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Supporting Information

Conjugates of Cryptophycin and RGD or *iso*DGR Peptidomimetics for Targeted Drug Delivery

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I. Biological evaluation

Solid-phase receptor binding assay

Recombinant human integrin $\alpha_v\beta_3$ (R&D Systems, Minneapolis, MN, USA) was diluted to 0.5 µg mL^{-1} in coating buffer containing 20 mM Tris-HCl (pH 7.4), 150 mM NaCl, 1 mM $MnCl_2$, 2 mM $CaCl_2$, and 1 mM $MgCl_2$. An aliquot of diluted receptor (100 µL $well^{-1}$) was added to 96-well microtiter plates (Nunc MaxiSorp) and incubated overnight at 4 °C. The plates were treated for additional 2 h at room temperature with blocking solution (coating buffer plus 1% bovine serum albumin) to block nonspecific binding and washed 2 times with the same solution. Different concentrations (10^{-5}–10^{-12} M) of the test compounds in the presence of 1 µg mL^{-1} biotinylated vitronectin were added to the plates, which were shaken for 3 h at room temperature. Vitronectin, (Molecular Innovations, Novi, MI, USA) was biotinylated using an EZ-Link Sulfo-NHS-Biotinylation kit (Pierce, Rockford, IL, USA). The plates were washed 3 times, and incubated with shaking for 1 h at room temperature, with streptavidin-biotinylated peroxidase complex (Amersham Biosciences, Uppsala, Sweden). The plates were washed again 3 times with blocking solution, and 100 µL $well^{-1}$ of Substrate Reagent Solution (R&D Systems, Minneapolis, MN, USA) were added before shaking in the dark for 30 min and stopping the reaction with the addition of 50 µL $well^{-1}$ 2 N H_2SO_4. Absorbance at λ = 415 nm was read in a SynergyTM HT Multi-Detection Microplate Reader (BioTek Instruments, Inc.). Each data point represents the average of triplicate wells; data analysis was carried out by nonlinear regression analysis with GraphPad Prism software. Each experiment was repeated twice.

Cell lines and culture conditions

The human melanoma cells M21 and M21-L were kindly provided by Dr. David Cheresh and The Scripps Research Institute (La Jolla, CA, USA). Cells were grown in RPMI 1640 supplemented with 10% foetal bovine serum (FBS), 1% L-glutamine and 1% Penicillin/Streptomycin in a humidified incubator at 37 °C and 5% CO_2.

In vitro cytotoxicity assay

Cell viability was quantified by MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) assay. Briefly, cells were plated at density of 3000 cells $well^{-1}$ and incubated

overnight to allow adherence. The following day, cells were treated with serial dilutions of each compound or 0.1% DMSO as control. After 2 h incubation, cells were centrifuged at 1200 rpm for 5 min, supernatant containing the compounds was removed and replaced with fresh medium. Cells were further incubated for 70 h as described above and at the end of treatment, 5 µL of MTT (5 mg mL^{-1} in deionized H_2O, Sigma #M5655) were added to each well and cells incubated for another 2 hours. Finally, 100 µL of lysis buffer (10% SDS, 10 mM HCl) were added, and cells placed in the incubator overnight for the formazan crystal solubilization. Absorbance at 540 nm was measured using FLUOstar Omega (BMG Labtech) instrument and the growth inhibition ratio was calculated. Blank controls detecting cell-free media absorbance were performed in parallel. Three experimental replicates were used. The half-maximal inhibitory concentration values (IC_{50}) were obtained from viability curves by fitting data to the four-parameter logistic equation using GraphPad Prism 6. The cell viability was expressed as percentage relative to the respective control conditions (0.1% of DMSO).

Flow cytometry analysis

The M21 and M21-L cells were plated at density of 80000 cells mL^{-1} $well^{-1}$ in a 12-well plate and incubated overnight at 37 °C. Next day, cells were detached and incubated with LM142 mouse anti-human integrin α_V monoclonal antibody (#MAB1978, Merck Millipore, Merck, 1:500 dilution) or with mouse anti-human CD51/CD61 (#555504, BD Biosciences) at 1 µg mL^{-1} for 15 minutes on ice. Subsequently, cells were washed with PBS and incubated with anti-mouse IgG-Alexa Fluor 488 (#A-11029, Thermo Fisher) at 8.3 µg mL^{-1} for 15 minutes on ice. Finally, cells were washed thoroughly with PBS, samples acquired using BD FACSCalibur™ flow cytometer and analysed using FlowJo software v.10. Anti-mouse IgG1 antibody was used as the negative isotope control.

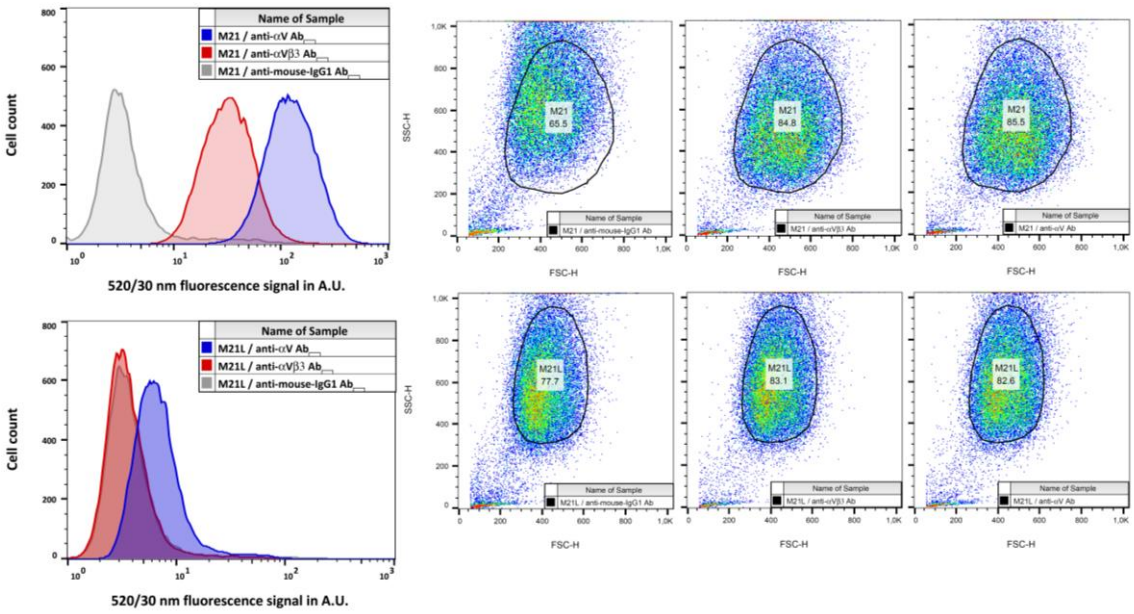


Figure S1. Flow cytometry analysis of integrin α_V and $\alpha_V\beta_3$ expression in M21, M21-L cell lines.

II. Chemical synthesis

General information

All experiments requiring anhydrous conditions were performed using oven-dried glassware under argon atmosphere, unless otherwise stated. DCM was distilled from CaH_2, THF was distilled from sodium/benzophenone, DMF was dried over 4Å molecular sieves. Chemicals and solvents (reagent-grade or analytical-grade) were purchased from commercial sources and used without further purification. Macherey-Nagel silica gel "Kieselgel 60" with 40 – 63 µm (230-400 mesh) was used as stationary phase for flash chromatography. Reactions were monitored by TLC (Merck Kieselgel 60, F254 on aluminium foil), spots were visualized with UV-light or by staining with potassium permanganate solution.

Liquid chromatography - mass spectrometry

Analytical HPLC-MS was performed using an Agilent 1200 series consisting of an autosampler, degasser, binary pump, column oven and diode array detector coupled to an Agilent 6220 accurate-mass TOF-MS, equipped with Phenomenex Luna® 3 C18(2) 100 Å (100 mm x 2 mm, 3 µm) column. Analyses were performed in positive ion mode.

Eluent A: $H_2O/CH_3CN/HCOOH$ = 95/5/0.1 and eluent B: $H_2O/CH_3CN/HCOOH$ = 5/95/0.1.

Method M1:

Flow rate: 300 µL min^{-1}

0 min	100% A	0% B
10 min	2% A	98% B
11 min	2% A	98% B
11.5 min	100% A	0% B
15 min	100% A	0% B

High resolution mass spectra (HRMS) were recorded on Agilent 6200 accurate mass TOF MS equipped with Agilent 1200 LC, Hypersil Gold C18 (50 x 2.1 mm, 1.9 µm) column and linear gradient from 0% to 98% B over 4 minutes, same solvents as before. External calibration using Agilent tuning mix was performed before measurements.

