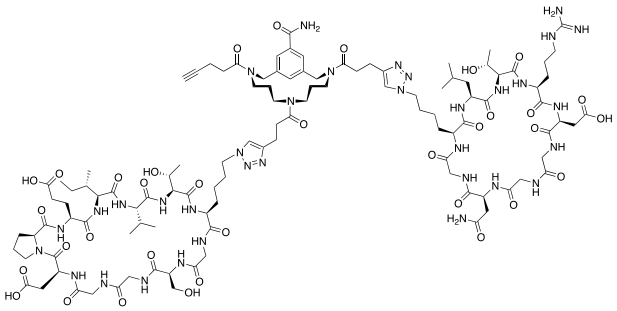


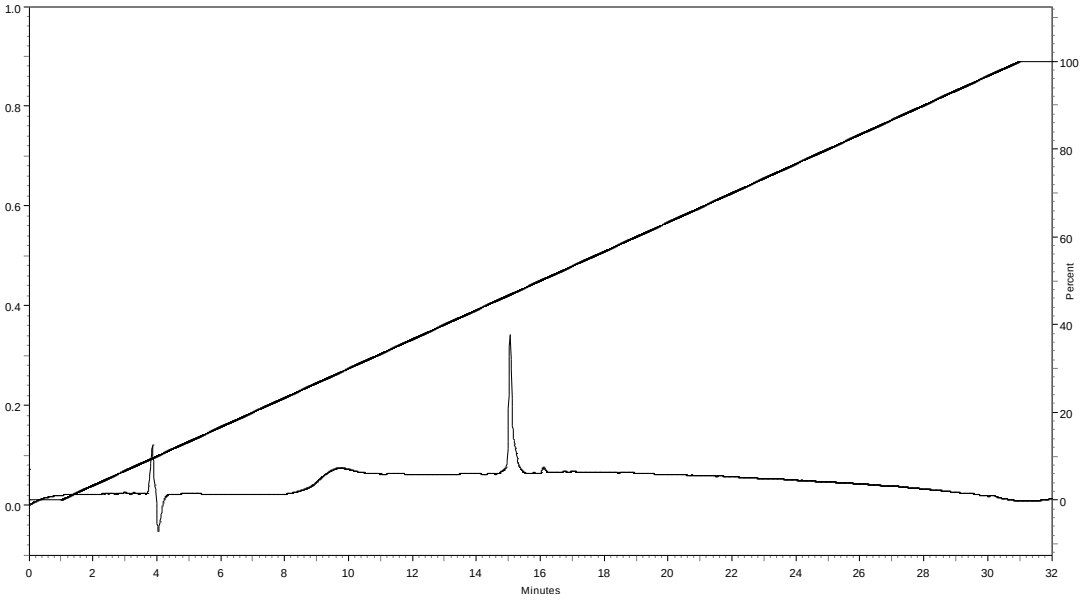
Scaffolded loops 1-3-0

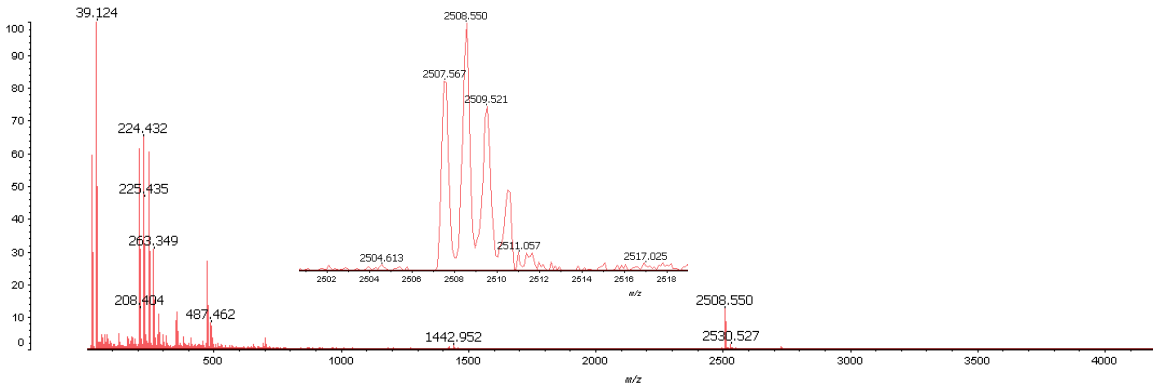
$C_{110}H_{166}N_{34}O_{34}$

$[M+H]^+$ monoisotopic calculated: 2508.2378

Found: 2507,567





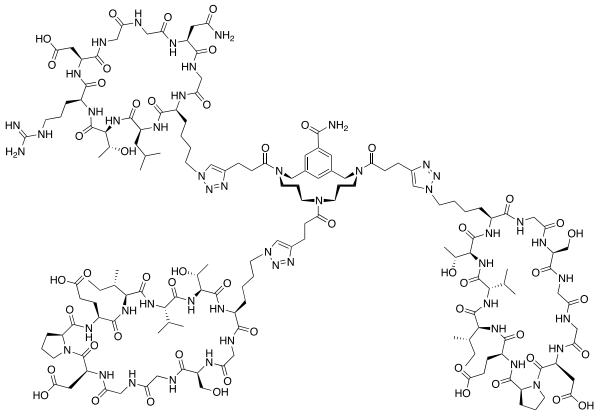


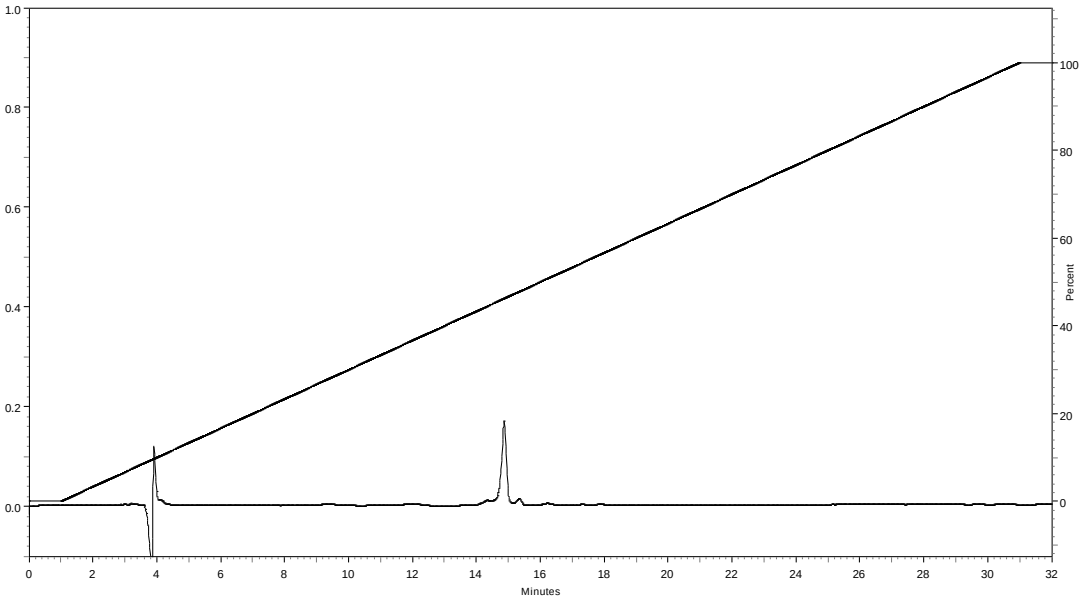