Semi-preparative RP-HPLC was performed on a Merck-Hitachi system (controller: D-7000, pump: L7150, detector: L7420, UV-absorption measured at λ = 220 nm), equipped with semi-preparative column: Macherey-Nagel Nucleosil 100-7 C18, 7 µm, 250 x 10 mm.

Eluent A: $H_2O/CH_3CN/TFA$ = 95/5/0.1 and eluent B: $H_2O/CH_3CN/TFA$ = 5/95/0.1

Method M2:

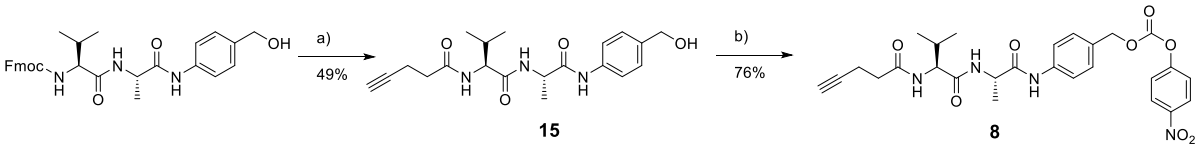
Flow rate: 4 mL min^{-1}

0 min	100% A	0% B
5 min	100% A	0% B
35 min	0% A	100% B
40 min	0% A	100% B
45 min	100% A	0% B

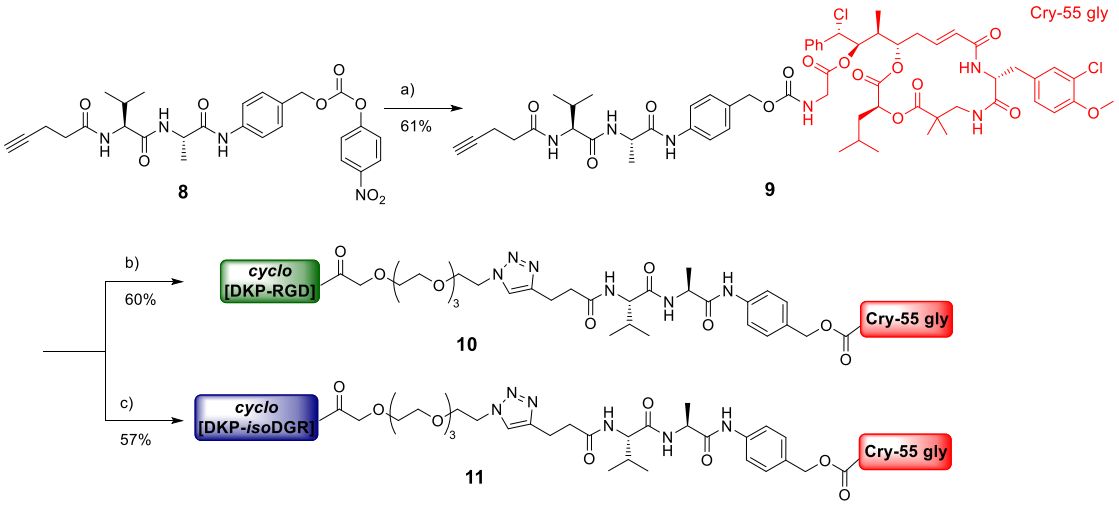
NMR spectroscopy

Proton NMR-spectra were recorded on a spectrometer operating at 400.16 MHz. Carbon NMR-spectra were recorded on a spectrometer operating at 100.63 MHz, with complete proton decoupling. Chemical shifts (δ) are reported in parts per million (ppm) and are referenced to residual nondeuterated solvent signal (DMSO-d_6: ^{1}H: 2.50 ppm; ^{13}C: 39.51 ppm; CD_3OD: ^{1}H: 3.33 ppm; ^{13}C: 49.05 ppm). Coupling constants (*J*) are reported in Hz with the following abbreviations used to indicate splitting: s = singlet, d = doublet, t = triplet, q = quartet, hept = heptet, m = multiplet, br = broad signal.

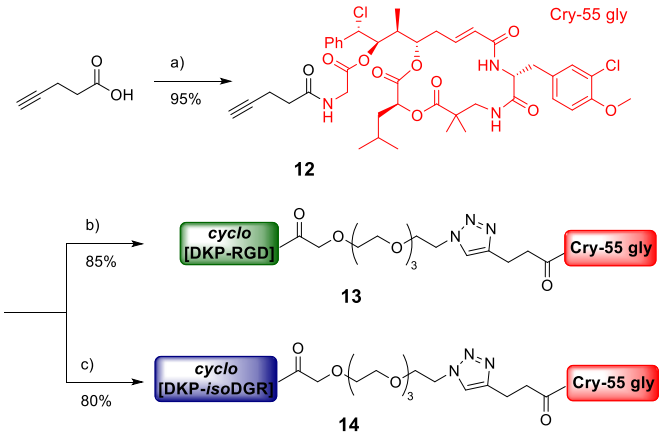
Synthesis schemes



Scheme S1. Synthesis of 4-Pentynamido-Val-Ala-PABC-PNP (**8**). Reagents and conditions: a) 1. piperidine/DMF, RT, 2 h; 2. 4-pentynoic acid, HATU, HOAt, DIPEA, DMF, RT, 4 h; b) bis(4-nitrophenyl) carbonate, DIPEA, DMF, RT, 3.5 h.



Scheme S2. Synthesis of *cyclo*[DKP-RGD]-PEG4-Val-Ala-PABC-Cry-55gly (**10**) and *cyclo*[DKP-isoDGR]-PEG4-Val-Ala-PABC-Cry-55gly (**11**). Reagents and conditions: a) **7**, DIPEA, DMF, RT, 4 h; b) *cyclo*[DKP-RGD]-PEG4-N_3, $CuSO_4 \cdot 5H_2O$, sodium ascorbate, 1:1 DMF/H_2O, 35 °C, 24 h; c) *cyclo*[DKP-*iso*DGR]-PEG4-N_3, $CuSO_4 \cdot 5H_2O$, sodium ascorbate, 1:1 DMF/H_2O, 35 °C, 24 h.

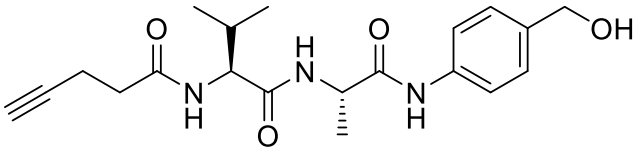


Scheme S3. Synthesis of *cyclo*[DKP-RGD]-PEG4-uncleavable-Cry-55gly (**13**) and *cyclo*[DKP-*iso*DGR]-PEG4-uncleavable-Cry-55gly (**14**). Reagents and conditions: a) **7**, PyBOP, HOBt, DIPEA, DMF, RT, 4 h; b) *cyclo*[DKP-RGD]-PEG4-N_3, $CuSO_4 \cdot 5H2O$, sodium ascorbate, 1:1 DMF/H_2O, 35 °C, 24 h; c) *cyclo*[DKP-*iso*DGR]-PEG4-N_3, $CuSO_4 \cdot 5H_2O$, sodium ascorbate, 1:1 DMF/H_2O, 35 °C, 24 h.

Synthetic procedures, characterization details

Synthesis of *cyclo*[DKP-RGD]-PEG4-Val-Ala-PABC-Cry-55gly (10) and *cyclo*[DKP-*iso*DGR]-PEG4-Val-Ala-PABC-Cry-55gly (11)

4-Pentynamido-Val-Ala-PABA (15)

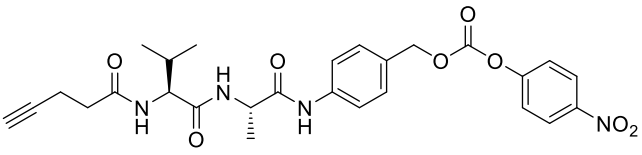


Fmoc-Val-Ala-PABA[1] (300 mg, 0.58 mmol, 1 eq) was dissolved in dry DMF (6 mL), piperidine (300 µL) was added and the reaction was stirred at RT for 2 h. The solvents were removed, and the residue was used for the next step.

4-pentynoic acid (88 mg, 0.87 mmol, 1.5 eq), HATU (375 mg, 0.99 mmol, 1.7 eq), and HOAt (134 mg, 0.99 mmol, 1.7 eq) were dissolved in dry DMF (4 mL) under N_2 atmosphere. DIPEA (404 µL, 2.32 mmol, 4 eq) was added and the mixture was stirred for 20 min at RT. A solution of NH_2-Val-Ala-PABA in dry DMF (6 mL) was added to the stirred mixture. After 4 h the solvent was removed, and the remained oil was purified by column chromatography using DCM/MeOH (95:5) to afford **15** as colorless solid (102 mg, 49% over two steps).