Scaffolded loops 1-3-3

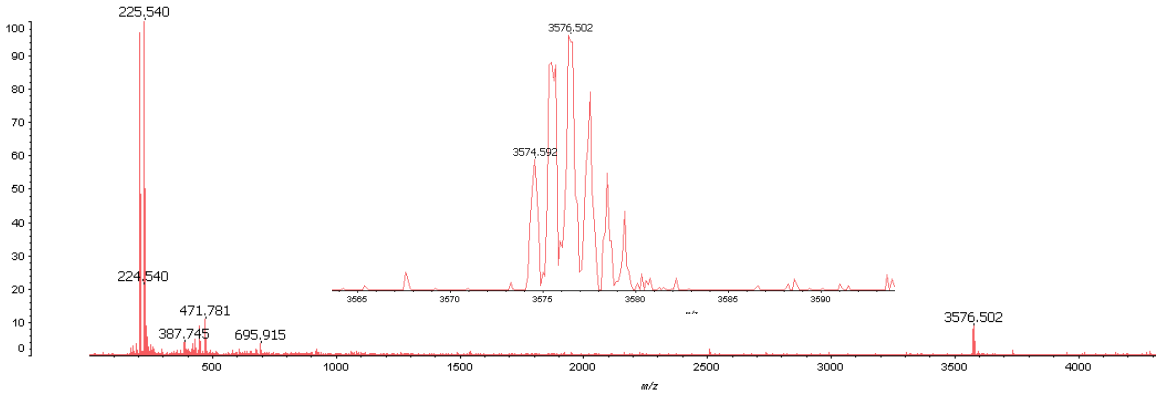
$C_{154}H_{236}N_{48}O_{51}$

$[M+H]^+$ monoisotopic calculated: 3574.7422

Found: 3574.592





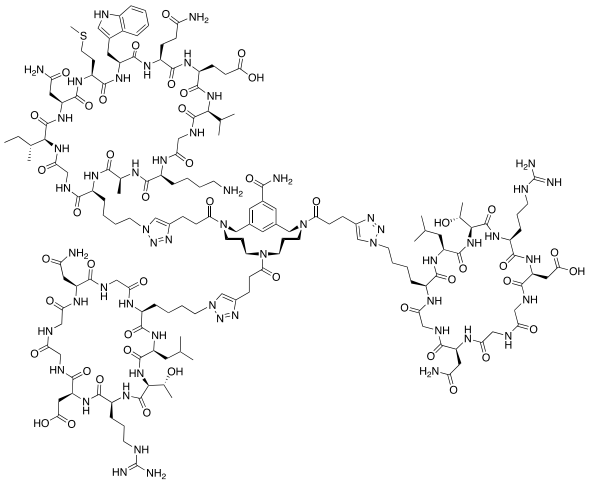


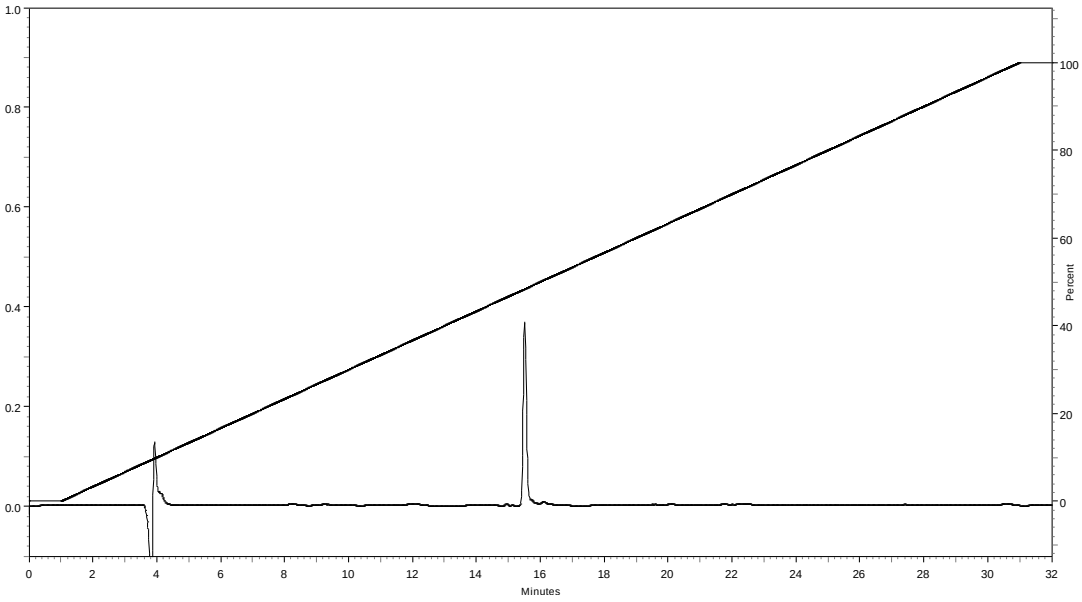
Scaffolded loops 1-1-2

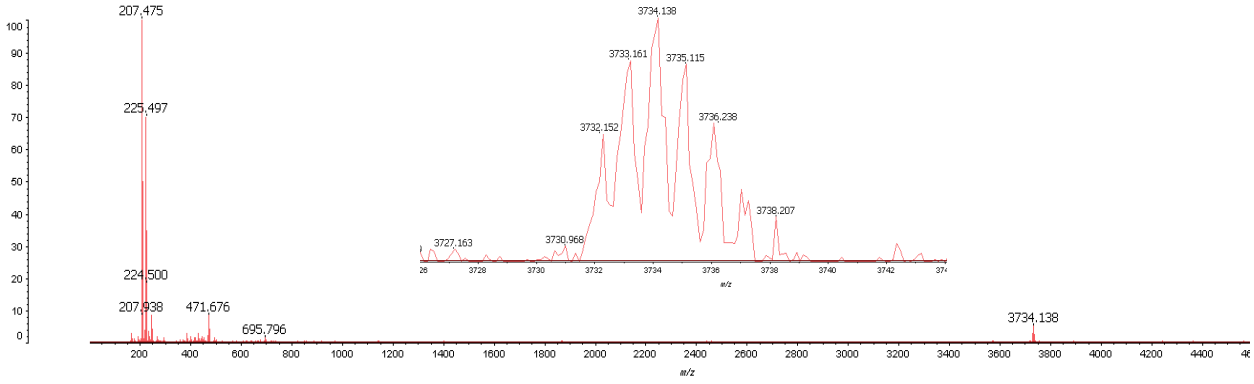