R_f = 0.11 (DCM/MeOH = 95:5); **^{1}H-NMR** (400 MHz, MeOD-*d*$_4$): δ (ppm) = 0.97 (d, *J* = 6.6 Hz, 3H), 0.99 (d, *J* = 7.0 Hz, 3H), 1.43 (d, *J* = 7.1 Hz, 3H), 2.09 (hept, *J* = 6.8 Hz, 1H), 2.26 (d, *J* = 2.5 Hz, 1H), 2.42-2.58 (m, 4H), 4.21 (d, *J* = 7.0 Hz, 1H), 4.48 (q, *J* = 7.1 Hz, 1H), 4.55 (s, 2H), 7.30 (d, *J* = 8.5 Hz, 2H), 7.55 (d, *J* = 8.5 Hz, 2H). **^{13}C-NMR** (100 MHz, MeOD-*d*$_4$): δ (ppm) = 15.6, 18.0, 18.7, 19.7, 31.8, 35.8, 51.1, 60.4, 64.8, 70.4, 83.7, 121.2, 128.6, 138.7, 138.7, 172.9, 173.6, 174.4.

4-Pentynamido-Val-Ala-PABC-PNP (8)

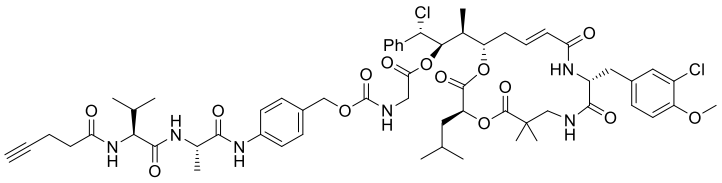


15 (35 mg, 0.094 mmol, 1 eq) and bis(4-nitrophenyl) carbonate (57 mg, 0.19 mmol, 2 eq) were dissolved in dry DMF (1 mL) under N_2 atmosphere. DIPEA (25 µL, 0.14 mmol, 1.5 eq) was

added and the mixture was stirred for 3.5 h at RT. The sample was concentrated under reduced pressure and the crude was purified by DCM/MeOH (98:2) affording compound **8** as colorless solid (39 mg, 76%).

R_f = 0.14 (DCM/MeOH = 98:2); **^{1}H-NMR** (400 MHz, DMSO-d_6): δ (ppm) = 0.87 (dd, J = 6.7, 15.7 Hz, 6H), 1.31 (d, J = 7.1 Hz, 3H), 1.97 (hept, J = 6.8 Hz, 1H), 2.30 – 2.48 (m, 4H), 2.74 (t, J = 2.2 Hz, 1H), 4.21 (dd, J = 6.7, 8.6 Hz, 1H), 4.39 (p, J = 7.0 Hz, 1H), 5.24 (s, 2H), 7.41 (d, J = 8.7 Hz, 2H), 7.57 (d, J = 9.1 Hz, 2H), 7.64 (d, J = 8.5 Hz, 2H), 7.94 (d, J = 8.6 Hz, 1H), 8.21 (d, J = 6.9 Hz, 1H), 8.31 (d, J = 9.1 Hz, 2H), 9.99 (s, 1H); **^{13}C-NMR** (100 MHz, DMSO-d_6): δ (ppm) = 14.3, 17.8, 18.1, 19.1, 30.5, 34.0, 49.1, 57.5, 70.2, 71.2, 83.8, 119.0, 122.6, 125.4, 129.2, 129.4, 139.4, 145.2, 151.9, 155.3, 170.5, 170.9, 171.2.

4-Pentynamido-Val-Ala-PABC-Cry-55gly (9)

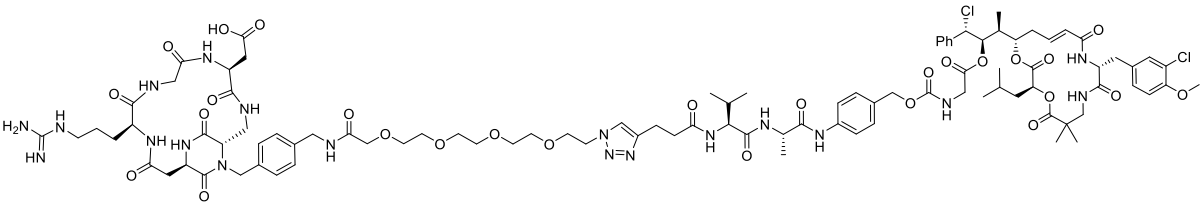


Cryptophycin-55 glycinate (**7**) was prepared as previously described.[2]

7 (8.1 mg, 10.6 µmol, 1 eq) and **8** (6.3 mg, 11.7 µmol, 1.1 eq) were dissolved in anhydrous DMF (300 µL). DIPEA (5.4 µL, 31.8 µmol, 3 eq) was added and the mixture was stirred for 4 h at RT, followed by RP-HPLC purification to yield **9** as colorless solid (7.5 mg, 61%).

LC-MS: t_R = 10.9 min, *m/z* calcd for $[C_{59}H_{75}Cl_2N_6O_{14}]^+$: 1161.47 $[M + H]^+$, found: 1161.49.

***cyclo*[DKP-RGD]-PEG4-Val-Ala-PABC-Cry-55gly (10)**



Ligand *cyclo*[DKP-RGD]-PEG4-N_3 (**16**) was prepared as previously described.[3]

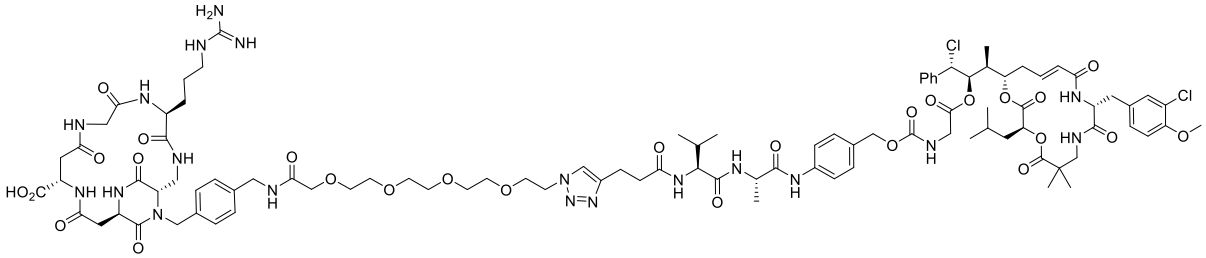
9 (2.8 mg, 2.4 µmol, 1 eq), **16** (2.1 mg, 2.4 µmol, 1 eq), $CuSO_4$ (0.35 mg, 1.4 µmol, 0.6 eq) and sodium ascorbate (0.19 mg, 0.95 µmol, 0.4 eq) were dissolved in DMF/H_2O (1:1, 1 mL,

degassed) and stirred for 24 h at 35 °C, followed by RP-HPLC purification to yield **10** as colorless solid (2.9 mg, 60%).

LC-MS: t_R = 7.9 min, *m/z* calcd for $[C_{96}H_{131}Cl_2N_{19}O_{27}]^{2+}$: 1025.94 $[M + 2H]^{2+}$, found: 1025.99.

HRMS (ESI-MS): *m/z* calcd for $[C_{96}H_{131}Cl_2N_{19}O_{27}]^{2+}$: 1025.9414 $[M + 2H]^{2+}$, found: 1025.9322.

***cyclo*[DKP-*iso*DGR]-PEG4-Val-Ala-PABC-Cry-55gly (11)**



Ligand *cyclo*[DKP-*iso*DGR]-PEG4-N_3 (**17**) was prepared as previously described.[4]

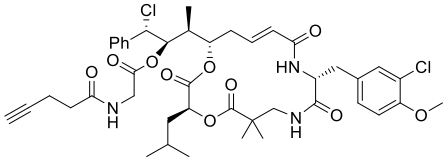
9 (3.5 mg, 3.2 µmol, 1 eq), **17** (3.1 mg, 3.5 µmol, 1.1 eq), $CuSO_4$ (0.40 mg, 1.1 µmol, 0.5 eq) and sodium ascorbate (0.38 mg, 1.9 µmol, 0.6 eq) were dissolved in DMF/H_2O (1:1, 200 µL, degassed) and stirred for 24 h at 35 °C, followed by RP-HPLC purification to yield **11** as colorless solid (3.8 mg, 57%).

LC-MS: t_R = 8.8 min, *m/z* calcd for $[C_{96}H_{131}Cl_2N_{19}O_{27}]^{2+}$: 1025.94 $[M + 2H]^{2+}$, found: 1025.93.

HRMS (ESI-MS): *m/z* calcd for $[C_{96}H_{131}Cl_2N_{19}O_{27}]^{2+}$: 1025.9414 $[M + 2H]^{2+}$, found: 1025.9439.