$C_{162}H_{249}N_{55}O_{46}S$

$[M+H]^+$ monoisotopic calculated: 3733.8628

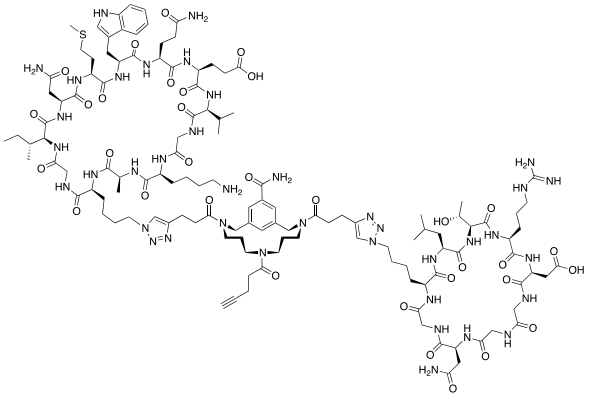
Found: 3732.152

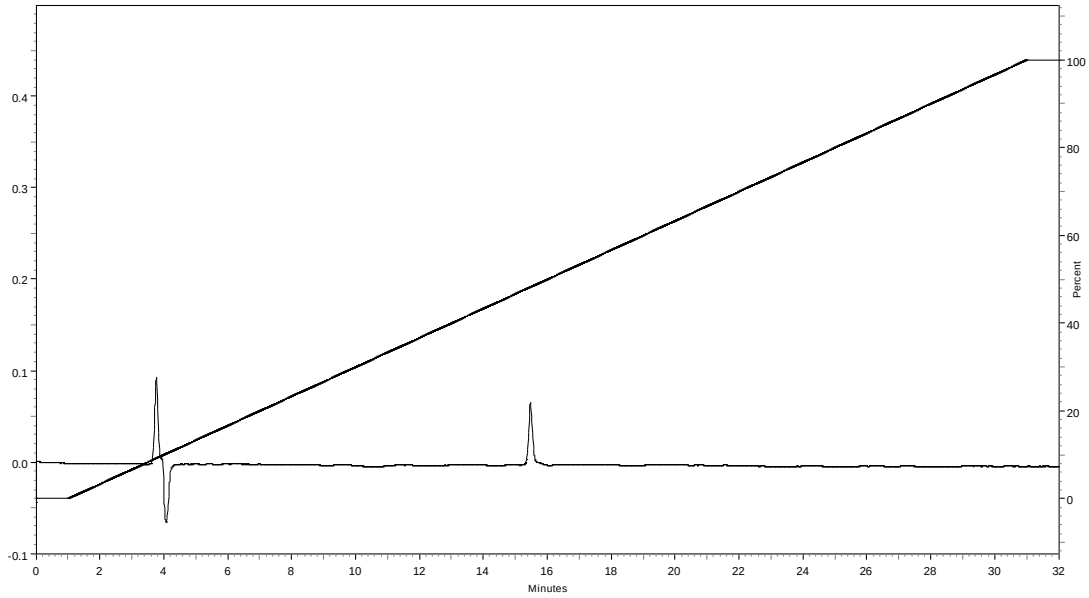


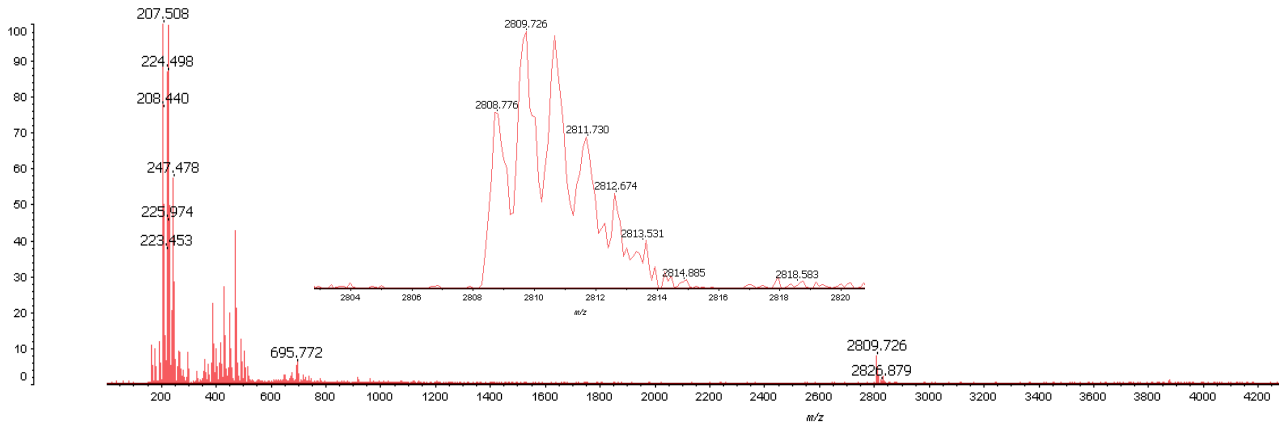




Scaffolded loops 1-2-0
$C_{126}H_{189}N_{39}O_{33}S$
$[M+H]^+$ monoisotopic calculated: 2809.4103
Found: 2808.776





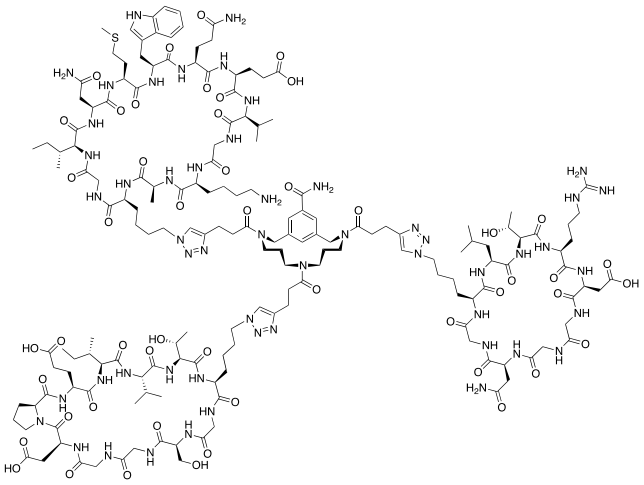


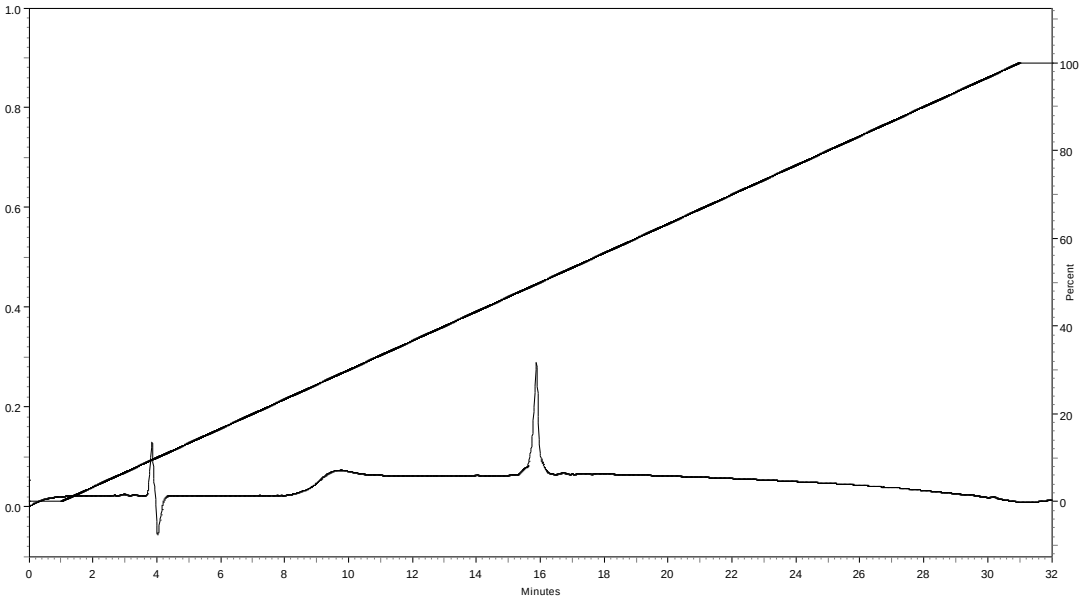
Scaffolded loops 1-2-3

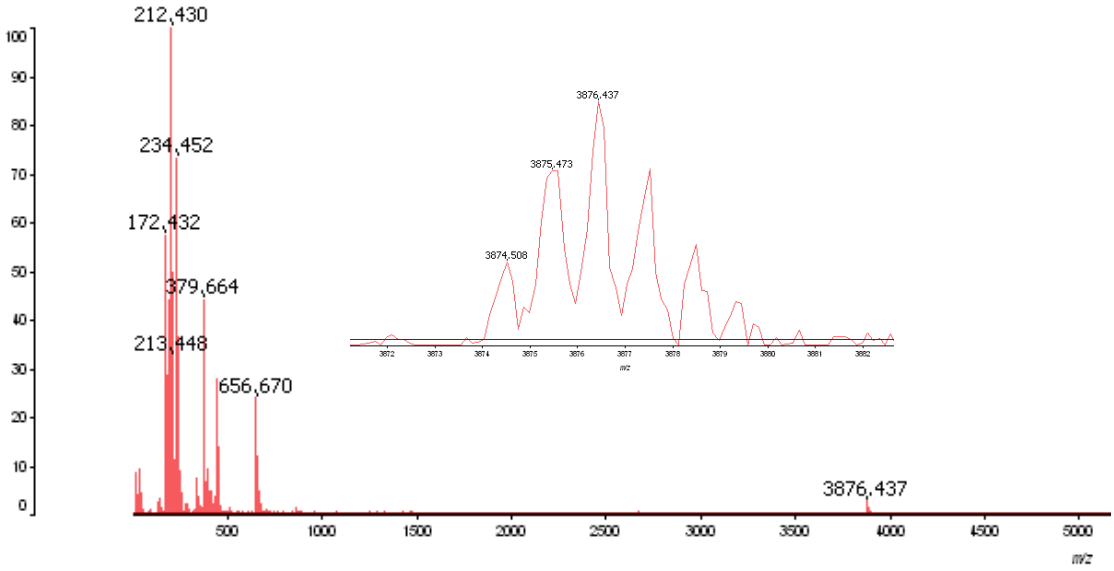
$C_{170}H_{259}N_{53}O_{50}S$

$[M+H]^+$ monoisotopic calculated: 3875.9147

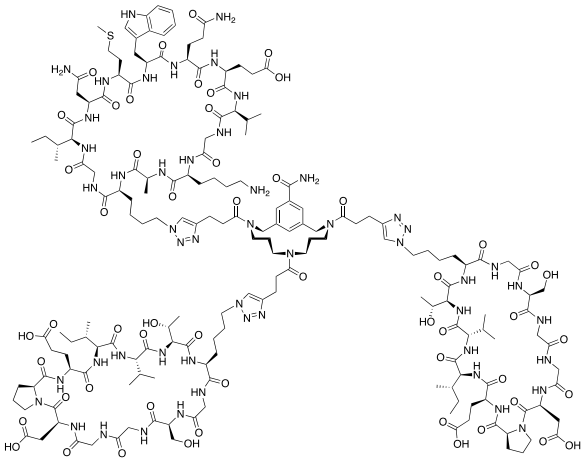
Found: 3874.508

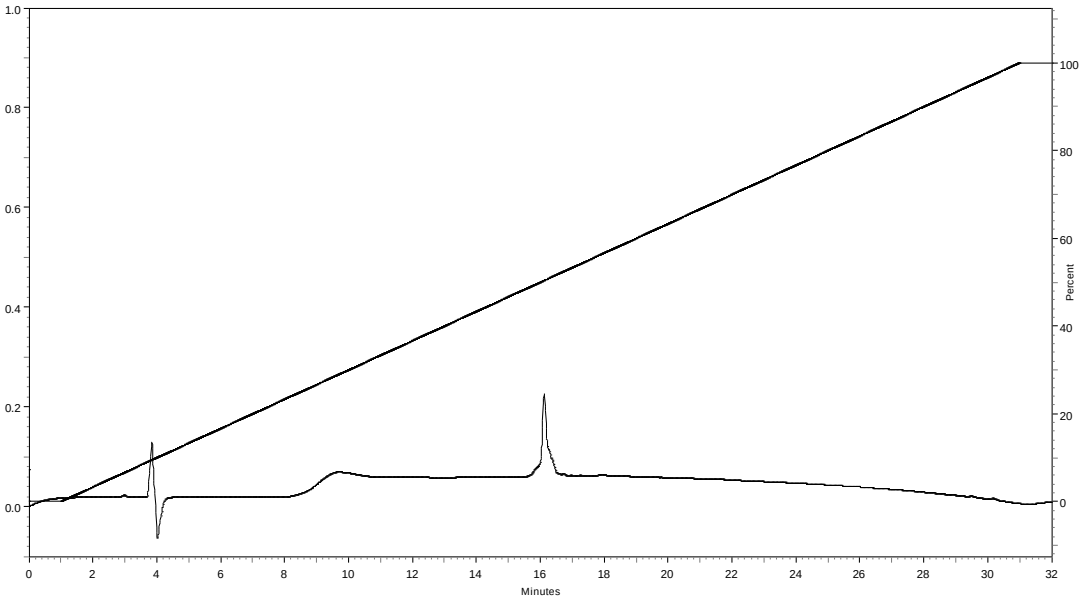


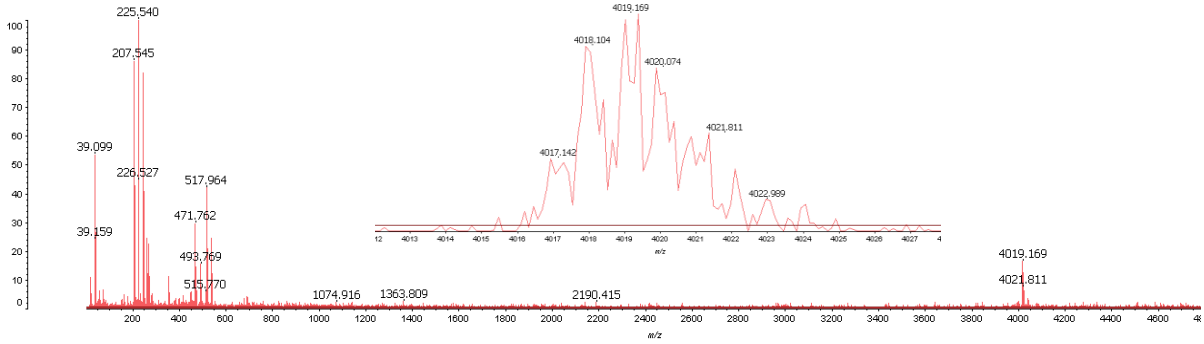




Scaffolded loops 2-3-3
$C_{178}H_{269}N_{51}O_{54}S$
$[M+H]^+$ monoisotopic calculated: 4017.9664
Found: 4017.142