Synthesis of *cyclo*[DKP-RGD]-PEG4-uncleavable-Cry-55gly (13) and *cyclo*[DKP-*iso*DGR]-PEG4-uncleavable-Cry-55gly (14)

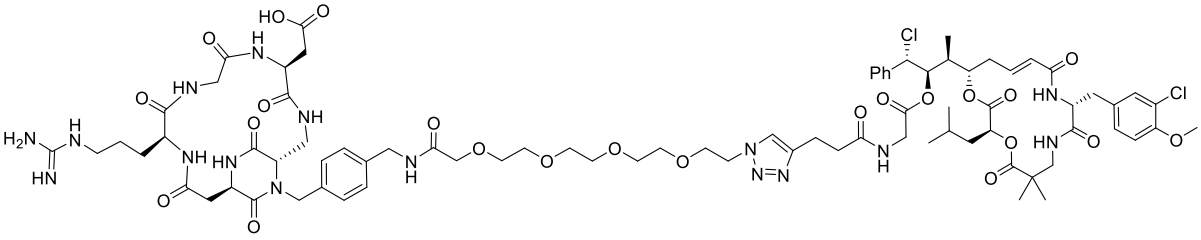
4-Pentynoil-Cry-55gly (12)



7 (6 mg, 7.8 µmol, 1 eq), 4-pentynoic acid (3 mg, 31.5 µmol, 4 eq), PyBOP (16 mg, 31.5 µmol, 4 eq) and HOBt (5.5 mg, 35.5 µmol, 4.5 eq) were dissolved in anhydrous DMF (500 µL) under argon. DIPEA (6 µL, 35.5 µmol, 4.5 eq) was added and the mixture was stirred for 4 h at RT, followed by RP-HPLC purification to yield **12** as colorless solid (6.2 mg, 95%).

LC-MS: t_R = 10.9 min, *m/z* calcd for $[C_{43}H_{53}Cl_2N_3O_{10}]^+$: 842.32 $[M + H]^+$, found: 842.34.

***cyclo*[DKP-RGD]-PEG4-uncleavable-Cry-55gly (13)**

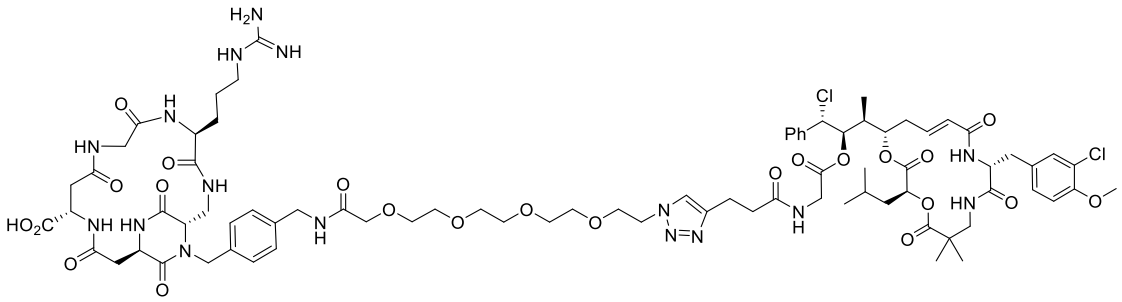


12 (2.5 mg, 3.0 µmol, 1 eq), **16** (2.7 mg, 3.0 µmol, 1 eq), $CuSO_4$ (0.45 mg, 1.8 µmol, 0.6 eq) and sodium ascorbate (0.24 mg, 1.2 µmol, 0.4 eq) were dissolved in DMF/H_2O (1:1, 600 µL, degassed) and stirred for 24 h at 35 °C, followed by RP-HPLC purification to yield **13** as colorless solid (4.4 mg, 85%).

LC-MS: t_R = 7.5 min, *m/z* calcd for $[C_{80}H_{109}Cl_2N_{16}O_{23}]^+$: 1731.72 $[M + H]^+$, found: 1731.81; *m/z* calcd for $[C_{80}H_{110}Cl_2N_{16}O_{23}]^{2+}$: 866.36 $[M + 2H]^{2+}$, found: 866.41.

HRMS (ESI-MS): *m/z* calcd for $[C_{80}H_{110}Cl_2N_{16}O_{23}]^{2+}$: 866.3648 $[M + 2H]^{2+}$, found: 866.3630.

***cyclo*[DKP-*iso*DGR]-PEG4-uncleavable-Cry-55gly (14)**



12 (1.5 mg, 1.73 µmol, 1 eq), **17** (1.7 mg, 1.90 µmol, 1.1 eq), $CuSO_4$ (0.22 mg, 0.87 µmol, 0.5 eq) and sodium ascorbate (0.20 mg, 1.04 µmol, 0.6 eq) were dissolved in DMF/H_2O (1:1, 200 µL, degassed) and stirred for 24 h at 35 °C, followed by RP-HPLC purification to yield **14** as colorless solid (2.4 mg, 80%).

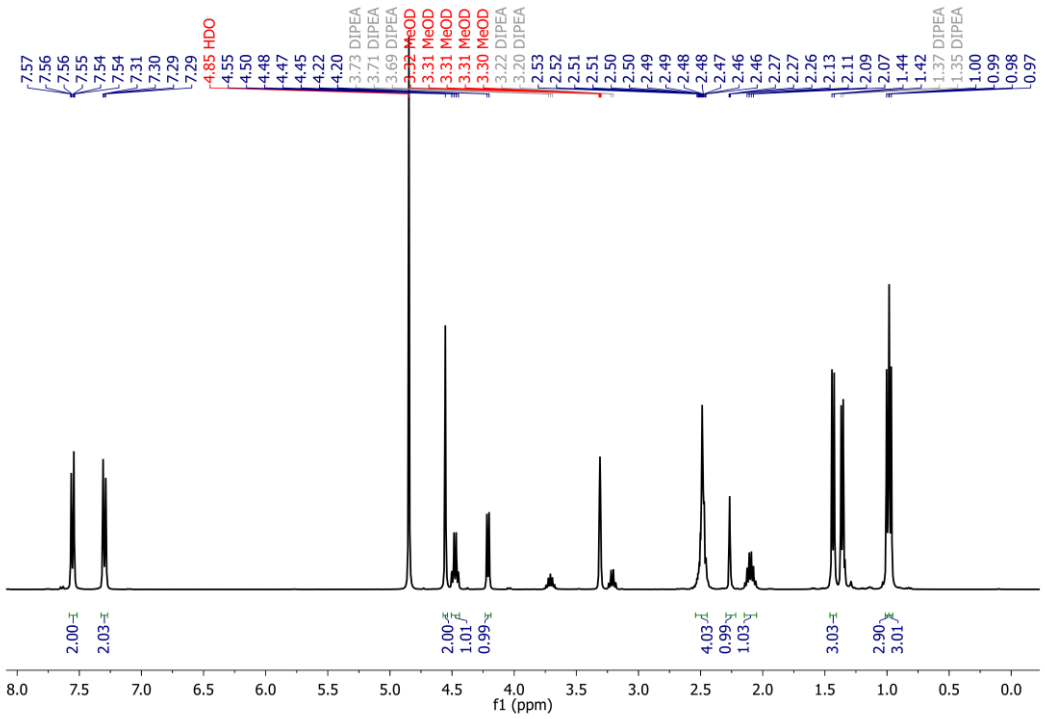
LC-MS: t_R = 8.4 min, *m/z* calcd for $[C_{80}H_{109}Cl_2N_{16}O_{23}]^+$: 1731.72 $[M + H]^+$, found: 1731.74; *m/z* calcd for $[C_{80}H_{110}Cl_2N_{16}O_{23}]^{2+}$: 866.36 $[M + 2H]^{2+}$, found: 866.38.

HRMS (ESI-MS): *m/z* calcd for $[C_{80}H_{110}Cl_2N_{16}O_{23}]^{2+}$: 866.3648 $[M + 2H]^{2+}$, found: 866.3654.