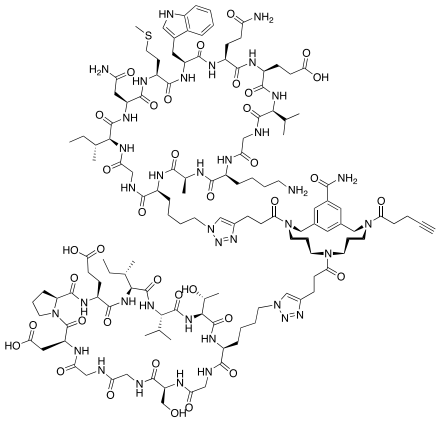


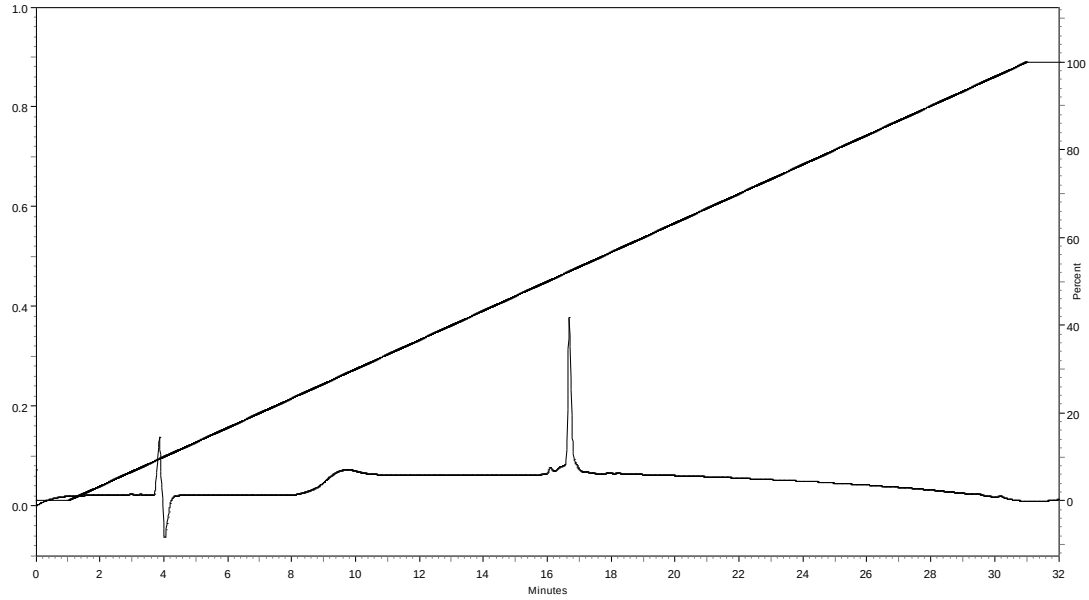
Scaffolded loops 2-3-0

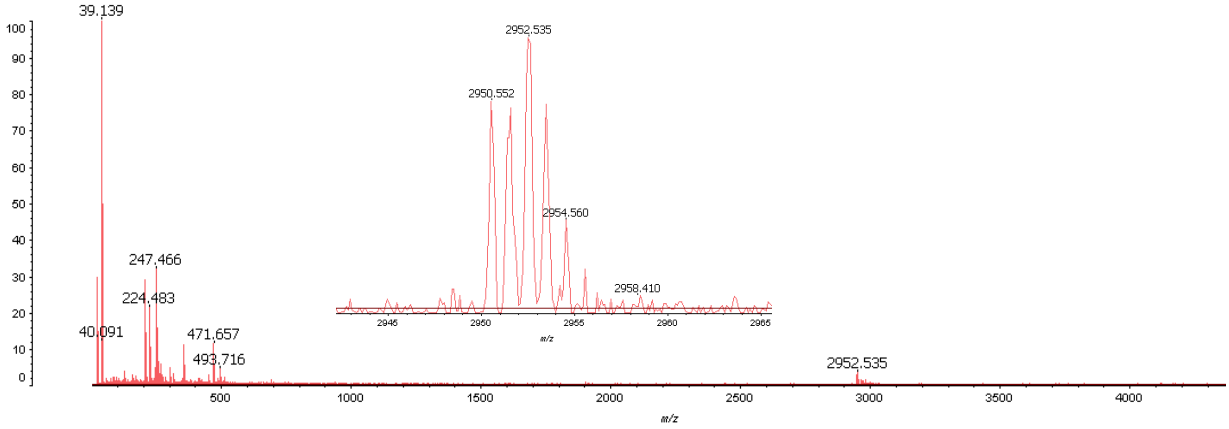
$C_{134}H_{199}N_{37}O_{37}S$

$[M+H]^+$ monoisotopic calculated: 2951.4621

Found: 2950.552







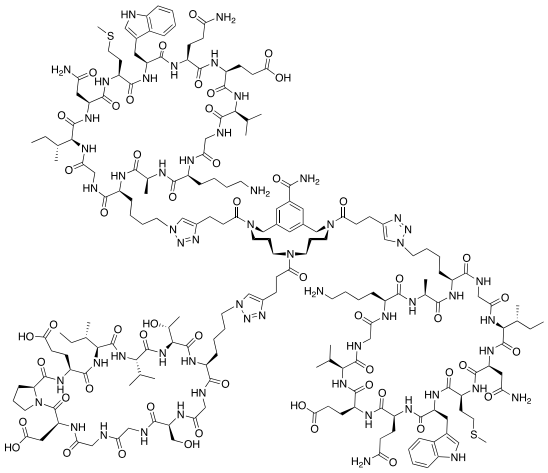
Scaffolded loops 2-2-3

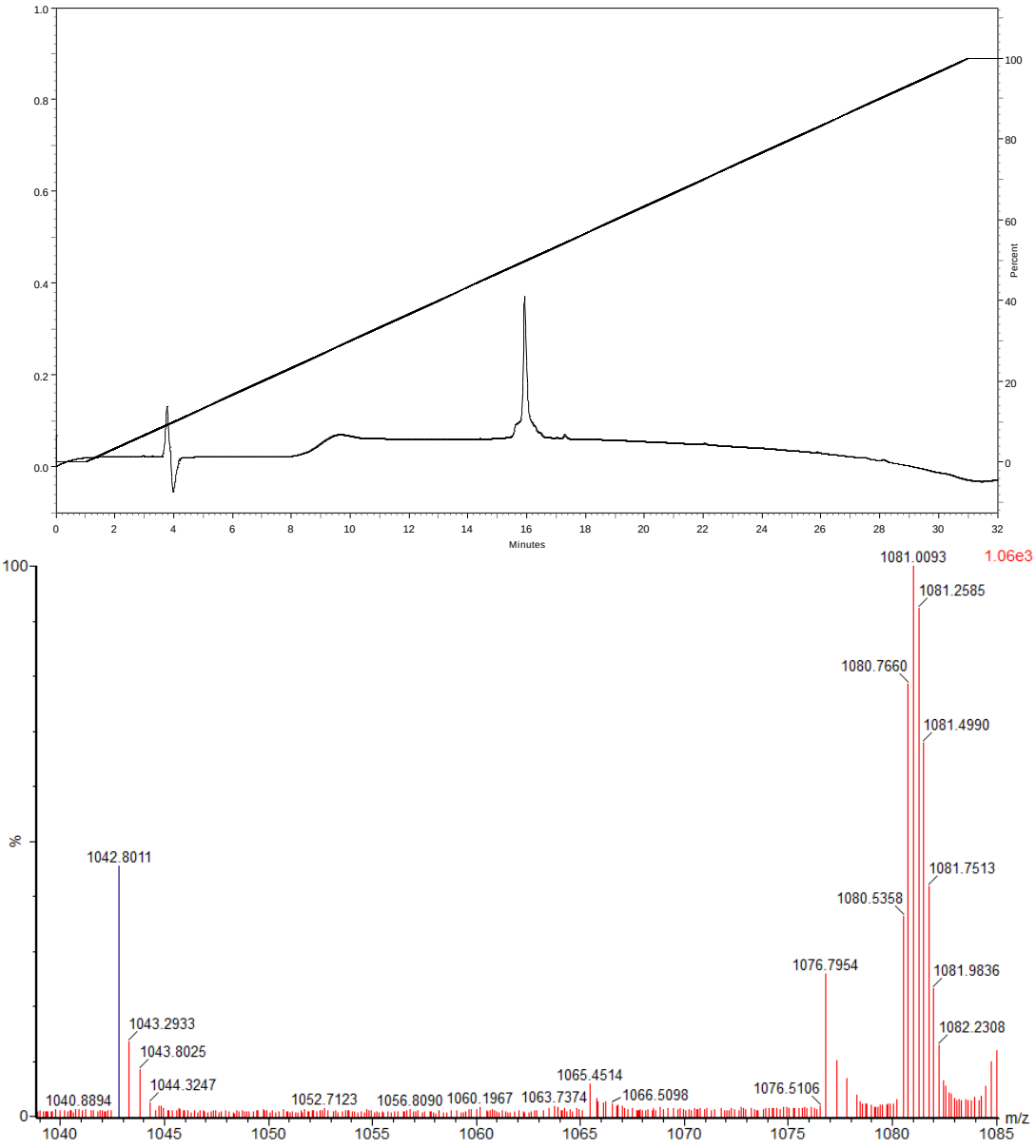
$C_{194}H_{292}N_{56}O_{53}S_2$

$[M+H]^+$ monoisotopic calculated: 4319.1389

$[M+4H]^{4+}$ calculated: 1080.5402

HRMS $[M+4H]^{4+}$ found: 1080.5358

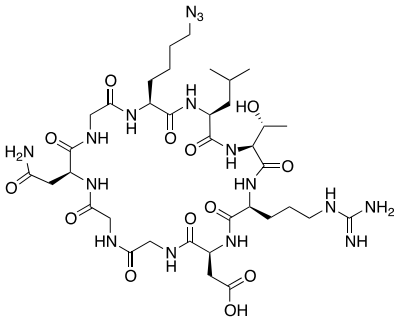




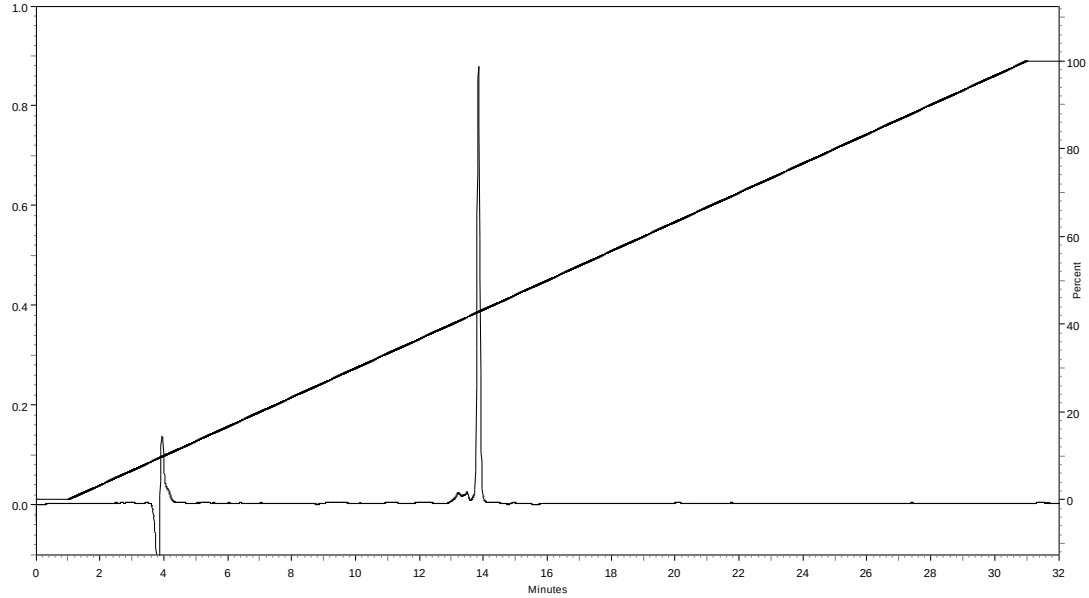
Compound 4a:

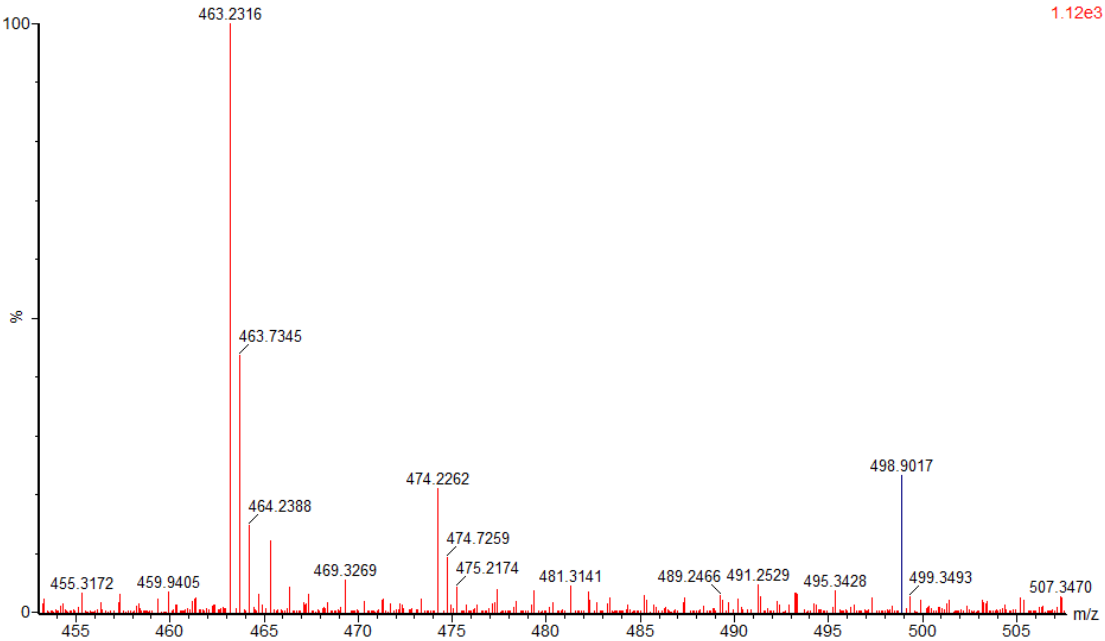
Loop 1: K(N_3)LTRDGGNG
$C_{36}H_{60}N_{16}O_{13}$
$[M+H]^+$ monoisotopic calculated: 925.4599
$[M+2H]^{2+}$calculated: 463.2336
HRMS $[M+2H]^{2+}$ found: 463.2316

Rt= 13.85 min
Purity= 94,5%



4a

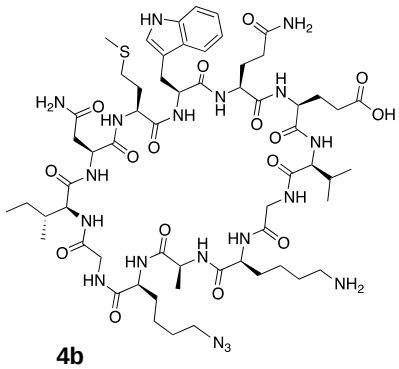




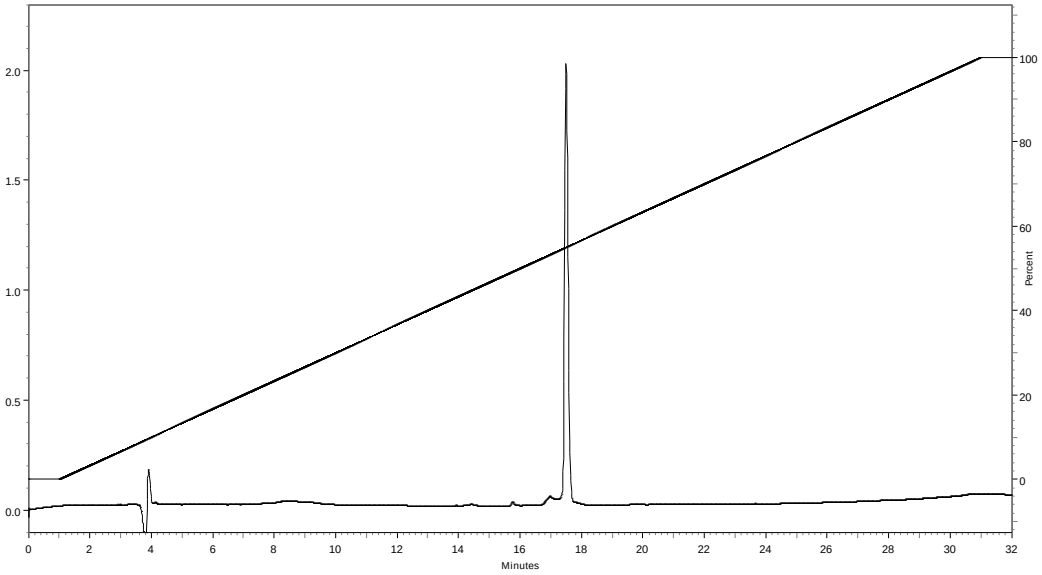
Compound 4b:

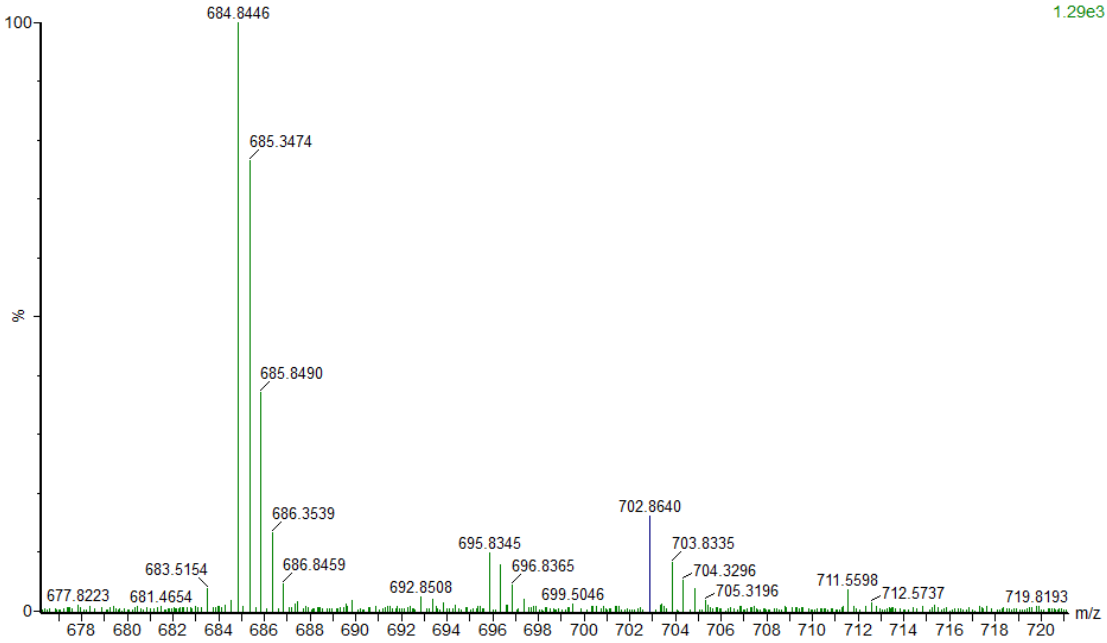
Loop 2: INMWQEVGKAK($_3$)G
$C_{60}H_{93}N_{19}O_{16}S$
$[M+H]^+$ monoisotopic calculated: 1368.6841
$[M+2H]^{2+}$calculated: 684.8457
HRMS $[M+2H]^{2+}$ found: 684.8446