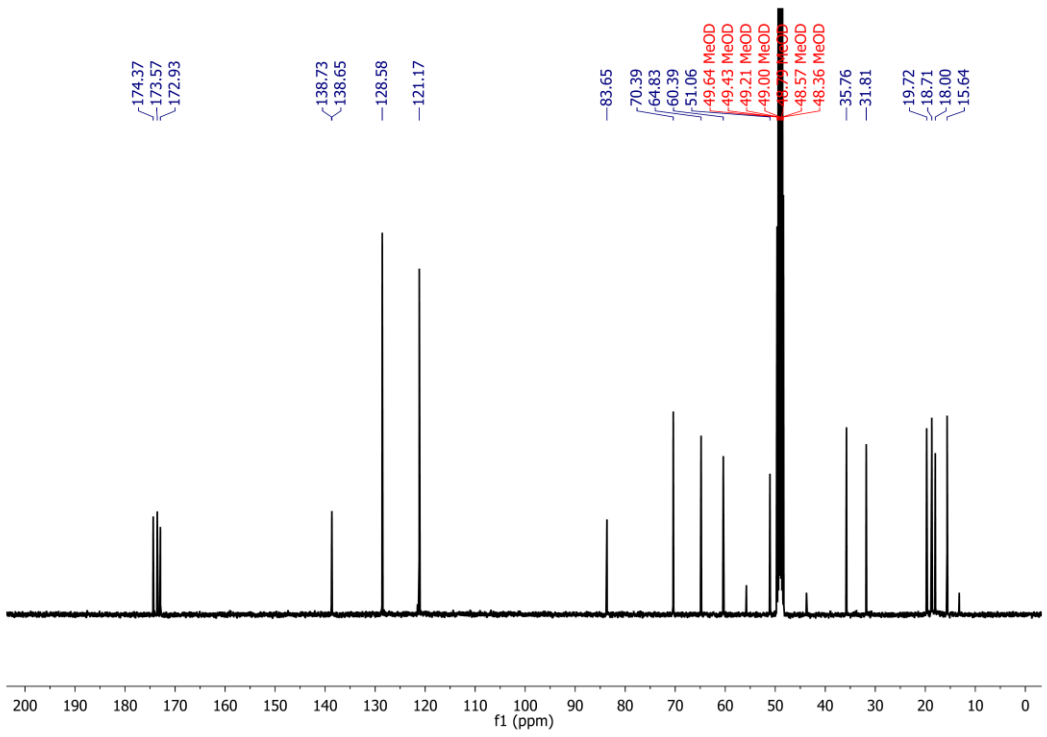
NMR, HPLC and mass spectra

4-Pentynamido-Val-Ala-PABA (15)

^{1}H-NMR (400 MHz, MeOD-d_4)

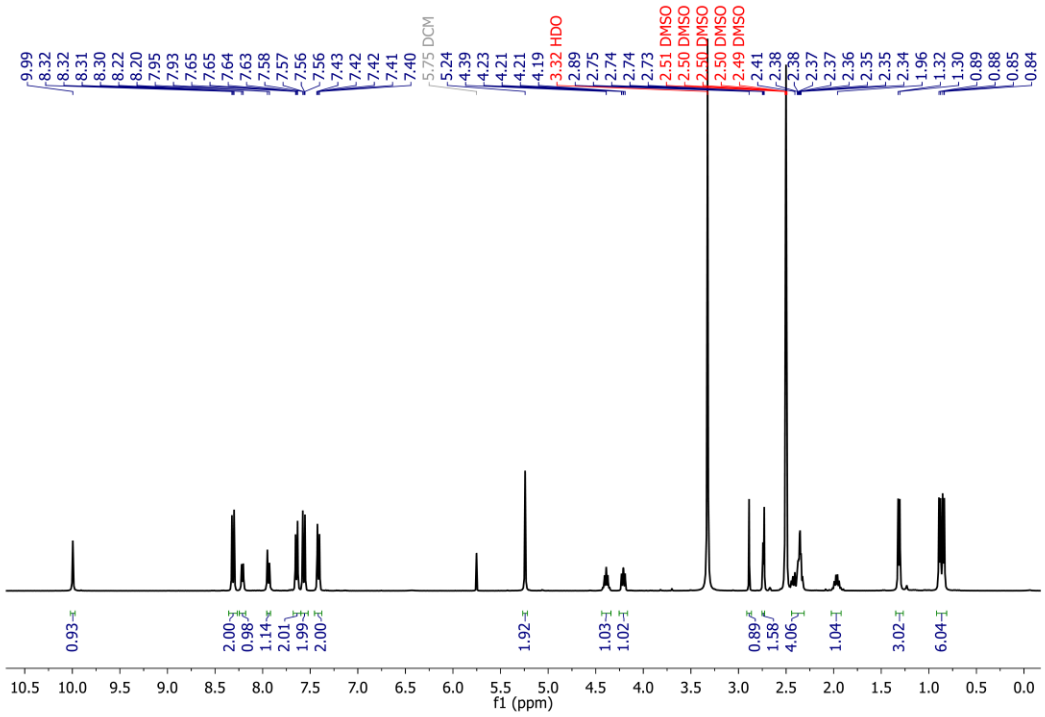


^{13}C-NMR (100 MHz, MeOD-d_4)

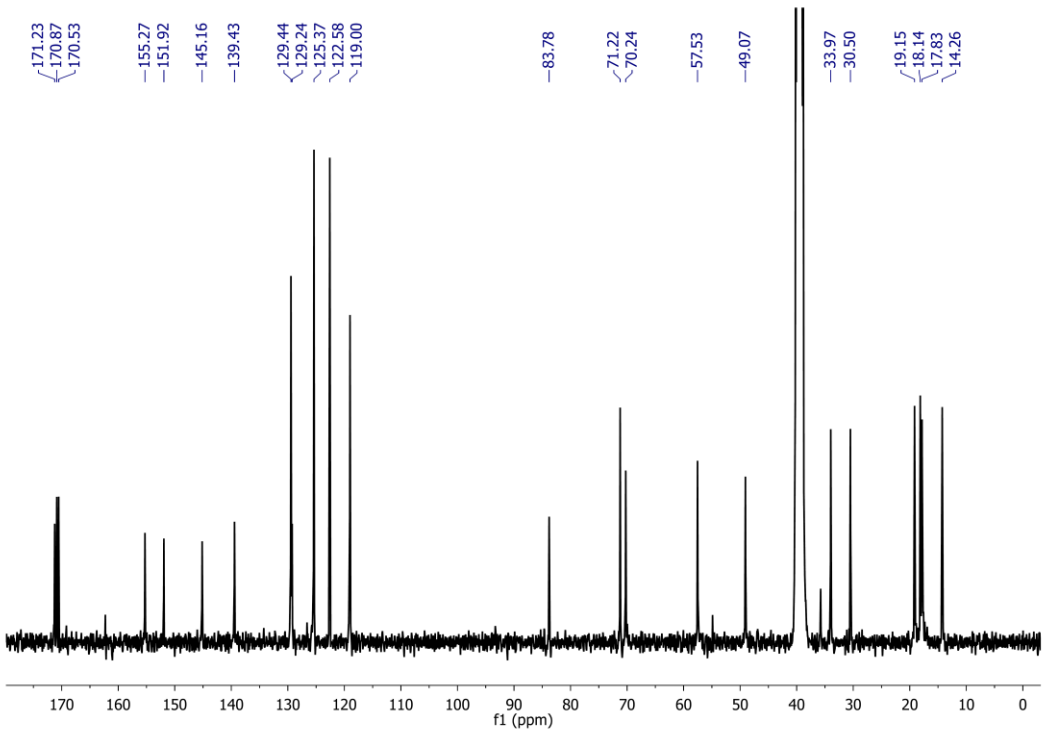


4-Pentynamido-Val-Ala-PABC-PNP (8)

^{1}H-NMR (400 MHz, DMSO-d_6)