Rt= 17.51 min
Purity= 95.3 %



4b





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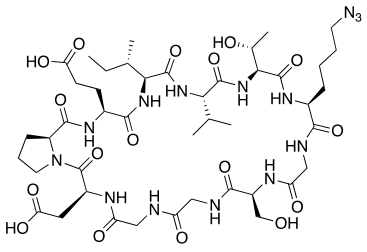
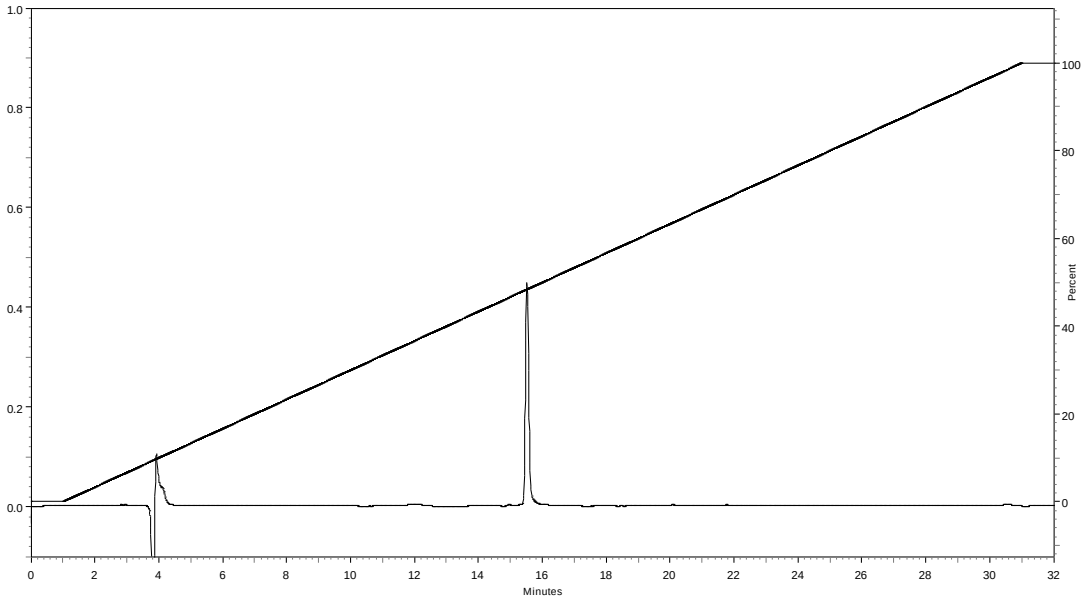
Compound 7:

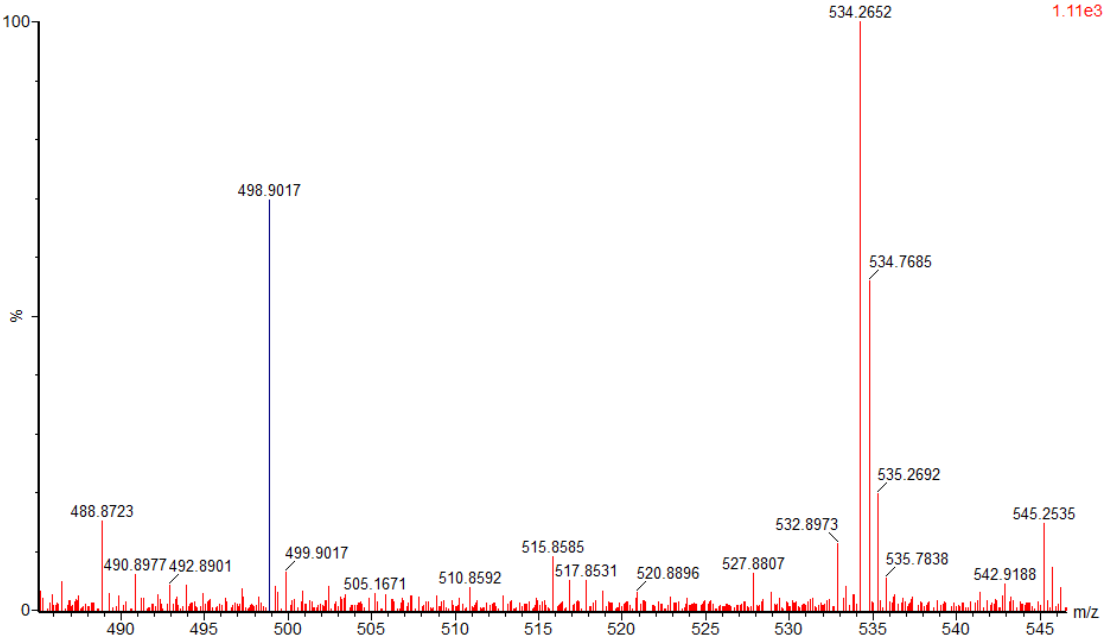
Loop 3: SGGDPEIVTK(N_3)G
$C_{44}H_{70}N_{14}O_{17}$
$[M+H]^+$ monoisotopic calculated: 1067.5116
$[M+2H]^{2+}$calculated: 534.2594
HRMS $[M+2H]^{2+}$ found: 534.2652

Rt= 15.52 min
Purity= 100%



4c





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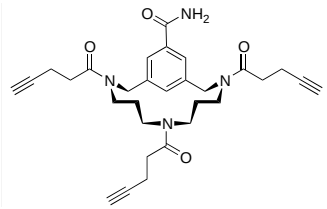
Compound 5:



$C_{30}H_{36}N_4O_4$
$[M+H]^+$ monoisotopic calculated: 517.2809
HRMS $[M+H]^+$ found: 517.2785

Rt= 23.87 min
Purity= 99.2%