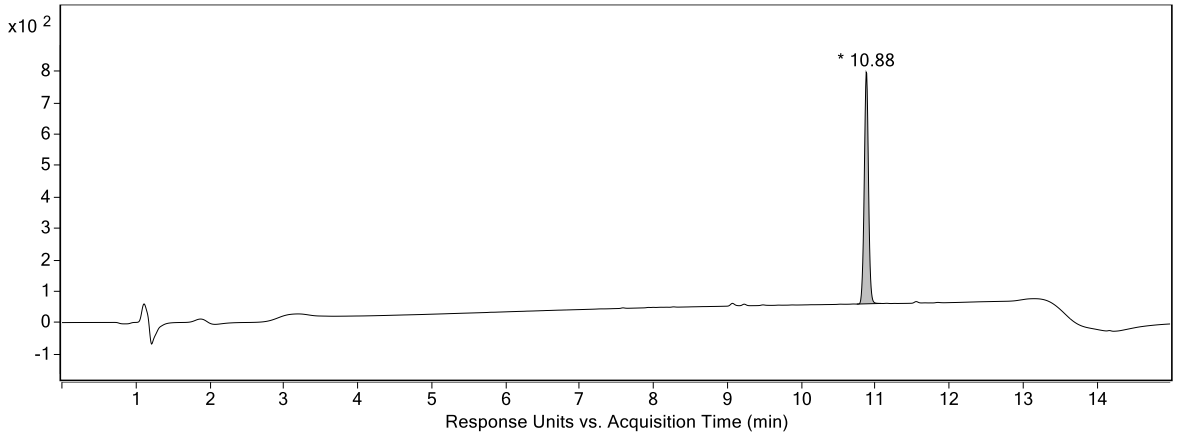


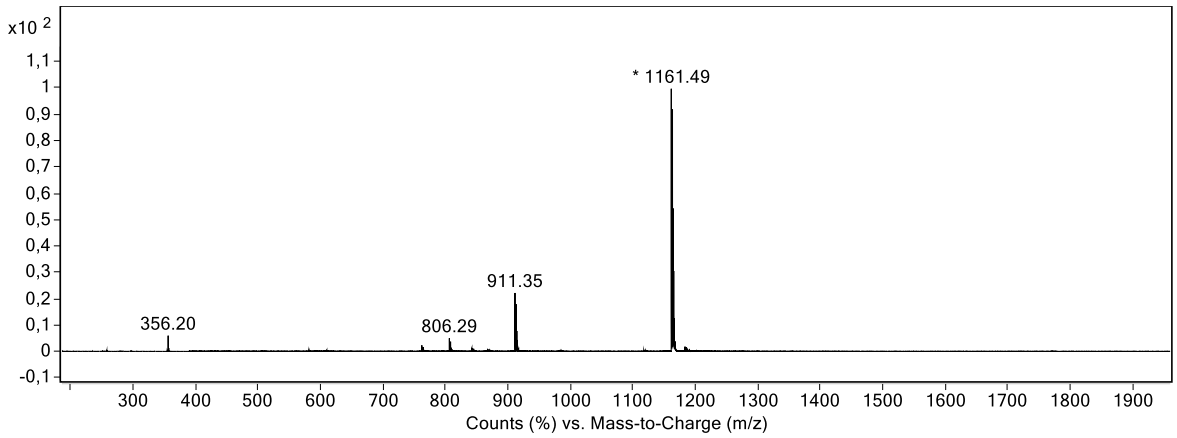
^{13}C-NMR (100 MHz, DMSO-d_6)



4-Pentynamido-Val-Ala-PABC-Cry-55gly (9)

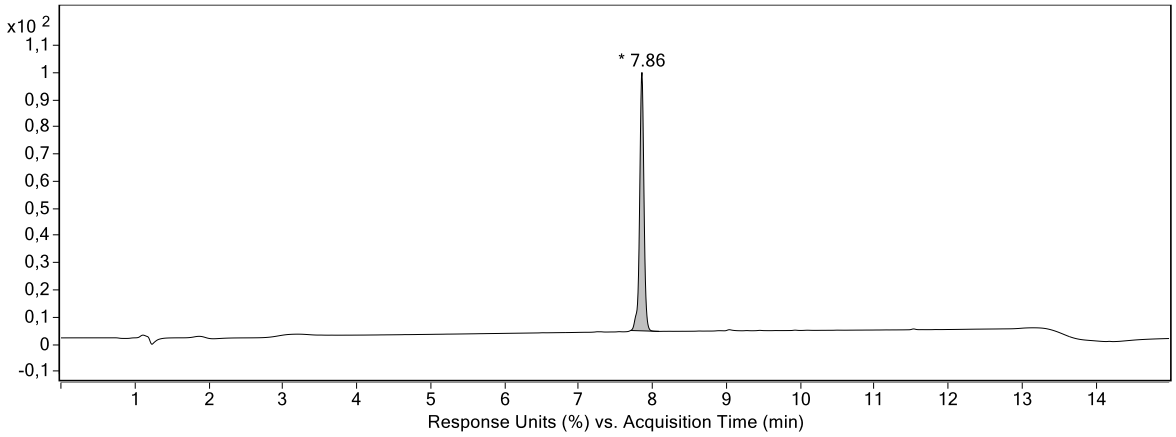
LC-MS:

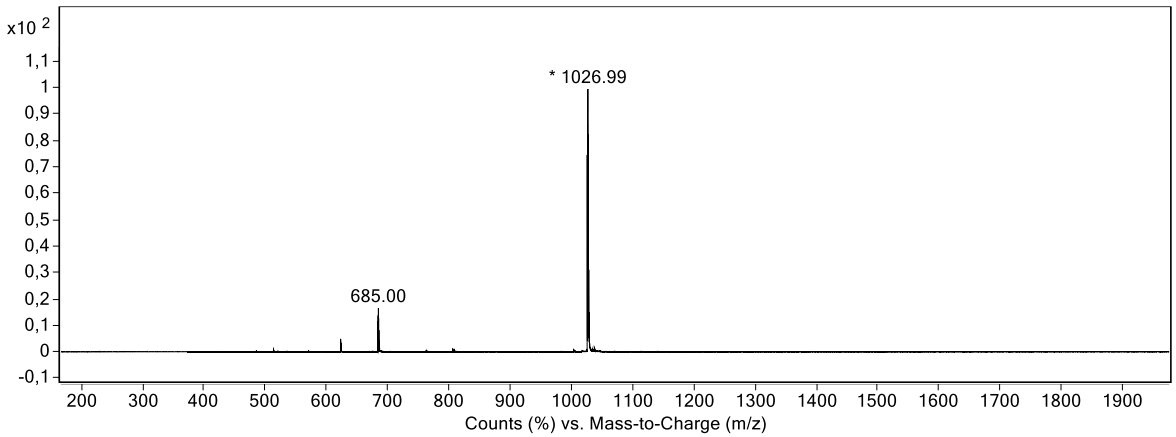


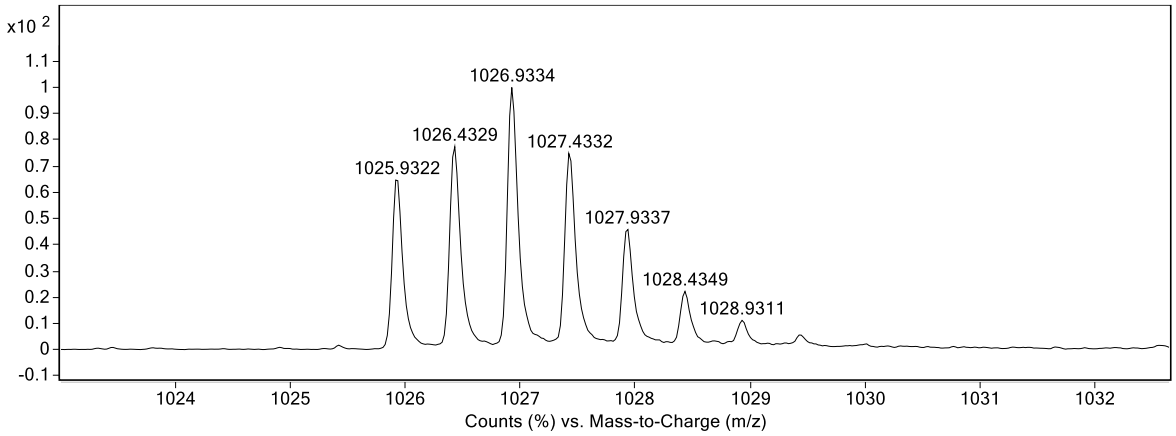


***cyclo*[DKP-RGD]-PEG4-Val-Ala-PABC-Cry-55gly (10)**

LC-MS, HRMS:

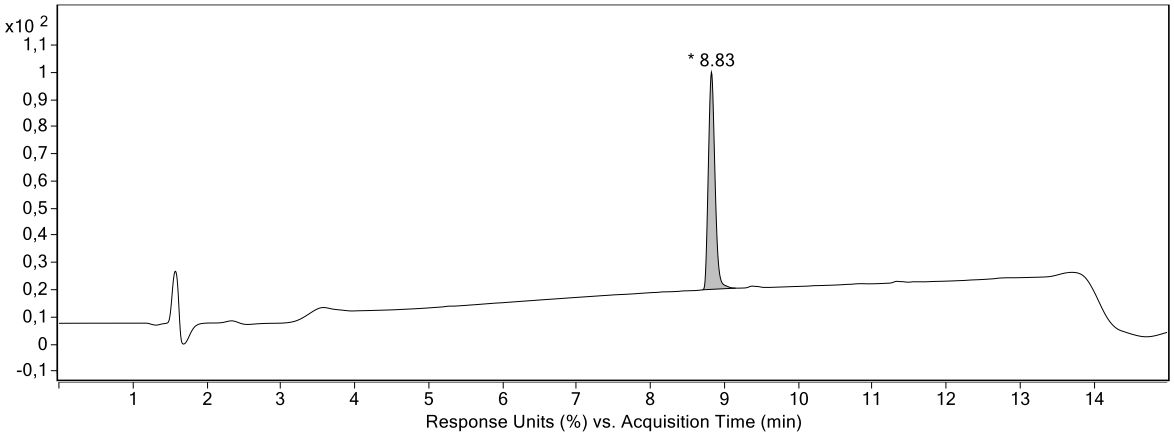


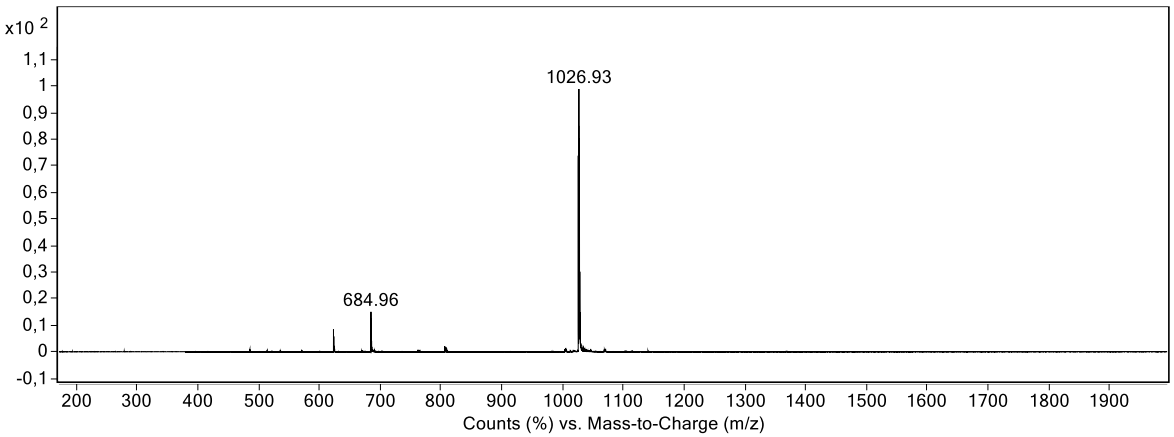


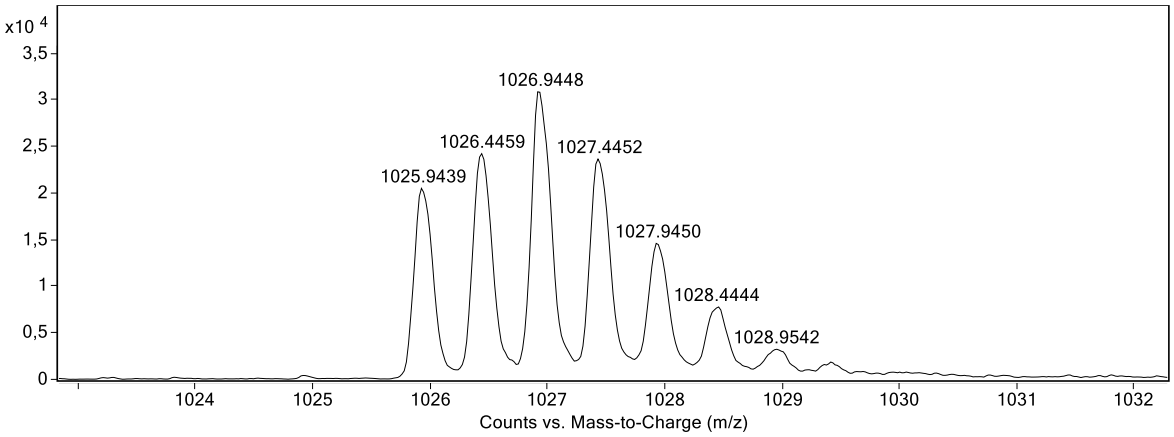


cyclo[DKP-*iso*DGR]-PEG4-Val-Ala-PABC-Cry-55gly (11)

LC-MS, HRMS:

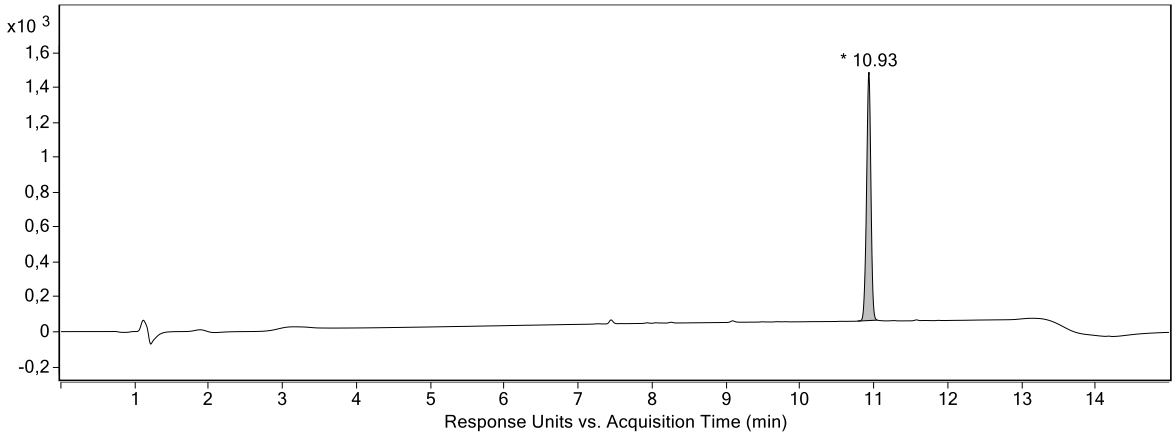


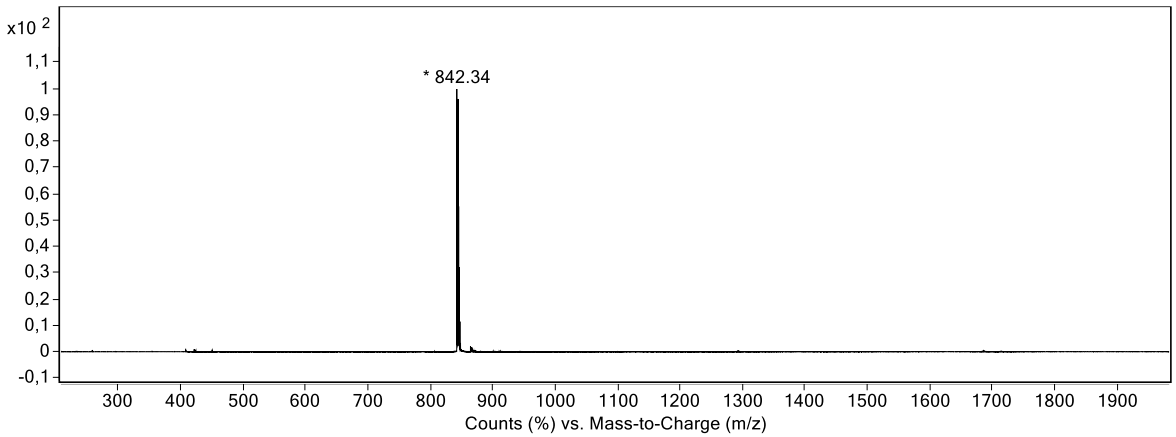




4-Pentynoil-Cry-55gly (12)

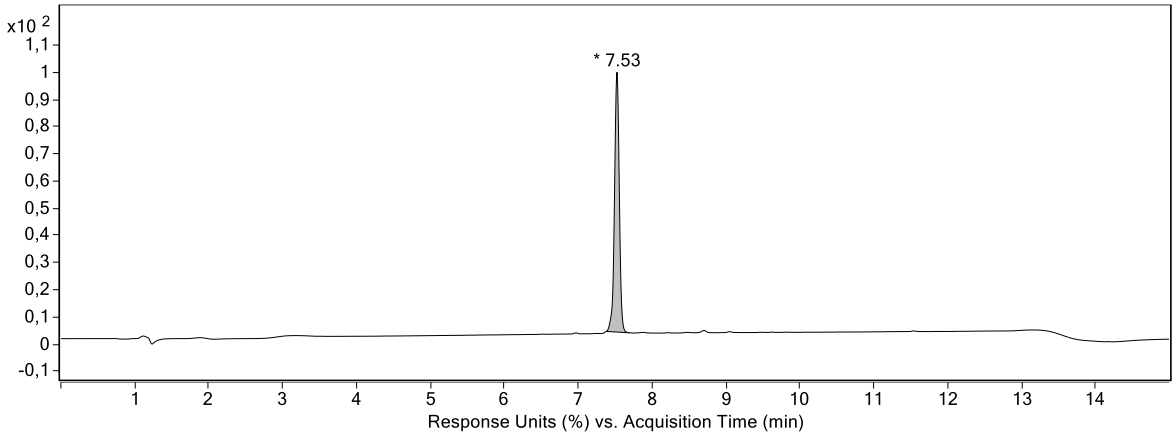
LC-MS:

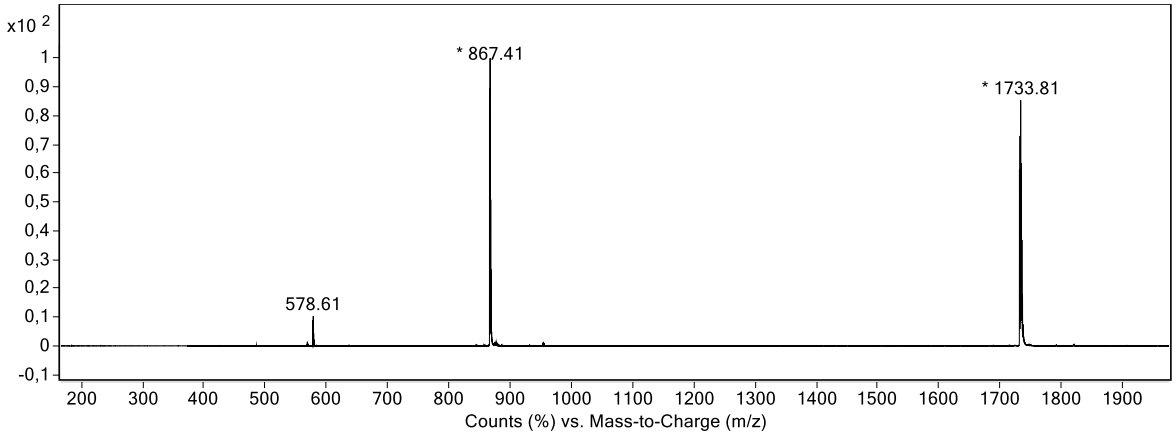


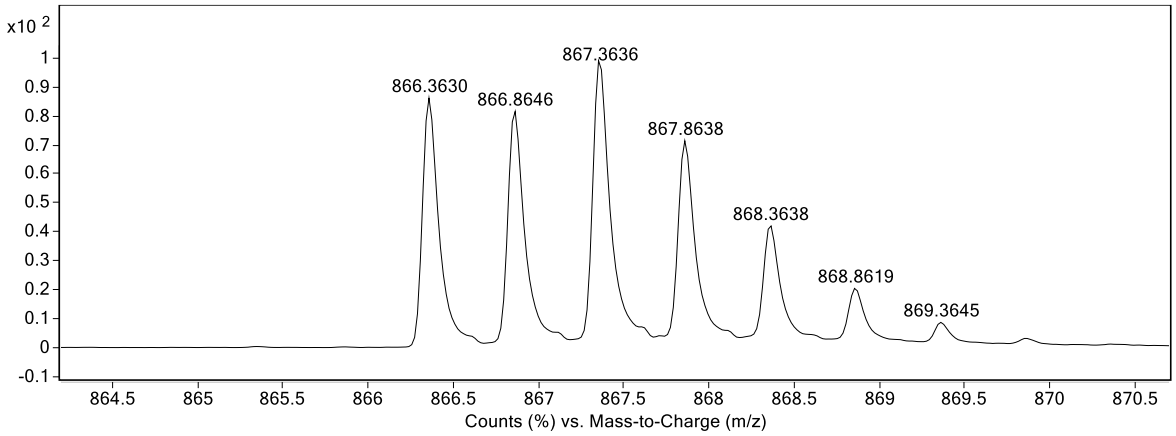


***cyclo*[DKP-RGD]-PEG4-uncleavable-Cry-55gly (13)**

LC-MS, HRMS:

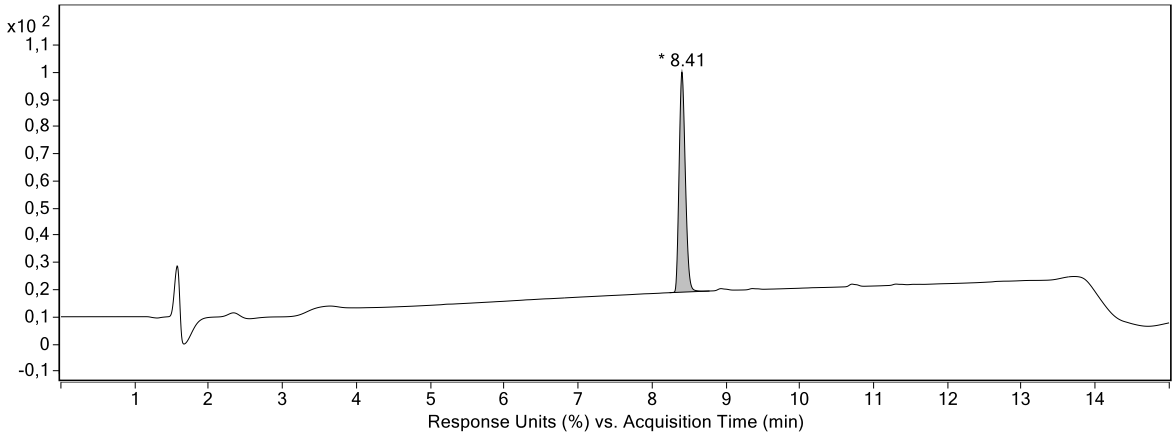


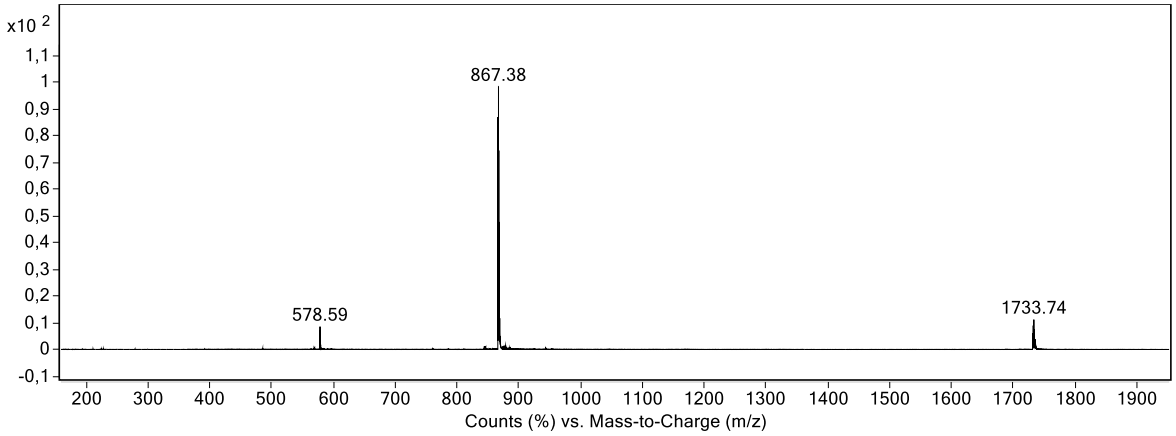


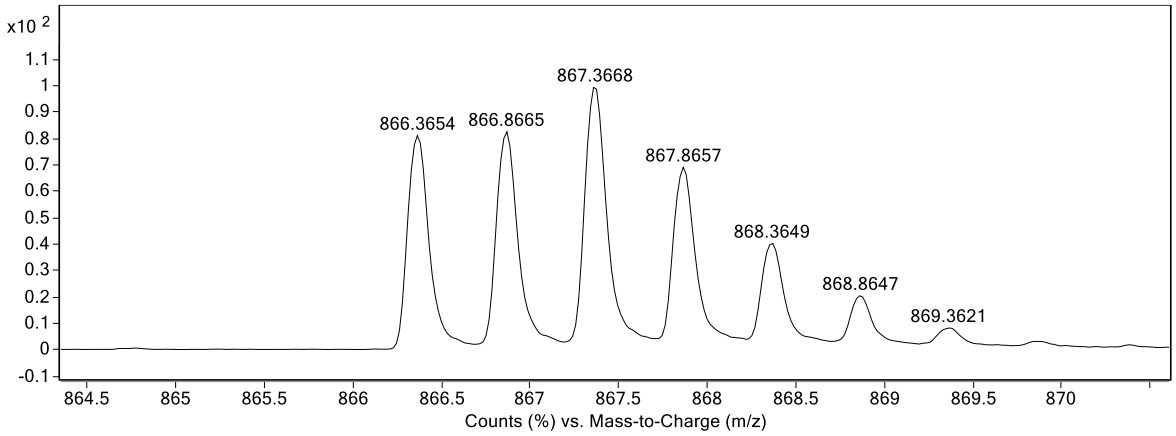


***cyclo*[DKP-*iso*DGR]-PEG4-uncleavable-Cry-55gly (14)**

LC-MS, HRMS:







III. References

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